Chemistry and Significance of Condensed Tannins

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PREFACE

This book was developed from the proceedings of the first North American Tannin Conference held in Port Angeles, Washington, August 1988. The objective of the conference was to bring together people with a common interest in condensed tannins and to promote interdisciplinary interactions that will lead to a better understanding of these important substances.

Another objective was the publication of this book because there has not been a monograph devoted to the chemistry and significance of tannins for several decades. The book is organized into sections dealing with the biosynthesis, structure, reactions, complexation with other biopolymers, biological significance, and use of tannins as specialty chemicals. The authors made a special attempt to focus on *what we don't know* as well as to provide a summary of what we do know in an effort to assist in planning future research.

Our thanks go to the authors who so kindly contributed chapters and so patiently responded to our requests. We also thank Rylee Geboski and the Conference Assistance Staff, College of Forestry, Oregon State University, for their assistance in planning and conducting the conference, and Julia Wilson, Debbie Wolfe, Helen Coletka, and Nancy Greene of the Southern Forest Experiment Station, Pineville, Louisiana, who typed the chapters. Linda Chalker-Scott was especially helpful in assisting us with editing. Dick Hemingway is indebted to the staff of the Alexandria Forestry Center at Pineville, Louisiana, for their exceptional patience, and particularly to Allan Tiarks and Timothy Rials who helped in solving problems with computers and LaTex applications. Gregory Safford of Plenum Publishing Company was very helpful in preparation of figures and developing the book format.

Both the tannin conference and preparation of this book have been rewarding endeavors. Most importantly, they have helped to bring the "tannin" research family together and serve as a start toward a series of such meetings and monographs that we expect will continue into the future.

Richard W. Hemingway Joseph J. Karchesy Susan J. Branham

April 27, 1989

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Introduction

HEMLOCK AND SPRUCE TANNINS: AN ODYSSEY

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ABSTRACT

In North America, the only significant chemical utilization of bark has been that of hemlock, which served as the major raw material for leather tanning for more than a century. This chapter traces the history of leather manufacture from its beginning as a colonial cottage industry through its emergence at the end of the nineteenth century as a major industrial activity based largely on a local eastern hemlock resource. The later availability of domestic (chestnut) and imported (quebracho and wattle) extracts caused a geographic shift in the industry to the source of hides rather than bark. Substitution of chromium salts for vegetable tannins also contributed to the demise of hemlock tannin utilization except for the production of sulfonated tannins from western hemlock used for oil well drilling, water treatment, agricultural trace metal treatments, etc. Modern chemical investigations of hemlock tannin composition began in 1954 with the advent of paper chromatographic separation techniques, which eventually showed that tannins were polymeric *cis* and *trans* procyanidins terminated by catechin and epicatechin. Recent work demonstrates the need for studies of polyphenolic polymers from morphologically distinct zones of bark. In spite of yet-to-be solved chemical structure problems in the field of spruce/hemlock tannins, utilization is not being held up. The major need is for the development of extraction technology that will remove the organic solvent-insoluble

*This chapter is based on Dr. Hergert's address in acceptance of the first North American Tannin Conference Award. Most of the research by the author and presented in this chapter was conducted at the ITT Rayonier research laboratory, Shelton, Washington.

procyanidin polymers without major structural alteration. The basic requirement appears to be a system that cleaves procyanidin polymers (without causing condensation with adjacent lignin molecules) and solubilizes them in an aqueous system. The best potential for use is as a resorcinol substitute in cold-setting adhesives, since domestic vegetable tannins are unlikely to again be of any major consequence in leather manufacture in North America.

INTRODUCTION

The word "odyssey" is defined in one dictionary as 1) a long wandering or voyage usually marked by many changes in fortune, and 2) an intellectual or spiritual wandering or quest. It seems, then, that this word is appropriate to describe the use and study of hemlock tannin (and to a much lesser extent, spruce tannin), a material whose utility was first discovered during colonial times in North America. It rose in importance to become the preferred raw material for leather manufacture by one of the top ten United States businesses at the turn of the century.[1] Its use then declined partly due to poor husbandry of the forest resource from which it was obtained, but mainly as a result of leather manufacturing process changes. For a brief period of two decades extending into the middle 1970's, the fortunes of this product seemed to have revived. It was used for oil-well drilling, mineral dispersion, cooling water treatment, adhesive formulations, chemical grouting, and agricultural trace metal carriers. Unfortunately, economics once again dictated the closures of hemlock tannin manufacturing plants. Nowadays, the use of hemlock tannin seems to be largely limited to potential grist for the mills of intellectual and scientific endeavor. This statement is not precisely correct because almost all hemlock and spruce bark generated as a byproduct of the forest products industry is consumed as fuel–an important but inelegant use for material containing tannins and other chemicals of such potentially useful structure.

The study of history is much more than a mere academic pursuit if one is willing to apply the lessons from the past to events of the present or future. Certainly, this is true of this particular odyssey, so we do not apologize for reviewing the hemlock tannin utilization of the past in connection with the chemical structural studies of the present.

Even today, the basic steps in preparing leather have not changed much from early times, although the types of chemicals used in each stage are quite different.[2] Hides are usually received by the tanner in a salt-cured condition, so the first step is to rehydrate and cleanse the skins to prepare them for the removal of hair. In the next stage, the hide is treated with lime or other substances, so that hair can be removed mechanically. Following this, extraneous proteins are removed enzymatically, and the pH is lowered. The skin is then ready to be treated with a tanning agent, the primary purpose of which is to stabilize the collagen fibers so that the skin will no longer be biodegradable. Finally, the tanned hides are washed, dried, and treated with various oils and finishing agents.

The first attempts to apply preservatives to hides and skins to make them useful for clothing and footwear are hidden in unwritten history. In the most ancient of times, they probably consisted of little more than cleansing and drying skins. Eventually, leather and tanning processes, as distinct from simple preservation, became well-developed in ancient civilizations from Babylon in the West to China in the East. Egyptian leather workers seemed to have reached a particularly high state of artistry and efficiency. A leather funeral tent of an Egyptian queen dated at 1100 B.C. is on display in a museum in Cairo. Egyptian, Greek, and Roman tanners utilized lime water to remove hair from hides, developed the use of the scraping knife and beam to complete the removal of hair, and packed hides flat with powdered oak bark between the layers. Analyses of an archeological specimen of an ancient Roman leather harness showed it to be representative of what is known currently as ordinary vegetable tanned leather.

In Medieval Europe, leather tanning was practiced by well-organized guilds. Raw hides were salted to check putrefaction, limed in weak lime liquor, and then brought to a suitable condition for dehairing and fleshing over a period of about 3 months. They were then placed between layers of coarsely ground oak bark in pits until full, and a thick top layer of bark was placed over them. No water or any kind of liquid was allowed to get into the pits. These packs were taken up and reversed several times with the introduction of fresh oak bark, the tanning process requiring about 18 months time. The hides were then transferred to a layer pit where they were turned over every day in a liquid or "ooze" made of oak bark. After 5 or 6 weeks, the skins were then immersed in a tanning-dyeing medium. Subsequent treatments of washing, finishing, etc., extended the process to more than 2 years from the receipt of the hide to the completion of the final product.

TANNING IN COLONIAL TIMES

When the first colonists arrived in America, they were confronted with the "tanning" practices of the Indians. One description of their procedure is as follows:[4]

> "As soon as the skin is removed from the animal by these aboriginal leather-dressers, it is stretched and dried. The brains are at this time taken out and also dried upon the grass, by exposure to the sun's rays. When the season of the chase is over, the squaws soak these skins in water, remove the hair from them with an old knife, and place them along with the brains, in a large earthen pot; the contents are then heated to about 95°, which converts the moistened brains into a kind of lather, and makes the skins exceedingly clean and pliable. They are then taken from the pot, wrung out, and stretched in every direction, by means of thongs, over a frame composed of upright stakes and crosspieces; and while drying they are constantly rubbed with a smooth stone, or a hard piece of wood, so as to expel the water and fat. One squaw can prepare eight to ten skins in a day."

The capability of preparing leather must have been extremely vital to the new immigrants from Europe, isolated as they were from the mother countries. The

rapid preparation of skins by the native Americans must have been in sharp contrast to leather tanning as practiced in England and Europe in the early 17th century. At first, leather was manufactured in America entirely for local demand by villagers who specialized in the art of tanning. The date of the establishment of the first tannery in North America is not certain, but from records of Plymouth, it is believed that the first tanners were Micah Richmond and Deacon Crumby of Plymouth. Experience Mitchell, a tanner, came to Plymouth in the good ship Ann in 1623 and established a tannery at Joppa, where "tanbark" was in plentiful supply. Here, he carried on an extensive business for 60 years. Subsequently, the firm of Experience Mitchell and Sons maintained the business for another 170 years.

The first tannery recorded in New Jersey was at Elizabethtown in 1660. It is believed to be the first tannery to manufacture leather for other than local needs. By the early part of the 18th century, Pennsylvania began to export leather to Europe. Practically every town north of Virginia had developed its own tannery, and the manufacture and working of leather was an integral part of life in every town. Adams[5] pictures an early tannery in Quincy, Massachusetts, as follows:

> "The early tanneries were strange primitive establishments. The vats were oblong boxes, sunken in the ground, close to the edge of the town brook at the point where it crossed the main street. They were without either covers or outlets. The beamhouse was an open shed, within which old, wornout horses circulated around while the bark was crushed at the rate of half a cord or so a day by alternate wooden and stone wheels, moving in a circular trough 15 feet in diameter."

The principal material used for tanning in New England and in New York during this period was hemlock bark along with small amounts of spruce bark. There are no records of conifer barks being used in England at this time, but small amounts of spruce (*Picea abies*) were used locally in Central Europe. In the central and Southern colonies, oak bark was the principal material used, but the southern States tanned only a part of their leather and bought the remainder from the north.

At the beginning of the 18th century, more than 200 tanneries were in operation in the Colonies. By 1750, more than a thousand tanneries had been established. Most were operated in conjunction with shoemaking shops, but somewhere near the time of the Revolution, the crafts of tanning and shoemaking were separated. By the end of the 18th century, there were 2,500 tanneries, but most of these continued to be small. Tanning was still a handicraft, the methods being basically the same as those used by the ancient Romans.

HEMLOCK TANNING IN NEW YORK AND PENNSYLVANIA

The real beginning of leather manufacture as a major industry in the United States dates back to various improvements that began in 1803 in Massachusetts.[4] These included the substitution of water power for manual labor in the softening and cleansing of hides before tanning, grinding of bark, pumping of tan liquor from one vat to another, and rolling and smoothing of leather. Controlling the use of lime for hair removal, application of heat to the leaching process, introduction of steam-power, and the employment of various machines for splitting, shaving, graining, and

finishing leather were soon to follow. By 1829, for example, over 36,000 sides of sole leather were tanned in just one tannery in the town of Hunter (Greene County), New York. By 1852, there were 6,263 tanneries in the United States, a third of them in Pennsylvania and New York. The total value of leather produced was 33 million dollars, a sum more than twice that of the value of leather produced in the major European industrial nations.

In a careful documentation of the role of technology in the history of forests of the Upper Delaware Valley, McGregor[6] has shown that tanning expanded "on a truly grand scale" during and after the late 1840's. Tanners used steam in connection with water power. Approximately 100 acres of land was consumed each year by a tannery. Eastern hemlock (*Tsuga canadensis*) bark was stripped from trees, and the logs were left in the forest to rot. Approximately 5 percent of the hemlock forest in the Upper Delaware Valley was destroyed between the years of 1845 and 1855 alone by this activity.

The operation of the Pratt tannery in Prattsville, N.Y., in 1850 is described in detail[7] and illustrates the usage of bark mentioned above. Six thousand tons of bark was consumed annually, requiring the destruction of at least 500 acres of land. The tannery was a large wooden building, 535 feet long, 43 feet wide, and two and one-half stories high. Associated with this were 300 vats tanning over 60,000 hides a year. A 40 x 80-foot building extended over the stream and contained 12 leaching vats for the production of "ooze" (i.e., water containing tannin). The tanning process was started by pressing water out of depilated skins and placing them in a vat with weak hemlock bark extract. After a certain amount of time, the skins were again pressed and returned to a vat containing stronger extract. After five or six treatments, the skins were ready for treatment with the strongest extract (i.e., ooze), where they were left for 3 or 4 months. Overall production time was just under 6 months per hide compared to 8-10 months in English tanneries.

Approximately 60 men were employed full time at this tannery, with pay averaging about $14 per month. The number of hides received at the tannery was 30,000 per year at a cost of $3 per hide, and 60,000 sides were produced (each hide produces two sides) at a selling price of slightly more than $13. The business was very profitable by 1845 standards.

After the Civil War, tanning was transformed from a handicraft to mass production. In 1870, almost 19 million hides and skins were tanned in 7,569 tanneries with an invested capital of $61 million. The effect of mechanical power and machine technique can be shown by the reduction of the number of tanneries to 741 in 1914, although the number of workers had increased from 35,000 to 56,000 and the capital invested to $331 million. Pennsylvania was the most important production State, largely because it had the most abundant eastern hemlock forest resource and was close to major East Coast markets.

The first amalgamation of the leather industry took place in 1892 with the formation of the United States Leather Company. This company incorporated most of the tanneries in Pennsylvania, producing 60 percent of the total sole leather tanned at this time, and was capitalized at $125 million. It was then the largest corporation in the United States and remained among the top ten until World War I.

Scientific research in the leather industry came upon the scene in 1887 with the employment of the first leather chemist. A method was perfected for doubling the output of sole leather for each pound of bark. In 1890, Proctor developed control of leather plumpness through his studies on the physical chemistry of proteins. Enzymes were discovered in 1898, which gradually did away with the need for the use of animal excrement in the bating process, the most obnoxious part of the tannery. In spite of these developments, almost all tanneries in Pennsylvania, New York, and farther west continued to harvest hemlock exclusively for bark, leaving the wood in the forests to rot. After the turn of the century, some of the wood was used for fuel, but there was never any such thing as the integration currently practiced in the wood products industry wherein byproducts of lumber manufacture are chipped and used for pulp production. Part of the reason for the waste of hemlock wood until 1900-1910 was the lack of suitable technology for converting it into marketable lumber.

COMPETITION FROM EXTRACTS

Two important developments for the future of North American tanning took place in the latter part of the 19th century. The first of these was the commercial introduction of chrome tanning in 1887. It was very rapid and could substantially reduce the investment in inventory for a given rate of production. More than 75 percent of all upper leather manufactured in the United States by the end of the 19th century was produced by the chrome process. A second development was the introduction of new and improved techniques for extracting tannins from bark or wood and concentrating them. This permitted tanneries to be located at sites far removed from the source of bark.[8] Thus, hides could be tanned in Texas and shipped overseas from the Gulf Coast ports, or they could be processed near the Chicago stockyards and manufactured into shoes, etc., for sale in the Midwest.

Hemlock extraction plants[9] began to appear after 1890. Bark was harvested in the late spring or early summer and then allowed to dry in stocks or piles for at least 6 months after peeling. The dried bark was ground and leached contercurrently in open vats or pressure autoclaves. The extract was then concentrated to about 25 percent solids for domestic shipment in tank cars or dried to a powder in a vacuum rotary drier. Standard hide powder analysis indicated 55 percent catechol tannins on an ovendry basis.

Meanwhile, the new extraction technology was being applied to chestnut domestically and to quebracho in Argentina and Paraguay and wattle in Natal. By the early 1950's, worldwide production of these three extracts amounted to 300,000 tons annually, about a third of which was exported to the United States.

The manufacture of quebracho extract from the heartwood of *Schinopsis balansae* and *S. lorentzii* began in 1889. By 1924, two dozen factories were operating with capacities of 250 to 2,500 tons of solid extract per month and a total annual output of 150,000 to 200,000 tons. The heartwood contains up to 25 percent tannin and yields a product with 80-85 percent tannin content. Leather tanned with quebracho has a yellow-brown color compared to the red color obtained with hemlock. By the late 1940's, quebracho was the predominant tannin used in the United States.

The development of the American chestnut extract industry took place rapidly at the turn of this century. Production reached approximately 140,000 tons annually and chestnut extract constituted two-thirds of all the tannin used in the United States by 1920. The tannins were mainly found in the heartwood and gave an extract with a very low nontannin content. Over-exploitation and the Oriental chestnut disease caused a rapid decline in chestnut tannin production beginning in the 1930's.

Wattle extract is derived primarily from the bark of black wattle (*Acacia mollissima*), a tree native to Australia but now extensively cultivated in the province of Natal in South Africa. Originally, the bark was shipped to England for extraction, but domestic production in South Africa began during World War I. A typical commercial extract contains about 75 percent tannin on a dry basis. Well over a half million acres are planted to wattle in South Africa. The trees are harvested on a short rotation basis, the bark being used for tannin extraction and the wood for dissolving pulp manufacture. For this reason, wattle tannin stands the best chance of being produced in the future, assuming that there is adequate demand for it.

CHEMICAL EXTRACTION OF WESTERN HEMLOCK

Meanwhile, the use of hemlock tannin declined to the point where it was less than 1 percent of the tannin used in the United States following World War II. Because of U.S. dependence upon imported tannins, major research programs were carried out by the U.S. Department of Agriculture at the Eastern Regional Research Laboratory in Philadelphia for two decades following World War II. This work was directed toward revival of eastern hemlock utilization as well as that of western hemlock, Douglas-fir, and Sitka spruce sawmill waste.[11] Rayonier Incorporated (now ITT Rayonier, Inc.) initiated research programs in 1950 to utilize waste western hemlock (*Tsuga heterophylla*) bark, which was a byproduct of their sulfite pulp and sawmills in the State of Washington and the province of British Columbia.

Most of the hemlock logs used at the Northwest mills had been transported and/or stored in salt water. This resulted in the leaching of part of the tannins from the bark and made it uneconomical to isolate tannin by simple hot water extraction as practiced on eastern hemlock by tanneries in the early part of the century. Rayonier scientists (primarily Lloyd VanBlaricom, Kenneth Gray, and John Steinberg) found that it was possible to obtain up to a third of the weight of the starting material as a water soluble extract by treatment of barks with aqueous sodium sulfite or bisulfite at temperatures above 125 °C. The extract was dark crimson red in color, and the tannins were assumed to be sulfonated. Leather tanning trials in the laboratory and at a commercial tannery were not particularly successful. The leather produced from the sulfonated western hemlock extract was not well plumped, had a hard surface, and was much too red-colored to compete with leather tanned with quabracho or wattle extracts.

Fortunately for Rayonier, exploration and drilling for oil were growing at a very rapid pace in the early 1950's. The sulfonated hemlock tannins were eminently suited for viscosity reduction of oil-well drilling fluids, or "drilling mud", as it is more commonly known.

Following the building of a 5-ton-per-day pilot plant at Hoquiam, Washington, in 1953 by Rayonier, a full-size manufacturing plant was built the next year in Vancouver, British Columbia, at a Rayonier Canada hemlock sawmill (Figure 1). Production from this plant was supplemented by the building of a second major facility adjacent to Rayonier's dissolving pulp mill in Hoquiam in the late 1960's. A group of research chemists and engineers, numbering between 20 to 45 people at any one time, was assembled at the company's research facilities in Shelton, Washington; Vancouver, British Columbia; and Whippany, New Jersey. Complete facilities for drilling mud testing, concrete admixture studies, particleboard and plywood adhesive research, greenhouses for trace metal applications, etc., were provided, as well as routine control testing at the manufacturing plants. The major products manufactured from western hemlock bark are shown in Table 1.

The Rayflo and Rayflo-C extracts were made by aqueous sodium sulfite/bisulfite extraction at 135 °C in a continuous horizontal extractor.[12–14] The extracts were clarified, concentrated in vacuum evaporators, spray-dried, and bagged for shipment

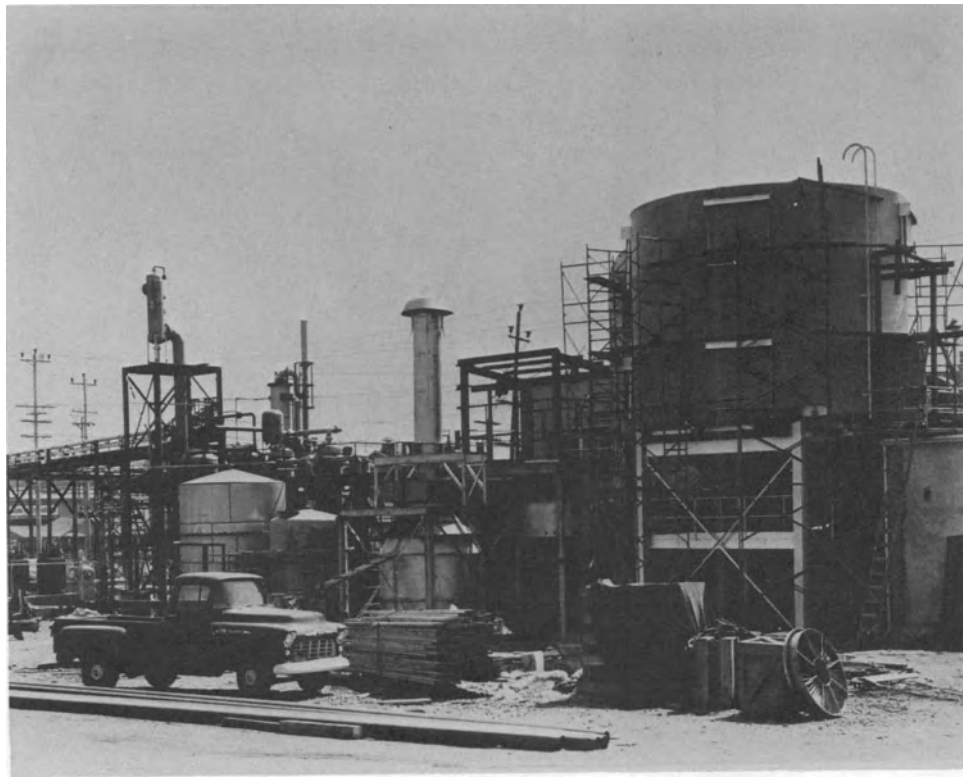

Figure 1. *Western hemlock bark extraction plant operated by Rayonier Canada Ltd. in Vancouver, British Columbia.*

Table 1. Commercial Hemlock Bark Extracts.[a]

Rayflo Drilling Mud Additives
Rayflo-C Boiler and Cooling Water Treatment
Rayplex Metal Complexes (Zn, Fe, Cu, Mn)
HT 115 and HT 193 Resin Intermediates
Terranier Chemical Grouting System

[a] Manufactured by ITT Rayonier Inc. at Hoquiam, Washington, and Rayonier Canada at Vancouver, B.C.

in 50-pound to carload lots. Rayplex metal complexes were made by reacting sulfonated extract with water-soluble zinc, iron, copper, or manganese salts and were primarily used for treating trace metal deficiencies in agricultural crops. HT 115 and HT 193 were resin intermediates prepared by ammonium hydroxide extraction of bark followed by displacement of the amonium ion by sodium or direct extraction of bark with sodium hydroxide.[15,16] Alkaline (unsulfonated) extracts were also found to be useful for chemical grouting ("Terranier").[17] A water solution of the bark extract was mixed with a complexing salt such as sodium dichromate or ferrous sulfate and with formaldehyde. The reaction mixture was then injected into porous soils or gravel to form an immobile gel, which added water impermeability and strength to the grouted structure.

Many other types of chemical extractions were investigated in Rayonier laboratories, some of which are described in the more than 20 patents issued to Rayonier covering its work on preparation and utility of bark extracts. Kraft liquor (sodium sulfide and sodium hydroxide), for example, was found to be suitable for preparation of an extract containing tannins and lignin from the bark in very high yields, up to 60 percent on a net basis. However, only the products listed in Table 1 found commercial acceptance.

Initially, the primary competition for the drilling mud additives was imported sulfited quebracho extract. Eventually, a more serious threat was chrome-complexed ligninsulfonates, so much so that the plant at Hoquiam was totally converted to the production of ligninsulfonate-derived materials. The plant at Vancouver, B.C., was faced with declining sales in the water treatment business, competition from EDTA and by NTA for metal agricultural additives as well as the need for substantial additional capital investment for effluent treatment. Reluctantly, Rayonier shut the doors of the Vancouver plant in 1975, thus ending, for all practical purposes, the chemical utilization of hemlock bark in North America.

STRUCTURE OF HEMLOCK AND SPRUCE TANNINS

Western Hemlock

When the utilization of western hemlock bark was begun by Rayonier, practically nothing was known about the chemical structure of western hemlock bark tannins other than they were "catecholic" (i.e., they gave a green color with ferric chloride solution). Chromatographic techniques, separation, and identification were in their infancy, and structural investigation of natural polymers, such as the con-

densed tannins, was dependent upon tedious and often imprecise functional group analyses because methods had not yet been found to cleave the polymers into their individual monomeric units. Against this backdrop, the author was employed by Rayonier in 1954 to provide a basic chemical understanding of the chemistry involved in the products, which were already in the process of becoming a commercial success. Rayonier officials coined the term "silvichemicals" to describe these chemicals from the forest; optimism for future growth was so high that programs were launched to explore production of high-temperature extracts from a whole range of coniferous bark species, especially those growing on the company's land in Georgia and Florida (i.e., slash and longleaf pine).

Since elegant separation techniques such as GPC or HPLC did not exist at that time, chemical investigation was primarily dependent upon chemical and spectral analyses of fractions isolated from bark by solvents of increasing polarity (Table 2). This approach to bark chemistry had been developed over the previous 5-year period, 1949-1954, by Ervin F. Kurth and his coworkers at the Oregon Forest Products Laboratory on the Oregon State University campus in Corvallis. Kurth's approach was modified and expanded at the Rayonier research laboratory in Shelton, Washington. Two early discoveries of particular importance were two-dimensional paper chromatography for separation and identification of the complex bark polyphenols, which were usually mixtures of 50 or more compounds,[18,19] and acidolytic cleavage of lignin with water at 170 °C, or refluxing with dioxane-hydrochloric acid[20] to give a series of guaiacyl monomers that clearly distinguished the bark lignins from the tannin polyphenols (for a summary of this work, see reference 21).

Table 2. Substances Extractable from Western Hemlock Bark with Solvents of Varying Polarity.

Substance	Percent in Bark	Solubility in Solvents A-E[a]				
		A	B	C	D	E
Wax	2.8		1		1	2
Flavonoids	3.6	3	3	3	4	4
Condensed Tannin	12.6	13	13	13	13	13
Phlobaphenes	5.3		5	3	5	5
Sugars, Gum, Ash	4.9	4	4	3	4	5
Phenolic Acid	16.0			14	16	16
Suberin	2.5				2	2
Lignin	15.0			2	3	12
Hemicellulose	17.7				7	12
Cellulose	19.5	20	26	38	55	73

[a] Solvents: A. Water, 100 °C; B. Acetone-water-sodium bisulfite (3:1:0.5), reflux; C. Sodium bisulfite, 0.10 SO_2 to bark, 150 °C.; D. 1% Sodium hydroxide, 100 °C.; E. Sodium hydroxide - sodium sulfide (1:1), 0.1 Na_2O to bark, 165 °C.

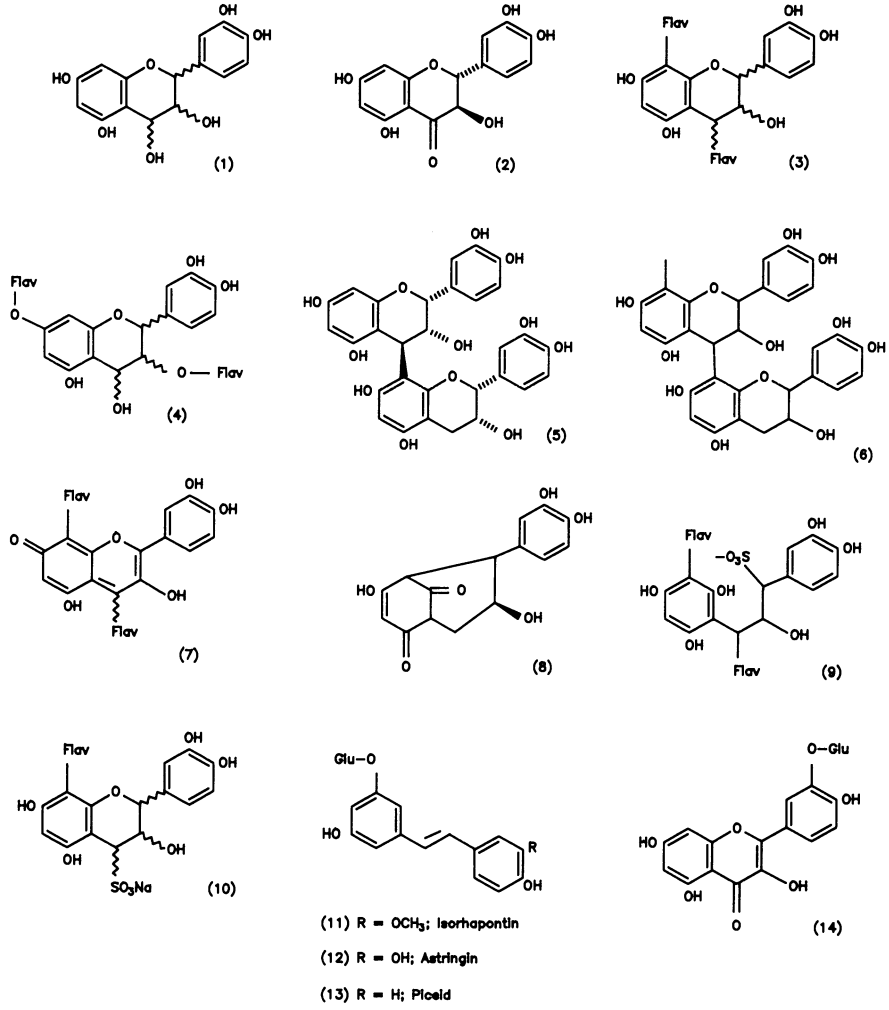

Figure 2. *Structures of products obtained from analysis of extracts from hemlock and spruce barks.*

Hot water extraction of whole bark gave a tannin extract with a "purity" (i.e., content of tannin absorbed on hide powder) of 55-65 percent depending upon the freshness and quality of the bark. Solvent fractionation of a tannin extract yielded a polymeric, pale red-colored solid as about 50 percent of the extract and an ethyl acetate-soluble fraction containing catechin and epicatechin, small amounts of gallocatechin, epigallocatechin, afzelechin, and epiafzelechin, and four compounds that yielded cyanidin upon acid hydrolysis. The latter were initially believed to be enantiomers of leucocyanidol (**1**), but attempts to reduce taxifolin (**2**, R = H) by sodium borohydride, lithium aluminum hydride, or palladized charcoal-catalyzed

hydrogenation failed to yield a stable form of (**1**). The reduction products condensed to form polymers containing one less mole of water per monomeric unit than (**1**) and were believed to have structure (**3**) on the basis of functional group analyses.

The synthetic polymers still yielded cyanidin upon acid hydrolysis as did the polymeric hemlock tannin, and the infrared spectra were very similar but not superimposable. Functional group analyses invariably gave phenolic contents too low for structure (**3**), so polymeric structure (**4**) was assigned to the natural polymers.[20,22] This structure was also preferred because it offered an explanation for the ready sulfonation of the molecule that would occur on the benzylic alcohol group in the 4-position. Subsequently, the late T.A. Geissman of the University of California at Los Angeles reported an extremely important piece of work insofar as condensed tannin chemistry is concerned. In 1965-1966, he proved that both natural and synthetic proanthocyanidin dimers involve two flavan-derived units joined in a carbon-carbon linkage.[23,24]

Geissman's work prompted the author to re-explore the structure of hemlock tannin and the cyanidin-yielding "monomers" in aqueous and organic solvent extract of the bark. One of these "monomers" was isolated in relatively pure form and found to be a biflavan, based on the molecular weight of a peracetylated derivative. Mild acid hydrolysis gave epicatechin (the lower unit in (**5**) and strong acid yielded cyanidin from the upper unit. Proton NMR spectroscopy showed the same chemical shifts for the aliphatic acetoxyl groups in either an acetate derivative or a derivative in which the phenolic groups in either an acetate derivative or a derivative in which the phenolic groups were methylated and the aliphatic hydroxyl groups acetylated. Model compound studies had shown sufficiently different shifts of the 3-acetoxyl group in the 2,3-*cis* and *trans* flavanols, so that splitting occurred in biflavans with mixed conformations at C-3 in the two units. Based on this evidence, the biflavan was assigned the structure (**5**)[25] [i.e., epicatechin-(4$\beta \rightarrow$ 8)-epicatechin or procyanidin B-2 in more recent parlance]. Stereochemistry at the 4-position of the leucoyanidin group and establishment of whether the carbon-carbon linkage was 4 $\rightarrow$ 8 or 4 $\rightarrow$ 6 could not be deduced from the PMR spectra at that time as is now possible with high resolution ^{13}C-NMR. Based on chromatographic Rf values and cleavage products, the other three related compounds were identified as procyanidins B1 [epicatechin-(4$\beta \rightarrow$ 8)-catechin], B3 [catechin-(4$\alpha \rightarrow$ 8)-catechin], and B4 [catechin-(4$\alpha \rightarrow$ 8)-epicatechin].

PMR spectra of the whole tannin polymer fraction isolated by salt precipitation from a hot water extract showed it to be contaminated with a methoxylated lignin-like polymer. Chromatographic separation of the mixed polymers was not successful, but treatment of the acetylated mixture in acetone or ethyl acetate with cyclohexylamine resulted in the precipitation of the amine salt of this lignin (for a description of the significance of this technique, see reference 26). The purified tannin was methylated, acetylated, etc., and the PMR spectra gave the correct ratio of A-ring to C-ring protons and phenolic OH to aliphatic OHs for structure (**3**). The chain extension units seemed to have equivalent amounts of 2,3-*cis* and 2,3-*trans* stereochemistry, based on the aliphatic acetoxyl PMR shifts. Mild acid hydrolysis of the purified polymeric tannin gave epicatechin, catechin, afzelechin,

and epiafzelechin in a ratio of about 5:1:0.5:0:1. Alkaline fusion yielded a mixture of phloroglucinol, protocatechuic acid, and pyrocatechol as the predominant products as well as small amounts of gallic acid, pyrogallol, *para*-hydroxybenzoic acid, and phenol. The yield ratio of these compounds suggested that the tannin monomer units were procyanidins, propelargonidins, and prodelphinidins in the ratio of 40:2:1 mainly terminated with epicatechin (**6**) and lesser amounts of catechin, afzelechin, and epiafzelechin, as already noted.

The number average molecular weight of the acetate derivative by vapor pressure osmometry was 2560, indicative of an average D.P. of five units. Gel permeation chromatogrphy on polystyrene[27] indicated a D.P. range of 2 to 8.

In the late 1960's, Rayonier research chemists K.D. Sears and R.L. Casebier made a major discovery that greatly assisted structural studies of condensed tannins.[28,29] They found a method to cleave the tannin into its monomeric units without disturbing the configuration of these units by cleavage of tannins with boiling thioglycolic acid followed by esterification to give methoxy-flavan-4-ylthio acetates. Their results indicated that the hemlock tannin was composed internally of equivalent amounts of 2,3-*cis* and *trans* leucocyanidin units and chemically confirmed the carbon-carbon linkages between them deduced from the NMR spectra. It is interesting to note that the infrared spectrum of purified hemlock tannin determined in Rayonier laboratories more than 30 years ago falls exactly in Foo's[30] Class B, a polymer mainly of the procyanidin type with monomers having both *cis* and *trans* configurations (i.e., the monomer units being of almost equal mixtures of the catechin and epicatechin type).

No further work has been published on the structure of the water-soluble tannins and flavanoids of western hemlock bark, since the bark research work was concluded at Rayonier in 1973. Thus, the many new tannin elucidation techniques developed by Hemingway, Karchesy, Porter, and others in the present decade still wait to be applied to western hemlock. Samejima and Yoshimoto[31] have, however, used some of the newer techniques to investigate the proanthocyanidins from *Tsuga sieboldii*, a Japanese species of hemlock. They found 0.2 percent catechin and epicatechin in a ratio of 35:65, and 0.3 percent dimeric proanthocyanidins with B1, B2, B3, and B4 being present in the ratio of 17:53:8:22. The polymeric proanthocyanidins had a molecular weight range of 1,000-7,800 with a Mn of 2,270 and a number average D.P. of 7.8. Only leucocyanidin units were present, the extension units being 11:89 and the termination units 44:56 2,3-*trans* to *cis* units, respectively. These results are remarkably similar to our work, especially given the differences in techniques used and the fact that two different *Tsuga* species were involved.

The organic solvent-soluble, hot water-insoluble fraction (termed "phlobaphene" in Table 2), was also reexamined in some detail in the late 1960's.[25,32] The ultraviolet and visible spectrum was nearly identical to cyanidin, but various chromatographic systems indicated it to be a mixture of polymers. Treatment of the acetate derivative with cyclohexyl-amine gave a brown-colored precipitate with 9 percent methoxyl content and an infrared spectrum similar to Braun's native lignin.[23] The unprecipitated, intensely red-colored fraction was concluded to be a polymer of cyanidin (**7**) based on the PMR spectra of derivatives, functional group analyses, and alkaline fusion products. Unfortunately, the experimental evidence was

not clearcut; there appears to be a possibility that the polycyanidin was covalently-linked to procyanidin units or to some guaicylic polymers. The work on this fraction needs to be repeated using more up-to-date chromatographic substrates to establish whether the phlobaphene fraction is a mixture of individual lignin and polycyanidin polymers or is a lignin-procyanidin-cyanidin copolymer.

The largest phenolic fraction of the whole bark is the mixture of polymers solublized by alkaline or high temperature sulfite extraction following organic solvent and hot water extraction (i.e., the so-called "phenolic acids"). Model experiments by the author in the late 1950's[34] had shown that alkaline treatment of bark tannins and phlobaphenes introduced an acidic group with an infrared absorption band at 1720cm^{-1} and presumed to be a carboxyl group. It was, therefore, concluded[22] that the carboxyl group was an artifact introduced into the phenolic acid fraction during isolation, and that this fraction prior to isolation only differed from the tannin in greater molecular size or a three-dimensional network. Sobolev[35] showed that hemlock tannins treated with dilute sodium hydroxide lost their formaldehyde reactivity associated with the phloroglucinol group, so it was evident that an alkaline rearrangement was taking place and that it involved the phloroglucinol group. Sobolev also showed that similar treatment of catechin was a good model for this reaction, since catechin readily rearranged to form catechinic acid. Attempts to determine the structure of the rearrangement product were only partly successful. Another effort was mounted in the early 1970's to determine the structure of the rearrangement product. It was established to be (**8**) through chemical and spectral methods and confirmed by x-ray crystallography.[36] This suggested that the polymeric bark phenolic acids contain an enolic hydroxyl rather than a carboxyl group as previously postulated. Work in 1987 by Laks and Hemingway on southern pine bark procyanidins[37] rearranged by alkali further supports this suggestion.

Treatment of extractive-free hemlock bark with sodium sulfite at 150 °C or 1 percent sodium hydroxide at 100 °C resulted in extracts that did not differ in their infrared spectra from similarly treated, purified hemlock tannins.[25,32] Dilute acid hydrolysis of the extractive-free bark gave mainly catechin along with much smaller amounts of afzelechin, phloroglucinol, protocatechuic acid, and *para*-hydroxybenzoic acid. Strong acid hydrolysis gave cyanidin and pelargonidin in a ratio of 9:1 along with a trace of delphinidin. Alkaline fusion gave phloroglucinol and protocatechnic acid along with traces of gallic acid, pyrogallol, catechol, homoprotocatechuic acid, and *para*-hydroxybenzoic acid. Hydrolysis with thioglycolic acid[29] gave a mixture of the same flavan derivatives obtained from the tannin except that the ratio of *cis* and *trans* isomers was 3:1. All this work indicated the solvent-insoluble polyphenolic polymers in the bark were closely similar in structure to the bark tannins (**3**), but not identical insofar as stereochemistry is concerned. This must mean that these polymers are, at least in part, biosynthesized independently from the tannins.

With regard to the sulfonation of the hemlock procyanidin polymers, model experiments on isolated, purified tannin treated under conditions used to prepare the commercial products showed that molecular weight was reduced very little (i.e., 10-15 percent at the most).[27] This indicated that the extraction of the "phenolic acid" fraction from the bark was primarily accomplished by solubilization through

sulfonation rather than cleavage of the polymers. Careful analysis following dialysis to remove sugars and unreacted sodium bisulfite or sulfite showed that the DP_n averaged about 6 and that there were slightly more than 2 flavan units per sulfonate group. The sulfonation of catechin was studied as a model reaction;[38] ring opening followed by sulfonation of the 2-carbon atom was verified. This suggested that the sulfonated polymers had (**9**) as their main structure. Wherever cleavage had taken place, it was likely that the sulfonate group entered the 4-position (**10**). Complexation with metals in the Rayplex series of products was primarily with the catecholic phenolic groups and secondarily with the sulfonate group based on color reaction experiments.

Sitka Spruce

The bark supply for the Rayonier silvichemical plants would occasionally contain up to 10 percent Sitka spruce (*Picea sitchensis*), so a minor investigation was made on the chemistry of the polyphenols from this source.[8] A hot water extract could be obtained in very good yield, up to 30 percent. Leather made from spruce tannin had a pleasing yellow-brown color, but the leather tended to be poorly plumped and quite harsh to the touch. This was a result of 20-25 percent of the extract consisting of substituted stilbene glucosides, which have been identified[8,38,39] as isorhapontin (**11**), astringin (**12**), piceid (**13**), and the corresponding aglycones present in the outer bark only: isorhapontigenin, astrigenin, and resveratrol. Co-occurring with these compounds were quercetin-3′-glucoside (**14**), taxifolin glucoside (**2**, R = glucose), catechin, gallocatechin, and biflavans B1 and B3. Acid hydrolysis of the polymeric tannin fraction from inner bark gave mainly catechin with traces of gallocatechin as the terminal units where the chain extender was procyanidin, prodelphinidin, and propelargonidin (20:1:1) in approximately equal 2,3-*cis* and *trans* configurations. Tannins from the outer bark were more complex in that they were admixed with lignin-like polymers (which gave coniferyl alcohol, coniferylaldehyde, and other guaiacylic derivations on hydrolysis) and seemed to also have end-units composed of the aglycones of stilbenes (**11**) and (**12**) and taxifolin (**2**, R = H).

Alkaline fusion of extractive-free outer bark gave resorcinol and resorcylic acid as well as the usual tannin degradation products: phloroglucinol, protocatechuic acid, and pyrocatechol. Dilute acid hydrolysis gave (**2**), the aglycones of (**11**), (**12**), and (**13**), catechin, and gallocatechin. This, coupled with summative analyses, strongly indicates that when outer bark is formed, some of the inner bark solvent-soluble tannins are converted to an insoluble form. Furthermore, additional procyanidins are formed along with considerable quantities of solvent-soluble and insoluble lignin. The procyanidins formed at the inner-outer bark boundary do not necessarily have the same stereochemistry as the inner bark procyanidins, and they incorporate a wider variety of chain termination units such as taxifolin, stilbenes, or even phloroglucinol. Biosynthesis of phenolic polymers upon conversion of the phloem (inner bark) to rhytodome (outer bark) was first reported by the author in 1976,[26] but it seems to have received little attention since that time. Proper studies of bark extractives and cell wall constituents should carefully distinguish between the various physiological parts of the bark (inner bark, cork, etc.). Much

of our chemical studies of bark constituents at Rayonier during the early years were difficult to interpret because we did not make this distinction (nor, incidentally, did most other chemists studying bark constituents at that time.)

Eastern Hemlock

During the preparation of this chapter, we belatedly recognized that there were no reports on the chemistry of eastern hemlock tannins in the literature in spite of the tremendous historical importance of this material. We intend to rectify this situation in the near future. In the meantime, we have conducted TLC chromatographic experiments on inner and outer bark extracts. Inner bark contains basically the same constituents as western hemlock (i.e., catechin, epicatechin, and the four biflavans, B1-B4, with B2 and B4 predominating). Surprisingly, the stilbene glucosides, (**12**) and (**13**), are also present, but in very much lower quantities than spruce. We wondered if these compounds had been missed during our earlier studies of western hemlock, so we re-examined fresh samples of western hemlock bark. We were unable to detect any stilbenes. Generally, when a genus contains stilbenes, they can be detected in all the species within that genus, so this preliminary finding will require additional study.

WHAT OF THE FUTURE?

Relatively large quantitites of clean, fresh eastern and western hemlock bark and spruce bark are available for utilization at inland pulp- and sawmills in the United States and Canada (coastal mills still tend to transport or impound their logs, rendering the bark marginally unsuitable for utilization). Since the shutdown of the ITT Rayonier bark extraction plants in 1974, there has been no attempt in North America to revive bark tannin extraction. Swan and coworkers[40] at the Vancouver Forest Products Laboratory (now Forintek Canada Corporation) initiated a research program in the late 1970's to revive interest in these polymers, but apparently they were not successful. The question may well be asked as to what further research needs to be done to encourage commercial isolation and utilization of our North American bark tannins. This brief summary certainly gives evidence that there are still many details concerning structure, etc., of the hemlock tannins that remain to be elucidated. And while much more is known about southern pine bark tannins, largely thanks to the work of Hemingway, Karchesy, Porter, and their colleagues at the Southern Forest Experiment Station, Oregon State University, and the Chemistry Division, DSIR in New Zealand, respectively, there is still much more information that might be gathered.

It is this author's opinion that, based on 35 years of research and executive experience with forest products utilization, additional structural studies are not the route to utilization. (This is not to say that such studies are not valuable. They are and will stand on their own merits). Rather, work directed toward better and more economical extraction methods seems to be needed. Future utilization is most likely confined to the resorcinolic activity of these materials for cold-setting resins and the like. Ammonia, caustic, or sulfite extraction suffers from the molecular rearrangements that are induced. Organic solvent or water extracts are generally

produced in too low a yield to be economic. Perhaps the answer lies in some combination of mechanical and chemical extraction processes. This challenge is thrown out to my fellow researchers not to discourage chemical structural studies, for surely they will continue to be needed, but to encourage more work on economic isolation techniques of the tannins.

REFERENCES

1. Hergert, H.L. The tannin extraction industry in the United States. *J. Forest History* 27(2):92 (1983).
2. Bailey, D.G.; Buechler, P.R.; Everett, A.L.; Feairheller, S.H. Leather. *In*: Kirk-Othmer Concise Encyclopedia of Chemical Technology. John Wiley and Sons, New York, pp. 694-5 (1985).
3. Watson, M.A. Economics of Cattlehide Leather Tanning. Rumpf Publishing Co., Chicago, pp. 1-28 (1950).
4. Mofit, C. The Arts of Tanning, Currying and Leather-Dressing. Henry Carry Baird, Philadelphia, 557 pp. (1852).
5. Adams, C.F. Three Episodes in Massachusetts History. Boston. Vol. II, Episode 2, p. 929 (1892).
6. McGregor, R.K. Changing technologies and forest consumption in the upper Delaware Valley, 1790-1880. *J. Forest History* 32:69 (1988).
7. Mofit, C. The Arts of Tanning, Currying and Leather-Dressing. Henry Carry Baird, Philadelphia, pp. 320-337 (1852).
8. Anonymous. Modern American Tanning: A Practical Treatise on the Manufacture of Leather. Jacobsen Publishing Co., Chicago. 292 pp. (1902).
9. Harvey, A. Tanning Materials with Notes on Tanning Extract Manufacture. Chemical Pub. Co., New York. 192 pp. (1921).
10. Howes, F.N. Vegetable Tanning Materials. Buttersworth Scientific Publications, London. 297 pp. (1953).
11. Roger, N.F.; Griffin, E.L.; Redfield, C.S.; Koepp, W.H. Paper chips and bark from hemlock slabs by air flotation. *For. Prod. J.* 5:400 (1955).
12. Herrick, F.W.; Hergert, H.L. Utilization of Chemicals from Wood: Retrospect and Prospect. *In*: Loewus, F.A.; Runeckles, V.C. (eds.) Recent Advances in Phytochemistry. p. 141 (1977).
13. Herrick, F.W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food Chem.* 28:228 (1980).
14. Hergert, H.L., Van Blaricom, L.E.; Steinburg, J.C.; Gray, K.R. Isolation and properties of dispersants from western hemlock bark. *For. Prod. J.* 15:485 (1965).
15. Herrick, F.W.; Bock, L.H. Thermosetting, exterior-plywood type adhesives from bark extracts. *For. Prod. J.* 8:269 (1958).
16. Herrick, F.W.; Conca, R.J. The use of bark extracts in cold-setting waterproof adhesives. *For. Prod. J.* 10:361 (1960).
17. Herrick, F.W.; Brandstrom, R.K. U.S. Patent 3,391,542 assigned to Rayonier Inc. (1968).
18. Hergert, H.L. Chemical composition of tannins and polyphenols from conifer wood and bark. *For. Prod. J.* 10:610 (1960).
19. Goldschmid, O.; Hergert, H.L. Examination of western hemlock for lignin precusors. *Tappi* 44:858 (1961).
20. Hergert, H.L. Abstracts of Papers, 131st Meeting, Am. Chem. Soc., 6E (1957); 133d Meeting, 7E (1958).

21. Sarkanen, K.V.; Hergert, H.L. Lignins and Phenolic Polymers in Tree Barks. *In*: Sarkanen, K.V.; Ludwig, C.H. (eds.) Lignins. Wiley-Interscience, New York, 916 p. (1971).

22. Hergert, H.L. The Economic Importance of Flavonoid Compounds: Wood and Bark. *In*: Geissman, T.A. (ed.) The Chemistry of Flavonoid Compounds. Macmillan, New York, 666 pp. (1962).

23. Geissman, T.A.; Dittmar, H.F.K. A proanthocyanidin from avocada seed. *Phytochemistry* 4:359 (1965).

24. Geissman, T.A.; Yoshimura, N.N. Synthetic proanthocyanidin. *Tetrahedron Letters* :2669 (1966).

25. Hergert, H.L. Polyphenols and tannins from hemlock bark. Abstracts of Papers, 155th Am. Chem. Soc. Meeting, 21D (1968).

26. Hergert, H.L. Secondary lignification in conifer trees. *In*: Arthur, J.C. (ed.) Cellulose Chemistry and Technology. ACS Symposium Series 48, Am. Chem. Soc., Washington, DC. p. 227 (1977).

27. Sears, K.D.; Engen, R.J. (unpublished Rayonier research report) (1971).

28. Sears, K.D.; Casbier, R.L. Cleavage of proanthocyanidins with thioglycolic acid. *J. Chem. Soc. Chem. Commun.* :1437 (1968).

29. Sears, K.D.; Casebier R.L. The reaction of thioglycolic acid with polyflavanoid bark fracations of *Tsuga heterophylla*. *Phytochemistry* 9:1589 (1970).

30. Foo, L.H. Proanthocyanidins: Gross chemical structures by infrared spectra. *Phytochemistry* 20:1397 (1981).

31. Samejima, M.; Yoshimoto, T. Systematic studies on the stereochemical composition of proanthocyanidins from coniferous bark. *Mokuzai Gakkaishi* 28:67 (1982).

32. Hergert, H.L. The chemistry and utilization of non-lignin phenolic polymers from conifers. Paper presented to 161st National Am. Chem. Soc. Meeting, Washington, DC. p. 66 CELL (1971).

33. Hergert, H.L. Infrared spectra. *In*: Sarkanen, K.V.; Ludwig, C.H. (eds.) Lignins - Occurrence, Formation, Structure, and Reactions. Wiley-Interscience, New York, p. 267 (1971).

34. Hergert, H.L. Chemical composition of cork from white fir bark. *For. Prod. J.* 8:335 (1958).

35. Sobolev, I. (internal Rayonier reports) (1958-1960).

36. Sears, K.D.; Casebier, R.L.; Hergert, H.L.; Stout, G.H.; McCandish, L.E. The structure of catechinic acid, a base rearrangement product of catechin. *J. Org. Chem.* 39:3244 (1974).

37. Laks, P.E., Hemingway, R.W. Condensed tannins. Structure of the 'phenolic acids.' *Holzforschung* 41:287 (1987).

38. Manners, G.D.; Swan, E.P. Stilbenes in the barks of five Canadian Picea species. *Phytochemistry* 10:607 (1971).

39. Hergert, H.L. (unpublished work) (1966-1970).

40. Frazer, H.S.; Swan, E.P. Phenolic character of sequential solvent extracts from western hemlock and white spruce barks. *Can. J. For. Res.* 9:495 (1979).

Biogenesis

TANNINS – THEIR PLACE IN PLANT METABOLISM

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ABSTRACT

Terrestrial vascular plants synthesize, in addition to structural polymers like cellulose and lignin, a rather bewildering array of metabolic products, such as lignans, phenolic acids, tannins, alkaloids, terpenoids etc. Excluding the structural polymers, the functions of many of these compounds (i.e., so-called secondary metabolites) are not well understood. This chapter presents an overview of the metabolism of phenylpropanoids, particularly hydrolyzable and condensed tannins, and then reexamines these pathways in the context of their relationships to general plant metabolism. Calculations show that the cost of diversion of biochemical energy of living plants into tannins is high compared to energy requirements for the structural cell wall polysaccharides. Hence, in many high-tannin-content plants, tannin synthesis contributes significantly to captured photosynthetic energy usage. Phenylpropanoid (hence, tannin) synthesis is also particularly important to nitrogen recycling.

INTRODUCTION

Since early recorded history, and presumably before that, mankind has sought to unravel the mysteries surrounding living processes. Plant metabolism has been no exception, particularly as regards the significance and function of the rather bewildering array of biochemical metabolites produced. These metabolites have somewhat arbitrarily been classified as either primary or secondary, according to perceived function. This distinction has been rather unfortunate, since initial usage of the term "secondary metabolites" tended to belittle their crucial role in many physiological and ecological functions. However, these perceptions have changed gradually, as our knowledge of the role of secondary metabolites in plant growth

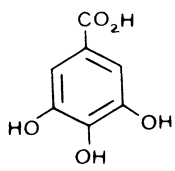
and development, defense strategies, and the like improves. In this respect, two recent informative reviews emphasize the importance of secondary metabolites in general defense strategies,[1] particularly with respect to tannins[2] that were previously viewed by some as simply being waste or extraneous compounds.[3] In terms of function, "condensed" tannins apparently act mainly against the action of microbes,[2] whereas "hydrolyzable tannins" defend against chewing phytophagus insects[2] or animals.[4]

In this chapter, the opportunity has been taken to put condensed tannins in context with other important metabolic pathways operational during normal plant growth and development. To do this, both experimental findings and speculative skills were drawn on, since substantial gaps remain in our knowledge of these complex metabolic routes.

Three areas are examined: (1) the biogenetic pathways leading to condensed and hydrolyzable tannins, (2) the utilization of stored energy (in ATP equivalents) for biosynthesis of condensed tannins and other major metabolic products, and (3) the importance of nitrogen recycling in the biosynthesis of condensed tannins.

BIOSYNTHESIS OF CONDENSED AND HYDROLYZABLE TANNINS

The terms "hydrolyzable" and "condensed" tannins have been used to distinguish between the two important classes of vegetable tannins,[5] namely gallic (**1**) or hexahydroxydiphenic (**2**) acid derived (hydrolyzable) and mainly the flavan-3,4-diol (**3**) derived (condensed) tannins. Although each class is represented in nature by what appears to be an innumerable array of fascinating structural variations, neither term (hydrolyzable or condensed) is very meaningful, since examples of both types can be hydrolytically degraded by acid. Thus, condensed tannins are now more correctly referred to as proanthocyanidins[6] or more broadly as polyflavanoids, and hydrolyzable tannins as gallo- or ellagitannins, or derivatives thereof. At this point, it is pertinent to note that both classes of tannins are derived from the shikimate/chorismate pathway and that some condensed tannins contain gallic acid substituents.



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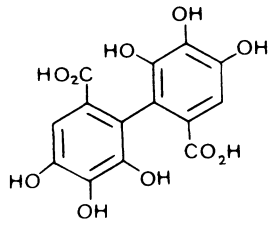

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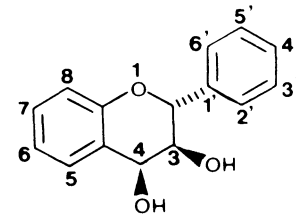

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The Shikimate – Chorismate Pathway

This is a major metabolic pathway operational in plants, fungi, and bacteria. Only essential features will be described, since there are excellent recent compre-

hensive reviews elsewhere.[7–9] Depending upon the organism in question, offshoots of the pathway can lead to both "hydrolyzable" and "condensed" tannins, as well as miscellaneous flavanoids, coumarins, lignins, lignans, hydroxycinnamic acids, aromatic amino acid-derived alkaloids, stilbenes, ubiquinones, plastiquinones, and tocopherols, for example.

The shikimate – chorismate pathway (Figure 1) begins with phosphoenolpyruvate (PEP) (**4**) and erythrose-4-phosphate (E-4-P) (**5**) that are products of the glycolytic and pentose phosphate pathways, respectively. These key intermediates then couple to afford 3-deoxy-*D*-arabinoheptulosonic acid-7-phosphate (DAHP) (**6**) in a reaction catalyzed by DAHP synthase (DS). This synthase can exist in either DS-Mn or DS-Co isozyme forms.[10] DAHP (**6**) is then converted into 3-dehydroshikimate (**8**) via 3-dehydroquinate (**7**). Subsequent conversions afford shikimic acid (**9**), shikimate-3-phosphate (**10**), 5-enolpyruvylshikimate-3-phosphate (**11**), and chorismic acid (**12**).[7–9]

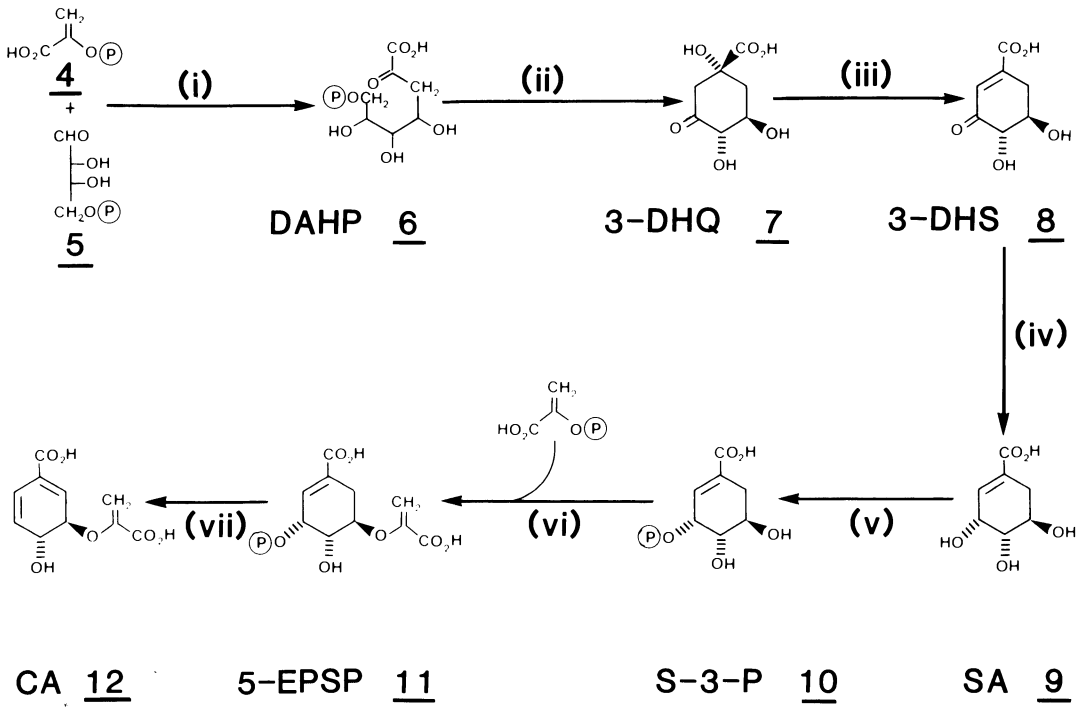
All of these enzymatic steps apparently occur in the plastid compartment of higher plants, as evidenced directly via detection of several of the enzymes, or indirectly from tracer experiments with isolated chloroplasts.[11] Additionally, the cytosol may also contain these enzymes, althougth at low levels of enzymatic activity. To date, however, only DAHP synthase,[11] shikimate dehydrogenase,[12] and perhaps 5-enolpyruvylshikimate-3-phosphate synthase[13] have been detected.

Pathway to *p*-Coumaric Acid via Phenylalanine and Tyrosine

The route affording *p*-coumaric acid (**20**) signalling the beginning of the general phenylpropanoid pathway, is shown in Figure 2. Chorismic acid first undergoes an unusual 3,3′ sigmatropic rearrangement to give prephenic acid (**13**)[7–9] in a reaction catalyzed by chorismate mutase (CM). Again, enzymatic activity has been detected in both plastid and cytosolic compartments for both isozyme forms (CM-1 and CM-2).[14,15]

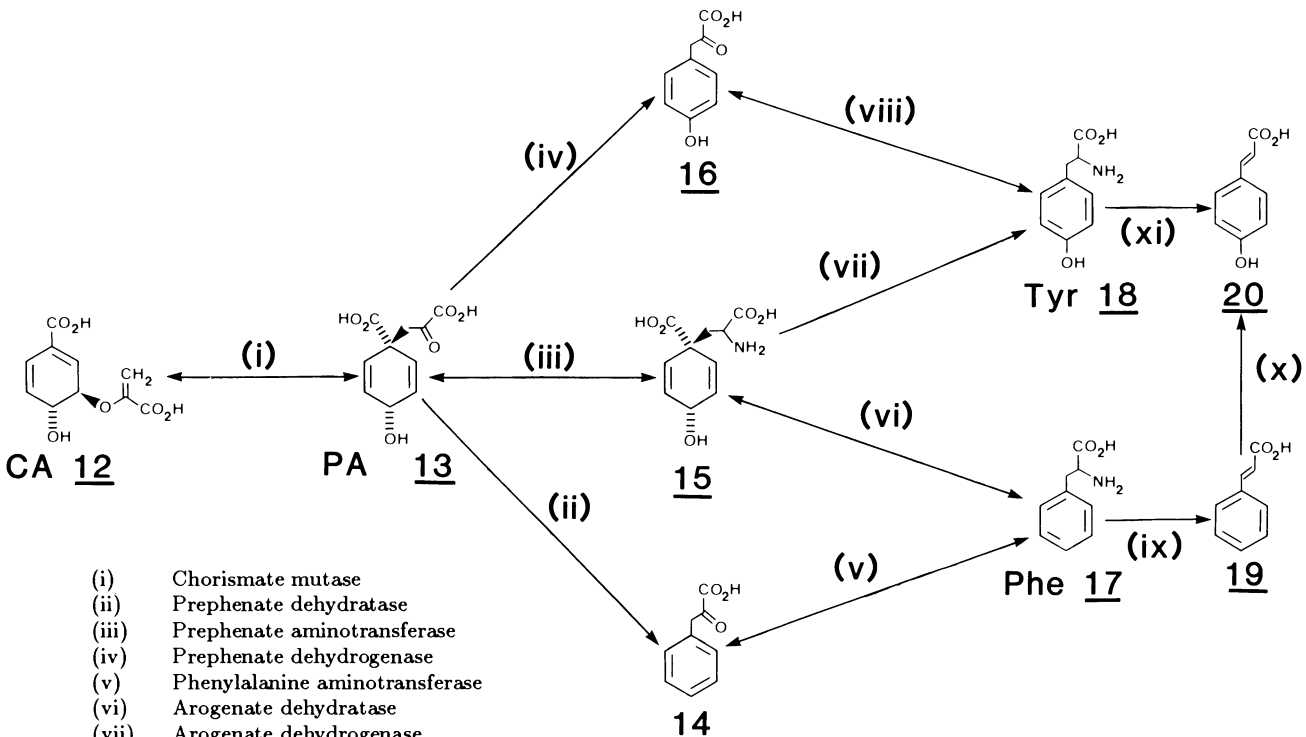
As far as subsequent conversions to phenylalanine (**17**) and tyrosine (**18**) are concerned, there is still some confusion over their formation from prephenic acid. Originally, it was more or less generally accepted that prephenate is converted into phenylalanine or tyrosine via transamination of phenylpyruvate (**14**) and 4-hydroxyphenylpyruvate (**16**), respectively.[16] However, for a variety of herbaceous plants, another pathway to phenylalanine/tyrosine utilizing arogenate (**15**) has been shown to occur (e.g., in tobacco (*Nicotiana sylvestris*) cultures,[17,18] sorghum (*Sorghum bicolor* L),[19,20] corn (*Zea mays*),[21] and spinach.[18,22] Indeed, in these plant species, this may be the sole pathway to phenylalanine and tyrosine. It, therefore, needs to be established as to whether, in woody plants, phenylalanine formation also results from arogenate, or phenylpyruvate, or both.

Beyond these amino acids, deamination affords *p*-coumaric acid either directly or via hydroxylation of cinnamic acid.[19] The importance of this deamination reaction cannot be overemphasized, since, as discussed later, this nitrogen can be reutilized in other primary metabolic processes. On the other hand, the carbon skeleton is essentially irreversibly committed, in an unidirectional manner, to the final product.



(i) 3-Deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP synthase)
(ii) 3-Dehydroquinate synthase
(iii) 3-Dehydroquinate dehydratase
(iv) Shikimate dehydrogenase
(v) Shikimate kinase
(vi) 5-Enolpyruvylshikimate-3-phosphate synthase
(vii) Chorismate synthase

Figure 1. *Biosynthetic pathway to shikimic* (**9**) *and chorismic* (**12**) *acids.*



(i) Chorismate mutase
(ii) Prephenate dehydratase
(iii) Prephenate aminotransferase
(iv) Prephenate dehydrogenase
(v) Phenylalanine aminotransferase
(vi) Arogenate dehydratase
(vii) Arogenate dehydrogenase
(viii) Tyrosine aminotransferase
(ix) Phenylalanine ammonia lyase
(x) Cinnamate-4-hydroxylase
(xi) Tyrosine ammonia lyase

Figure 2. *Biogenetic pathways to p-coumaric acid* (**20**) *from chorismic acid* (**21**).

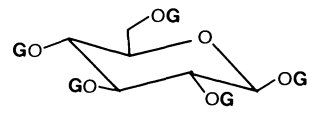
Gallo- and Ellagitannins (Hydrolyzable Tannins)

In contrast to proanthocyanidins (condensed tannins), hydrolyzable tannins are of limited distribution in nature.[23–30] Gallo- and ellagitannins are abundant in leaves, fruits, pods, and galls (and in some cases, wood and bark) of dicots such as oak, chestnut, sumac, and others, but have not been detected in monocots.[31]

Gallotannins. The gallotannins have a polyol core (normally glucose) with pendant esterified gallic acid constituents. The most frequently found member is β-glucopentagallin (**21**). Additional galloyl units can be added (most commonly at C-3) via depside linkages as illustrated in the *meta*-depside ester (**22**).

The biogenetic pathway leading to gallic acid is somewhat enigmatic, and three different pathways have been proposed (Figure 3). In the first, 3-^{14}C [*D,L*] phenylalanine was administered to *Rhus typhina*, and radioactive gallic acid was isolated.[32] It was concluded that this transformation occurred via side-chain cleavage of the postulated intermediate, 3,4,5-trihydroxycinnamic acid (**23**) (Figure 3, route a). However, (**23**) has never been shown to be a natural plant product.

An alternate pathway, based upon labelling experiments with cinnamic (**19**), caffeic (**24**), and protocatechuic (**25**) acids, was also proposed (Figure 3, route b).[33] In that study, 3-^{14}C cinnamic acid was administered to *Gaultheria procumbens* and *Hydrangea macrophylla* and 3-^{14}C caffeic acid to *Orzya sativa*. In all cases, radiolabelled protocatechuic acid was isolated. Next, [^{14}C-carboxy]-protocatechuic acid was administered to *Pelargonium hortorum*, and radiolabelled gallic acid was obtained. It is important to note, however, that the complete pathway (cinnamic → caffeic → protocatechuic → gallic acid) has not been demonstrated in any one plant species.



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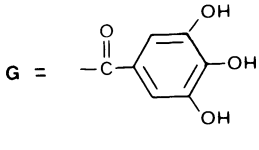

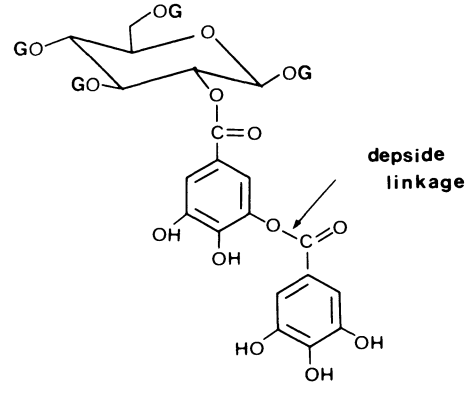

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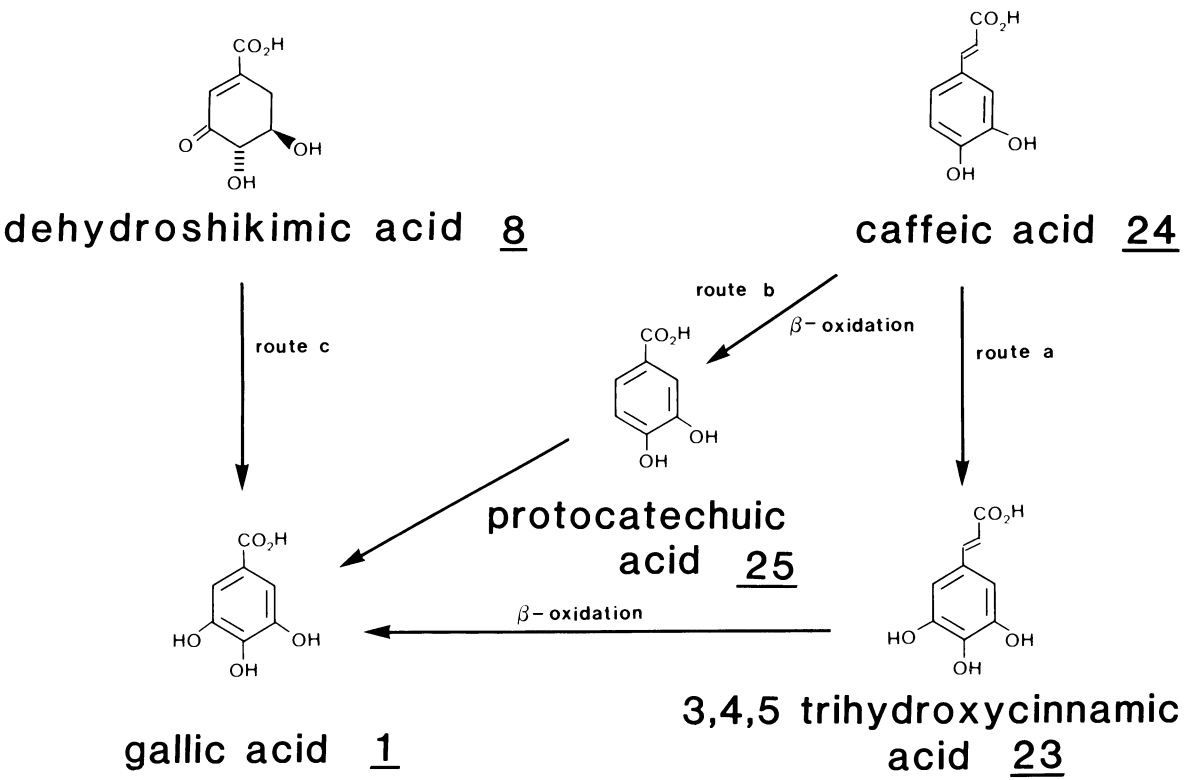
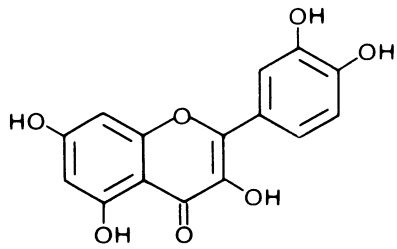


Figure 3. *Proposed biosynthetic routes to gallic acid* (**1**).

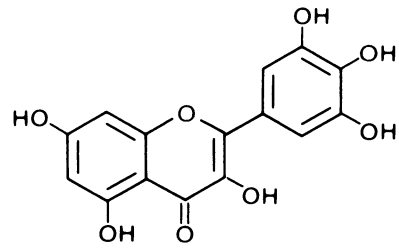
Prior to these studies, Conn and Swain[34] showed that U-^{14}C phenylalanine was efficiently incorporated into both quercetin (**26**) and myricetin (**27**), but not into gallic acid, in *Geranium pyrenaicum*. On the other hand, U-^{14}C glucose significantly radiolabelled gallic acid, and the authors reasoned that it was formed directly from shikimic acid via dehydroshikimic acid (**8**) (Figure 3, route c).

Subsequent studies by Dewick and Haslam[35] demonstrated that [G-^{14}C] shikimic acid was a more efficient precursor of gallic acid in *Rhus typhina* than (U-^{14}C)-L-phenylalanine (0.85 vs. 0.03 percent incorporation). Importantly, degradation of gallic acid, from the phenylalanine feeding experiment, suggested uniform labelling of all seven carbons, indicating intact incorporation. Recent experiments by Saijo[36] confirmed and extended these observations in the tea plant, *Camellia sinensis*, where gallic acid was found esterified to epicatechin (**28**) and epigallocatechin (**29**). Again labelled [G-^{14}C]-shikimic acid was more efficiently incorporated into gallic acid than [U-^{14}C]-phenylalanine (2.5 versus 0.2 percent incorporation). More importantly, however, [^{14}C-CO_2H]-shikimic acid was also incorporated into gallic acid (1.3 percent incorporation). This suggests, but does not prove (without appropriate degradation to establish the position of the radiolabel), that the carboxylic acid group of shikimic acid was retained during the formation of gallic acid.

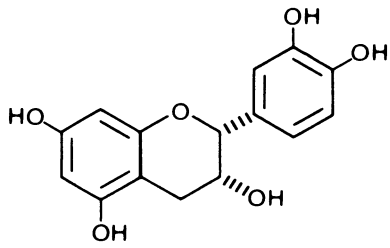
Additional evidence favoring the dominant biogenesis of gallic acid from shikimic acid, and not phenylalanine, was obtained from experiments with *Quercus robur* and *Rhus typhina*.[37] This evidence was obtained by administering ^{14}C-shikimic acid to *Quercus robur* cell suspensions in the presence of *L*-2-aminooxy-3-phenylpropionic



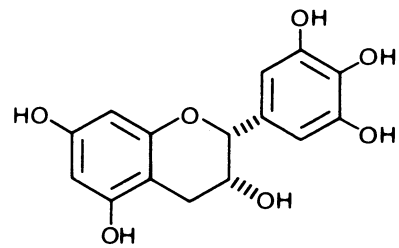
26, QUERCETIN



27, MYRICETIN



28, EPICATECHIN



29, EPIGALLOCATECHIN

acid (AOPP) and N-(phosphonomethyl) glycine (glyphosate), respectively. AOPP is known to inhibit phenylalanine ammonia lyase activity,[38,39] and glyphosate inhibits both 5-enolpyruvylshikimate-3-phosphate synthase[40] and the Co (but not Mn) DAHP isozyme.[11] It was thus demonstrated that glyphosate treatment resulted in a fivefold enhancement of the gallic acid content of *Q. robur*, whereas, AOPP had no noticeable effect. Additionally, $^{14}CO_2$ incorporation into gallic acid in the leaves of *Rhus typhina* was enhanced in the presence of glyphosate, but not AOPP. These experiments would have been more meaningful had parallel experiments been carried out using ^{14}C-phenylalanine to determine the relative importance of the alternate β-oxidation pathway in these plant systems. Nevertheless, the current body of evidence seems to favor gallic acid being formed from shikimate directly.

The subsequent formation of the gallotannins has received considerable attention in recent years. With the use of crude enzyme preparations from tannin-producing oak leaves, it was established that the first enzymatic product was β-*O*-glucogallin (**30**), where gallic acid was activated for transesterification via its

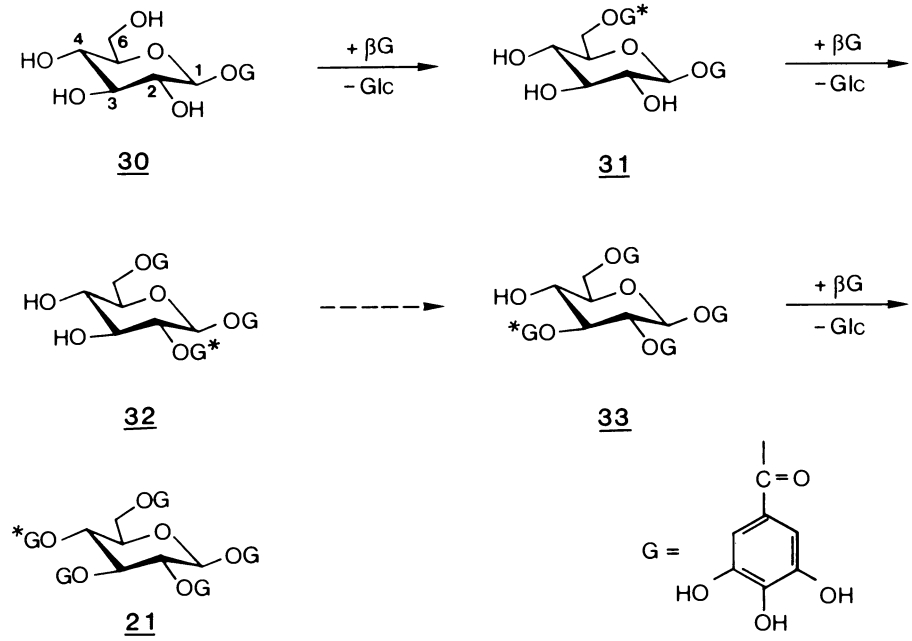


Figure 4. *Biosynthetic pathway to β-glucopentagallin*(**21**). *βG = β-O-glucogallin* (**30**). *Reproduced from Gross, G.G. Enzymology of gallotannin biosynthesis. In: Lewis, N.G.; Paice, M.G., (eds) Plant Cell Wall Polymers: Biogenesis and Biodegradation. ACS Symposium Series (in press) with permission from the American Chemical Society. (The transformation of* (**32**) *to* (**33**) *has not yet been demonstrated with crude enzyme preparations).*

UDP-D-glucose, and not CoA, derivative.[24-26] It was subsequently shown that β-*O*-glucogallin served as both donor and acceptor in the sequential biosynthetic steps leading to β-glucopentagallin (Figure 4).[41-44] Interestingly, it is currently thought that these individual steps are catalyzed by different enzymes.

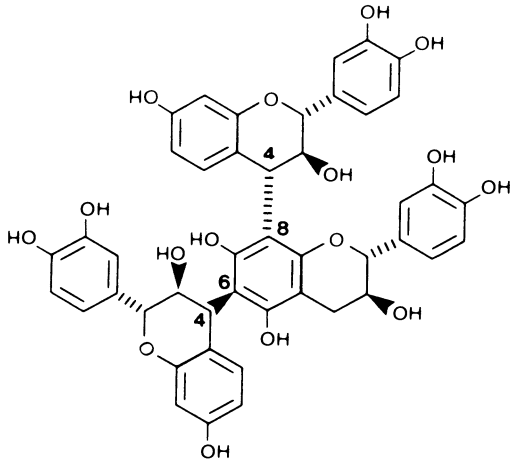
Thus, although significant progress is being made in elucidating the enzyme-mediated steps to β-glucopentagallin, there is still inadequate information regarding formation of the *meta*-depside bonds, as in ester (**22**). In this case, activation of gallic acid may occur via its CoA ester, rather than the glucose conjugate.[44]

Ellagitannins and related structures. The ellagitannins and their derivatives are mainly found in oak, eucalyptus, chestnut, and some *Caesalpina sp.*, and *Terminilia chebula.*[23] To date, nothing is known about the coupling of galloyl residues to yield hexahydroxydiphenic acid. It does need to be established, though, whether there is a specific coupling enzyme involved in this reaction. Beyond hexahydroxydiphenic acid, which gives the dilactone ellagic acid for which these compounds are named, a number of tannins is formed, apparently via fission of the aromatic ring following oxidative and/or reductive processes.[23] The enzymology of these steps, as well as the sequence of intermediates, is also unknown.

Condensed Tannins

The terms proanthocyanidins and polyflavanoids are generic names used to define the more abundant class of tannins, which are widely distributed in the plant kingdom, particularly in the conifers.[23] All of these compounds contain the familiar flavonoid skeleton, linked together in apparently infinite arrangements, depending upon the nature of the interflavonoid linkage, hydroxylation pattern, stereochemistry at carbons 2, 3, and 4 of the pyran ring, as well as the presence of additional substituents, (e.g., ester-linked gallic acid). Hence, well over a hundred oligomeric and polymeric proanthocyanidins with subtle, but well-defined, structural variations have been isolated from many different plant species. These structural variations are illustrated by the four dimeric species (**34**) - (**37**) shown below. For the most part, interflavonoid linkages occur at C-4, C-6, and C-8, but other unusual variations (e.g., at C-2 and C-7) can also occur. Note also the pendant gallic acid residues in structure (**36**) linking the biogenetic pathways between hydrolyzable and condensed tannins. These structural variations are discussed in more detail by Hemingway in Chapter 5 of this volume.

In proanthocyanidins, the flavonoid skeleta can readily be divided into two broad categories, namely, those containing either phloroglucinol or resorcinol (so-called 5-deoxy) substitution patterns (Figure 5). These broad categories can also be further subdivided into individual families depending upon the degree of hydroxylation in either the "B" ring or at C-3. Thus, phloroglucinol "A" ring type proanthocyanidins are represented by the proapigeninidins (**38**), propelargonidins (**39**), proluteolinidins (**40**), procyanidins (**41**), and prodelphinidins (**42**), of which the latter two are the most commonly found in nature (see Chapter 5). Resorcinol "A" ring proanthocyanidins are represented by the proguibourtinidin (**43**), proteracacidin (**44**), profisetinidin (**45**), promelacacidin (**46**), and prorobinetinidin (**47**) families. Of these, only proteracacidins have not been found in plants.



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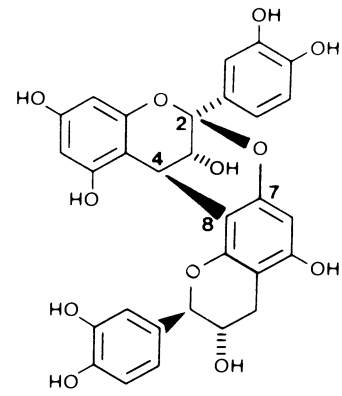

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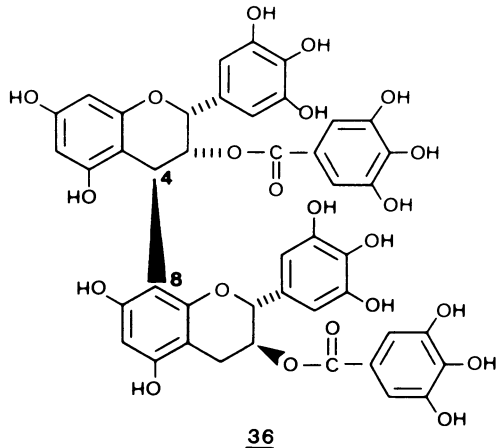

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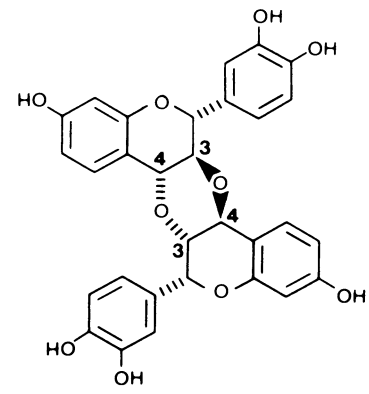

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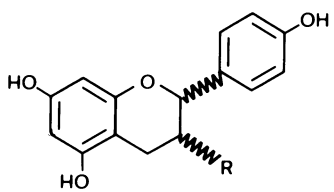
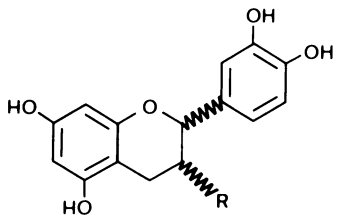
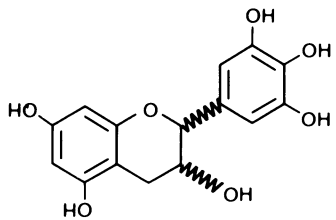
Biogenesis. As previously mentioned, one of the most important distinctions between the various proanthocyanidin groups is the lack (or presence) of an hydroxyl group at C-5 on the "A" ring. Until recently, only the pathways affording proanthocyanidins with phloroglucinol "A" rings have been elucidated and these only partly. The currently accepted biogenesis route is shown in truncated form (Figure 6, pathway a) because it is covered in detail by Stafford in Chapter 3. Thus, three molecules of malonyl-CoA (**48**) and one molecule of *p*-coumaryl CoA (**49**) combine together to form naringenin chalcone (**50**) (6′-hydroxychalcone) in a reaction catalyzed by chalcone synthase.[7] All three hydroxyl groups (on the "A" ring) are derived from the carbonyl oxygen of acetyl CoA, the latter being formed via decarboxylation of malonate. In the presence of chalcone isomerase (CHI), cyclization to afford naringenin (**51**) occurs, which then serves as the precursor of



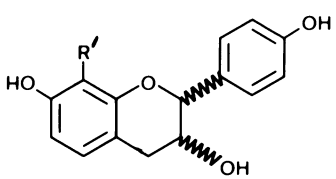
38, R=H, PROAPIGENINIDINS
39, R=OH, PROPELARGONIDINS



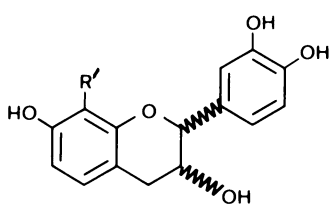
40, R=H, PROLUTEOLINIDINS
41, R=OH, PROCYANIDINS



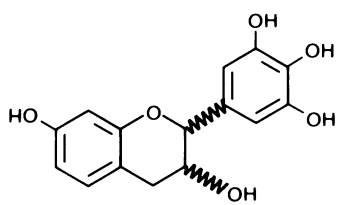
42, PRODELPHINIDINS



43, R'=H, PROGUIBOURTINIDINS
44, R'=OH, PROTERACACIDINS



45, R'=H, PROFISETINIDINS
46, R'=OH, PROMELACACIDINS



47, PROROBINETINIDINS

Figure 5. *Classification of proanthocyanidins according to hydroxylation patterns.*

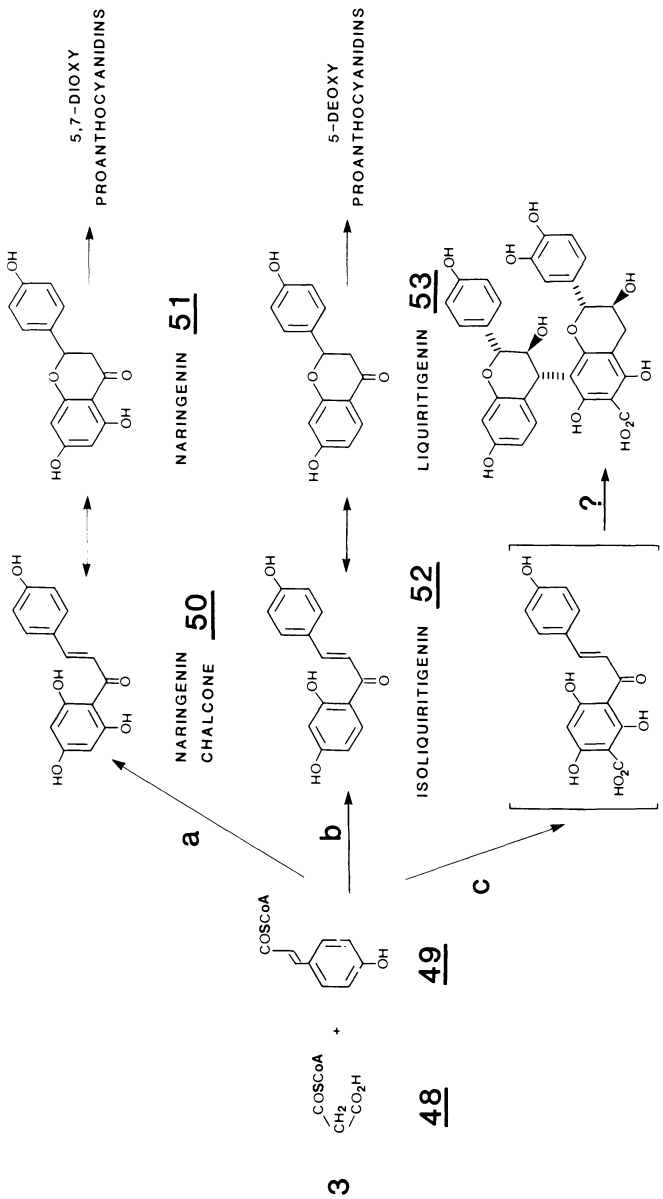


Figure 6. *Proposed biogenetic pathways to 5,7-dihydroxy- and 5-deoxyproanthocyanidins.*

the procyanidins/prodelphinidins (Chapter 3). It is assumed, but not yet proven, that naringenin is converted into the flavan-3,4-diol and that this intermediate is the ultimate precursor of the oligomeric and polymeric chain extender units in procyanidins and prodelphinidins. This is concluded because in vitro experiments demonstrated that proanthocyanidin-like polymers can be obtained on treatment of (**3**) with acid[45] or base.[46]

However, it must be emphasized that such a transformation has not been demonstrated in vivo. Additionally, the sequences of hydroxylation steps, the control of stereochemistry at carbons 2-4, and the various different (but stereochemically controlled) interflavanoid linkages indicate considerable enzymatic control during proanthocyanidin formation. All of these reactions now need to be demonstrated at the cell-free level.

As far as the 5-deoxyproanthocyanidins are concerned, there is a growing body of evidence suggesting that they are formed from liquiritigenin (5-deoxychalcone) (**53**) via isoliquiritigenin (6′-deoxychalcone) (**52**) (Figure 6, route b).[47,48] This was concluded from a study using protoplasts from *Glycyrrhiza echinata* cells.[48] When these were incubated with [^{14}C]-phenylalanine, only radiolabelled liquiritigenin (**53**), and not naringenin (**51**), was formed. However, in vitro chalcone synthase assays produced naringenin, whereas, when high concentrations of NADH (6.3mM) were added, both naringenin and liquiritigenin were produced. As a result, the authors suggested a dual catalytic activity for chalcone synthase where product formation was influenced by the presence or absence of NADPH.[47]

However, Welle and Grisebach[49] have since demonstrated, using highly purified chalcone synthase preparations, that formation of these 5-deoxy analogues is not dependent upon NADPH addition to this enzyme. Instead, a NADPH-dependent reductase is also required, which acts in concert with the chalcone synthase. Interestingly, it was demonstrated (using appropriately labeled ^{3}H **R** and **S** forms of NADPH) that only the pro **R** hydrogen of NADPH was transferred during the stereospecific reduction (Figure 7). Subsequent "A" ring closure and dehydration affords (**52**), which can then be converted into (**53**) by the action of chalcone isomerase.

Finally, the formation of the unusual proguibourtinidin (**55**) in *Acacia luderitzii*, which contains a carboxylic acid group at carbon 6,[50] is also worthy of mention (Figure 6, route c). This is because whether or not the carboxylic acid group(s) are mevalonate derived needs to be established, as well as whether (**54**) is a biosynthetic intermediate. Alternatively, formation of the carboxyl functionality could be via methylation and subsequent oxidation.

UTILIZATION OF ENERGY FOR PROANTHOCYANIDIN BIOGENESIS

It is important to first recall that all living cells (plants included) have three fundamental requirements: energy (ATP), reducing power (NADPH), and starting materials for biosynthesis. To assess the amount of captured photosynthetic energy used for the formation of any given metabolite, Atkinson's[51] method was applied to classify metabolic products (including NADPH) in terms of the number of moles of ATP (**56**) required per mole of product formed. In such cases, the unit of energy

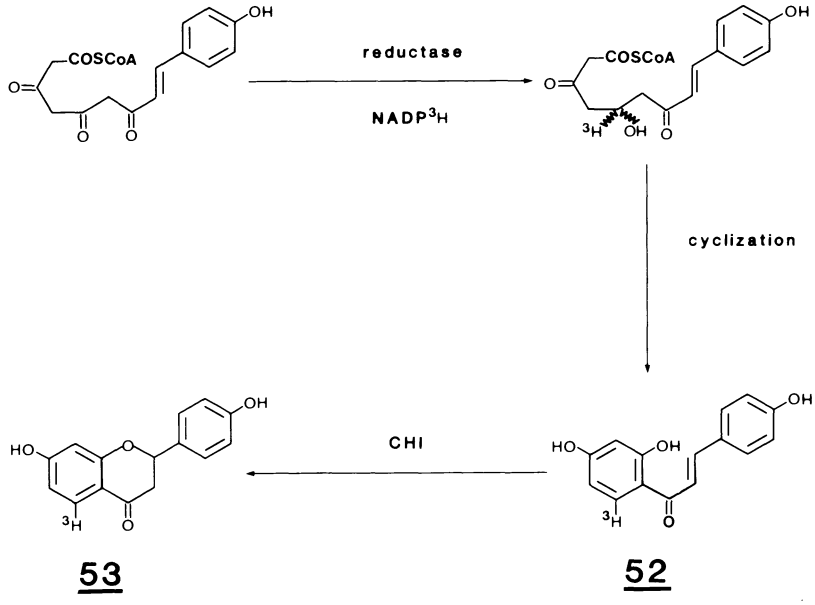
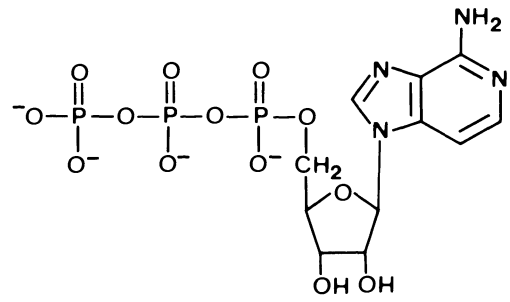


Figure 7. *Proposed mechanism for formation of 5-deoxyproanthocyanidins.*

exchange was defined as an ATP equivalent, with one equivalent being equal to the energy released during the conversion of ATP to ADP. In this analysis, energy requirements have been tabulated in those terms.

Before embarking upon such energy calculations (in ATP equivalents) for proanthocyanidins and other major metabolic products, let us first consider the basic stages of plant growth and development, namely, photosynthesis, catabolism, biosynthesis, and growth (Figure 8). In this regard, we need to draw attention to the functional relationships connecting the processes of catabolism and biosynthesis. As can be seen, these blocks are linked together by the *energy coupling* agents, ATP and NADPH (i.e., catabolism provides the coupling agents for energy exchange needed to fuel the biochemical reactions of biosynthesis).



(56)

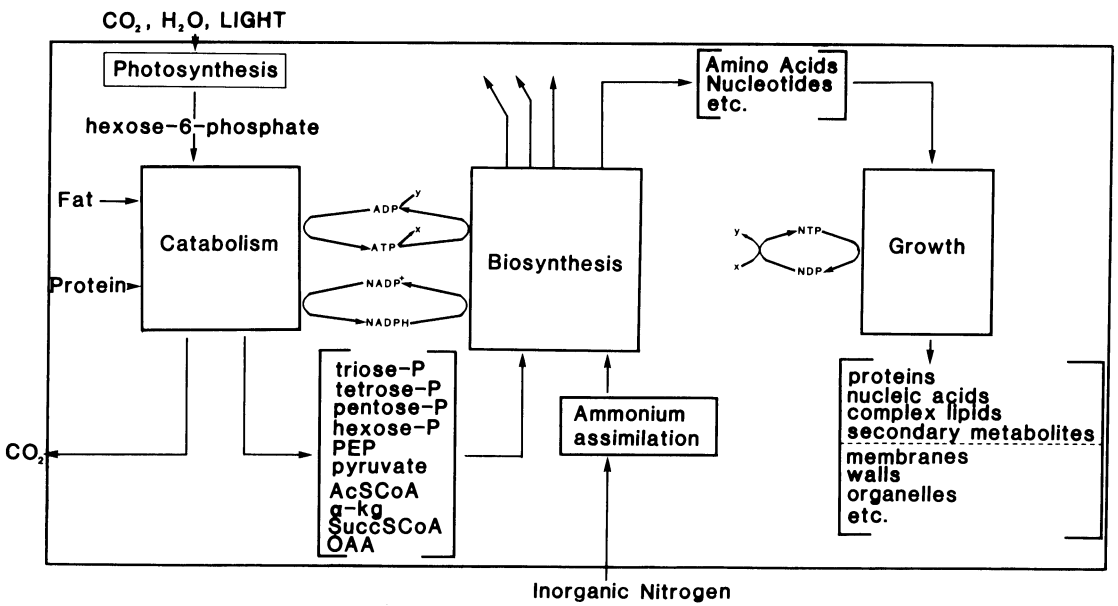


Figure 8. *Schematic block diagram of plant metabolism (modified from Atkinson*[51]*).*
Legend: triose → hexose-P = triose → hexose phosphate;
PEP = phosphoenolpyruvate; AcSCoA = acetyl CoA; α-kg = α-ketoglutarate;
SuccSCoA = succinyl CoA; OAA = oxaloacetic acid.

Additionally, during catabolism, hexose-6-phosphate is converted into 10 starting materials (e.g., triose phosphate and tetrose phosphate) needed as substrates for all subsequent biosynthetic reactions.[51] Atkinson previously calculated prices, in ATP equivalents, for each product of catabolism. The reader is encouraged to read this comprehensive treatment. For our purposes, two examples will suffice: complete combustion of pyruvate in the tricarboxylic acid cycle and electron transport chain affords 15 moles of ATP per mole of pyruvate synthesized; (i.e., pyruvate has a metabolic price of 15 ATP equivalents). Similarly, NADPH can be equated in terms of 4 ATP equivalents.

As regards proanthocyanidin biogenesis, we can begin by examining the ATP energy requirements for phenylalanine formation. As previously noted, this molecule is constructed from erythrose-4-phosphate (26 ATP equivalents) and two molecules of PEP (2x16 ATP equivalents) in a series of enzymatic reactions requiring the following cofactors: one NADPH (4 ATP equivalents), one ATP to convert dehydroshikimate into shikimate-3-phosphate and 5 ATP equivalents for transamination. Hence, the overall cost for phenylalanine biosynthesis is 26 + (16 x 2) + 1 + 4 + 5 = 68 ATP equivalents. Beyond phenylalanine, deamination and hydroxylation to *p*-coumaric acid requires one NADPH (4 ATP equivalents). Subsequent formation of the CoA ester consumes 2 ATP equivalents for the ligase reaction. The *p*-coumaryl-CoA (74 ATP equivalents) formed can then condense with 3 malonyl-CoA molecules (3 x 13 ATP equivalents) to afford naringenin chalcone (113 ATP equivalents). Subsequent conversion to the flavan-3,4-diol (121 ATP equivalents), considered to be the last precursor for proanthocyanidin formation, requires only two moles of NADPH (2 x 4 ATP equivalents) for hydroxylation and reduction. Note, however, that the energy requirements for polymerization cannot be estimated, since this process has not yet been delineated. Thus, proanthocyanidin formation can only be expressed at this point in terms of formation of the flavan-3,4-diol. This value of 121 ATP equivalents can also be described in terms of a specific cost (0.395 ATP equivalents per g of product produced) by dividing by the formula weight (see Table 1).

Table 1. Comparison of Metabolic Costs of Plant Polymer Biosynthesis.

Polymer	Structural Type	Specific Cost (ATP equiv./gram)
Lignin	guaiacyl	0.511
	syringyl	0.466
	p-hydroxyphenyl	0.573
Proanthocyanidin	(flavan-3,4-diol)	0.395
Hydrolyzable Tannin	β-glucopentagallin	0.270
Polysaccharide[a]	cellulose	0.247
Protein	hypothetical[b]	0.385
	wheat leaf	0.354
Lipid	tripalmitin[b]	0.622

[a]Specific costs for other polysaccharides (e.g., hemicelluloses and pectin) do not vary > ± 3% of that of cellulose.

[b]From Atkinson.[51]

In a similar manner, the ATP energy requirements for some of the other plant polymers were also calculated (Table 1), where it was found that only lignin and lipids exceeded the energy cost for proanthocyanidin formation. Lipids, generally found only in small amounts, are a rather special case, since they can be recycled, thus retrieving the captured photosynthetic energy; on the other hand, the energy flow into lignins and proanthocyanidins is apparently unidirectional.

The value of 0.395 ATP equivalents/g for proanthocyanidins is significantly higher than that calculated for the hydrolyzable tannins (0.270 ATP equivalents/g). The latter figure is based upon the energy requirements for the formation of β-glucopentagallin. In this calculation, dehydroshikimate was considered to serve as the direct precursor of gallic acid. As before, in the case of the proanthocyanidins, it was not possible to estimate the requirements for either gallotannin or ellagitannin polymer formation, since these reactions are still not adequately understood.

Another important finding from this analysis is the high cost for both proanthocyanidins and lignins, and which is comparable to that of proteins. This is not too surprising when their formation is considered in terms of phenylalanine requirements, since this amino acid is the second most expensive amino acid for protein synthesis next to tryptophan (81 ATP equivalents) (Table 2). Thus the high ATP equivalents for lignin and proanthocyanidin biogenesis are due to their being mainly derived from phenylalanine, whereas, the lower energy requirements for proteins reflect the low aromatic amino acid content of these biopolymers (Table 3). Recently, the importance of phenylalanine metabolism into phenylpropanoids (such as proanthocyanidins and lignins) was shown by treatment of several herbaceous plants with *L*-2-aminooxy-3-phenylpropionic acid (AOPP),[52] a specific inhibitor of phenylalanine ammonia lyase (PAL).[38,39] This resulted in an up to fortyfold increase in phenylalanine levels, since its subsequent conversion into cinnamic acid was now reduced substantially.

Finally, it is worthwhile mentioning that in some plant species, such as those belonging to the Gramineae, formation of *p*-coumaric acid can occur also from tyrosine via mediation of tyrosine ammonia lyase (TAL).[53] Calculations, as before, gave a value of 65 ATP equivalents for *p*-coumaric acid via this route, and this was some 7 ATP equivalents less than that obtained for its formation via phenylalanine. In spite of its apparent lower cost, the tyrosine/p-coumarate route appears to be restricted only to certain plant families.

Table 2. Relative Costs of Amino Acids in ATP Equivalents.

Group	Cost (ATP equiv./mole)	Amino Acid
I	12-29	Gly, Ser, Cys, Ala, Asp/Asn, Thr, Glu/Gln
II	39-53	Pro, Val, His, Arg, Met, Leu, Lys, Ile
III	65-81	Tyr, Phe, Trp

Table 3. Phenylalanine Requirements for Plant Polymers.

Polymer		g Phe/g Polymer
Protein		0.033 – 0.066
Lignin	guaiacyl	0.915
	syringyl	0.776
	p-hydroxyphenyl	1.099
Proanthocyanidin		0.540

NITROGEN RECYCLING IN PHENYLPROPANOID BIOSYNTHESIS

Since large quantities of proanthocyanidins and lignins are deposited in plant tissues, the products of the phenylalanine-cinnamate pathway serve as a major metabolic sink for assimilated carbon. Because of these deposition processes, essentially all vascular plants have high phenylalanine requirements. It should, therefore, be self-evident that if deamination and nitrogen recycling did not occur, then plants would suffer from nitrogen deficiencies.

It is also worth emphasizing that the carbon:nitrogen balance for other (general) metabolic processes can be regulated to some extent by activation/deactivation of the phenylpropanoid pathway.[53–55] For example, when plant discs were incubated with an exogenously provided carbon source (sucrose or some other sugar), experimental evidence indicated activation of the phenylpropanoid pathway.[53] This was shown for buckwheat (*Fagopyrum esculentum*), which underwent a fiftyfold increase in PAL activity,[53,54] and for the grapevine (*Vitis vinifera* L)[55] by a substantial increase in its anthocyanin production. On the other hand, addition of nitrate to grapevine leaf discs inhibited the anthocyanin "overproduction."

It can thus be argued that activation of the phenylpropanoid pathway is to ensure an adequate supply of available nitrogen (as ammonia) for other metabolic processes. Such nitrogen provision thereby ensures both smooth carbon and energy flow, even under high carbon-to-nitrogen growth conditions. This postulate has further experimental support, since when plants are grown under nitrogen-limiting conditions, they respond by producing more phenolics (presumably phenylpropanoids).[56] For example, *Lotus pedunculatus* only produced flavolans under nitrogen-free conditions but did not when an adequate supply of nitrogen (as ammonium nitrate) was provided.[57] Thus, under conditions of high C:N ratios, further activation of the phenylpropanoid pathway occurs. In this way (i.e., via deamination of phenylalanine), optimum use is made of the plant's available nitrogen.

CONCLUSIONS

Figure 9 schematically illustrates the metabolic relationship between condensed tannins (proanthocyanidins) and the other major plant biopolymers; the diagram is modified from that presented by of Minamikawa and Yoshida.[58] As can be seen,

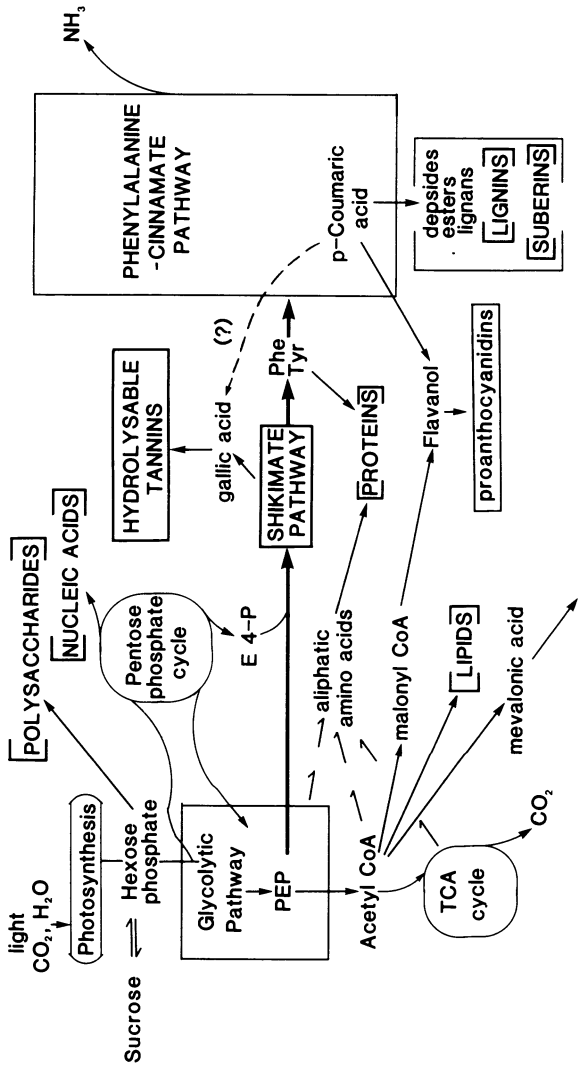


Figure 9. *Metabolic relationship between proanthocyanidins and other biopolymers. (Modified from Minamikawa and Yoshida.)*[58]

proanthocyanidins are products of the general phenylpropanoid and polyketide (malonyl-CoA) pathways. As previously mentioned, their involvement in general phenylpropanoid metabolism results in proanthocyanidins being classified as one of the most expensive major plant metabolites next to lignin and proteins (i.e., that their formation contributes significantly to the utilization of captured photosynthetic energy). Finally, the phenylpropanoid pathway, leading to products such as proanthocyanidins, and the like, also plays an important role in other general metabolic processes. Activation of the pathway helps to maintain inorganic nitrogen availability, under either nitrogen-limiting or high organic carbon conditions.

ACKNOWLEDGMENTS

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REFERENCES

1. Wink, M. Plant breeding: Importance of secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* 75:225 (1988).
2. Zucker, W.V. Does structure determine function? An ecological perspective. *Am. Nat.* 121:335 (1983).
3. Stewart, C.M. Excretion and heartwood formation in living trees. *Science* 153:1068 (1966).
4. Itakura, Y.; Habermehl, G.; Mebs, D. Tannins occurring in the toxic Brazilian plant *Thiloa glaucocarpa*. *Toxicon* 25(12):1291 (1987).
5. Freudenberg, K. *Die Chemie der Naturlischen Gerbstoffe*. Springer-Verlag (1920).
6. Weinges, K.; Marb, H.-D.; Goritz, K. Die rotationsbehinderung-an der $C(sp^2)$-$C(sp^3)$ bindung der 4-arylsubstituierten polymethoxy-flavane. *Chem. Ber.* 103:2336 (1970).
7. Dewick, P.M. The biosynthesis of shikimate metabolites. *Nat. Prod. Rep.* 2:495 (1985).
8. Ganem, B. From glucose to aromatics – recent developments in natural products of the shikimic acid pathway. *Tetrahedron* 34:3353 (1978).
9. Floss, H.G. The shikimate pathway - an overview. *In*: Conn, E.E. (ed.). Recent Advances in Phytochemistry **20**. Plenum Press pp. 13-56 (1986).
10. Rubin, J.L.; Jensen, R.A. Differentially regulated isozymes of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase from seedlings of *Vigna radiata* [L] Wilczek. *Plant Physiol.* 79:711 (1985).
11. Jensen, R.A.; Morris, P.; Bonner, C.; Zamir, L.L. Biochemical interface between aromatic amino acid biosynthesis and secondary metabolism. *In*: Lewis, N.G.; Paice, M.G. (eds.) Plant Cell Wall Polymers: Biogenesis and Biodegradation. ACS Symposium Series 1989 (in press).
12. Mousdale, D.M.; Campbell, M.S.; Coggens, J.R. Purification and characterization of bifunctional dehydroquinase - shikimate = NADP oxidoreductase from pea seedlings. *Phytochemistry* 26:2665 (1987).
13. Ream, J.E.; Steinrucken, H.C.; Porter, C.A.; Sikorski, J.A. Purification and properties of 5-enolpyruvylshikimate-3-phosphate synthase from dark-grown seedlings of *Sorghum bicolor*. *Plant Physiol.* 87:232 (1988).
14. Singh, B.K.; Connelly, J.A. Chorismate mutase isozymes from *Sorghum bicolor*: Purification and properties. *Arch. Biochem. Biophys.* 243:374 (1985).

15. d'Amato, T.A.; Ganson, R.; Jensen, R.A. Subcellular localization of chorismate-mutase isozymes in protoplasts from mesophyll and suspension-cultured cells of *Nicotiana sylvestris*. *Planta* 162:104 (1984).

16. Shimada, M. Biochemical studies on bamboo lignin and methoxylation in hardwood and softwood lignins. *Wood Res.* 53:19 (1972).

17. Gaines, C.G.; Byng, G.S.; Whitaker, R.J.; Jensen, R.A. L-Tyrosine regulation and biosynthesis via arogenate dehydrogenase in suspension-cultured cells of *Nicotiana sylvestris* Speg et Comes. *Planta* 156:233 (1982).

18. Jung, E.; Zamir, L.O.; Jensen, R.A. Chloroplasts of higher plants synthesize L-phenylalanine via L-arogenate. *Proc. Nat. Acad. Sci. USA* 83:7231 (1986).

19. Connelly, J.A.; Conn, E. E. Tyrosine biosynthesis in *Sorghum bicolor*: isolation and regulatory properties of arogenate dehydrogenase. *Z. Naturforsch. Biosci.* 41:69 (1986).

20. Siehl, D.L.; Connelly, J.A.; Conn, E.E. Tyrosine biosynthesis in *Sorghum bicolor*: Characteristics of prephenate aminotransferase. *Z. Naturforsch. Biosci.* 41:79 (1986).

21. Byng, G.; Whitaker, R.; Flick, C.; Jensen, R.A. Enzymology of L-tyrosine biosynthesis in corn (*Zea mays*). *Phytochemistry* 20:1289 (1981).

22. Jensen, R.A. Tyrosine and phenylalanine biosynthesis: Relationships between alternative pathways, regulation and subcellular location in the shikimic acid pathway. *Recent Adv. Phytochem.* 20:57 (1985).

23. Hillis, W.E. Biosynthesis of tannins. *In*: Higuchi, T. (ed.) Biosynthesis and Biodegradation of Wood Components. Academic Press, pp. 325-347. (1985).

24. Haddock, E.A.; Gupta, R.K.; Al-Shafi, S.M.K.; Layden, K.; Haslam, E.; Magnolato, D. The metabolism of gallic acid and hexahydroxydiphenic acid in plants: biogenetic and molecular taxonomy considerations. *Phytochemistry* 21:1049 (1982).

25. Haslam, E. The metabolism of gallic acid and hexahydroxydiphenic acid in higher plants. *Fortschr. Chem. Org. Naturst.* 41:1 (1982).

26. Haddock, E.A.; Gupta, R.K.; Haslam, E. The metabolism of gallic acid and hexahydroxydiphenic acids in plants. Part 3. Esters of (**R**)- and (**S**)-hexahydroxydiphenic acids and dehydrohexahydroxy acid with D-glucopyranose. *J. Chem. Soc. Perkin Trans. 1* :2535 (1982).

27. Gupta, R.K.; Al-Shafi, S.M.K.; Layden, K.; Haslam, E. The metabolism of gallic acid and hexahydroxydiphenic acid in plants. Part 2. Esters of (**S**)-hexahydroxydiphenic acid with D-glucopyranose ($^4C_1$). *J. Chem. Soc. Perkin Trans. 1* :2525 (1982).

28. Haddock, E.A.; Gupta, R.K.; Al-Shafi, S.M.K.; Haslam, E. The metabolism of gallic acid and hexahydroxydiphenic acid in plants. Part 1. Introduction. Naturally occurring galloyl esters. *J. Chem. Soc. Perkin Trans. 1* :2515 (1982).

29. Haslam, E. Vegetable tannins. *In*: Conn, E.E. (ed.) The Biochemistry of Plants. Vol. 7. Secondary Plant Products. Academic Press, New York, pp 527-56 (1981).

30. Haslam, E. Vegetable tannins. *Rec. Adv. Phytochem.* 12:475 (1979).

31. McMillan, C. The condensed tannins (proanthocyanidins) in seagrasses. *Aq. Bot.* 20:351 (1984).

32. Zenk, M.H. Zur frage der biosynthese von gallussaure. *Z. Naturforsch.* 196:83 (1964).

33. El-Basyouni, S.Z.; Chen, D.; Ibrahim, R.K.; Neish, A.C.; Towers G.H.N. The biosynthesis of hydroxybenzoic acids in higher plants. *Phytochemistry* 3:485 (1964).

34. Conn, E.E.; Swain, T. Biosynthesis of gallic acid in higher plants. *Chem. Ind.* :592 (1961).

35. Dewick, P.M.; Haslam, E. Phenol biosynthesis in higher plants. Gallic acid. *Biochem. J.* 113:537 (1969).

36. Saijo, R. Pathway of gallic acid biosynthesis and its esterification with catechins in young tea shoots. *Agric. Biol. Chem.* 47(3):455 (1983).

37. Amrhein, N.; Topp, H.; Joop, O. The pathway of gallic acid biosynthesis in higher plants. *Plant Physiol. Suppl.* 75:18 (1984).

38. Amrhein, N.; Frank, G.; Leming, G.; Luhmann, H.-B. Inhibition of lignin formation by L-α-aminooxy-β-phenylpropionic acid, an inhibitor of phenylalanine ammonia lyase. *Eur. J. Cell. Biol.* 29:139 (1983).

39. Smart, C.C.; Amrhein, N. The influence of lignification on the development of vascular tissue in *Vigna radiata* L. *Protoplasma* 124:87 (1985).

40. Rubin, J.L.; Gaines, C.G.; Jensen, R.A. Glyphosate inhibition of 5-einolpyruvylshikimate-3-phosphate synthase from suspension cultured cells of *Nicotiana sylvestris*. *Plant Physiol.* 75:839 (1984).

41. Gross, G.G. Synthesis of mono-, di-, and trigalloyl-β-D-glucose by β-glucogallin dependent galloyltransferases from oak leaves. *Z. Naturforsch.* 38c:519 (1983).

42. Gross, G.G.; Schmidt, S.W.; Denzel, K.J. β-glucogullin dependent acyltransferase from oak leaves. 1. Partial purification and characterisation. *J. Plant Physiol.* 126:173 (1986).

43. Schmidt, S.W.; Denzel, K.; Schilling, G.; Gross, G.G. Enzymatic synthesis of 1,6-digalloylglucose from β-glucogallin: β-glucogallin 6-*O*-galloyltransferase from oak leaves. *Z. Naturforsch.* 42c:87 (1987).

44. Gross, G.G. Enzymology of gallotannin biosynthesis. *In*: Lewis, N.G.; Paice, M.G. (eds.) Plant Cell Wall Polymers: Biogenesis and Biodegradation. ACS Symposium Series 1989 (in press).

45. Delcour, J.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 9. The condensation sequence of leucocyanidin with (+)-catechin and with the resultant procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1711 (1983).

46. Hemingway, R.W.; Laks, P.E. Condensed tannins: A proposed route to 2**R**, 3**R** (2,3-*cis*)-proanthocyanidins. *J. Chem. Soc. Chem. Commun.* :746 (1985).

47. Ayabe, S.; Udagawa, A.; Furuya, T. NAD(P)H-dependent 6′-deoxychalcone synthase activity in *Glycyrrhiza echinata* cells induced by yeast extract. *Arch. Biochem. Biophys.* 261(2):458 (1988).

48. Ayabe, S.; Udagawa, A.; Furuya, T. Stimulation of chalcone synthase activity by yeast extract in cultured *Glycyrrhiza echinata* cells and 5-deoxyflavone formation by isolated protoplasts. *Plant Cell Reports* 7:35 (1988).

49. Welle, R.; Grisebach, H. Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6′-deoxychalcone. *FEBS Lett.* 236(1):221 (1988).

50. duPreez, I.C.; Rowan, A.C.; Roux, D.G. A biflavonoid proanthocyanidin carboxylic acid and related biflavonoids from *Acacia luederitzii* Engl. var *retinens* (Sim.) J. Ross + Brenan. *J. Chem. Soc. Chem. Commun.* :492 (1970).

51. Atkinson, D.E. Functional stoichiometric coupling and metabolic prices. *In*: Cellular Energy Metabolism and its Regulation. Academic Press, New York, pp. 31-83 (1977).

52. Laber, B.; Kiltz, H.-H.; Amrhein, N. Inhibition of phenylalanine ammonia lyase *in vitro* and *in vivo* by (1-amino-2-phenylethyl) phosphonic acid, the phosphonic analogue of phenylalanine. *Z. Naturforsch.* 41c:49 (1986).

53. Camm, E.L.; Towers, G.H.N. Phenylalanine ammonia lyase. *Phytochemistry* 12:961 (1973).

54. Amrhein, N.; Zenk, M.H.. Untersuchungen zur rolle der phenylalanin-ammonium-lyase (PAL) bei der regulation der flavonoidesynthese in buchweitzen (*Fagopyrum esculentum* Moench). *Z. Pflanzenphysiol.* 64:145 (1971).

55. Pirie, A.; Mullins, M.G. Changes in anthocyanin and phenolics content of grapevine leaf and fruit tissues treated with sucrose, nitrate and abscisic acid. *Plant Physiol.* 58:468 (1976).

56. Graham, R.D. Effects of nutrient stress on susceptibility of plants to disease with particular reference to the trace elements. *In*: Woolhouse, H.W., (ed.), Advances in Botanical Research. Academic Press, New York, Vol. 10, pp. 221-276 (1983).

57. Pankhurst, C.E.; Jones, W.T. Effectiveness of *Lotus* root nodules III. Effect of combined nitrogen on nodule effectiveness and flavolan synthesis in plant roots. *J. Exp. Bot.* 30:1109 (1979).

58. Minamikawa, T.; Yoshida, S. Kotoshokubutsu no nizitaisha Kenkyuho. (In Japanese) Gakkaishuppan Center, Tokyo (1980).

THE ENZYMOLOGY OF PROANTHOCYANIDIN BIOSYNTHESIS

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ABSTRACT

The cell-free enzymology of the biosynthesis of the 2,3-*trans* forms of flavan-3-ols and the oligomeric 2,3-*trans* proanthocyanidins is now known except for the final condensation step to the oligomers. The individual enzymic steps at the C-15 level are described. A speculative model to explore the origin of the still unknown 2,3-*cis* pathway and its relationship to the 2,3-*trans* pathway is presented. This model accounts for the radioactive phenylalanine feeding experiments published by various laboratories. The pathway is also discussed in terms of the regulation of proanthocyanidin biosynthesis in gymnosperms, especially in Douglas-fir.

INTRODUCTION

The word tannin was used in the 1800's to designate unknown compounds isolated from the tan oak galls that were employed in the tanning of leather.[1] It was not until much later that the distinction between hydrolyzable and the flavonoid-based condensed tannins (now called proanthocyanidins) became clear enough to show that, although both were phenolic compounds capable of precipitating proteins, their biosynthetic pathways and distribution within the plant kingdom must be quite different.[2] Until recently, the chemistry of the proanthocyanidins was better known than their biosynthetic pathway. Gymnosperms are of primary interest as biological tools in the study of proanthocyanidins because the presence of these compounds in gymnosperms is probably universal, and no hydrolyzable tannins have been detected so far.

The biological tool studied by our laboratory has been mainly Douglas-fir (*Pseudotsuga menziesii* Franco). We have examined the proanthocyanidins in needles,

bark, and in cell suspension cultures derived from cotyledons and young needles. Summaries of the total amounts of proanthocyanidins as μg per mg of dry weight and the ratio of procyanidins to prodelphinidins are shown in Table 1.[3,4] A relatively high percent are soluble in 70 percent methanol. The very high concentration in the cell suspension cultures was one of the reasons that such cell cultures were vital in our discovery of a part of the cell-free enzymology of the pathway to proanthocyanidins.[5,6] Another reason that the cell cultures were so valuable for cell-free enzymology is that the cells produced only proanthocyanidins and their related flavan-3-ols as major secondary phenolic products. The other flavonoids present in intact needle tissues, mainly flavonol glycosides and C_6-C_3 products such as chlorogenic acid, were not detected. Although needles produce both procyanidins and prodelphinidins, our cell cultures produce only procyanidins.

Table 1. Comparison of Proanthocyanidins in Bark, Needles, and Cell Suspension Cultures.[a]

	Methanol Soluble %	Total PAs	PC:PD[b]
Stem Bark:			
6-month hypocotyls	78	212 ±12	1:1.3
3-year plants	58	118 ±75	1:1
80-year-old trees	70	116 ±25	1:0
Needles	80	140 ±12	1:1
Cell Suspension Cultures	80	575 ±112	1:0

[a] Unpublished data and reference (4)
[b] Procyanidin:prodelphinidin ratio

OVERVIEW OF MAJOR STEPS

The basic chemical unit of proanthocyanidins and their related flavan-3-ols is the C-15 flavonoid molecule that arises via a dual biosynthetic pathway, involving malonyl-CoA and 4-coumaroyl-CoA (Figure 1).[7,8] The hydroxyl groups on the A-ring arise from acetate units of the malonyl-CoA, whereas, those on the B- and C-rings arise from specific hydroxylases. Two stereochemical forms are commonly found, a 2,3-*trans* isomer (**2R**,**3S**) as in (+)-catechin, and a 2,3-*cis* isomer (**2R**,**3R**) as in (-)-epicatechin, due to changes in the stereochemistry at C-3. Although compounds with a **2S** configuration have been isolated from plants,[9] this discussion will be limited to the more common **2R**, stereochemical forms. In addition, emphasis will be on the more common pathway involving the 5,7-dihydroxy A-ring and the common 4 $\rightarrow$ 8 linkage between flavonoid units (Figure 1). The initial steps in the pathway leading to proanthocyanidins with a 5,7-dihydroxy A-ring from the first C-15 intermediate, the naringenin chalcone, are now relatively well known. However, only some of the enzymatic reactions unique to proanthocyanidins have been demonstrated in cell-free systems. The basic flavan-3-ol units are synthesized in the following series of steps (Figure 1): a) the condensation of 3[C_2] units arising from malonyl-CoA with a monophenolic phenylpropane (C_6-C_3) unit to form the basic

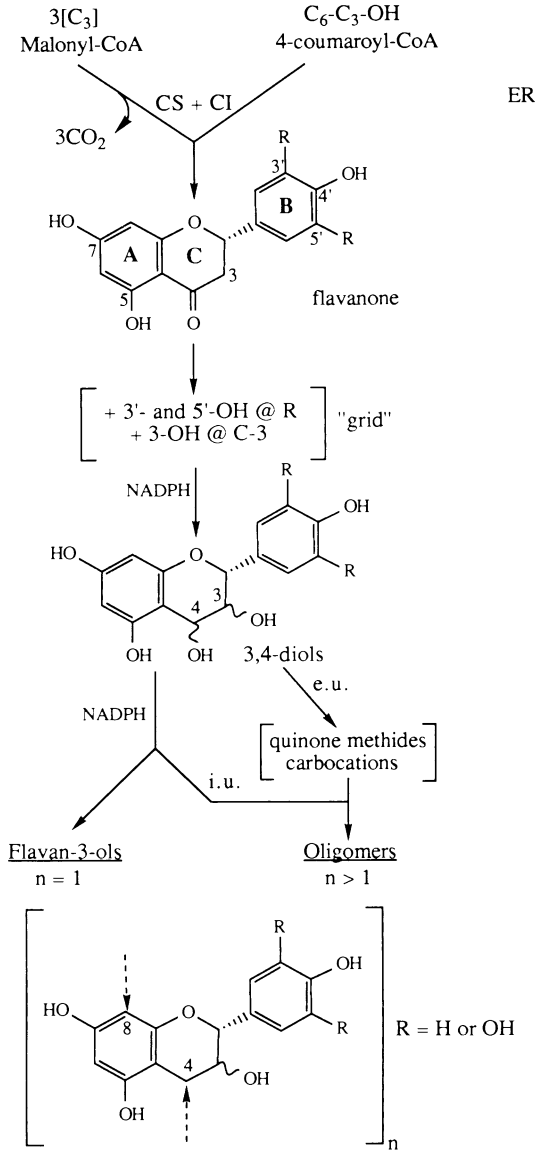


Figure 1. *The overall pathway to flavan-3-ols and proanthocyanidins R = H or OH; i.u. = initiating unit; e.u. = extension unit.*

C_{15} or C_6-C_3-C_6 flavonoid molecule (Chapter 1 by Lewis), followed by isomerization to produce the C-ring and to establish the stereochemistry of the B-ring at C-2; b) the hydroxylation of both the 3 and 3′ carbon positions, plus that of 5′ in some cases; c) the reduction of the 3-hydroxyflavanone to a 3,4-diol (leucoanthocyanidin), followed by a bifurcation in the pathway so that d) some of the 3,4-diol units are reduced to flavan-3-ols, whereas, e) others are converted to "extension" units via carbocations or quinone methides, culminating in f) the condensation at C-4 of one or more "extension" units to a pre-existing chain or to a flavan-3-ol at C-8 that initiates a new chain (or teminates polymer growth). Since other diol units can subsequently be added only to the previous diol-derived unit to form a linear chain, it seems preferable to consider that the flavan-3-ol initiates rather than terminates a chain during biosynthesis.

All the steps leading to 3,4-diols are shared with the anthocyanin pathway. The only steps unique to the synthesis of flavan-3-ols and proanthocyanidins are the terminal NADPH-dependent reduction step(s) and the condensation step(s).

FEEDING AND LABELING EXPERIMENTS

Before any cell-free enzymology of the flavonoid pathway was known, crucial information concerning the overall pathway was obtained by feeding isotopically labeled precursors to plant tissues (Table 2). In the initial work of Neish's laboratory in 1957,[10] the dual aspects of the pathway were demonstrated by showing that all of the carbons of ^{14}C-labeled phenylalanine were incorporated into the B- and C-rings, whereas, the acetate carbons of malonyl Co-A were conserved in the A-ring of the C-15 flavonoid molecule[7,11] (Figure 1). In 1970, it was shown that the hydrogen at the appropriate carbon of the chalcone was directly transferred to the C-3 of the flavanone.[12] In 1973, labeled phenylalanine and dihydrokaempferol (the stereochemistry was not reported) fed to tea plants were incorporated into (-)-epicatechin.[13] Subsequently, Haslam's laboratory demonstrated that there was unequal incorporation of labeled phenylalanine and cinnamic acid into the initiating ("lower" or "terminal") units of dimeric proanthocyanidins, compared with the "upper" or "extension" units, in shoots of various angiosperms.[14] Much more radioactivity was incorporated into the extension units. We corroborated these results in feeding experiments with cell suspension cultures of Douglas-fir[15] and also extended these results to higher oligomers. In addition, we demonstrated that the pools of the free flavan-3-ol monomers, (+)-catechin and (-)-epicatechin, had a much higher specific radioactivity than the comparable initiating (or terminal) unit of the dimers. A higher percent of the label was, however, incorporated into the catechin molecule compared with epicatechin. Haslam's group also demonstrated that the tritium label of phenylalanine associated with the C-2 position of the flavanone was largely retained, whereas, most of that associated with C-3 was lost.[14] Haslam interpreted the latter as indicating a symmetrical intermediate at the flavan-3,4-diol level, but other possibilities of exchange such as at the 3-hydroxylation step can occur.[16,17] Critical experiments with labeled flavanones and 3-hydroxyflavanones are still necessary to explain the loss of the label at C-3.

Table 2. Summary of ^{14}C-Tracer Studies of Incorporation into Proanthocyanidins.

1.	^{14}C-U.L. labled phenylalanine – all C's conserved in aromatic B-ring and central C-ring.[8,10,11]
2.	^{14}C-U.L. labled acetate – all C's conserved in aromatic A-ring.[8,10,11]
3.	^{2}H (α)-chalcone – direct transfer of H to C-3 of flavanone via isomerase activity.[12]
4.	Unequal labeling of ^{14}C-phenylalanine in proanthocyanidin oligomers: a) greater specific radioactivity and total activity in extension ("upper") units than in initiating ("lower") unit.[14,15] b) greater specific radioactivity in flavan-3-ol pools than in initiating units.[15]
5.	^{3}H-phenylalanine– retention of H that is on C-2 of proanthocyanidin unit, but loss at C-3.[14]

ENZYMOLOGY OF THE MAJOR STEPS IN THE PATHWAY COMMON TO OTHER FLAVONOIDS

Pre-C-15 Level

The pre-C-15 pathway to proanthocyanidins involves the shikimate pathway that gives rise to phenylalanine,[18] followed by the phenylpropanoid pathway to *p*-coumaric acid (4-hydroxycinnamic acid) via the enzymes phenylalanine ammonia-lyase and cinnamic-4-hydroxylase, respectively.[7,19] By the action of 4-coumarate:-CoA ligase, the *p*-coumarate is converted to the CoA ester[20,21] that is required to form the B- and C-rings of the flavonoid unit. The malonyl CoA required to form the A-ring is synthesized from acetyl-CoA and HCO_3^- by acetyl-coenzyme A:bicarbonate ligase.[22,23] Lewis discusses the pre-C-15 level in more detail in Chapter 2.

Chalcone Synthase and Isomerase to Form Flavanones

The first C-15 intermediate of flavanoids with a 5,7-dihydroxy A-ring is naringenin chalcone, an open-chain flavonoid with a *p*-hydroxy B-ring formed by the condensation of 3 malonyl-CoA molecules with *p*-coumaroyl-CoA, via chalcone synthase[7,11] (Figures 1 and 2). The three carbonyl groups of malonyl-CoA are released as carbon dioxide. Isozymes of chalcone synthase and isomerase have been demonstrated. Although some of the isolated chalcone synthases are also capable of using the *o*-dihydroxy caffeoyl-CoA ester to produce eriodictyol directly, the *p*-hydroxy *p*-coumaroyl:CoA ester, is generally considered the physiological

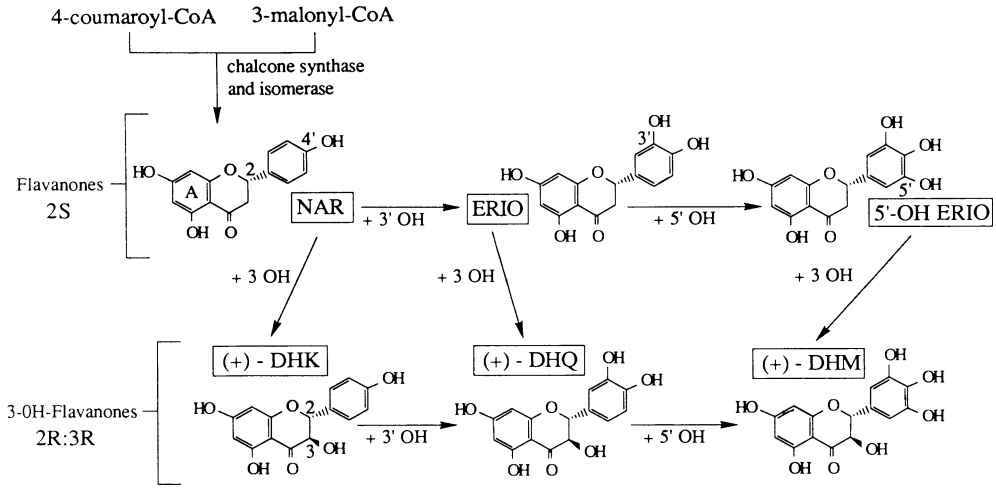


Figure 2. *The "grid" pathway to flavanones and 3-hydroxyflavanones (dihydroflavonols). (NAR = naringenin, ERIO = eriodictyol, DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin).*

substrate. Parsley and spinach chalcone synthases have been shown to be dimers with subunits of a molecular weight of 42,000 to 45,000.[11,24,25] Since the genetics controlling chalcone synthase are known in several flower types, this is now an active area of research in molecular genetics.[8] The subunits of the cloned genes are similar to the extracted enzyme. A chalcone synthase multigene family from *Petunia hybrida*, containing seven members, is believed to be the result of very recent gene duplications.[26] Six to eight genes, differentially activated by wounding, infection, or illumination have been identified in *Phaseolus vulgaris*.[27] Although the enzyme is easily solubilized in usual extraction buffers, immunological and immunocytochemical evidence indicates that a chalcone synthase is associated with isolated portions of the endoplasmic reticulum.[28] However, there are also recent claims of only a cytosolic or cytoplasmic localization with immunological techniques on frozen sections of spinach leaves.[24,25]

The synthesis of 6′-deoxy chalcones that form flavanones with a 5-deoxy A-ring has recently been demonstrated in crude cell-free extracts of a legume by a reduction step requiring NADPH. These extracts convert malonyl-CoA and 4-coumaroyl-CoA to naringenin via naringenin chalcone (6′-hydroxychalcone) and to liquiritigen via isoliquiritigenin (6′-deoxychalcone). So far, only the 5-deoxyflavonoids have been found in these cultures, and the authors considered that only one bifunctional chalcone synthase was involved.[29] Subsequently, a novel NADPH reductase of about 34,000 molecular mass that coacts with chalcone synthease to produce the deoxy forms was isolated and purified.[30] The addition of the reductase to chalcone synthase from parsley, a plant that does not produce 5-deoxy flavonoids,

produced the deoxychalcone. The authors concluded, therefore, that deoxychalcone synthesis depends only on the presence of the reductase rather than on the nature of the chalcone synthase.

A chalcone isomerase converts naringenin chalcone to naringenin, a 5,7-dihydroxy flavanone.[7,8] The stereochemistry at C-2 is established at this step and is **2S** in most flavanones (Figure 1).[12] (Note that the configuration assignment at C-2 becomes **R** at the 3-hydroxyflavanol and the proanthocyanidin levels without a change in stereochemistry). No special cofactors are required for either of these activities, and the equilibrium of the reaction is such that the flavanone, naringenin, can be considered the first stable intermediate of flavonoid biosynthesis. Some chalcone isomerases catalyze the isomerisation of both 6′-hydroxy and 6′-deoxy chalcones (various legumes), whereas, others function only with 6′-hydroxychalcones (*Petunia hybrida*).[31] (The 6′-hydroxyl group becomes the 5-hydroxyl group in the A-ring upon formation of the C-ring). Molecular weights of the single polypeptide reported vary from about 25,000 to 60,000. Different isozymes were found in the corolla and anther tissues.[32] The enzyme from soybeans is highly stereoselective for the formation of (2**S**)-flavanones over the (2**R**)-flavanones.[33]

The "Grid" Pathway of Hydroxylation Steps of Flavanones and 3-Hydroxyflavanones

The common pathway from the initial flavanone, naringenin, to 3-hydroxyflavanones with mono-, di- and tri-hydroxy B-rings is sometimes referred to as the "grid" system,[34] since the hydroxylases involved are apparently non-specific, and hydroxylation at the 3 and 3′ positions can occur in either sequence in the same cell (Figure 2). For instance, the 3-hydroxyflavanone (or dihydroflavonol) dihydroquercetin can be formed from naringenin via either dihydrokaempferol or eriodictyol, depending on whether the 3′- or 3-hydroxylation step occurs first (Figure 2).[35] A mechanism involving membrane-associated multienzyme complexes that permits only one of these pathways to function will be discussed later.

The two basic types of hydroxylases involved in the 2,3-*trans* pathway are a microsomal cytochrome P-450 monooxygenase, requiring oxygen and NADPH, for the 3′ and 5′ hydroxylations of the B-ring,[35,36] and a "soluble" cytosolic dioxygenase requiring O_2, 2-oxo-glutarate, Fe^{2+} and ascorbate for the hydroxylation of the 3-position on the C-ring.[37] The latter hydroxyl group is inserted stereospecifically at C-3 (β-hydroxylation) to produce the common 2,3-*trans* isomer (2**R**,3**R**) of 3-hydroxyflavanones. Experiments with tritium labeled substrates are needed to determine which hydrogen of the flavanone, eriodictyol, is replaced by the hydroxyl group during the synthesis of dihydroquercetin. This information might account for the loss of the hydrogen at C-3 in previous studies.[14] Other possibilities to explain this loss have been discussed,[16,17] but labeling experiments probably rule out the prior isomerase step as the site of the loss.[12] The 2,3-*cis* pathway will be discussed later.

In angiosperm flowers such as *Petunia* or *Verbena* that produce flavonoids with a trihydroxy B-ring, genetic, biochemical, and enzymological data indicate that the same microsomal enzyme is responsible for both the 3′ and 5′ hydroxylations and is controlled by a single gene.[8,36,38] Two other gene loci control the enzymes

specific for 3′-hydroxylation of the B-ring and for 3-hydroxylation of the C-ring in other *Petunia* mutants.[36,38] The soluble 3-hydroxylase has been purified and has a molecular weight of 74,000.[37] Flavanones with *p*- and 3,4-dihydroxy (but not trihydroxy) B-rings were substrates. The hydroxylation of the B-ring is controlled at least in part at the phenylpropanoid level in a few plants.[8]

The cell-free enzymology of the above steps has been demonstrated so far only in angiosperms. Isotopic feeding data, however, indicate that the pathway must be similar in gymnosperms,[15] but any special characteristics and substrate specificities of the enzymes involved still need to be determined. The hydroxylation steps are common to practically all major flavonoid endproducts. Aglycones are intermediates in the pathways leading to these other flavonoids, but are also accumulated after glycosylation in the central vacuole as various glycosides. They must be hydrolyzed, however, to remove the sugars before further metabolism, since the enzymes of the pathway react only with the aglycones.

Synthesis of 2,3-*trans*-3,4-*cis*-Leucocyanidin from 2,3-*trans*-(+)-Dihydroquercetin by a 3-Hydroxyflavanone Reductase

The reduction of the 3-hydroxyflavanone, (+)-dihydroquercetin, to its 3,4-diol or leucocyanidin was first demonstrated in cell-free extracts of cell suspension cultures of the gymnosperm, *Pseudotsuga menziesii* (Douglas-fir), in 1982[39] and subsequently in *Ginkgo biloba*[40] (Figure 3).

The 3,4-diols were suspected as intermediates in proanthocyanidin biosynthesis based on the stereochemical studies from Roux's laboratory in which it was demonstrated that the 2,3-*trans* stereochemistry of (+)-dihydroquercetin was retained upon its chemical reduction by $NaBH_4$ and condensation in a second step to form proanthocyanidins.[41] Roux's group did not isolate the intermediate involved in the above two-step process. In 1982, Porter and Foo[42] isolated and identified this nonenzymic reduction product as the 2,3-*trans*-3,4-*trans* diol (2**R**,3**S**,4**R**)-(+)-3,4,5,7,3′,4′-hexahydroxyflavan.

In our own enzymic work cited above, we initially demonstrated that soluble extracts of cell suspension cultures of Douglas-fir were capable of reducing (+)-dihydroquercetin in the presence of NADPH to a 3,4-diol (a leucocyanidin) that was a different isomer than the above nonenzymic reduction product, but which was slowly converted to the nonenzymic product during its isolation by paper chromatography or high performance liquid chromatography on C-18 columns. Subsequently, proof of the stereochemistry of the enzymic product as the corresponding 3,4-*cis* diol was established independently by two groups, one in intact and cell-free extracts of barley grains[43,44] and the other in Douglas-fir preparations.[45] A similar enzyme and 3,4-diol product was demonstrated later in the *Cryptomeria* cultures.[46] All the above sources contained proanthocyanidins and in some cases anthocyanins. Subsequently, a similar dihydroquercetin reductase activity producing the same 3,4-diol was found to be a key step in the anthocyanidin pathway in the aleurone layer of *Zea mays* kernels,[47] *Dahlia* flowers,[48] and in *Matthiola* flowers.[49] The genetic locus, A1, that controls anthocyanin biosynthesis in *Zea* has been cloned,[50] and the protein synthesized from the isolated mRNA (from cDNA) was shown to catalyze the

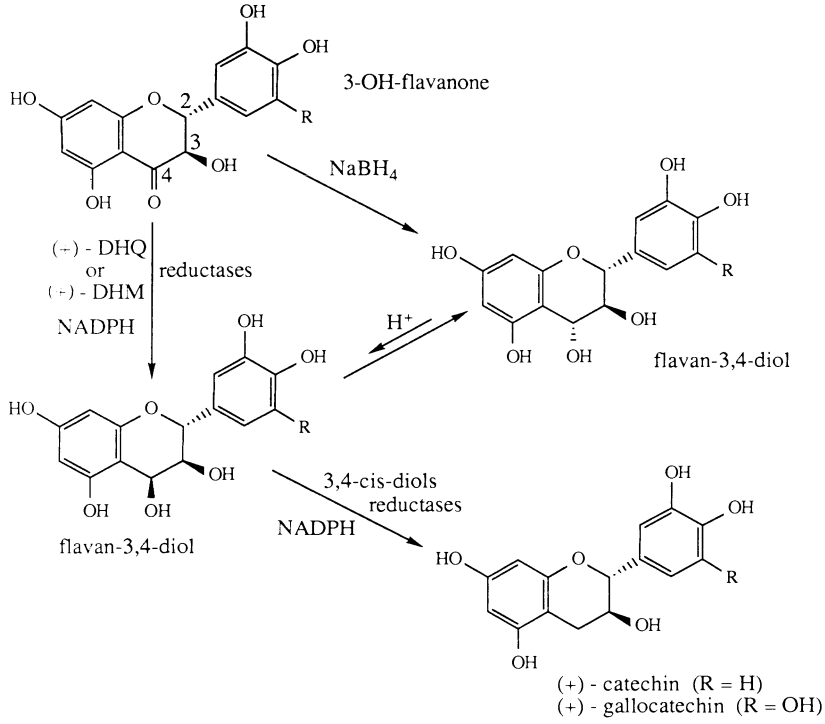


Figure 3. *The conversion of (+)-dihydroquercetin (DHQ) and its 2,3-trans-3,4-cis diol to (+)-catechin by NADPH-dependent reductases compared to the nonenzymic reduction by $NaBH_4$ that can be followed by acid epimerization to the 3,4-trans diol.*

same dihydroquercetin reductase activity.[47] See Heller and Forkmann, 1988, for a good reveiw of anthocyanin biosynthesis.[8]

These NADPH-dependent reductases that form leucoanthocyanidins (flavan-3,4-diols) are only beginning to be studied, but there is already evidence that there are substrate specificity differences among the 3-hydroxyflavanone reductases (or dihydroflavonol reductases) in the anthocyanidin pathway. Reductases from *Petunia* flowers reduce dihydromyricetin more effectively than dihydroquercetin, but do not reduce dihydrokaempferol. Others such as from *Zea* aleurone reduce dihydrokaempferol and dihydroquercetin.[51] The *Dahlia* reductase, purified to apparent homogeneity and with a molecular weight of about 41,000, is similar to the *Zea* enzyme in being more effective with dihydrokaempferol and dihydroquercetin, with only a weak activity with dihydromyricetin. It was also capable of reducing flavanones to their corresponding flavan-4-ols, but with much lower maximal velocities. Differences in substrate affinities of the reductase(s) in the flavan-3-ol and proanthocyanidn pathways are still unknown, since the proteins have not yet been highly purified.

We have been unsuccessful in purifying the Douglas-fir (+)-dihydroquercetin reductase beyond a fivefold range, due to instability of the enzyme (Stafford, unpublished data). The high concentrations of proanthocyanidins in the original cells probably will make such purification very difficult in comparison with the enzymes from flower petals. However, the recovery in the purest preparation from *Dahlia* flowers discussed above was only about 1 percent. Possible enzymic interrelationships between proanthocyanidins and both the 3-hydroxyanthocyanins and the rarer 3-deoxyanthocyanins derived from flavan-4-ols in tissues such as sorghum grains and corn kernels need to be studied.[52,53]

Synthesis of 2,3-*trans*-3,4-*cis*-Leucodelphinidin from 2,3-*trans*-(+)-Dihydromyricetin by a 3-Hydroxyflavanone Reductase

The conversion of (+)-dihydromyricetin to its flavan-3,4-diol (presumably also the 3,4-*cis* isomer) by a NADPH-dependent reductase has also been demonstrated in cell cultures of *Ginkgo biloba* that accumulate large amounts of prodelphinidins in addition to procyanidins (Figure 3).[40] Such a conversion was expected in the case of *Ginkgo* cultures, since they form relatively large amounts of prodelphinidins in addition to procyanidins. The Douglas-fir cultures, however, do not form prodelphinidins, although needles from which they were derived synthesize substantial amounts of prodelphinidins. These results could be explained by assuming that the 3-hydroxyflavanone (dihydroflavanol) reductases are not specific for the *o*-dihydroxy B-ring, but function as well as, or at least partially with, the vicinal trihydroxy B-ring. As in the case of the leucocyanidin reductase activity, attempts to purify the reductase activity from either source beyond a fivefold range have been unsuccessful due to the great instability of the enzyme (Stafford, unpublished data). The absence of pyrogallol B-ring moieties in Douglas-fir cell cultures may be due to a lack of synthesis of dihydromyricetin and its 3,4-diol, rather than to a lack of a specific reductase.

STEPS UNIQUE TO THE FLAVAN-3-OL AND PROANTHOCYANIDIN PATHWAYS

The subsequent conversion of the flavan-3,4-diols to flavan-3-ols and to their oligomeric proanthocyanidin forms is the beginning of the pathway unique to the biosynthesis of these secondary products (Figures 3 and 4).

Biosynthesis of (+)-Catechin and (+)-Gallocatechin by 3,4-Diol (Leucoanthocyanidin) Reductases

The NADPH-dependent reduction of the 2,3-*trans*-3,4-*cis*-diol with a dihydroxy B-ring to the flavan-3-ol, (+)-catechin was first shown in soluble extracts of Douglas-fir cell cultures.[54] This is actually the only step unique to the flavan-3-ol pathway. The comparable reduction to (+)-gallocatechin, synthesized from the corresponding 3,4-diol by dihydromyricetin reductase activity, was demonstrated with a soluble enzyme requiring NADPH in extracts of *Ginkgo biloba*.[40]

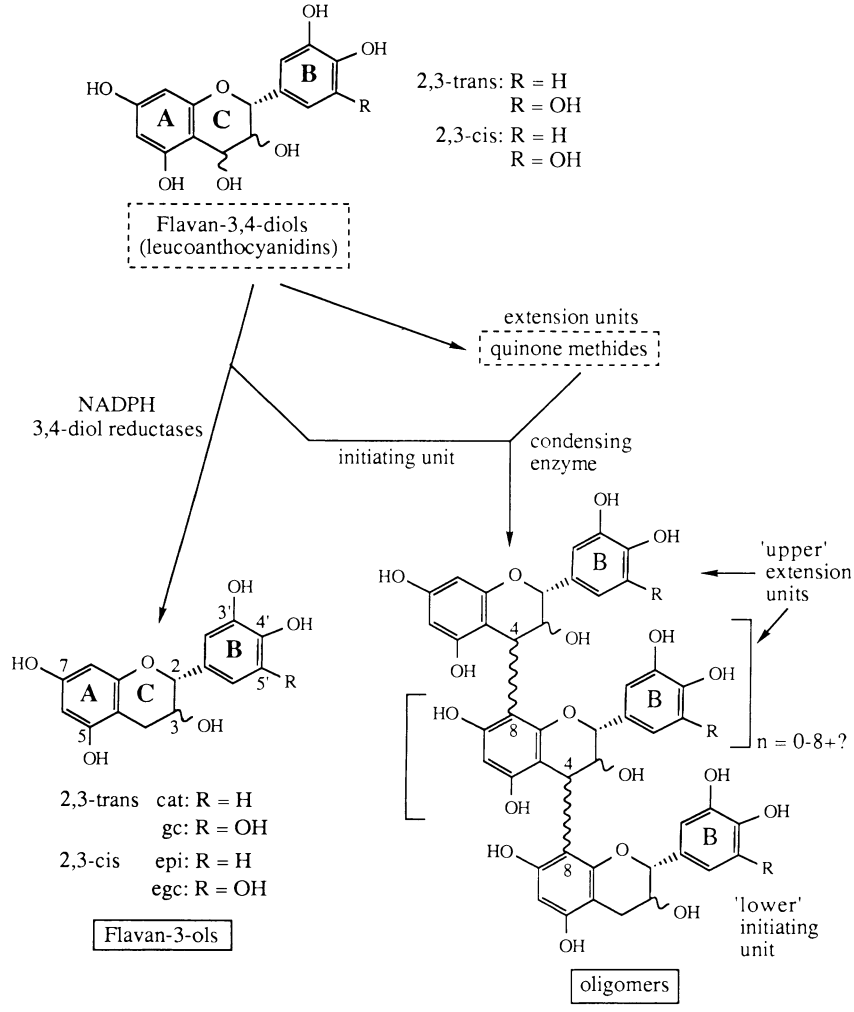


Figure 4. *Steps unique to the flavan-3-ol and proanthocyanidin pathways.*

The 2,3-*cis* Pathway to the Flavan-3-ols, (-)-Epicatechin and Epigallocatechin, and the Corresponding Units of Oligomers

This pathway is still unknown. Neither critical isotopic feeding nor cell-free enzymological data are available. Although Douglas-fir cell cultures produce large amounts of 2,3-*cis* units in the oligomeric proanthocyanidins,[3] attempts to demonstrate cell-free synthesis of 2,3-*cis* units starting with 2,3-*trans*-eriodictyol or 2,3-*trans*-dihydroquercetin with a variety of different co-factors have all been unsuccessful.

Three possible routes for the biosynthesis of the 2,3-*cis*-dihydroquercetin were postulated earlier;[16] the first is a stereospecific α-hydroxylation at position 3 at the level of the flavanone in addition to the β-hydroxylation (Figure 5); the other two involve epimerase activity at the level of dihydroquercetin or its 3,4-diol. Epimerization at the 3,4-diol level is now unlikely, since the 3-glycoside of 2,3-*cis*-dihydroquercetin has recently been identified as a natural product.[55,56] The isotopic evidence that dihydrokaempferol (2,3-*trans*-?) gave rise to the 2,3-*cis*-(-)-epicatechin would support the epimerization of dihydroquercetin,[13] but the results are equivocal, since the substrate used in these studies may not have consisted solely of the 2,3-*trans* isomer (**2R**,**3R**).

Both Foo[17] and Porter[57] prefer the pathway involving an α-hydroxylation at C-3 of a flavanone to produce the 2,3-*cis* isomer, in contrast to a β-hydroxylation to produce the more commonly accumulated 2,3-*trans*-isomer that would have to undergo epimerization. This preference is based on the fact that some proanthocyanidins contain only the 2,3-*cis* isomers. The absence of the 2,3-*trans* isomers such as the 3,4-*trans* diols and (+)-catechin could be due to the loss through mutation of the 2,3-*trans* dihydroquercetin reductase activity (Figure 5). Although substrate

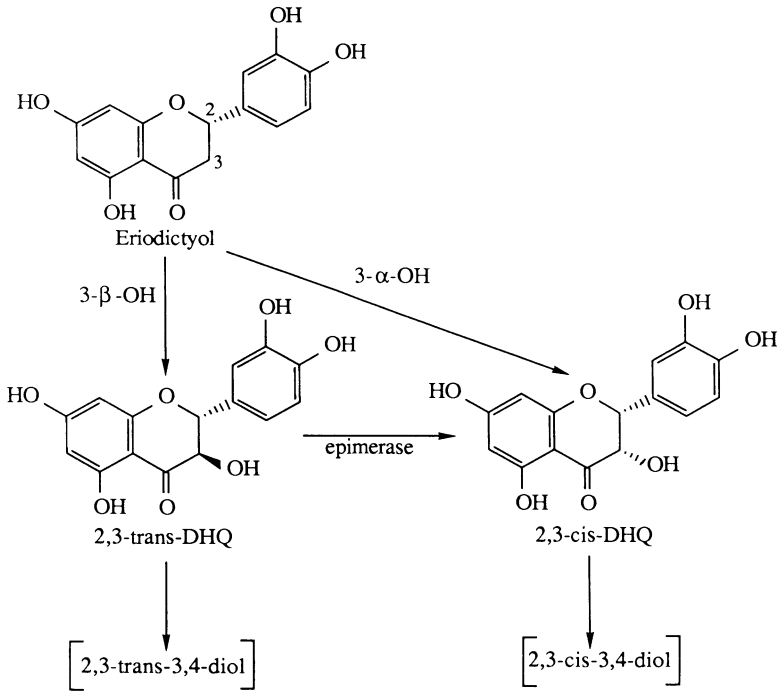


Figure 5. *Two possible pathways from eriodictyol to 2,3-cis-(2**R**,3**S**)-(-)-dihydroquercetin via either an α-hydroxylase or a C-3 epimerase. A similar set of reactions can be postulated to produce both isomers of dihydromyricetin.*

specificity studies have not been made, all other flavonoid pathways probably produce the 2,3-*trans*-(+)-dihydroquercetin. Therefore, even the above plants probably produce this 2,3-*trans* isomer of 3-hydroxyflavanones when other flavonoids are synthesized. A speculative scheme starting with 2,3-*trans*-(+)-dihydroquercetin will be presented later to account for the predominance of the 2,3-*cis* pathway in Douglas-fir.

Roux and Ferreria have argued for a role of α-hydroxychalcone (α-2′,3,4,4′-pentahydroxychalcone) in the pathway leading to both 2,3-*trans* and 2,3-*cis* flavanoids.[58,59] Cyclization of the *trans*-enolic isomers would permit the formation of both stereochemical forms nonenzymically. So far, the natural α-hydroxychalcones identified have only the 6′-deoxy A-ring. It is difficult to reconcile this mechanism with known enzymology, since the β-hydroxylation of flavanones to their respective 3-hydroxyflavanones with the 2,3-*trans* stereochemistry is well known, and the stereochemistry at C-2 was determined at the preceding isomerase step (see the earlier discussion of the "grid" portion of the pathway. So far, 2,3-*cis*-(2**R**,3**S**)-(-)-dihydromyricetin has not been identified as a natural product, but such an intermediate must exist at least on the surface of an enzyme to synthesize the (-)-epigallocatechin unit. If all four possible isomers of the 3-hydroxyflavanones are natural products as 3-glycosides (as indicated by Nonaka et al for dihydroquercetin[55]), and if they are all enzymically produced, the biosynthetic pathway and analysis of oligomeric sequences will be complicated tremendously. The possibility, however, of nonenzymic racemization during storage of the needles used or the extraction procedures needs to be eliminated. Such artifacts are discussed by Lundgren and Theander.[56] In addition, racemization and epimerization might occur nonenzymically upon senescence and cell death during normal development in tissues such as the bark.

Speculations about the Enzymology of the Subsequent Condensation Steps

It is well known now that the oligomeric forms of proanthocyanidins are synthesized by the sequential addition of an intermediate derived from the flavan-3,4-diols to a preexisiting chain or to a flavan-3-ol that initiates (or terminates) a chain rather than being self-condensation products of the diols (Figure 4).[54] Although the condensation step can be easily done nonenzymically in so-called biomimetic syntheses, the presence of one or more condensing enzymes is very probable for regulatory reasons. However, no good evidence of the enzymes involved has yet been demonstrated, perhaps because of the ease of the nonenzymic synthesis. We have attempted to gain evidence under a variety of conditions, but with nothing more than trace amounts of dimer formation above the nonenzymic controls. A speculative model to account for enzymic control of the condensation step will be shown later.

Many biomimetic syntheses of proanthocyanidins by a condensation of 3,4-diol and flavan-3-ol precursors have been published.[41,60,61] The nature of the actual extension unit or intermediate that adds on to a flavan-3-ol or chain is described either as a carbocation (or carbenium ion)[59] or a quinone methide intermediate.[62,63] Typical pH values in vesicular compartments of the cytoplasm might easily be

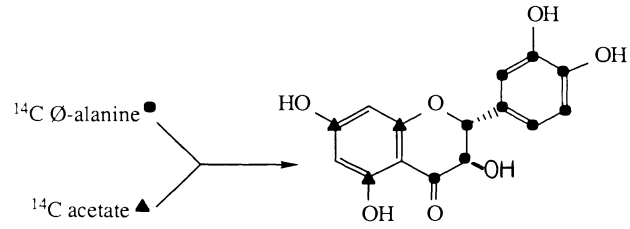
expected to be in the range of pH 5-8. The actual extension unit involved, however, might exist only on the surface of a protein rather than being soluble in an aqueous cytoplasmic environment as in the above mechanisms. The mechanism of catalysis in such a 'solid state' environment could be different.

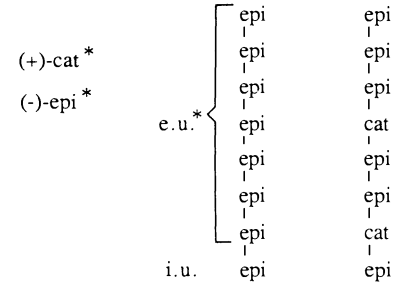
REGULATION

The biosynthesis of flavan-3-ols and various oligomeric forms of proanthocyanidins is the most complex pathway within the flavonoids, except possibly for that of the pterocarpans. Although it is well documented that the proanthocyanidins are synthesized on the endoplasmic reticulum and accumulated in the large central vacuole of living cells,[64] secondary changes may occur, especially as cells die during normal development. Except for the relatively rare dimers known as biflavonoids,[65] proanthocyanins are the only oligomeric (or polymeric) group of flavonoids. Dimers, trimers, and tetramers have been well described, but the sequences of extender units and the location of the interflavanoid bonds in higher oligomers still remain largely unknown as well as the degree of branching and their shape or physical state. Porter[57] reports that little branching has been detected in oligomers with 5,7-dihydroxy A-rings, and these compounds are predominantly "linear" molecules even though they may contain both 4 → 8 and 4 → 6 linkages. Those with the 5-deoxy A-ring are usually angular at a catechin or gallocatechin initiating (or terminal) group[53] Although some flavan-3-ols and lower oligomers have recently been found to exist as 3-glycosides,[66] requiring glucosyltransferases, evidence that such covalent bonds exist with high molecular weight carbohydrates is lacking. (See Chapter 15 in this volume by Haslam for a more recent review). The presence of A-ring hydroxylation patterns other than the common 5,7-dihydroxy type, rare methylated forms, complexes or covalent bonding with non-flavan-3-ol units, and especially of the so-called *ent*-isomers (2S), can make the biosynthetic picture very complex (Chapter 5 in this volume and other references).[53,57,65]

For the purpose of the model to be discussed later, matters can be simplified by considering just the procyanidin type of proanthocyanidin, and the corresponding flavan-3-ols, consisting of 2,3-*trans* isomers with an *o*-dihydroxy B-ring and 5,7-dihydroxy A-ring, such as are found in Douglas-fir cell cultures. Presumably, only 4 → 8 interflavanoid linkages are present.[67] The lower or initiating units can be either (+)-catechin (2,3-*trans*) or (-)-epicatechin (2,3-*cis*), whereas, the upper units in bark preparations and in cell cultures are predominantly 2,3-*cis* procyanidin units.[3,15,68]

Figure 6 illustrates the basic composition of such a simplified system as is found in Douglas-fir cell cultures. The flavan-3-ol pool of (+)-catechin was generally much larger than that of (-)-epicatechin. Of the four possible dimers, the epicatechin-(4β → 8)-catechin, and sometimes catechin-(4α → 8)-catechin were present in higher concentrations relative to the other two with epicatechin "lower" units. Except for the fact that the 2,3-*cis* isomer was probably prevalent in the "upper" or extension units and also in the "lower" initiating units, nothing is known about the actual sequence of the flavanoid units of the higher molecular weight oligomers or polymers. ^{13}C-NMR analyses of the polymers isolated from cell cultures indicated





*High specific radioactivity

	Flavan-3-ols	Dimers	Oligomers
Concentration per mg dry weight	cat > epi	epi-cat > cat-cat	high 2,3-cis

Figure 6. *Incorporation of ^{14}C-phenylalanine into flavan-3-ols and proanthocyanidins isolated from cell suspension cultures of Douglas-fir. Top: conservation of all the nine carbons of phenylalanine and all six carbons of acetate into dihydroquercetin and subsequently into the rings of flavan-3-ols and proanthocyanidins. Middle: higher specific activity found in both the flavan-3-ol pools and the extension units (e.u.) compared with the initiating (i.u.) units that can be either epicatechin as shown or catechin; Bottom: higher concentration of the 2,3-trans isomer in the flavan-3-ol pools, but the higher concentration of 2,3-cis isomers in oligomeric proanthocyanidins.*

a M_n value of 2500-3000 (L.J. Porter, unpublished data). This is in the same range reported by Foo and Karchesy in bark extracts.[68] If the biosynthesis in needles is considered, the story is complicated by the approximately 1:1 ratio of procyanidin to prodelphinidin, and the presence of at least a small (+)-gallocatechin pool. The concentration of flavan-3-ols and dimers is very small compared with that of the total amount of oligomeric forms of proanthocyandins.

A model needs to accommodate the isotopic tracer work cited earlier (Table 2). For instance, when labeled precursors such as phenylalanine were fed to cell suspension cultures of Douglas-fir, both the extension units and the free flavan-3-ol pool had higher specific radioactivities than the lower initiating flavan-3-ol group (Figure 6). What intracellular arrangement could produce such results?

Hypothetical Model of Multienzyme Complexes to Explain the Asymmetric Labeling and the 2,3-cis Predomination

Although the "grid" arrangement of flavanone and 3-hydroxyflavanone intermediates permits two or more pathways to the same compound (Figure 2), any one cell may use only one pathway because the enzymes involved may be arranged in unidirectional sequences in multienzyme complexes associated with vesicles and membranes of the endoplasmic reticulum. Intermediates could be passed directly from one protein to another. The "tighter" the complex, the less possibility of diversion of an intermediate to another pathway (Figures 7 and 8).

C-15 precursors that accumulate might shed some light on the sequence used by a particular cell. While aglycones are the substrates for the enzymes involved, rather than the glycosides, pools of flavonoid-*O*-glycosides are found in gymnosperms. Some of the intermediates, therefore, are drained away from the aglycone sequence for glycosylation and storage ultimately in the large central vacuole. Hydrolysis must occur before they reenter the proanthocyanidin pathway. Douglas-fir cell cultures and needles accumulate only the flavanone eriodictyol as the 3′- or 7-glucosides, and its 3-hydroxy derivative dihydroquercetin as the 3′-glucoside. Possible traces of naringenin-7-glucoside were found[3,6] (Table 3). The main route to

Table 3. Precursors of Proanthocyanidins in Douglas-fir Needles and Cell Suspension Cultures Involving Total Amount and Procyanidin:Prodelphinidin Ratios.[a]

Tissue	Total PAs	PC:PD	Precursor	Yield	
				μg/mg dry weight	
Needles	140	1:1	Eriodictyol-7-G	0.3	
			Dihydroquercetin-3′-G	2.0	
				NAA	2,4D
CSC[b]	575	1:0	Eriodictyol-7-G	3.9	(2.0)
			Eriodictyol-3′-G	0.7	(9.4)
			Dihydroquercetin-3′-G	2.9	(91.0)

[a] Unpublished data and in reference (3)

[b] Cell suspension culture with auxin NAA or 2,4D

2,3-*trans*-procyanidins, therefore, might be expected to proceed as follows: naringenin → eriodictyol → dihydroquercetin → leucocyanidin (Figures 2 and 3). No dihydromyricetin glycoside accumulated even in needles that produce prodelphinidins. The absence of a precursor could mean that the enzyme complex involved is "tight", rather than that a particular sequence is involved. The relative amounts of precursor glycosides in the "grid" portion of the pathway varied in the tissues studied and in cell suspension cultures grown in the presence of different auxins[3]. For instance, higher concentrations of eriodictyol glycosides were found in both cell suspension cultures and needles, perhaps an indication of a "looser" complex or a greater binding capacity of the glycosyl transferases. The auxin added, whether naphthaleneacetic acid (NAA) or 2,3-dichlorophenoxyacetic acid (2,4-D), altered the relative amounts accumulated (Table 3). However, the mechanism of the regulation of the accumulation of these glycosidic precursors is unknown.

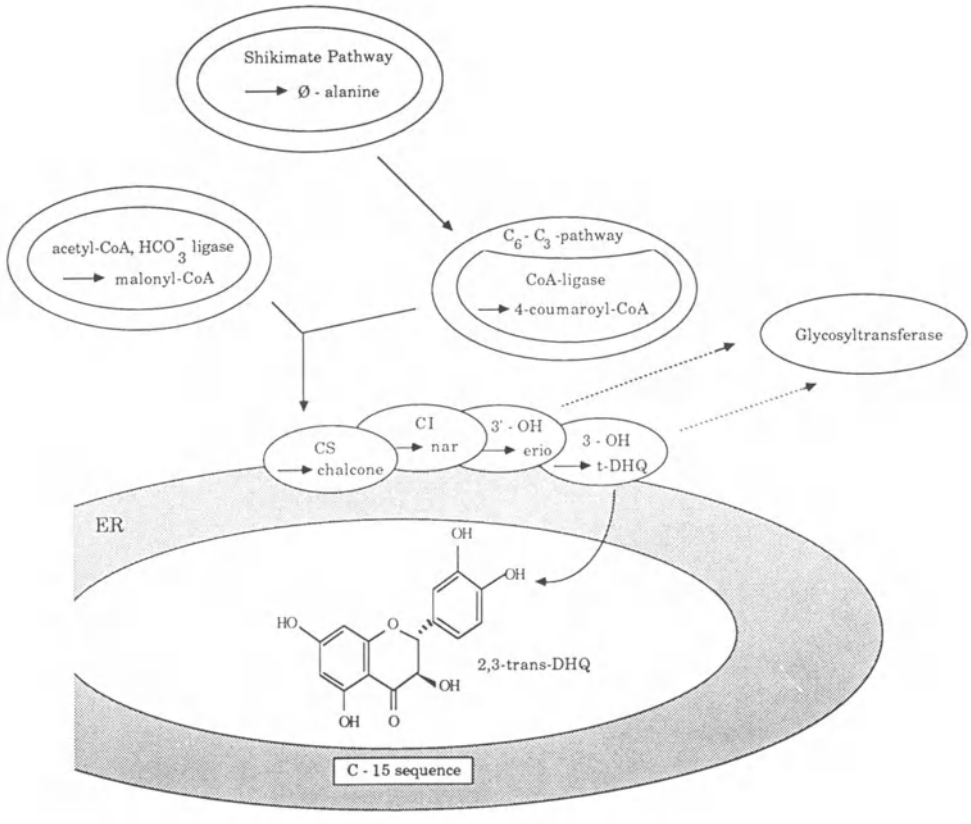


Figure 7. *A model of a series of vesicle-associated multienzyme complexes capable of synthesizing (+)-dihydroquercetin.*

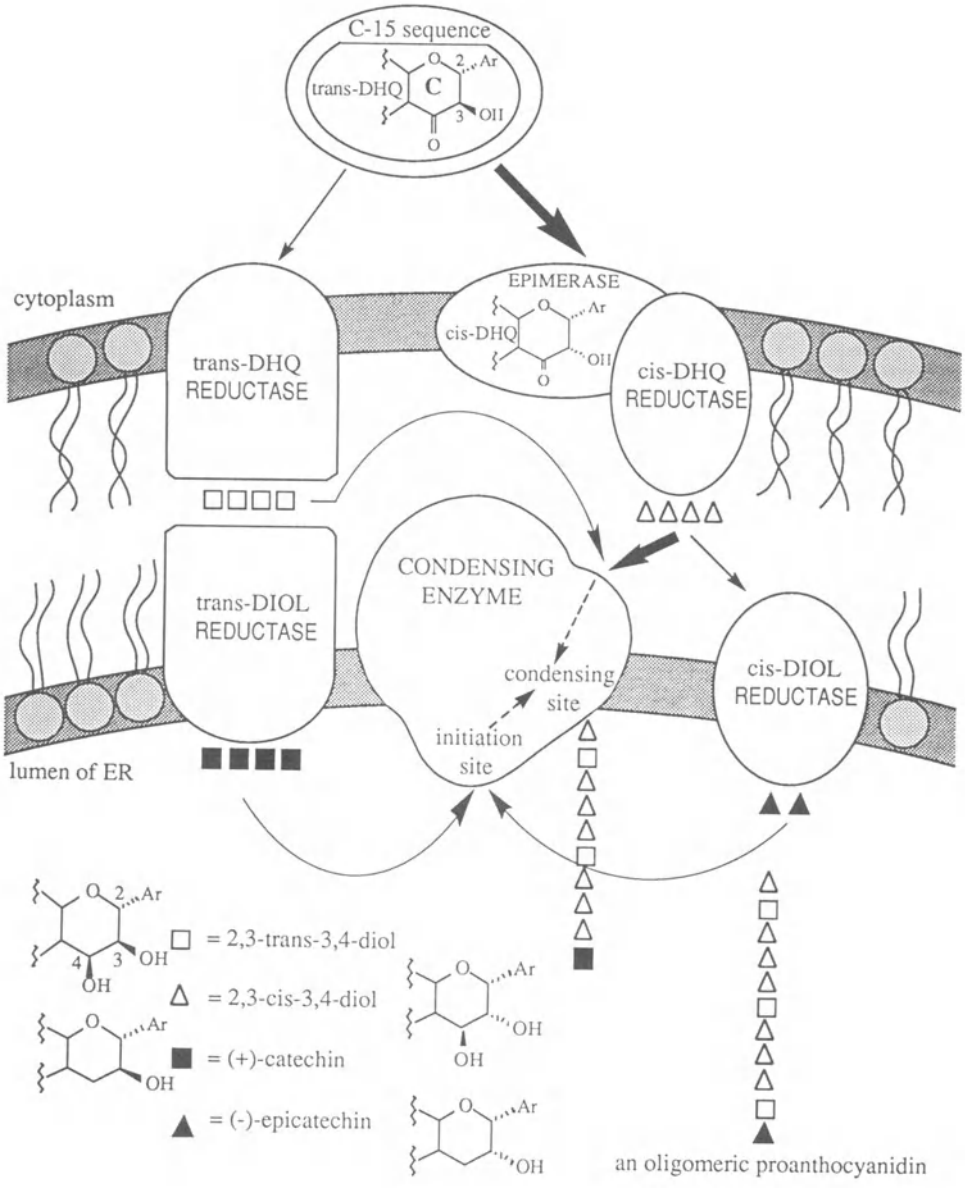


Figure 8. *A model of an ER-derived vesicle capable of converting 2,3-trans-(+)-dihydroquercetin to flavan-3-ols and oligomeric proanthocyandins with either 2,3-trans or 2,3-cis stereochemistry. Flavan-3-ols form the initiating unit of an oligomeric chain, with the 3,4-diols, as carbocations or quinone methides, forming the extension units .*

So far, most of the enzymes of the flavonoid pathway demonstrated in cell-free extracts are easily solubilized and, therefore, would be considered cytosolic enzymes. However, in the past, there have been claims of localization in chloroplasts. The older literature has been reviewed by Hrazdina and Wagner.[69] Although controversy still exists,[28] recent evidence from Hrazdina's laboratory indicates that the chalcone synthase is localized on the endoplasmic reticulum.[27] Such localization could also explain the chloroplast localization as a contaminant of this system. When we extracted the reductase enzymes in borate buffer at pH 8, the activities were found in the soluble rather than in the particulate fraction.[39,40,45,54] Since the high pH and the borate buffer would break up membranes and release associated enzymic activities, the soluble activities we observed could be artifacts of the extraction. Unfortunately, it is difficult in such studies to determine just which result is the artifact, since procedures could solubilize a particulate enzyme or cause a "soluble" enzyme to become adsorbed onto a membrane.

The concept of the so-called cytosolic or cytoplasmic compartment as being a "bag" full of soluble enzymes is no longer held by many workers, even with such easily solubilized systems as the glycolytic pathway. Instead, polyisozymic complexes[70] and aggregations permitting enzyme-enzyme interactions[71] have been postulated for these so-called soluble unidirectional pathways (see the Discussion Forum in TIBS for comments regarding this somewhat controversial area).[72] Such complexes permit the direct transfer of metabolites from one enzyme site to another, rather than being dependent upon dissociation and random diffusion through an aqueous environment.[73] Complexes can be considered to vary in "tightness" or "looseness," depending on the closeness of the proteins involved and on the ability of other pathways to intercept the intermediates. Since any one plant synthesizes more than one flavonoid end product, and isozymes of key enzymes such as chalcone synthase and isomerase have been found, regulation of these pathways with many common steps is likely to consist of such complexes. Competition for substrates would occur prior to the flavonoid pathway rather than for C-15 intermediates in a common metabolic pool. Regulation, therefore, would involve the entire sequence at the C-15 level. The coordinated *de novo* synthesis of key enzymes of the flavonoid pathway after UV induction would support this concept.[74] The preceding two diagrams (Figures 7 and 8) represent an attempt to speculate on the nature of such complexes that are membrane-associated or enclosed within vesicles. Present technology will permit experimental verification of such hypotheses, although unequivocal data will be hard to obtain as the enzymes are so easily solubilized.

Figure 7 portrays a series of hypothetical vesicles budded off from the endoplasmic reticulum (ER). One accumulates phenylalanine via the shikimate pathway; another, 4-coumaroyl-CoA via the phenylpropanoid pathway; and a third, malonyl-CoA via acetyl-CoA:HCO_3^--ligase. The fourth one containing the C-15 level enzymes accumulates 2,3-*trans*-dihydroquercetin. The fate of the latter vesicle is shown in Figure 8.

Figure 8 illustrates several aspects of this membrane-associated complex: (1) a tight complex of the two NADPH-dependent reductases that permits efficient (+)-catechin synthesis via channeling and accumulation in the cisternae or lumen of the endoplasmic reticulum; (2) an epimerase, with a higher affinity for 2,3-*trans*-

dihydroquercetin than the first reductase that forms 2,3-*cis*-dihydroquercetin; (3) an epimerase that is attached to only the *cis*-DHQ reductase, permitting the less stable 2,3-*cis* diol to be preferentially bound to sites on the condensing protein; (4) a limited number of initiation binding sites on the condensing protein for flavan-3-ol binding. Finally, upon condensation of the extension units to the initiating flavan-3-ol unit and subsequently to the growing chain, the chain is pushed out into the lumen of the endoplasmic reticulum.

The condensing protein is hypothesized to contain two binding sites, one for the initiating flavan-3-ol, the other for the quinone methide or carbocation precursor of the extension units. The sites for the flavan-3-ols are limited in the number. When they are filled, no further uptake of radioactive tracers can occur. This could explain the low specific radioactivity of the initiating units compared with the free flavan-3-ols and the extension units. The chains break off randomly at different lengths, and a new flavan-3-ol can now occupy the initiating site. The small vesicles containing flavan-3-ols and proanthocyanidins ultimately fuse with the vacuolar membrane (tonoplast) and empty their contents into the large central vacuole of the plant cell.

Some key questions remain. Do the same reductases use substrates with both di- and trihydroxy B-rings? Or, if different ones are involved, what are their substrate specificities? Little is known about the distribution of prodelphinidin units within the oligomers. Are there separate vesicles that accumulate 2,3-*trans*-dihydromyricetin? How is 2,3-*cis*-dihydromyricetin formed? Do the dihydroxy and trihydroxy B-ring intermediates have separate sites for binding on the condensing protein(s)?

Competition with Anthocyanidin Biosynthesis?

Although anthocyanidins are not major components of most gymnosperm needles or leaves, they are present in some tissues. For instance, cyanidin-3-glucoside is found in Douglas-fir strobili[75] and in the seedling hypocotyl.[4,76] Both proanthocyanidins and anthocyanins are produced in the epidermal cells of the hypocotyl at approximately the same time.[4] Since they have a common pathway through the 3,4-diol level, one might expect competition for the 2,3-*trans*-flavan-3,4-diol, after which their paths diverge. However, if a separate endoplasmic reticulum associated multienzyme sequence for the anthocyanidin pathway is also postulated, regulation would involve the entire sequence rather than just the terminal common step, and competition might be for the substrates of the first enzyme in the C-15 sequence, chalcone synthase.

CONCLUSIONS

The cell-free enzymology of the pathway to 2,3-*trans* flavan-3-ols and the oligomeric 2,3-*trans* proanthocyanidins is known except for the final condensation step to the oligomers. The steps up to the 3,4-diols (leucoanthocyanidins) are shared with anthocyanin biosynthesis. The only steps unique to the flavan-3-ol and proanthocyanidin pathways are the NADPH-dependent reductase(s) to form flavan-3-ols and the postulated condensation step(s) forming oligomers. The enzymology of the

2,3-*cis* pathway, on the other hand, is unknown. The unknown condensing enzyme catalyzing the last step with either stereoisomer is assumed to contain binding sites for both the flavan-3-ol that forms the initiating or terminal unit of a chain and the carbocation or quinone methide intermediate derived from the 3,4-diols. Regulation of the flavan-3-ol and proanthocyanidin pathway from the first synthesis of the C-15 unit by chalcone synthase is postulated to involve linear multienzyme complexes associated with vesicles derived from the endoplasmic reticulum.

REFERENCES

1. Haslam, E. Vegetable tannins. *In*: Swain, T.; Harbonne, J.B.; VanSumere, C. F. (eds.) Biochemistry of Plant Phenolics (Recent Advances in Phytochemistry). Plenum Publishing Co., New York, 12:475 (1979).
2. Freudenberg, K.; Weinges, K. Catechins and flavonoid tannins. *In*: Geissman, T.A. (ed.) The Chemistry of Flavonoid Compounds. MacMillin, New York, p. 197 (1962)
3. Stafford, H.A.; Kreitlow, K.S.; Lester, H.H. Comparison of proanthocyanidins of *Pseudotsuga menziesii* Franco, and *Ribes sanguineum* Pursh. *Plant Physiol.* 82:1132 (1986).
4. Stafford, H.A.; Smith, E.C.; Weider, R.M. The development of proanthocyanidins (condensed tannins) and other phenolics in bark of *Pseudotsuga menziesii* Franco. *Can. J. Bot.* (in press).
5. Stafford, H.A.; Lester, H.H. Proanthocyanidins (condensed tannins) in green cell suspension cultures of Douglas fir compared with those in strawberry and avocado leaves by means of C-18-reversed phase chromatography. *Plant Physiol.* 66:1085 (1980).
6. Stafford, H.A.; Lester H.H. Proanthocyanidins and potential precursors in needles of Douglas-fir and in cell suspension cultures derived from seedling shoot tissues. *Plant Physiol.* 68:1035 (1981).
7. Grisebach, H. Biosynthesis of flavonoids. *In*: Higuchi, T. (ed.) Biosynthesis and Biodegredation of Wood Components. Academic Press, New York. p. 291 (1985).
8. Heller, W.; Forkmann, G. Biosynthesis. *In*: Harborne, J.B. (ed.) The Flavonoids, Advances in Research Since 1980. Academic Press, New York, p. 399 (1988).
9. Ellis, C.J.; Foo, L.Y.; Porter, L.J. Enantiomerism: a characteristic of the proanthocyanidins of the monocotyledonae. *Phytochemistry* 22:483 (1983).
10. Neish, A.C. Major pathways of biosynthesis of phenols. *In*: Harborne, J.B. (ed.) Biochemistry of Phenolic Compounds. Academic Press, New York, p. 295 (1964).
11. Heller, W.; Hahlbrock, K. Highly purified 'flavanone' synthase from parsley catalyzes the formation of naringenin chalcone. *Arch. Biochem. Biophy.* 200: 617 (1980).
12. Hahlbrock, K.; Zilg, H.; Grisebach, H. Stereochemistry of the enzymatic cyclization of 4,2',4'-trihydroxychalcone to 7,4-dihydroxyflavanone by isomerases from mung bean seedlings. *Eur. J. Biochem.* 15:13, (1970).
13. Zapromotov, M.N.; Grisebach, H. Dihydrokaempferol as precursor of catechins in the tea plant. *Z. Naturforsch.* 28c, 113, (1973).
14. Jacques D.; Opie, C.T.; Porter, L.J.; Haslam, E. Plant proanthocyanidins. Part 4. Biosynthesis of procyanidins and observations on the metabolism of cyanidin in plants. *J. Chem. Soc. Perkin Trans. 1* :1637 (1977).
15. Stafford, H.A.; Shimamoto, M.; Lester, H.H. Incorporation of [^{14}C] phenylalanine into flavan-3-ols and procyanidins in cell suspension cultures of Douglas fir. *Plant Physiol.* 69:1055 (1982).
16. Stafford, H.A. Enzymic regulation of procyanidin biosynthesis: lack of flav-3-en-3-ol intermediate. *Phytochemistry* 22:2643 (1983).

17. Foo, L.Y. Configuration and conformation of dihydroflavonols from *Acacia melanoxylon*. *Phytochemistry* 26:813 (1987).

18. Jensen, R. Tyrosine and phenylalanine biosynthesis: relationship between alternative pathways, regulation and subcellular location. *In*: Conn, E.E. (ed.) The Shikimic Acid Pathway. Recent Advances in Phytochemistry. Plenum Publishing Co., New York, 20:57 (1986).

19. Jahnen, W.; Hahlbrock, K. Differential regulation and tissue-specific distribution of enzymes of phenylpropanoid pathways in developing parsley seedlings. *Planta* 173:453 (1988).

20. Knoblock, K.; Hahlbrock, K. 4-Coumarate:CoA ligase from cell suspension cultures of *Petroselinim hortsense*. Hoffm. Partial purification, substrate specificity, and further properties. *Arch. Biochem. Biophys.* 184:237 (1977).

21. Grand, C.; Boudet, A.; Boudet, A.M. Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems: properties and tissue distribution. *Planta* 158:225 (1983).

22. Stumpf, P.K. Biosynthesis of saturated fatty acids. *In*: Stumpf, P.K.; Conn, E.E. (eds.) The Biochemistry of Plants. Academic Press, New York, 9:121 (1981).

23. Finlayson, S.A.; Dennis, D.T. Acetyl-coenzyme A carboxylase from the developing endosperm of *Ricinus communis*. *Arch. Biochem. Biophys.* 225:586 (1983).

24. Beerhues, L.; Wiermann, R. Chalcone synthases from spinach (*Spinacea oleracea* L.I.) Purification, peptide patterns, and immunological properties of different forms. *Planta* 173:532 (1988).

25. Beerhues, L.: Robenek, H.; Wiermann, R. Chalcone synthases from spinach (*Spinacea oleracea* L.). II. Immunofluorescence and immunogold localization. *Planta* 173:544 (1988).

26. Koes, R.E.; Spelt, C.E. Mol. J.N.M.; Gerata, G.M. The chalcone synthase multigene family of *Petunia hybrida* (V30): sequence homology, chromasomal localization and evolutionary aspects. *Plant Molec. Bio.* 10:159 (1987).

27. Ryder, T.B.; Hedrick, S.A.; Bell, J.N.; Liang, X.; Clouse, S.D.; Lamb, C.J. Organization and differential activation of gene family encoding the plant defense enzyme chalcone synthase in *Phaseous vulgaris*. *Mol. Gen. Genet* 210:219 (1987).

28. Hrazdina, G.; Zobel, A.M.; Hoch, H.C. Biochemical, immunological, and immunocytochemical evidence for the association of chalcone synthase with endoplasmic reticulum membranes. *Proc. Natl. Acad. Sci.* 84:8966 (1987).

29. Ayabe, S.; Udagawa, A.; Furuya, T. NAD(P)H-dependent 6'-deoxychalcone synthase activity in *Glycyrrhiza* cells induced by yeast extract. *Arch. Biochem. Biophys.* 261:458 (1988).

30. Welle, R.; Grisebach, H. Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6'-deoxychalcone. *FEBS Lett.* 236:221 (1988).

31. Dixon, R.A.; Blyden, E.R.; Robbins, M.P.; Van Tunen, A.J.; Mol, J.N. Comparative biochemistry of chalcone isomerases. *Phytochemistry* 27:2801 (1988).

32. van Tunen, A.J.; Koes, R.E.; Spelt, C.E.; van der Krol, A.R.; Stuitje, R.; Mol, N.M. Cloning of two chalcone flavanone isomerase genes from *Petunia hybrida:* coordinate, light-regulated and differential expression of flavonoid genes. *EMBO J.* 7:1257 (1988).

33. Bednar, R.A.; Hadcock, J.R. Purification and characterization of chalcone isomerase from soybeans. *J. Biol. Chem.* 263:9582 (1988).

34. Haslam, E. Secondary metabolism - fact and fiction. *Natural Product Reports* 3:217 (1986).

35. Forkman, G.; Heller, W.; Grisebach, H. Anthocyanin biosynthesis of *Matthiola incana* flavanone 3- and flavonoid 3'-hydroxylases. *Z. Naturforsch.* 35c:691 (1980).

36. Stotz, G.; Forkmann, G. Hydroxylation of the B-ring of flavonoids in the 3'- and 5'-position with extracts from flowers of *Verbena hybrida*. *Z. Naturforsch.* 37c:19 (1982).

37. Britsch L.; Grisebach, H. Purfication and characterization of (2**S**)- flavanone-3-hydroxylase from *Petunia hybrida*. *Eur. J. Biochem.* 156:569 (1986).

38. Stotz, G.; de Vlaming, P.; Wiering, H.; Schram, A.W.; Forkmann, G. Genetic and biochemical studies on flavonoid 3'-hydroxylation in flowers of *Petunia hybrida*. *Theor. App. Gen.* 70:300 (1985).

39. Stafford, H.A.; Lester, H.H. Enzymic and non-enzymic reduction of (+)-dihydroquercetin to its 3,4-diol. *Plant Physiol.* 70:695 (1982).

40. Stafford, H.A.; Lester, H.H. Flavan-3-ol biosynthesis, The conversion of (+)-dihydromyricetin to its flavan-3,4-diol (leucodelphinidin) and to (+)-gallocatechin by reductases extracted from tissue cultures of *Ginkgo biloba* and *Pseudotsuga menziesii*. *Plant Physiol.* 78:791 (1985).

41. Botha, J.J.; Ferreira, D.; Roux, D. Synthesis of condensed tannins. Part 4. A direct biomimetic approach to [4,6]- and [4,8]-biflavanoids. *J. Chem. Soc. Perkin Trans. 1* :1235 (1981).

42. Porter, L.J.; Foo, L.Y. Leucocyanidin: synthesis and properties of (2**R**,3**S**,4**R**)-(+)-3,4,5,-7,3',4'-hexahydroxyflavan. *Phytochemistry* 21:2947 (1982).

43. Kristiansen, K.N. Biosynthesis of proanthocyanidins in barley: genetic control of the conversion of dihydroquercetin to catechin and procyanidins. *Carlsberg Res. Commun.* 49:503 (1984).

44. Kristiansen, K.N. Conversion of (+)- dihydroquercetin to (+)-2,3-*trans*-3,4-*cis*-leucoyanidin and (+)-catechin with an enzyme extract from maturing grains of barley. *Carlsberg Res. Commun.* 51:51 (1986).

45. Stafford, H.A.; Lester, H.H.; Porter, L.J. Chemical and enzymatic synthesis of monomeric procyanidins (leucocyanidins or 3',4',5',7- tetrahydroxyflavan-3,4-diols) from (2**R**, 3**R**)-dihydroquercetin. *Phytochemistry* 24:333 (1985).

46. Ishikura, N.; Murakami, H.; Fujii, Y. Conversion of (+)-dihydroquercetin to 3,4-*cis*-leucocyanidin by a reductase extracted from cell suspension cultures of *Cryptomeria japonica*. *Plant Cell Physiol.* 29:795 (1988).

47. Reddy, A.R.; L Britsch, F.; Salamini, F.; Saedler, H.; Rohde, W. The A1 (anthocyanin-1) locus in *Zea mays* encodes dihydroquercetin reductase. *Plant Sci.* 52:7 (1987).

48. Fischer, D.; Stich, K.; Britsch, L.; Grisebach, H. Purification and characterization of (+)-dihydroflavanol 4-reductase from flowers of *Dahlia variabilis*. *Arch. Biochm. Biophys.* 264:40 (1988).

49. Heller, W.; Forkman, G.; Britsch, L.; Grisebach, H. Enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols with flower extracts from *Matthioila incana* and its role in anthocyanin biosynthesis. *Planta* 165:284 (1985).

50. Schwarz-Sommer, Z.; Shepherd, N.; Tacke, E.; Gierl, A.; Rohde, W.; Leclercq, L.; Mattes, M.; Berndtgen, R.; Peterson, P.A.; Saedler, H. Influence of transposable elements on the structure and function of the A1 gene of *Zea mays*.. *EMBO J.* 6:287 (1987).

51. Meyer, P.; Heidmann, I.; Forkmann, G.; Saedler, H. A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature* 330:677 (1987).

52. Guyer, R.; Magnoloto, D.; Self, R. Glucosylated flavanoids and other phenolic compounds from sorghum. *Phytochemistry* 25:1431 (1986).

53. Porter, L.J. Flavans and proanthocyanidins. *In*: Harborne, J.B. (ed.) The Flavonoids, Advances in Research Since 1980. Chapman and Hall Ltd., London, pp. 21-62 (1988).

54. Stafford, H.A.; Lester, H.H. Flavan-3-ol biosynthesis: the conversion of (+)-dihydroquercetin and flavan-3,4-*cis*-diol (leucocyanidin) to (+)-catechin by reductase extracted from cell suspension cultures of Douglas-fir. *Plant Physiol.* 76:184 (1984).

55. Nonaka, G.; Goto, Y.; Kinjo, J.; Nohara, T.; Nishioka, I. Tannins and related compounds. LII. Studies on the constituents of leaves of *Thujopsis dolabrata* Sieb. et Zucc. *Chem. Pharm. Bull.* 35:1105 (1987).

56. Lundgren, L.N.; Theander, O. *Cis*- and *trans*-dihydroquercetin glucosides from needles of *Pinus sylvestris*. *Phytochemistry* 27:829 (1988).

57. Porter, L.J. Condensed tannins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, New York, (in press).

58. Roux, D.G.; Ferreira, D. α-Hydroxychalcones as intermediates in flavonoid biogenesis: the significance of recent chemical analogies. *Phytochemistry* 13:2039 (1974).

59. Roux, D.G.; Ferreira, D. Rationalization of divergent condensation sequences in flavanoid oligomerization. *Ann. Proc. Phytochem. Soc. Eur.* 25:221 (1985).

60. Gupta, R.K.; Haslam, E. Plant proanthocyanidins. Part 7. Prodelphinidins from *Pinus sylvestris*. *J. Chem. Soc. Perkin Trans. 1* :1148 (1981).

61. Delcour, J.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 9. The condensation sequence of leucocyanidin with (+)-catechin and with the resultant procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1711 (1983).

62. Hemingway, R.W.; Laks, P.E. Condensed tannins: a proposed route to 2**R**, 3**R**-(2,3-*cis*)-proanthocyanidins. *J. Chem. Soc. Chem. Commun.* :746 (1985).

63. Attwood, M.R.; Brown, B.R.; Lisseter, S.G. Spectral evidence for the formation of quinone methide intermediates from 5- and 7- hydroxyflavonoids. *J. Chem. Soc. Chem. Commun.* :177 (1984).

64. Stafford, H.A. Proanthocyanidins and the lignin connection. *Phytochemistry* 27:1 (1988).

65. Hemingway, R.W. Biflavonoids and proanthocyanidins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, New York (in press).

66. Porter, L.J.; Foo, L.Y.; Furneaux, R.H. Isolation of three naturally occurring 0-β-glucopyranosides of proanthocyanidin polymers. *Phytochemistry* 24:567 (1985).

67. Foo, L.Y.; Karchesy, J.J. Procyanidin dimers and trimers from Douglas-fir inner bark. *Phytochemistry* (in press).

68. Foo, L.Y.; Karchesy, J.J. Procyanidin polymers of Douglas-fir bark: structure from degration with phloroglucinol. *Phytochemistry* (submitted).

69. Hrazdina, G.; Wagner, G.J. Compartmentation of plant phenolic compounds: sites of synthesis and accumulation. *Ann. Proc. Phytochem. Europe* 25:119 (1985).

70. Ureta, T. The role of isozymes in metabolism: a model of metabolic pathways as the basis for the biological role of isozymes. *Current Topics in Enzyme Regulation* 13:233 (1978).

71. Friedrich, P. Supramolecular Enzyme Organization. Pergamon Press, Oxford (1984).

72. Welch, G.R.; Keleti, T. Is cell metabolism controlled by a 'molecular democracy' or by a 'supramolecular socialism'? *TIBS* 12:216 (1987).

73. Srivasta, D.K.; Bernhard, S.A. Metabolite transfer via enzyme-enzyme complexes. *Science* 234:1081 (1986).

74. Halbrock, K. Flavonoids. *In*: The Biochemistry of Plants. 7:425 (1981).

75. Santamour, F.S. Anthocyanidins of conelets in the Pinaceae. *Forest Science* 12:429 (1966).

76. Ching, K.K.; Aft, H.; Highley, T. Color variation in strobili of Douglas-fir. *In*: Proc. Western Forest Genetics Assoc. Olympia, WA, December 6-7. 37. (1965).

BIOGENESIS OF CONDENSED TANNINS – AN OVERVIEW

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ABSTRACT

There are currently four hypotheses as to how condensed tannins are produced in nature. In order to explain the 2,3-*cis* configuration of frequently encountered proanthocyanidin polymers, each of these biosynthetic proposals invoke isomerase enzymes or chemical intermediates not yet encountered in nature. Thus, substantial further study is required to clarify the pathways involved. An experimental program is proposed to achieve this objective.

INTRODUCTION

Perusal of the table of contents of this book should convince even the most dispassionate reader that there is a wide variety of aspects to the study of condensed tannins. Each individual reader will probably arrange the topics in some order of importance depending upon his or her orientation. The technologist might be mainly concerned with utilization of these materials; the organic chemist would concern himself with chemical structure; the biologist might pursue environmental interactions; and so on. It is quite likely that most would not place biosynthesis at the top of their lists. The convenors of the North American Tannin Conference, however, not only placed the papers on biosynthesis at the very beginning of the conference, but when they changed their role to editors of this book, they continued that same order of importance.

The reasons for this may not be self-evident considering the imperfect state of our knowledge of enzymic pathways and intermediates involved in the biogenesis of proanthocyanidin polymers. Nevertheless, a good case can be made for amplified attention to this subject. In the present chapter, the major yet-to-be solved biosynthesis problems will be discussed as well as the benefits that could accrue from their elucidation.

LIGNIN BIOGENESIS STUDIES AS A PRECEDENT FOR CORRESPONDING WORK ON CONDENSED TANNINS

The phenolic polymer, lignin, is the second most abundant polymer (cellulose is first) on our planet and is a key ingredient in the science of pulp and paper production, which, in turn, is one of the major manufacturing activities in North America. Thousands of investigations on the structure and reactions of lignin were conducted over the first half of this century, but they generated mostly heat and very little light. In the middle 1950s, Karl Freudenberg[1,2] showed that addition of mushroom phenoloxidase and hydrogen peroxide to an aqueous solution of coniferyl alcohol (**1**) yielded an isolated polymer remarkably similar to spruce lignin in composition, spectral properties, reactions, and degradation products. Subsequent work showed that the reaction involved coupling of quinone methides such as (**2**) to yield dimers (**3**), (**4**), and (**5**) that further polymerized to the "dehydrogenation polymers" (DHP) as they were designated (Figure 1).

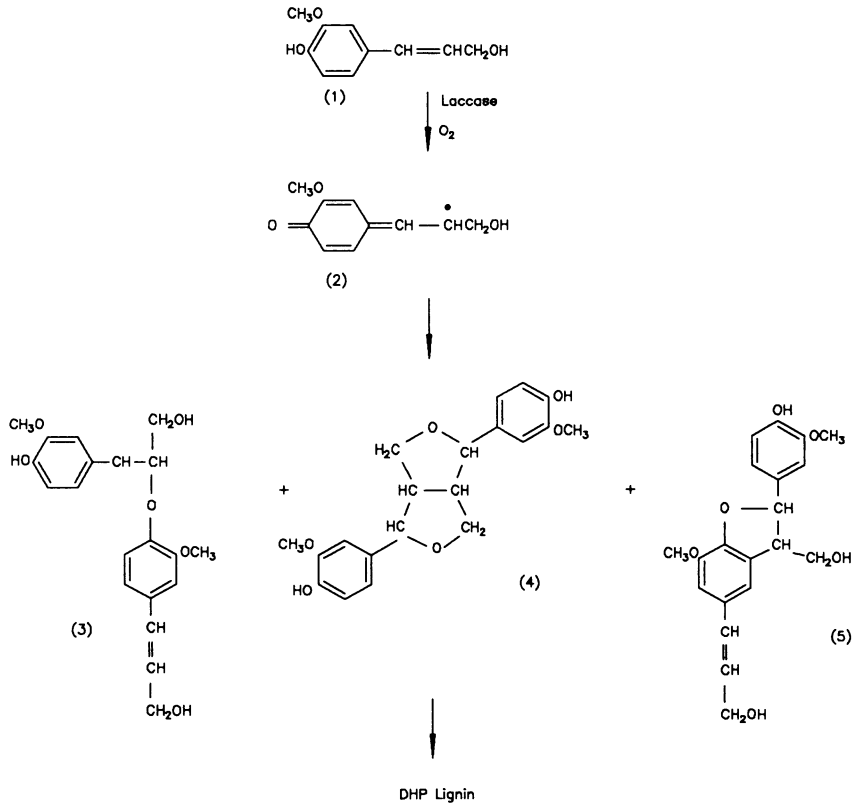

Figure 1. *In vitro synthesis of lignin by Freudenberg and coworkers.*

The presence of coniferin, the β-glucoside of (**3**), in the cambium of woody tissues adjacent to the zones where lignification takes place had been discovered many years earlier. The compound was extremely difficult to isolate and consequently not particularly suitable for large-scale in vitro experiments. The discovery in Freudenberg's laboratory of a practical synthetic method to produce coniferyl alcohol was, in retrospect, the probable key to the eventual success of the polymerization experiments.

Many lignin investigators were strongly influenced by this work. Some took the approach of examining the tree for the presence of intermediates,[3] especially compound (**3**), because it seemed to represent the primary structural unit in lignin. Other work involved the feeding of labelled compounds to tissues elaborating lignin and detailed comparisons of the degradation products from synthetic lignins with those from wood. A kind of interactive process in investigation can be discerned in which in vitro experiments seem to have spawned in vivo experiments. As new results from these were generated, alterations and amplifications to the in vitro approaches took place. This work is still continuing albeit with modern analytical techniques such as HPLC, FTIR, ^{13}C-NMR, etc. Most of what is known today about the structure of lignin is the result of this work. It is very doubtful that it would have been obtained without Freudenberg's initial series of "biomimetic" experiments; e.g., in vitro experiments carried out under physiological conditions replicating those presumed to exist in nature.

The early success of the biosynthetic approach to determining lignin structure lead to analogous studies on condensed tannins by the Freudenberg school, Hathway, Hasegawa, the author, and others.[4] Labelled *D*-catechin was biosynthesized from radioactive acetate and shikimic acid and fed to *Chamaecyparis pisifera* cuttings.[5] Some catechin was converted into tannins and highly polymerized substances deposited on the cell walls, but mainly it was concluded to be an end-product not converted into other flavonoids. Catechin and related flavanols were treated with various polyphenol oxidase or peroxidase enzymes to yield colored polymers with ultraviolet and visible spectra "identical" with isolated condensed tannins. These polymers generally contained quinone groups not present in carefully purified tannins.[4]

Several problems afflicted this early work. The invariable co-occurrence of large quantities of catechin (or related flavanols) with tannins had led to the conviction that catechin must be the precursor of the tannins. When the focus shifted in the early 1960s to flavan-3,4-diols as tannin precursors, the propensity of synthetic, monomeric flavan-3,4-diols to polymerize under very mild conditions resulted in abandonment of hypotheses that invoked enzyme mediation in the polymerization process. This is unfortunate because recent work has shown that the tannins have species- and organ-specific conformations and molecular weight distributions.[6] The whole process of synthesis, from initial precursor to ultimate polymer must, therefore, be under enzymic control. Repetition of the earlier in vitro work but with more appropriate substrates, such as Haslam's hypothesized flav-3-en-3-ol or α-hydroxychalcone intermediates,[7] could be very instructive (see below).

CURRENT HYPOTHESES OF CONDENSED TANNIN BIOSYNTHESIS

Speculation as to the mode of biosynthesis of condensed tannins has evolved, as might be expected, with new major pieces of information. The first of these was characterization of the low molecular weight compounds invariably co-occurring with the tannins, which yielded cyanidin or related compounds upon oxidative, acidic hydrolysis and were thought to have the structure of leucocyanidin [(**9**), Figure 2] or to be dimers with labile ether linkages. Work in a number of laboratories in the late 1960s – early 1970s established that these compounds were 4 → 6 or 4 → 8 carbon-carbon linked dimers as were the higher molecular weight polymeric tannins [(**16**), Figure 2]. The discovery of new chain cleavage techniques in the 1970s and the application of ^{13}C-NMR showed that the stereochemistries at the **2** or **3** carbons in the chain were often different from those of the terminal group. All of these observations have been consolidated into two major alternative biosynthetic sequences by Roux[7,8] and Haslam[9,10] with modifications subsequently proposed by Stafford[11,12] and Hemingway.[13]

These biosynthetic sequences were preceded by the work of a number of investigators who have demonstrated that plants incorporate acetate and shikimic acids into flavanones such as [(**6**), Figure 2], which cyclizes to the flavanone (**7**), adds a hydroxyl group to the 3′ position to form eriodictyol, which can subsequently be hydroxylated in the 3 position to give (**8**). The 3-hydroxy flavanones in nature invariably have a 2,3-*trans* stereochemistry that would be retained in the reduction step to flavan-3-ols (**10**) and flavan-3,4-diols (**9**). Roux[7] has suggested an α-hydroxychalcone intermediate (**12**) that could cyclize to either *cis* or *trans* forms of (**8**). Reduction of the subsequently formed flavan-3,4-diol (**9**) would yield the flavan-3-ol (**10**) of appropriate stereochemistry and could act as a nucleophile to terminate a polymerizing molecule of (**9**) giving dimers (**11**), trimers (**16**), and larger molecules.

Haslam[10,11] invokes the formation of a flavan-3-en-3-ol (**13**) that might be derived from the sequence (**12**) → (**8**) → (**9**) → (**13**). Stereospecific reduction would result in 2,3-*cis* or 2,3-*trans* carbocations (**14**) that would either polymerize to (**15**) or be further reduced into a pool of flavan-3-ols that would act as chain stoppers. Hemingway[14] questioned the presence of carbocations under mildly acidic to slightly alkaline pH, as later verified by Brown and coworkers,[15] and proposed the modification to Roux's and Haslam's schemes as shown in Figure 3. The 3-hydroxyflavanone (**8**) is enzymatically reduced to the flavan-3,4-diol (**9**), which then eliminates water to form a 2,3-*trans*-quinone methide (**17**).[13] This intermediate can polymerize to form a 2,3-*trans* polymer (**20**) or form Haslam's flav-3-en-3-ol intermediate (**10**) by an enzyme-mediated rearrangement parallel to the base-catalyzed tautomerism observed in the reactions of diarylpropanoids. Compound (**10**) can then be converted back into a 2,3-*cis*-quinone methide (**19**), which can polymerize to form polymers with *cis* stereochemistry.

Stafford[11,12] has postulated a C-3 epimerase, which acts on *trans* (**8**) to yield *cis* (**8**). This would then be reduced to the corresponding *cis* 3,4-flavan-diol (**9**) to give 2,3-*cis* polymers. Thus far, enzyme preparations from Douglas-fir callus

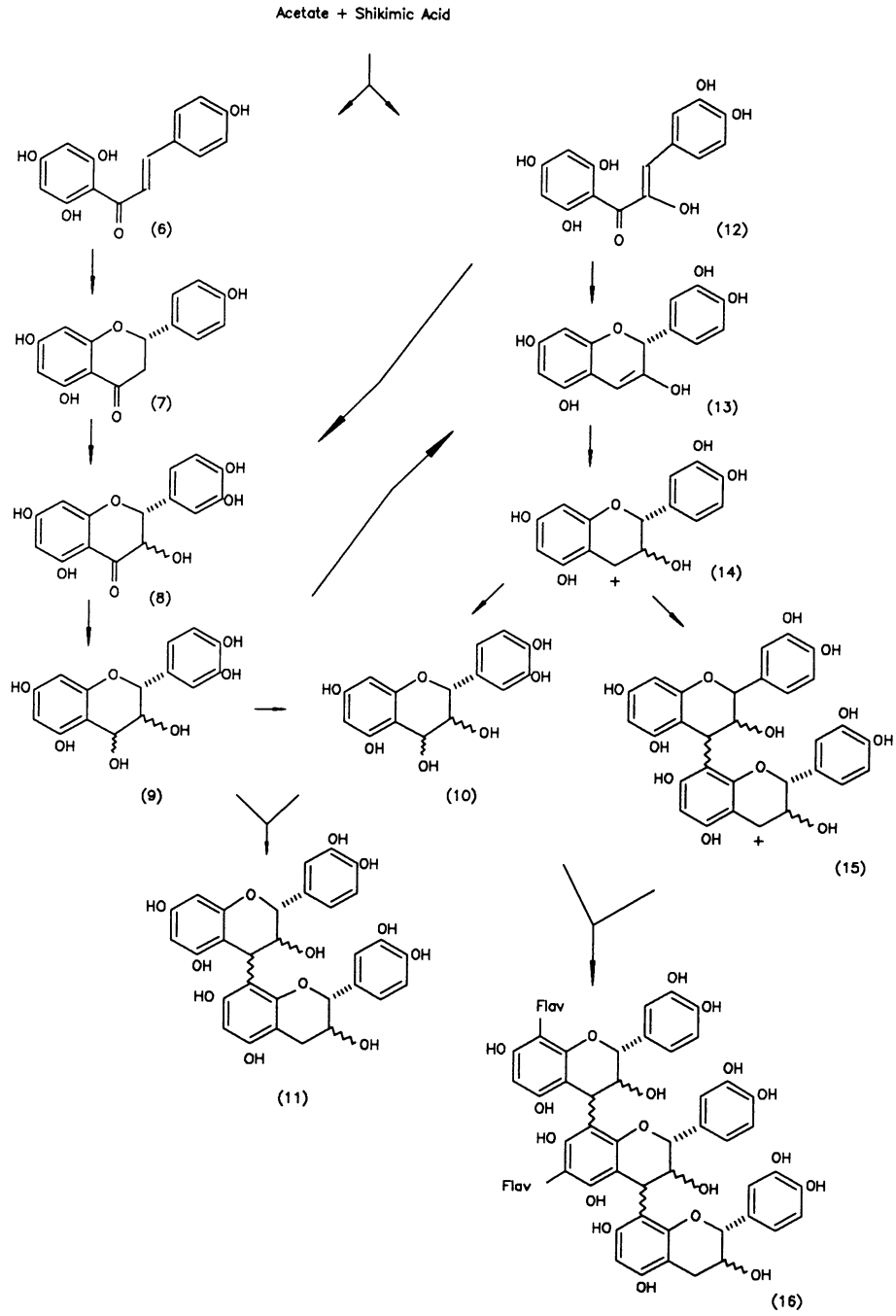

Figure 2. *Sequences proposed for the biosynthesis of condensed tannins.*

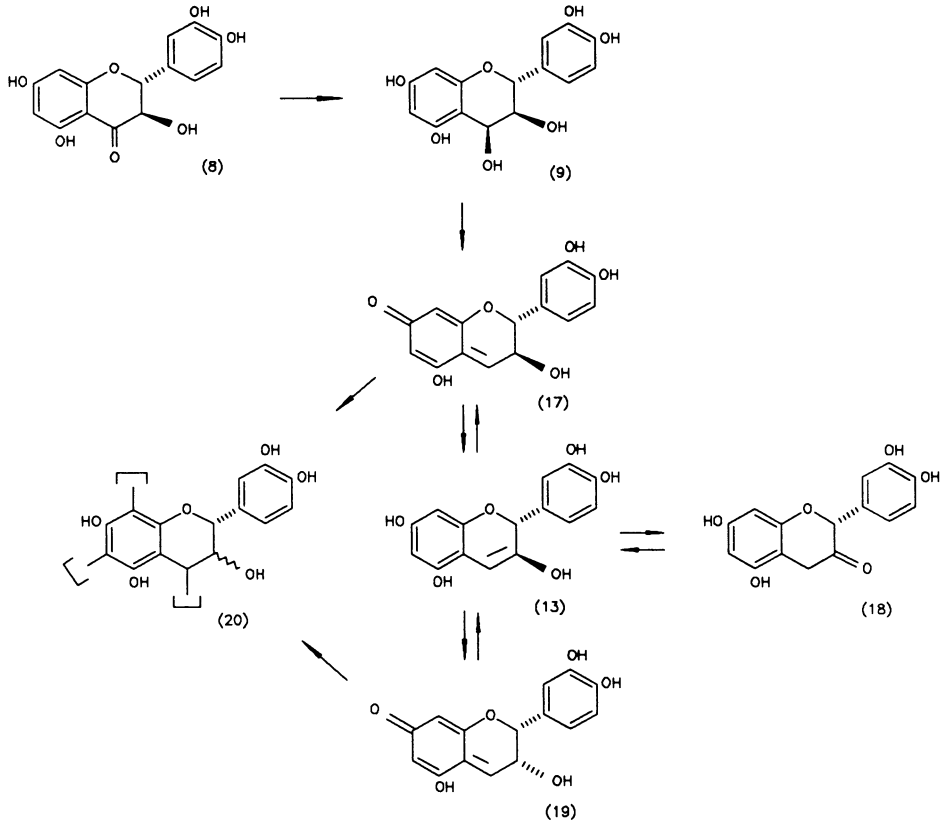

Figure 3. *Hemingway's route to account for control of 2,3-cis or 2,3-trans stereochemistry through quinone methide rearrangements.*

have not provided any 2,3-*cis* polymers. Furthermore, careful examination of the dihydroflavonol (**8**) in Douglas-fir wood or bark has failed to reveal even traces with 2,3-*cis* stereochemistry. Wong and Birch[16] have demonstrated that labelled aromadendrin, a 3-hydroxyflavanone of 2,3-*trans* stereochemistry, when fed to raspberry (*Rubus idaeus*) stems elaborated the flavanol (-)-epicatechin (2,3-*cis* stereochemistry). It may be, therefore, that Douglas-fir callus employed by Stafford lost the epimerase originally present in the Douglas-fir tissue from which the callus cells were cultured.

INDICATIONS FOR FUTURE WORK

Ferreira, Haslam, Stafford, Lewis, and Hemingway were present at a round table discussion at the North American Tannin conference to describe their respective views of procyanidin biosynthesis. It is believed that the sequences in Figures 2 and 3 represent each of their current views. All agreed that there was a lack of

hard data to establish rigorously which of the biogenetic schemes is correct. The possibility of multiple mechanisms was discussed including postmortem oxidative reactions, which are not included in this overview. The panelists and some in the audience disagreed with Stafford's designation of flavan-3-ols as chain initiators. The reason for this is that no mechanism has been proposed for how these compounds might initiate polymerization. Labelling experiments strongly suggest that these compounds are formed in a second metabolic pool at a much slower rate than the main polymeric units. Furthermore, a growing number of oligomeric proanthocyanidins with "terminal" units other than flavan-3-ols are now being isolated from plants (Chapter 5). Thus, it is much more probable that they are chain terminators rather than initiators.

Haslam has at various times expressed a philosophy that should be heeded by all workers who propose biosynthetic sequences. Briefly stated, it is that suggested biochemical conversions should be demonstrably shown to be possible on solid chemical grounds. Keeping this and the foregoing biosynthetic schemes in mind, the following experiments are suggested.

1. Tissues in the process of elaborating procyanidin polymers should be carefully searched for the presence of proposed intermediates in the process, in particular compounds of types (**6**), (**12**), and (**13**). As already noted, *cis*-3-hydroxyflavanones should be looked for. Out of the more than 40 genera from which dihydroflavonols had been reported up to 1988, all were of *trans* configuration[17] except 3-*O*-methyl-2,3-*cis*-fustin from *Peltogyne*[18] and 2,3-*cis*-3′,4′,7,8-tetrahydroxydihydroflavonol from *Acacia melanoxylon* heartwood.[19] Both of these are 5-deoxy flavonoids so their finding is problematical with regard to the yet-to-be found *cis*-dihydroquercetin, (**8**) as the aglycone. Lundgren and Theander[20] have recently isolated small amounts of 2,3-*cis*-dihydroquercetin glucosides together with much larger amounts of the 2,3-*trans* isomers from *Pinus sylvestris* needles. Perhaps this report will be a precursor of many more observations of 2,3-*cis*-dihydroflanonols. Foo[19] notes that the 2,3-*cis* compound from *Acacia* is relatively unstable compared to the *trans* isomer. Nevertheless, he favors an α- or β-hydroxylase for stereospecific hydroxylation of the flavanone at C-3 and rejects Haslam's flav-3-en-3-ol hypothesis for biosynthesis of proanthocyanidins.

2. Attempts should be made to polymerize chalcones, α-hydroxychalcones or reduced forms of cyanidin such as (**13**) directly to procyanidins by the action of an isolated enzyme (polyphenol oxidase?). Many of the woody species of conifers seem to elaborate procyanidins and flavan-3-ols without having any co-occurring dihydroflavonols in the vicinity.

3. Procyanidin polymers need to be examined for other chain terminators than the flavan-3-ols. There are some reports of oligomers and some suggestions from spectral investigations of crude tannin fractions that dihydroquercetin (**8**), phloroglucinol, and substituted stilbenes or stilbene glucosides may also serve as nucleophilic chain-stoppers (Chapters 1, 5, and 6).

4. Extrapolating results from Sephadex LH-20 fractionation of crude polymers needs to be done with caution since the resultant fractions eluted from columns may give an oversimplified picture of the tannins because they represent only a part of the original mixture. At the Tannin Conference, Hemingway made note of

this deficiency in current procyanidin analytical practice, and this author believes his point is well taken.

5. Much more information is needed on the uniformity (or lack thereof) of stereochemistry in polymers isolable from different types of cells in the same part of the plant. For instance, are the tannins that are synthesized in parenchma cells the same as those formed in tracheids of the xylem? Are the procyanidins biosynthesized at the heartwood-sapwood boundary the same as those present in the sapwood? Similarly, are the procyanidins in the periderm identical with those in the phloem? And, what about the large amounts of organic solvent-insoluble polymers in (on?) the cells of outer phloem or in the rhytidome? Structural differences in the polymers may provide much additional insight about biological mechanisms.

It seems to this author that substantial benefits would accrue to the whole field of condensed tannins if we had more thoroughly substantiated biosynthetic mechanisms to undergird our understanding of these materials. Admittedly, the discipline is difficult, and the work tends to be ignored by structural organic chemists. It can be said to its advantage, however, that the field is not overcrowded, and each new investigation can contribute significantly to the work already conducted by dedicated workers such as Stafford (Chapter 3).

CONCLUSIONS

The sequences involved in the biosynthesis of procyanidins have been speculated upon and partly proven by the work of Stafford, Haslam, Roux, Hemingway, and others. The biggest deficiency is solid demonstration of how *cis* configuration can be initiated in the polymers. Most of the current schemes invoke the mediation of dihydroflavonols, which are invariably of *trans* configuration in nature. Further advances may be inhibited by convictions as to the role of the dihydroflavonols. Further progress might well be achieved by going further back in the proposed sequences to chalcones, flav-en-ols, or related compounds and performing polymerization experiments in vitro with isolated enzymes.

REFERENCES

1. Freudenberg, K.; Neish, A.C. Constitution and Biosynthesis of Lignin. Springer-Verlag, Heidelberg. pp 82-97 (1968).
2. Sarkanen, K.V. Precursors and their polymerization. *In*: Sarkanen, K.V.; Ludwig, C.H. (eds) Lignins. Wiley-Interscience, New York. pp. 95-163 (1971).
3. Goldschmid, O.; Hergert, H.L. *TAPPI* 44(12):858 (1961).
4. Hergert, H.L. Economic importance of flavonoid compounds: wood and bark. *In*: Geissman, T.A. (ed.) The Chemistry of Flavonoid Compounds. Pergamon Press, Oxford. pp 553-592 (1962).
5. Hasegawa, M.; Hasue, M. *J. Jap. For. Soc.* 44(9):244 (1962).
6. Samejima, M.; Yoshimoto, T. Systematic studies on the stereochemical composition of proanthocyanidins from coniferous bark. *Mokuzai Gakkaishi* 28(1):67 (1982).
7. Roux, D.G.; Ferreira, D. α-hydroxychalcones as intermediates in flavanoid biogenesis: the significance of recent chemical analogies. *Phytochemistry* 13:2039 (1974).
8. Hillis, W.E. Biosynthesis of tannin. *In*: Higuchi, T. (ed.) Biosynthesis and Biodegradation of Wood Components. Academic Press, New York, pp. 325-347 (1985).

9. Haslam, E. Proanthocyanidins. *In*: Harborne, J.B.; Mabry, T.J. (eds.) The Flavonoids: Advances in Research. Chapman and Hall, London. pp. 417-447 (1982).

10. Platt, R.V.; Opie, C.T.; Haslam, E. Plant proanthocyanidins. Part 8. Biosynthesis of flavan-3-ols and other secondary plant products from 2S-phenylalanine. *Phytochemistry* 23:2211 (1984).

11. Stafford, H.A. Enzymic regulation of procyanidin biosynthesis: lack of a flav-3-en-3-ol intermediate. *Phytochemistry* 22:2643 (1983).

12. Stafford, H.A.; Lester, H.H.; Porter, L.J. Chemical and enzymatic synthesis of monomeric procyanidins (leucocyanidins or 3',4',5,7-tetrahydroxyflavan-3,4-diols) from (2R,3R)-dihydroquercetin. *Phytochemistry* 24:333 (1985).

13. Hemingway, R.W., Laks, P.E. Condensed tannins: a proposed route to 2R,3R-(2,3-*cis*)-proanthocyanidins. *J. Chem. Soc. Chem. Commun.* :746 (1985).

14. Hemingway, R.W., Foo, L.Y. Condensed tannins: quinone methide intermediates in procyanidin synthesis. *J. Chem. Soc. Chem. Commun.* :1035 (1983).

15. Attwood, M.R., Brown, B.R., Lisseter, S.G., Torrero, C.L., Weaver, P.M. Spectral evidence for the formation of quinone-methide intermediates from 5- and 7-hydroxyflavonoids. *J. Chem. Soc. Chem. Commun.* :177 (1984).

16. Wong, E.; Birch, E.J. *J. Chem. Soc. Chem. Comm.* :979 (1975).

17. Bohm, B.A. The minor flavonoids. *In*: Harborne, J.B.; Mabry, T.J. (eds.) The Flavonoids: Advances in Research. Chapman and Hall, London. pp 313-416 (1982).

18. Malan, E.; Roux, D.G. (+)-trans-Pubeschin, the first catechin analogue of peltygynoids from *Peltogyne pubescens* and *P. venosa*. *Phytochemistry* 13:1575 (1974).

19. Foo, L.Y. Configuration and conformation of dihydroflavonols from *Acacia melanoxylon*. *Phytochemistry* 26(3):813 (1987).

20. Lundgren, L.N., Theander, O. *Cis* and *trans*-dihydroquercetin glucosides from needles of *Pinus sylvestris*. *Phytochemistry* 27:829 (1988).

Structure

STRUCTURAL VARIATIONS IN PROANTHOCYANIDINS AND THEIR DERIVATIVES

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ABSTRACT

Structural complexity in the condensed tannins (polymeric proanthocyanidins) is centered principally on variations in hydroxylation patterns of the flavan chain extender units, the stereochemistry at the three chiral centers of the heterocyclic ring, the location and type of interflavanoid bond, and the structure of the terminal unit. Superimposed on these basic structural features are derivatizations such as *O*-methylation, *C*- and *O*-glycosylation, and *O*-galloylation. Because of the strong nucleophilicity of the resorcinolic or phloroglucinolic A-rings, some unusual conjugates linked through the C-6 or C-8 positions have also been found. A further level of structural diversity results from facile rearrangements of these compounds. Although great progress has been made in elucidation of the structure of oligomeric proanthocyanidins, our knowledge of the structure of higher polymers rests primarily on interpretations of their ^{13}C-NMR spectra.

INTRODUCTION

Our knowledge of the structure of condensed tannins is based primarily on the structures of oligomeric proanthocyanidins of which over 100 compounds, ranging from dimers to hexamers, have now been described.[1,2] The hypothesis that condensed tannins are equivalent to polymeric proanthocyanidins has been supported in a sufficient number of studies of different tannin isolates that the structures of the higher polymers are often projected based on study of the low molecular weight oligomers.[3] At times, this projection has been made without sufficient caution. There are examples where the structural features of the low molecular weight

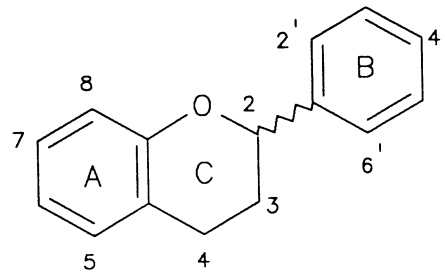
oligomers are not found in the higher polymers isolated from the same plant extracts. Unfortunately, our current state of knowledge is such that there usually is no other choice but to make predictions from the structures of low molecular weight proanthocyanidins. Therefore, this review must of necessity deal in large measure with the structural variations that have been shown to exist in the oligomeric proanthocyanidins. Projections from the features of oligomers will usually not lead to error because most data that have been obtained from studies of the higher polymers indicate that the structures of the oligomers are reasonable reflections of the essential structural features of the higher polymers.

Although the past 15 years has also seen important advances in our understanding of the higher polymers, these studies have been largely confined to polymers of the procyanidin and prodelphinidin classes.[4] Most important to this progress has been the application of ^{13}C-NMR spectral analyses that have been used to define the hydroxylation patterns of the A- and B-rings, the stereochemistry of the heterocyclic rings, the degree of branching within the polymer, and the number average molecular weight.[3–7] As will be seen in Chapter 6 by Mattice and Chapter 9 by Ferreira, much attention is currently being directed to the conformation of these polymers.[8,9] These studies, like applications of ^{13}C-NMR, rest on definitive results on the structures of the oligomers. Therefore, what might seem to be a preoccupation with the structures of oligomers is clearly justified considering our present state of knowledge of these polymers. More attention needs to be given to structure elucidation of the higher polymers.

NOMENCLATURE

The vegetable tannins are made up of two groups of polyphenolic polymers. The condensed tannins that are flavanoid polymers are the compounds reviewed here. The hydrolyzable tannins give gallic and/or ellagic acids on acid hydrolysis. The condensed tannins are further classified on the basis of the hydroxylation patterns of the A- and B-rings. The propelargonidins (**1**), procyanidins (**2**), and prodelphinidins (**3**) all carry phloroglucinolic A-rings in the chain extender units and are named for the anthocyanidins generated on treatment with acid (Figure 1). Other types of oligomeric flavanols (i.e., B → B or B → A linked oligomers) also belong to the condensed tannins but may not be called proanthocyanidins so the terms are not synonymous. The tannins isolated from most plants are made up of one or more of the above classes of proanthocyanidins. The 5-deoxy analogs, with resorcinolic A-rings in the chain extender units, are the proguibourtinidins (**4**), profisetinidins (**5**), and prorobinetinidins (**6**). They are named for their corresponding flavan-3,4-diols. Other classes of proanthocyanidins are the proteracacidins (**7**) and promelacacidins (**8**). These two classes of compounds are rarely found. The profisetinidins and prorobinetinidins are likewise restricted to plants in the Leguminosae and Anacardiaceae. Proanthocyanidins lacking a hydroxyl group at C-3 such as the proapigeninidins (**9**) and proluteolinidins (**10**) are rare.

A variety of systems has been used to name various compounds within these classes. They range from the trivial notation of B-1 to B-8 etc. for the various procyanidins, first introduced by Weinges[10,11] and extended by Haslam,[12–14] to the use of formal IUPAC nomenclature that defines the absolute stereochemistry at



Class	Hydroxylation Pattern	Compound No.
Propelargonidin	3,4',5,7	(**1**)
Procyanidin	3,3',4',5,7	(**2**)
Prodelphinidin	3,3',4',5,5',7	(**3**)
Proguibourtinidin	3,4',7	(**4**)
Profisetinidin	3,3',4',7	(**5**)
Prorobinetinidin	3,3',3',5',7	(**6**)
Proteracacidin	3,4',7,8	(**7**)
Promelacacidin	3,3',4',7,8	(**8**)
Proapigeninidin	4',5,7	(**9**)
Proluteolinidin	3',4',5,7	(**10**)

Figure 1. *Variation in hydroxylation patterns of principal classes of condensed tannins.*

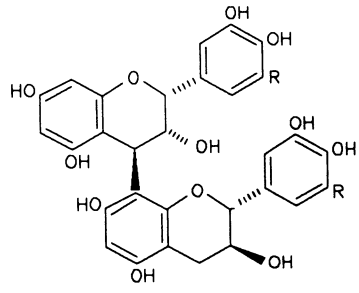
all chiral centers, such as was generally used by Roux's school.[*eg.*15,16] Assignment of the absolute stereochemistry through sequence rules for **R** and **S** can become extremely difficult in some of the higher oligomers. In an attempt to simplify and yet preserve accuracy in definition of the stereochemistry, a nomenclature analogous to the IUPAC system for naming of carbohydrates was developed.[17] In this system, the basic structural units are named from their familiar monomeric flavan-3-ols for the 2**R** isomers, and the prefix *ent-* is used for the less common 2**S** isomers. The location and stereochemistry (α or β) of the interflavanoid bond are denoted within brackets as in the carbohydrates. With this system, the procyanidin B-1 is named epicatechin-(4β → 8)-catechin (**11**), the analogous prodelphinidin is named epigallocatechin-(4β → 8)-gallocatechin (**12**), the corresponding 2**S** enantiomer is named *ent*-epicatechin-(4α → 8)-*ent*-catechin (**13**), and the angular trimers are named such as fisetinidol-(4α → 8)-catechin-(6 → 4β)-fisetinidol (**14**) (Figure 2). Porter[2] has recently suggested a simplification for naming the A-type linked compounds as epicatechin-(2β → 7;4β → 8)-catechin rather than as epicatechin-(4β → 8:2β → O → 7)-catechin, for example, for the dimer (**15**) that is a major constituent of peanut skins.[18] This nomenclature is used throughout this chapter.

At the North American Tannin Conference, from which this volume was derived, there was considerable discussion, stimulated by Professor Haslam, about the proper terminology to describe these compounds. It is true that many, if not

most, of the compounds being isolated today do not give anthocyanidins on treatment with acid and, hence, are not appropriately named proanthocyanidins. In this chapter, these compounds are included in a section titled proanthocyanidin derivatives, since most of these compounds are derived from proanthocyandins. Perhaps, polyflav(a)noids is a more accurate term with the term polyflav(o)noids being reserved for the flavonoid oligomers dominated by compounds with a carbonyl at the C-4 position. As progress continues to be made in elucidation of new compounds, the problem of definition of compound classes with appropriate terminology will no doubt continue to plague us.

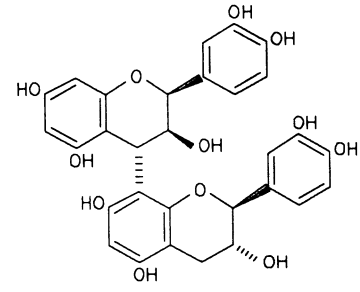
OLIGOMERIC PROANTHOCYANIDINS

Detailed accounts of all known oligomeric proanthocyanidins and their distribution in plants have recently been prepared and should appear in print simultaneously with this book.[1,2] The present chapter focuses on some of the more interesting structural variations that have been found in these compounds and is not meant



(11) R = H

(12) R = OH



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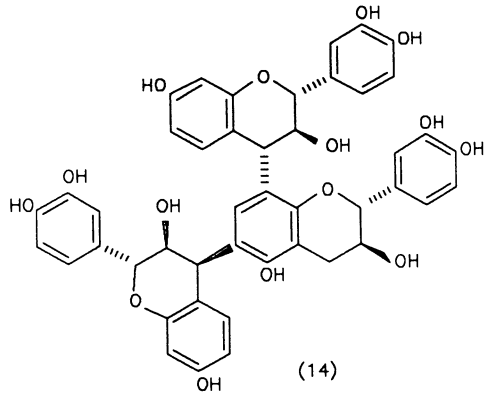
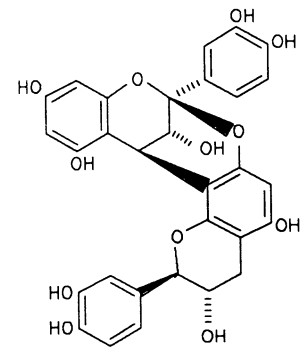


(14)



(15)

Figure 2. *Structures of some common proanthocyanidin oligomers used to illustrate nomenclature* (**11**) through (**15**).

to be a thorough treatment of all known compounds. In addition to the recent chapters by Hemingway[1] and Porter,[2,3] earlier reviews[11–16] of these compounds are recommended reading for those embarking on study of the chemistry of condensed tannins. The biflavonoids[19] and isoflavonoids[20–22] are not covered in this chapter.

Hydroxylation Patterns

Propelargonidins. The oligomeric 3,4′,5,7-tetrahydroxyflavans, Figure 1-(**1**), are comparatively rare. Eleven oligomers have been described as natural products. Afzelechin dimers terminated with afzelechin, catechin, and epicatechin have recently been isolated from *Kandelia candel* bark.[23] An epiafzelechin-(4β → 8)-catechin dimer was isolated from *Uncaria gambir* where it occurs in low concentration together with the unusual C-2 substituted "gambirins" discussed more fully below.[24] Two epiafzelechin-4-*O*-methylgallocatechin dimers have been isolated from the root bark of plants in the genus *Elaeodendron*.[25,26] Also, two *ent*-epiafzelechins linked through (2β → 7;4β → 8) interflavanoid bonds have been isolated from roots of peach trees.[27,28] They are of particular interest because of their phytotoxicity toward rice seedlings. *Ephedra* sp. roots also contain interesting mixtures of 2**R** and 2**S** epiafzelechin dimers linked with the A-type linkages at both the C-8 and C-6 positions (**16**) and (**17**) (Figure 3). Further diversity of these compounds occurs in the location of the interflavanoid bond and the type of terminal unit [cf. the epiafzelechin unit in (**16**) and a kaempferol unit in (**17**)[29–31]]. Work on definition of the structure of these compounds emphasizes the importance of the new NMR experiments to progress in elucidation of their structures.

Procyanidins. The 3,3′,4′,5,7-pentahydroxyflavans, Figure 1-(**2**), are the proanthocyanidins found most widely in the plant kingdom. About 50 procyanidins ranging from dimers to hexamers have now been described.[1,2] Most of the natural compounds isolated to date have 2**R**,3**R** (2,3-*cis*) chain extender units. These compounds are common in the barks of woody plants. Nearly all of these compounds are terminated with either a catechin or epicatechin unit. *Wisteria* sp. contains the unusual catechin-(4α → 8)-epiafzelechin dimer (Figure 3-**18**).[32] An epicatechin-(4β → 8)-epiafzelechin dimer has also been recently isolated from *Saraca asoca*.[33] Although mixtures of procyanidins and prodelphinidins as well as oligomers with both types of chain extenders in the same molecule have been isolated as natural products (**19**), to date, no procyanidin has been isolated with a gallocatechin terminal unit.[34] These compounds contain a number of important structural variations that are discussed below.

Prodelphinidins. The 3,3′,4′,5,5′,7-hexahydroxyflavans, Figure 1-(**3**), are the major constituents in the leaves[35] of conifers, whereas, the procyanidins predominate in bark.[36] Prodelphinidins are also found in the male flowers of gymnosperms and angiosperms.[37,38] They are important commercially because of their presence in barley and their influence on the flavor and solution properties of beer.[39,40] The prodelphinidins are often found with galloyl substituents (**20**) as in the extracts from *Myrica* and *Quercus* species used as tanning agents in Asia (Figure 3).[41–44]

The prodelphinidins are commonly terminated with catechin rather than gallocatechin or epigallocatechin.

Proguibourtinidins. The 3,4′,7-trihydroxyflavans, Figure 1-(**4**), are comparatively rare, having been isolated from *Acacia luderitzii*,[45] *Julbernadia globiflora*,[46] *Cassia fistula*,[47] and most recently from *Colophospermum mopane* that also contains much higher proportions of profisetinidins.[48] The proguibourtinidins in *Acacia luderitzii* (**21**) are unusual in the occurrence of derivatives carrying C-6 and C-8 carboxylic acid functions[45] (Figure 4). Oligomers isolated from *Guibourtia coleospermum* are terminated with a stilbene rather than a flavan-3-ol (**22**).[49] The stereochemistry and conformation of these compounds (linked through C-4 of the

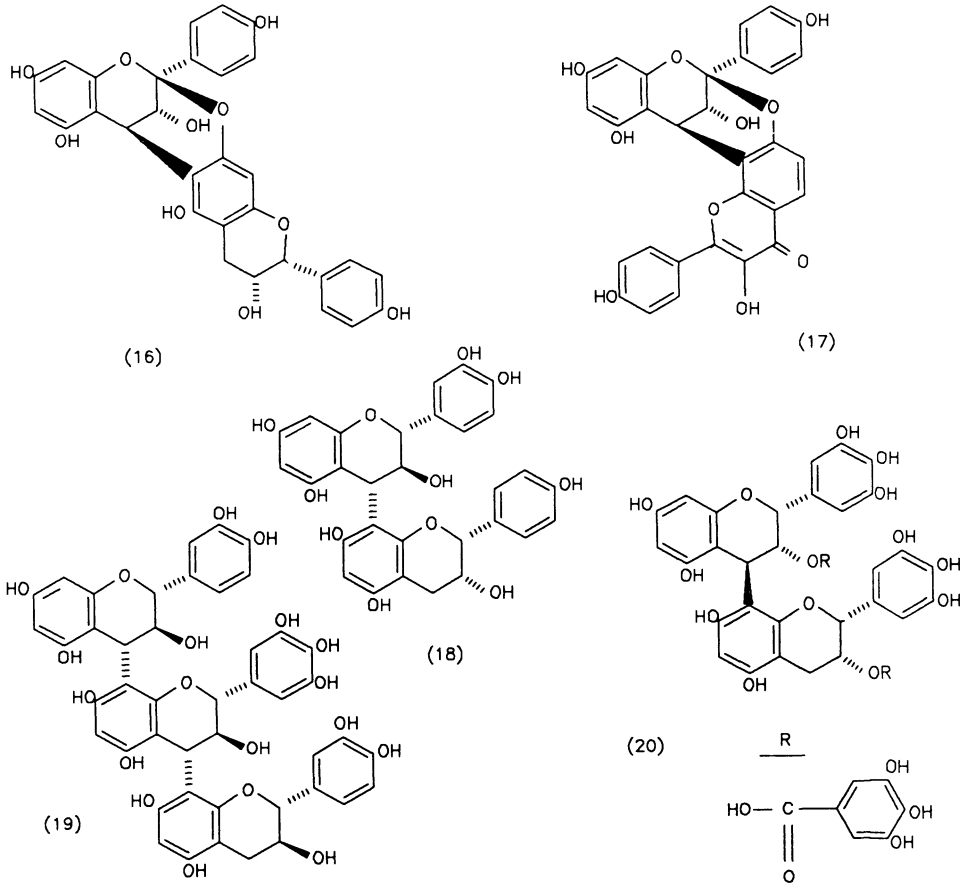


Figure 3. *Examples of structural variation in the propelargonidins, procyanidins, and prodelphinidins* (**16**) through (**20**).

flavan to the C-2 and C-6 positions on the 3,5-dihydroxy ring of the stilbene) have recently been studied by ^{1}H-n.O.e. difference spectroscopy.[50]

Profisetinidins. Although predominantly restricted to plants in the Leguminosae and Anacardiaceae, the 3,3′,4′,7-tetrahydroxyflavans, Figure 1-(**5**), are among the best known proanthocyanidins and represent a wide range of structures.[15,16,51–57] Approximately 40 compounds are known, largely through the efforts of Dr. David G. Roux and his colleagues at the University of the Orange Free State, Bloemfontein, South Africa. Profisetinidin dimers and trimers isolated from *Colophospermum mopane* and *Acacia mearnsii* are of special interest because some of these compounds are terminated with the corresponding flavan-3,4-diols

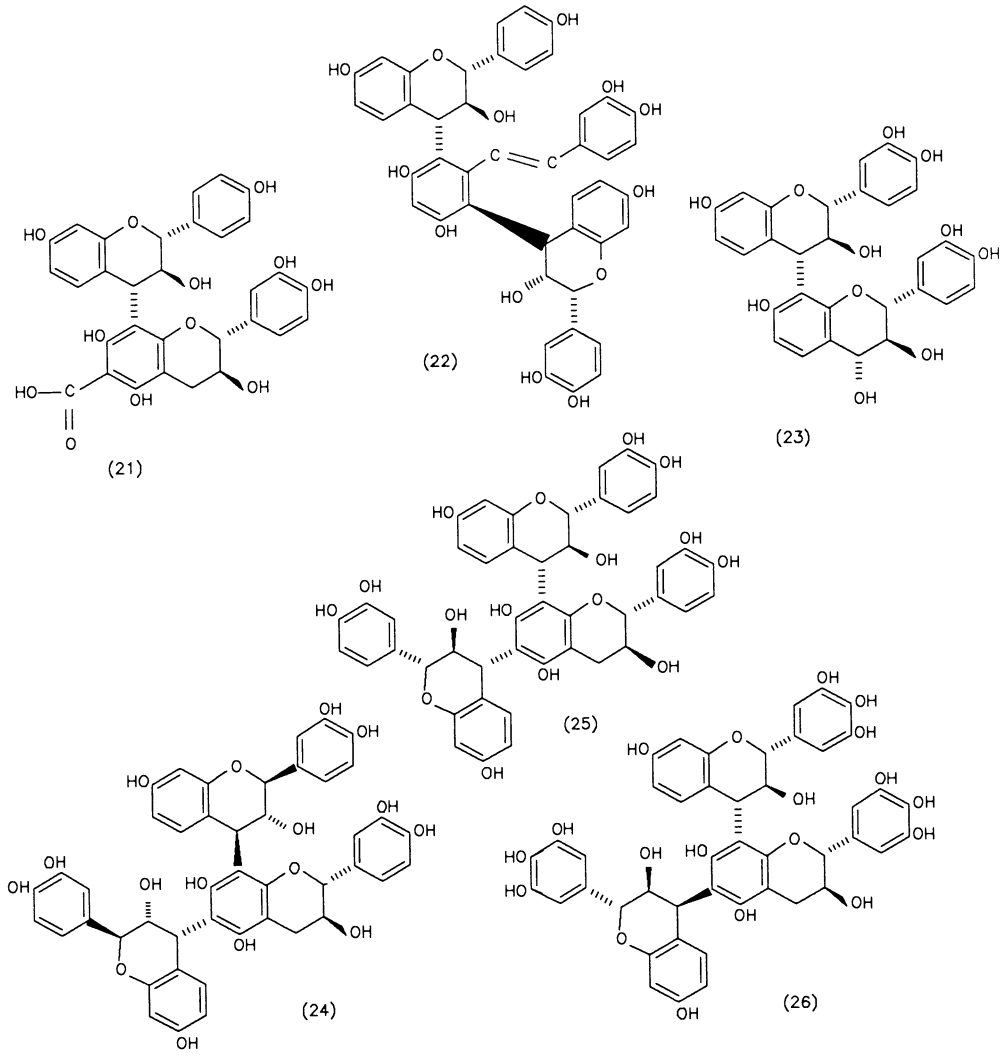


Figure 4. *Examples of structural variation in the proguibourtinidins, profisetinidins, and prorobinetinidins* (**21**) through (**26**).

(Figure 4, **23**).[55] Conformational analyses by ^{1}H-n.O.e. difference spectroscopy of methylether acetate derivatives of angular profisetinidin tetramers reveal cyclo-conformations of these oligomers.[56] The 2**S** profisetinidins (**24**) represent the commercially important "quebracho" (*Schinopsis*),[57] and the 2**R** analogues (**25**) represent the tannins in black wattle (*Acacia mearnsii*) heartwood.[15,52] The heartwood of *Colophospermum mopane* contains profisetinidin dimers with both 2**R**,3**S** (-)-fisetinidol and 2**S**,3**S** (+)-epifisetinidol terminal units as well as a fisetinidol-(4α $\rightarrow$ 8)-afzelechin dimer.[48] The heartwood of *Acacia baileyana* var *purpurea* does not contain only profisetinidins, but rather a mixture of profisetinidins, procyanidins, and prodelphinidins.[58]

Prorobinetinidins. The (3,3′,4′,5′,7-pentahydroxyflavans, Figure 1-(**7**) also occur in *Acacia mearnsii*, but in the bark.[59,60] Compounds terminated with either (+)-catechin or (+)-gallocatechin (**26**) are present in the same extract (Figure 4).[59] These compounds represent the structure of the commercially important "wattle" or "mimosa" tannins used in leather manufacture[61] and wood adhesives.[62]

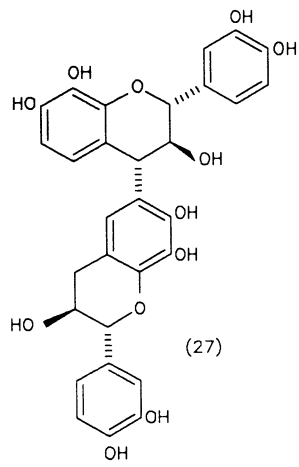
Proteracacidins and Promelacacidins. Two proteracacidin dimers have been synthesized, but there are no known natural compounds based on 4′,7,8-trihydroxyflavans, Figure 1-(**7**).[53,59] Only a few promelacacidins with the 7,8,3′,4′-tetrahydroxyflavan structure are known, Figure 1-(**8**). A mesquitol-(4α $\rightarrow$ 6)-mesquitol (i.e., a melacacidin named "prosopin" in the nomenclature proposed by Porter[2]) dimer (**27**) has been isolated from the heartwood of *Prosopis glandulosa* "mesquite" where it occurs in small amounts together with large quantities of oxidatively coupled compounds that will be discussed later (Figure 5).[63] Foo[64] isolated an epimesquitol-(4α $\rightarrow$ 6)-epimesquitol-4α-ol (alternatively named epiprosopin by Porter[2]) dimer (**28**) from *Acacia melanoxylon* that is the only natural product that has the 2**R**,3**R**,4**R**- (2,3-*cis*-3,4-*cis*)-stereochemistry.

Proapigeninidins and Proluteolinidins. Although apparently rare, proanthocyanidins with 3-deoxyflavan chain extender units have been isolated as well. A proapigeninidin with a (2β $\rightarrow$ 7;4α $\rightarrow$ 8)interflavanoid bond (**29**) has been isolated from *Ephedra* roots[31] and a series of proluteolinidin-*O*-glycosides (**30**) has been isolated from *Sorghum vulgare*.[65]

Stereochemistry

The three chiral centers in the heterocyclic ring lead to much of the complexity (and fun) involved in the study of these compounds. Most of the proanthocyanidins isolated from plants are of a 2**R** absolute stereochemistry. The exceptions (2**S**) include the proanthocyanidins from many monocotyledonous plants,[66] and those from *Uncaria*,[24] *Polygonum*,[67] *Rapheolepsis*,[68] *Rhus*,[69] as well as the commercially important "quebracho" (*Schinopsis*) tannins[57] among the dicotyledonous plants.

Nearly all proanthocyanidins of the 5-deoxy classes including the proguibourtinidins, profiesetinidins, prorobinetinidins, and the promelacacidins, with the exception of Foo's compound, have 2,3-*trans* stereochemistry in the chain extender units.



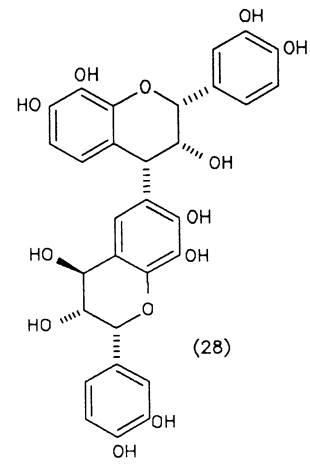
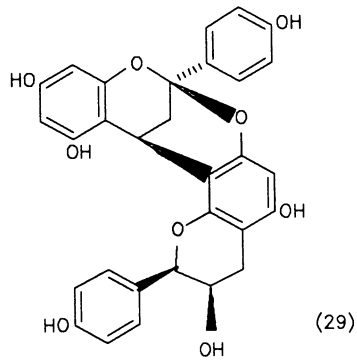


Figure 5. *Example promelacacidins* (**27**) *and* (**28**).

An exception is the 2,3-*cis*-3,4-*cis* [2**R**,3**R**,4**R**] epimesquitol-(4α → 6)-epimesquitol-4α-ol dimer isolated from *Acacia melanoxylon*.[64] In contrast, most plants outside the Leguminosae and Anacardiaceae contain 2,3-*cis* [2**R**,3**R**] procyanidins and prodelphinidins. Here is an example of where the distribution of dimeric procyanidins may not reflect the constitution of the higher polymers. In both oak bark[44] and southern pine bark,[70] significant amounts of the dimer catechin-(4α → 8)-catechin are present, but the higher polymers contain only small amounts of 2,3-*trans* chain extenders. Some examples of tannin sources with exceptionally high proportions (>70 percent) of 2,3-*trans* chain extenders are: *Ribes sanguineum* (leaf),[3] *Watsonia pyramidata* (leaf),[66] *Juniperous chinensis* (bark),[36] *Thuja standishii* (bark),[36] *Cryptomeria japonica* (bark),[36] and *Pinus radiata* (outer bark, not the inner bark).[71] Of the species listed in Porter's[4] tabulations, approximately 25 percent of the tannins



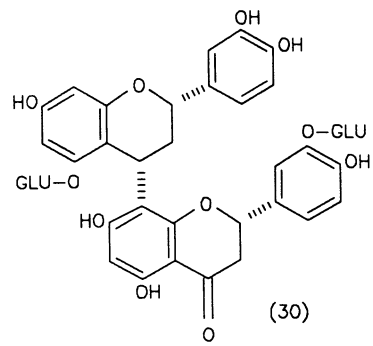


Figure 6. *Example 3-deoxy proanthocyanidins* (**29**) *and* (**30**).

contained at least 90 percent of 2,3-*cis* units as chain extenders. The vast majority of the tannins in the procyanidin and prodelphinidin classes are of mixed stereochemistry with 2,3-*cis* to 2,3-*trans* ratios between 90:10 and 50:50.

The stereochemistry at C-4 (the interflavanoid bond) is highly dependent on the hydroxylation pattern of the A-ring. In the 5-deoxy proanthocyanidins, both 2,3-*trans*-3,4-*trans* and 2,3-*trans*-3,4-*cis* isomers are found in similar ratios. However, interflavanoid bond formation is strongly stereoselective in the procyanidins and prodelphinidins. For many years, no procyanidin (either natural or synthetic) had been found with 2,3-*trans*-3,4-*cis* stereochemistry. However, Delcour et al[72] and Kolodziej[73] recently synthesized catechin-(4$\beta \rightarrow$ 8)-epicatechin and catechin-(4$\beta \rightarrow$ 8)-catechin in yields of about 10 percent of their corresponding (4$\alpha \rightarrow$ 8) diasteriomers. In addition, small proportions of 2,3-*trans*-3,4-*cis* procyanidins were recently isolated from *Potentilla erecta* extracts.[74]

With one exception, all natural and synthetic 2,3-*cis* proanthocyanidins have 3,4-*trans* stereochemistry. Foo's promelacacidin dimer discussed earlier[64] remains the only natural product with a 2,3-*cis*-3,4-*cis* stereochemistry. It is interesting to note that only the 2,3-*cis*-3,4-*trans* isomer was obtained by synthesis. This again brings to question enzymic control in the polymerization of flavan intermediates in tannin formation that has been addressed by Stafford in Chapter 3.

Interflavanoid Bonds

Most proanthocyanidins are linked simply by bonds between the benzylic C-4 of the heterocyclic ring to either the C-6 or C-8 of the flavan A-ring. In the 5-deoxy profisetinidins and prorobinetinidins, C-4 to C-6 linkages between the chain extender units are favored.[15,16] Because of the greater nucleophilicity of the phloroglucinol ring of the terminal catechin or gallocatechin units, these compounds are normally "angular"(i.e., flavanyl substituents at the C-6 and C-8 positions of a unit with no substituent at C-4) polymers. Steenkamp and coworkers have recently synthesized a "branched" (i.e., flavanyl substituents at C-4, C-6 and C-8 positions of a unit) profisetinidin tetramer.[54]

In the procyanidins and prodelphinidins, the C-4 $\rightarrow$ C-8 interflavanoid bonds are most common, but usually both C-4 $\rightarrow$ C-6 and C-4 $\rightarrow$ C-8 interflavanoid bonds are present in relative proportions of about 1:3.[17,75] Polymers with only C-4 $\rightarrow$ C-8 interflavanoid bonds are apparently rare exceptions.[76,77] All natural procyanidins and prodelphinidins isolated to date are "linear" polymers. An angular trimeric procyanidin representative of a branch point has been synthesized,[78] but no analogous oligomers or cleavage products from higher polymers have been isolated. This suggests that these polymers are not extensively, if at all, branched. ^{13}C-NMR spectral and molecular weight distribution studies also suggest that there is little branching in these polymers.[7,79]

In contrast to the biflavonoids that have a carbonyl function at C-4 and are normally diarylether or biphenyl linked oligomers,[1,19,80,81] proanthocyanidins with these linkages are rare. Dibenzyl ether linkages were proposed as the dominant mode of linkage in early studies[82] because of the extreme lability of proanthocyanidins to acid-catalyzed thiolysis. However, Sears and Casebier[83] demonstrated that catechin-(4$\alpha \rightarrow$ 2)-phloroglucinol was readily cleaved under mild reaction con-

ditions, and linkages through oxygen have since been shown to be rare. Both propelargonidins[29–31] and procyandins[18,84–89] linked by (4α- or $4\beta \rightarrow 8$ $2\beta \rightarrow O \rightarrow 7$;) interflavanoid bonds have been isolated from a wide variety of plants, particularly from nutshells. These compounds are interesting because of their phytotoxic[27,90] and anti-inflammatory properties.[87] Examples of other unusual ether-linked oligomers include the dioxane-linked profisetinidins isolated from the heartwood of *Acacia mearnsii*.[91,92] The "gambirins" are related chalcan-flavan oligomers that are produced by opening the pyran ring of flavan-3-ols through the formation of a quinone methide in the B-ring with condensation at the C-6 or C-8 of the A-ring.[24] Analogous compounds were synthesized from (+)-catechin in the early studies of tannins by Freudenburg and Weinges at Heidelberg.[11,93,94]

Oxidative coupling of flavan-3-ols, studied intensively by Freudenberg and Weinges,[11] formed the basis for most of the early research directed to the chemistry of condensed tannins. Although the proanthocyanidin structures discussed previously are now known to represent the basic structural features of most condensed tannins, oxidative coupling products are important natural products, no doubt, more than is appreciated to date. These compounds were studied intensively by Weinges[95,96] and by Ollis[97,98] and their colleagues. This type of linkage is prominent in the theaflavins found in black tea[97] and result from oxidative coupling of the thearubigins that are predominantly prodelphinidins and their gallate esters.[98] Considerable efforts were directed to the elucidation of the structure of dehydro-dicatechin A, and its structure was finally confirmed by X-ray crystallography in 1971.[99] The best known examples of oxidatively coupled flavans are found in the tannins of mesquite (*Prosopis glandulosa*) in which 2,3-*trans*-7,8,3′,4′-tetrahydroxyflavan-3-ols are oxidatively coupled alone (through the C-5 and C-6 positions) or with catechin (through the C-5 and C-8 positions) to form biphenyl and *m*-terphenyl (through C-5 and C-6 + C-8 positions) linked oligomers (Figure 7-**31**).[63,100,101] The absolute configurations of the atropisomers have been studied by ^{1}H n.O.e difference spectroscopy.[100–103] The 2′,2′-linked catechin → taxifolin dimer isolated from willow bark[104] is a recent example of an oxidative coupling product between a flavan-3-ol and a dihydroflavonol. Biphenyl linkages were proposed in the procyanidins of *Quercus robur*,[105,106] but analogous compounds have not been found in *Q. falcata*,[44] *Q. dentata*,[43] or *Q. miyagii*.[107] Profisetinidins oxidatively coupled to dihydroflavonols are further examples of these types of products.[102] Young et al[103] have synthesized a number of biphenyl and O → C linked catechin dimers by treatment of catechin with $K_3[Fe(CN)_6]$ in acetonitrile-glycine buffer. As in the previous work,[100] pairs of atropisomeric dimers were produced. In the [catechin]$_2$ dimers, linkages were through the B-ring of the upper unit [i.e., C-2′ to C-8; C-6′ to C-8; C-2′ to C-6; and either C-3′ to C-8 or C-4′ to C-8 through ether linkages, Figure 7-(**32**)]. However, Kolodziej (personal communication) found that all attempts at synthesizing [2′,2′]-(+)-catechin-(+)-taxifolin by direct oxidative couplings failed. He suggested that this may indicate that oxidation of the C-4 methylene group of a hypothetical [2′,2′]-bi-(+)-catechin might account for the biogenesis of this compound rather that direct oxidative coupling between flavan-3-ols and dihydroflavanols.

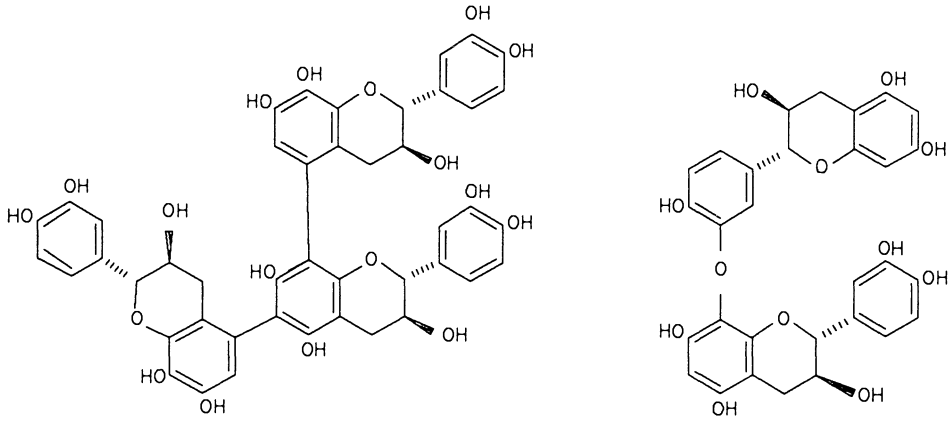


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Figure 7. *Examples of oxidative coupling products* (**31**) *and* (**32**).

Terminal Units

Most natural proanthocyanidins contain flavan-3-ols as chain terminating units. The tannins in *Colophospermum mopane* and *Acacia mearnsii* heartwood contain profisetinidins mainly terminated with the flavan-3,4-diol, fisetinidol (**23**).[55] Work on the profisitinidins of *Burkea africana* by Ferreira's group at Bloemfontein highlights the wide range of terminating nucleophiles that can sometimes be found in natural extracts.[102] Profisetinidin dimers terminated with the flavan-3-ols catechin-3-gallate and robinetinidol, the flavonol robinetin (linked through the C-2′ of the B-ring), the dihydroflavanols taxifolin and ampelopsin (linked through the C-6 position) have all been isolated from the heartwood of this tree.[102]

Absence of natural procyanidins terminated with the corresponding leucocyanidins (i.e., flavan-3,4-diols) is fully understandable because of the extreme reactivity of these compounds.[108] This is especially true because enzymic reduction of dihydroquercetin gives the 2,3-*trans*-3,4-*cis* isomer that would be expected to be particularly reactive.[109,110] These compounds are such good electrophiles that it is surprising that more proanthocyanidins terminated with a broader range of compounds have not been found. The propelargonidin terminated with kaempferol,[30] proguibortinidins terminated with 3,4,3′,5′-tetrahydroxystilbenes,[49,50] and the procyanidin terminated with phloroglucinol from *Nelia meyeri*[111] together with the profisetinidins from *Burkea africana* mentioned earlier[102] are example compounds on a list that seems certain to grow in the future.

Within the proanthocyanidins terminated with flavan-3-ol units, most compounds have (+)-catechin as the terminal unit. This is true of the profisetinidins and prodelphinidins as well as the procyanidins. Homopolymers of (-)-epicatechin are found in species such as *Aesculus hippocastanum* (horse chestnut), *Crataegus oxycanthus, Cinnamomum, Malus pumila, Theobroma cacao* (cacao), *Cotoneaster*

sp., *Cydonia oblonga, Prunus cerasus, Chaenomeles chinensis, Persea americana* and *Actinidia chinensis.*[4] Haslam recognized the requirement for a separate metabolic pool for the chain extender and terminal units because most plants containing 2,3-*cis* procyanidins have oligomers and polymers terminated with (+)-catechin.[112] Only three proanthocyanidins outside the propelargonidins are terminated with epiafzelechin: the guibourticacidol-epiafzelechin dimer[47] and both epicatechin-(4β → 8)- and catechin-(4α → 8)-epiafzelechin.[32,33] Prorobinetinidins terminated with gallocatechin are found in *Acacia mearnsii* bark and prodelphinidins terminated with epigallocatechin are found in *Myrica rubra.*[47] However, most prodelphinidins are terminated with a (+)-catechin unit.[1,2,4]

Proanthocyanidin Derivatives

Although both *C*- and *O*-methylated derivatives are common in the mono-[113] and biflavonoids,[19,80,81] methylated flavan-3-ols and proanthocyanidins are rare.[1,2] The only known natural methylated proanthocyanidins are two epiafzelechin → 4-*O*-methyl-gallocatechin dimers.[27,28] Although prenylated flavan-3-ols were found in *Illicium anisafum* bark, the procyanidins isolated from the same extract were not prenylated.[114] The spectral properties of methylated and acetylated proanthocyanidins are, however, well known, particularly for the profisetinidins and prorobinetinidins studied by Roux's school, because these derivatives are normally made to assist in their purification and structure elucidation.

Proanthocyanidin *C*- and *O*-glycosides have recently been described.[115–117] A complex mixture of glucosides was isolated from rhubarb (*Rheum*),[116] and analogous compounds have now been isolated from the bark of Cinnamomum.[117] A proanthocyanidin → polysaccharide complex from mangrove (*Rhizophora stylosa*) leaves[118] was eventually separated to give a proanthocyanidin-glucoside[119] apparently similar to that described in *Larix gmelini* bark.[120] These polymers are similar to the 3-*O*-β-*D*-glucoside isolated earlier from *Cydonia oblonga* where the glucose function is attached only to the terminal epicatechin unit.[115] Procyanidin-glucosides isolated from *Pinus brutia* and *Picea abies* appear to have the glucose attached to the phenolic hydroxyl groups.[115] *Quercus miyagii* bark contains both the 3-*O*-α-**L**- and the 3″-*O*-α-**L**-rhamnopyranosides of catechin-(4α → 8)-catechin.[107] Among the most interesting proanthocyandins are the proluteolinidin glycosides isolated from *Sorghum vulgare.*[65] Recent work on condensed tannin → carbohydrate complexes[121] is reviewed by Haslam and his coworkers in Chapter 6.

Gallate esters of prodelphinidins are, in comparison, more common. They are present in high concentrations in bayberry *Myrica rubra*[42] and *Myrica esculenta*,[41] the latter particularly prized as a leather tannage in China. ^{13}C-NMR spectra of the polymers from *Myrica esculenta* indicate 3-*O*-galloyl functions on the epigallocatechin chain extender and on the epigallocatechin terminal units. Gallate esters are also commonly found in bark extracts of oaks. *Quercus dentata*[43] contains the prodelphinidin, 3-*O*-galloyl-epigallocatechin-(4β → 8)-catechin, whereas, *Quercus falcata* and *Q. miyagii* barks contain the procyanidin 3-*O*-galloyl-epicatechin-(4β → 8)-catechin.[44,107] In contrast to Sun's results on the tannins of *Quercus dentata*, galloyl functions were not prominent features of the higher polymers in *Quercus falcata.*[44] Gallate esters of procyanidins are also present in *Vitis* spp. (grapes),[122]

Rheum palmatum (rhubarb),[123] *Polygonum multiflorum*, a climbing vine native to China,[67] *Sanguisorba officinalis*,[124] a Japanese plant that also contains the gambirins discussed above, and *Ceratonia siliqua* (carob pods).[125] Gallate esters are also the most common tannins in green tea.[98] Another interesting type of acylated proanthocyanidin is made up of 1-hydroxy-6-oxo-2-cyclohexene carboxylic acid ester of procyanidin isolated from the bark of *Salix sieboldiana*.[126] Here, acylation is restricted to the 3-hydroxyl of the terminal catechin unit.

The high degree of nucleophilicity of the resorcinolic or phlorglucinolic A-rings results in a variety of interesting proanthocyanidin conjugates. The C-6 and C-8 carboxylated proguibourtinidins (**21**) found in *Acacia luderitzii*[45,127] and the propelargonidin-based gambrins found in *Uncaria gambir*[24] have been described previously. Another example is the series of caffeic acid condensation products such as with the epicatechin chain extender units named "cinchonains" from their first isolation from *Cinchona succirubra*.[128] These compounds have also been isolated from *Kandelia candel* bark.[23] Some diverse examples of flavan-3-ols substituted at the C-6 or C-8 positions, such as kopsirachin (catechin substituted at both C-6 and C-8 by the alkaloid skytanthine)[129] and the stenophyllanins (catechin substituted at C-6 or C-8 by hydrolysable tannins),[130] are no doubt forerunners on a growing list of proanthocyanidin derivatives obtained from electrophilic substitution of the A-ring.

When used in applications such as wood adhesives, condensed tannins are usually subjected to alkaline conditions, either during extraction or during resin formulation.[62] Alkaline treatment of procyanidin-based tannins results in a reduction in formaldehyde reactivity with an increase in carbonyl functionality.[131] Sears et al[132] explained these results by demonstrating that (+)-catechin rearranged to an enolic form of 6-(3,4-dihydroxyphenyl)-7-hydroxybicyclo[3.3.1]nonane-2,4,9-trione. This compound is further converted to an analogue with a hydroxy function at C-9 on further heating.[133] Laks and Hemingway[134] examined base-catalyzed rearrangements further, using condensed tannins from southern pine bark rather than catechin in order to define the lability of the interflavanoid bond (see Chapter 17). When procyanidins are reacted at pH 12 and ambient temperature in the presence of toluene-α-thiol, a series of diarylproanoid derivatives was obtained that, based on the stereochemistry of the products, indicated that the interflavanoid bond cleavage preceded opening of the pyran ring. A diarylpropanone was subsequently produced from the dibenyzlthio derivative from the chain extender units by loss of the phenylsulfide function α to the phloroglucinol ring with tautomeric rearrangement to the ketone. Similar loss of the phenylsulfide function α to the catechol ring in the product obtained from the terminal unit also gave a diarylpropanone that further rearranged to an indan derivative. This work most importantly demonstrated the extreme lability of the interflavanoid bond to base-catalyzed cleavage and provided a chemical analogy[135] to the flav-3-en-3-ol proposed by Haslam as a central intermediate in the biogenesis of condensed tannins.[136,137]

When the same reaction conditions were examined but with phloroglucinol as a capture nucleophile, the phloroglucinol adduct obtained from chain extender units rearranged to an enolic form of 8-(3,4-dihydroxyphenyl)-7-hydroxy-6-(2,4,6- trihydroxyphenyl)-bicyclo[3.3.1]nonane-2,4,9-trione.[138] The stereochemistry

of this compound was also consistent with initial cleavage of the interflavanoid bond. A further rearrangement product, an enolic form of 3′-8-(3,4-dihydroxyphenyl)-7-hydroxy-2,4,9-trioxo-bicyclo[3.3.1]nonan-6-yl-4-(3,4-dihydroxyphenyl)-2′,3,4′,5,-6′,-7-hexahydroxyflavan demonstrated another rearrangement reaction leading to a new flavan ring system. ^{13}C-NMR spectra of reaction products of procyanidin-based tannins produced without the addition of an external nucleophile could be interpreted to a large extent from the above rearrangement products. A generalized structure of the base-catalyzed rearrangement products of polymeric procyanidins has been proposed [Figure 8-(**33**)] on the basis of this spectral data.[139]

Rearrangement products are also found in commercial wattle tannin extracts used in leather manufacture and wood adhesives.[140–145] In contrast to reactions of the polymeric procyanidins, based-catalyzed rearrangements of the 5-deoxy profisetinidins or prorobinetinidins do not involve interflavanoid bond cleavage but lead to a "liberation" of resorcinolic functionality that could result in improved reactivity with formaldehyde.[142,143] Here, opening of the pyran ring with rotation and recyclization to the more nucleophilic phloroglucinol ring leads to mixture of "phlobatannin" rearrangement products that carry the resorcinolic rings as C-4 adducts to the newly formed pyran ring rather than as the A-ring [Figure 8-(**34**)].

These "phlobatannin" rearrangement products have been isolated from *Colophospermum mopane*, *Guibourtia coleosperma* (profisetinidins), and *Baikiaea plurijuga* (prorobinetinidins), emphasizing the facile nature of these rearrangements.[141–143] Steynburg et al[144,145] have greatly extended our understanding of the mechanisms of these rearrangement reactions as well as defined the conformation of these products through use of ^{1}H n.O.e. difference spectroscopy.

POLYMERIC PROANTHOCYANIDINS

The profisetinidins and prorobinetinidins representative of wattle and quebracho tannins are thought to have a number average degree of polymerization of about 4 to 5,[140] so the oligomeric proanthocyanidins described by Roux's (now

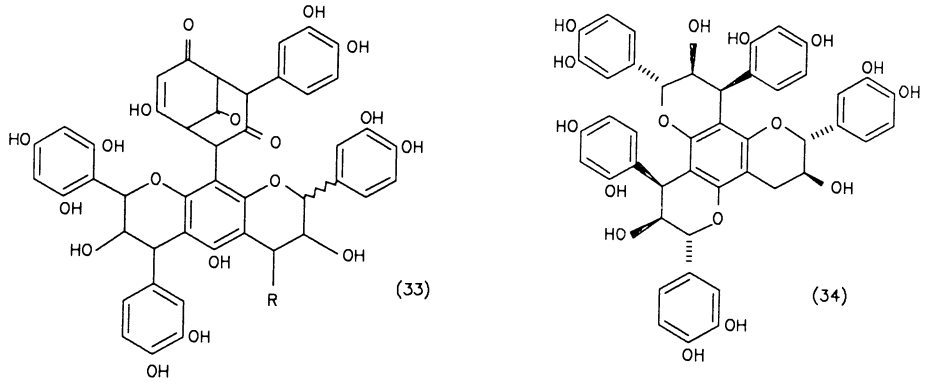

Figure 8. *Base-catalyzed rearrangement products of procyanidins* (**33**) *and profisetinidins* (**34**).

Ferreira's) group at the University of the Orange Free State, in Bloemfontein, South Africa, have defined the structural features of a substantial proportion of species in commercial extracts. Because of the stability of the interflavanoid bond in the 5-deoxy proanthocyanidins, cleavage reactions such as the formation of thiol or phloroglucinol adducts, are not useful in the definition of the polymer structure in this class of tannins. However, evaluation of high field ^{13}C-NMR spectra of wattle and quebracho tannin extracts did not reveal any significant structural features not previously described by the structures of the oligomers.[146] The absolute stereochemistry of these compounds (as well as those with phloroglucinolic A-rings) can be established from the CD spectrum.[147,148] More recent work on the structure of the 5-deoxy proanthocyanidins has centered on the conformation of the methyletheracetate derivatives as revealed by ^{1}H n.O.e. difference spectroscopy.[149,150] This work is described in detail by Ferreira in Chapter 10.

Most progress in elucidation of the structures of the higher polymers has occurred in the procyanidin and prodelphinidin classes of tannins. The number average molecular weight and dispersivity of the molecular weight distribution are both substantially higher than in the quebracho or wattle tannins with Dp's in the range of 9-11 commonly found in tannin extracts of the former.[151,152] ^{13}C-NMR spectral studies can readily provide information on the hydroxylation patterns of the A- and B-rings, the stereochemistry of the heterocyclic rings in the chain extender and terminal units, and in favorable cases, can estimate number average molecular weight.[3,4] Even at moderate field strengths (62.5 MHz), detailed information on the location of interflavanoid bond and an estimate of the extent of branching can be obtained by interpretation of the fine structure of ^{13}C-NMR spectra.[6,7]

When working with tannins that carry a phloroglucinolic A-ring and are therefore amenable to cleavage reactions, it is usually possible to isolate partial cleavage products that will substantiate conclusions reached from interpretation of the ^{13}C-NMR spectra. For example, partial thiolytic cleavage was the essential proof of the heterogeneity of interflavanoid bond location in polymeric procyanidins in *Pinus palustris*,[17] *Photinia glaubrescens*,[17] *Pinus taeda*,[70] *Areca catechu*,[75] and most recently, *the lack thereof* in the tannins from *Crataegus oxyacantha*[77] and *Cinnamomum cassia*.[153] The conclusion that polymeric procyanidins contain little branching (as derived from interpretation of the ^{13}C-NMR spectra[7] and from consideration of their molecular weight distributions[79]) is supported by failure to isolate the angular trimer epicatechin-(4β $\rightarrow$ 8)-catechin-(6 $\rightarrow$ 4β)-epicatechin[78] from partial thiolytic cleavage of tannins from the southern pines. Definition of the location of interflavanoid bonds in these polymers by bromination followed by thiolytic cleavage has not been fully successful because of facile debromination.[89]

A problem of considerable concern to many tannin chemists is the comparatively low yield (often only about 30 percent) of acid-catalyzed cleavage products that are obtained from cleavage of most tannin isolates. Other structural features not evident in the ^{13}C-NMR spectra need to be considered. Like work on the profisetinidins,[149,150] efforts to define the conformation of profisetinidins and prodelphinidins have increased substantially in recent years. Here, time-resolved fluorescence spectra of the oligomers have been important in defining the distribution of rotational isomers in the phenolic state[154] and the relationships of molec-

ular weight to fluorescence quantum yield.[155] The current state of our knowledge of the conformations of procyanidins and prodelphinidins is reviewed by Mattice in Chapter 7.

CONCLUSIONS

Progress in definition of the structure of oligomeric proanthocyanidins, their derivatives, and rearrangement products has been truly phenomenol over the past 15 years, particularly when considering the comparatively few laboratories studying these compounds. Our understanding of the higher molecular weight polymers remains, however, comparatively sparse. Perhaps the recent increases in the cost of phenol (currently at prices comparable to those during the petroleum crisis of the early 1970's) will rekindle interest in the condensed tannins as renewable sources of phenolic compounds for applications such as adhesives (Kreibich, Chapter 29 and Hamed Chapter 30) and other specialty chemical applications such as biocides (Laks Chapter 32). If a resurgence of interest and funding for research should occur, more attention certainly needs to be given to the properties of the polymers.

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REFERENCES

1. Hemingway, R.W. Biflavonoids and proanthocyanidins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag (in press).
2. Porter, L.J. Flavans and proanthocyanidins, *In*: Harborne, J.B. (ed.) The Flavanoids. Advances in Research Since 1980. Chapman and Hall, London, pp. 21-62 (1988).
3. Czochanska, Z.; Foo, L.Y.; Newman, R.H.; Porter. L.J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J. Chem. Soc. Perkin Trans. 1* :2278 (1980).
4. Porter, L.J. Condensed tannins, *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag (in press).
5. Karchesy, J.J.; Hemingway R.W. Loblolly pine bark polyflavonoids. *J. Agric. Food Chem.* 28:222 (1980).
6. Porter, L.J; Newman, R.H; Foo, L.Y; Wong, H.; Hemingway, R.W. Polymeric proanthocyanidins: ^{13}C-nmr studies of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1217 (1982).
7. Newman, R.H.; Porter, L.J.; Foo, L.Y.; Johns, S.R.; Willing, R.I. High resolution ^{13}C NMR studies of proanthocyanidin polymers. *Mag. Res. Chem.* 25:118 (1982).
8. Viswanathan, V.N.; Mattice, W.L. Preferred conformations of the sixteen (4 → 6) and (4 → 8) linked dimers of (+)-catechin and (-)-epicatechin with axial or equatorial dihydroxyphenyl rings at C(2). *J. Chem. Soc. Perkin Trans. 2* :739 (1987).
9. Young, D.A.; Cronje, A.; Botes, A.L.; Ferreira, D.; Roux D.G. Synthesis of condensed tannins. Part 14. Biflavanoid profisetinidins as synthons. The acid-induced 'phlobaphene' reaction. *J. Chem. Soc. Perkin Trans. 1* :2521 (1985).
10. Weinges, K.; Kaltenhauser, W.; Marx, H.-D.; Nader, E.; Nader. F.; Perner, J.; Seiler, D. Procyanidine aus fruchten. *Liebigs Ann. Chem.* 711:184 (1968).

11. Weinges, K.; Bahr, W.; Ebert, W.; Goritz, K.; Marx, H.-D. Konstitution, entstehung, und bedeutung der flavonoid gerbstoffe. *Fortschr. Chem. Org. Naturst.* 27:158 (1969).

12. Thompson, R.S.; Jacques, D.; Haslam, E.; Tanner, R.J.N. Plant proanthocyanidins. Part I. Introduction: the isolation, structure and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1387 (1972).

13. Haslam, E. Natural proanthocyanidins. *In*: Harborne, J.B.; Mabry, T.J.; Mabry, H. (eds.) The Flavonoids. Academic Press, New York, p. 505-559 (1975).

14. Haslam, E. Proanthocyanidins. *In*: Harborne, J.B.; Mabry, T.J.; Mabry, H. (eds.) The Flavonoids: Advances in Research. Chapman and Hall, London, p. 417-447 (1982).

15. Roux, D.G.; Ferreira, D. The direct biomimetic synthesis, structure and absolute configuration of angular and linear condensed tannins. *Fortschr. Chem. Org. Naturst.* 41:48 (1982).

16. Roux, D.G.; Ferreira, D. Structure and function in biomimetic synthesis of linear, angular and branched condensed tannins. *Rev. Pure and Applied Chem.* 54:2465 (1982).

17. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Linkage isomerism in trimeric and polymeric 2,3-*cis* procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1209 (1982).

18. Karchesy, J.J.; Hemingway, R.W. Condensed Tannins: $(4\beta \rightarrow 8;2\beta \rightarrow O \rightarrow 7)$-linked procyanidins in *Arachis hypogaea*. L. *J. Agric. Food Chem.* 34:966 (1986).

19. Geiger, H.; Quinn, C. Biflavonoids. *In* Harborne, J.B.; Mabry, T.J. (eds.) The Flavonoids: Advances in Research. Chapman and Hall Ltd., London, p. 505-539 (1982).

20. Dewick, P.M., Isoflavonoids. *In* Harborne, J.B., Mabry, T.J. (eds.) The Flavonoids. Advances in Research, Chapman and Hall Ltd. London, p. 535-632 (1982).

21. Bezuidenhoudt, B.C.B.; Brandt, E.V., Steenkamp, J.A., Roux, D.G., Ferreira, D., Oligomeric isoflavanoids. Part 1. Structure and synthesis of the first (2,3′) isoflavone-isoflavan dimer. *J. Chem. Soc. Perkin Trans. 1* :1227 (1988).

22. Bezuidenhoudt, S.E., Bezuidenhoudt, B.C.B.; Brandt, E.V., Ferreira, D., Oligomeric isoflavanoids. Part 2. Structure and synthesis of xanthocercin A and B. The first isoflavonoid-lignols. *J. Chem. Soc. Perkin Trans. 1* :1237 (1988).

23. Hsu, F.; Nonaka, G.; and Nishioka, I. Tannins and related compounds. XXXI. Isolation and characterization of proanthocyanidins in *Kandelia candel* (L.) Druce. *Chem. Pharm. Bull.* 33:3142 (1985).

24. Nonaka, G.; Nishioka, I. Novel biflavonoids, chalcan-flavan dimers from gambir. *Chem. Pharm. Bull.* 28:3145 (1980).

25. Delle-Monache, F.; Pomponi, M.; Marini-Bettolo, G.B.; D'Albuquerque, I.L.; Delima, O.G. A methylated catechin and proanthocyanidin from the *Celastraceae*. *Phytochemistry* 15:573 (1976).

26. Weeratunga, G.; Bohlin, L.; Verpoorte, R.; Kumar, V. Flavonoids from *Elaeodendron balae* root bark. *Phytochemistry* 24:2093 (1985).

27. Ohigashi, H.; Minami, S.; Fukui, H.; Koshimizu, K.; Mizutani, F.; Sugiura, A.; Tomana, T. Flavanols, as plant growth inhibitors from the roots of peach, *Prunus persica* Batsh. cv. 'Hakato'. *Agric. Biol. Chem.* 46:2555 (1982).

28. Porter, L.J. (personal communication).

29. Hikino, H.; Shimoyama, N.; Kasahara, Y.; Takahashi, M.; Konno, C. Structures of mahuannin A and B, hypotensive principles of *Ephedra* roots. *Heterocycles* 19:1381 (1982).

30. Hikino, H.; Takahasi, M.; Konno, C. Structure of ephedrannin A, a hypotensive principle of *Ephedra* roots. *Tetrahedron Letts.* 23:673 (1982).

31. Kasahara, Y.; Hikino, H. Structure of mahuannin D, a hypotensive principle of *Ephedra* roots. *Heterocycles* 20:1953 (1983).

32. Weinges, K.; Goritz, K.; Nader, F. Zur kenntnis der proanthocyanidine XI. Konfigurationsbestimmung von $C_{30}H_{26}O_{12}$-procyanidinen und struckturauf-klarung eines neuen procyanidin. *Leibigs Ann. Chem.* 715:164 (1968).

33. Middlekoop, T.B.; Labadie, R.P. Proanthocyanidins in the bark of *Saraca asoca* Roxb. de Wilde. *Z. Naturforsch.* 40B:855 (1985).

34. Outtrup, H.; Schaumburg, K. Structure elucidation of some proanthocyanidins in barley by ^{1}H-270 MHz-NMR spectroscopy. *Carlsberg Res. Commun.* 46:43 (1981).

35. Lebreton, Ph.; Thivend, S.; Boutard, B. Distribution des proanthocyanidines chez les gymnospermes. *Plants Med. Phytotherapie* 14:105 (1980).

36. Samejima, M.; Yoshimoto, T. Systematic studies on the stereochemical composition of proanthocyanidins from coniferous bark. *Mokuzai Gakkashi* 28:67 (1982).

37. Gupta, R.K.; Haslam, E. Plant proanthocyanidins. Part 7. Prodelphinidins from *Pinus sylvestris*. *J. Chem. Soc. Perkin Trans. 1* :1148 (1981).

38. Foo, L.Y.; Porter, L.J. Prodelphinidin polymers: Definition of structural units. *J. Chem. Soc. Perkin Trans. 1* :1186 (1978).

39. Brandon, M.J.; Foo, L.Y.; Porter, L.J.; Meredith, P. Proanthocyanidins of barley and sorghum; composition as a function of maturity of barley ears. *Phytochemistry* 21:2953 (1982).

40. Delcour, J.A.; Tuytens, G.M. Structure elucidation of three dimeric proanthocyanidins isolated from a commercial Belgian pilsner beer. *J. Inst. Brew.* 90:153 (1984).

41. Sun, D.; Zhao, A.; Wong, H.; Foo, L.Y. Tannins and other phenolics from *Myrica esculenta* bark. *Phytochemistry* 27:579 (1988).

42. Nonaka, G.; Muta, M.; Nishioka, I. Myricatin, a galloyl-flavanol-sulfate and prodelphinidin gallate from *Myrica rubra*. *Phytochemistry* 22:237 (1983).

43. Sun, D.; Wong, H.; Foo. L.Y. Proanthocyanidin dimers and polymers from *Quercus dentata*. *Phytochemistry* 26:1825 (1987).

44. Ohara, S.; Hemingway, R.W. The phenolic extractives of southern red oak *Quercus falcata* var. *falcata* bark. *Holzforschung* (in press).

45. duPreez, I.C.; Rowan, A.C.; Roux, D.G. A biflavanoid proanthocyanidin carboxylic acid and related biflavonoids from *Acacia luderitzii*, Engl. var. retinens, J. Ross and Brenan. *J. Chem. Soc. Chem. Commun.* :492 (1970).

46. Pelter, A.; Amenechi, P.I.; Warren, R. The structures of two proanthocyanidins from *Julbernadia globiflora*. *J. Chem. Soc. (C)* :2572 (1969).

47. Patil, A.D.; Desphande, V.H. A new dimeric proanthocyanidin from *Cassia fistula* sapwood. *Indian J. Chem.* 21B:626 (1982).

48. Steenkamp, J.A., Malan, J.C.S.; Roux, D.G.; Ferreira, D., Oligomeric flavanoids. Part 1. Novel dimeric profisetinidins from *Colophospermum mopane*. *J. Chem. Soc. Perkin Trans. 1* :1325 (1988).

49. Steynberg, J.P.; Ferreira, D.; Roux, D.G. The first condensed tannins based on a stilbene. *Tetrahedron Letts.* 24:4147 (1983).

50. Steynberg, J.P.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins: Part 18. Stilbenes as potent nucleophiles in regio- and stereospecific condensations: novel guibourtinidol-stilbenes from *Guibourtia coleosperma*. *J. Chem. Soc. Perkin Trans. 1* :1705 (1987).

51. Botha, J.J.; Viviers, P.M.; Young, D.A.; duPreez, I.C.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 5. The first angular (4,6:4,8)-triflavonoids and their natural counterparts. *J. Chem. Soc. Perkin Trans. 1* :527 (1982).

52. Young, D.A.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 15. Structure of natural 'angular' profisetinidin tetraflavanoids: Asymetric induction during oligomeric synthesis. *J. Chem. Soc. Perkin Trans. 1* :2529 (1985).

53. Botha, J.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins, Part 4. A direct biomimetic approach to (4,6) and (4,8) biflavanoids. *J. Chem. Soc. Perkin Trans. 1* :1235 (1981).

54. Steenkamp, J.A.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 8. The first branched (4,6:4,8:4,6)-tetraflavonoid. Coupling sequence and absolute configuration. *J. Chem. Soc. Perkin Trans. 1* :23 (1983).

55. Viviers, P.M.; Young, D.A.; Botha, J.S.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 6. The sequence of units, coupling positions and absolute configuration of the first linear (4,6:4,6) triflavanoid with terminal 3,4-diol function. *J. Chem. Soc. Perkin Trans. 1* :535 (1982).

56. Brandt, E.V.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 20. Cycloconformations and conformational stability amoung derivatives of 'angular' tetraflavanoid profisetinidins. *J. Chem. Soc. Perkin Trans. 1* :2353 (1987).

57. Viviers, P.M.; Kolodziej, H.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 11. Intramolecular enantiomerism of constituent units of tannins from the *Anacardiaceae*: Stoichiometric control in direct synthesis, derivation of ^{1}H nuclear magnetic resonance parameters applicable to higher oligomers. *J. Chem. Soc. Perkin Trans. 1* :2555 (1983).

58. Foo, L.Y. Condensed tannins: co-occurrence of procyanidins, prodelphinidins and profisetinidins in the heartwood of *Acacia baileyana*. *Phytochemistry* 23:2915 (1984).

59. Botha, J.J.; Ferreira, D.; Roux, D.G. Condensed tannins: Direct synthesis, structure and absolute configuration of four biflavanoids from black wattle ('mimosa') extract. *J. Chem. Soc. Chem. Commun.* :700 (1978).

60. Viviers, P.M.; Botha, J.J.; Ferreira, D.; Roux, D.G.; Saayman, H.M. Synthesis of condensed tannins. Part 7. Angular (4,6:4,8)- prorobinetinidin triflavanoids from black wattle ('mimosa') bark extract. *J. Chem. Soc. Perkin Trans. 1* :17 (1983).

61. Sparrow, N.A.; Russell, A.E.; Glasser, L. The measurement of the binding of tannin subunits to soluble collagen by continuous-flow dynamic dialysis. *J. Soc. Leather Tech. Chem.* 66:97 (1982).

62. Pizzi, A. Tannin-based adhesives. *In*: Pizzi, A., (ed.). Wood Adhesives, Chemistry and Application. Marcel Dekker, New York p. 177-246 (1982).

63. Jacobs, E.; Ferreira, D.; Roux, D.G. Atropisomerism in a new class of condensed tannins based on biphenyl and o-terphenyl. *Tetrahedron Letts.* 24:4627 (1983).

64. Foo, L.Y. A novel pyrogallol A-ring proanthocyanidin dimer from *Acacia melanoxylon*. *J. Chem. Soc. Chem. Commun.* :236 (1987).

65. Gujer, R.; Magnolato, D.; Self, R. Glycosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry* 25:1431 (1986).

66. Ellis, C.J.; Foo, L.Y.; Porter, L.J. Enantiomerism: a characteristic of the proanthocyanidin chemistry of the *Monocotyledonae*. *Phytochemistry* 22:483 (1983).

67. Nonaka, G.; Miwa, N.; Nishioka, I. Stilbene glycoside gallates and proanthocyanidins from *Polygonum multiflorum*. *Phytochemistry* 21:429 (1982).

68. Nonaka, G.:Ezaki, E.; Hayashi, K.; Nishioka, I. Flavanol glucosides from rhubarb and *Rhaphiolepsis umbellata*. *Phytochemistry* 22:1659 (1983).

69. Young, D.A.; Kolodziej, H., Ferreira, D.: Roux, D.G. Synthesis of condensed tannins. Part 16. Stereochemical differentiation of the first 'angular' (**2S**,3**R**-profisetinidin tetraflavanoids from *Rhus lancea* (Karree) and the varying dynamic behavior of ther derivatives. *J. Chem. Soc. Perkin Trans. 1* :2537 (1985).

70. Hemingway, R.W.; Karchesy, J.J.; McGraw, G.W.; Wielesek, R.A. Heterogeneity of interflavanoid bond location in loblolly pine bark procyanidins. *Phytochemistry* 22:275 (1983).

71. Porter, L.J.; Hemingway, R.W. (unpublished results).

72. Delcour, J.A.; Serneels, E.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins Part 13. The first 2,3-*trans*-3,4-*cis* procyanidin: sequence of units in a trimer of mixed stereochemistry. *J. Chem. Soc. Perkin Trans. 1* :669 (1985).

73. Kolodziej, H. The first 2,3-*trans*-3,4-*cis* procyanidin. *Phytochemistry* 24:2460 (1985).

74. Schleep, S.; Friedrich, H.; Kolodziej, H. The first natural procyanidin with a 3,4-*cis* configuration. *J. Chem. Soc. Chem. Commun.* :392 (1986).

75. Nonaka, G.; Hsu, F-L.; Nishioka, I. Structures of dimeric, trimeric and tetrameric procyanidins from *Areca catechu*. *J. Chem. Soc. Chem. Commun.* :781 (1981).

76. Gupta, R.K.; Haslam, E. *J. Chem. Soc. Perkin Trans. 1* :892 (1978)

77. Kolodziej, H.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 12. Direct access to [4,6]- and [4,8]- all *cis* procyanidin derivatives from (-)-epicatechin: Assessment of bonding positions in oligomeric analogues from *Crataegus oxyacantha* L. *J. Chem. Soc. Perkin Trans. 1* :343 (1985).

78. Foo, L.Y.; Hemingway, R.W. Condensed tannins: synthesis of the first 'branched' procyanidin trimer. *J. Chem. Soc. Chem. Commun.* :85 (1984).

79. Mattice, W.L.; Porter, L.J. Molecular weight averages and ^{13}C-nmr intensities provide evidence for branching in proanthocyanidin polymers. *Phytochemistry* 23:1309 (1984).

80. Locksley, H.D. The chemistry of biflavonoid compounds. *Fortschritt. Chem. Org. Naturst.* 30:207 (1973).

81. Geiger, H.; Quinn, C. Biflavonoids, *In*: Harborne, J.B.; Mabry, T.J. (eds.) The Flavonoids. Academic Press, New York, p. 692-742 (1975).

82. Betts, M.J.; Brown, B.R.; Shaw, M.R. Reactions of flavonoids with mercaptoacetic acid. *J. Chem. Soc. (C)* :1178 (1969).

83. Sears, K.D.; Casebier, R.L. Cleavage of proanthocyanidins with thioglycollic acid. *J. Chem. Soc. Chem. Commun.* :1437 (1968).

84. Geissman, T.A.; Dittmar, H.F.K. A proanthocyanidin from avocado seed. *Phytochemistry* 4:359 (1965).

85. Mayer, W.; Goll, L.; von Arndt, E.M.; Mannschreck, A. Procyanidino-(-)-epicatechin, ein zweiarmig verknupftes condensiertes proanthocyanidin aus *Aesculus hippocastanum*. *Tetrahedron Letts.* 4:429 (1966).

86. Jacques, D.; Haslam, E.; Bedford, G.R.; Greatbanks, D. Plant proanthocyanidins. Part II. Proanthocyanidin A2 and its derivatives. *J. Chem. Soc. Perkin Trans. 1* :2663 (1974).

87. Otsuka, H.; Fujioka, S.; Komiya T.; Mizuta, E.; Takamoto, M. Studies on anti-inflammatory agents: VI Anti-inflammatory constitutents of *Cinnamomum sieboldei*. Meissn. *Yakugaku Zasshi* 102:162 (1982).

88. Schilling, C.; Weinges, K.; Muller, O.; Mayer, W. ^{13}C-NMR spektroskopische konstitutionsermittlung der $C_{30}H_{24}O_{12}$-procyanidine. *Liebigs Ann. Chem.* :1471 (1973).

89. Nonaka, G.; Morimoto, S.; Nishioka, I. Tannins and related compounds. Part 13. Isolation and structures of trimeric, tetrameric and pentameric proanthocyanidins from cinnamon. *J. Chem. Soc. Perkin Trans. 1* :2139 (1983).

90. Fisher, N. (personal communication).

91. Drewes, S.E.; Ilsley, A.H. Dioxane-linked biflavanoid from the heartwood of *Acacia mearnsii*. *J. Chem. Soc. (C)* :897 (1969).

92. Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 10. Dioxane linked profisetinidins. *J. Chem. Soc. Perkin Trans. 1* :2031 (1983).

93. Freudenberg, K.; Alonso deLama, J.M. Zur kenntnis der catechin gerbstoffe. *Liebigs Ann. Chem.* 612:78 (1958).

94. Freudenberg, K; Weinges, K. Zur Kenntnis der flavonoid-gerbstoffe. *Liebigs Ann. Chem.* 668:92 (1963).

95. Weinges, K.; Ebert, W. Isolierung eines kristallisierten dehydriemungs dimeren aus (+)-catechin. *Phytochemistry* 7:153 (1968).

96. Weinges, K.; Ebert, W.; Huthwelker, D.; Mattauch, H.; Perner J. Konstitution und bildungsmechanisim des dehydro-dicatechin A. *Liebigs Ann. Chem.* 726:114 (1969).

97. Brown, A.G.; Eyton, W.B.; Holmes, A.; Ollis, W.D. The identification of the thearubigins as polymeric proanthocyanidins. *Phytochemistry* 8:2333 (1969).

98. Coxon, D.T.; Holmes, A.; Ollis, W.D.; Vora, V.C.; Grant, M.S.; Tee, J.L. Flavanol digallates in green tea leaf. *Tetrahedron* 28:2819 (1972).

99. van Soest, T.C. Aufklarung der molekularstrukur structur des dehydro-dicatechins A durch rontgenstrukturanalyse seines bromoheptamethylathers. *Liebig Ann. Chem.* 754:137 (1971).

100. Young, E.; Brandt, E.V., Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 17;. Oligomeric (2**R**,3**S**)-3,3′,4′,7,8-pentahydroxyflavans: atropisomerism and conformation of biphenyl and *m*-terphenyl analogues from *Prosopis glandulosa* (Mesquite). *J. Chem. Soc. Perkin Trans. 1* :1737 (1986).

101. Brandt, E.V.; Young, D.A.; Young, E.; Ferreira, D. Absolute configuration of atropisomeric *m*-terphenyl-type flavan-3-ols. *J. Chem. Soc. Perkin Trans. 2* :1365 (1987).

102. Malan, J.C.S., Young, D.A., Steenkamp, J.A., Ferreira, D. Oligomeric flavanoids. Part 2. The first profisetinidins with dihydroflavonols constituent units. *J. Chem. Soc. Perkin Trans. 1* (in press).

103. Young, D.A.; Young, E., Roux, D.G., Brandt, V., Ferreira, D. Synthesis of condensed tannins. Part 19. Phenol oxidative coupling of (+)-catechin and (+)-mesquitol. Conformation of bis-(+)-catechins. *J. Chem. Soc. Perkin Trans. 1* :2345 (1987).

104. Kolodziej, H. [2′,2′]-(+)-Catechin-(+)-taxifolin from commercial willow bark: structure, bonding positions and oxidative cleavage. *J. Chem. Soc. Perkin Trans. 1* :219 (1988).

105. Ahn, B-Z; Gstirner, F. Uber catechin dimere der eichenrinde. *Arch. Pharmaz.* 304:666 (1971).

106. Ahn, B-Z. Ein catechin trimer aus der eichenrinde. *Arch. Pharmaz.* 307:186 (1974).

107. Ishimura, K.; Nonaka, G.; Nishioka, I. Flavan-3-ol and procyanidin glycosides from *Quercus miyagii*. *Phytochemistry* 26:1167 (1987).

108. Porter, L.J.; Wong, R.Y.; Chan, B.G. The molecular and crystal structure of (+)-2,3-*trans*-3,4-*trans*-leucocyanidin [(2**R**,3**S**,4**R**)-(+)-3,3′,4,4′,5,7-hexahydroxyflavan] dihydrate and comparison of its heterocyclic conformation in solution and the solid state. *J. Chem. Soc. Perkin Trans. 1* :1413 (1985).

109. Stafford, H.A.; Lester, H.H. Flavan-3-ol biosynthesis, the conversion of (+)-dihydroquercetin and flavan-3,4-*cis*-diol (leucocyanidin) to (+)-catechin by reductases extracted from cell suspension cultures of Douglas fir. *Plant Physiol.* 76:184 (1984).

110. Stafford, H.A.; Lester, H.H.; Porter, L.J. Chemical and enzymic synthesis of monomeric procyanidins (leucocyanidins or 3',4',5,7-tetrahydroxyflavan-3,4-diols) from 2**R**,3**R**-dihydroquercetin. *Phytochemistry* 24:333 (1985).

111. Kolodziej, H. The first naturally occurring 4-arylflavan-3-ol. *Tetrahedron Letts.* 24:1825 (1983).

112. Haslam, E. Symmetry and promiscuity in procyanidin biochemistry. *Phytochemistry* 16:1625 (1977).

113. Wollenweber, E. Flavones and flavonols, *In*: Harborne, J.B.; Mabry, T.J. (eds.) The Flavonoids: Advances in Research, Chapman and Hall, London, New York, pp. 189-259 (1982).

114. Morimoto, S.; Tanabe, H.; Nonaka, G.; Nishioka, I. Prenylated flavan-3-ols and procyanidins from *Illicium anisatum*. *Phytochemistry* 27:907 (1988).

115. Porter, L.J.; Foo, L.Y.; Furneaux, R.H. Isolation of three naturally occurring *O*-β-*D*-glucopyranosides of procyanidin polymers. *Phytochemistry* 24:567 (1985).

116. Kashiwada, Y.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XLV. Rhubarb (5). Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chem Pharm. Bull.* 34:3208 (1986).

117. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds XXXIX. Procyanidin C-glycosides and an acylated flavan-3-ol glucoside from the barks *of Cinnamomum cassia* Blume and *C. obtusifolium* Nees. *Chem. Pharm. Bull.* 34:643 (1986).

118. Neilson M.J.; Painter, T.J.; Richards, G.N. Flavologlycan: a novel glycoconjugate from leaves of mangrove (*Rhizophora stylosa*, Griff.). *Carbohydrate Res.* 147:315 (1986).

119. Richards, G.N. (personal communication).

120. Shen, Z.; Haslam, E.; Falshaw, C.P.; Begley, M.J. Procyanidins and polyphenols of *Larix gmelini* bark. *Phytochemistry* 25:2629 (1986).

121. McManus, J.P.; Davies, K.G.; Bent, J.E.; Godfrey, S.H.; Lilley, T.H.; Haslam, E. Polyphenol interactions. Part 1. Introduction: some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc. Perkin Trans. 2* :1429 (1985).

122. Foo, L.Y.; Porter, L.J. The phytochemistry of proanthocyanidin polymers. *Phytochemistry* 19:1747 (1980).

123. Nonaka, G.; Nishioka, I.; Nagasawa, T.; Oura, H. Tannins and related compounds I. Rhubarb. *Chem. Pharm. Bull.* 29:2862 (1981).

124. Tanaka, T.; Nonaka, G.; Nishioka, I. 7-*O*-galloyl-(+)-catechin and 3-*O*-galloyl-procyanidin B-3 from *Sanguisorba officinalis*. *Phytochemistry* 22:2576 (1983).

125. Tamir, M.; Nachtomi, E.; Alumot, E. Degradation of tannins from carob pods (*Ceratonia siliqua*) by thioglycolic acid. *Phytochemistry* 10:2769 (1971).

126. Hsu, F., Nonaka G., Nishioka. I. Acetylated flavanols and proanthocyanidns from *Salix sieboldiana*. *Phytochemistry* 24: 2089 (1985).

127. Ferreira, D.; duPreez, I.C.; Wijamaalen, J.C.; Roux, D.G. Biflavanoid proquibourtinidin carboxylic acids and the biflavanoid homologues from *Acacia luderitzii*. *Phytochemistry* 24:2415 (1985).

128. Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. VIII. A new type of proanthocyanidin, cinchonains IIa and IIb from *Cinchona succirubra*. *Chem. Pharm. Bull.* 12:4277 (1982).

129. Homberger, K.; Hesse, M. Kopsirachin, ein ungewohnliches alkaloid aus der Apocynaceae *Kopsia dasyrachis* Ridl. *Helv. Chim. Acta.* 67:237 (1984).

130. Nonaka, G.; Nishimura, H.; Nishioka, I. Tannins and related compounds. Part 26. Isolation and structures of stenophyllanins A, B, and C, novel tannins from *Quercus stenophylla*. *J. Chem. Soc. Perkin Trans. 1* :163 (1985).

131. Herrick, F.W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food Chem.* 28:228 (1980).

132. Sears, K.D.; Casebier, R.L.; Hergert, H.L.; Stoudt, G.H.; McCandlish, L.E. The structure of catechinic acid, a base-rearrangement product of catechin *J. Org. Chem.* 39:3244 (1975).

133. Courbat, P.; Weith, A.; Albert, A.; Pelter, A. Contribution a l'etude du comportment de la catechin en mileau alcalin. *Helv. Chem. Acta.* 60:1665 (1977).

134. Laks, P.E.; Hemingway, R.W. Condensed Tannins: Base-catalyzed reactions of polymeric procyanidins with toluene-α-thiol. Lability of the interflavanoid bond and pyran ring. *J. Chem. Soc. Perkin Trans. 1* :465 (1987).

135. Hemingway, R.W.; Laks, P.E. Condensed tannins: a proposed route to 2**R**,3**R**-(2,3-*cis*)-proanthocyanidins. *J. Chem. Soc. Chem. Commun.* :746 (1985).

136. Jacques, D.; Opie, C.T.; Porter, L.J.; Haslam, E. Plant proanthocyanidins. Part 4. Biosynthesis of procyanidins and observations on the metabolism of cyanidin in plants. *J. Chem. Soc. Perkin Trans. 1* :1637 (1977).

137. Platt, R.V.; Opie, C.T.; Haslam, E. Biosynthesis of flavan-3-ols and other secondary plant products from (2**S**)-phenylananine. *Phytochemistry* 23:2211 (1984).

138. Laks, P.E.; Hemingway, R.W.; Conner, A.H. Condensed tannins: Base-catalyzed reactions of polymeric procyanidins with phloroglucinol. Intramolecular rearrangements. *J. Chem. Soc. Perkin Trans. 1* :1875 (1987).

139. Laks, P.E.; Hemingway, R.W. Condensed tannins. Structure of the 'phenolic acids'. *Holzforschung* 41:287 (1987).

140. Roux, D.G.; Ferreira, D.; Hundt, H.K.L.; Malan, E. Structure, stereochemistry and reactivity of natural condensed tannins as basis for their extended industrial application. *J. Appl. Polym. Sci. Polym. Symp.* No. 28:335 (1975).

141. Steynberg, J.P., Young, D.A.; Burger, J.F.W.; Ferreira, D.; Roux, D.G. Phlobatannins via facile ring isomerizations of profisetinidin and prorobinetinidin condensed tannin units. *J. Chem. Soc. Chem. Commun.* :1013 (1986).

142. Roux, D.G., Activation of some condensed tannins via facile ring isomerizations: potential adhesive applications. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.). Adhesives from Renewable Resources. ACS Symposium Series No. 385. pp. 217-228 (1989).

143. Steenkamp, J.A.; Steynberg, J.P.; Brandt, E.V.; Ferreira, D.; Roux, D.G. Phlobatannins, a novel class of ring isomerized condensed tannins. *J. Chem Soc. Chem. Commun.* :1678 (1985).

144. Steynburg, J.P., Burger, J.F.W., Young, D.A., Brandt, E.V., Steenkamp, J.A., Ferreira, D. Oligomeric flavanoids. Part 3. Structure and synthesis of phlobatannins related to (-)-fisetinidol (4α,6) and (4α,8)-(+)-catechin profisetinidins. *J. Chem. Soc. Perkin Trans. 1* (in press).

145. Steynburg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V.; Steenkamp, J.A.; Ferreira, D. Oligomeric flavanoids. Part 4 Base-catalyzed conversions of (-)-fisetindiol-(+)-catechin profisetinidins with 2,3-*trans*-3,4-*cis*-flavan-3-ol constituent units. *J. Chem. Soc. Perkin Trans. 1* (in press).

146. Porter, L.J.; Hemingway, R.W. (unpublished results).

147. Barrett, M.W.; Klyne, W.; Scopes, P.M.; Fletcher, A.C.; Porter, L.J.; Haslam E. Plant proanthocyanidins. Part 6. Chiroptical studies. Part 95. Circular dichroism of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :2375 (1979).

148. Botha, J.J.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 1. Stereoselective and stereospecific syntheses of optically pure 4-arylflavan-3-ols, and assessment of their absolute stereochemistry at C-4 by means of circular dichroism. *J. Chem. Soc. Perkin Trans. 1* :1213 (1981).

149. Brandt, E.V.; Young, D.A.; Kolodziej, H.; Ferreira, D.; Roux, D.G. Cycloconformations of two tetraflavanoid profisetinidin condensed tannins. *J. Chem. Soc. Chem. Commun.* :913 (1986).

150. Brandt, E.V.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 20. Cycloconformations and conformational stability among derivatives of 'angular' tetraflavanoid profisetinidins. *J. Chem. Soc. Perkin Trans. 1* :2353 (1987).

151. Williams, V.M.; Porter, L.J.; Hemingway, R.W. Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry* 22:569 (1983).

152. Porter, L.J. Number- and weight-average molecular weights for some proanthocyanidin polymers (condensed tannins). *Aust. J. Chem.* 39:557 (1986).

153. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXVIII. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamomum cassia* Blume). *Chem. Pharm. Bull.* 34:633 (1986).

154. Bergmann, W.R.; Barkley, M.D.; Hemingway, R.W.; Mattice, W.L. Heterogeneous fluorescence decay of 4→6 and 4→8 linked dimers of (+)-catechin and (-)-epicatechin as a result of rotational isomerism. *J. Am. Chem. Soc.* 109:6614 (1987).

155. Cho, D.; Porter, L.J.; Hemingway, R.W.; Mattice, W.L. Fluorescence quantum yields for oligomers of (2**R**,3**S**)-[(+)-catechin] and (2**R**,3**R**)-[(-)-epicatechin]. *Polymer* (in press).

CHEMICAL NATURE OF PHLOBAPHENES

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ABSTRACT

Phlobaphenes are reddish-colored, water-insoluble phenolic substances that are believed to be related to co-occurring condensed tannins. The term is also used to describe the red insoluble material produced by treating condensed tannins with mineral acid. The chemical composition of the "natural" phlobaphenes is complex and linked to other extraneous materials in addition to condensed tannins. Phlobaphenes are variable from plant source to plant source and display a variety of functional groups not seen in the condensed tannins. Douglas-fir phlobaphenes are composed of a mixture of polymeric procyanidins, dihydroquercetin, carbohydrate (glucosyl), and methoxyl moieties. Water insolubility appears to be due to the abundance of methoxyl groups.

INTRODUCTION

Although considerable advances have been made in the chemistry of condensed tannins, the chemical constitution of phlobaphenes remains an enduring problem. This state of affairs is perhaps not unexpected as the definition of phlobaphenes is highly nebulous and can encompass many widely different plant constituents that vary from plant to plant. Over the years, two different definitions for phlobaphenes have come into common use in the literature. The first usage, preferred by many natural products chemists, refers to phlobaphenes as the reddish-colored phenolic substances extracted from plants that are alcohol soluble but water insoluble.[1,2] These "natural" phlobaphenes are believed to be structurally related to co-occurring

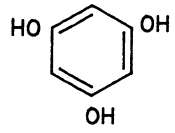
condensed tannins. Both phlobaphenes and condensed tannins commonly occur in woody plants. It has often been assumed that the natural phlobaphenes are formed by oxidation and/or polymerization of condensed tannins. Extraneous materials may be incorporated into the phlobaphene polymer during this process, which results in the observed heterogeniety of phlobaphenes from source to source.

The second common use of the term phlobaphenes stems from the tanning industry where phlobaphenes are identified with the red-colored water-insoluble reaction products – also called tannin reds – that result from treatment of tannin extracts with mineral acid.[3,4] Condensed tannins are the common link between these two types of phlobaphenes, and we are only beginning to understand some of the transformations leading to the two kinds of phlobaphenes. A priori, one would expect some significant differences between the two types of phlobaphenes because of the obviously different reaction circumstances. In this paper, the literature on phlobaphenes is surveyed with emphasis centered on the research reported since Hergert's (1962) review[2] of this subject. In addition, our studies on Douglas-fir bark phlobaphenes are reported.

PREVIOUS RESEARCH

The heterogeniety of "natural" phlobaphenes was noted earlier by Hergert,[2] who classified them into two general types. Some types differ only slightly in color and analyses from co-occurring condensed tannins, whereas, others were more deeply colored and believed to be cyanidin-like polymers.[5,6] Mullick[7] later found that the highly colored, reddish-purple pigments are specifically found in the secondary periderm of amabilis fir, western hemlock, and western red cedar and in fact are themselves each mixtures of several distinct pigments as indicated by thin layer chromatography.

A variety of functional groups has been reported to be present in phlobaphenes. For example, Swan,[8] in an extensive investigation of the phlobaphenes from western red cedar bark, concluded that they were heterogeneous polyphenolic polyesters containing both organic cations and organic anions. This material gave phloroglucinol (**1**) and protocatechuic acid (**2**) on alkali fusion as well as small amounts of fatty acids. Pyrolysis gave phenols and an unidentified yellow oil. The chemical complexity of this material no doubt was a factor in its lack of response to a suitable chromatographic system. Magnesol, florisil, nylon, charcoal, and Dowex 50W either degraded or permanently absorbed the phlobaphenes, which were finally chromatographed on a silicic acid-celite column. Methoxyl groups have been reported in some phlobaphenes[9] and variously ascribed to soluble native lignin,[10] a methylation reaction that accompanies phlobaphene formation, and co-polymerization of methoxyl-containing substances with condensed tannins.[11] In a series of elegant pyrolysis studies, Zavarin et al[9,11,12] concluded that the presence of polymers with guaiacol (**3**) or veratrol (**4**) moieties different from soluble native lignin is mainly responsible for the methoxyl content. Their results also indicated a large variability of phlobaphenes isolated from various sources, which led to the conclusion that chemical constitution of the phlobaphenes is linked to other extraneous materials as well as to condensed tannins. Douglas-fir outer bark is well known for the large



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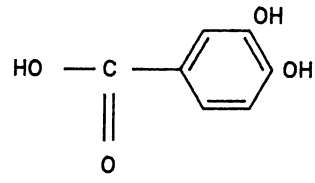

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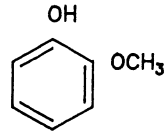

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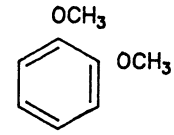

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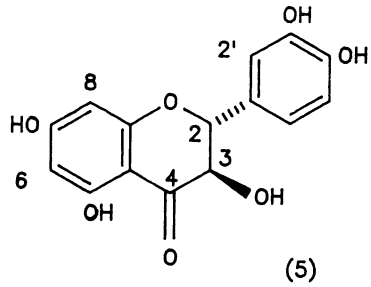


(5)

amount of dihydroquercetin (**5**) found there.[13,14] It should not then be surprising that Kurth et al[15] found that dihydroquercetin was liberated by ethanolysis of Douglas-fir outer bark phlobaphenes and proposed that dihydroquercetin is involved in phlobaphene formation in Douglas-fir by either being incorporated into the phlobaphene polymer structure or attached to it by an ether or ester linkage.

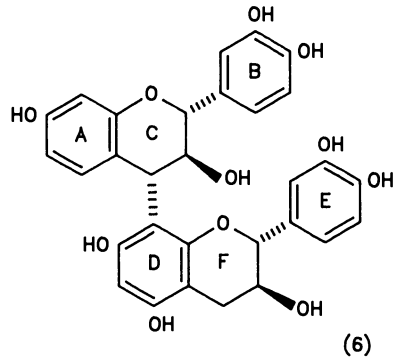
Endres et al[16] and Ishak[17] have shown that phlobaphenes from spruce and *Acacia nilotica* bark are readily solubilized in water by sulfitation, which explains the increased tannin yields when these barks are sulfite-extracted. The solubilizing effect of sulfite treatment on phlobaphenes has been known for some time and in fact was a major discovery for the tanning industry when first applied to quebracho extract.[3] A soluble quebracho extract was prepared by sulfite treatment of a tannin liquor, and its value was that the phlobaphenes did not precipitate when the extract was made ready for tanning. Tanning experiments carried out on delimed goatskin indicated good quality leather using the sulfited *Acacia nilotica* bark extract.[17] However, unsulfited phlobaphenes seem to have a negative effect on leather quality.[18] Murko

et al[19] found that tanning materials from leaves of *Salvia officinalis* L. undergo changes during longterm storage that lead to phlobaphenes.

Phlobaphenes and anthocyanidins are the two flavonoid pigments commonly found in maize.[20] While the anthocyanidins can be found in almost any tissue, the phlobaphenes are found predominantly in the cob and pericarp tissues. Genetic studies have shown that cob phlobaphenes are red in Pr plants and orange in pr plants. Anthocyanidin production is controlled by the R locus, whereas, the P locus controls production of the phlobaphenes.[21] Phlobaphenes have also been found in maize embryo tissue along with anthocyanidins.[22] The red color found in seedcoats and the red or black color of the glumes of mature grain in certain strains of sorghum have also been ascribed to phlobaphenes.[23]

Phlobaphenes have also been isolated from Bija wood (*Pterocarpus marsupium* Roxb)[24] and *Pinus radiata* bark.[25–27] The *Pinus radiata* phlobaphenes have been used in a series of interesting chemical studies. Duran et al[25] have shown that when these phlobaphenes are subjected to peroxidation with alkaline hydrogen peroxide, the reaction is accompanied by chemiluminescence in the spectral region of 500-700 nm. Condensed tannins and catechin behave similarly. When subjected to photolysis[26] in the presence of oxygen, the pine phlobaphenes undergo rapid bleaching with formation of carboxylic acid groups. A product with identical TLC behavior was obtained when catechin was similarly reacted. A mechanism is proposed for the attack of the catechin A ring by singlet oxygen to give the observed reaction products. Baeza et al[27] have also compared the pine phlobaphenes with condensed tannins and model compounds by conductimetric titration for determination of their phenolic groups.

Young et al[28] have recently investigated the acid-induced transformations of profisetinidins in order to understand the transformations taking place during the acid induced generation of phlobaphenes from condensed tannins; i.e., the "phlobaphene reaction," in tannery parlance. Treatment of the dimeric profisetinidin (**6**) with acetic acid in ethanol gave reaction products that reflected cleavage of the interflavanoid bond followed by alternative secondary reactions of anthocyanidin formation, positional rearrangement of the interflavanoid bond, or self-condensation. In addition to these products, the ring isomerized product (**7**) was also identified, and its formation can be explained by initial protonation of the C-ring heteroatom followed by ring opening to form a 2-carbenium ion that then is attacked by the 7-OH of the lower catechin unit. The natural occurrence of C-ring isomerized condensed tannins, termed phlobatannins, was demonstrated by the isolation of (**7**) and (**8**) from the heartwoods of *Guibourtia coleosperma* and *Colophospermum mopane*, respectively.[29] These ring isomerization reactions are suggestive of some of the possible transformations that could be taking place in phlobaphene formation. Steynberg et al[30] have shown that the above ring isomerization reaction readily takes place under alkaline conditions as well. The apparent conformational stability and relative planarity of the central core in (**8**) led Steenkamp et al[29] to postulate that these factors contribute to reduced water solubility and enhancement of collagen affinity.



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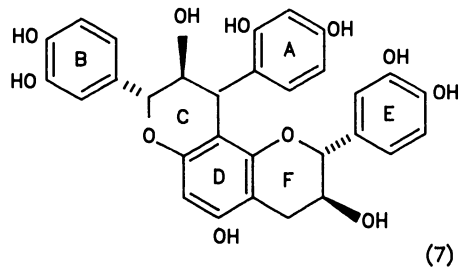

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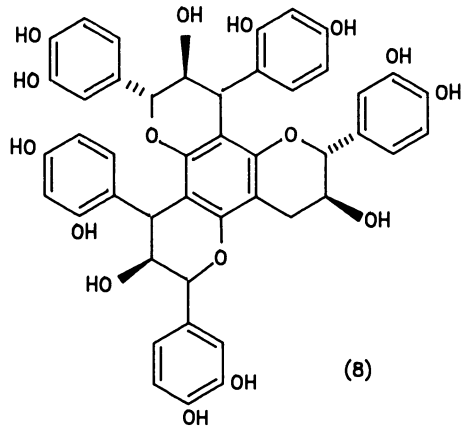

(8)

DOUGLAS-FIR BARK PHLOBAPHENES

Phlobaphenes were confined to the outer bark of Douglas-fir. Inner bark methanol extracts were found to be totally water soluble. The methanol extracts of the outer bark were purified partially by washing the reddish-brown solid material with cold hexane, ethyl acetate, and then water to give the crude phlobaphenes. Fractionation of the phlobaphenes was achieved by chromatography on Sephadex LH-20 using methanol as an eluting solvent. Six main fractions were obtained and characterized by ^{13}C-NMR and IR. A substantial portion of the crude phlobaphenes was irreversibly retained on the column.

Although six chromatographic fractions of the Douglas-fir phlobaphenes were isolated and differed from one another spectroscopically in some respects, only the chromatographic fraction C will be discussed here in some detail. The ^{13}C-NMR spectrum of this fraction shown in Figure 1 exhibits all of the features that are

present in varying degrees in the other fractions: condensed tannins (polymeric procyanidins), carbohydrate, methoxyl, and dihydroquercetin fragments. The condensed tannin moiety is evident from the key resonances, which closely parallel those observed for Douglas-fir[31] and other polymeric procyanidins[32]: (ppm) 30 (C-4 of terminal flavan unit), 38 (C-4 of extending flavan unit), the series of signals 68-83 (heterocyclic ring carbons C-2 and C-3 for both catechin and epicatechin units), B-ring signals at 116 (C-2′,5′), 119 (C-6′), 132 (C-1′), 146 (oxygen bearing C-3′,4′), and phloroglucinol A-ring oxygen-bearing carbons (C-5, 7, 9) at 154-157. The signals at 96 and 97 ppm are characteristic of the proton-bearing carbons (C-6, 8) for a phloroglucinol A-ring. However, their relative abundance seems out of proportion to the rest of the signals attributed to polymeric procyanidins and in addition is likely due to the C-6 and C-8 carbons of a dihydroquercetin moiety. Other diagnostic resonances that substantiate the presence of a dihydroquercetin moiety are the C-4 carbonyl at 198 ppm and the downfield position of the phloroglucinol A-ring oxygen-bearing carbons (C-5, 7, 9), at 162-167 ppm. A strong signal at 83 ppm

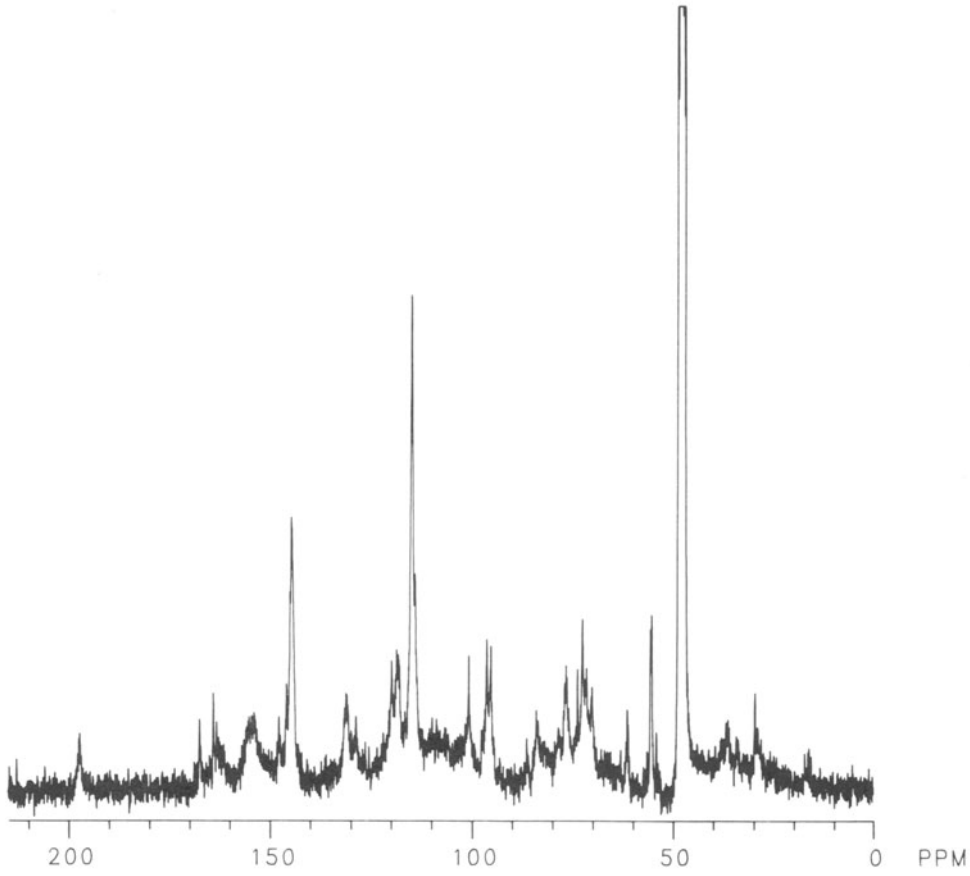

Figure 1. *^{13}C-NMR spectrum of a phlobaphene fraction from Douglas-fir bark.*

also is consistent with the C-2 of the hetrocyclic C ring of dihydroquercetin, which has 2,3-*trans* stereochemistry. Other B-ring carbon signals for dihydroquercetin are similar to the condensed tannin signals noted above.

In the spectrum of dihydroquercetin,[33] the unsubstituted C-6 and C-8 resonances are of equal intensity, very much like those observed in the phlobaphene spectrum (Figure 1). Free dihydroquercetin is unlikely to be present in the phlobaphene sample, at least to the extent indicated from the ^{13}C-NMR spectrum for the following reasons. Initial exhaustive extraction of the bark extract with ethyl acetate would have taken out most, if not all, of the monomeric dihydroquercetin. Chromatography over Sephadex LH-20 with methanol would also have readily eluted the dihydroquercetin in different fractions than the isolates collected. Thus, the observed signals could only be attributed to dihydroquercetin that is covalently bound through the B-ring to other moieties.

The carbohydrate moiety is strongly suggested by the series of relatively sharp signals at 62 (C-6), 71 (C-4), 74 (C-2), 77 (C-3), 78 (C-5), and 102 ppm (C-1), which are essentially identical to the glucosyl carbon signals for a series of phenolic glycosides isolated from Douglas-fir inner bark.[34] These glycosides included those of catechin, epicatechin, 3′-O-methyl epicatechin, dihydroquercetin, and dihydrokaempferol, all of which could presumably be incorporated into phlobaphene structures. The methoxyl moiety is readily apparent from the signals at 56 and 57 ppm and could be due to a 3′-*O*-methyl epicatechin-7-*O*-β-*D*-glucopyranoside residue, which has been isolated from Douglas-fir bark[34] and/or to the presence of lignans. The remaining resonances of many common lignans would coincide with (and could be hidden by) the other signals described above.[35]

All phlobaphene fractions showed the presence of a condensed tannin moiety and methoxyl functional groups. However, all fractions differed in the relative amounts of these and the other moieties observed in fraction C. For example, while the ^{13}C-NMR spectrum of fraction A shows resonances that are readily identified with the methoxyl, carbohydrate, and condensed tannin moieties, the spectrum is completely devoid of resonances that could be ascribed to a dihydroquercetin moiety. The later chromatographic fractions D and E, on the other hand, show evidence for the presence of all of these fractions except for the carbohydrate. The relative intensity of the dihydroquercetin resonances increases from fractions B through E.

It is clear that the phlobaphenes of Douglas-fir bark are a complex mixture of condensed tannins, carbohydrates (most likely as glucosides), methoxyl groups (likely as flavanoid methyl ethers and/or lignans), and dihydroquercetin. The question of how and in what order these entities are linked together is unclear at present. The lack of solubility in water is probably largely due to the high level of methoxyl groups, which amount to 11 percent in fraction B. While chromatography over Sephadex LH-20 gave chemically different fractions, it is still not certain if these fractions are homogeneous or a mixture of different types of polymers or reaction products. Clearly, improved chromatographic systems are needed before significant advances in structural elucidations can be made.

Our results clearly support the idea that phlobaphenes are the reaction products of condensed tannins and other extraneous materials in the bark. Douglas-fir

phlobaphenes may be unique when compared to other bark phlobaphenes because of the presence of large amounts of dihydroquercetin in the outer bark and its subsequent incorporation into the phlobaphenes structures. This situation exemplifies a problem with phlobaphene nomenclature that must be kept in mind. By definition, phlobaphenes are likely to be a crude extract preparation that is complex in structure, heterogeneous in composition, and variable from source to source.

EXPERIMENTAL METHODOLOGY

The outer bark (1.0 kg) of a 120-year-old Douglas-fir tree was extracted exhaustively with methanol and the solvent evaporated under reduced pressure to give 149 gm of a solid. The solid was extracted with cold hexane (5X, 1 liter), ethyl acetate (6X, 1 liter), and then water (1 liter) to give a final insoluble material of crude phlobaphenes (32 gm). The crude phlobaphenes were dissolved in a minimal amount of methanol and applied to a Sephadex LH-20 column. Elution with methanol gave six 2-liter fractions, which were evaporated to give the following:

Fraction A: 2.9 g. ν_{max} (cm^{-1}) 3416, 2931, 1703 (sh), 1615, 1522, 1448, 1367, 1282, 1116, 1068, 1028, 821, 780. ^{13}C-NMR gave broad peaks in the general region (MeOH-d_4, ppm): 157, 145, 133, 119, 115, 97-112, 82-84, 68-79, 61-63, 56, 38, 35, 30, 25.

Fraction B: 6.5 g. found (%) C = 58.45, H = 5.10, methoxyl = 11.67; ν_{max} 3550-3250, 2938, 1720 (sh), 1620 (br), 1518, 1448, 1365, 1285, 1164, 1114, 1028, 822, 780. ^{13}C-NMR (MeOH-d_4, ppm): 154-157, 149, 145-148, 129-132, 119, 121, 116, 111, 110, 102, 101, 97, 96, 88, 87, 81-84 (br), 79, 78, 75, 74, 73, 71, 68 (w), 62, 57, 56, 38, 35, 32, 30.

Fraction C: 2.85 mg. ^{13}C-NMR (see Figure 1).

Fraction D: 3.3 g. ν_{max} (cm^{-1}), 3416, 2931, 1615, 1525, 1445, 1364, 1285, 1248, 1203, 1160, 1113, 1066, 822, 779.

Fraction E: 2.5 g. ν_{max} (cm^{-1}) 3570-3260, 1635, 1517, 1450, 1359, 1288, 1180, 1162, 1119, 1082, 834, 779. ^{13}C-NMR (MeOH-d_4, ppm): 198, 168, 164-166, 157, 146, 133, 130, 119-120, 116, 102, 98, 97, 85, 78, 71-74, 57, 38, 30.

Fraction F: 1.0g. ν_{max} (cm^{-1}) 3430, 1692 (sh), 1611, 1519, 1445, 1364, 1284, 1240, 1204, 1162, 1114, 1070, 820, 778.

^{13}C-NMR spectra were obtained on a GE QE-300 spectrometer, and IR spectra were recorded on KBr pellets using a Nicolet 5DXB FT-IR spectrometer. Elemental and methoxyl analyses were done by Galbraith Laboratories, Knoxville, Tennessee.

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REFERENCES

1. Stahelin, C.; Hofstetter, J. Chemische untersuchung einiger Rinden. *Annal. d. Chemis. u. Pharm.* 51:63 (1844).
2. Hergert, H.L. Economic importance of flavonoid compounds: wood and bark. *In*: Geissman, T.A. (ed.) The Chemistry of Flavonoid Compounds, The MacMillan Co., New York, pp. 553-592 (1962).
3. Harvey, A. Tannin Materials with Notes of Tanning Extract Manufacture. Crosby, Lockwood and Son. London, 182p. (1921).
4. Haslam, E. Chemistry of Vegetable Tannins. Academic Press, New York, 179 p. (1966).
5. Becker, E.S.; Kurth, E.F. The chemical nature of the extractives from the bark of red fir. *Tappi* 41:380 (1958).
6. Hergert, H.L.; Blaricom, L.E.; Steinberg, J.C.; Grey, K.R. Isolation and properties of dispersants from western hemlock bark. *For. Prod. J.* 15:485 (1965).
7. Mullick, D.B. Reddish purple pigments in the secondary periderm tissues of western North American conifers. *Phytochemistry* 8:2205 (1969).
8. Swan, E.P. Characteristics of a phlobaphene from western red cedar bark. *For. Prod. J.* 13:195 (1963).
9. Zavarin, E.; Snajberk, K. The chemistry of the natural phlobaphenes. III. Pyrolysis of the phlobaphenes from five representative softwood species. *Tappi* 48:612 (1965).
10. Buchanan, M.A.; Lewis, H.F.; Kurth, E.F. Chemical nature of redwood tannin and phobaphene. *Ind. Eng. Chem.* 36:907 (1944).
11. Zavarin, E.; Snajberk, K.; Smith, R.M. The chemistry of the natural phlobaphenes. II. Further pyrolysis studies of the phlobaphenes from redwood, (*Sequoia sempervirens* Endl.) *Tappi* 48:574 (1965).
12. Zavarin, E.; Snajberk, K. The chemistry of the natural phlobaphenes. I. Pyrolysis of the phlobaphenes from redwood (*Sequoia sempervirens* Endl.) *Tappi* 46:320 (1963).
13. Hubbard, J.K.; Kurth, E.F. Douglas-fir bark tannin. *J. Am. Leather Chem. Assoc.* 44:604 (1949).
14. Hergert, H.L.; Kurth, E.F. The chemical nature of the cork from Douglas-fir bark. *Tappi* 35:59 (1952).
15. Kurth, E.F.; Aida. K.; Fujii, M. Alcoholysis products from bark flavonoids and polymeric phenolics. *Tappi* 51:461 (1968).
16. Endres, H.; Stadler, P.; Crone, F.V.D. Uber die Gerbstoffe der Fichtenrinde. X. Mitteilung. Untersuchung uber die Sulfitierung der Phlobaphene des Fichtenrindengerbstoffes. *Das Leder* 11:1 (1960).
17. Ishak, M.S. Phlobaphenes of *Acacia nilotica* bark and their sulphitation. *Leather Sci.* 20:41 (1973).
18. Ishak, M.S. Some aspects on the chemistry of phlobaphenes from *Acaia nilotica* bark. *Egypt. J. Chem.* 17:699 (1974).
19. Murko, V.D.; Ramic, S.; Kekic, M. Die Gerbstoffe der Dalmatinischen *Salvia officinalis* und deren Veranderung wahrend der Lagerung. *Planta Medica* 25:295 (1974).
20. Styles, E.D.; Oldriska, C. Genetic control of 3-hydroxy- and 3-deoxy- flavonoids in *Zea mays*. *Phytochemistry* 14:413 (1975).
21. Styles, E.D.; Oldriska, C. The genetic control of flavonoid synthesis in maize. *Can. J. Genet. Cytol.* 19:289 (1977).

22. Mathur, J.M.S.; Sharma, N.D. Unusual occurrence of phlobaphenes: the non-anthocyanic co-pigments in maize embryo. *Maize Genetics Cooperation Newsletter* 50:48 (1976).

23. Bate-Smith, E.C. Luteoforol (3′,4,4′,5,7-pentahydroxyflavan) in *Sorghum vulgare* L. *Phytochemistry* 8:1803 (1969).

24. Dhake, J.D.; Girhe, S.N. Chemical nature of extraneous components of Bija *Pterocarpus marsupium* Roxb. *IPPTA* 21:63 (1984).

25. Duran, N.; Rojas, H.; Nascimento, M.G.; Freer, J.; Baeza, J. Biomass photochemistry II. Chemiluminescence in the peroxidation of tannin, phlobaphene and catechin. *Cellulose Chem. Technol.* 18:411 (1984).

26. Duran, N.; Baeza, J.; Freer, J.; Rojas, N.; Nascimento, M.G. Biomass photochemistry, VI: Light-induced oxidation of phlobaphene from wood. *Polymer Photochem.* 6:393 (1985).

27. Baeza, J.; Freer, J.; Rojas, N.; Duran, N. Conductimetric method for the determination of phenolic groups in phlobaphene and tannin from *Pinus radiata* D. Don: solvent effect. *Analyst* 110:1407 (1985).

28. Young, D.A.; Cronje, A.; Botes, A.L.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 14. Biflavanoid profisetinidins as synthons. The acid induced 'phlobaphene' reaction. *J. Chem. Soc. Perkin Trans. 1* :2521 (1985).

29. Steenkamp, J.A.; Steynberg, J.P.; Brandt, E.V.; Ferreira, D.; Roux, D.G., Phlobatannins, a novel class of ring-isomerized condensed tannins. *J. Chem. Soc. Chem. Commun.* :1678 (1985).

30. Steynberg, J.P.; Young, D.A.; Burger, J.F.W.; Ferreira, D.; Roux, D.G. Phlobatannins via facile ring isomerizations of profisetinidin and prorobinetinidin condensed tannin units. *J. Chem. Soc. Chem. Commun.* :1013 (1986).

31. Foo, L.Y.; Karchesy, J.J. Procyanidin polymers of Douglas-fir bark. Structure from degradation with phloroglucinol. *Phytochemistry* (in press).

32. Porter, L.J.; Newman, R.H.; Foo, L.Y.; Wong, H.; Hemingway, R.W. Polymeric proanthocyanidins. ^{13}C-NMR studies of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1217 (1982).

33. Markham, K.R.; Chari, V.M.; Mabry, T.J. Carbon-13 NMR spectroscopy of flavonoids. *In*: Harborne, J.B.; Mabry, T. J. (eds.) The Flavonoids: Advances in Research. Chapman and Hall, London, pp. 19-134 (1982).

34. Foo, L.Y.; Karchesy, J.J. Polyphenolic glycosides from Douglas-fir inner bark. *Phytochemistry* (in press).

35. Agrawal, P.K., Thakur, R.S., ^{13}C NMR spectroscopy of lignin and neolignan derivatives. *Magnetic Resonance in Chemistry* 23:389 (1985).

CONFORMATIONAL ANALYSIS OF OLIGOMERIC PROANTHOCYANIDINS

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ABSTRACT

The conformational behavior of polymers of 2,3-*trans*- and 2,3-*cis*-flavan-3-ols [(+)-catechin and (-)-epicatechin] depends on the population of dihedral angles at the interflavan bond and the conformation of the heterocyclic rings. The behavior of the heterocyclic rings can be determined by study of the monomers and dimers. The results obtained from x-ray diffraction and MM2 calculations are reviewed here. Conformations at the interflavan bond are studied by MM2 calculations, high resolution ^{1}H-NMR, and time-resolved fluorescence. The implications of these results are that $4\beta \rightarrow 8$ linked polymers are random coils with dimensions somewhat smaller than those of unperturbed polystyrene chains of the same molecular weight. The dimensions of polymers with $4\beta \rightarrow 6$ links are not much different.

INTRODUCTION

Polymer scientists have long recognized three major classes of chain conformations that are experimentally distinguishable by virtue of the manner in which the mean square radius of gyration, denoted by $< s^2 >$, depends on the molecular weight, M. For monodisperse samples of high molecular weight, the relationship between $< s^2 >$ and M is of the form shown in Equation (1).

Rigid rods, which are usually helices obtained by rigorous propagations of a specified conformation by all monomer units in the chain, are characterized by $a = 2$. The collapsed state, which is best exemplified by globular proteins, has

$$< s^2 > \sim M^a \quad (1)$$

a = 2/3. The third major class, which is usually referred to as a random or statistical coil, has a = 1.0-1.2. The value of 1.0 is obtained in the unperturbed state, which is experimentally accessible in the bulk amorphous polymer or in a dilute solution in a rather poor solvent where the second virial coefficient is zero. The larger value of 1.2 is obtained with a random coil in dilute solution in a good solvent.

Application of the classical methodology to polymeric proanthocyanidins would require the measurement of $<s^2>$ for several monodisperse samples of high molecular weight and constant local covalent structure. This approach has not yet been feasible because of the lack of monodisperse samples with a specified type of monomer and interflavan linkage and a large range of molecular weights.

An alternative approach to the specification of the conformation of a polymer exploits detailed knowledge of the conformational properties of the monomer and small oligomers. Rotational isomeric state theory provides the formalism for the construction of a realistic model for the conformation of a polymer of high molecular weight if sufficient information is available for the monomer and small oligomers.[1] The rotational isomeric state approach is currently being implemented for polymers of catechin and epicatechin. The database is provided by crystal structures determined by x-ray diffraction,[2-8] conformational energy calculations,[8,10-12] high resolution ^{1}H-NMR,[7-9,13-16] and time-resolved fluorescence.[16,17] The present status of the database is reviewed here. Selected rotational isomeric state extensions to high polymers are also summarized.[17-20]

STRUCTURES IN THE CRYSTALLINE STATE

The angles between the C(4)-H bonds in a monomer and the mean plane of the fused ring system are strongly dependent on the dihedral angle at the C(4)-C(10) bond. The numbering scheme for the carbon atoms and the definitions of the A- and B-rings are shown in Figure 1. One or the other of the C-H bonds is replaced by the interflavan bond in a dimer with an α or β linkage at C(4). The conformational properties of a polymer are sensitive to the dihedral angle at C(4)-C(10), and hence, to the puckering of the heterocyclic ring, because this dihedral angle

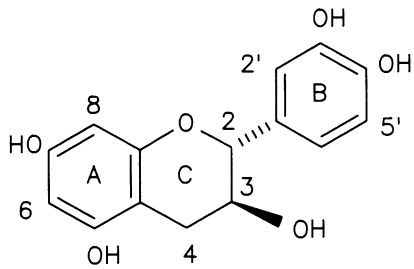

Figure 1. *Numbering system used for (+)-catechin and definition of the A- and B-rings.*

affects the relative orientation of monomer units that are nearest neighbors. X-ray diffraction studies of monomers and their derivatives yield important information about the conformation of the heterocyclic ring.

Two independent studies of the structure of crystalline (-)-epicatechin were reported in 1984.[3,4] Both accounts describe the same basic structure, but considerably higher resolution was obtained by Fronczek et al.[3] The structure obtained at higher resolution is depicted in Figure 2. The conformation of the heterocyclic ring places the B-ring in a pseudo-equatorial position. The value of the dihedral angle defined by C(3)-C(4)-C(10)-C(9) is -18.2°. Atoms C(2) and C(3) are 26.3 pm above and 49.5 pm below, respectively, the mean plane of the A-ring. The heterocyclic ring can be described as a half-chair that is distorted toward a C(3) sofa. There are no intramolecular hydrogen bonds, but four of the five hydroxyl groups participate in intermolecular hydrogen bonds in the crystal. No crystal structure is available for (+)-catechin. There are, however, several crystal structures for derivatives of (+)-catechin[2,5-8] and (-)-epicatechin.[9] Three of these structures deserve special mention for historical reasons,[2] for the demonstration of an unusual conformation of the heterocyclic ring,[7] and for insight into the conformation adopted in oligomers with an α linkage at C(4).[8] The first structure reported for this class of molecules is the description in 1978 of 8-bromotetra-*O*-methyl-(+)-catechin.[2] The heterocyclic ring is a half-chair that is distorted toward a C(2) sofa. It places the B-ring in a pseudo-equatorial position. Six years were to pass before the publication of the next detailed crystal structure for a molecule in this class. That structure was the one adopted by (-)-epicatechin and described at the beginning of this paragraph.[3] Penta-*O*-acetyl-(+)-catechin is unique in that the heterocyclic ring adopts a geometry that places the B-ring in a pseudo-axial position,[7] rather than the more commonly observed pseudo-equatorial position. The dihedral angle defined by C(3)-C(9)-C(10)-C(9) is 16°,[7] in contrast with the value of -18.2° found

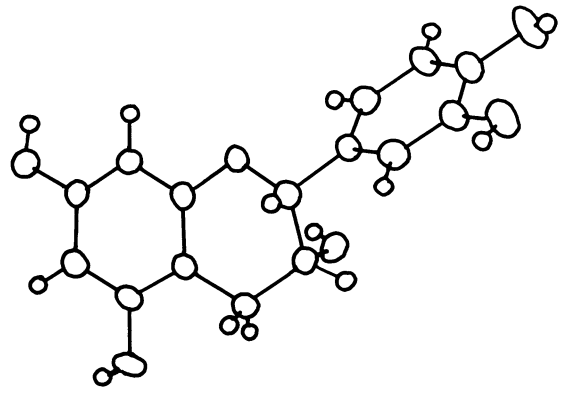

Figure 2. *Structure of (-)-epicatechin in the crystal. (Figure reproduced from J. Chem. Soc. Perkin Trans. 2: 1611, 1984).*

in (-)-epicatechin.[3] This structure is also noteworthy in that there is some disorder in penta-*O*-acetyl-(+)-catechin in the crystal, with two distinct positions accessible to the acetyl group at C(5). The structure of (+)-catechin-(4α → 2)-phloroglucinol heptamethyl ether is noteworthy because it is the first description of a molecule that has a substituent at C(4) that provides a model for the interflavan bond found in dimers with a 4α linkage.[8] The C(3)-C(4)-C(10)-C(9) dihedral angle is -8°, the B-ring occupies a pseudo-equatorial position, and the heterocyclic ring is a half-chair that is distorted toward a C(2) sofa.

CONFORMATIONAL ENERGY CALCULATIONS

A thorough analysis of the conformations of the heterocyclic rings in monomers[8,12] and dimers[10-12] has been performed using the computer program known as MM2[21] with the addition of several parameters.[10,22] Comparison of the optimized structures obtained from the MM2 calculations and the conformations revealed by x-ray diffraction of crystals provides grounds for confidence in the ability of MM2 to determine the optimized conformations of the heterocyclic ring. MM2 correctly predicts that the heterocyclic ring in (-)-epicatechin is a half-chair with distortion toward a C(3) sofa.[8] It also predicts that (+)-catechin should have a virtually identical conformation. (This prediction is not susceptible to comparison with the results of an x-ray diffraction study due to the absence of suitable crystals.) Attachments of a phloroglucinol ring at C(4) of (+)-catechin via an α linkage, however, cause the optimized heterocyclic ring to flip to a half-chair that is distorted toward a C(2) sofa, in agreement with experiment.[8] Finally, optimization by MM2 always favors a heterocyclic ring that places the B-ring in a pseudo-equatorial position over a heterocyclic ring that assigns the B-ring to a pseudo-axial position.[11] This result is consistent with all but one of the available crystal structures. The exception is penta-*O*-acetyl-(+)-catechin, in which the B-ring occupies a pseudo-axial position in the crystal.[7] This apparent disagreement between MM2 and experiment may be due to the influence of intermolecular interactions that are unique to the crystalline environment. High resolution ^{1}H-NMR spectra strongly suggest that dissolution of the crystal is accompanied by a change in conformation. The interpretation of the high resolution ^{1}H-NMR spectra in dilute solution is that the conformation might be described as a nearly equimolar mixture of pseudo-axial and pseudo-equatorial conformations.[7,8]

MM2 can provide a much more comprehensive picture of the conformational characteristics of the heterocyclic ring than can experiment because the computation can be applied to molecules for which samples are not available. This algorithm has been used to identify the optimized conformations of 128 heterocyclic rings in a variety of dimers.[10,11] The dimers examined exhaust all combinations of (+)-catechin or (-)-epicatechin for the top unit, (+)-catechin or (-)-epicatechin for the bottom unit, α or β linkage, 4 → 6 or 4 → 8 linkage, positive or negative rotamer at the interflavan bond, and pseudo-axial or pseudo-equatorial position for the B-rings.The data are conveniently summarized by depicting the distances of C(2) and C(3) from the mean plane of the fused ring system. This information is summarized in Figure 3. The points describe two families of conformations.[11] Changes within a family are achieved by coordinated movement of C(2) and C(3) with

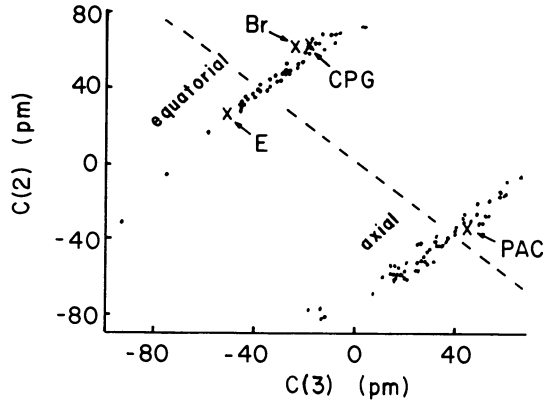

Figure 3. *Distances of C(2) and C(3) from the mean plane of the fused ring system in (+)-catechin and (-)-epicatechin units in dimers with B-rings in pseudo-axial or pseudo-equatorial positions. The unlabelled filled circles denote the conformations in structures optimized by MM2. The four X's denote the conformations observed in four crystal structures: (-)-epicatechin (E), 8-bromotetra-O-methyl-(+)-catechin (Br), (+)-catechin-(4α → 2)-phloroglucinol heptamethyl ether (CPG), and penta-O-acetyl-(+)-catechin (PAC). The dashed line is C(2) = -C(3). (Figure reproduced from J. Chem. Soc. Perkin Trans. 2, 739, 1987).*

respect to the mean plane of the fused ring system. The conformations found in the crystal structures of 8-bromotetra-*O*-methyl-(+)-catechin,[2] (-)-epicatechin,[3,4] and (+)-catechin-(4α → 2)-phloroglucinol heptamethyl ether[8] are located well within the family of conformations found in dimers in which the B-rings occupy pseudo-equatorial positions.[10] The MM2 calculations define another parallel family for dimers in which the B-rings occupy pseudo-axial positions.[11] The only relevant crystal structure, for penta-*O*-acetyl-(+)-catechin,[7] is found here. The MM2 calculations show that the optimal structures of a heterocyclic ring in a dimer vary over a range that requires coordinated movements of 40 pm or more by C(2) and C(3) with respect to the mean plane of the fused ring system. Detailed descriptions of the individual conformations can be found in the original accounts.[10,11] The conformations of the heterocyclic rings are affected very little by the selections of a 4′-hydroxy, 3′,4′-dihydroxy, or 3′,4′,5′-trihydroxy B-ring.[12] All dimers exhibit a twofold rotation about the interflavan bond. The two conformation energy minima are quite narrow due to the rapid onset of repulsive steric interactions as the dihedral angle at the interflavan bond rotates away from an equilibrium position.[10,11] The ranges of the dihedral angles at the two conformational energy minima are 74° to 102° and -80° to -102° in 4 → 6 linked dimers, with the atoms defining the dihedral angle being C(3)-C(4)-C(6)-C(5). With 4 → 8 linked dimers, the ranges are

74° to 104° and -78° to -104°, with the angle here defined by C(3)-C(4)-C(8)-C(9). In the 32 dimers considered, the difference in conformational energy, ΔE, between the rotational isomer with an interflavan dihedral angle near 90° and the isomer with an interflavan dihedral angle near -90° ranges from -3.9 to 3.6 kcal/mol,[11] according to the MM2 calculations. These results are best interpreted as suggesting that some dimers will exhibit a strong preference for one rotational isomer or the other, but there will also be dimers that have significant populations of both rotational isomers (the absolute value of the calculated ΔE is smaller than RT at 300 °K for $\simeq 1/4$ of the dimers studied).

Unfortunately, MM2 is much more effective in defining the optimal conformation of the heterocyclic ring and the optimum dihedral angle at the interflavan bond than in defining the difference in conformational energies of the two rotational isomers at the interflavan bond. The origin of this difficulty is best explained with the aid of Figure 4. Here ϕ denotes the conformational variables that define the conformations of the heterocyclic rings and the interflavan bond. It excludes the other conformational variables, such as the dihedral angles

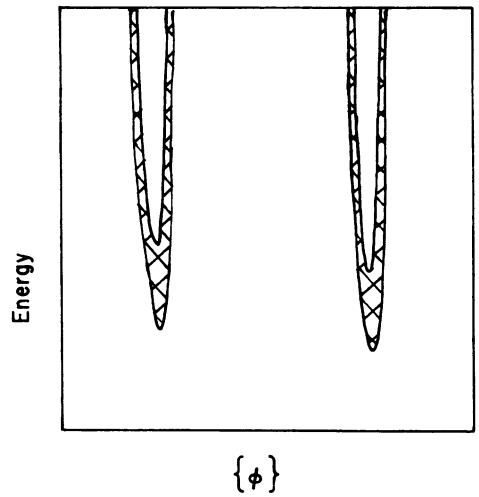

Figure 4. *Schematic of the relationship between the conformational energy of a dimer and the conformation. The set of dihedral angles that defines the conformations of the heterocyclic rings and the dihedral angle of the interflavan bond is denoted by ϕ. The two curves are drawn in the regions of ϕ that correspond to the two rotational isomers at the interflavan bond. Variation in the remaining dihedral angles, denoted by ψ, is responsible for the range of conformational energies at each value of ϕ.*

about C-OH bonds and the dihedral angle at the bond from C(2) to the B-ring. These other conformational variables are collectively denoted by ψ. The minimum at the left in Figure 4 is in the region of ϕ where the dihedral angle at the interflavan bond is near -90°, and the minimum at the right is in the region of ϕ where this dihedral angle is near 90°. Small changes in any of the variables that contribute to ϕ bring about large changes in the calculated conformational energy. Therefore, the set of ϕ at each minimum is well defined. The cross-hatched region depicts the uncertainty in the calculated conformational energy that arises from the difficulty in dealing with the large number of additional conformational variables denoted by ψ. The width of each cross-hatched band is larger than RT. Dipolar interactions make an important contribution to the uncertainty in conformational energy associated with ψ.[3,12,23] The size of this cross-hatched band makes it difficult to confidently predict the relative populations of the two rotational isomers, even though the conformation of the heterocyclic rings and the dihedral angles at the interflavan bond are well defined. For this reason, the relative populations of the two rotational isomers cannot be determined by the MM2 calculations. It must be determined by an appeal to experimentation.

HIGH RESOLUTION ^{1}H-NMR

The coupling constants for vicinal protons in the high resolution ^{1}H-NMR spectra provide experimental evidence for the conformation of the heterocyclic ring in dilute solution. The analysis of (-)-epicatechin shows that the ratio of pseudo-equatorial to pseudo-axial heterocyclic ring conformations is 86:14.[8] The dominance of the conformation with a pseudo-equatorial B-ring is consistent with the structure observed in the crystal[3,4] and obtained by optimization with MM2.[8] The pseudo-equatorial conformation is also favored by (+)-catechin, but it is not as dominant as was the case with (-)-epicatechin. The ratio is 62:38.[8]

Acetylation of the hydroxyl group at C(3) brings the ratio to 48:52.[8] Experimentally, there are nearly equal amounts of the two conformations in dilute solution, but the pseudo-axial conformation is the only one found in the crystal of penta-*O*-acetyl-(+)-catechin,[7] and optimization of the structure of an isolated molecule of penta-*O*-acetyl-(+)-catechin leads to a pseudo-equatorial conformation. The delicate balance between pseudo-axial and pseudo-equatorial in solution is tipped toward pseudo-axial by intermolecular interactions in the crystal, but it is tipped toward pseudo-equatorial by the parameters in the MM2 force field. Of particular relevance for the structures of the polymers are the effects seen upon the introduction of a substituent at C(4). This substitution strongly favors the pseudo-equatorial conformation of the B-ring at C(2).[8] The heterocyclic ring favors a half-chair in an (-)-epicatechin substituted at C(4), and it favors a C(2) sofa in the similarly substituted (+)-catechin,[8] according to the ^{1}H-NMR measurements. Of course, the much more extensive range of samples amenable to study by MM2 shows that there is substantial overlap in the ranges of conformations adopted by (+)-catechin and (-)-epicatechin with substitutents at C(4).[10,11]

High resolution ^{1}H-NMR also can provide evidence for rotational isomerism at the interflavan bond in selected dimers by the appearance of doublets, often of unequal intensity.[13–16] The ability to detect rotational isomers by ^{1}H-NMR is

enhanced in the presence of substituents that increase the energy barrier that must be crossed in interconversion from one rotational isomer to the other. For example, high resolution ^{1}H-NMR can detect rotational isomers at 25 °C for (-)-epicatechin-(4β → 8)-(+)-catechin decaacetate at 25 °C in nitrobenzene[14,16] and dioxane,[16] with the fraction of the dimers populating the dominant rotational isomer being 0.84 ± 0.01. In contrast, a first-order spectrum is observed at 25 °C in acetone if the ten acetate groups are removed.[13] Evidence for rotational isomers in the biologically important free phenol form of the dimer is present in ^{1}H-NMR spectra at lower temperatures in suitable solvent systems, such as acetone at 0 °C.[13] In the free phenol forms, the interconversion of the two rotational isomers at 25 °C is "fast" on the NMR time scale, and a first-order spectrum is observed. The more severe steric interactions generated by the acetate groups cause an increase in the barrier height in the decaacetates, and the interconversion at 25 °C is "slow" on the NMR time scale. Two distinct sets of resonances are then observed. This behavior suggests that ^{1}H-NMR may be unsuitable for detection of rotational isomers in the free phenol forms at ambient temperatures. Attainment of this objective requires the use of a technique that has a faster time scale.

STATIONARY STATE FLUORESCENCE

Unionized (+)-catechin and (-)-epicatechin in dilute solution in dioxane or water exhibit an absorption band with a maximum near 280 nm. Separate contributions to this band by the A- or B-rings cannot be resolved. A single fluorescence emission band at 310-321 nm is observed upon excitation at 280 nm.[16,24] Distinct contributions by the A- and B-rings cannot be resolved in this broad structureless emission band. The fluorescence quantum yields in a particular solvent are indistinguishable for (+)-catechin and (-)-epicatechin. These quantum yields are about 0.3 in dioxane and 0.1 in water. The larger quantum yield in dioxane makes it the more attractive solvent for fluorescence work.

Study of several dimers[16,24] supports three generalizations. Dimers exhibit emission in the same spectral region as the monomers, but with reduced quantum yields for fluorescence. The quantum yields are lower for peracetylated dimers than for the free phenol forms of the same molecules. Quantum yields measured in dioxane are larger than those in water.

TIME-RESOLVED FLUORESCENCE

Following excitation by an extremely short pulse of light, the time dependence of the fluorescence from the monomers can be described by a single exponential decay (equation 2), where I(t) is the fluorescence intensity at time t, τ is the

$$I(t) = \alpha \cdot exp(-t/\tau) \tag{2}$$

fluorescence lifetime, and α is the preexponential factor.[16] Both monomers have the same value of τ in a common solvent, but this value depends on the solvent. It is 2 ns in dioxane, and 0.6-0.7 ns in water. Consequently, the fluorescence quantum yield, Q, is described by equation (3):

$$Q = \tau/\tau_o \tag{3}$$

where τ_o is the intrinsic radiative lifetime, which has a value of about 7 ns in both solvents. Additional nonradiative decay processes for the electronic excited state in water are responsible for the lower quantum yield in this solvent.

Dimers that can populate two rotational isomers have a more complicated fluorescence decay than the one described by equation (2). For the two dimers depicted in Figure 5, an adequate description is provided by a decay law that is the sum of two exponentials (equation 4).

$$I(t) = \alpha_1 \cdot exp(-t/\tau_1) + \alpha_2 \cdot exp(-t/\tau_2) \tag{4}$$

When this decay law is applied to the time-resolved fluorescence of (-)-epicatechin-(4$\beta \rightarrow$ 8)-(+)-catechin decaacetate, the value of $[\alpha_1/(\alpha_1 + \alpha_2)]$ is found to be 0.90 $\pm$ 0.05.[16] This result suggests that the dominant rotational isomer, for which the high resolution ^{1}H-NMR finds a population of 0.84 in this solvent, is responsible for that portion of the decay characterized by τ_1. The value of τ_1 is 0.48 $\pm$ 0.05 ns. The remainder of the decay, with τ_2 = 1.4 $\pm$ 0.2 ns, is contributed by the peracetylated dimers that populate the other rotational isomer.This interpretation of the time-resolved fluorescence is supported by the observation of a simpler decay, described by equation (2), in the dimer (-)-epicatechin-(4$\beta \rightarrow$ 8; 2$\beta \rightarrow$ 7)-(+)-catechin.[16] The bridging oxygen atom enforces the population of a single rotational isomer, and the result is a fluorescence decay that can be described by a single exponential term, equation (2).

The most exciting fluorescence decay results are obtained with the free phenol forms of unbridged dimers. Typically, these dimers exhibit first-order high resolution ^{1}H-NMR spectra at ambient temperatures. Nevertheless, the fluorescence

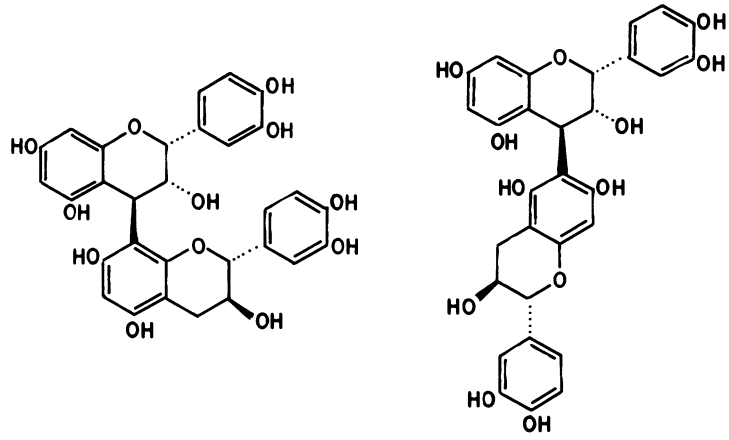

Figure 5. *Structures of the dimers studied by the time-resolved fluorescence.*

decay of (-)-epicatechin-(4β → 6)-(+)-catechin and (-)-epicatechin-(4β → 8)-(+)-catechin cannot be described by a single exponential term (equation 2), but instead requires the use of a sum of two exponential terms (equation (4)). The values of $[\alpha_1/(\alpha_1 + \alpha_2)]$ in dioxane are 0.85 ± 0.08 and 0.75 ± 0.15, respectively, for these two dimers.[16] These values provide a measurement of the population of the dominant rotational isomer in the free phenol forms at 25 °C. The rotational isomers are detectable by the time-resolved fluorescence, even though they cannot be resolved by ^{1}H NMR, because of the faster time scale (ns) of the fluorescence measurement.

The importance of the time-resolved fluorescence in the conformational analysis of oligomeric procyanidins lies in its ability to resolve the populations of the rotational isomers at the interflavan bond in the biologically important free phenol forms at ambient temperatures. These populations cannot be assigned by MM2 calculations because of the complication introduced by ψ, and the rapid interconversion of the rotational isomers prevents their resolution by ^{1}H NMR spectra.

IMPLICATIONS FOR HIGH POLYMERS

Rotational isomeric state theory[1] permits estimation of the conformational properties of higher polymers from the information provided by MM2 calculations and time-resolved fluorescence measurements. The bond lengths and bond angles, all of which fall within the usual ranges, are taken from the MM2 calculations.[10,11] MM2 also provides the values of the dihedral angles, including the crucial dihedral angles in the heterocyclic ring and at the interflavan bond. The relative population of the two rotational isomers at the interflavan bond is provided by the pre-exponential factors in the analysis of the time-resolved fluorescence by equation (4).[16]

Application to polymers that contain 4β → 6[19] or 4β → 8[18] linkages shows that the dimensions of the high polymers are extremely sensitive to the relative population of the two rotational isomers at the interflavan bond. The polymers form helices if one rotational isomer is populated to the exclusion of the other. The handedness of the helix is determined by the selection of the rotational isomer. For example, in the polymers with 4β → 8 linkages, propagation of one rotational isomer produces a right-handed helix with 3.0 residues per turn and a translation per residue of 0.31 nm.[18] Selection of the other rotational isomer produces a more extended (translation per residue of 0.47 nm) left-handed helix with 4.1 residues per turn. Clearly, the two helices are not mirror images! If population of both rotational isomers is allowed, there is a marked collapse in the dimensions of the chain. If the relative population of the two rotational isomers is 0.75 ± 0.15, as required by the time-resolved fluorescence in dioxane, the value of $< s^2 >$ for a large 4β → 8 linked polymer is somewhat smaller than that found for an unperturbed polystyrene chain of the same molecular weight in cyclohexane at 35 °C.[18] The conformation is an example of a random coil. The behavior of the circular dichroism is consistent with this interpretation.[25,26]

Further work with other oligomers will be required before generalizations can be drawn that would apply to all conceivable polymers of the catechin and epicatechin types. At this time, one cannot exclude the possibility that other types of linkages between the monomer units might produce polymers with $< s^2 >$ quite different from those described for chains in which the linkages are 4β → 6 or 4β → 8. A

thorough study of the fluorescence of addition oligomers should provide the missing information required for a more general description of the conformations of the polymers. Detailed knowledge of the conformational properties of small oligomers will also be of great use in developing an understanding of the detailed structures of their complexes with polypeptides.[27]

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REFERENCES

1. Flory, P.J. Foundations of rotational isomeric state theory and general methods for generating configurational averages. *Macromolecules* 7:381 (1974).
2. Engel, D.W.; Hattingh, M.; Hundt, H.K.L.; Roux, D.G. X-ray structure, conformation, and absolute configuration of 8-bromo-tetra-*O*-methyl-(+)-catechin. *J. Chem. Soc. Chem. Commun.* :695 (1978).
3. Fronczek, F.R.; Gannuch, G.; Mattice, W.L.; Tobiason, F.L.; Broeker, J.L.; Hemingway, R.W. Dipole moment, solution conformation and solid state structure of (-)-epicatechin, a monomer of procyanidin polymers. *J. Chem. Soc. Perkin Trans. 2* :1611 (1984).
4. Spek, A.L.; Kojic-Prodic, B.; Labadie, R.P. Structure of (-)-epicatechin: (2**R**,3**R**)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran -3,5,7-triol, $C_{15}H_{14}O_6$. *Acta Crystallogr.* C40:2069 (1984).
5. Einstein, F.W.B.; Kiehlmann, E.; Wolowidnyk, E.K. Structure and nuclear magnetic resonance spectra of 6-bromo-3,3′,4′,5,7-penta-*O*-methylcatechin. *Can. J. Chem.* 63:2176 (1985).
6. Porter, L.J.; Wong, R.Y.; Chan, B.G. The molecular and crystal structure of (+)-2,3-*trans*-3,4-*trans*-leucocyanidin (2**R**,3**S**,4**R**)-(+)-3,3′,4,4′,5,7-hexahydroxyflavan)-dihydrate and comparison of its heterocyclic conformation in solution and the solid state. *J. Chem. Soc. Perkin Trans. 1* :1413 (1985).
7. Fronczek, F.R.; Gannuch, G.; Mattice, W.L.; Hemingway, R.W.; Chiari, G.; Tobiason, F.L.; Houglum, K.; Shanafelt, A. Preference of occupancy of axial positions by substituents bonded to the heterocyclic ring in penta-*O*-acetyl-(+)-catechin. *J. Chem. Soc. Perkin Trans. 2* :1383 (1985).
8. Porter, L.J.; Wong, R.Y.; Benson, M.; Chan, B.G.; Viswanadhan, V.N.; Gandour, R.D.; Mattice, W.L. Conformational analysis of flavans: ^{1}H-NMR and molecular mechanical (MM2) studies for the benzpyran ring of 3′,4′,5,7-tetrahydroxyflavan-3-ols: the crystal and molecular structure of the procyanidin: (2**R**,3**S**,4**R**)-3',4',5,7-tetramethoxy-4-(2,4,6-trimethoxyphenyl)-flavan-3-ol. *J. Chem. Res.* (S):86, (M):830 (1986).
9. Boeyens, J.C.A.; Denner, L.; Kolodziej, H.; Ferreira, D.; Roux, D.G. Structure, conformation and absolute configuration of 3-*O*-acetoxy-6-bromo-3′,4′,5,7-tetra-*O*-methyl-(-)-epicatechin. *J. Chem. Soc. Perkin Trans. 2* :301 (1986).
10. Viswanadhan, V.N.; Mattice, W.L. Conformational statistics of (4 → 6) and (4 → 8) β-linked homopolymers of (+)-catechin or (-)-epicatechin. *J. Comput. Chem.* 7:711. (1986).

11. Viswanadhan, V.N.; Mattice, W.L. Preferred conformations of the sixteen (4 → 6) and (4 → 8) linked dimers of (+)-catechin and (-)-epicatechin with axial or equatorial dihydroxyphenyl rings at C(2). *J. Chem. Soc. Perkin Trans. 2* :739 (1987).
12. Viswanadhan, V.N.; Mattice, W.L. Conformation of monomers and dimers of 2,3-*trans* and 2,3-*cis*-flavan-3-ols with differing hydroxylation patterns in the B-ring. *Int. J. Biol. Macromol.* (in press).
13. Fletcher, A.C.; Porter, L.J.; Haslam, E.; Gupta, R.K. Plant proanthocyanidins. Part 3. Conformational and configurational studies of natural procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1628 (1977).
14. Rauwald, H.W. ^{1}H-NMR studie zur analytik rotamerer procyanidinie. *J. Med. Plant Res.* 46:110 (1982).
15. Foo, L.Y.; Porter, L.J. Synthesis and conformation of procyanidin diastereoisomers. *J. Chem. Soc. Perkin Trans. 1* :1535 (1983).
16. Bergmann, W.R.; Barkley, M.D.; Hemingway, R.W.; Mattice, W.L. Heterogeneous fluorescence decay of 4 → 6 and 4 → 8 linked dimers of (+)-catechin and (-)-epicatechin as a result of rotational isomerism. *J. Am. Chem. Soc.* 109:6614 (1987).
17. Mattice, W.L. The role of fluorescence in the determination of the unperturbed dimensions of polymers of (+)-catechin and (-)-epicatechin *ACS Symp. Ser.* (in press).
18. Viswanadhan, V.N.; Bergmann, W.R.; Mattice, W.L. Configurational statistics of (4 → 6) β-linked homopolymers of (+)-catechin or (-)-epicatechin. *Macromolecules* 20:1539 (1987).
19. Bergmann, W.R.; Viswanadhan, V.N.; Mattice, W.L. Conformations of polymeric proanthocyanidins composed of (+)-catechin or (-)-epicatechin joined by 4-6 interflavan bonds. *J. Chem. Soc. Perkin Trans. 2* :45 (1988).
20. Viswanadhan, V.N.; Mattice, W.L. Configurational statistics of C(4)-C(8) linked polymers of (+)-catechin or (-)-epicatechin with mixed axial/equitorial substituents at C(2). *Int. J. Biol. Macromol.* 10:9 (1988).
21. Allinger, N.L.; Yuh, Y.H. *Quantum Chemistry Program Exchange* 12:395 (1980).
22. Allinger, N.L., (personal communication).
23. Mattice, W.L.; Tobiason, F.L.; Houglum, K.; Shanafelt, A. Conformational analysis and dipole moments of tetra-*O*-methyl-(-)-epicatechin. *J. Am. Chem. Soc.* 104:3359 (1982).
24. Bergmann, W.R.; Mattice, W.L. Specific interactions of (+)-catechin and (-)-epicatechin with polymers that contain the L-prolyl residue. *ACS Symp. Ser.* 358:162 (1987).
25. Czochanska, Z.; Foo, L.Y.; Newman, R.H.; Porter, L.J. Polymeric proanthocyanidins, stereochemistry, structural units and molecular weight. *J. Chem. Soc. Perkin Trans. 1* :2278 (1980).
26. Gaffield, W.; Foo, L.Y.; Porter, L.J. Chiroptical properties of tannins – intense split Cotten effects of dimeric procyanidins at low wavelength.*Proc. Fed. Eur. Chem. Soc. Int. Conf. Circular Dichroism* 6:338 (1985).
27. Tilstra, L.F.; Maeda, H.; Mattice, W.L. Interaction of (+)-catechin with the edge of the β sheet formed by poly(S-carboxymethyl-L-cysteine). *J. Chem. Soc., Perkin Trans. 2* :1213 (1988).

AN OVERVIEW OF CONDENSED TANNIN STRUCTURE

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ABSTRACT

The proanthocyanidins are an important class of natural products forming the basis for various classes of flavonoid polymers such as the condensed tannins, phlobaphenes, and phenolic acids. Refining our understanding of the structure of polyflavonoids will lead to a better appreciation of their properties and help in developing their use as a renewable source of commodity and specialty chemicals. Considerable progress has already been made in the last two decades, but more information is needed on structural elaborations during, and particularly after, their biosynthesis. Armed with further information on the structure of polyflavonoids, we should be able to understand better their interactions with other biopolymers and hence their biological significance.

INTRODUCTION

The purpose of this overview is not to summarize the chapters within this section on structure, but to provide a context into which they fit. Condensed tannins are not an isolated group of compounds, but a part of the vast collection of compounds and chemistries that make up the chemicals called natural products. In plants, these include the primary metabolites such as proteins, lignin, structural and storage carbohydrates, ribonucleic acids and various lipids, as well as the secondary metabolites in which the polyflavonoids are grouped along with terpenes, alkaloids, polyacetylenes, phenylpropanoids, stilbenes, and other phenolics. In order to appreciate the current—and especially the potential—importance of polyflavonoids, some discussion of the current trends in natural products research is necessary.

By any classification system, the polyflavonoids must be considered to be an important group of plant natural products. They are the second most abundant natural phenolic materials after lignin and are found in species throughout the plant kingdom; often in high concentrations. For example, they are ubiquitous in conifers, being found in the bark and foliage of most species and in the heartwood of many.[1] Despite their widespread occurrence, the functions of the polyflavonoids have not yet been clearly elucidated (Chapters 3, 22-28). It is generally accepted, however, that they are involved in defense against pathogens and/or herbivores. Whatever their function, clearly they would not be so ubiquitous nor accumulated in such high concentrations, and at a high energy cost (Chapter 2), if they did not benefit the plant.[2]

WHY STUDY CONDENSED TANNIN STRUCTURE?

There are many reasons for studying condensed tannin structure. Perhaps the most obvious practical reason is that they are an abundant chemical resource with many real and potential applications (Chapters 29-33). The greater use of renewable biomass as a source of commodity and specialty chemicals is inevitable. If the cost of petrochemicals does not increase for political reasons, it will be because the more easily accessible petroleum reserves are depleted, and more expensive deposits have to be used. We need to be ready for comparatively expensive petrochemicals by increasing our understanding of the structure and chemistries of the biomass resource. Because of their structure, and resulting wide ranging reactivities, the condensed tannins are potentially a valuable source of a variety of industrial materials, especially specialty chemicals. However, this resource can only be used efficiently if the structures of the tannins are well understood.

As with most other biomass chemicals, condensed tannins are intrinsically a variable resource. Their structure and properties depend on the particular plant species being used, the time of harvest during the year, and the age of the material being extracted. This variability is of particular concern in North America where most interest is in the procyanidins. This class of polyflavonoids is more reactive than the resorcinolic profisetinidins and prorobinetinidins, resulting in more structural variation occurring during biosynthesis, as well as after deposition in the plant tissues. A number of these post-synthesis modifications to a regular proanthocyanidin structure is described in Chapter 6 on Douglas-fir phlobaphenes. Upon exposure to atmospheric oxygen, portions of the polyflavonoid structure can be oxidized to reactive intermediates (Chapter 15) that can either rearrange or react with other chemical species present to give a wide range of derivative structures. How much the regular polyflavonoid structure is altered and the nature of the alteration will affect how appropriate the plant extract is for a given application. For example, if a tannin preparation with a relatively regular proanthocyanidin structure is desired, the best source may be plant materials produced on an annual basis such as nut residues, rather than tree barks that are exposed to the environment for many years and probably undergo a large amount of structure alteration. Information on the nature of these modifications is generally lacking and is important in making decisions on the suitability of a condensed tannin source for a given use.

Another way in which an understanding of the structure and function of natural products can be valuable is through their acting as models for synthetic chemical products, especially pharmaceuticals and biocides. Due to increasing EPA regulations reflecting environmental and mammalian toxicity concerns, there has been a dramatic change in the types of biocides used in the U.S. The trend has been to go from broad spectrum, persistent chemicals to products with more selective toxic activities and shorter lifetimes. Some of the latter biocides are either of a biological origin (e.g., *Bacillus thuriengensis*) or modeled on natural products. A recent example of this evolution in biocides is in soil treatment insecticides for control of termites. Up until 1987, the principal chemicals for this application were chlorinated hydrocarbons (e.g., chlordane). Due to toxicity concerns, the use of these chemicals has been greatly restricted. One group of pesticides now being used for soil treatment is the pyrethroids, analogues of a natural insecticide, pyrethrum. In 1988, about one-third of the pest control companies in the U.S. were using synthetic pyrethroids as their principal insecticide for the termite soil treatment market, an increase from only a few percent in 1986.[3,4]

There is also considerable, and growing, regulatory pressure on the conventional wood preservatives — creosote, pentachlorophenol, and the waterborne arsenicals. Condensed tannins are natural wood preservatives. In our own laboratory, we are investigating wood preservative systems based on procyanidins and materials with analogous properties (Chapter 32).[5–7] Others are working with prorobinetinidins.[8] There are, no doubt, other biocidal applications for polyflavonoids besides wood preservatives that have not been investigated as yet.

Anyone reading the scientific literature has to be impressed with the current emphasis on biotechnology as a means to solving technological problems. This emphasis and interest have spread to natural products chemistry as well. The 1989 annual meeting of the American Society of Pharmacognosy will include a symposium on the biotechnology of natural products. An understanding of the structure and properties of natural products, including the condensed tannins, is essential for the full development of biotechnology. The tannins in sorghum are an example of this. Although the relatively high tannin content of sorghum seed reduces their human digestibility, varieties bred for low tannin content have been unsuccessful in certain locations because they were prone to bird predation.[9] Knowledge of the structure of tannins and their relationship to their digestability and bird resistance (Chapter 24) should facilitate gene manipulation to produce an "ideal" sorghum variety. Without this kind of detailed understanding of the structure and resulting function of natural products, we can neither truly understand how plants work, nor realize the potential of biotechnology.

CONDENSED TANNIN STRUCTURE

In much of the older literature on natural products, "tannin" was the term given to the water-soluble, phenolic, heterogeneous material left over in a plant extract after the interesting chemicals (usually alkaloids and terpenes) had been removed. There has been considerable progress during the last two decades on elucidating condensed tannin structure.[10,11] The state of our knowledge is summarized in Chapter 5. Most of this recent literature, however, deals with oligomers

that are relatively easy to isolate or are carefully purified "clean" flavonoid polymer preparations. Often the bulk of the extract, containing tannin-like materials, is still not well characterized. This is especially true for more persistent plant tissues like tree barks where the flavonoids are more likely to undergo post-biosynthesis modifications due to oxidation, UV exposure, etc. Of course, elucidation of the basic polyflavonoid structure is a necessary first step, but what is most often needed is an understanding of the structures of polyflavonoid components that have undergone derivatization and/or rearrangement. Too often, however, investigators are still skimming off the relatively easy to characterize, simpler polyflavonoids and calling the remainder phlobaphenes or phenolic acids.

It is now appreciated that proanthocyanidins can be derivatized in nature with various substituents including *C*- and *O*-glycosides, methyl groups, and gallate esters. A number of compounds with atypical flavonoid, or even non-flavonoid, lower terminal units have also been described (Chapter 5). The list of oligomeric flavonoids with both proanthocyanidin and biphenyl linkages continues to grow. It is likely that atypical structures such as these make up a significant proportion of most crude polyflavonoid preparations.

SOME FUTURE TRENDS

Monomeric flavonoids have been isolated with structures and substituents not normally associated with proanthocyanidins. These include *C*-methyl, isoprenyl, and various aryl groups as substituents superimposed on neoflavonoid, rotenoid and pterocarpan carbon skeletons. It seems likely that more flavonoid polymers with terminal units such as these will be found in appropriate plants, due to the high reactivity of the electrophilic end of the polyflavonoid polymer while it is being synthesized. The increasingly common occurrence of these types of compounds indicates that, in some plants at least, the growing flavonoid polymer is not completely compartmentalized. This would mean that the electrophilic terminal end of the polymer could react with a wide variety of nucleophilic plant cell wall constituents as well. This would be especially likely to occur during the formation of polyflavonoid heartwood extractives at the sapwood/heartwood interface in trees (for a discussion of heartwood formation, see Bamber and Fukazawa).[12] Polyflavonoids could also be incorporated in the cell wall structure during the biosynthesis of lignin by oxidative copolmerization with lignin precursors.[13] Either or both of these possibilities may account for the decay resistance of some species' heartwoods even after the extractives have been removed — the unextracted, covalently-bound flavonoids still conferring some decay resistance.[6]

As mentioned above, the extent and type of post-synthesis oxidative reactions of polyflavonoids are important factors that need to be addressed. Oxidations (Chapter 15) could result in intramolecular bonds within a polyflavonoid molecule or intermolecular bonds to another polyflavonoid or other nucleophilic species. Prodelphinidins and prorobinetinidins are more sensitive to oxidation because of their pyrogallol-type B-rings. More understanding of these reactions could be important in determining the useful lifetime of condensed tannins in some longterm use applications as well as their exploitation for the development of specialty chemicals.

CONCLUSIONS

There are undoubtedly many structural features of condensed tannins that have not been elucidated yet. The variable, and often low, yields of monomer derivatives from acidic nucleophilic solvolysis of apparently pure condensed tannin preparations suggest that there may be structural features in even relatively simple polyflavonoid extracts that have not been clarified. The presence of oxidative coupling bonds would be relatively difficult to determine by ^{13}C-NMR, the primary technique used for structural determination of procyanidin polymers, perhaps explaining why bonding of this type has not been found yet.

The polyflavonoids isolated in recent years that incorporate derivatized flavonoids and nonflavonoids as co-monomers are probably only the beginning of a wide range of such compounds that will eventually be found. The study of polymeric flavonoids is a relatively specialized area. Harborne lists over 4,000 flavonoid structures of which only 143 are proanthocyanidins.[14] Most of the researchers involved in flavonoid chemistry are concerned only with monoflavonoids. Unique polymeric flavonoids are probably being missed because of the difficulty in working with these compounds.

It seems likely that flavonoids are strongly bound to the cell wall in some plant tissues. Characterization of these non-extractable flavonoid derivatives would probably help in our understanding of the role of proanthocyanidins in plants and perhaps aid in developing new applications for these fascinating natural products.

REFERENCES

1. Gottlieb, O.R. Evolution of natural products. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, Chapter 2 (in press).
2. Loehle, C. Tree life history strategies; the role of defenses. *Can. J. For. Res.* 18:209 (1988).
3. Mix, J. Dursban TC again named top termiticide. *Pest Control*, February, 56 (1989).
4. Mix, J. Termiticide market stabilizes. *Pest Control*, February, 22 (1987).
5. Laks, P.E.; McKaig, P.; Hemingway, R.W. Flavonoid biocides: wood preservatives based on condensed tannins. *Holzforschung* 42(5):299 (1988).
6. Laks, P.E. Wood preservation as trees do it. Proceedings of the American Wood Preservers' Association, Minneapolis :147 (1988).
7. Laks, P.E.; Putman, L.J.; Pruner, M.S. Natural products as wood preservatives. Proceedings of the Canadian Wood Preservation Association, Toronto, 1988 (in press).
8. Schmidt, E.L.; Lotz, W.R. Tropical wood extracts as preservatives for southern pine. Proceedings of the American Wood Preservers' Association, Minneapolis :173 (1988).
9. Bullard, R.W.; Garrison, M.V.; Kilburn, S.R.; York, J.O. Laboratory comparisons of polyphenols and their repellent characteristics in bird-resistant sorghum grains. *J. Agric. Food Chem.* 28:1006 (1980).
10. Porter, L.J. Condensed tannins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, Chapter 6.7 (in press).
11. Porter, L.J. Flavans and proanthocyanidins. *In*: Harborne, J.B. (ed.) The Flavonoids, Advances in Research since 1980. Chapman and Hall, pp. 21-62 (1988).
12. Bamber, R.K.; Fukazawa, K. Sapwood and heartwood: a review. *For. Prod. Abstr.* 8(9):265 (1985).

13. Kodera, M.; Tanahashi, M.; Higuchi, T. Dehydrogenative co-polymerization of d-catechin and coniferyl alcohol. *Wood Res.* 65:1 (1979).

14. Harborne, J.B. The Flavonoids, Advances in Research Since 1980. Chapman and Hall, pp. 539-596 (1988).

Analytical Methods

CHROMATOGRAPHY OF PROANTHOCYANIDINS

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ABSTRACT

Current trends in chromatographic isolation and analyses of proanthocyanidins are reviewed. Preparative isolations by low pressure column chromatography can be carried out using a variety of gel types. Often, repeated separations are required to obtain pure compounds, and it has been found advantageous to alternate each separation with a different gel type. Counter-current separation methods have seen limited application; however, with the development of new apparatus, the situation could change in the future. Paper and thin layer cellulose chromatography remain widely used for qualitative analyses of lower molecular weight oligomers. Quantitative analyses of oligomers can be carried out by high performance liquid chromatography with a variety of reversed-phase columns. Molecular weight profiles of either derivatized or underivatized proanthocyanidins can now be obtained by gel permeation chromatography.

INTRODUCTION

Proanthocyanidins in plants are complex polyphenolic mixtures that present a special challenge to those who wish to separate or analyze them chromatographically. Because of their chemical nature, these compounds readily undergo both intra- and inter-molecular hydrogen bonding. They also readily associate with proteins, carbohydrates, and metals. A significant amount of energy is involved in

these associations, even if one only considers the 3-6 Kcal/Mol associated with each OH → H and NH → H bond. The strength of such associations can greatly affect proanthocyanidin conformational and chemical properties; consequently, their chromatographic behavior. Separation methods must also take the chemical reactivity of proanthocyanidins into account. They are susceptible to oxidation, thermally labile, and can undergo facile molecular rearrangements and decompositions with acidic or basic catalysts. In short, we are dealing with difficult separations that often require delicate handling. Much success has been achieved with the separation and analysis of the lower molecular weight oligomers. The challenge for the future is in separating the more complex, larger oligomers where, for example, either a 3,4-*cis* or 3,4-*trans* stereochemistry in one of the monomer units or a single C-4 → C-6 vs. a C-4 → C-8 interflavanoid bond occurs as a subtle difference between two large molecules whose properties are dominated by other forces. This chapter is a review of some of the more recent trends in the isolation and analysis of proanthocyanidins.

COLUMN CHROMATOGRAPHY

The preparative isolation of proanthocyanidin oligomers has most commonly been achieved using Sephadex LH-20 column chromatography with elution by ethanol or alcohol-water solvents. Two strategies have emerged that appear to work equally well. In the first, crude phenolic mixtures are applied to the column, and the compounds are eluted with ethanol.[1-5] Appropriate fractions are then often rechromatographed with an alcohol-water solvent. It is not uncommon to have to rechromatograph a sample many times in order to obtain pure compounds. A second approach is to subject the plant extract to chromatography over Sephadex LH-20 with water containing increasing amounts of methanol[6-9] to obtain a preliminary fractionation. As an alternative to Sephadex, chromatography on cellulose with water as an eluent has also been used for a first separation of profisetindin oligomers,[10-12] which were then further purified by Sephadex LH-20, and finally preparative TLC.

As the complexity of the oligomers isolated has increased, several groups have found it advantageous to employ schemes that alternate separations between columns of Sephadex LH-20 and other column materials. Nonaka et al[6,13] used an alternating combination of Sephadex LH-20 and MCI-gels to isolate first a series of B-type (i.e., 4β → 8) linked procyanidin dimers, trimers, and tetramers and then a series of trimeric, tetrameric, and pentameric compounds containing some A-type (i.e., 4β → 8, 2β → 0 → 7) linkages. Manufactured in several particle sizes, MCI-gels are highly porous styrene-divinylbenzene copolymers carrying macropores.[14] Separations of proanthocyanidins on these reversed-phase columns are usually accomplished with methanol-water solvents.[6,13,15-19]

Other examples of alternating separation sequences on different column materials are separations on columns of Sephadex LH-20, MCI-gels, and a Bondapak C-18/Porasil B.[8,9,20,21] Fractogel TSK with methanol as an eluent was also useful for the separation of malt and hop proanthocyanidin dimers and trimers after an initial purification on Sephadex LH-20.[22] Similar uses of Fractogel TSK (marketed in the United States as "Toyopearl") are reported by Sun et al[19] and Delcour et

al.[23] Use of this gel in our laboratory gave excellent separation of Douglas-fir bark procyanidin C-4 → C-8 linked dimers, using an ethanol-water gradient of 15 percent to 50 percent ethanol.[68] A distinct advantage of this gel system is its ability to operate with low back-pressure while using ethanol-water solvents.

The preparative isolation of homogeneous polymeric proanthocyanidins is most commonly done by applying the crude polymer preparation to a Sephadex LH-20 column in 50 percent aqueous methanol.[24,25] Purification is achieved by washing the column with 50 percent aqueous methanol to remove carbohydrates and low molecular weight phenolics. Because 50 percent aqueous methanol is such a poor solvent for proanthocyanidin polymers, they are absorbed on the stationary phase. The purified polymers are recovered by elution with acetone-water (7:3 or 1:1) as a single narrow band. Acetone-water mixtures have tremendous solvating powers for proanthocyanidin polymers and can even displace tannin-carbohydrate and tannin-protein associations. Because hydrolyzable tannins have the same solubility and mobility characteristics on Sephadex, they will elute with the proanthocyanidins if present.[25] Czochanska et al[24] proposed checking for hydrolyzable tannins in freeze-dried proanthocyanidin polymer preparations by analysis for the presence of carbonyl groups either by IR or ^{13}C-NMR spectroscopy. However, a complication can arise because proanthocyanidins with carbonyl groups such as gallate esters are known.[26–28]

COUNTER-CURRENT CHROMATOGRAPHY

The separation process in counter-current chromatography (CCC) is based upon the partitioning of solute between two immiscible liquids. The separation occurs without the presence of a solid stationary phase, so that the irreversible column binding typically associated with crude tannin mixtures does not occur. There are relatively few solvent mixtures that provide the proper conditions for high selectivity, thus, the most common use of CCC is for the preliminary separation of crude extracts.

Condensed tannins from sorghum grain have been separated from a crude methanol extract with 1-butanol - 0.1 M sodium chloride or 0.1 M sodium chloride with 1 mM phytic acid, pH 4.0 aqueous phase.[29] Young et al[30,31] have used counter-current distribution as a preliminary purification method prior to column chromatography on Sephadex LH-20 for profisetindin tetraflavanoids and related compounds. Solvent systems were water-butan-2-ol-hexane (5:3:2 v/v) and water-butan-2-ol-hexane (5:4:1 v/v). Prorobinetinidin triflavanoids were similarly cleaned up by CCC (water-butan-2-ol-isohexane 5:45:0.5 v/v) prior to isolation via preparative paper chromatography.[32] The recently reported separation of flavanoids with an analytical high-speed CCC apparatus[33] indicates that this technique may find further applications to the quantitative analysis of condensed tannins in the near future.

PAPER AND THIN LAYER CHROMATOGRAPHY

Two-dimensional paper chromatography has proven to be a powerful technique for the qualitative analyses of proanthocyanidin dimers, trimers, and associated

monomers. Although the higher oligomers and polymers are often poorly defined, this method is still quite useful in following separations on column chromatography, monitoring reactions, or to give distinctive "fingerprints" that may be used as a taxonomic guide to patterns of occurrence of different types of proanthocyanidins in certain plants. While a number of different solvent pair systems have been used for the development of two-dimensional paper chromatographs of polyphenols, two systems have been used most commonly for proanthocyanidins. In the system described by Haslam[34] and used by Thompson et al[1] for the analyses of procyanidin oligomers, chromatograms were developed in the first direction with 6-percent acetic acid (solvent A) and then in the second direction with butan-2-ol-acetic acid-water (14:1:5), (solvent B). The use of this solvent pair takes advantage of separations based on different mechanisms for each direction. Separations with dilute acetic acid on paper are based on differential absorption and water solubility, whereas, separations in the second direction are based on a partitioning of the compounds between the solvent and the stationary aqueous phase of the paper.

A similar solvent pair system of water-saturated butan-2-ol-(solvent A) for the first direction and 2 percent acetic acid (solvent B) for the second direction has been used for the analyses of wattle and quebracho tannins and associated compounds.[35–39] More recently, this system has been used to monitor the separations of synthetic procyanidins,[40] profisetinidin oligomers,[10,30] and oligomeric pentahydroxyflavans.[31]

In many laboratories, two-dimensional (2-D) cellulose thin layer chromatography (TLC) has replaced paper chromatography. The solvent system pair consists of t-butanol-acetic acid-water (3:1:1) for development in the first direction and 6-percent acetic acid for development in the second direction.[3,5,18,19,41,42] The relative separations of procyanidin oligomers by this technique is shown in figure 1. A definite advantage of the cellulose plates is that they can be cut into small sizes (e.g., 7.6 or 10 sq. cm), and development times are greatly reduced over paper chromatography. Hemingway et al[3] have demonstrated the effectiveness of using 2-D cellulose TLC to monitor partial thiolysis reactions of procyanidin trimers. Linkage isomerization in the trimers was readily revealed by the distinctively different Rf values for C-4 → C-8 and C-4 → C-6 linked dimers produced during the reactions.

Detection of polyphenols on both paper chromatograms and cellulose TLC's is often accomplished with a freshly prepared aqueous solution of ferric chloride (0.2 percent), potassium ferricyanide (0.2 percent), and a trace of potassium permanganate.[1] After application of this spray reagent, the papers or cellulose plates are washed with dilute hydrochloric acid and then water to reveal the easily oxidized phenols as prussian blue spots on a white background. Selective visualization of procyanidins and other compounds with a reactive phlorglucinol A ring can be achieved using a vanillin – hydrochloric acid[43] or vanillin – *para*-toulene sulfonic acid spray reagents.[44] The vanillin – hydrochloric acid spray reagent is prepared in our laboratories by dissolving 1 gm of vanillin in 50 ml of absolute ethanol, and adding 10 ml of conc. hydrochloric acid. Chromatograms are lightly sprayed and then heated in an oven at 110 °C for a minute or two until spot color develops, usually a bright pink to red. Depending on sample concentration, color will develop

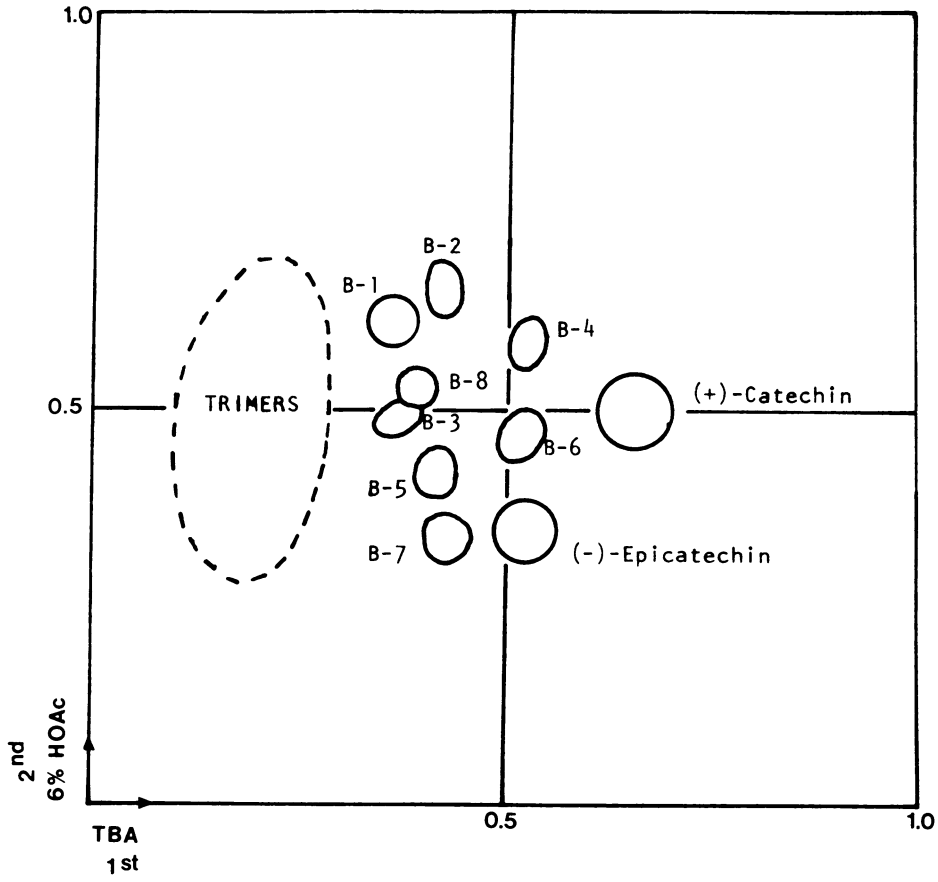

Figure 1. *Two-dimensional cellulose TLC of procyanidin oligomers.*

without heating. Readers are referred to earlier reviews by Haslam[34] or Roux and Maihs[44] for details on additional spray reagents.

TLC of acetylated or methylated proanthocyanidin derivatives is frequently carried out on silica as a means of purification.[1] A benzene-acetone (8:2 v/v) solvent system has been used frequently to isolate procyanidin dimer[45] and trimer acetates,[3] whereas, chloroform-methanol (500:1 and 40:1) has been useful for isolation of their respective methyl ether derivatives.[1,3] Roux and Ferreira's group at the University of the Orange Free State in Bloemfontein, South Africa, has employed a wide variety of additional TLC solvent systems for the isolation of methylated proanthocyanidin oligomers and related compounds, some of which are benzene-acetone modified solvent systems and others that do not contain benzene.[10,11,23,30–32] Benzene-acetone in various proportions gives excellent separations of proanthocyanidin derivatives on silica. However, benzene as a chromatography solvent has been of some concern because sufficient evidence of its carcinogenicity to humans has been demonstrated.[46]

Hemingway's laboratory now uses toluene-acetone (8:2 v/v or 7:3 v/v) for separations of acetate derivatives.

Proanthocyanidins in their free phenolic form have been analyzed by TLC on silica using acidified solvents. The solvent system ethyl acetate-water-formic acid (90:5:5) has been used to monitor isolations by column chromatography.[23,27] The solvent system toluene-acetone-formic acid (30:30:10) can be used with silica TLC to obtain a visual pattern of the molecular weight distribution of procyanidin oligomer mixtures.[47,48] In this case, the Rf values of the compounds were found to be in reverse order of molecular weight. Resolution is obtained for monomers through hexamers, and C-4 → C-6 linked dimers are also distinguished from C-4 → C-8 linked dimers. A useful method of spot detection of these phenolic compounds on silica has been the sulfuric acid-formalin (40:1) spray reagent.[23,27] Such reagents are not always necessary, however. After TLC development with the toluene-acetone-formic acid solvent system, easily oxidized compounds such as the procyanidins and catechins will slowly appear as brown spots as the plates are dried.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) is increasingly used for both analysis and preparative isolation or purification of proanthocyanidins. A general trend has been to use reversed-phase columns with acidified methanol-water or similar acidified solvent systems, which leads to better peak resolution than with nonacidified solvent systems. However, because of the concern for acid catalyzed reactions during workup, many prefer to use neutral solvents for preparative isolations, although this has not always been done. The successful chromatographer must ascertain whether or not a suggested acidified solvent system will alter the compounds of interest.

Table 1 selectively surveys some of the HPLC analyses of proanthocyanidins reported for plant extracts and agricultural commodities. Readers are also referred to additional informational sources in the review by Daigle and Conkerton,[49] and to the procedural paper by Vande Casteele et al.[50] Lea[51] originally surveyed a variety of packing materials when studying cider procyanidins and found the best results were achieved with reversed-phase materials using acidified aqueous-methanol solvents. A pH shift technique was later developed that allowed for improved and direct analysis of procyanidins in fresh and oxidizing apple juices.[52] Samejima and Yoshimoto[67] have found, however, that direct analysis of coniferous bark extracts was unsuitable, and pre-chromatography on Sephadex LH-20 was necessary to obtain accurate results. Hemingway et al[3] similarly pre-chromatographed the procyanidin trimers of loblolly pine phloem over Sephadex G-50 (acetone-water 1:1 v/v) prior to HPLC analysis. The columns reported in Table 1 are based on either pellicular- or silica-coated materials. In our laboratory, the polymer based (styrene-divinylbenzene co-polymer) PLRP-S 100 Å reversed-phase column was found to be quite useful. Satisfactory analyses of procyanidin dimers, trimers, and associated monomers from Douglas-fir bark tissues have been achieved using methanol-water solvents isocratically.[68] Pre-chromatography on Sephadex LH-20 also was required prior to HPLC analyses.

Table 1. Selected References to HPLC Analyses of Proanthocyanidins in Plant Extracts and Agricultural Commodities.

Substance	Column	Solvents	Reference
Cider	Variety surveyed	acidified MeOH-H_2O	51
Ciders and wine	Lichrosorb RP-8 Spherisorb Hexyl Hypersil SAS	acidified MeOH-H_2O gradient	52
Apple juice	Spherisorb Hexyl	MeOH-H_2O pH-Shift Tech.	53
Apple juice	Zorbax CN μBondapak C-18	acidified THF-hexane MeOH-aq.KH_2PO_4	54
Wine	Micropak C-18	acidified MeOH-H_2O	55
Wines	Altex C-18	MeCN-aq.buffer	56
Grape skins and fruit residues	Spherisorb Hexyl	acidified MeOH-H_2O	57
Barley and hops	μBondapak C-18	acidified MeOH-H_2O	58
Barley	Polyamide-6 Sil C-18 HL	MeOH-H_2O H_2O-HOAc	59
Barley, hops, beer	Sil C-18 HL	H_2O-HOAc,	60
Cell cultures	μBondapak C-18	MeOH-H_2O	61-63
Rose stem and cell culture	μBondapak C-18	acidified MeOH-H_2O,	64
Sorghum	Lichrosorb Si60	acidified THF-MeOH	65
Sorghum	Lichrosorb RP-8	acidified MeCN-H_2O	66
Bark	Lichrosorb RP-8	acidified MeOH-H_2O,	67
Pine bark	Zorbax CN μBondapak C-18	MeOH-H_2O acidified MeOH-H_2O	3
Douglas-fir bark	PLRP-S (100 Å)	MeOH-H_2O	68

Other analytical HPLC procedures of interest include the use of Zorbax ODS (methanol-5 percent acetic acid 30:70 v/v)[67,69] and μBondapak C-18 (methanol-water-acetic acid, 20:79:1 v/v)[3] columns to analyze the degradation products from thiolysis of procyanidins. Beart et al[70] followed the kinetics of the acid-catalyzed decomposition of some procyanidin dimers using a Zorbax NH_2 column with the solvent system acetonitrile-water-phosphoric acid (950:50:1). Detection of the proanthocyanidins in HPLC analyses is traditionally done by U.V. methods at 254 or 280 nm. Lunte et al[56] and Chiavari et al[71] have recently reported on the use of dual-electrode liquid chromatography-electrochemistry detection and electrochemical detection of procyanidins as an alternative to U.V. detection. In dual-electrode liquid chromatography-electrochemistry, compounds are identified based on a combination of retention times and voltammetric behavior. Electrochemical detection offers enhanced selectivity and sensitivity over U.V. detection.

Preparative isolations of procyanidin dimers,[45,72,73] trimers,[3] and phloroglucinol-tannin degradation products[3] have been reported using a Zorbax CN column and methanol-water eluents. Guier et al[66] used a Lichrosorb RP-8 column with an acetonitrile-water gradient for proanthocyanidin purification.

GEL PERMEATION CHROMATOGRAPHY

Standard techniques for estimating the molecular weights of proanthocyanidin polymers include ^{13}C-NMR, vapor phase osmometry, and gel permeation chromatography (GPC). GPC is perhaps the most convenient of these methods for many laboratories. The separation process in GPC (sometimes called size exclusion chromatography, SEC), is theoretically based solely on the entropic behavior between solute and gel. Molecular weight distribution data are obtained when the system is calibrated against monodisperse and well-characterized standards. Thus, the molecular weight distribution is a secondary method limited by the availability of proper calibration on standards. Calculations are customarily accomplished with commercially available GPC computer software.

GPC analyses of proanthocyanidin polymers to date have ordinarily been done on methyl ether or acetylated derivatives. Samejima and Yoshimoto[69] used an HSG-15 column and THF solvent for analysis of methylated (by diazomethane) procyanidin polymers. The system was calibrated with polystyrene standards. Williams et al[74] employed a series of μStyragel columns ($10^3\mathring{A}$ and $10^4\mathring{A}$) with a tetrahydrofuran eluting solvent to analyze acetylated proanthocyanidin polymers. Calibration was accomplished with a combination of lower molecular weight procyanidin acetates and higher molecular weight polystyrene standards. Polystyrene calibration standards are useful for the estimation of apparent molecular weights of polymers whose structures are not known or where standards with the appropriate structures are not available. Caution must be advised, however, as in our laboratory, it has been found that a system calibrated with polystyrene standards does not give the same molecular weight data for polymeric procyanidin acetates when calibrated with procyanidin oligomer acetates (monomer to pentamer).[75] Figure 2 compares the calibration curves for each type of standard on the same series of μStyragel columns.

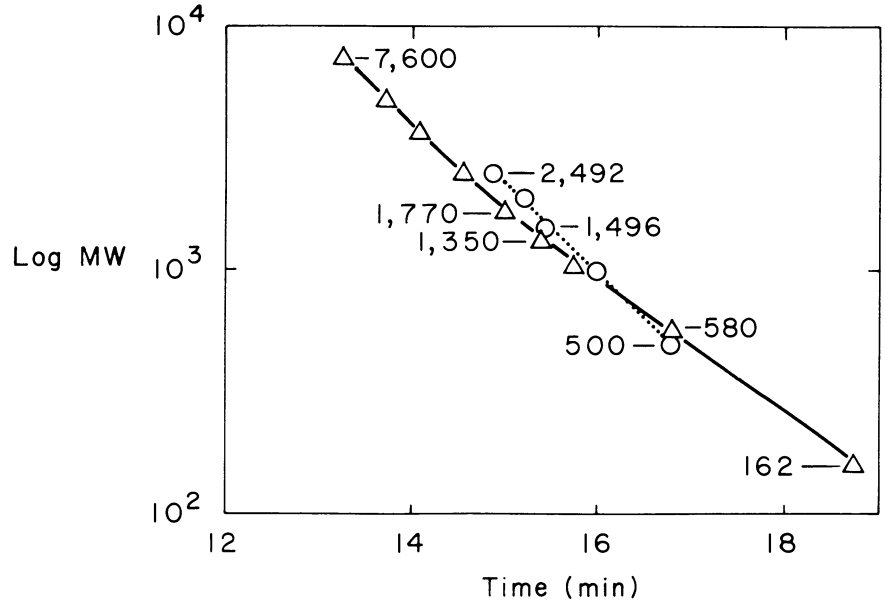

Figure 2. *Comparison of calibration curves for polystyrene standards* △ – △ *and procyanidin peracetate standards* ○ – ○ *on a μStyragel column set (10⁴, 10³, 500, 100 Å) with THF solvent (2 ml/min).*

Bae[75] has found that GPC analyses can be readily accomplished for procyanidin polymers in the free phenolic form using a dimethylformamide solvent system and a two-column set (500, 10^3Å) of PL gel columns (5μM polystyrene-divinylbenzene copolymer). Calibration of the system was accomplished with procyanidin oligomers. Because the polymers do not need to be derivatized, the technique offers a rapid method of determining molecular weight and also of following the progress of isolations from large preparative columns.

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REFERENCES

1. Thompson, R.S.; Jacques, D.; Haslam, E.; Tanner, R.J.N. Plant proanthocyanidins. Part 1. Introduction: the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkin 1* :1387 (1972).
2. Gupta, R.K.; Haslam, E. Plant proanthocyanidins. Part 7. Prodelphinidins from *Pinus sylvestris*. *J. Chem. Soc. Perkin 1* :1148 (1981).
3. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Linkage isomerism in trimeric and polymeric 2,3-cis-procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1209 (1982).
4. Foo, L.Y.; Porter, L.J. Prodelphinidin polymers: definition of structural units. *J. Chem. Soc. Perkin Trans. 1* :1186 (1978).

5. Foo, L.Y. Condensed tannins: co-occurrence of procyanidins, prodelphinidins and profisetinidins in the heartwood of *Acacia baileyana*. *Phytochemistry* 23:2915 (1984).

6. Nonaka, G.; Morimoto, S.; Nishioka, I. Tannins and related compounds. Part 13. Isolaton and structures of trimeric, tetrameric, and pentameric proanthocyanidins from cinnamon. *J. Chem. Soc. Perkin Trans. 1* :2139 (1983).

7. Hsu, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXIII. Isolation and characterization of procyanidins in *Dioscorea cirrhosa* Lour. *Chem. Pharm. Bull.* 33:3293 (1985).

8. Kashiwada, Y.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XLV. Rhubarb. (5). Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chem. Pharm. Bull.* 34:3208 (1986).

9. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. LX. Isolation and characterization of proanthocyanidins with a doubly-linked unit from *Vaccinium vitis-idaea* L. *Chem. Pharm. Bull.* 36:33 (1988).

10. Viviers, P.M.; Kolodziej, H.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 11. Intramolecular enantiomerism of the constituent units of tannins from the Anacardiaceae: stoichiometric control in direct synthesis: derivation of ^{1}H nuclear magnetic resonance parameters applicable to higher oligomers. *J. Chem. Soc. Perkin Trans. 1* :2555 (1983).

11. Young, D.A.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 15. Structure of natural 'angular' profisetinidin tetraflavanoids: asymmetric induction during oligomeric synthesis. *J. Chem. Soc. Perkin Trans. 1* :2529 (1985).

12. Steenkamp, J.A.; Malan, J.C.S.; Roux, D.G.; Ferreira, D. Oligomeric flavanoids. Part 1. Novel dimeric profisetinidins from *Colophospermum mopane*. *J. Chem. Soc. Perkin Trans. 1* :1325 (1988).

13. Nonaka, G.; Hsu, F.; Nishioka, I. Structures of dimeric, trimeric, and tetrameric procyanidins from *Areca catechu* L. *J. Chem. Soc., Chem. Commun.* :781 (1981).

14. Mitsubishi Chemical Industries, MCI GEL for Liquid Chromatography. Technical Bulletin 603, Tokyo, Japan. Sept. 1984. 14 pp.

15. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXV. Proanthocyanidins with a doubly linked unit from the root bark of *Cinnamomum sieboldii* Meisner. *Chem. Pharm. Bull.* 33:4338 (1985).

16. Ezaki-Furuichi, E.; Nonaka, G.; Nishioka, I.; Hayashi, K. Isolation and structures of procyanidins (condensed tannins) from *Rhaphiolepis umbellata*. *Agric. Biol. Chem.* 50:2061 (1986).

17. Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. VIII. A new type of proanthocyanidin, cinchonains IIa and IIb from *Cinchona succirubra*. (2). *Chem. Pharm. Bull.* 30:4277 (1982).

18. Foo, L.Y.; Wong, H. Diastereoisomeric leucoanthocyanidins from the heartwood of *Acacia melanoxylon*. *Phytochemistry* 25:1961 (1986).

19. Sun, D.; Wong, H.; Foo, L.Y. Proanthocyanidin dimers and polymers from *Quercus dentata*. *Phytochemistry* 26:1825 (1987).

20. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXVIII. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamomum cassia* Blume). *Chem. Pharm. Bull.* 34:633 (1986).

21. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXIX. Procyanidin C-glucosides and an acylated flavan-3-ol glucoside from the barks of *Cinnamomum cassia* Blume and *C. obtusifolium* Nees. *Chem. Pharm. Bull.* 34:643 (1986).

22. Derdelinckx, G.; Jerumanis, J.; Separation of malt and hop proanthocyanidins on Fractogel TSK HW-40 (S). *J. Chromatography* 285:231 (1984).

23. Delcour, J.A.; Serneels, E.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 13. The first 2,3-*trans*-3,4-*cis* procyanidins: sequence of units in a 'trimer' of mixed stereochemistry. *J. Chem. Soc. Perkin Trans. 1* :669 (1985).

24. Czochanska, Z.; Foo, L.Y.; Newman, R.H.; Porter, L.J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J. Chem. Soc. Perkin Trans. 1* :2278 (1980).

25. Foo, L.Y.; Porter, L.J. The phytochemistry of proanthocyanidin polymers. *Phytochemistry* 19:1747 (1980).

26. Nonaka, G.; Muta, M.; Nishioka, I. Myricatin, a galloyl flavanonol sulfate and prodelphinidin gallates from *Myrica rubra*. *Phytochemistry* 22:237 (1983).

27. Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. *Chem. Pharm. Bull.* 31:3906 (1983).

28. Kashiwada, Y.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XLVIII. Rhubarb. (7). Isolation and characterization of new dimeric and trimeric procyanidins. *Chem. Pharm. Bull.* 34:4083 (1986).

29. Putman, L.J.; Butler, L.G. Fractionation of condensed tannins by counter-current chromatography. *J. Chromatography* 318:85 (1985).

30. Young, D.A.; Kolodziej, H.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 16. Stereochemical differentiation of the first "angular" (2S,3R)-profisetinidin tetraflavanoids from *Rhus lancea* (Karree) and the varying dynamic behaviour of their derivatives. *J. Chem. Soc. Perkin Trans. 1* 2537 (1985).

31. Young, E.; Brandt, E.V.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 17. Oligomeric (2R,3S)-3,3',4',7,8-pentahydroxyflavans: atropisomerism and conformation of biphenyl and m-terphenyl analogues from *Prosopis glandulosa* ("Mesquite"). *J. Chem. Soc. Perkin Trans. 1* :1737 (1986).

32. Viviers, P.M., Botha, J.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 7. Angular [4,6: 4,8]-prorobinetinidin triflavanoids from black wattle ("Mimosa") bark extract. *J. Chem. Soc. Perkin Trans. 1* :17 (1983).

33. Zhang, T.Y.; Xiao, R.; Xiao, Z.Y. Pannel, L.K.; Ito, Y. Rapid separation of flavanoids by analytical highspeed counter-current chromatography. *J. Chromatography* 445:199 (1988).

34. Haslam, E. Chemistry of Vegetable Tannins. Academic Press, New York pp. 14-30 (1966).

35. Roux, D.G.; Evelyn, S.R. Condensed tannins. 1. A study of complex leuco-anthocyanins present in condensed tannins. *Biochem. J.* 69:530 (1958).

36. Roux, D.G. Methods of fractionation and identification of constituents of condensed tannins. *J. Am. Leather Chem. Assn.* 53:384 (1959).

37. Drewes, S.E.; Roux, D.G.; Saayman, H.M.; Eggers, S.H.; Feeny, J. Some stereochemically identical biflavanols from the bark tannins of *Acacia mearnsii*. *J. Chem. Soc.* (C) :1302 (1967).

38. Fourie, T.G.; DuPreez, I.C.; Roux, D.G. 3',4',7,8-tetrahydroxyflavonoids from the heartwood of *Acacia nigrescens* and their conversion products. *Phytochemistry* 11:1763 (1972).

39. Malan, E.; Roux, D.G. Flavonoids and tannins of *Acacia* species. *Phytochemistry* 14:1835 (1975).

40. Delcour, J.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 9. The condensation sequence of leucocyanidin with (+)–catechin and with the resultant procyanidin. *J. Chem. Soc. Perkin Trans. 1* :1711 (1983).

41. Karchesy, J.J.; Hemingway, R.W. Condensed tannins: ($4\beta \rightarrow 8;2\beta \rightarrow 0 \rightarrow 7$)-linked procyanidins in *Arachis hypogea* L. *J. Agric. Food Chem.* 34:966 (1986).

42. Laks, P.E.; Hemingway, R.W. Condensed tannins: base catalysed reactions of polymeric procyanidins with toluene-α-thiol. Lability of the interflavanoid bond and pyran ring. *J. Chem. Soc. Perkin Trans. 1* :465 (1987).

43. Bate-Smith, E.C. Colour reactions of flowers attributed to (a) flavanols and (b) carotenoid oxides. *J. Exper. Bot.* 4:1 (1953).

44. Roux, D.G. Maihs, A.E. Selective spray reagents for the identification and estimation of flavonoid compounds associated with condensed tannins. *J. Chromatog.* 4:65 (1960).

45. Hemingway, R.W.; Karchesy, J.J.; McGraw, G.W.; Wielesek, R.A. Heterogeneity of interflavanoid bond location in loblolly pine bark procyanidins. *Phytochemistry* 22:275 (1983).

46. World Health Organization, International Agency for Research on Cancer, IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. IARC Monographs Supplement 4:56-67 (1982).

47. Lea, A.G.H. The phenolics of ciders: oligomeric and polymeric procyanidins. *J. Sci. Food Agric.* 29:471 (1978).

48. Lea, A.G.H.; Bridle, P.; Timberlake, C.F.; Singleton, V.L. The procyanidins of white grapes and wines. *Am. J. Enol.* 30:289 (1979).

49. Daigle, D.J.; Conkerton, E.J. Analysis of flavonoids by HPLC. *J. Liquid Chromatography* 6:105 (1983).

50. Vande Casteele, K.; Geiger, H.; DeLoose, R.; Van Sumere, C.F. Separation of some anthocyanidins, anthocyanins, proanthocyanidins and related substances by reversed-phase high-performance liquid chromatography. *J. Chromatog.* 259:291 (1983).

51. Lea, A.G.H. High performance liquid chromatography of cider procyanidins. *J. Sci. Food Agric.* 30:833 (1979).

52. Lea, A.G.H. Reversed-phase gradient high-performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimised by Snyder's procedures. *J. Chromatog.* 194:62 (1980).

53. Lea, A.G.H. Reversed-phase high-performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. *J. Chromatog.* 238:253 (1982).

54. Wilson, E.L. High-pressure liquid chromatography of apple juice phenolic compounds. *J. Sci. Food Agric.* 32:257 (1981).

55. Salagoity-Auguste, M.; Bertrand, A. Wine phenolics – analysis of low molecular weight components by high performance liquid chromatography. *J. Sci. Food Agric.* 35:1241 (1984).

56. Lunte, S.M.; Blankenship, K.D.; Read, S.A. Detection and identification of procyanidins and flavanols in wine by dual-electrode liquid chromatography-electro-chemistry. *Analyst* 113:99 (1988).

57. Galletti, G.C.; Self, R. The polyphenols (syn. vegetable tannins) of grape skins and pressed fruit residues. *Annaldi di Chimica* 76:195 (1986).

58. McMurrough, I. High-performance liquid chromatography of flavonoids in barley and hops. *J. Chromatography* 218:683 (1981).

59. Mulkay, P.; Touillaux, R.; Jerumanis, J. Proanthocyanidins of barley: separation and identification. *J. Chromatography* 208:419 (1981).

60. Jerumanis, J. Quantitative analysis of flavanoids in barley, hops and beer by high-performance liquid chromatography (HPLC). *J. Inst. Brew.* 91:250 (1985).

61. Stafford, H.A.; Lester, H.H. Procyanidins (condensed tannins) in green cell suspension cultures of Douglas fir compared with those in strawberry and avocado leaves by means of C_{18}-reversed-phase chromatography. *Plant Physiol.* 66:1085 (1980).

62. Stafford, H.A.; Lester, H.H. Proanthocyanidins and potential precursors in needles of Douglas fir and in cell suspension cultures derived from seedling shoot tissues. *Plant Physiol.* 68:1035 (1981).

63. Stafford, H.A.; Kreitlow, K.S.; Lester, H.H. Comparson of proanthocyanidins and related compounds in leaves and leaf-derived cell cultures of *Ginkgo bioloba* L., *Pseudotsuga menziesii* Franco, and *Ribes sanguineum* Pursh. *Plant Physiol.* 82:1132 (1986).

64. Muhitch, M.J.; Fletcher, J.S. Isolation and identification of the phenols of Paul's scarlet rose stems and stem-derived suspension cultues. *Plant Physiol.* 75:592 (1984).

65. Glennie, C.W.; Kaluza, W.Z.; Van Niekerk, P.J. High-performance liquid chromatography of procyanidins in developing sorghum grain. *J. Agric. Food Chem.* 29:965 (1981).

66. Gujer, R.; Magnolato, D.; Self, R. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry* 25:1431 (1986).

67. Samejima, M.; Yoshimoto, T.; High performance liquid chromatography of proanthocyanidins and related compounds. *Mokuzai Gakkaishi* 27:658 (1981).

68. Chalker-Scott, L.; Karchesy, J.J. (unpublished results).

69. Samejima, M.; Yoshimoto, T. Procyanidins from the inner bark of sugi (*Cryptomeria japonica* D.Don). *Mokuzai Gakkaishi* 25:671 (1979).

70. Beart, J.E.; Lilley, T.H.; Haslam, E. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations on some polymeric proanthocyanidins. *J. Chem. Soc. Perkin Trans. 2* :1439 (1985).

71. Chiavari, G.; Vitali, P.; Galletti, G.C. Electrochemical detection in the high-performance liquid chromatography of polyphenols (vegetable tannins). *J. Chromatog.* 392:426 (1987).

72. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Polymeric proanthocyanidins: interflavanoid linkage isomerism in (epicatechin-4)-(epicatechin-4)-catechin procyanidins. *J. Chem. Soc. Commun.* :320 (1981.

73. Foo, L.Y.; Porter, L.J. Enantiomerism in natural procyanidin polymers: use of epicatechin as a chiral resolution reagent *J. Chem. Soc., Chem. Commun.* :241 (1981).

74. Williams, V.M.; Porter, L.J.; Hemingway, R. W. Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry* 22:569 (1983).

75. Bae, Y.S.; Douglas-fir inner bark procyanidins: sulfonation, isolation, and characterization. PhD Thesis, Oregon State University, Corvallis, (1989).

NEW NMR EXPERIMENTS APPLICABLE TO STRUCTURE AND CONFORMATION ANALYSIS

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ABSTRACT

Recent advances in the field of NMR spectroscopy have been primarily responsible for the rapid progress achieved in the study of proanthocyanidins over the past decade. This chapter summarizes these advances by describing how techniques including n.O.e. difference spectroscopy, homonuclear J-resolved and chemical shift correlation methods, and ^{13}C- and heteronuclear experiments have been applied to analyses of some example proanthocyanidins. These NMR experiments have provided information regarding molecular structure, configuration, and conformation that was previously inaccessible or extremely difficult to obtain.

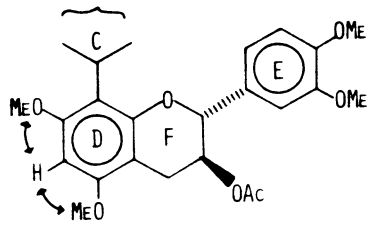
INTRODUCTION

The advent of pulsed Fourier Transform (FT) NMR provided vast increases in sensitivity and the ability to observe less abundant nuclei of the Periodic Table. Since the debut of FT-NMR, contemporary technology has been responsible for the introduction of a vast number of innovations including superconducting magnets with high magnetic fields, ultra-fast computers capable of manipulating large quantities of data, and pulse transmitters programmed to relay intricate pulse sequences. These advances led to the invention of a wealth of NMR experiments that have been applied to resolve questions about molecular structure and stereochemistry previously difficult or impossible to approach. Appropriate elements of this methodology have progressively been introduced to the study of flavanoids and analogous classes of compounds with increasing success.

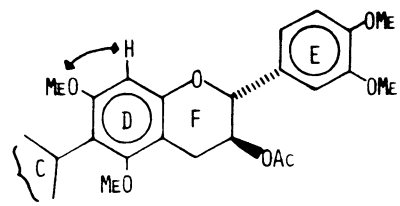
NUCLEAR OVERHAUSER EFFECT DIFFERENCE SPECTROSCOPY

Few NMR techniques have displayed the impact on structural flavanoid chemistry than those relying on the nuclear Overhauser effect (n.O.e.) phenomenon have.[1] This holds true especially for the homonuclear case, which, by dependence of dipolar relaxation on non-bonded internuclear distances, is capable of supplying substantial information regarding steric relationships unobtainable by other methods. The phenomenon is preferentially observed as 1D difference spectroscopy[2–4] as opposed to the more contemporary 2D NOESY experiment[5] mainly due to difficulties associated with the choice of an appropriate "mixing" time required by the latter.

Initial recognition of the general usefulness of the n.O.e. difference technique in structural flavanoid chemistry was demonstrated by its elegant application to the differentiation between the C-8 (**1**) and C-6 (**2**) substituted (+)-catechin moities of condensed tannins.[6]



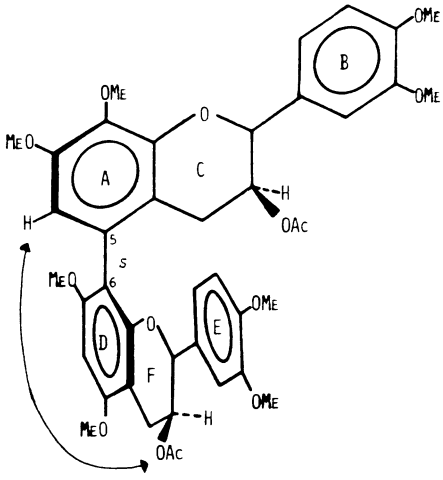
(**1**)



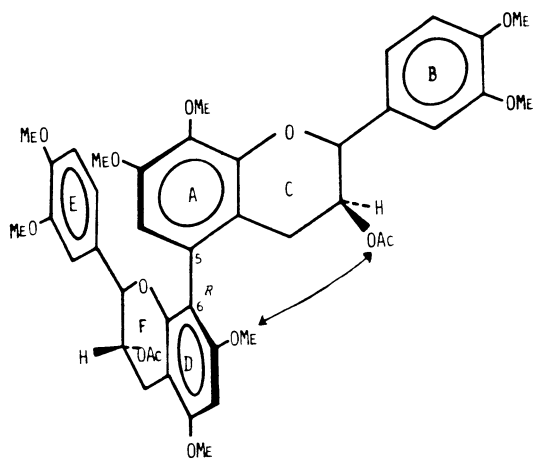
(**2**)

In contrast to earlier dependence on dubious methods based on progressive shielding of methoxy-proton resonances with change of solvent composition[7,8] or the absolute values of chemical shifts of 6- and 8-H(A) in $CDCl_3$[9,10] (both temperature- and solvent dependent), the present method allows the problem to be solved directly by n.O.e. association of the residual proton with either two methoxy groups (6-H → 5,7-OMe) for 8-C- (**1**) or one methoxy group (8-H → 7-OMe) for 6-C-linked (**2**) units.[6] This is illustrated by the differentiation of the derivatized (5,8)- and (5,6)-(+)-mesquitol-(+)-catechin biphenyl-type oligomers (**3**), (**4**), and (**5**) [cf. Figure 1 for spectrum of (**3**)], synthesized by direct oxidative phenol coupling of (+)-mesquitol and (+)-catechin as evidence supporting the structures of the naturally occurring atropisomers.

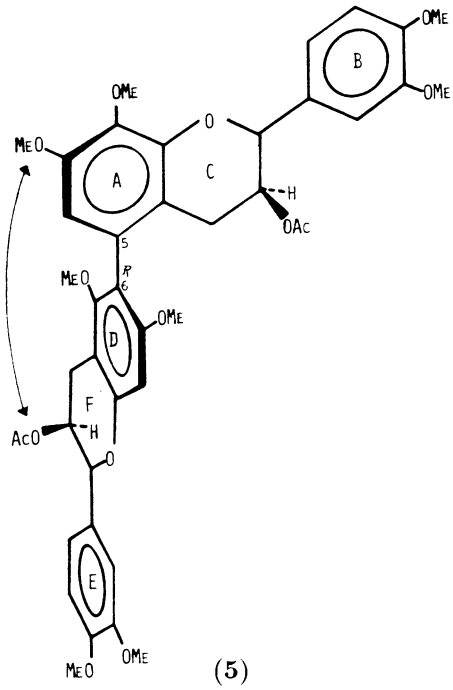
Assessment of the absolute configurations of the derivatives (**3**), (**4**), and (**5**), whose relative stabilities are attributed to rotational restrictions imposed by the rigidity of the 4-CH_2(C) function, *o*-disubstitution of the D-ring relative to the bond, and also to the combined "buttressing effect" of the 7,8-dimethoxy function on 6-H(A), is permitted by extending the concept to interflavanyl associations. Thus, correlation of 8-OMe(A) with 2-, 5-, and 6-H(E) and of 6-H(A) with 2-H(F) establishes [P]-helicity (**S**-configuration) for (**3**) and indicates a dihedral angle of ca. 90° between the biphenyl A- and D-rings. The corresponding isomer (**4**), where



(3)



(4)



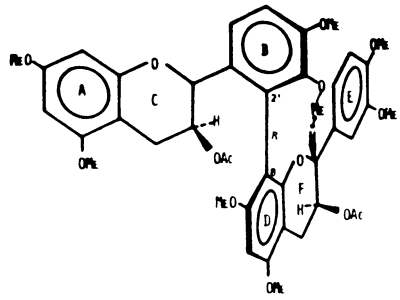
(5)

such associations were absent, therefore exhibits [M]-helicity and **R**-absolute configuration. N.O.e. associations originating from ^{1}H dipolar interaction between aromatic, heterocyclic, and methoxy protons of relevance to stereochemical assignments were absent in the (5,6)-atropisomer (**5**). To eliminate ambiguities regarding configurational assignments based on the absence of n.O.e. effects, this approach was elaborated to include heterocyclic acetoxy protons;[11] such experiments, how-

ever, required an increased number of scans for each experiment. Thus, in the (**S**)-atropisomer (**3**), the anticipated weak n.O.e. association between 3-OAc(F) and 6-H(A) was observed, whereas, the effect between 7-OMe(D) and 3-OAc(C) confirmed [M]-helicity and an (**R**)-configuration for atropisomer (**4**). The weak association of 7-OMe(D) and 3-OAc(C) similarly defines [M]-helicity and thus (**R**)-absolute configuration for the (+)-mesquitol-(5,6)-(+)-catechin (**5**). The same methodology also facilitated the definition of absolute configuration about the biphenyl bond in a series of synthetic B-D-ring linked bis-(+)-catechin atropisomers[11] (**6**)-(**10**) as well as those of the four "trimeric" *m*-terphenyl (+)-mesquitol-(+)-catechins (**12**)-(**15**) of type (**11**). Whereas, the spectra of the former were fully assignable [e.g., (**6**)-Figure 1], only a limited number of key signals could be allocated unambiguously for the *m*-terphenyls [e.g., (**15**)-Figure 1]. The well-separated A- and G-ring "residual" proton singlets, the strongly shielded 5- and 7-methoxy resonances of ring D, and the three separate acetoxy signals in all four cases provided reference signals for observation of potentially stereochemically significant interflavanyl n.O.e. associations. Owing to anisotropic shielding by both A- and G-rings, 7-OMe(D) is anticipated to resonate at higher field than 5-OMe(D). This was confirmed by observation of an n.O.e. effect from 7-OMe(D) to both residual A- and G-ring singlets and from 5-OMe(D) to the G-ring singlet only, thus unequivocally defining four of the resonances of paramount importance to assigning the absolute configuration about the biphenyl bonds. These results clearly demonstrate the elegance in which n.O.e. difference spectroscopy may be utilized in solving problems previously confined to the more classical X-ray crystallographic method.

Such an approach is not restricted to oxidative coupled oligomers but applies equally to conventional analogues possessing predominant stable conformations. Two known natural "tetrameric" profisetinidins[13,14] [i.e., the (-)-fisetinidol-(+)-catechin (**16**) with three (2**R**,3**S**)-flavan-3-ol constituent units and the diastereomer based on (2**S**,3**R**)-flavan-3-ol moieties] display this property.

This unique thermodynamic stability is attributable to the relative configurations of constituent flavanyl units, steric repulsion by functional groups *ortho* to interflavanyl bonds, and steric inhibition of mobility about these linkages due to partial overlap of the terminal units. The stability (on the NMR timescale) of the dominant conformer of each of the diastereomers is evident from sharp ^{1}H NMR spectra (Figure 2) over a wide temperature range (-50 to ca. 80 °C). Coexistence of at least two minor conformers at ambient temperatures was estimated by integration of concomitant subordinate peaks. Preponderance (85-88 percent abundance), however, of a "stable" conformer permits conformational analysis of such high-molecular condensed tannins and hence assessment of absolute configurations about each of the sp^2-sp^3 C-C interflavanyl bonds. The n.O.e. analysis of (**16**)[15,16] is based on interactions of the 5- and 7-methoxy functions of the (+)-catechin moiety and also of 5-H(D) of the (4β,8)-(-)-fisetinidol unit. These associations indicate the close proximity of 7-OMe(G) to 5-H(D),4-H(F), 5-H(J), and 4-H(L), and of 5-H(D) to 2-H-(C) permitted by a single possible arrangement of flavanyl units [(**17**)-Figure 3]. In order to satisfy these n.O.e. associations, the DEF and JKL (-)-fisetinidol units are required to approach right angles to the general plane of the GHI (+)-catechin moiety and the ABC (-)-fisetinidol unit to occupy a plane at



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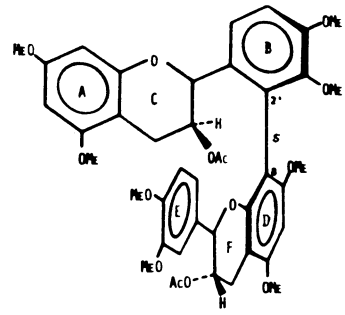

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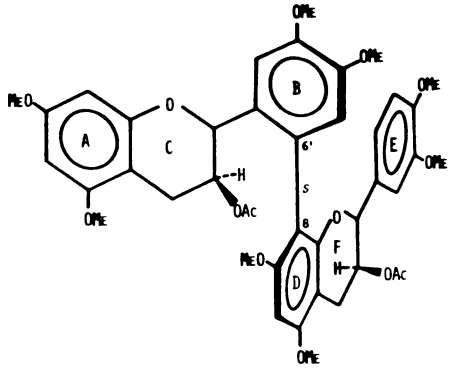

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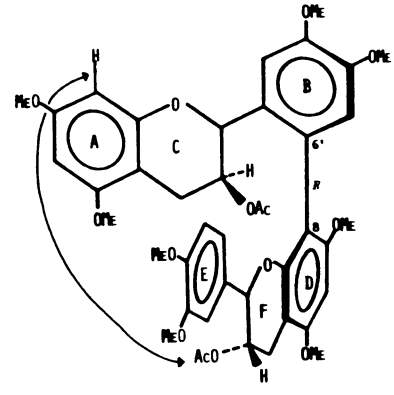

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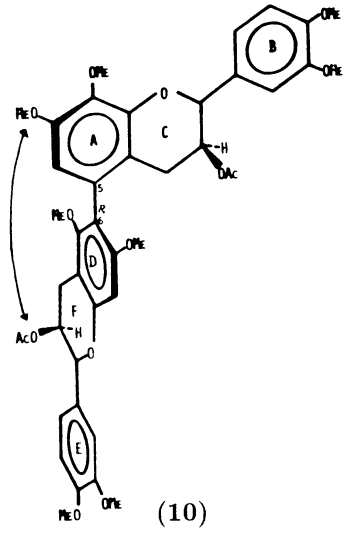

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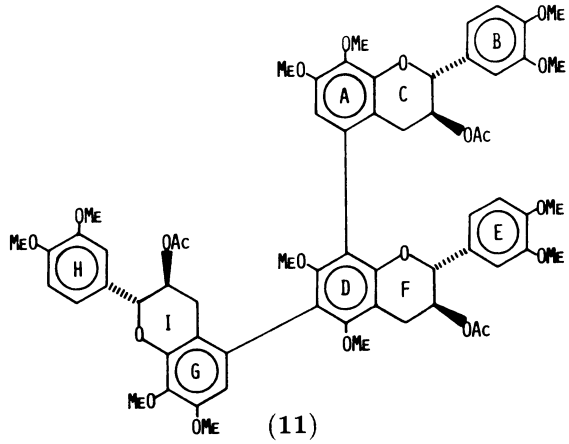

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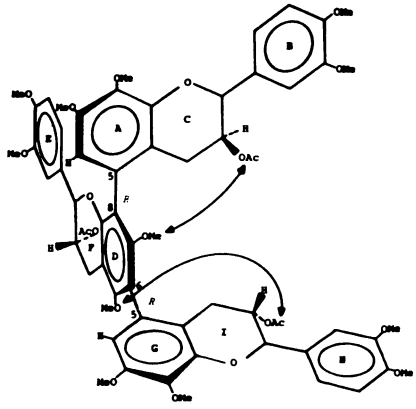

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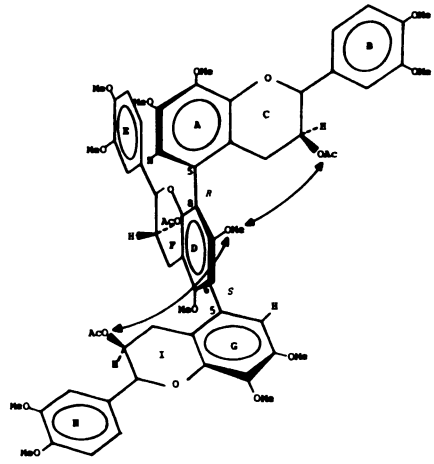

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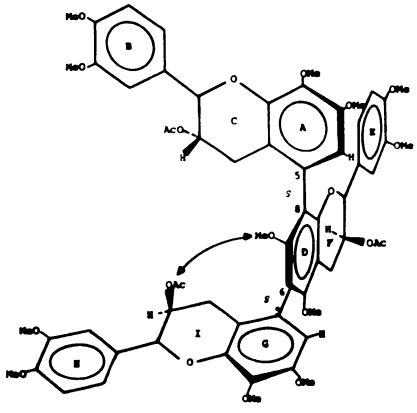

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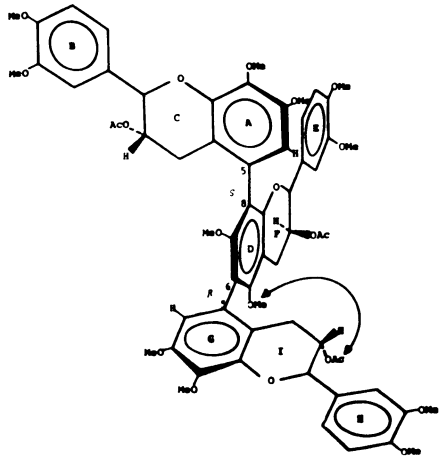

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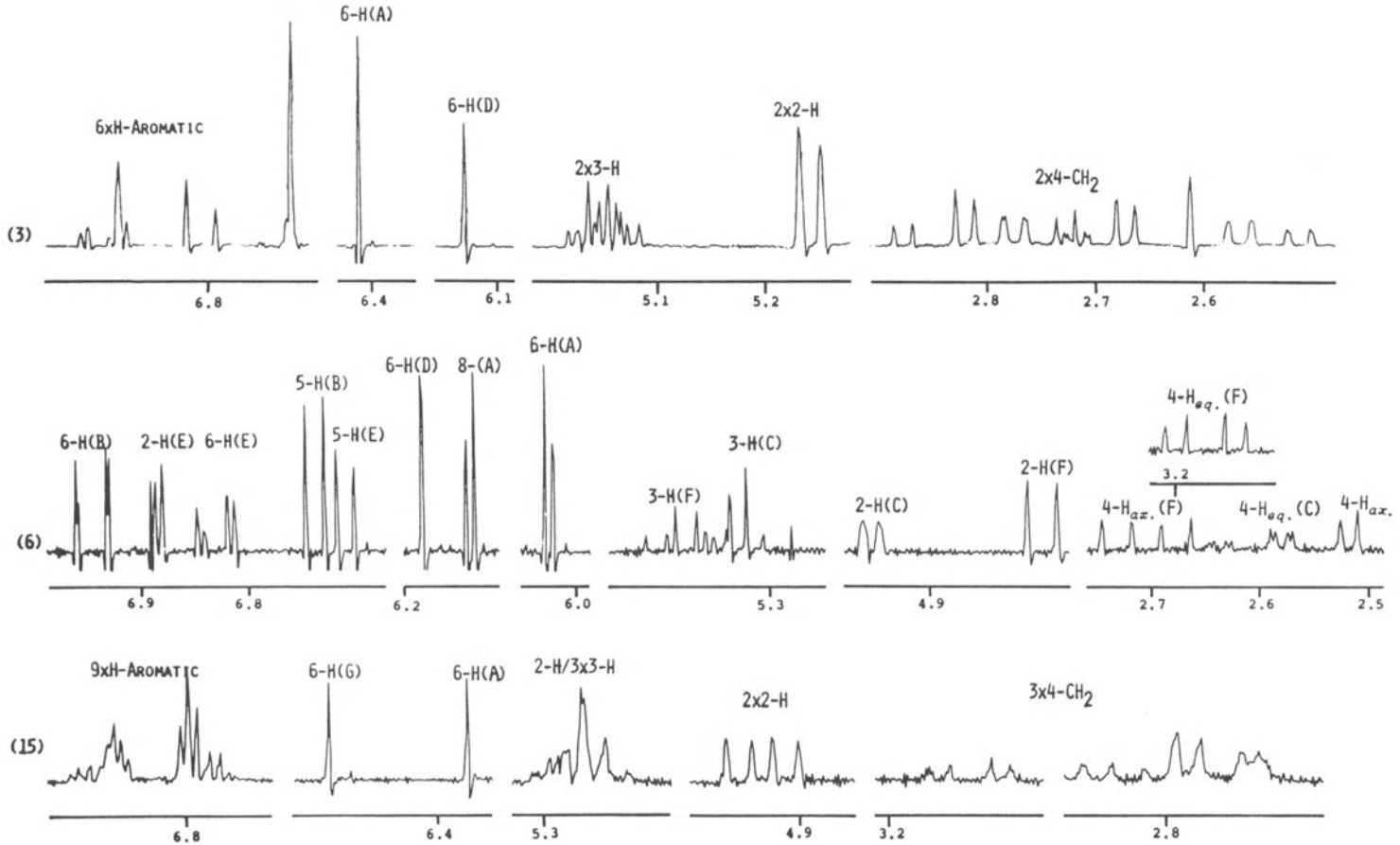
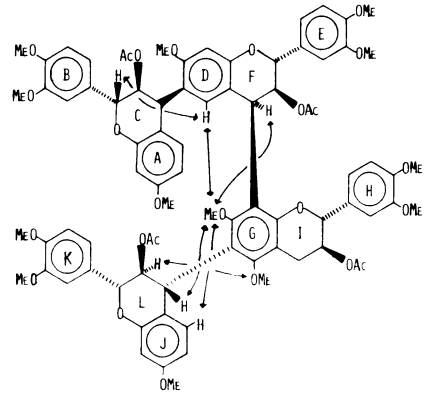


Figure 1. *Aromatic- and heterocyclic regions of the* ^{1}*H-NMR spectra of atropisomeric methyl ether acetates representative of the (+)-mesquitol-(+)-catechins* (**3**), *bis-(+)-catechins* (**6**), *and m-terphenyl-type (+)-mesquitol-(+)-catechins* (**15**) *in* $CDCl_3$ *at 300 MHz.*



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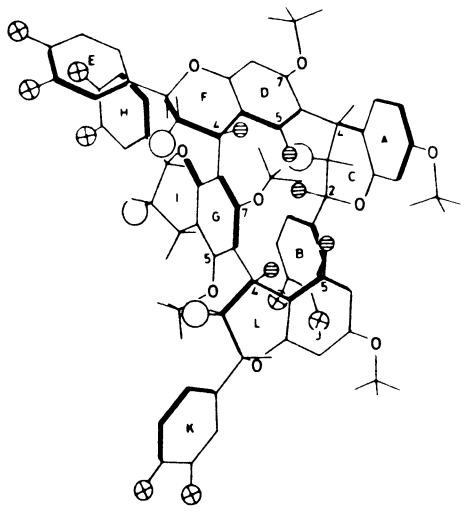

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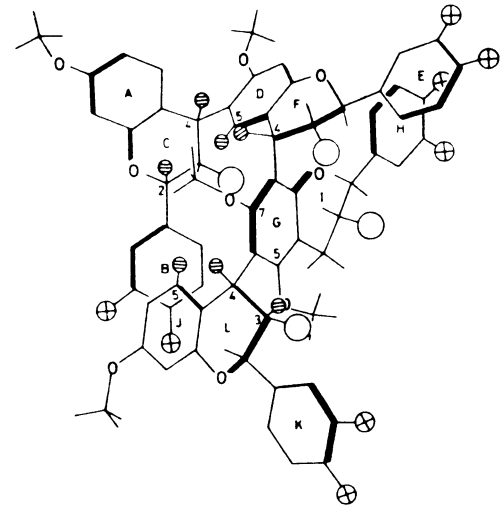


(18)

Figure 2. *The cycloconformations of the tridecamethyl ether tetra-acetates of "angular" profisetinidin tetramers* (**16**) *and its (2***S***,3***R***)-diastereomer (C-, F-, and L-rings) with* ⊖ *representing protons involved in n.O.e. difference interactions with methoxy function, and* O *and* ⊕*, respectively, indicative of 3-acetoxy and 3′,4′-methoxy functions on pyrocatechol units.*

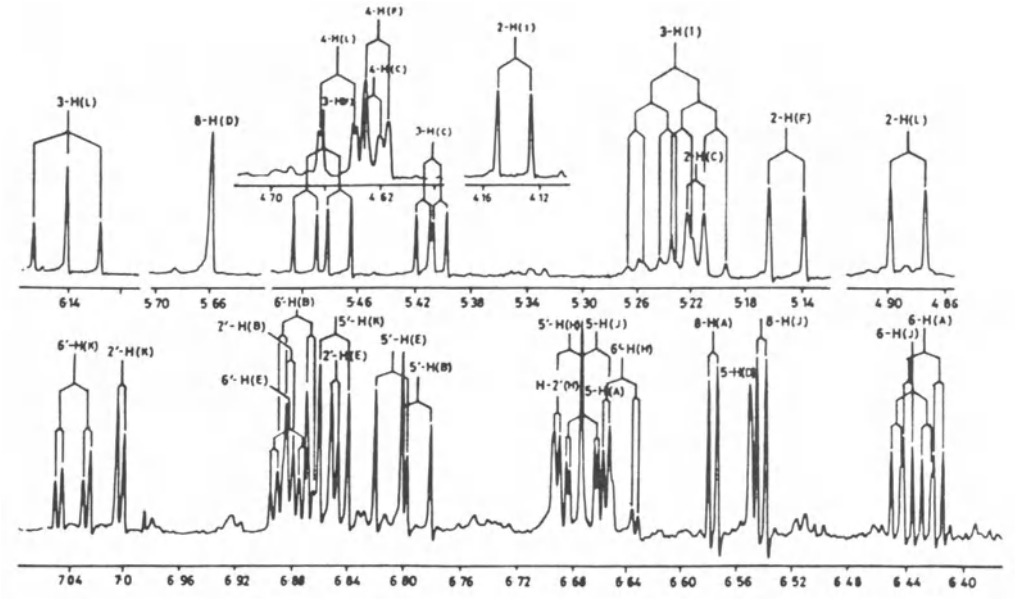
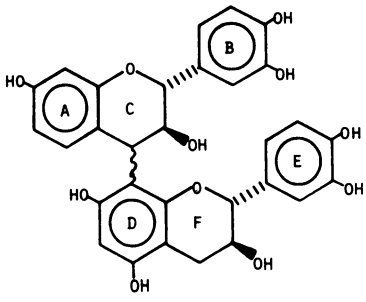


Figure 3. *Aromatic- and heterocyclic regions of the* ^{1}*H-NMR spectrum of the tetraflavanoid tridecamethyl ether tetra-acetate* (**16**) *in* $CDCl_3$ *at 500 MHz.*

right angles to its DEF counterpart. The general conformation of the tetraflavanoid molecule is, therefore, cyclic with the pyrocatechol B-ring abutting on the resorcinol J-ring, and the pyrocatechol E-, H-, and K-rings radiating out in three different directions. The overall stable conformation of (**16**) is thus of [P]-helicity, with the reverse [M]-helicity evident for its diastereomer[15,16] [(**18**)-Figure 2]. Analogous supporting evidence is available from three synthetic profisetinidin isomers which, however, lack natural counterparts.[17]

The natural occurrence of a novel series of C-ring isomerized profisetinidins, termed phlobatannins, has recently been demonstrated.[18] These compounds are conveniently synthesized by treatment of appropriate biflavanoids under mild basic conditions (pH 10, 3-5 h at 50 °C).[19] (4,8)-Profisetinidins of the (2**R**, 3**S**)-series (C-ring with 2,3-*trans*-3,4-*trans* constituent units) (**19**) are subject to stereospecific C-ring isomerization to 8,9-*trans*-9,10-*cis*-tetrahydropyrano-[2,3-h]-chromenes (**23**) via intermediacy of B-ring quinone-methides[20] of type (**24**).

The (4α,6)-analogues (**21**) are similarly converted to a mixture of tetrahydropyrano[2,3-g]-(**25**) and [2,3-f]-(**26**) chromenes. (-)-Fisetinidol units with 2,3-*trans*-3,4-*cis* configuration [(**20**), (**22**)] are susceptible to stereoselective ring isomerization to tetrahydropyranochromenes with all-*trans*- and *cis-trans* stereochemistry of their C-rings [(**27**), and (**28**)] as well as analogues in which the pyrocatechol B- and resorcinol A-rings are interchanged [e.g., (**29**), (**30**)] relative to



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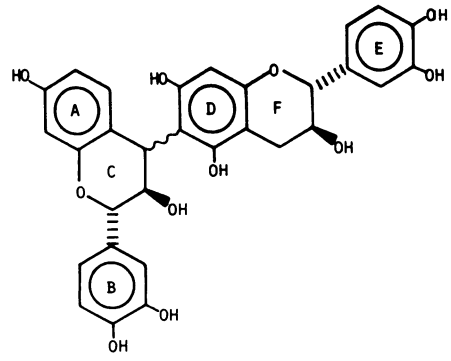
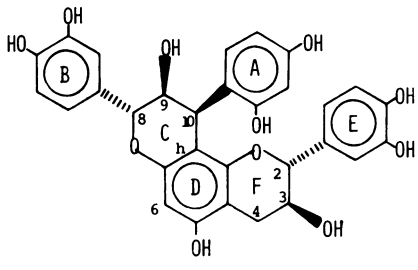


(21) ≡

(22) ≡



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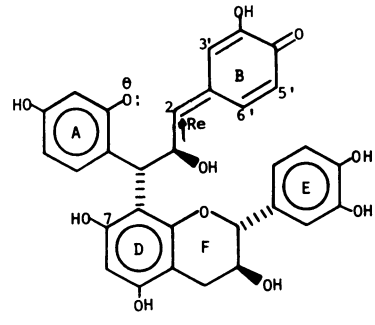


(24)

their positions in the more common analogues [e.g., (**23**)].[21,22] Such a ring-interchange invariably occurs with inversion of the absolute configuration at 3-C (C-ring) of the parent biflavanoid.

The structures of these novel metabolites were established by application of n.O.e. difference spectroscopy to their heptamethylether diacetates, the ^{1}H NMR spectra [Figure 4, heterocyclic regions of [2,3-h] analogues (**23**), (**27**) - (**30**] of which are conspicuously free of the effects of dynamic rotational isomerism at ambient temperatures.[18] N.O.e. effects of 2-OMe(A) with 3-H(A) and of 4-OMe(A) with both 3- and 5-H(A) indicate resorcinol units that are "liberated" from the A/C-ring system in the biflavanoid precursors. Association between the methoxy protons and one-proton singlet of ring D in the tetrahydropyrano [2,3-h]- and [2,3-f]-chromenes (but absence of similar effects in the [2,3-g]-analogues) clearly distinguishes these groupings of the phlobatannins. Differentiation of the [2,3-h] and the [2,3-f]-regiomers is similarly affected by the selective n.O.e. association of the D-ring methoxy group and 8-H(C) in the latter instance only.[23] The n.O.e. technique was also elegantly applied to establish the relative configuration of the C-ring of the *cis-trans* analogues [e.g., (**28**)] unambiguously via association of 8-H(C) and the *o*-proton(s) of the aryl group at 10-C.[21,22]

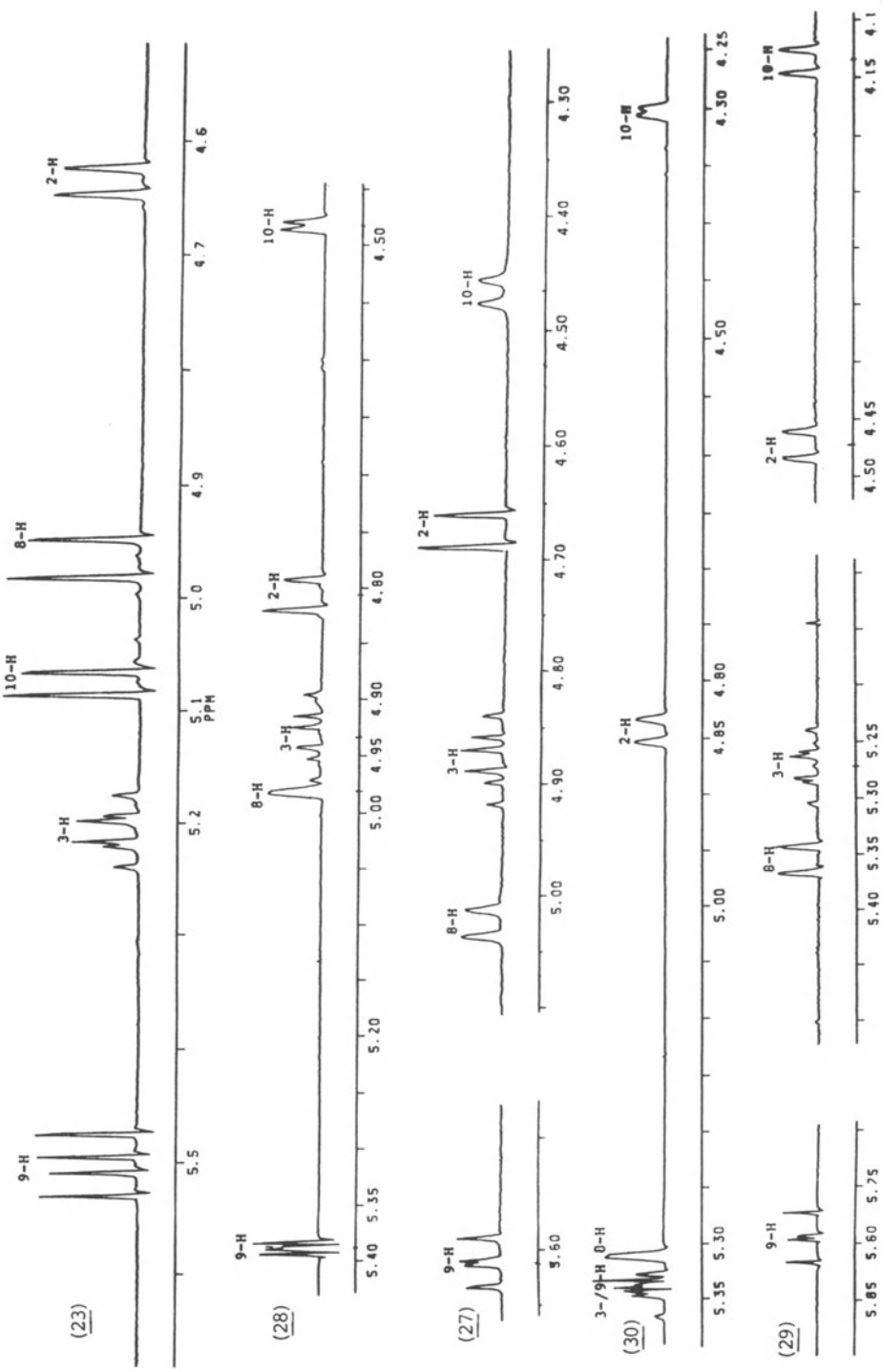
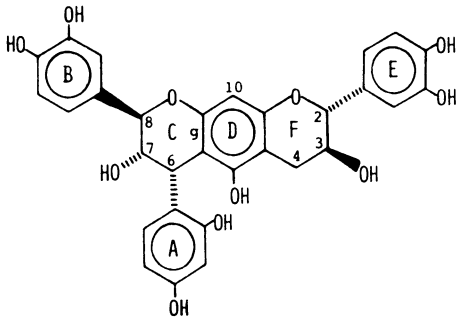


Figure 4. *Splitting patterns of heterocyclic protons of the heptamethylether diacetates of the isomeric terahydropyrano[2,3-h]chromenes* (**23**), (**27-30**) *in* $CDCl_3$ *at 300 MHz.*



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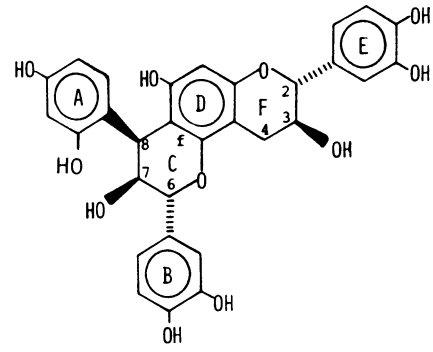
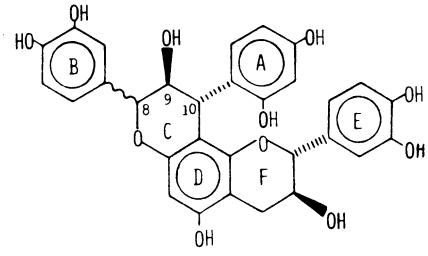
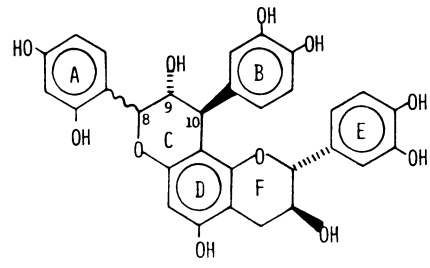


(26)



(27) ≡ ≡

(28) ≡ ▲

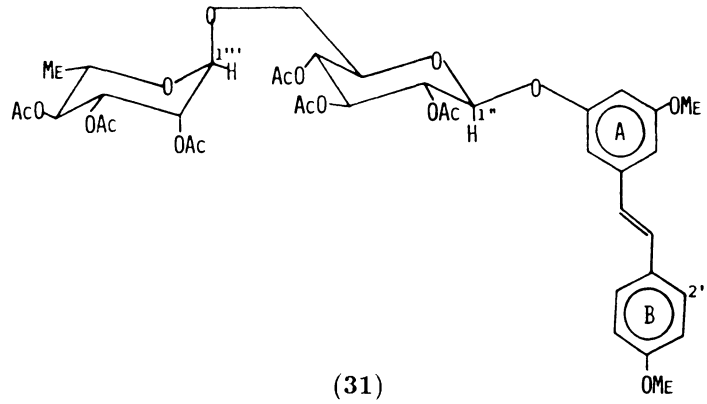


(29) ≡ ▲

(30) ≡ ≡

Evolution in the structural elucidation of glycosides has hitherto been hampered by two fundamental problems; i.e., determination of the position and mode of linkage, both of the glycosyl to the aglycone moiety as well as interglycosidic. Owing to its versatility, n.O.e. difference spectroscopy, by extension of applications in the structural elucidation of condensed tannins,[6,15,18] provides a relatively simple solution to these problems as opposed to previous methodology involving tedious chemical procedures or spectroscopic methods requiring substantial sample quantities.[24]

Such an approach is substantiated by the determination of glycosidic connectivities in several stilbene- and other glycosides[25] such as the (E)-3,4′-dimethoxy-5-[*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glycopyranosyloxy]stilbene (**31**).[26] The n.O.e. association of the anomeric proton (1‴-H) of the *O*-α-L-rhamnopyranosyl unit with the 6″-C methylene proton of the *O*-β-D-glucopyranosyl moiety defines C-6″-O-C-1‴ connectivity of the sugar units. Linkage of the rutinosyl residue to 3-OH(A) of the stilbene is confirmed by association of the glucosyl anomeric proton to both 2- and 4-H(A). Prominent association of the anomeric protons with mutual diaxial hydrogens in the same glycosyl moiety affords an additional convenient probe in the unravelling of more complex glycosidic spinning patterns.



(31)

HOMONUCLEAR J-RESOLVED AND CHEMICAL SHIFT CORRELATION SPECTROSCOPY

Positions of resonance lines in conventional NMR-spectra are dependent on chemical shift and scalar coupling. 2D J-resolved experiments[27,28] have in common that these two parameters are separated along the two axes of a 2D-spectrum, allowing direct and unambiguous determination of both J and δ. Although prone to artifacts that may complicate interpretation[28] and are probably responsible for a hitherto limited application, the technique is capable of excellent results, providing that the overlap remains first-order. Its application is elegantly illustrated by the ^{1}H NMR data (Figure 5) of the bioside derivative (**31**)[25] in which only 1‴-H and 5‴-Me of the *O*-α-L-rhamnopyranosyl- and 1″-H of the *O*-β-D-glucopyranosyl units are clearly defined. The remaining glycosidic protons on the *O*-acetyl bearing carbons resonate in a narrow region, thus leading to severe signal overlap. These multiplicities are elucidated by extraction of the appropriate columns from a 2D J-resolved experiment, allowing complete rationalization of the spectrum as discussed in the previous section. The more familiar 2D homonuclear shift correlation experiment[28–30] (COSY), initially proposed as an alternative to homonuclear decoupling[31] for the determination of scalar coupling in homonuclear spin systems, has often been regarded as superfluous and time-consuming especially in the case of "small" molecules. Although such criticism may occasionally be justified, the procedure's merits have to be judged not merely in the determination of connectivities but also in its additional proficiency. Thus, identification of overlapping protons [e.g., 2-CH_2(F) of the bi-isoflavan (**32**)],[32] one overlapping with 2-CH_2(C) and the other with 3-H(C), is entirely dependent on their respective connectivities, as indicated by off-diagonal peaks with 3-H(F), which, in turn, are coupled to both of the methylene protons at 4-C(F). A similar strategy applied to xanthocercin-A triacetate (**33**), the first isoflavonolignoid,[33] not only allows definition of the connectivities of the four aliphatic protons but also defines the

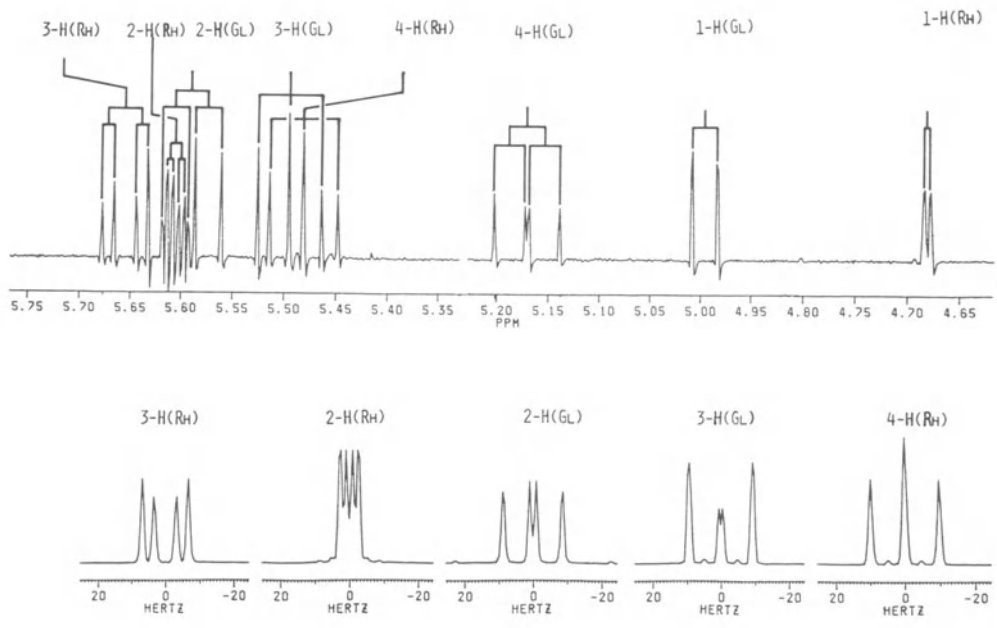
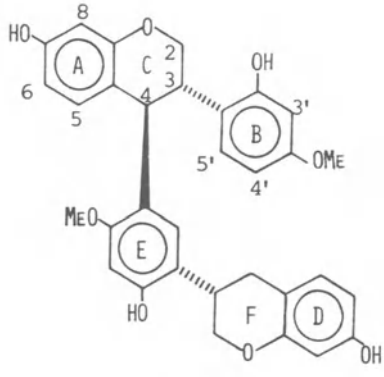
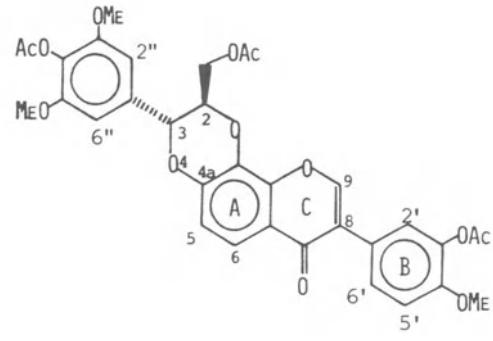


Figure 5. *Multiplicities of glycosidic protons of stilbene bioside* (**31**) *from a 2D J-resolved experiment.*



(**32**)



(**33**) – single enantiomer

substitution pattern of the B-ring through long-range scalar coupling of methoxy protons with 5-H(B) only. The presence of such long-range coupling is characteristic of COSY experiments, which may be optimized accordingly,[28] and also affords evidence regarding the bonding position for the bi-isoflavan (**32**) by coupling of 4-CH_2(F) with 5-H(D) and thus by inference, interflavanoid coupling via 5′-C of ring E, the protons of which exhibit ρ-coupled singlets.

^{13}C- AND HETERONUCLEAR TECHNIQUES

Application of ^{13}C-NMR spectroscopy in flavanoid chemistry is firmly established and includes the investigation of a variety of monomers,[34–42] glycosides,[35,44] biflavanoids,[43] and tannins.[45–47] These revolve mainly about chemical shifts, their differences and compatibilities between similar systems, and the effects induced by substituents.[39,48,49] Despite their undisputed merits, these methods occasionally lead to certain anomalies due to the ambiguous assignment of signals, which thus subsequently require further elucidation. This is accomplished most conveniently by determination of their heteronuclear association via 1-4 bond scalar coupling with protons that may be unequivocally defined.

Advantages involving the observation of ^{1}J multiplicities by off-resonance decoupling or SPI[50] experiments, which have lately been superseded by the more sensitive INEPT[51] or DEPT[52] pulse sequences for the characterization of carbon atoms, have long been recognized. These, however, lack specificity regarding the precise heteronuclear association between atoms, an aspect currently accessible only by 2D heteronuclear shift correlation techniques.[53–56] The elegance of the HETCOR experiment is exemplified by application to phlobatannins in which the resorcinol A- and pyrocatechol B-rings are interchanged, e.g., (**29**) and (**30**). The benzylic connection of 8- and 10-H(C) and the *o*-proton(s) of these rings is subject to the correct allocation of the former resonances. Confirmation of the chemical shifts of 8- and 10-H(C) and thus unambiguous proof for the ring-interchange is obtained via 2D correlation of these protons with 8- and 10-C[21,22] (Figure 6). The same approach was also adopted by Hemingway et al[57] for the structural elucidation of related compounds obtained by base-catalyzed reactions of polymeric procyanidins with phloroglucinol.

Heteronuclear scalar coupling over more than one bond (J $\sim$ 1-15Hz) is, in contrast to the above, capable of providing information regarding non-protonated carbons. The detection of such coupling may effectively be determined by selective low-power decoupling[58] or by deuterium exchange where ^{13}C-^{1}H coupling to hydroxyl-protons is involved.[40] Such methodology allows distinction between the different orientations of substituents on the 1,4-dioxane nucleus of cleomiscosin A(**34**) and B(**36**)[59] by collapse of $^{3}J_{CH}$ at the same carbon (7-C) during discrete decoupling of 5- and 7′-H in isomer (**34**), whereas, identical irradiation of (**36**) leads to the loss of $^{3}J_{CH}$ at different carbons (7- and 8-C). Application of a similar strategy toward solving comparable problems regarding coumarinolignoids[60] also facilitates structural elucidation of xanthocercin A triacetate (**33**)[33] via selective $^{3}J_{CH}$-association of 3-H with 4a-C. This, however, is subject to the unambiguous assignment of 4a-C by adopting a variation of the HETCOR experiment (COLOC)[61] optimized for long-range $^{3}J_{CH}$, allowing 4a-C to be defined by association with 6-H. The technique was earlier also successfully applied to the determination of interglycosidic linkages of glycopeptides.[24]

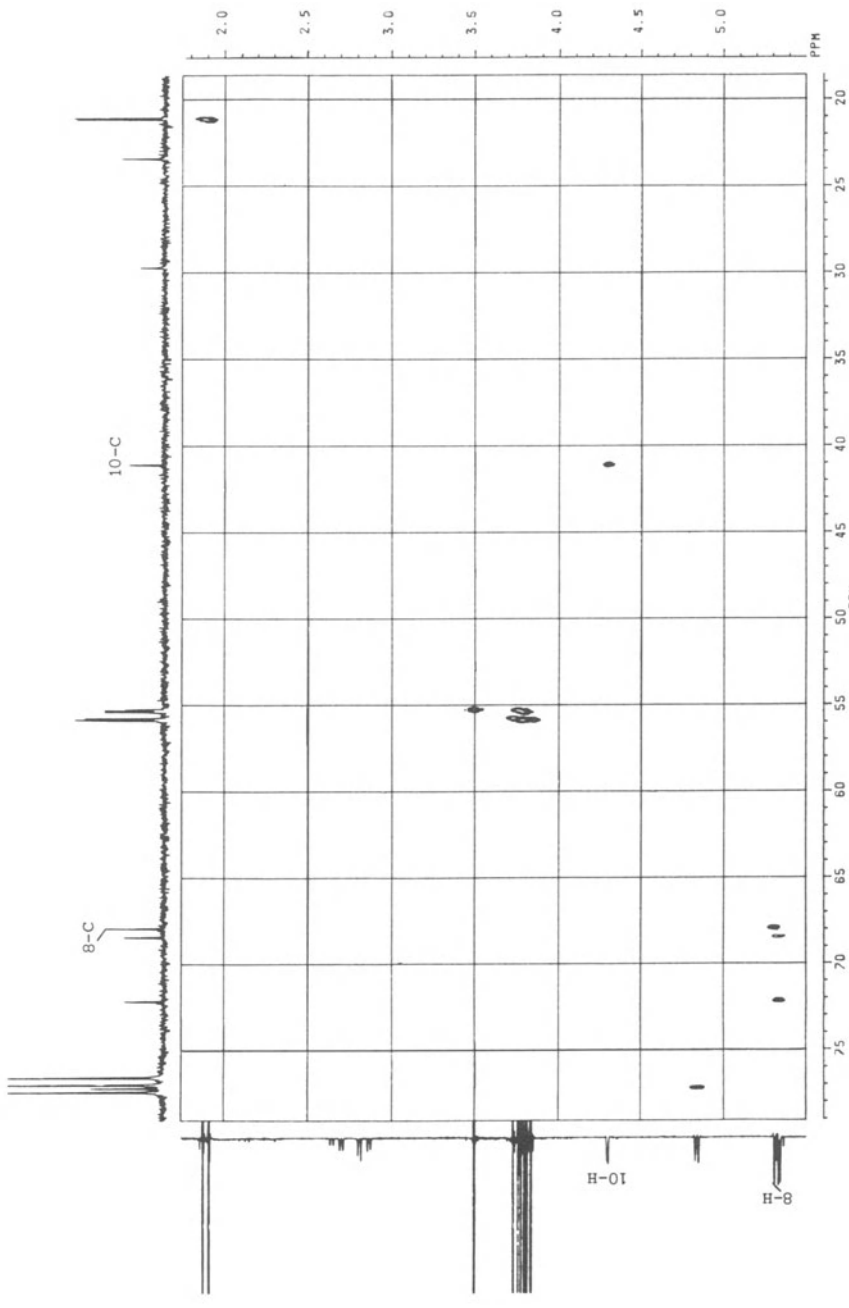
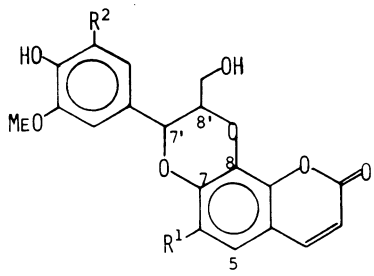
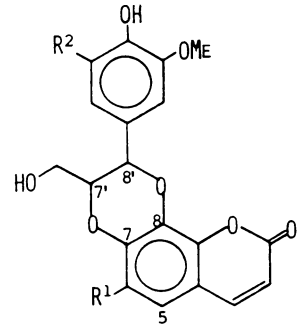


Figure 6. *Heterocyclic region of the HETCOR spectrum of the heptamethyl ether diacetate of the 8,9-cis-9,10-trans-tetrahydropyrano[2,3-h]chromene* (**30**) *in* $CDCl_3$ *at 300 MHz.*



(**34**) R^1 = OMe, R^2 = H
(**35**) R^1 = H, R^2 = OMe



(**36**) R^1 = OMe, R^2 = H
(**37**) R^1 = H, R^2 = OMe

Despite the obvious significance associated with the observation of long-range J_{CH}, the method suffers from the disadvantage of the effect becoming difficult to observe for small values of J_{CH}. This phenomenon adversely affects the observation of often important $^3J_{CH}$ (vicinal) couplings for dihedral angles approaching 90°. Such a problem has partly been overcome by the recent introduction of a novel selective INEPT pulse sequence (SINEPT)[62] allowing three-bond couplings to be emphasized, thus leading to highly simplified but diagnostic spectra. This technique could conveniently be optimized for ^3J = 1Hz to allow differentiation between daphneticin (**35**) and its regiomer (**37**)[63] by determination of the respective $^3J_{CH}$-correlations between 7′-H and 7-C, and 8′-H and 8-C for (**35**). Corresponding, but opposite, data were observed for isomer (**37**).

The examples described here clearly demonstrate the considerable impact that recent advances in the field of NMR spectroscopy have had on the progress of the chemistry of flavanoids and related compounds. We all look forward to the development of additional novel pulse sequences to assist in unravelling the complex structures nature has burdened us with.

ACKNOWLEDGMENTS

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REFERENCES

1. Noggle, J.H.; Schirmer, R.E. The Nuclear Overhauser Effect. Academic Press, New York (1971).
2. Hall, L.D.; Saunders, J.M.K. Complete analysis of ^{1}H-nmr spectra of complex natural products using a combination of one- and two-dimensional techniques. 1. Dehydrotestosterone. *J. Amer. Chem. Soc.* 102:5704 (1980).
3. Hull, W.E. Aspect 2000 Applications Note No. 1. *Bruker Report* 1:4 (1978).

4. Chapman, E.G.; Abercrombie, D.B.; Carey, P.D.; Bradbury, E.M. The measurement of small nuclear Overhauser effects in the proton spectra of proteins and their application of lysozyme. *J. Magn. Reson.* 31:459 (1978).

5. Jeener, J.; Meier, B.H.; Bachman, P.; Ernst, R.R. Investigation of exchange processes by two-dimensional nmr spectroscopy. *J. Chem. Phys.* 71:4546 (1979).

6. Young, E.; Brandt, E.V.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 17. Oligomeric (2R,3S)-3,3′,4′,7,8-pentahydroxyflavans: Atropisomerism and conformation of biphenyl and *m*-terphenyl analogues from *Prosopis glandulosa* ('Mesquite'). *J. Chem. Soc., Perkin Trans. 1* :1737 (1986).

7. Pelter, A.; Amenechi, P.I. Isoflavonoid and pterocarpinoid extractives of *Lanchocarpus laxiflorus J. Chem. Soc. (C)* :887 (1969).

8. Pelter, A.; Amenechi, P.I.; Warren, R.; Harper, S.H. The structures of two proanthocyanidins from *Julbernadia globiflora. J. Chem. Soc. (C)* :2572 (1969).

9. Hundt, H.K.L.; Roux, D.G. Condensed tannins: Determination of the point of linkage in 'terminal' (+)-catechin units and degradative bromination of 4-flavanylflavan-3,4-diols. *J. Chem. Soc., Chem. Commun.* :696 (1978).

10. Hundt H.K.L.; Roux, D.G. Synthesis of condensed tannins. Part 3. Chemical shifts for determining the 6- and 8- bonding positions of 'terminal' (+)-catechin units. *J. Chem. Soc., Perkin Trans. 1* :1227 (1981).

11. Young, D.A.; Young, E.; Roux, D.G.; Brandt, E.V.; Ferreira, D. Synthesis of condensed tannins. Part 19. Phenol oxidative coupling of (+)-catechin and (+)-mesquitol. Conformation of bis-(+)-catechins. *J. Chem. Soc., Perkin Trans. 1* :2345 (1987).

12. Brandt, E.V.; Young, D.A.; Young, E.; Ferreira, D. Absolute configuration of atropisomeric *m*-terphenyl-type flavan-3-ols. *J. Chem. Soc., Perkin Trans. 1* :1365 (1987).

13. Young, D.A.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 15. Structure of natural 'angular' profisetinidin tetraflavanoids: Asymmetric induction during oligomeric synthesis. *J. Chem. Soc., Perkin Trans. 1* :2529 (1985).

14. Young, D.A.; Kolodziej, H.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 16. Stereochemical differentiation of the first 'angular' (2S,3R)-profisetinidin tetraflavanoids from *Rhus lancea* (Karree) and the varying dynamic behaviors of their derivatives. *J. Chem. Soc., Perkin Trans. 1* :2537 (1985).

15. Brandt, E.V.; Young, D.A.; Kolodziej, H.; Ferreira, D.; Roux, D.G. Cycloconformations of two tetraflavanoid profisetinidin condensed tannins. *J. Chem. Soc., Chem. Commun.* :913 (1986).

16. Brandt, E.V.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 20. Cycloconformations and conformational stability among derivatives of 'angular' tetraflavanoid profisetinidins. *J. Chem. Soc., Perkin Trans. 1* :2353 (1987).

17. Young, D.A.; Ferreira, D.; Roux, D.G. Stereochemistry and dynamic behavior of some synthetic 'angular' profisetinidin tetraflavanoid derivatives. *J. Polym. Sci., Polym. Chem. Ed.* 24:835 (1986).

18. Steenkamp, J.A.; Steynberg, J.P.; Brandt, E.V.; Ferreira, D.; Roux, D.G. Phlobatannins, a novel class of ring-isomerized condensed tannins. *J. Chem. Soc., Chem. Commun.* :1678 (1985).

19. Steynberg, J.P.; Young, D.A.; Burger, J.F.W.; Ferreira, D.; Roux, D.G. Phlobatannins via facile ring isomerizations of profisetinidin and prorobinetinidin condensed tannin units. *J. Chem. Soc., Chem. Commun.* :1013 (1986).

20. Steynberg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V. Steenkamp, J.A.; Ferreira, D. Oligomeric flavanoids. Part 3. Structure and synthesis of phlobatannins related to (-)-fisetinidol-(4α,6) and (4α,8)-(+)-catechin profisetinidins. *J. Chem. Soc., Perkin Trans. 1* :3323 (1988).

21. Steynberg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V.; Steenkamp, J.A.; Ferreira, D. Novel base-catalysed rearrangements of (-)-fisetinidol-(+)-catechin profisetinidins with 2,3-*trans*-3,4-*cis* flavan-3-ol constituent units. *J. Chem. Soc., Chem. Commun.* :1055 (1988).

22. Steynberg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V.; Steenkamp, J.A.; Ferreira, D. Oligomeric flavanoids. Part 4. Base-catalysed conversions of (-)-fisetinidol-(+)-catechin profisetinidins with 2,3-*trans*-3,4-*trans*-flavan-3-ol constituent units. *J. Chem. Soc., Perkin Trans. 1* :3331 (1988).

23. Burger, J.F.W.; Steynberg, J.P.; Young, D.A.; Brandt, E.V.; Ferreira, D. Oligomeric flavanoids. Part 5. Base-catalysed C-ring isomerization of (+)-fisetinidol-(+)-catechin profisetinidins. *J. Chem. Soc., Perkin Trans. 1* 8/02280A/P1P (1988).

24. Kessler, H.; Bermel, W.; Griesinger, C.; Kolar, C. The elucidation of the constitution of glycopeptides by the nmr spectroscopic COLOC technique. *Angew. Chem. Int. Ed. Engl.* 25:342 (1986).

25. Steynberg J.P.; Brandt E.V.; Burger J.F.W.; Bezuidenhoudt, B.C.B.; Ferreira, D. Stilbene glycosides from *Guibourtia coleosperma*: determination of glycosidic connectivities by homonuclear nuclear Overhauser effect difference spectroscopy. *J. Chem. Soc., Perkin Trans. 1* :37 (1988).

26. Bombardelli, E.; Martinelli, E.M.; Mustich, G. Plants of Mozambique IX. New hydroxystilbene glycoside form *Terminalia sencea*. *Fitoterapia* 46:199 (1975).

27. Aue, W.P.; Karhan, J.; Ernst, R.R. Homonuclear broad band decoupling and two-dimensional J-resolved nmr spectroscopy. *J. Chem. Phys.* 64:4226 (1976).

28. Bax, A.D.; Two-dimensional Nuclear Magnetic Resonance in Liquids. Delft University Press, Holland p. 99 (1982).

29. Jencer, J. Ampere International Summer School, Basko Polje, Yugoslavia, (1981).

30. Aue, W.P.; Bartholdi, E.; Ernst, R.R. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *J. Chem. Phys.* 64:2229 (1976).

31. Anderson, W.A.; Freeman, R. Influence of second radio-frequency field on high resolution nuclear magnetic resonance spectra. *J. Chem. Phys.* 37:85 (1962).

32. Bezuidenhoudt, B.C.B.; Brandt, E.V.; Roux, D.G. Synthesis of isoflavanoid oligomers using a pterocarpan as inceptive electrophile. *J. Chem. Soc., Perkin Trans. 1* :2767 (1984).

33. Bezuidenhout, S.C.; Bezuidenhoudt, B.C.B.; Brandt, E.V.; Ferreira, D. Oligomeric isoflavonoids. Part 2. Structure and synthesis of xanthocercin A and B, the first isoflavono-lignoids. *J. Chem. Soc., Perkin Trans. 1* :1237 (1988).

34. Ternai, B.; Markham, K.R. Carbon-13 nmr studies of flavonoids. I. Flavones and flavonols. *Tetrahedron* 32:565 (1976).

35. Markham, K.R.; Ternai, B. ^{13}C nmr of flavonoids. II. Flavonoids other than flavone and flavonol aglycones. *Tetrahedron* 32:2607 (1976).

36. Wagner, H.; Chari, V.M.; Sonnenbichler, J. ^{13}C-nmr Spectren naturlich verkommender Flavonoide. *Tetrahedron Letters* 21:1799 (1976).

37. Kingsburg, C.A.; Looker, J.H. Carbon-13 spectra of methoxyflavones. *J. Org. Chem.* 40:1120 (1975).

38. Joseph-Nathan, P.; Mares, J. Hernandez, Ma.C.; Schoolery, J.N. Proton and carbon-13 nuclear magnetic resonance studies of flavone and deuterated analogues. *J. Magn. Reson.* 16:447 (1974).

39. Pelter, A.; Ward, R.S.; Gray, T.I. The carbon-13 nuclear magnetic resonance spectra of flavonoids and related compounds. *J. Chem. Soc., Perkin Trans. 1* :2475 (1976).

40. Wherli, F.W. Proton coupled ^{13}C nuclear magnetic resonace spectra involving ^{13}C-^{1}H spin-spin coupling to hydroxyl-protons, a complementary assignment aid. *J. Chem. Soc., Chem. Commun.* :663 (1975).

41. Solaniova, E.; Toma, S.; Gronowitz, S. Investigation of substituent effects of chalcones by carbon-13 nmr spectroscopy. *Org. Magn. Reson.* 8:439 (1976).

42. Chang, C. Carbon-13 proton long range couplings of phenols. Hydrogen bonding and stereospecificity. *J. Org. Chem.* 41:1881 (1976).

43. Chari, V.M.; Ilyas, M.; Wagner, H.; Neszmelyi, A.; Chen, L.-K.; Lin, Y.-C.; Lin, Y.-M. ^{13}C nmr spectroscopy of biflavonoids. *Phytochemistry* 18:1273 (1977).

44. Chari, V.M.; Jordan, M.; Wagner, H.; Theis, P.W. A ^{13}C-nmr study of the structure of an acyl-linarin from *Valeriana wallichii*. *Phytochemistry* 16:1110 (1977).

45. Karchesy, J.J.; Hemingway, R.W. Loblolly pine bark polyflavanoids. *J. Agric. Food Chem.* 28:222 (1980).

46. Czochawska, Z.; Foo, L.Y.; Newman, R.H.; Porter, L.J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J. Chem. Soc., Perkin Trans. 1* :2278 (1980).

47. Porter, L.J.; Newman, R.H.; Foo, L.Y.; Wong, H.; Hemingway, R.W. Polymeric proanthocyanidins. ^{13}C nmr studies of procyanidins. *J. Chem. Soc., Perkin Trans. 1* :1217 (1982).

48. Wenkert, E.; Buckwalter, B.L.; Burfitt, I.R.; Gasic, M.J.; Gottlieb, H.E.; Hagaman, E.W.; Schell, F.M.; Wovkulich, P.M.; Heleva, A.Z. *In*: Levy, G.C. (ed.) Topics in Carbon-13 NMR Spectroscopy. Wiley-Interscience, New York, II:2 (1976).

49. Philipsborn, W. von. Applications of double resonance and Fourier transform nmr spectroscopy in organic chemistry. *Pure Appl. Chem.* 40:159 (1974).

50. Chalmers, A.A.; Pachler, K.G.R.; Wessels, P.L. Difference selective population inversion spectra and their application to the study of carbon-13 – hydrogen coupling constants in 2,3-dibromothiophene. *Org. Magn. Reson.* 6:445 (1974).

51. Morris, G.A.; Freeman, R. Enhancement of nuclear magnetic resonance signals by polarization transfer. *J. Amer. Chem. Soc.* 101:760 (1979).

52. Doddrell, D.M.; Pegg, D.T.; Bendall, M.R. Distortionless enhancement of nmr signals by polarization transfer. *J. Magn. Reson.* 48:323 (1982).

53. Maudsley, A.A.; Ernst, R.R. Indirect detection of magnetic resonance by heteronuclear two-dimensional spectroscopy. *Chem. Phys. Lett.* 50:368 (1977).

54. Bodenhausen, G.; Freeman, R. Correlation of proton and carbon-13 nmr spectra by heteronuclear two-dimensional spectroscopy. *J. Magn. Reson.* 28:471 (1977).

55. Maudsley, A.A.; Mueller L.; Ernst, R.R. Cross-correlation of spin-decoupled nmr spectra by heteronuclear two-dimensional spectroscopy. *J. Magn. Reson.* 28:463 (1977).

56. Bax, A.; Morris, G. An improved method for heteronuclear chemical shift correlation by two-dimensional nmr. *J. Magn. Reson.* 42:501 (1981).

57. Laks, P.E.; Hemingway, R.W.; Conner, A.H. Condensed tannins. Base-catalyzed reactions of polymeric procyanidins with phloroglucinol. Intramolecular rearrangements. *J. Chem. Soc., Perkin Trans. 1* :1975 (1987).

58. Kalyanasundaram, K. Use of long range ^{1}H-^{13}C couplings in structure determination: shellatin, a novel dihydroisocoumarin from *Aspergillus varicolor*. *J. Chem. Soc., Chem. Commun.* :628 (1978).

59. Arisawa, M.; Handa, S.S.; McPherson, D.D.; Laukin, D.C.; Cordell, G.A.; Wong, H.H.S.; Farnsworth, N.R. Plant anticancer agents. XXIX. Cleomiscosin A from *Simaba multiflora, Soulanea soulameopides* and *Matayba arborescens*. *J. Nat. Prod.* 47:300 (1984).

60. Ray, A.B. Chasttopadhyay, S.K.; Kumar, S.; Konno, C.; Kiso, Y.; Hinkino, H. Structures of cleomisconsias, coumarino-lignoids of *Cleome miscosa* seeds. *Tetrahedron* 41:209 (1985).

61. Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H.R. Assignment of carbonyl carbons and sequence analysis in peptides by heteronuclear shift correlation via small coupling constants with broadband decoupling in t_1 (COLOC). *J. Magn. Reson.* 57:331 (1984).

62. Bax, A. Structure determination and spectral assignment by pulsed polarization transfer via long-range proton-carbon-13 couplings. *J. Magn. Reson.* 57:314 (1984).

63. Lin, L.-J.; Cordell, A. Applications of the SINEPT pulse progamme in the structure elucidation of coumarinolignans. *J. Chem. Soc., Chem. Commun.* :377 (1986).

FAB-MS APPLICATIONS IN THE ELUCIDATION OF PROANTHOCYANIDIN STRUCTURES

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ABSTRACT

Fast atom bombardment mass spectrometry (FAB-MS), which requires no chemical derivatization prior to mass spectral analysis, has become a powerful tool for studying the structures of biopolymers. FAB and similar forms of mass spectrometry have the potential for achieving the same degree of importance in the elucidation of proanthocyanidin structure currently accorded nuclear magnetic resonance. The extent and state of research on the use of FAB-MS to determine molecular weight, adduct identity, sequence, branching, and linkage type are reviewed in this paper. Experimental considerations (such as sample introduction, sample matrices, and instrument modes) and mass spectral features associated with the characterization of oligomeric proanthocyanidins are surveyed.

INTRODUCTION

Proanthocyanidins are well-known, ubiquitous constituents of woody plants.[1,2] These compounds have been the subject of much study because their chemical structures and their roles in the biology of plants are intrinsically interesting and because they have commercial value. The vegetable tannins complex with proteins, carbohydrates, nucleic acids, alkaloids, and minerals.[3,4] These properties affect the flavor and palatability,[4,5] nutritional value[6,7] pharmacological and toxic effects, and resistance to microbial and insecticidal attack[8,9] of many plant products. The condensed or nonhydrolyzable tannins are potential replacements for petroleum-derived phenolic polymers used in industry as adhesives, dispersants, and ion exchange materials.[10–12] Their molecular size and polyphenolic character make the structure of proanthocyanidins refractory to analysis. The early colorimetric assays[13,14] and methods involving protein precipitation[15–18] yielded questionable results.[19] In

the last two decades, electron impact mass spectrometry,[20–24] chemical ionization mass spectrometry,[22,25–27] field desorption mass spectrometry,[22,28–33] proton[34,35] and carbon-13[36,37] nuclear magnetic resonance (NMR) spectroscopies, and to a lesser degree, microcalorimetry[38] have been effectively employed to elucidate many of the principal structural features of vegetable tannins.

Electron impact mass spectrometry was employed in 1968 by Weinges et al to study peracetylated and permethylated procyanidins.[20] Use of EI mass spectrometry is summarized through 1980 in reviews by Mabry and Markham[21] and by Mabry and Ulubelen;[22] more recently, the technique has been employed to study methylated gallotannins[23] and kaki tannins.[24] Wherein considerable structural data have resulted from the EI analysis of monomers and a few dimers, information on oligomeric proanthocyanidins has been forthcoming in only a few isolated instances; this is because of the compounds' low thermal stability and lack of volatility.[21,22] Attempts to alleviate these problems through use of trimethylsilyl ethers or polymethylated/acetylated derivatives are common, but the spectra generally fail to exhibit molecular ions. Chemical ionization mass spectrometry[22,25–27] and field desorption mass spectrometry[22,28–33] have been used to obtain more intense molecular ion signals from flavonoids, including oligomeric procyanidins[30,33] and related compounds.[28] However, these methods produce too few diagnostic fragments for structural analysis. Chemical ionization mass spectrometry suffers additionally from the requirement for derivitization of the proanthocyanidin oligomers to increase their volatility. Both proton[34,35] and carbon-13[36,37] NMR spectrometries have contributed significantly to the elucidation of proanthocyanidin structures. Nonetheless, resonance multiplicity and broadening associated with rotational and conformational isomerism often severely complicate the interpretation of NMR spectra of large oligomers. Recent advances in techniques for the structural analysis of proanthocyanidins are reviewed by D. Ferreira in the preceding chapter of this volume.

Despite the advances made in elucidating the structures of proanthocyanidins over the past 20 years, questions still exist concerning the distribution of molecular weights, the occurrence and extent of branching, the relative proportions of different interflavanoid linkage types, and the sequential order of different monomers in the polymers. It may be possible to address these questions, either partially or completely, via the relatively new mass spectrometric technique of fast atom bombardment (FAB)[39,40] mass spectrometry. This technique, which requires no chemical derivitization prior to mass analysis, has become a powerful tool for analyzing the structures of biopolymers.[41] Both experimental considerations and mass spectral features associated with the characterization of oligomeric proanthocyanidins by FAB mass spectrometry are surveyed in this review.

FAB MASS SPECTROMETRY – GENERAL

FAB belongs to a family of particle-induced desorption ionization techniques[42–44] that in the main produce mass spectra with strikingly similar features, viz. abundant molecular ion species and relatively few fragment ions. The techniques – FAB mass spectrometry, secondary ion mass spectrometry, plasma desorption mass spectrometry, and laser desorption mass spectrometry – have revolutionized our

ability to analyze biopolymers for molecular weights and for certain structural information.[41,45]

The FAB method is distinct from its desorption ionization relatives in two ways: 1) neutral atoms (Ar or Xe) having translation energies in the range of 5,000-10,000 eV are employed as primary particles and 2) the analyte is dissolved or suspended in a liquid matrix. The widespread analytical utility of the technique is now generally attributed to the second feature-the liquid matrix.[43,46] In fact, when a liquid matrix is used, it is essentially impossible to distinguish the mass spectra produced by neutral primary particles from those produced by ionic primary particles having the same translational energy and comparable mass.[47]

A schematic representation of a FAB ion source and its operation are shown in Figure 1. In practice, one or two microliters of a viscous liquid, typically (though not necessarily) glycerol, are applied onto the end of the sample probe. From one to several micrograms of sample, either as solid or in solution, are added to the support liquid (sometimes with and sometimes without stirring) to complete the matrix. The probe with sample is inserted through a vacuum lock into the ion source. The primary beam – atomic or ionic – is turned on to generate the secondary

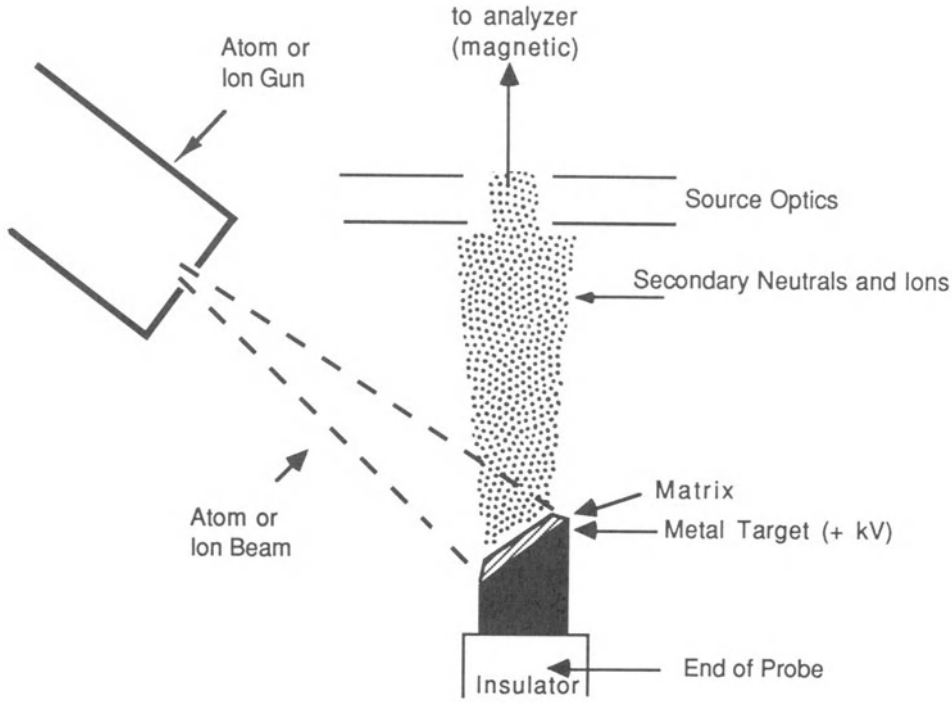


Figure 1. *Schematic representation of the geometry and operation of a FAB or Liquid SIMS source. In the case of Liquid SIMS, where ions are used as primary particles, the ion beam is generally focused to some degree onto the sample matrix.*

ions that are mass analyzed. The sheer simplicity of this source arrangement and sample handling – devoid as they are of critical geometrical alignment and manual manipulation – accounts, in part, for FAB's rapid transition from a novel to a routine technique. The technique's operational compatibility with sector instruments has also contributed to its widespread success.[48] During atom or ion bombardment, both positive and negative secondary ions are simultaneously ejected from the sample matrix; the relative abundances of these ions depend on the nature of the analyte, the inherent properties of the support liquid, and any additives (e.g., acid or base) used to alter the ionic character of the liquid matrix. Which ions are mass analyzed is at the discretion of the mass spectroscopist; examples of both positive and negative mass spectra are common in the literature. Frequently, useful emission of sample ions can be sustained for 10-30 minutes, permitting high resolution mass spectrometry and tandem mass spectrometry to be performed on sector instruments.[49,50]

Sample purity can have a profound effect on the production of mass spectra.[51] Most of the secondary ions ejected from the sample matrix result from direct desorption of precharged species.[43,44,46] Variations in solution chemistry and differential surface activities lead to a high degree of selectivity in the FAB process of desorption ionization; frequently, a mixture of two or more species, which generate good spectra when mass analyzed individually, will produce a mass spectrum of only one of the components. Hence, the presence of unknown impurities (e.g., incompletely separated residual compounds, reaction products, and salts from sample isolation and purification stages) can and frequently do suppress desorption ionization of analytes.

In general, FAB mass spectra are characterized by the appearance of even-electron cations or anions; radical cations or anions are less frequently observed.[43,44,46] Molecular ion species formed by desorption of precharged compounds (salt-derived organic cations or anions), by the gain or loss of a proton (protonation or deprotonation), and by adduction or clustering with available inorganic ions (cationization or anionization) are usually quite intense. As a rule, fragment ions are less intense than molecular ion species and can be traced to their even-electron precursors via the loss of a neutral molecule.

The interested reader is referred to earlier reviews for a more comprehensive treatment of the mechanistic aspects of FAB processes[43,44,46] and for a broader survey of the applications of FAB mass spectrometry.[48,52–56]

FAB MASS SPECTROMETRY – PROANTHOCYANIDINS

Experimental Factors

Several laboratories have investigated the applicability of FAB to the mass analysis of vegetable tannins. Surprisingly little experimentation with the liquid matrix has been performed, and present knowledge about the effects of different liquid matrices on the production of molecular ion species and fragment ions is meager. In nearly all cases reported, neat glycerol was used as the matrix liquid. Domon and Hostettmann[57] indicated use of both gylcerol and thiogylcerol but did not comment on the relative merits of the two liquids. Karchesy and coworkers[58,59] have consis-

tently found that for oligomeric procyanidins a eutectic mixture of dithiothreitol and dithioerythritol (5:1), commonly referred to as "magic bullet," is superior to glycerol as a FAB matrix; both molecular and fragment ion species are produced in greater abundances. After dissolving their favonol samples in glycerol, de Koster and coworkers[60] acidified the matrix with acetic acid, presumably to obtain more intense mass spectra. Clearly, more research on the chemical environment of the samples (i.e., the liquid matrix) during FAB analyses is necessary in the future.

Both positive and negative FAB mass spectra have been reported in the literature. Figure 2 exhibits typical positive and negative ion FAB mass spectra of B-7 procyanidin (**III**, Figure 5); both spectra exhibit abundant molecular ion species and coherent fragment ions. Large mass peaks attributable to matrix cluster and adduct ions are also prevalent in the mass spectra; Domon and Hostettman[57] and de Koster et al[60] have both pointed out the well-known problem of these matrix peaks superimposing on peaks due to analyte ions and thereby interfering with interpretation of mass spectra. It should also be noted that less abundant ion species from the liquid sample matrix contribute small peaks at virtually every nominal mass in a spectrum; this background, commonly referred to as chemical noise, is a limiting factor for sensitivity in FAB analyses.

Conclusions drawn regarding the relative utility of the positive and negative ionization modes vary among investigators. Nishioka and coworkers,[61–67] who did not indicate the liquid matrix and other FAB conditions they used, have only reported positive FAB ion species including molecular ion species for several underivatized pentaflavanoids. Not surprisingly, de Koster and coworkers[60] found the absolute abundances of protonated flavonols desorbed from an acidified liquid matrix to be greater than the abundances of deprotonated flavonols. Karchesy and coworkers[58,59] reported fairly comparable positive and negative FAB emission for oligomeric procyanidins. As a rule, these researchers have found molecular ion species to be slightly more abundant in the negative ion mode, a tendency that becomes more pronounced as the oligomers become larger, while fragment ions are slightly more pronounced in the positive ion mode, Figure 2. Domon and Hostettmann's[57] results with monomeric flavonoid glycosides match those of Karchesy et al in that positive and negative ion modes gave similar mass spectra but differed in that both molecular ion species and higher mass fragment ions were more intense in the negative ion mode. Gujer et al,[68] Galletti and Self,[69] and Self et al[70] tested both positive and negative ion FAB modes on several classes of compounds; they found that both modes contained comparable molecular weight information through protonated and deprotonated molecular ions, respectively, but that the positive ion spectra studied, regardless of the class of compound, contained only sparse, structurally coherent fragmentation. Self and his coworkers themselves profess that their observation, particularly in regard to the non-glycosidic procyanidin oligomers, the flavanol gallates, and the dimers and depsides of the gallotannins they have studied, has no precedent outside their own experience.[70] The routine reports of Nishioka and coworkers notwithstanding, the bulk of current evidence points to the negative FAB mode as being superior to its positive counterpart in most mass analyses of vegetable tannins and related compounds.

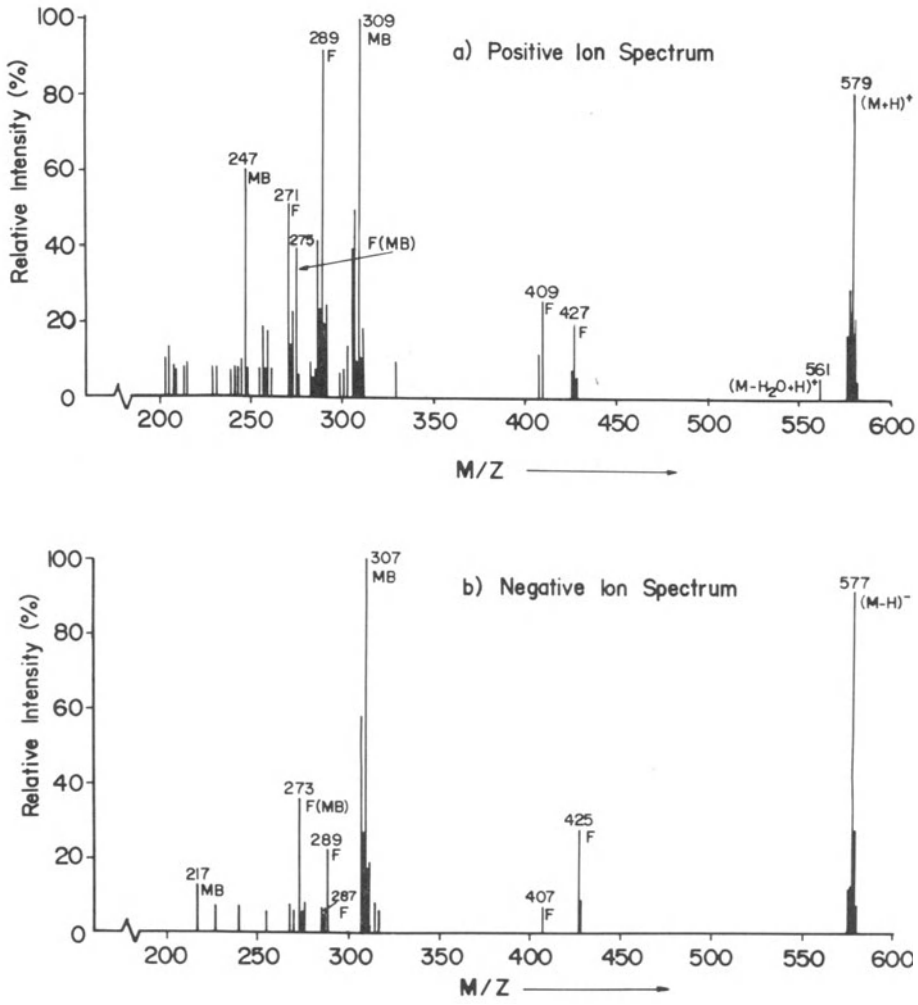


Figure 2. *Positive and negative FAB mass spectra of B-7 procyanidin dimer (***III***, Figure 5); background mass peaks with less than 5% relative intensity are not shown. Protonated and deprotonated molecular ion species are indicated as* $(M + H)^+$ *and* $(M - H)^-$ *respectively. Mass peaks due to fragment ions are designated with a F (refer to Figure 6 for interpretation), and mass peaks due to the matrix ions (magic bullet – 5:1 mixture of dithiothreitol and dithioerytritol) are designated with MB.*

Determination of Individual Molecular Weights

Molecular weight is one of the most important pieces of information that can be obtained from mass analysis. Knowledge of a condensed tannin's molecular weight can reveal its degree of polymerization; i.e., the number of monomeric units, and the presence (possibly even the identity) of any carbohydrate, nuclei acid, or other covalently bound functionalities. Determining the molecular weight of a proanthocyanidin oligomer is, however, a difficult task. The monomeric units in condensed tannins have molecular weights on the order of 300 daltons. Thus, even with a small degree of polymerization, the oligomers have relatively large individual molecular weights. In addition to being large, polyphenolic compounds are polar and thermally labile.

Before proanthocyanidin oligomers can be mass-analyzed by electron impact or chemical ionization techniques, the compounds must be derivatized to increase their volatility and stability. Peracetylation, pertrifluoroacetylation, permethylation, and pertrimethysilyation have, for example, been used in attempts to mass analyze a variety of flavonoids including proanthocyanidins. Unfortunately, several problems accompany these methods. Given the large number of hydroxyl and phenolic groups that these compounds possess, derivatization subtantially increases their already considerable molecular weights and effectively reduces the upper limit of molecular size that can be accommodated on any particular mass spectrometer. Partially derivatized compounds that require additional purification steps for their elimination and, in some cases, chemically altered structures that complicate interpretation are undesirable byproducts of derivatization methods. Most discouraging, however, is the fact that, for vegetable tannins larger than dimers, the effort to derivatize and repurify them results in only weak or even undetectable molecular ion signals under electron impact conditions and only slightly enhanced ion intensities under chemical ionization conditions.[21,22]

Field desorption, which is actually a member of the desorption ionization family, was the first mass spectrometric technique to yield molecular weights of nonderivatized flavonoids.[29–33] Field desorption mass spectrometry of flavonoids has been reviewed up to 1980 by Mabry and Ulubelen.[22] Because of a general perception that field desorption mass spectrometry is relatively difficult to practice, the method has never been widely applied in the analysis of vegetable tannins. In fact, field desorption is quite simple to use with modern instruments, and descriptions of its routine use to determine molecular weights occasionally are found in the current literature.[33,57,62–66]

The advent of FAB mass spectrometry[39,40] brought with it two advantages over field desorption mass spectrometry: facile operation and prolonged ion production. Some laboratories have already incorporated FAB techniques into the cadre of analytical procedures they routinely use for analyses of vegetable tannins.[61–67,70,71] Under FAB conditions, abundant molecular ion species have been reported for monomeric flavonols,[60] monomeric[57,63,65,68,70] and oligomeric[64,65,68,70] polyphenolic glycosides, procyanidins,[58,59,61–65,67–71] and oligomeric hydrolyzable tannins[70,72] and related monomeric glycosylated phenolic compounds.[73,74] Small abundances of doubly charged ions of the molecular species of flavanol mono- and digalates and a

monomeric ellagitannin,[70] of metastable decomposition ions of flavanol mono- and digallates,[70] and of cluster ions, $(2M \pm H)^{\pm}$, of flavanol mono- and digallates[70] and monomeric flavonoid glycosides[57] have also been reported and found useful in identifying unknown polyphenols.

There is no indication of an upper limit on the mass of a pure proanthocyanidin that could be analyzed by FAB or related techniques. Molecular ions from a protein with a molecular weight of 24,000 daltons have been produced and recorded under FAB-like conditions on a modern sector instrument equipped with a high field magnet and a diode-array detector.[75] Molecular ions of a protein with a molecular weight approaching 35,000 daltons have been produced and recorded using plasma desorption ionization on a time-of-flight instrument.[76] There is no intrinsic reason why similar results could not be achieved with condensed tannins. Gujer et al,[68] Morimoto et al.,[62,63] and Foo et al[77] have already measured the masses of individual molecular ion species corresponding to pentameric procyanidins ($m/z \sim 1{,}400$) by FAB mass spectrometry. The limiting factor for measuring the masses of condensed tannins with high molecular weights seems at present to lie with the isolation and purification of individual compounds and not with the FAB method itself. Galletti and Self point out that high order oligomeric proanthocyanidins may be present in extracted material as insoluble complexes with proteins or other molecules and, as such, would be undetectable; they call for improvements over the standard methods for purification and analysis of polyphenols.[69]

Determination of Molecular Weight Distributions

Sufficient evidence exists to indicate that proanthocyanidin polymers are polydisperse in individual plant species with sizes ranging from two to hundreds of monomeric units and with molecular weight distributions symmetrical in some plants and bimodal in others.[78] The molecular weight of an individual condensed tannin, isolated from its original preparation, can be used to estimate its degree of polymerization but not the range of molecular weights or dispersivity of the condensed tannins in its plant-source. In principle, gel-permeation chromatography could be used to determine the distribution of molecular weights in a mixture of condensed tannins, but it has not been possible to do this directly because of the high polarity and strong tendency toward hydrogen bonding characteristic of phenolic polymers.[78]

The feasibility of applying FAB mass spectrometry to molecular weight distribution has been tested to a limited degree by Galletti et al and Self et al., who have produced negative ion FAB mass spectra of polyphenol mixtures (from grape skins) derivatized with phloroglucinol and not subjected to further chromatographic separation,[69] of extracts of hydrolyzable tannins from oak galls and from *Myrobalans*,[70] and of HPLC fractions containing mixtures of procyanidins[69,70] (Figure 3). Self and coworkers issue two caveats in regard to quantification from such mixture-spectra: 1) with the exception of the highest molecular weight ion peak in a spectrum, which can reasonably be assumed to correspond to a molecular ion species (e.g., m/z 1153, Figure 3b,c), each major ion peak (e.g., m/z 865, m/z 577, and m/z 289) receives contributions both from the molecular ion species of an oligomer in the mixture and from fragment ions of larger molecular ion species, and

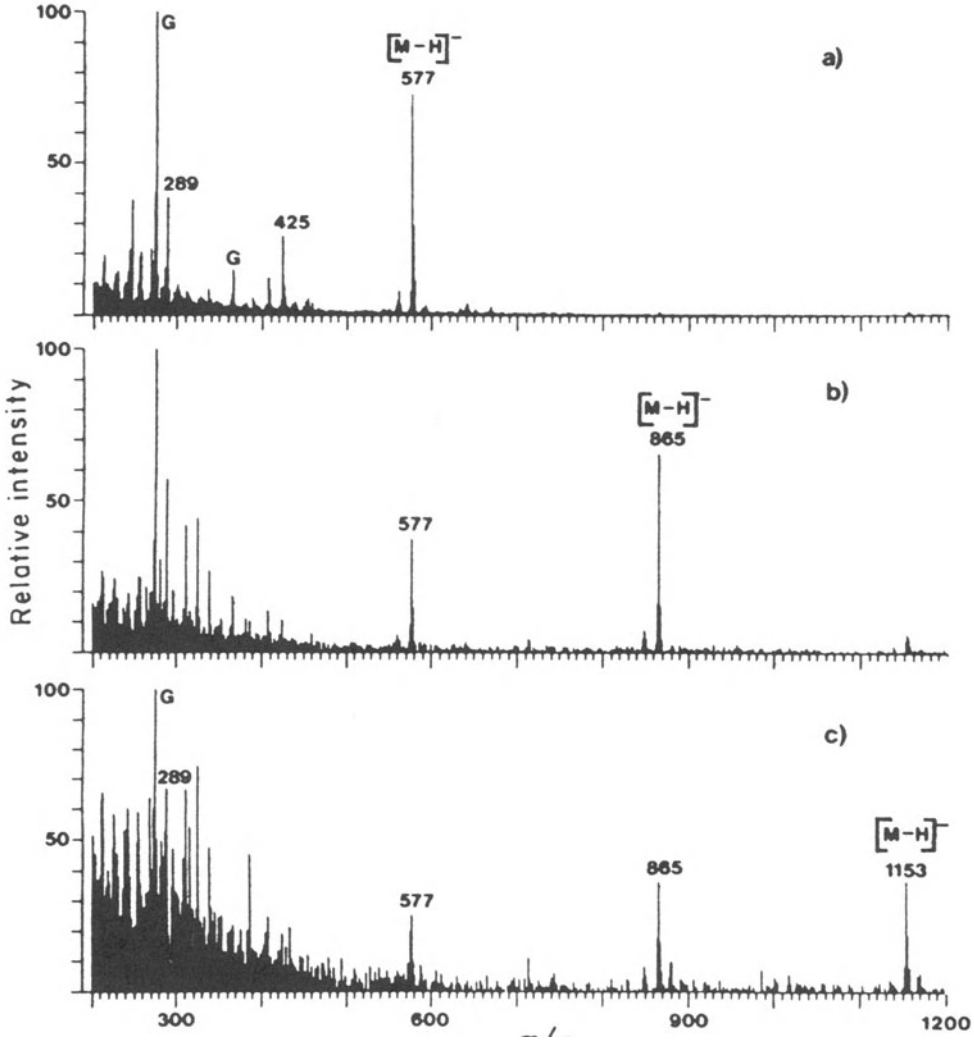


Figure 3. *Negative ion FAB mass spectra of some procyanidin fractions extracted from cider apples: a) dimeric, b) trimeric, d) tetrameric. Reprinted with permission from R. Self, J. Eagles, G.C. Galletti, I. Mueller-Harvey, R.D. Hartley, A.G.H. Lea, D. Magnolato, U. Richli, R. Gujer, and E. Haslam, Biomed. Environ. Mass Spectrom. 13: 449 (1986). Copyright 1986, John Wiley and Sons Ltd.*

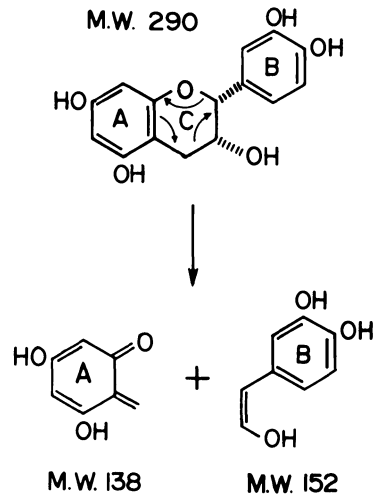
2) the relative FAB ionization efficiencies and, hence, overall instrument sensitivities for the individual oligomers are unknown. These investigators see no reason why standard compounds, once available, cannot be used to overcome some of the problems associated with quantitative mixture analysis via FAB mass spectrometry.[70] Continuous-flow-FAB,[79] in which FAB is performed directly off the end of a capillary liquid chromatography column terminated in the ion source, presents another possibility for qualitative and quantitative analyses of mixtures of condensed tannins.

Both Karchesy et al[58] and Self et al[70] have suggested that FAB coupled with some form of tandem mass spectrometry could permit direct qualitative analysis of proanthocyanidin oligomers without recourse to chromatographic separation of pure compounds. The potential of this powerful technique has already been demonstrated on a number of biopolymer systems;[80,81] it should be equally applicable to investigating the polydisperse nature of vegetable tannins in plants.

Elucidation of Structure

The primary fragment peaks in mass spectra of flavonoid compounds by whatever method arise from direct retro-Diels-Alder (RDA) fission of the heterocyclic ring and from cleavage of the interflavanoid bonds.[21,22] The basic mechanisms for fission of the heterocyclic ring were suggested by Pelter and coworkers after a thorough study of several types of flavanoids under electron impact conditions.[82,83] Following this early work, a myriad of studies (mostly employing electron impact ionization) ensued to examine the effects of different A-ring and B-ring substitution patterns on the basic fragmentation pathways.[21,22]

Relatively few studies of condensed tannins have been conducted using FAB ionization, but is clear that the principal fragmentations in FAB mass spectra follow the same patterns observed in the other ionization modes. Cleavage of the heterocyclic ring via RDA fission is depicted in Scheme 1 for (-)-epicatechin. Charge retention on either fragment in the form of protonation or deprotonation accounts for most of the structurally coherent ion species observed respectively in positive or negative FAB mass spectra of monomeric flavonoids. Thus, for example, a positive ion mass spectrum of (-)-epicatechin would show a protonated molecule $(M+H)^+$ at m/z 291 and protonated RDA fragments at m/z 139 and m/z 153, whereas, a negative ion mass spectrum would show a deprotonated molecule $(M-H)^-$ at m/z 289 and the deprotonated RDA fragments at m/z 137 and m/z 151. Positive FAB mass analyses of three monomeric flavonols (kaempferol, quercetin, and robinetin) by de Koster and coworkers produced the expected protonated RDA fragment peaks for their respective A-rings, but instead of the corresponding protonated dienophiles, mass peaks characteristic of the B-rings resulted via an alternate fission pathway.[60] Several relatively intense mass peaks corresponding to the loss of small neutral molecules, such as H_2O and CO, were also observed in the spectra of these three flavonols. Structurally uniformative peaks such as these are commonly observed in the FAB mass spectra of many organic compounds. Using collisional activation[84] in helium and daughter ion linked scanning,[84] de Koster et al demonstrated that the isomers quercetin and robinetin could be



Scheme 1. *Retro-Diels-Alder fission of (-)-epicatechin.*

distinguished on the basis of the different relative intensities of their A-ring and B-ring mass peaks; they suggest that this method could be used in general to differentiate between isomeric aglycones having differently substituted A-rings and B-rings.[60]

In addition to ions that result from one or two stages of RDA fission, both positive and negative FAB mass spectrometry of oligomeric proanthocyanidins produce ions indicative of the sequence of the monomeric units. The positive ion mass spectrum of the purified branched procyanidin trimer (IV, Figure 5) shown in Figure 4a exhibits strong peaks at m/z 579 and m/z 289 corresponding respectively to the dimeric and monomeric units making up the original molecule. Karchesy and coworkers[58] established the origin of the sequence ions by daughter ion linked scanning from the molecular ion (Figure 4b). Only ions formed by unimolecular gas phase decompositions of the same ion species can contribute to a daughter ion linked scan mass spectrum of that parent species; thus, linked scanning substantially reduces the noise from background ions (chemical noise) in the spectrum (Figure 4b) and permits unambiguous identification of a fragment ion's parent. Self and coworkers have applied this powerful technique to mixtures of procyanidins to estimate the relative contributions of sequence ions originating from higher order oligomers and of molecular ions of lower order oligomers to the peak heights at their common masses (Figure 3).[70]

The linked scan in Figure 4 shows that, besides the sequence ions, essentially only the RDA fission fragment at m/z 715, $(M+H-152)^+$, comes directly from the molecular ion; linked scans on the ions species at m/z 715, m/z 579, and m/z 427 established that loss of H_2O from m/z 715 contributes the peak at m/z 697, that RDA fission and interflavanoid bond cleavage of the dimer ion are responsible,

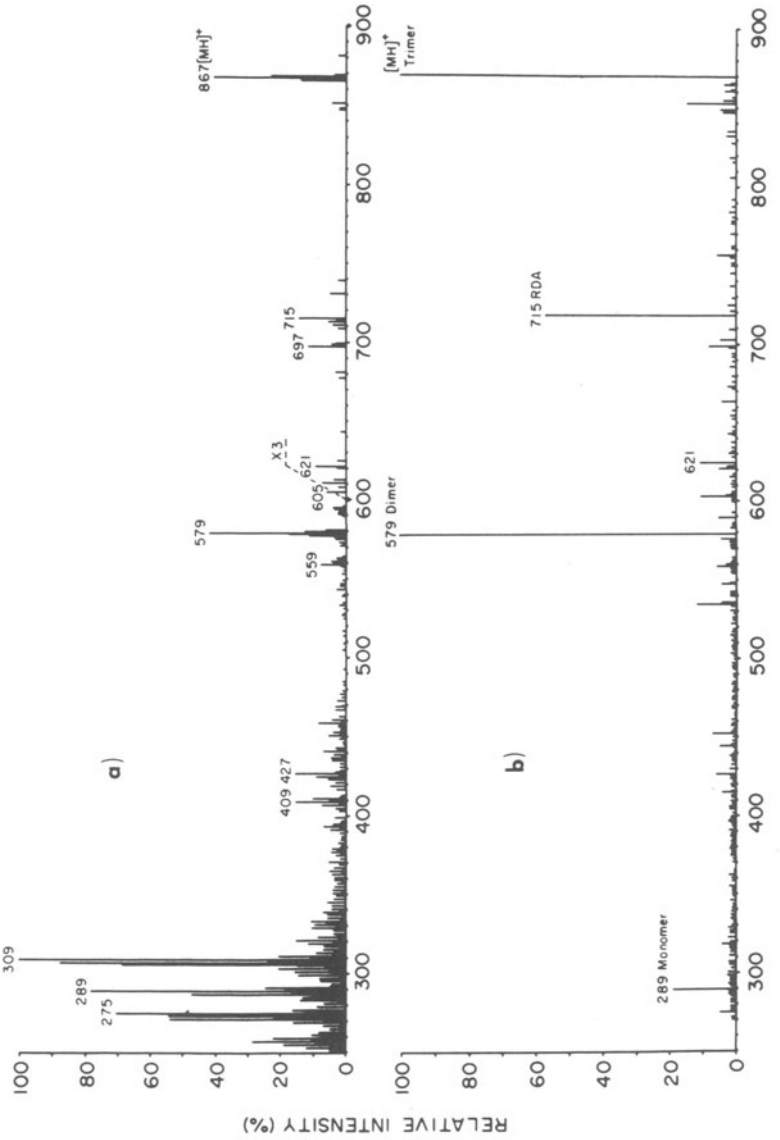


Figure 4. *a) FAB mass spectrum of a branched procyanidin trimer (IV, Figure 5). b) Daughter ion linked scan of $(M+H)^+$, m/z 867. Reprinted with permission from J.J. Karchesy, R.W. Hemingway, L.Y. Foo, E. Barofsky, and D.F. Barofsky, Anal. Chem. 58:2563 (1986). Copyright 1986, American Chemical Society.*

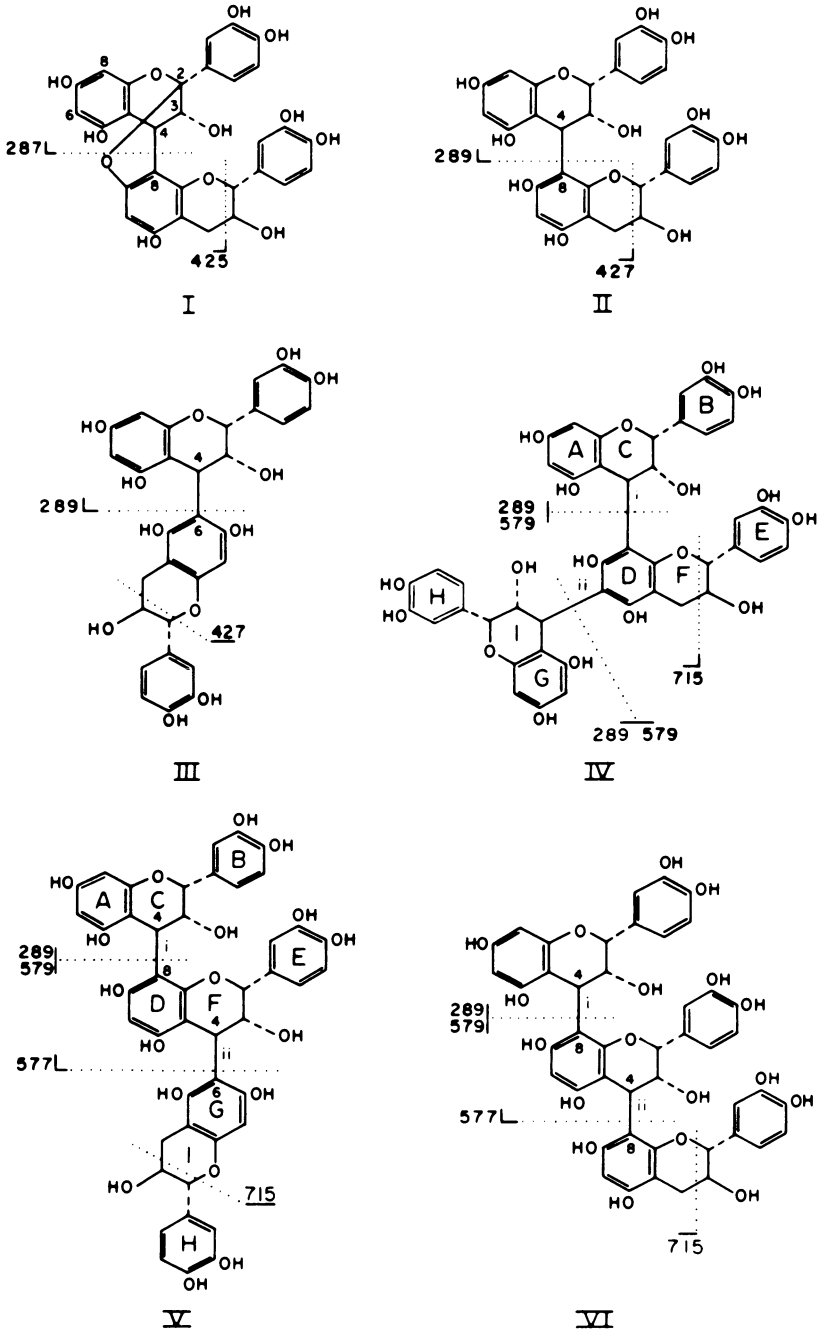


Figure 5. *Structures of a selected series of proanthocyanidin dimers and trimers indicating the principal fragmentations observed in their FAB mass spectra.*

respectively, for the entire peak at m/z 427 and part of the peak at m/z 289, and that loss of H_2O from the dimer's RDA fragment gives rise to the peak at m/z 409.

It is not possible to deduce hydroxylation patterns, detect stereoisomerism among constituent monomeric units, distinguish oligomeric isomers, or differentiate between the acid labile 4-6 and 4-8 interflavanoid bonds via FAB mass spectrometry of proanthocyanidin oligomers. However, by using both daughter and parent (in which only parents appear in the mass spectrum of a given fragment ion) ion linked scanning to map the fragmentation pathways of the series of procyanidin dimers and trimers shown in Figure 5 in both positive[58] and negative[59] FAB ionization modes, Karchesy and coworkers have shown that sequence ions from procyanidin oligomers can be used both to differentiate A-type and B-type interflavanoid bonds and to distinguish trimeric branched and linear isomers. Referring to the positive ion pathways for dimers in Figure 6, the monomer ion at m/z 289 cleaves directly from the B-type dimer ion – probably following protonation of the aromatic ring at the bonding carbon (C6 or C8) – to give the carbonium ion at C4. By contrast, the A-type dimer ion first undergoes RDA fission to give an ion at m/z 425, and that ion subsequently decomposes to produce the monomer ion at m/z 287. These gas phase ion decomposition processes parallel solution chemistry in which the B-type procyanidin linkages readily undergo acid-catalyzed cleavage to give carbonium ions at the C4-position of the upper flavanoid unit,[2,85] but the A-type linkages resist acid-catalyzed cleavage.[71,86] In the negative ion mode, a quinone methide mechanism[35,87–92] leading to the formation of carbanions has been proposed to account for the different masses of the sequence ions that are produced from the upper and lower flavan units (Figure 6, Scheme 2, Scheme 3).[59] The ability to distinguish between these isomeric units after cleavage of the bond between them could facilitate identification of points of attachment by carbohydrates, nucleic acids, or other functionalities. As seen in Figure 6, both positive and negative FAB modes provide fragmentation pathways that can be used to distinguish branched from linear procyanidin trimers.[58,59]

Several laboratories have studied or simply used FAB mass spectrometry of underivatized polyphenolic glycosides and related compounds. In general, it has been found that the identity of an aglycone as well as the class (hexose, deoxyhexose, pentose, etc.) of the monosaccharide units attached to it can be ascertained from FAB mass spectra.[57,60,65,68,70] Even glucuronides, which are very polar and thus refractory to mass analysis, are amenable to either positive or negative FAB mass spectrometry.[57] Differentiation between isomeric hexose substituents, determination of the types of linkage between monosaccharides, and identification of the glycosidation positions on the aglycone are not possible by FAB mass spectrometry alone.[60] Gujer et al[68] and Self et al[70] have demonstrated that, in addition to loss of the sugar moieties, the FAB mass spectra of oligomeric glucosylated dimers and trimers exhibit sequence ions characteristic of cleavage of the interflavan bonds. Unlike the case with procyanidin dimers, however, glycosylation can make it possible to differentiate between monomeric units.[70] In an investigation of material extracted from a species of Ethiopian browse, Self and coworkers[70] used the losses of the galloyl moiety, $(M\text{-}H\text{-}152)^-$, RDA fragment ions,

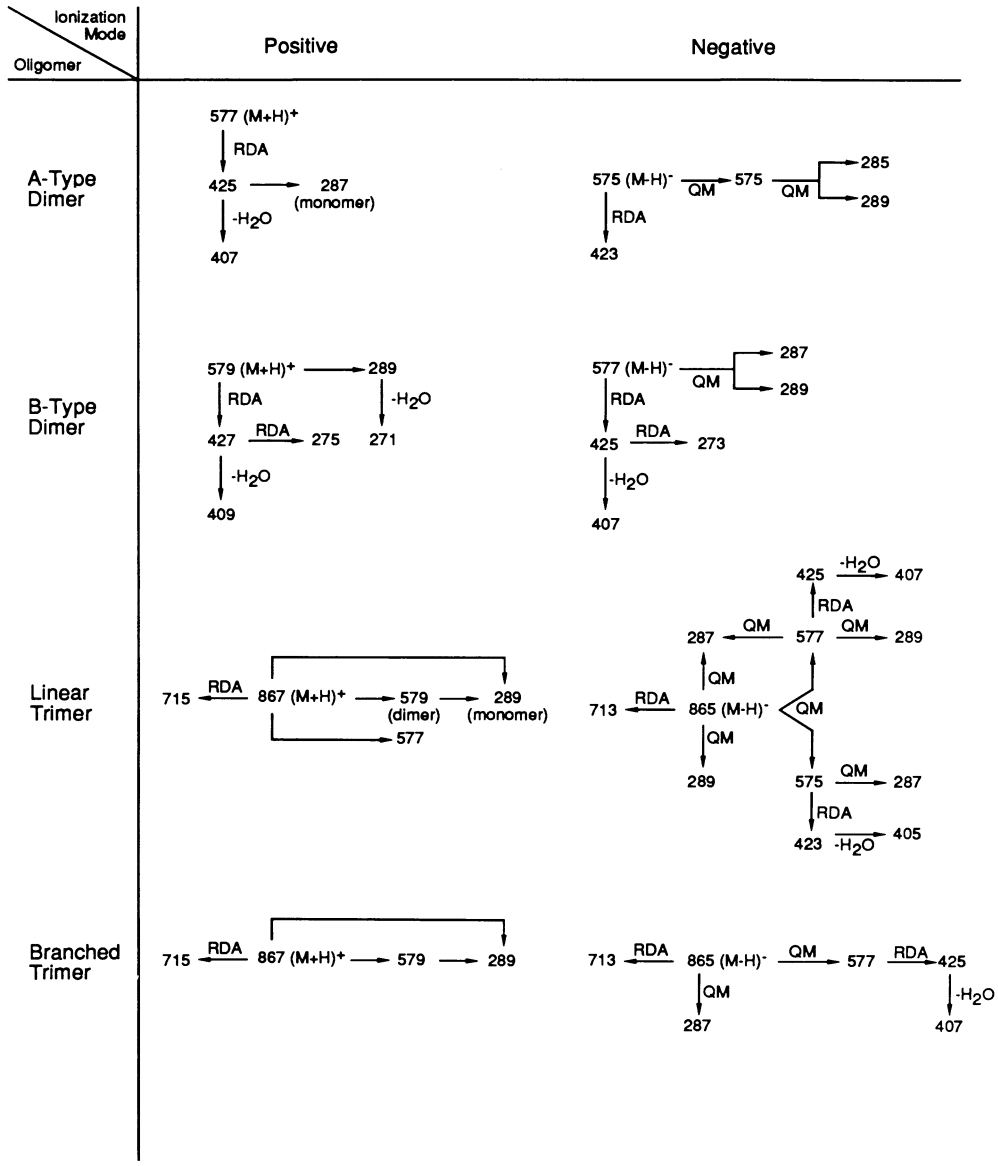
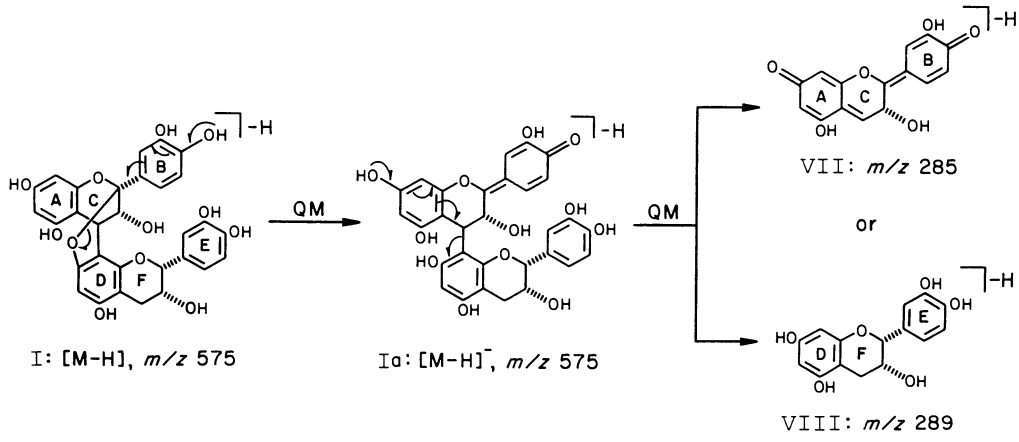
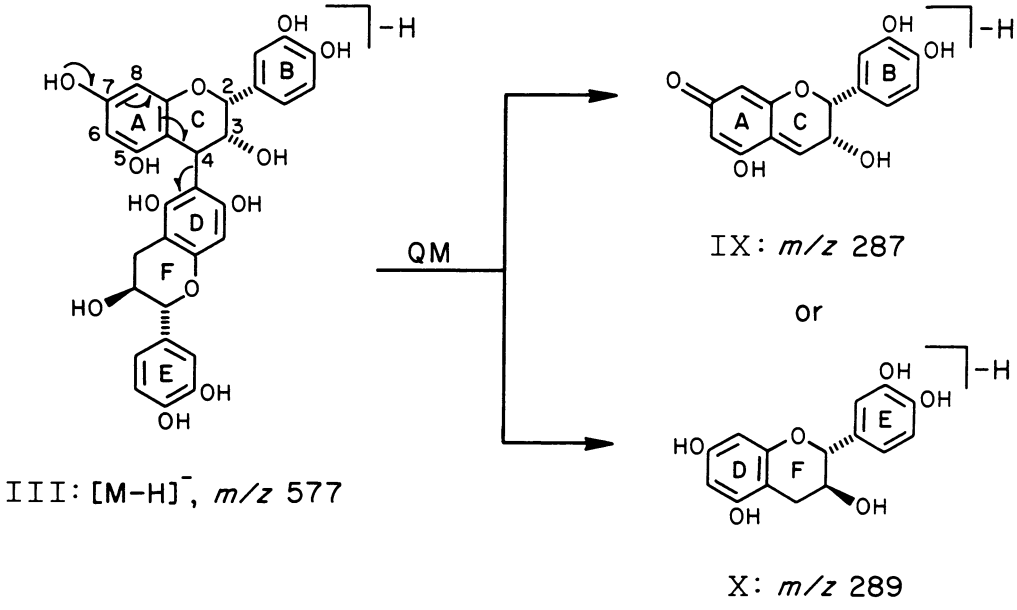


Figure 6. *Metastable decomposition pathways leading to the major fragment ions observed in FAB mass spectra of procyanidin dimers and trimers.*



Scheme 2. *Quinone-methide mechanism for cleavage of an A-type interflavanoid bond in a procyanidin dimer* (**I**, Figure 5).



Scheme 3. *Quinone-methide mechanism for cleavage of a B-type interflavanoid bond in a procyanidin dimer* (**III**, Figure 5).

metastable decomposition ions, and doubly charged ions appearing in negative ion FAB mass spectra as an aid to identifying flavanol monogallates and digallates. These same researchers have also shown that under FAB conditions cleavages of galloyl residues from the dimers and depsides of gallo-tannins (hydrolyzable tannins) yield structurally coherent ions.[70]

CONCLUDING REMARKS

Utilization of FAB mass spectrometry for the elucidation of proanthocyanidin structures is in its infancy. Only a few systematic investigations of its application to various classes of vegetable tannins, both synthetic and naturally occurring, have been carried out.

There can be little doubt that FAB mass spectrometry and its related techniques can be used to add significantly to exisiting knowledge of the structures of proanthocyanidins and their distribution in plants. In order to advance the utility of FAB mass spectrometry for this task, research must be conducted in several areas. Advances in mass spectrometry, FAB being no exception, reflect in large part the availability of model compounds. Hence, efforts to obtain pure proanthocyanidin oligomers, particularly with a high degree of polymerization, should be promoted, and whenever available, these compounds should be used: 1) to find more optimal matrix conditions for producing mass analyzable ions from both individual compounds and mixtures and 2) to learn how to identify structural features in their mass spectra. Alternate conditions and methods, such as continuous flow FAB mass spectrometry and tandem mass spectrometry, for analyzing both homologous and heterologous mixtures of vegetable tannins should be investigated. These efforts should include attempts with different combinations of ionization mode and mass analyzer, such as secondary ionization or plasma desorption time-of-flight mass analyses, to test the upper limits of the mass range as well as their potential for analyzing mixtures.

In this author's opinion, FAB and similar forms of mass spectrometry have the potential for achieving the same level of importance in the elucidation of proanthocyanidin structure currently accorded nuclear magnetic resonance.

REFERENCES

1. Haslam, E. Natural proanthocyanidins. *In*: Harborne, J.B.; Mabry, T.J.; Mabry, H. (eds.) The Flavonoids, Academic Press, New York, pp. 505-559 (1975).
2. Haslam, E. Proanthocyanidins. *In*: Harbone, J.B.; Mabry, T.J. (eds.) The Flavonoids: Advances in Research, Chapman and Hall, London, pp. 417-447 (1982).
3. Salunkhe, D.K.; Jadhav, S.J.; Kadam, S.S.; Chavan, J.K. Chemical, biochemical, and biological significance of polyphenols in cereals and legumes. *CRC Crit. Rev. Food Sci. Nutr.* 17:277 (1982).
4. Haslam, E. Symmetry and promiscuity in procyanidin biochemistry. *Phytochemistry* 16:1625 (1977).
5. Delcour, J.A.; Vandeberghe, M.M.; Dondeyne, P.; Schrevens, E.L.; Wijnhoven, J. Flavor and haze stability differences in unhopped and hopped all-malt Pilsner beers brewed with proanthocyanidin-free and with regular malt. *J. Inst. Brew.* 90:67 (1984).
6. Kumar, R.; Singh, M. Tannins: their adverse role in ruminant nutrition. *J. Agric. Food. Chem.* 32:447 (1984).

7. Butler, L.G.; Riedl, D.J.; Lebryk, D.G.; Blytt, H.J. Interaction of proteins with sorghum tannin: mechanism, specificity and significance. *J. Am. Oil Chem. Soc.* 61:916 (1984).
8. Klocke, J.A.; Chan, B.C. Effects of cotton condensed tannin on feeding and digestion in the cotton pest *Heliothis zea*. *J. Insect Physiol.* 28:911 (1982).
9. Zucker, W.V. Tannins: does structure determine function? An ecological perspective. *Am. Nat.* 121:335 (1983).
10. Hemingway, R.W. Bark: its chemistry and prospects for chemical utilization. *In*: Goldestein, I.S. (ed.) Organic Chemicals from Biomass, CRC Press, Boca Raton, Florida pp. 189-248 (1981).
11. Pizzi, A. Tannin-based wood adhesives. *In*: Pizzi, A. (ed.) Wood Adhesives, Chemistry and Technology, Marcel Dekker, New York, pp. 177-246 (1983).
12. Hemingway, R.W.; Laks, P.E.; McGraw, G.W.; Kreibich, R.E. Reactions of condensed tannins and the development of tannin-based adhesives. *In*: Proceedings IUFRO Conference, Forest Products Research International - Achievements and the Future, Vol. 6, Pretoria, South Africa, pp. 17.11-17.20 (1985).
13. Price, M.L.; Van Scoyoc, S.; Butler, L.G. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* 26:1214 (1978).
14. Price, M.L.; Butler, L.G. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* 25:1268 (1977).
15. Hagerman, A.E.; Butler, L.G. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* 26:809 (1978).
16. Hagerman, A.E.; Butler, L.G. Condensed tannin purification and characterization of tannin-associated proteins. *J. Agric. Food Chem.* 28:947 (1980).
17. Bate-Smith, E.C. Hemanalysis of tannins. Concept of relative astringency. *Phytochemistry* 12:907 (1973).
18. Haslam, E. Polyphenol-protein interactions. *Biochem. J.* 139:285 (1974).
19. Maxson, E.D.; Rooney, L.W. Evaluation of methods for tannin analysis in sorghum grain. *J. Cereal Chem.* 49:719 (1972).
20. Weinges, K.; Kaltenhauser, W.; Marx, H.-D.; Nadar, E.; Nader, F.; Perner, J.; Seiler, D. Proanthocyanidins. X. Procyanidins from fruit. *Justus Leibigs Ann. Chem.* 711:184 (1968).
21. Mabry, T.J.; Markham, K.R. Mass spectrometry of flavonoids. *In*: Harborne, J.B.; Mabry, T.J.; Mabry, H. (eds.) The Flavonoids, Academic Press, New York (1975).
22. Mabry, T.J.; Ulubelen, A. Mass spectrometry of flavonoids and related plant phenolics. *In*: Waller, G.R.; Dermer, O.C. (eds.) Biochemical Applications of Mass Spectrometry, 1st Suppl. Vol. Wiley, New York (1980).
23. Ozawa, T.; Kobayashi, S.; Seki, R.; Imagawa, H. A new gallotannin from bark of chesnut tree *Castanea crenata* Sieb. et Zucc. *Agric. Biol. Chem.* 48:1411 (1984).
24. Piretti, M.V.; Pistore, R.; Razzoboni, C. On the chemical constitution of kaki tannin. *Ann. Chim.* 75:137 (1985).
25. Kingston, D.G.I.; Fales, H.M. Methane chemical ionization mass spectrometry of flavonoids. *Tetrahedron* 29:4083 (1973).
26. Itokawa, H.; Oshida, Y.; Ikuta, A.; Shida, Y. In-beam electron impact, chemical ionization and negative ion chemical ionization of flavonoid glycosides. *Chem. Lett.* :49 (1982).
27. Bankova, V.S.; Mollova, N.N.; Popov, S.S. Chemical ionization mass spectrometry with amines as reactant gases. *Org. Mass Spectrom.* 21:109 (1986).
28. Karchesy, J.J.; Laver, M.L.; Barofsky, D.F.; Barofsky, E. Structure of oregonin, a natural diarylheptanoid xyloside. *J. Chem. Soc. Chem. Commun.* :649 (1974).
29. Schulten, H.R.; Games, D.E. High resolution field desorption mass spectrometry. II. Glycosides. *Biomed. Mass Spectrom.* 1:120 (1974).

30. Karchesy, J.J.; Loveland, P.M.; Laver, M.L.; Barofsky, D.F., Barofsky, E. Condensed tannins from the barks of *Alnus rubra* and *Pseudotsuga menzies II. Phytochemistry* 15:2009 (1976).

31. Biswas, K.M.; Ali, M.E.; Jackson, A.H.; Games, D.E. Application of field desorption and electron impact mass spectrometry and NMR spectroscopy in the study of flavonoid O-glycosides. *J. Ind. Chem. Soc.* 55:1240 (1978).

32. Geiger, H.; Schwinger, G. Field desorption mass spectrometery and thermal fragmentation of flavonoid glucosides. *Phytochemistry* 19:897 (1980).

33. Nonaka, G.-I.; Morimoto, S.; Nishioka, I. Tannins and related compounds. Part 13. Isolation and structure of trimeric, tetrameric and pentameric proanthocyanidins from cinnamen. *J. Chem. Soc. Perkin Trans. 1.* :2139 (1983).

34. Foo, L.Y.; Porter, L.J. Prodelphinidin polymers: definition of structural units. *J. Chem. Soc. Perkin Trans. 1* :1186 (1978).

35. Foo, L.Y.; Hemingway, R.W. Condensed tannins: synthesis of the first branched procyanidin trimer. *J. Chem. Soc. Chem. Commun.* :85 (1984).

36. Yoshida, T.; Hatano, T.; Okudo, T.; Memon, M.; Shingu, T.; Inoue, K. Spectral and chromatographic analyses of tannins. I. Carbon-13 nuclear magnetic resonance spectra of hydrolyzable tannins. *Chem. Pharm. Bull.* 32:1790 (1984).

37. Nonaka, G.-I.; Kawahara, O., Nishioka, I. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates from green tea leaf. *Chem. Pharm. Bull.* 31:3906 (1983).

38. Beart, J.E.; Lilley, T.H.; Haslam, E. Plant polyphenols – secondary metablolism and chemical defense: some observations. *Phytochemistry* 24:33 (1985).

39. Surman, D.J.; Vickerman, J.C. Fast atom bombardment quadrapole mass spectrometry. *J. Chem. Soc. Chem. Commun.* :324 (1981).

40. Barber, M.; Bordoli, R.S.; Sedgwick, R.D.; Tyler, A.N. Fast atom bombardment of solids (F.A.B.): a new ion source for mass spectrometry. *J. Chem. Soc. Chem. Commun.* :325 (1981).

41. Burlingame, A.L.; Baillie, T.A.; Derrick, P.J. Mass spectrometry. *Anal. Chem.* 58:165R (1986).

42. Day, R.J.; Unger, S.E.; Cooks, R.G. Molecular secondary ion mass spectrometry. *Anal. Chem.* 52:557A (1980).

43. Williams, D.H.; Findeis, A.F.; Naylor, S.; Gibson, B.W. Aspects of the production of FAB and SIMS mass spectra. *J. Am. Chem. Soc.* 109:1980 (1987).

44. Pachuta, S.J.; Cooks, R.G. Mechanisms in molecular SIMS. *Chem. Rev.* 87:647 (1987).

45. Williams, D.H.; Smith, R.J.; Santikarn, S.; Maggio, J.E.; Daley, D.J.; Bradley, C.V. FAB mass spectrometry of some biopolymers. *Spectros. Int. J.* 2:232 (1983).

46. Fenseleau, C.; Cotter, R.J. Chemical aspects of fast atom bombardment. *Chem. Rev.* 87:501 (1987).

47. Aberth, W.; Straub, K.M.; Burlingame, A.L. Secondary ion mass spectrometry with cesium ion primary beam and liquid target matrix for analysis of bioorganic compounds. *Anal. Chem.* 54:2029 (1982).

48. DePauw, E. Liquid matrices for secondary-ion mass spectrometry. *Mass Spectrom. Rev.* 5:191 (1986).

49. Carr, S.A.; Reinhold, V.N.; Green, B.N.; Hass, J.R. Enhancement of structural information in FAB ionized carbohydrate samples by neutral gas collision. *Biomed. Mass Spectrom.*, 12:288 (1985).

50. Barber, M.; Bordoli, R.S.; Elliott, G.J.; Tyler, A.N.; Bill, J.C.; Green, B.N. Fast atom bombardment (FAB) mass spectrometry: a mass spectal investigation of some of the insulins. *Biomed. Mass Spectrom.* 11:182 (1984).

51. Martin, S.A.; Costello, C.E.; Biemann, K. Optimization of experimental procedures for fast atom bombardment mass spectrometery. *Anal. Chem.* 54:2362 (1982).

52. Barber, M.; Bordoli, R.S.; Elliott, G.J.; Sedgwick, R.D.; Tyler, A.N. Fast atom baombardment mass spectrometry. *Anal. Chem.* 59:645A (1982).

53. Fenselau, C. Fast atom bombardment. *In*: Benninghoven, A. (ed.) Formation from Organic Solids, Springer-Verlag, Berlin (1983).

54. Rinehart, K.L. Fast atom bombardment mass spectrometry. *Science* 218:254 (1982).

55. Miller, J.M. Fast-atom bombardment mass spectrometry and related techniques. *Adv. Inorg. Chem. Radiochem.* 28:1 (1984).

56. Cochran, R.L. Fast atom bombardment/mass spectrometry (FAB/MS) and its industrial applications. *Appl. Spectrosc. Rev.* 22:137 (1986).

57. Domon, B.; Hostettman, K. Mass spectrometric studies of underivatized polyphenolic glycosides. *Phytochemistry* 24:575 (1985).

58. Karchesy, J.J.; Hemingway, R.W.; Foo, Y.L.; Barofsky, E.; Barofsky, D.F. Sequencing procyanidin oligomers by fast atom bombardment mass spectrometry. *Anal. Chem.* 58:2563 (1986).

59. Karchesy, J.J.; Foo, L.Y.; Barofsky, E.; Arbogast, B.; Barofsky, D.F. Negative ion fast atom bombardment mass spectrometry of procyanidin oligomers. *Wood Chem. Technol.* (in press).

60. de Koster, C.G.; Heerma, W.; Dijkstra, G.; Niemann, G.J. Fast atom bombardment of flavonols. *Biomed. Mass Spectrom.* 12:596 (1985).

61. Hsu, F.L.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXIII. Isolation and characterization of procyanidins in *Dioscorea cirrhosa* Lour. *Chem. Phar. Bull.* 33:3293 (1985).

62. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXXV. Procyanidins with a doubly linked unit from the root bark of *Cinnamomum sieboldii* Meisner. *Chem. Pharm. Bull.* 33:4338 (1985).

63. Morimota, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXVIII. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamomum cassia* Blume). *Chem. Pharm. Bull.* 34:633 (1986).

64. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXIX. Procyanidin c-glucosides and acylated flavan-3-ol glucoside from barks of *Cinnamomum cassia* Blume and *C. obtusifolium* Nees. *Chem. Pharm. Bull.* 34:643 (1986).

65. Kashiwada, Y.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XLV. Rhubarb. Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chem. Phar. Bull.* 34:3208 (1986).

66. Nonaka, G.; Morimoto, S.; Kinjo, J.; Nohara, T.; Nishioka, I. Tannins and related compounds. L. Structures of proanthocyanidin A-1 and related compounds. *Chem. Pharm. Bull.* 35:149 (1987).

67. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. LX. Isolation and characterization of proanthocyanidins with a doubly-linked unit from *Vaccinium vitis-idaea* L. *Chem. Pharm. Bull.* 36:33 (1988).

68. Gujer, R.; Magnolato, D.; Self, R. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry* 25:1431 (1986).

69. Galletti, G.C.; Self. R. The polyphenols (*syn* vegetable tannins) of grape skins and pressed fruit residues. *Annali di Chimica* 76:195 (1986).

70. Self, R.; Eagles, J.; Galletti, G.C.; Mueller-Harvey, I.; Hartley, R.D.; Lea, A.G.H.; Magnolato, D.; Richli, U.; Gujer, R.; Haslam, E. Fast atom bombardment mass spectrometry of polyphenols (*syn.* vegetable tannins). *Biomed. Environ. Mass Spectrom.* 13:449 (1986).

71. Karchesy, J.J.; Hemingway, R.W. Condensed tannins: $4\beta \rightarrow 8{:}2\beta \rightarrow 0 \rightarrow 7$)-linked procyanidins in *Arachis hypogea* L. *J. Agric. Food Chem.* 34:966 (1986).

72. Yoshida, T.; Hatano, T.; Okuda, T.; Memon, M.V.; Shingu, T.; Inoue, K.; Fukushima, K. Nuclear magnetic resonance and mass spectral analyses of oligomeric hydrolyzable tannins and related tannins. *Tenne Yuki Kagobutsu Toronkai Koen Yushishu* 26:158 (1983).

73. Sakushima, A.; Hisada, S.; Nishibe, S.; Brandenberger, H. Application of fast atom bombardment mass spectrometry to chlorgenic acids. *Phytochemistry* 24:325 (1985).

74. Mueller-Harvey, I.; Hartley, R.D.; Harris, P.J.; Curzon, E.H. Linkage of *p*-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. *Carbohydr. Res.* 148:71 (1986).

75. Barber, M.; Green, B.N. The analysis of small proteins in the molecular weight range 10-24 kDa by magnetic sector mass spectrometry. *Rapid Commun. Mass Spectrom.* 1:80 (1987).

76. Craig, A. G.; Engstrom, A.; Bennich, H.; and Kamensky I. Enhancement of molecule ion yields in plasma desorption mass spectrometry. *In*: 35th ASMS Conference on Mass Spectrometry and Allied Topics. Denver, Colorado, (1987).

77. Foo, L.Y.; Karchesy, J.J.; Arbogast, B.A.; Barofsky, D.F. +FAB of a mixture of water soluble procyanidins from the inner bark of Douglas-fir/magic bullet/oligomers up to pentamer (m/z 1441), unpublished data (1988).

78. Porter, L.J. Condensed tannins, *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, New York, (in press).

79. Caprioli, R. Analysis of biochemical reactions with molecular specificity using fast atom bombardment mass spectrometry. *Biochemistry* 27: 513 (1988).

80. McLafferty, F. W. (ed.) Tandem Mass Spectrometry. Wiley-Interscience, New York (1983).

81. Gross, M.L.; Jensen, N.J.; Lippstreu-Fisher, D.L.; Tomer, K.B. Tandem mass spectrometry and fourier transform mass spectrometry for analysis of biomolecules, *In*: Burlingame, A.L.; Castagnoli, N. Sr. (eds.) Mass Spectrometry in the Health and Life Sciences. Elsevier, New York (1985).

82. Pelter, A.; Stainton, P.; Johnson, A.P.; Barber, M. The mass spectra of oxygen heterocycles. I. The 4-hydroxy-3-phenylcoumarins (*iso*flavonols). *J. Heterocyclic Chem.* 2:256 (1965).

83. Pelter, A.; Staintor, P.; Barber, M. The mass spectra of oxygen heterocycles. II. The mass spectra of some flavonoids. *J. Heterocyclic Chem.* 2:262 (1965).

84. Watson, J.T. Introduction to Mass Spectrometry, Raven Press, New York (1985).

85. Thompson, R.S.; Jacques, D.; Haslam, E.; Tanner, R.J.N. Plant proanthocyanidins. I. Introduction, isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1387 (1972).

86. Jacques, D.; Haslam, E.; Bedford, G.R.; Greatbanks, D. Plant proanthocyanidins. II. Proanthocyanidin A2 and its derivatives. *J. Chem. Soc. Perkin Trans. 1* :2663 (1974).

87. Attwood, M.R.; Brown, B.R.; Lisseter, S.G.; Torrero, C.L.; Weaver, P.M. Spectral evidence for the formation of quinone methide intermediates from 5- and 7-hydroxyflavonoids. *J. Chem. Soc. Chem. Commun.* :177 (1984).

88. Foo, L.Y.; Wong, H. Diastereoisomeric leucoanthocyanidins from the heartwood of *Acacia melanoxylon*. *Phytochemistry* 25:1961 (1986).

89. Laks, P.E.; Hemingway, R.W. Condensed tannins: base-catalyzed reactions of polymeric procyanidins with toluene-α-thiol. Stability of the interflavanoid bond and pyran ring. *J. Chem. Soc. Perkin Trans. 1* :465 (1987).

90. Hemingway, R.W.; Foo, L.Y. Condensed tannins: quinone methide intermediates in procyanidin synthesis. *J. Chem. Soc. Chem. Commun.* :1035 (1983).

91. Delcour, J.A.; Tuytens, G.M. Structure elucidation of three dimeric proanthocyanidins isolated from a commercial Belgian pilsner beer. *J. Inst. Brew.* 90:153 (1984).

92. Karchesy, J.J.; Foo, L.Y.; Hemingway, R.W.; Barofsky, E.; Barofsky, D.R. Fast atom bombardment mass spectrometry of condensed tannin sulfonate derivatives. *Wood Fiber Sci.* 21:155 (1989).

ANALYTICAL METHODS: AN OVERVIEW

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ABSTRACT

A wide variety of analytical methods is currently being used to provide a diversity of information about proanthocyanidins. Rapid advances are being made in the ability to determine structure and conformation due to recent developments in NMR, MS, and other spectrometric techniques. In the future, these methods will play major roles in the study of conformational changes caused by associations with proteins, metals, and carbohydrates. More needs to be learned about the structure of phlobaphenes, the nature of the molecules that serve as building blocks, and the reaction mechanisms that lead to phlobaphene formation. Absence of effective chromatographic separation methods for phlobaphene components is a limiting factor. Isolations of proanthocyanidins are primarily done using low-pressure column chromatography with a variety of gel types. HPLC is presently under-utilized for such purposes, and metal-free HPLC columns need to be investigated because of the metal complexing ability of proanthocyanidins. Quantitative analysis of condensed tannins can sometimes suffer from lack of reproducibility due to details of the procedure, sample collection, and handling.

INTRODUCTION

"Chemists have usually divided the varieties of tannin, which occur so abundantly in the vegetable kingdom, into two kinds — those which give *black* and those which give *green* precipitates with salts of iron. The propriety of this distinction has of late been called in question by Berzelius, who seems to think that tannic acid in all plants is essentially the same, and that the green and grey colours of the precipitates with salts of iron are owing to the presence of free acid. Professor Liebig appears also to hold a similar opinion." — John Stenhouse, November 15,

1842[1]. Stenhouse goes on to describe the analytical procedures of the day as they were applied to distinguish the tannins of alder, birch, and oak bark, catechu, nutgalls, sumach, divi-divi and others. His results did not support the conclusions of Berzelius and Liebig.

The analytical methods at hand have stimulated major advances in our understanding of condensed tannins: their structures, reaction products, biological significance, and potential utilization. A hundred and thirty-seven years after John Stenhouse's paper, our tools are of course much more advanced. They cover a wide range of methods and degrees of sophistication, e.g., nuclear magnetic resonance (NMR) is used to establish structure whereas, "old-fashioned wet chemistry" is used to quantify the condensed tannins in tissues. Such tools or methods at any given time in history have provided great advances in the field, whereas, others may have limited progress. In the first North American Tannin Conference, the organizers decided to cover three important and fast-developing areas that are having a great impact on our knowledge of the structure and chemistry of condensed tannins.

New chromatographic strategies (Chapter 9) are allowing us to isolate new compounds whose structures can then be quickly elucidated by impressive new techniques in NMR (Chapter 10) and FAB-MS (Chapter 11). The chapter on chromatography also covers existing qualitative (TLC, PC) and quantitative (HPLC, GPC) procedures. It is apparent when reading that chapter that chromatographic-based quantitative analyses are limited in scope and certainly are not providing biologists, in particular, with the needed tools to make significant advancements in their fields as NMR and FAB-MS are doing for chemists. The discussions at the Conference highlighted the fact that accurate and reproducible quantitative analyses of condensed tannins are of vital importance to the biologists (Chapter 26) whose research is aimed at unraveling the biological significance of tannins. Quantitative analyses should have been covered as a topic in itself at the Conference and will need to be addressed at future meetings.

STRUCTURE AND CONFORMATION

The recent advances that have taken place in NMR (Chapter 10) and MS (Chapter 11) techniques have provided the tannin chemist with powerful tools to determine the structures and conformations of exceptionally complex molecules. Today, structural elucidations of new compounds can take place with unprecedented speed and accuracy compared to our abilities of only 5 years ago. In many cases, the combined results of NMR and FAB mass spectrometry are all that is needed to define a molecular structure. A thorough structural determination, as a matter of good practice, however, is needed to confirm these results with infra-red and ultraviolet spectroscopy, elemental analysis, and derivatization. Times are exciting for those of us who use these structural tools, and it is fully expected that new NMR pulse sequences and mass spectrometric ionization and detection techniques will continue to become available in the future as a result of present ongoing research in physics, computer engineering, and physical chemistry aimed at advancing these fields.[2,3]

In the area of NMR spectroscopy,[2] rapid developments are being made in a variety of techniques that tantalize the imagination and may eventually find application in tannin analyses. The list includes improved analyses of solid samples, imaging of in-situ samples, coupling to chromatography, NMR microscopy, new solvent suppression techniques, utilization of nuclei other than ^{13}C to establish heteronuclear couplings, and of course, a wide range of new pulse techniques for use in structural assignments. Because solid-state NMR has proven to be such a powerful technique for the study of surfaces, it seems likely the method will also find application in the study of solid tannin complexes at some point. A technique for the quantitative determination of phenols and other compounds with OH groups by derivatization and analysis by ^{31}P-NMR has also been reported.[4]

FAB-MS is rapidly achieving the same level of importance for structural elucidation as NMR (Chapter 11). However, there are certain items that need to be further researched. FAB-MS of proanthocyanidins has been done so far, for the most part, only on pure isolated compounds in spite of the potential for mixture analysis via tandem mass spectrometry or MS-MS techniques.[5–7] Slight impurities seem to cause problems with obtaining strong molecular ions. This is not completely understood as there are a number of questions about proanthocyanidin ionization and its relationship to other variables such as the liquid matrix used when analyzing these compounds. The effects of the liquid matrix need to be studied in a systematic fashion. We also don't fully understand the effect of slight changes in matrixes and resulting changes in the types of molecular ions observed. For example, in the negative ion spectrum of a C4 → C8 linked dimer such as procyanidin B-2, the $[M\text{-}H]^-$ ion is almost exclusively produced in the molecular ion region.[8] The same holds true of procyanidin sulfonates when $[M\text{-}H]^-$ and $[M\text{-}Na]^-$ ions predominate.[9] However, "phlobatannin" sulfonates and similar structures give molecular ion envelopes containing $[M\text{-}H]^-$, M^- and $[M+H]^-$ species.[10] Their proportion seems to be influenced at least in part by the liquid matrix used. This phenomenon is also observed with other types of compounds.[11,12] Clearly, more research needs to be done with regard to how proanthocyanidins ionize under FAB-MS conditions and how other variables affect molecular ion formation. Other aspects of FAB-MS need to be further investigated. HPLC coupled FAB-MS systems for analyses are under development. FAB-MS is known to induce fragmentation that mimics known reaction products in solution chemistry. Acid- and base-catalyzed reactions of procyanidins could be studied on a preliminary basis while using very little compound by this approach. The potential for use of FAB and other types of mass spectrometry for providing a number of new and unique analytical tools is just beginning to emerge.

Structural analyses, in general, will likely continue to focus on topics such as conformation and the conformational changes that take place when tannins form associations with other compounds such as proteins, metals, and carbohydrates. NMR spectroscopy has played a major role in conformational elucidation, and that likely will continue to be the case in the future. However, one must consider the contributions that can be made by other methods. In particular, time resolved

fluorescence spectroscopy has the ability to resolve the populations of rotational isomers about the interflavanoid bond even when they cannot be resolved by ^1H NMR (Chapter 7). The method has an advantage over NMR by the faster time scale of the fluorescence measurement. Hagerman (Chapter 20) has noted the need to analyze soluble proanthocyanidin complexes and has described an electrophoresis technique that shows promise.

More needs to be learned about the structure of phobaphenes, the nature of the molecules that serve as the building blocks, and the reaction mechanisms that lead to phobaphene formation. The recent isolation of psuedotsuganol[13] (a biphenyl-linked dihydroquercetin-pinoresinol flavonolignan) from Douglas-fir outer bark gives support to the concept of oxidative coupling of catechol rings as a mechanism leading to phlobaphene formation. Chromatographic procedures to separate individual compounds are presently a limitation. The phobaphenes exhibit significantly different solubility and chromatographic characteristics compared to the associated procyanidins (Chapter 6). Tannin-metal complexes will provide similar challenges to chromatography and structural elucidation.

CHROMATOGRAPHY

As powerful as NMR and mass spectrometric techniques are, there has been no significant application to the analyses of mixtures of tannins in vivo. In fact, the major limitation to the identification of new complex and higher molecular weight compounds is in our ability to isolate and purify them chromatographically prior to spectrometric analyses (Chapter 9). Low pressure column and preparative TLC (usually derivatives) are methods that are effective for such isolations. The newer synthetic polymer-based gels, such as MCI and Toyopearl, have not yet been fully explored for their range of separation abilities with tannin compounds. Counter current chromatography applications are promising, but this method seems to play a support role for other purification techniques. HPLC has simply failed to meet its potential for preparative isolations of tannin and related compounds. This seems to be due, in part, to at least two factors. First is the poor peak shape with excessive tailing when neutral solvents are used. Secondly, there is the problem of low compound recovery and product instability when metal columns and frits are used. Perhaps this should not be surprising when considering the metal complexing ability of tannins (Chapter 15) and the ability of ferric ion to catalyze anthocyanidin formation (Chapter 16).[14] In fact, we consistently have found the UV-Vis spectra of compounds isolated by use of metal HPLC columns to exhibit a small peak in the 520-550 nm region. A metal-free preparative HPLC column is certainly needed to take advantage of the rapid separations that automated HPLC equipment would allow.

It becomes apparent that a study of complexes, such as the carbohydrate-polyphenol systems presented in Chapter 19, may also provide valuable information about the chromatographic behavior of tannins and associated compounds. In fact, it would be helpful to study the interactions of tannins or polyphenols with other synthetic polymers that may be used for chromatography. Little is known about what is actually going on when we chromatograph these compounds, and much time is spent in trial and error procedures when using new column packing materials.

QUANTITATIVE ANALYSES

Quantitative analyses of condensed tannins are most commonly carried out using either an acidified-vanillin method, as described by Broadhurst and Jones,[15] or the acid-catalyzed generation of anthocyanidins[16] (Chapter 16). Both methods of tannin estimation are colorimetric. Associated procedures often employed by biologists to supplement the estimation of condensed tannin content include a Folin-type reagent to estimate total phenols[17] and an astringency procedure (e.g., BSA as described in Chapter 20) that measures the ability to precipitate a protein.

Shultz (Chapter 26) quite appropriately outlines the many problems that can arise when using these procedures to quantify "condensed tannins" and the resulting frustrations that must be felt by many other biologists. In short, these methods can suffer from lack of reproducibility, and there are many reasons for this, including not only the analytical procedure itself, but also details of sample collection, preparation, and handling. Schultz is also correct in noticing that there needs to be better interaction between the chemists and biologists in applying these analyses and in the development of any new procedures. Accurate analyses are essential if we are to make advancements in our understanding of the biological significance of condensed tannins.

Some of the problems leading to a lack of reproducibility in condensed tannin analyses can be noted as follows, and perhaps a better understanding of some of the limitations will help. The color generating reactions for both the acidified-vanillin method and anthocyanidin are not quantitative. All of the reaction mechanisms and variables are not completely understood. For instance, Porter et al[14] found the yield of anthocyanidin to be dependent on water concentration of the reaction mixture, mole fraction of proanthocyanidin in the polymer chain, and the age of the proanthocyanidin preparation. The reaction products of the acidified-vanillin method have not been well characterized. Color is given with compounds that have a reactive phloroglucinolic or resorcinolic A ring. Presumably, this is the same reaction that generates the various shades of orange to pink by spraying TLC plates with the vanillin-hydrochloric acid spray reagent (Chapter 9). One concern is that in our laboratory we observe different shades of color depending on the structure of the compound, in particular with different substituents at the C-4 carbon. The choice of standards for calibration of this method is important. Standards need to be well-characterized and of determined purity.

Care also needs to be taken in handling the samples before analysis. Condensed tannins are thermally and photochemically labile; they readily undergo acid- or base-catalyzed rearrangements. Storage of plant material prior to extraction can lead to loss of condensed tannins and increased amounts of phlobaphenes (Chapter 6), which do not respond in the same way to anthocyanidin generation or acidified-vanillin. The choice of solvent for extraction is also important. Tannins are commonly extracted with anhydrous methanol or acetone-water (1:1 or 7:3). Differing amounts and slightly different molecular weight distributions can result with extraction by each solvent type. Methanol-water has been shown to be a poor solvent choice for extracting condensed tannins.[18] The development of standard procedures would be helpful. There should also be a minimal characterization of the condensed

tannins being analyzed (i.e., are they procyanidins or prodephinidins?) Molecular weight profiles, which can be obtained by GPC, combined with the quantitative determination of condensed tannins, might also provide some valuable insight to biological function.

REFERENCES

1. Stenhouse, J. XXIII. On some astringent substances as sources of pyrogallic acid. *Memoirs and Proc. of the Chem. Soc. London* 1:132 (1843).
2. Rabenstein, D.L.; Guo, W. Nuclear magnetic resonance spectroscopy. *Anal. Chem.* 60:1R (1988).
3. Burlingame, A.L.; Maltby, D.; Russell, D.H.; Holland, P.T. Mass spectrometry. *Anal. Chem.* 60:249R (1988).
4. Schiff, D.E.; Verkade, J.G.; Metzler, R.M.; Squires, T.G.; Venier, C.G. Determination of alcohols, phenols, and carboxylic acids using P-31 NMR spectroscopy. *Appl. Spectr.* 40:348 (1986).
5. Karchesy, J.J.; Hemingway, R.W.; Foo, Y.L.; Barofsky, E.; Barofsky, D.F. Sequencing procyanidin oligomers by fast atom bombardment mass spectometry. *Anal. Chem.* 58:2563 (1986).
6. McLafferty, F.W. (ed.) Tandem Mass Spectometry. Wiley-Interscience, New York (1983).
7. Gross, M.L.; Jensen, N.J.; Lippstreu-Fisher, D.L.; Tomer, K.B. Tandem mass spectrometry and fourier transform mass spectrometry for analysis of biomolecules, *In*: Burlingame, A.L.; Castagnoli, N. Sr. (eds.) Mass Spectrometry in the Health and Life Sciences. Elsevier, New York. (1985).
8. Karchesy, J.J.; Foo, L.Y.; Barofsky, E.; Arborgast, B.; Barofsky, D.F. Negative ion fast atom bombardment mass spectrometry of procyanidin oligomers. *J. Wood Chem. Technol.* (submitted).
9. Karchesy, J.J.; Foo, L.Y.; Hemingway, R.W.; Barofsky, E.; Barofsky, D.F. Fast atom bombardment mass spectrometry of condensed tannin sulfonate derivatives. (in press). *Wood and Fiber Science* 21: (1989).
10. Bae, Y.S. Douglas-fir inner bark procyanidins: Sulfonation, isolation and characterization. Ph.D. Thesis, Oregon State University, Corvallis, Oregon (1989).
11. Fenselau, C. Fast atom bombardment and middle molecule mass spectrometry. *J. Nat. Prod.* 47:215 (1984).
12. Dass C.; Ramakrishnan, S.; Israel, M.; Desideris, D.M. Fast atom bombardment mass spectrometric analysis of anthracyclines and anthracyclinones. *Biomed. and Environ. Mass Spectrom.* 17:37 (1988).
13. Foo, L.Y.; Karchesy, J.J. Pseudotsuganol, a biphenyl-linked pinoresinol-dihydroquercetin from Douglas-fir bark: Isolation of the first true flavonolignan. *J. Chem. Soc. Chem. Commun.* (in press).
14. Porter, L.J.; Hrstich, L.N.; Chan, B.G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223 (1986).
15. Broadhurst, R.B.; Jones, W.T. Analysis of condensed tannins using acidified vanillin. *J. Sci. Ed. Agric.* 29:788 (1978).
16. Pigman, W.; Anderson F.; Fischer, R.; Buchanan, M.; Browning, B.L. Color precursors in spruce and western hemlock woods and inner barks. *Tappi* 36:4 (1953).
17. Swain, T.; Goldstein, J.L. The quantitative analysis of phenolic compounds. *In*: Pridham, J.B. (ed.) Methods in Polyphenol Chemistry. Pergamon Press, New York (1964).
18. Foo, L.Y.; Porter, L.J. The phytochemistry of proanthocyanidin polymers. *Phytochemistry* 19:1747 (1980).

Reactions

MNDO MOLECULAR ORBITAL ANALYSES OF MODELS FOR PROANTHOCYANIDIN-METHYLOLPHENOL REACTIONS

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ABSTRACT

The MNDO molecular orbital method has been applied to molecular structures that model or react with proanthocyanidins. Atom net charge distribution, structural parameters, heats of formation, and ionization potentials are evaluated for: (+)-catechin, (-)-epicatechin, catechol, resorcinol, phloroglucinol, *o*- and *p*-methylolphenol, and *o*- and *p*-benzoquinone methide in an investigation of chemical reactivity under different pH conditions. Other MO calculations are reviewed, and the optimized MNDO MO parameters are compared with existing literature data. The total net and HOMO electronic charge distribution is evaluated in terms of nucleophilicity and chemical reactivity. The HOMO frontier orbitals in polyhydroxybenzene and the LUMO orbitals in the benzoquinone methides play an important role in chemical reactivity.

INTRODUCTION

The formation of wood adhesives based on condensed tannins is an important research area to the wood products industry.[1] An especially significant development, first advocated by MacLean and Gardner[2] and later implemented by Herrick and Bock,[3] is the cross-linking of condensed tannins with polymethylolphenols rather than formaldehyde to make tannin-based adhesives for bonding of plywood and particleboard. Very little is known about these reactions, however.[4]

Tannin-polymethylolphenol reactions have been modeled using the chemical kinetics of methylolphenols reacting with resorcinol, phloroglucinol, or (+)-catechin.[5] Reaction rates and reactant conversion were followed by proton and ^{13}C-NMR under different pH conditions to examine the nucleophilicity of the phenols and the role of steric hindrance in the reaction.[4,5] This work concluded that the reactions

were not charge controlled by the polyhydroxybenzene, but rather that the stability of the quinone methide or the carbocation was important.

In order to answer questions regarding the relative reactivity and the influence of steric hindrance in compounds like catechol, resorcinol, and phloroglucinol as models for tannins reacting with *o*- and *p*-methylolphenol, information on the quantum mechanical electronic charge densities on the atom sites and the MO coefficients of the outer frontier orbitals would be needed.[6,7,8]

The partial charge on the carbon atoms about the benzene ring can be examined relative to further electrophilic or nucleophilic substitution. The specific charge density on a particular atom, and especially that arising from the outer filled MO's, can add insight into the kinetics and mechanism of further substitution,[6,9] although this is complicated by uncertainty as to the exact nature of the transition state structure.

Consequently, it is of interest to compare the charge densities on phenol monomers relative to each other and to the hydroxybenzyl alcohols and the benzoquinone methide intermediates. The reaction kinetics for these compounds should be influenced by the relative charge densities on the carbon atoms *ortho* or *para* to the hydroxyl groups in the phenols and on the methylol carbon atom for the methylolphenols in question.

There have been a number of theoretical studies done on phenol and the dihydroxybenzenes, with most of them not structure optimized. Several were investigations of the conformational properties of the hydroxyl groups and intramolecular hydrogen bonding based on the *ab initio* and the CNDO methods,[10–15] and one was a fully optimized structure calculation.[14] Most of the CNDO methods used did not include structure optimization. There has been one MNDO study[16] examining the reaction between formaldehyde and phenol and another comparing phenol and cresol optimized structural properties with x-ray results.[17]

Studies have been conducted on the *o*- and *p*-quinone methides (6-methylene-2,4-cyclohexadien-1-one and 4-methylene-2,5-cyclohexadien-1-one)[18–21] primarily using the CNDO/S method to examine their electronic photoionization or ultraviolet spectra. The quinone methides are often suggested as intermediates in phenolic chemistry.[1] There has been, however, no systematic examination by any method concerning the reactions between methylolphenols or quinone methides and resorcinol, phloroglucinol, and (+)-catechin. That is the goal of this study.

This study includes a review of the existing literature and reports the quantum mechanical MNDO molecular orbital charge density calculations for some of the phenol monomers used in model reactions of aducts formed in tannin adhesives. A correlation is made to the relative rates of reaction kinetics observed under different pH conditions. The quantitative relationships are discussed in terms of their potential application to condensed tannins in adhesive systems.

CALCULATIONS OF CHARGE DENSITIES

The charge densities of the phenolic compounds were calculated by the modified neglect of diatomic overlap (MNDO) method developed by Dewar and Thiel.[23.24] A computer program with full structure optimization has been developed by Stewart and is available from the QCPE.[25] The calculations were carried out on a Digital

VAX 11/750 computer. Although the MNDO method is used for consistency, some comparison has been done using the AM1 method.[26]

In all the calculations except for (+)-catechin and (-)-epicatechin, the values were checked with the keyword "precise" turned on.[27] The coordinates were found by orienting each molecule with the benzene ring in the xy plane of a right-handed cartesian axis system. The x, y, and z values for the coordinates were calculated from a Z-matrix with the starting molecular geometries taken from microwave or crystal structure data.[10,28–33] Representative molecular structures and the atom counting used for charge densities and orbital correlation given in the tables are shown in Figure 1. The numbering for the anions is the same except that the phenolic hydroxyl protons are removed. When more than one conformation is possible, the symmetry group the molecule belongs to is noted for identification.

The net atomic charges are evaluated from the coefficients of the LCAO molecular orbitals. Since the molecular orbital squared is the charge density, the atomic charges are computed from summing the coefficients squared for the (i)th atom over all (p) occupied molecular orbitals, and multiplied by 2, since each is filled with two electrons.[9] This is given by equation 1.

$$Q_i = 2\Sigma_p C_{pi}^2 \tag{1}$$

The net charge comes from comparing this value with the free atom charge value. The same equation is used for LUMO (lowest unoccupied molecular orbital) and HOMO (the highest occupied molecular orbital) charge densities with their respective coefficients from that orbital used. Here, a single atomic coefficient is squared and multiplied by 2.

In dipole moment computations, when considering compounds in which more than one conformer contributes to the overall dipole moment, a mole fractional average of each conformation's dipole moment is calculated by equation 2.[34]

$$< \mu^2 >= \Sigma_i X_{ti} \mu_{ti}^2 \tag{2}$$

The dipole moment of each conformer is designated by μ_{ti}, and X_{ti} is the mole fraction of each conformer present in the gas phase. The Boltzmann populations used to calculate the mole fractions are taken from the ground state energies relative to the most stable conformer as evaluated by MNDO. In addition to the computational check that can be made by comparing the calculated and experimental dipole moments, the heats of formation and the ionization potentials can be calculated from the molecular orbital energy levels by Koopmans' theorem[35] and compared with experimental values.

RESULTS

Table 1 shows net atomic electron charges on each atom for all of the conformers of hydroquinone, catechol, resorcinol, and phloroglucinol. When the OH group

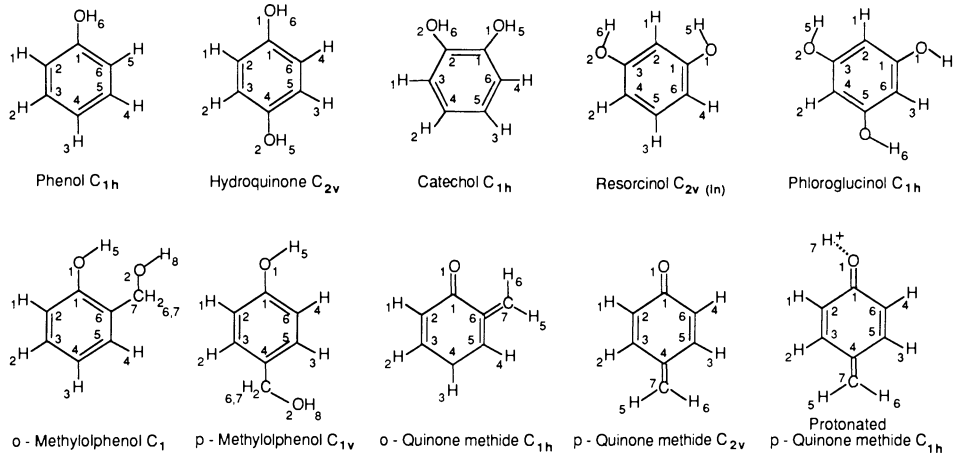


Figure 1. *Molecular structures, hydrogen atom configuration, and atom numbering system for model phenols and quinone methides. The anion numbering system is the same, except that the phenolic hydroxy protons are removed.*

orientation in phenol is changed by 180°, the residue charges are just reversed from those listed in the table. Conformation C_{1h} of catechol and resorcinol is when the OH groups are oriented in the same direction about the ring, see Figure 1; and these are the most stable conformations. Conformation C_{2v}(out) for catechol and resorcinol indicates that the OH groups are oriented away from each other. Conformation C_{2v} (in) for resorcinol occurs when the hydrogens in the OH groups are directed toward each other. This conformation for catechol is not energetically allowed. In the optimized structures, all OH groups are always oriented at 0° or 180° in the plane of the benzene ring. Conformation C_{2h} for hydroquinone occurs when the OH groups are aligned in the same direction about the ring (where the dipole moment is zero), and conformation C_{2v} is the case when the OH groups are directed toward each other as in conformation C_{2v}(in) for resorcinol. The heats of formation in kcal/mol, dipole moments in Debyes, ionization potentials, and relative energies in electron volts for the conformers are also listed. From the relative energy values and the Boltzmann factor, populations can be computed and compared to those obtained by the CNDO/2 method.[11]

Table 2 shows the net electron charge on each atom for the most stable model phenol conformers (signified by its Schoenflies point group) compared to the *o*- and *p*-hydroxymethylphenol (*ortho*-HMP, *para*-HMP). The charge densities shown in the table are residue point charges for the most stable conformers as determined from the minimum MNDO total energies. The values shown for hydroquinone and phloroglucinol are for the conformation in which the dipole moment is zero. The *o*- and *p*-hydroxymethylbenzyl alcohols each have several potential conformations;

Table 1. MNDO Atomic Charges, Ionization Potentials and Heats of Formation for Stable Conformers of Neutral Model Phenol Compounds.

Atom#	Phenol	Hydroquinone		Catechol[a]		Resorcinol			Phloroglucinol	
	C1h	C2v	C2h	C2v(out)	C1h	C2v(in)	C2v(out)	C1h	C1h	C3h
C(1)	0.1091	0.0749	0.0739	0.0867	0.0273	0.1489	0.1414	0.1494	0.1882	0.1806
C(2)	-0.0859	-0.0530	-0.1092	0.0867	0.0972	-0.2296	-0.1177	-0.1749	-0.2108	-0.2109
C(3)	-0.0275	-0.0529	-0.0459	-0.1140	-0.0614	0.1489	0.1414	0.1423	0.1722	0.1806
C(4)	-0.0962	0.0749	0.0739	-0.0549	-0.0589	-0.1226	-0.1808	-0.1228	-0.1525	-0.2109
C(5)	-0.0189	-0.1033	-0.1092	-0.0549	-0.0595	0.0031	0.0215	0.0120	0.1794	0.1806
C(6)	-0.1433	-0.1033	-0.0459	-0.1141	-0.0977	-0.1226	-0.1808	-0.1807	-0.2670	-0.2109
O(1)	-0.2485	-0.2483	-0.2489	-0.2313	-0.2666	-0.2463	-0.2460	-0.2484	-0.2464	-0.2467
O(2)		-0.2483	-0.2489	-0.2313	-0.2491	-0.2463	-0.2460	-0.2466	-0.2442	-0.2467
O(3)									-0.2441	-0.2467
H(1)	0.0758	0.0775	0.0625	0.0622	0.0772	0.0618	0.0921	0.0773	0.0791	0.0791
H(2)	0.0605	0.0775	0.0761	0.0617	0.0630	0.0779	0.0633	0.0779	0.0739	0.0791
H(3)	0.0619	0.0613	0.0625	0.0617	0.0619	0.0618	0.0588	0.0603	0.0637	0.0791
H(4)	0.0591	0.0612	0.0761	0.0622	0.0608	0.0780	0.0633	0.0634	0.1960	0.1979
H(5)	0.0611	0.1909	0.1914	0.1897	0.1990	0.1935	0.1946	0.1953	0.1972	0.1979
H(6)	0.1926	0.1909	0.1914	0.1897	0.2067	0.1935	0.1946	0.1955	0.1952	0.1979
μ, D	1.165	2.243	0.000	1.574	1.940	1.755	2.207	1.142	2.271	0.000
I_p, eV	8.889	8.523	8.514	8.581	8.660	8.746	8.880	8.807	8.882	8.957
ΔH_f, Kcal/mole	-26.671	-73.930	-74.002	-72.651	-74.078	-74.716	-74.762	-74.949	-123.047	-123.460
ΔE_c, eV[b]	0.0000	0.0032	0.0000	0.0618	0.0000	0.0101	0.0081	0.0000	0.0179	0.0000

[a] Energy for catechol C2v(in) is not favorable for stability.

[b] Energy of conformer relative to the lowest energy one.

however, the dominant optimized planar structure is shown for the *p*-methylolphenol (C_{1v}), with the methylol group carbon, oxygen, and hydroxyl hydrogen laying in the plane. The methylol group in *o*-HMP is rotated out of the plane, but it is oriented in a hydrogen bonded position toward the phenolic oxygen. The time-averaged charge densities for phenol can be taken from the average of the charge densities when the OH group takes the 0° and 180° conformations in the plane of the benzene ring. This amounts to averaging positions 2 and 6, and 3 and 5.

Table 3 shows the residue atom electron charges of the atoms in the anion phenol monomers. Since a molecule is either ionized or not, only one set of data is necessary for each compound. The mono charged anions for the polyhydroxybenzenes are not included here. These calculations depict how the electron density about the aromatic ring changes when the hydroxyl protons are removed, and consequently illustrate how these compounds may behave in a basic medium. Table 4 shows the charge densities for the quinone methide structures thought to be generated from methylolphenols in both the acidic and basic mediums. In the acidic medium, the charge is shown on the proton, Figure 1, but in fact this can be considered a carbocation, since the charge is distributed strongly to carbon atom 7.

Table 5 shows a comparison of the calculated gas phase and experimental dipole moments for the neutral monomers. The conformer populations are compared to those computed from the CNDO/2 method.[11] Table 6 gives a comparison of some heats of formation and ionization potentials calculated from the MNDO and AM1 to experimental values. Table 7 compares the optimized structural parameters for catechol obtained from MNDO and AM1 methods with *ab initio* values and the microwave and crystal structure experimental results. Table 8 gives a preliminary examination of the correlation of ^{13}C-NMR shift data with averaged atomic electron density data on the carbon atom ortho to the hydroxyl group. MNDO values for (+)-catechin, (-)-epicatechin and a protonated (-)-epicatechin are illustrated in Table 9. Only selected net charges and HOMO charge densities are illustrated for the A-, B-, and flavan-rings. Tables 10, 11, and 12 give the HOMO charge densities for the HOMO and LUMO atomic orbital coefficients along with the energies of those frontier orbitals.

DISCUSSION

Examination of atomic charges on the positions adjacent to the hydroxyl groups in resorcinol and phloroglucinol in Table 1 reveals several distinct differences. In resorcinol, the charge density of -0.175 between the hydroxyl groups on carbon atom 2 (C_{1h}) is definitely less in magnitude than the -0.211 found in phloroglucinol. Note that these atom sites are both more electron-rich than the average of the -0.0614 and -0.097 found on the 3 and 6 positions in catechol (C_{1h}). It would be expected that in near neutral conditions, phloroglucinol would show the greatest nucleophilic reaction characteristics if the total charge density were dominant in the transition state formation. It also appears from the charge densities that the resorcinol has more nucleophilicity than the catechol as well as hydroquinone in positions adjacent to the hydroxyl group. However, the differences between these isomers are not always large.

Table 2. MNDO Atomic Charges for the Most Abundant Conformer in Neutral Model Phenol Compounds Compared to *ortho*- and *para*-Hydroxymethylphenol.

Atom#	Phenol C1h	Hydroquinone C2h	Catechol C1h	Resorcinol C1h	Phloroglucinol C3h	*p*-HMP C1h	*o*-HMP C1
C(1)	0.1091	0.0739	0.0273	0.1494	0.1806	0.1204	0.1511
C(2)	-0.0859	-0.1092	0.0972	-0.1749	-0.2109	-0.0947	-0.1563
C(3)	-0.0275	-0.0459	-0.0614	0.1423	0.1806	-0.0126	-0.0021
C(4)	-0.0962	0.0739	-0.0589	-0.1228	-0.2103	-0.1360	-0.1073
C(5)	-0.0189	-0.1092	-0.0595	0.0120	0.1806	0.0275	-0.0027
C(6)	-0.1433	-0.0459	-0.0977	-0.1807	-0.2109	-0.1571	-0.1737
C(7)						0.2211	0.2221
O(1)	-0.2485	-0.2489	-0.2666	-0.2484	-0.2467	-0.2469	-0.2404
O(2)		-0.2489	-0.2491	-0.2466	-0.2467	-0.3266	-0.3181
O(3)					-0.2467		
H(1)	0.0758	0.0625	0.0772	0.0773	0.0791	0.0761	0.0633
H(2)	0.0605	0.0761	0.0630	0.0779	0.0791	0.0562	0.0611
H(3)	0.0619	0.0625	0.0619	0.0603	0.0791	0.0730	0.0639
H(4)	0.0591	0.0761	0.0608	0.0634	0.1979	0.0624	0.0574
H(5)	0.0611	0.0914	0.1990	0.1953	0.1979	0.1939	0.1956
H(6)	0.1926	0.0914	0.2067	0.1955	0.1979	-0.0195	0.0343
H(7)						-0.0202	-0.0242
H(8)						0.1825	0.1761
μ, D	1.165	0.000	1.940	1.142	0.000	0.797	2.415
I_p, eV	8.888	8.514	8.660	8.807	8.957	8.829	9.000
ΔH_f, Kcal/mole	-26.671	-74.002	-74.078	-74.949	-123.460	-75.806	-75.535

Table 3. The MNDO Calculated Heats of Formation, Ionization Potentials, and Atomic Partial Charges for Fully Ionized Model Phenol Anions.

Atom#	Phenol	Hydroquinone	Catechol	Resorcinol	Phloroglucinol	*p*-HMP	*o*-HMP
C(1)	0.3034	0.1972	0.2237	0.3325	0.3384	0.3109	0.3198
C(2)	-0.2904	-0.2256	0.2237	-0.4862	-0.5400	-0.3006	-0.2671
C(3)	0.0285	-0.2256	-0.4088	0.3325	0.3384	0.0769	0.0253
C(4)	-0.2985	0.1972	-0.1250	-0.4089	-0.5400	-0.3561	-0.2957
C(5)	0.0236	-0.2253	-0.4088	0.0452	0.3384	0.0500	0.0750
C(6)	-0.2904	-0.2253	-0.1248	-0.4089	-0.5400	-0.2892	-0.3986
C(7)						0.2699	0.2617
O(1)	-0.5283	-0.6977	-0.6158	-0.6546	-0.7550	-0.5129	-0.5216
O(2)		-0.6977	-0.6158	-0.6546	-0.7550	-0.3300	-0.3652
O(3)					-0.7550		
H(1)	0.0249	-0.0242	-0.0230	-0.0033	-0.0434	0.0276	0.0294
H(2)	-0.0029	-0.0242	-0.0513	-0.0174	-0.0434	0.0143	0.0029
H(3)	0.0131	-0.0242	-0.0510	-0.0591	-0.0434	-0.0038	0.0180
H(4)	-0.0029	-0.0242	-0.0232	-0.0174		0.0282	0.0025
H(5)	0.0249					-0.0672	-0.0314
H(6)						-0.0672	-0.0365
H(7)						0.1491	0.1814
μ, D	3.235	0.000	3.541	3.227	0.000	7.058	2.341
I_p, eV	2.526	-3.077	-3.045	-1.999	-6.416	2.732	2.905
ΔH_f, Kcal/mole	-42.228	3.680	4.383	-7.151	116.606	-94.437	-99.099
E_T, eV	-1160.5760	-1464.8708	-1464.8404	-1465.3406	-1766.2596	-1639.5333	-1639.6354

Table 4. Partial Atomic Charges, Heats of Formation, and Ionization Potentials for *ortho*- and *para*-Quinone Methides in Acidic and Basic Media.

Atom#	*o*-Quinone Methide		*p*-Quinone Methide	
	Acidic	Basic	Acidic	Basic
C(1)	0.3392	0.2986	0.3486	0.2849
C(2)	-0.2291	-0.1404	-0.2120	-0.1360
C(3)	0.1892	-0.0034	0.1594	0.0000
C(4)	-0.1217	-0.0820	-0.2345	-0.1075
C(5)	0.0833	-0.0207	0.1344	0.0000
C(6)	-0.2221	-0.1743	-0.1501	-0.1361
C(7)	0.2770	0.0642	0.2506	0.0215
O(1)	-0.1927	-0.3076	-0.1602	-0.2940
H(1)	0.1126	0.0790	0.1124	0.0792
H(2)	0.1092	0.0630	0.0989	0.0583
H(3)	0.1147	0.0649	0.1005	0.0583
H(4)	0.0983	0.0564	0.1277	0.0792
H(5)	0.0876	0.0410	0.0837	0.0462
H(6)	0.0946	0.0613	0.0851	0.0461
H(7)	0.2599		0.2555	
μ, D	1.047	2.831	2.762	4.057
I_p, eV	14.129	9.110	14.298	9.356
ΔH_f, Kcal/mole	163.11	9.432	161.56	10.095
E_T, eV	-1307.9141	-1300.4124	-1307.9810	-1300.3836

Taking the combined charge densities from Table 1, one finds the average mole fraction weighted charge density on carbons 4 and 6 for the primary conformers of resorcinol to be -0.152. These values found in the average conformer of resorcinol are substantially less than the charge of -0.211 found adjacent to the OH's, positions 2, 4, and 6 in phloroglucinol. It would appear that resorcinol would show less nucleophilicity in positions 4 and 6 as compared to phloroglucinol in positions 2, 4, and 6. Both of these molecules would be better nucleophiles than catechol, with conformer C_{1h} as the most dominant. Next, consider how these phenol monomers might react with *o*- or *p*-methylolphenol near pH 7; see Table 1. The *p*-HMP and *o*-HMP methylol carbons carry large positive charges and could therefore act as electrophiles; note the relative charges on carbon 7 of 0.221 and 0.222 in Table 2. One would predict that these two electrophiles would behave similarly and with reasonably large charge interaction. This similarity in relative reactivity is in agreement with trends measured by proton NMR of product formation from phloroglucinol and resorcinol reacting with *o*- and *p*-methylolphenols.[4] This is in the pH range, however, where the reaction is the slowest.

Although it has been reported that benzyl alcohol has the methylol group rotated 60° out of the plane of the benzene ring,[42] the MNDO optimized structure leaves the methylol group in the plane of the ring for *p*-HMP. The *o*-HMP methylol group is oriented for intramolecular hydrogen bonding, but the OH group is rotated out of the benzene ring plane.

Table 5. Comparison of Experimental and MNDO Dipole Moments in Debyes.

Cpd/isomer	Expl.[a]	Mole Fract.		Calc.	CNDO[c]
		MNDO	CNDO[b]		
Phenol	1.53				
	1.28 (gas)[d]			1.16	
Catechol	2.65				3.07
	2.15 (gas)[e]			1.93	
C1h		0.96	1.00	1.94	
C2v(OH in)		0.0	0.0	1.94	
C2v(OH out)		0.04	0.0	1.57	
Resorcinol	2.05			1.55	2.26
C1h		0.59	0.50	1.14	
C2v(OH in)		0.20	0.25	1.76	
C2v(OH out)		0.21	0.25	2.21	
Hydroquinone	1.78			1.63	1.64
C2h		0.53	0.50	2.24	
C2v		0.47	0.50	0.00	
Phloroglucinol	2.70			1.47	
C1h		0.42		2.27	
C3h		0.58		0.0	

[a] Reference 36.
[b] Reference 37.
[c] Reference 11.
[d] Reference 30.
[e] Reference 31.

Table 6. Comparison of MNDO and AM1 Heats of Formation and Ionization Potentials with Gaseous Experimental Results.

Compound	Experiment		MNDO/AM1	
	IP, eV	ΔH_f, Kcal/mol	IP, eV	ΔH_f, Kcal/mol
Phenol	8.50	-23.04[a]	8.89/9.12	-26.67/-22.25
Hydroquinone	8.44[b]	-63.41[c]	8.51/8.72	-74.00/-65.71
Resorcinol	8.63[b]	-65.65[c]	8.81/9.05	-74.95/-66.80
o-Quinone methide	8.80[d]		9.11/9.23	9.43/14.57
o-HMP	8.58[d]		9.00/9.22	-75.54/-73.69

[a] Reference 24.
[b] Reference 37.
[c] Reference 38.
[d] Reference 19.

Table 7. Comparison of Experimental and Theoretical Structural Parameters for Catechol (C1h).

Parameters[a]	Microwave[b]	Crystal[c]	MNDO	AM1	*ab initio*[d]
C_1C_2	1.3970[e]	1.384[f]	1.436(1.427)[f]	1.412(1.398)[f]	1.3895[f]
C_1O_1	1.323	1.369	1.357	1.374	1.3948
C_2O_2	1.406	1.373	1.363	1.381	1.4006
O_1H	0.9896[e]	0.80	0.947	0.970	0.9896
O_2H	0.9871[e]	0.81	0.947	0.969	0.9871
CH	1.0840	0.99	1.090	1.099	1.0817[f]
$C_2C_1O_1$	118.95	121.0	124.6	122.8	120.84
$C_3C_2O_2$	122.35	123.2	122.8	123.5	124.98
C_1O_1H	108.28	106	114.3	108.0	104.01
C_2O_3H	109.57	111	112.8	107.7	106.01
CCC	120.00[e]	120.0[f]	120.2[f]	120.1[f]	120.00

[a] Bond lengths in Å, bond angles in degrees.
[b] Reference 31.
[c] Reference 29.
[d] Reference 10.
[e] Fixed values.
[f] Mean value.

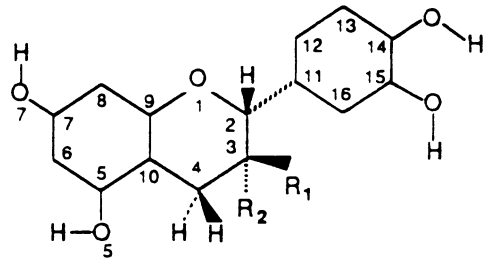
Table 8. Comparison of ^{13}C-NMR Chemical Shifts and Electron Charge Densities on Carbons Adjacent to the Hydroxyl Groups.

Compound/Atom#	Charge Density[a]	Chemical Shift
Phloroglucinol/6	-0.211	95.8[b]
(+)-Catechin/6	-0.217	96.7[c]
Resorcinol/6	-0.150	108.5[b]
Phenol/6	-0.115	115.4[d]
Hydroquinone/6	-0.078	116.3[e]
Catechol/6	-0.074	117.2[d]
Benzoquinone	-0.090	136.6[e]

[a] Conformation weighted average.
[b] Calculated in ppm from additive properties in $CDCl_3$.
[c] Reference 40, acetone:water (1/1).
[d] Reference 41.
[e] Reference 39.

Table 9. Comparison of Atomic Charges and HOMO Charge Densities for Selected Atoms on (-)-Epicatechin, (+)-Catechin, and Protonated-(-)-Epicatechin.

Atom#	(-)-Epicatechin	(+)-Catechin	Protonated-(-)-Epicatechin[a]
O(1)	-0.264(0.006)	-0.265(0.009)	-0.095(0.006)
C(2)	0.170(0.006)	0.173(0.002)	0.241(0.007)
C(4)	0.039(0.003)	0.076(0.000)[b]	0.027(0.002)
C(6)	-0.214(0.001)	-0.218(0.000)[c]	-0.157(0.000)
C(8)	-0.210(0.000)	-0.214(0.002)[c]	-0.195(0.000)
C(11)	-0.085(0.370)	-0.095(0.385)	-0.223(0.400)
C(13)	-0.076(0.056)	-0.069(0.055)	-0.091(0.098)
C(16)	-0.076(0.002)	-0.058(0.002)	-0.056(0.040)
μ, D	2.67	2.08	4.72
I_p, ev	8.66	8.68	11.77
ΔH_f, Kcal/mole	-225.11	-223.20	-42.85



(+)-catechin R_1 = OH; R_2 = H
(-)-epicatechin R_1 = H; R_2 = OH

[a] Proton stabilized on the flavanoid oxygen.
[b] The LUMO charge density for C(4) is 0.004 on catechin.
[c] On catechin, the subadjacent orbitals, a degenerate pair, give C(6) and C(8) charge densities of 0.001 and 0.560, respectively.

The anion charge density data in Table 3 can be examined for reactivity in an alkaline medium. To consider how these phenol anions might react with the hydroxybenzyl alcohols, note the charge densities of both the *para-* and *ortho-*hydroxybenzyl alcohols as listed in Table 3. The net charges on the methylol carbons (atom 7) are both positive, 0.270 and 0.262, and larger than in the non-charged molecular species. However, they remain nearly the same, which implies that, if total charge control were dominant, there would be no selectivity between the *o*-HMP(-1) and *p*-HMP(-1). The resorcinol is clearly less nucleophilic at carbon 2 than the phloroglucinol, -0.486 vs. -0.540, but not by a significant amount. The resorcinol anion with a charge of -0.409 at positions 4 and 6, however, is considerably below the phloroglucinol value of -0.540. The catechol and phenol sites appear to have more nucleophilicity than found for hydroquinone. The mono anions that were evaluated (but not included in this paper) show considerably more stability (i.e., much lower heats of formation and bound ionization potentials) than the fully

Table 10. The HOMO Charge Density on Carbon Atoms for the Most Abundant Conformers of Model Phenols.

Atom#	Phenol C1h	Hydroquinone C2h	Catechol C1h	Resorcinol C1h	Phloroglucinol[a] C3h	*p*-HMP C1v	*o*-HMP C1
C(1)	0.4646	0.4455	0.4235	0.2883	0.3424	0.4343	0.4396
C(2)	0.2853	0.1330	0.4548	0.0004	0.7868	0.2600	0.2208
C(3)	0.0619	0.2048	0.0604	0.2663	0.3425	0.0671	0.1130
C(4)	0.5697	0.4455	0.2432	0.5885	0.7870	0.5879	0.5652
C(5)	0.1329	0.1332	0.3791	0.0037	0.3425	0.1359	0.0673
C(6)	0.2123	0.2048	0.0019	0.5429	0.7868	0.1953	0.3041
C(7)						0.0091[b]	0.0006[b]
E(HOMO)	-8.888	-8.514	-8.660	-8.807	-8.957	-8.829	-8.999
E(LUMO)[c]	0.2460	0.1040	0.1572	0.1878	0.1821	0.1624	0.0132

[a] The charge density is summed over two degenerate levels.
[b] The LUMO charge densities on C(7) for *p*-HMP and *o*-HMP are approximately 0.0.
[c] LUMO, energy of lowest unoccupied molecular orbital.

Table 11. The HOMO Charge Densities for Model Phenol Anions.

Atom#	Phenol (-)	Hydroquinone (-2)	Catechol (-2)	Resorcinol (-2)	Phloroglucinol[a] (-3)	*p*-HMP (-)	*o*-HMP (-)
C(1)	0.0533	0.1829	0.1076	0.0307	0.0878	0.0457	0.0504
C(2)	0.4725	0.1829	0.1076	0.0000	1.0222	0.4604	0.4176
C(3)	0.0053	0.2493	0.4196	0.0306	0.0878	0.0017	0.0026
C(4)	0.6419	0.2499	0.2269	0.8209	1.0221	0.6868	0.6309
C(5)	0.0053	0.2489	0.2269	0.0000	0.0877	0.0034	0.0133
C(6)	0.4744	0.2489	0.4196	0.8204	1.0195	0.4400	0.5341
C(7)						0.0000[b]	0.0000[b]
E(HOMO)	-2.527	3.077	3.045	1.999	6.417	-2.732	-2.904
E(LUMO)[c]	5.525	9.846	10.76	10.42	15.76	5.454	5.100

[a]The charge density is summed over two degenerate energy levels.
[b]The LUMO charge densities for C(7) of *p*-HMP and *o*-HMP are 0.001 and 0.008, respectively.
[c]E(LUMO) is the energy in eV of the lowest unoccupied molecular orbital.

Table 12. The HOMO and LUMO MO Orbital Coefficients for Normal and Protonated *ortho*- and *para*-Quinone Methides.

Atom#	HOMO/LUMO Coefficients			
	o-Quinone Methide	*p*-Quinone Methide	Prot-*o*-Quinone Methide	Prot-*p*-Quinone Methide
C(1)	0.025/ 0.281	0.002/-0.302	-0.322/ 0.499	-0.255/ 0.391
C(2)	0.456/ 0.359	0.403/-0.317	-0.478/ 0.055	-0.303/ 0.129
C(3)	0.265/-0.352	0.266/ 0.339	-0.037/-0.460	-0.020/-0.399
C(4)	-0.468/-0.324	-0.406/ 0.285	0.533/-0.042	0.378/-0.165
C(5)	-0.467/ 0.383	0.266/ 0.339	0.483/ 0.352	-0.262/-0.365
C(6)	0.276/ 0.239	0.403/-0.317	-0.168/ 0.051	-0.539/ 0.103
C(7)	0.375/-0.518	-0.528/-0.542	-0.175/-0.592	0.365/ 0.677
E(HOMO), eV	-9.110	-9.357	-14.130	-14.298
E(LUMO), eV	-0.935	-1.005	-6.658	-6.617

charged anions of catechol, resorcinol, and phloroglucinol. However, it would be anticipated that the ionized structures would be stabilized by the high dielectric in an aqueous medium.

With the methylolphenol anions, the methylol group in *p*-HMP is optimized planar, and the O and H in the *o*-HMP methylol are oriented for hydrogen bonding with the phenolic oxygen, but rotated out of the plane of the benzene ring. Elder and Worley[16] found the methylol group planar in the *o*-HMP anion and in a hydrogen bonding orientation.

The charges on the potential quinone methide intermediates in both acidic and basic media are shown in Table 4. In condensation reactions with *o*- and *p*-hydroxybenzyl alcohols in both acidic or basic media, carbocations or quinone methides are predicted to form. The MNDO optimized structures were found to be planar to within 0.10° in all cases. The *o*- and *p*-quinones have benzoquinone structural features with the CO, C_1C_2, C_2C_3 and CC_7 equal to 1.229, 1.495, 1.355, and 1.354 Å, respectively. Considering the basic medium, it is interesting that the *o*- and *p*-quinone methides have methylene charges of 0.064 and 0.022, respectively. These charges are not large enough in themselves to explain the increased reactivity of the methylolphenols at high pH or the *p*-HMP selectivity reported.[4] In an acidic medium where the carbocation could form, the optimized structures are planar with considerably increased charge on the methylene carbon 7; see Table 4. Again, the total charge difference between the *o*- and *p*-quinone methide carbocations is not significant in looking for differences in electrophilic attack, although the total energy for the *p*-carbocation is about 1.5 kcal/mol lower. The dipole moments are larger for the *p*-quinone methide and the *p*-quinone carbocation relative to the *o*-quinone structures. Consequently, even though the charge on the carbocation may play a role in the reaction, there must also be some other explanation for the selectivity of the *p*-methylolphenol, especially in an acidic medium. This could be due to outer orbital properties, to the influence of dipolar attraction, or to steric factors.

In order to check the validity of the MNDO molecular orbital calculations, several comparisons with experimental observations were evaluated. There is rea-

sonable agreement in computing the dipole moments observed for these phenols, especially in light of the necessity of weighting the population of the monomers as shown in Table 5. There are limited gaseous dipole moment data available, but the phenol and catechol microwave data agree well and conform to the estimated 0.2-0.3 D decrease in going from solution to the gaseous state. The populations described for these molecules appear to be satisfactory and similar to those estimated from CNDO/2 results.[11] CNDO methods typically do not give good dipole moments. The general quality of the MNDO results lends confidence to the relative atomic charges calculated. Although there are few experimental results available, comparisons of some ionization potentials and heats of formation are shown in Table 6. The MNDO values are for structures shown in Tables 2 and 4. Values calculated using AM1 and the precise keyword are also included for comparison. Note that the MNDO calculated heats of formation are considerably more negative than those from AM1. However, the ionization potentials are all better reproduced by MNDO. The ultraviolet spectral transitions are not produced very well from MNDO or AM1. Table 7 compares one structural optimization with existing data. Although most aromatic CC bonds conform to experiment, even if a little larger, there are problems when considerable strain exists between adjacent carbon atoms, especially as on catechol or *o*-HMP(-). The CO bond lengths and average bond angles are pretty good, except for bond angles involving hydroxyl groups. In this case, it is noted that the AM1 model gives a better overall structural fit, although MNDO gave better CO and CH bond lengths. The CO and average CC bond lengths found for phenol (e.g., 1.359 and 1.408 $\AA$, respectively) compare to the 1.374 an 1.393 $\AA$ found from microwave spectroscopy.[30]

Table 8 shows the ^{13}C-NMR chemical shift data calculated for the site adjacent to the O atom for the neutral phenols compared to the residue charge density at that site. There appears to be general correlation of the chemical shift and calculated electron density for the unsubstituted carbon 6. The catechin chemical shift value is for a different solvent, which may account for its small deviation. On the other hand, according to Levy,[43] for varying structures there generally can be considerable scatter in a correlation curve. Notice in Table 8 how the benzoquinone structure chemical shift is out of line. This type of positive correlation, however, could have implications for using ^{13}C data for an atom site to predict chemical reactivity in complex tannin mixtures, especially for comparable structures. In light of these general agreements which support the charge density values, it is interesting to examine the significance of these charge densities in more detail with regard to chemical reactivity.

Most current mechanisms involving hydroxybenzyl alcohols go through a quinone methide intermediate structure, as shown in Figure 2. Since the total net charge does not correlate with much of the chemical reactivity or selectivity observed, the HOMO and LUMO orbital coefficients or charge densities are included from Tables 10, 11, and 12. The HOMO coefficients for resorcinol and LUMO coefficients for *p*-HMP and *p*-quinone methide are given in parentheses in Figure 2. The first striking thing noted is that resorcinol has a nodal plane through carbons 2 and 5. Consequently, even though the net charge density is high at that site, the frontier orbital suggests, with the near zero coefficient when neutral and zero when ionized,

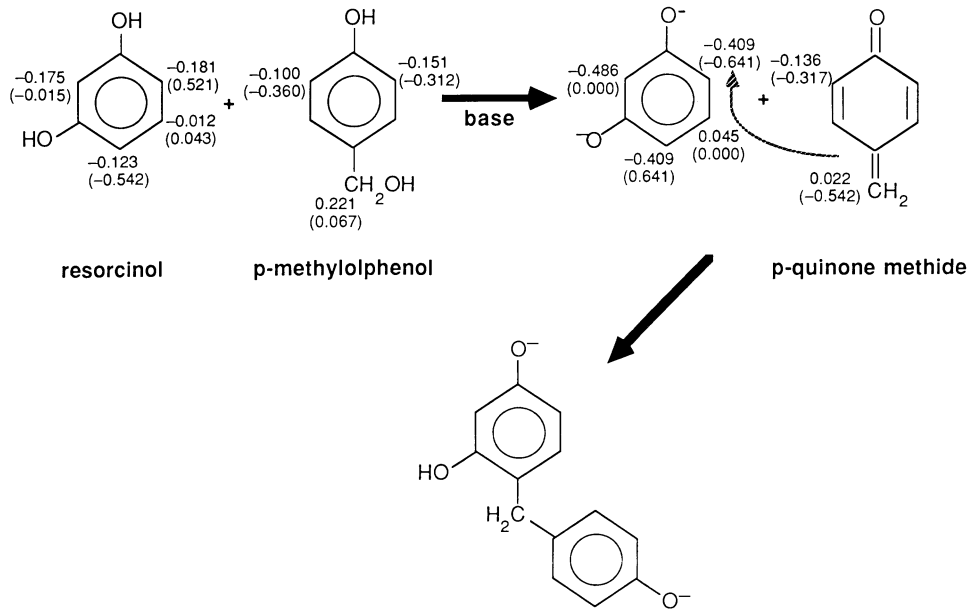


Figure 2. *The reaction between resorcinol and p-HMP in a neutral and basic medium. The HOMO atomic coefficients for resorcinol and the LUMO atomic coefficients for the quinone methide are given in parentheses below the net atomic charges.*

that no reaction would take place on carbon 2 in either neutral or basic media. This is found to be true experimentally.[46]

In the neutral medium, the *p*- and *o*-HMP molecular species dominate, and the HOMO charge density as shown on carbon atom 7 is small (Table 10). Likewise, the LUMO coefficients are found to be near zero for carbon 7. The reacting molecular region is also nonplanar. In this case, the reaction would be expected to be slow. Perhaps dimerization with ether linkage formation is an expectation. The reaction is found to be the slowest under these conditions.[4]

In Figure 2, when the medium is basic, the resorcinol becomes a better nucleophile as measured by both the total charge density and frontier HOMO orbital density at carbons 4 and 6. In addition, the quinone methide structure becomes stable. The methylene carbon of the quinone methide and the 4 and 6 carbons of resorcinol or the 2, 4, or 6 carbons on phloroglucinol, for example, are well suited for molecular orbital interaction. Notice the high frontier orbital LUMO coefficient on the methylene carbon for the quinone methide; see Table 12. The methylene carbon atomic orbital from the LUMO is from a π_z orbital and is oriented perpendicular to the benzene ring. It is ideal for bonding with the HOMO π_z orbital from resorcinol or phloroglucinol. The HOMO-LUMO orbital combination selected is that having the closest energies. This gives the largest orbital perturbation.[6,7]

A bonding approach is depicted in Figure 3, which could explain the increased reactivity in the basic medium and the *p*-HMP selectivity.[44] This transition state approach is no doubt aided by the large dipolar attraction that would be present. The *o*-quinone methide is probably not as reactive since the LUMO coefficient is just a little smaller, the dipole moment is considerably smaller, and there is also more steric hindrance. In this case, the steric hindrance in the structure alignment could be a large factor.

However, as the medium turns acidic and the carbocation form of the quinone methides becomes stable, the methylene carbon LUMO coefficients for both molecules become large (Table 12); furthermore, the *p*-quinone methide carbocation LUMO coefficient is larger. This frontier effect along with excellent dipolar alignment probably favors the *p*-HMP reactions with resorcinol and phloroglucinol in acidic media. These frontier orbitals are also π_z orbitals, which are ideal for interaction. Since the phloroglucinol has a sizable number of molecular conformers in a nonpolar form, the transition state may form differently; however, its large HOMO coefficient (Tables 10 and 11) would predict a more favorable bond interaction than with resorcinol. The selection of *p*-HMP for phloroglucinol over resorcinol is found experimentally in both acidic and basic media.[4,5] The orbital interaction and structure alignment are depicted in Figure 4. The *o*-quinone carbocation coefficient and dipole moment are given on the figure.

Finally, examination of the (+)-catechin and (-)-epicatechin data in Table 9 shows that the charge densities on carbons 6 and 8 are much more like phloroglucinol than resorcinol. Although the HOMO for (+)-catechin is localized on the B-ring,

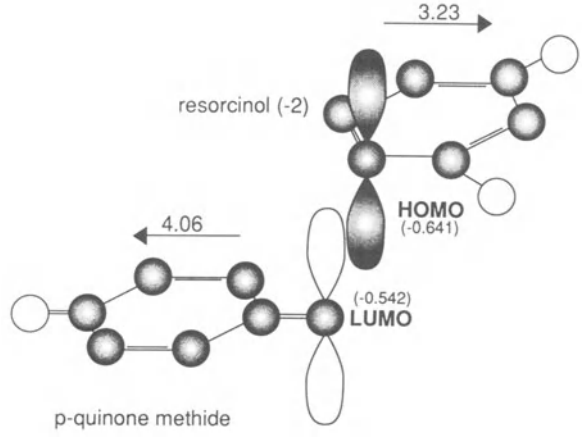


Figure 3. *A depiction of the approach of the p-quinone methide and resorcinol anion for bonding between the quinone LUMO and resorcinol HOMO orbitals. The alignment of the dipole moments is given by the arrows.*

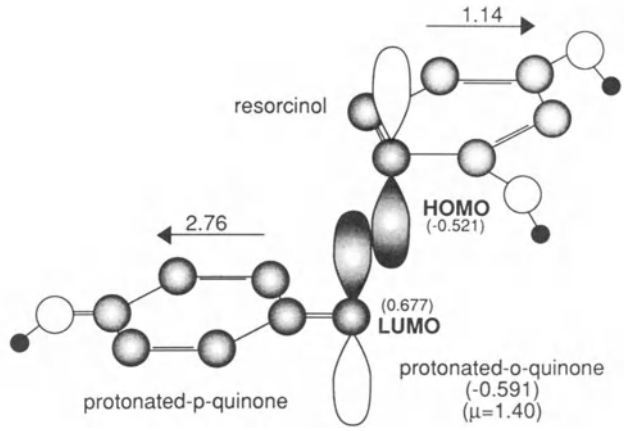


Figure 4. *The depiction of the p-quinone carbocation and resorcinol molecular alignment in bonding. The shaded HOMO and LUMO depict orbital overlap matching. The o-quinone carbocation coefficient and dipole moment are also shown.*

the subadjacent filled orbital has a large coefficient (0.560) on carbon 8. This would predict a high reactivity at this site. The characteristics of the (-)-epicatechin structure are found to be very similar on both the A- and B-rings to (+)-catechin. Reasons for a large *o*-HMP selectivity for position 8 over *p*-HMP are not apparent.[4,5] It may, however, be a combination of less steric hindrance and a possible transition state ring stabilization through the *o*-quinone carbonyl group and the C(7)-OH proton of (+)-catechin.

CONCLUSIONS

In summary, these calculations support the proposition that charge densities computed by the MNDO method can be used to help interpret phenolic-tannin chemical reactions. The electron charge densities on basic phenol structures were calculated, showing that resorcinol and phloroglucinol had very similar charge densities on carbons adjacent to the O atoms. Based on total atomic charge, phloroglucinol would have the greatest nucleophilicity under both acidic and basic conditions. It was found that the methylol carbon of *p*-hydroxybenzyl alcohol has the identical electro-positive character as the methylol carbon in *o*-hydroxybenzyl alcohol in a neutral medium. The positive values computed for the carbon atom in the methide group for the quinone methides suggest in both cases that the protonated *o*- and *p*-quinone methide structures would be good electrophiles in acidic media and that both carbocations according to net atomic charge would behave similarly. The frontier orbitals, however, show definite differences between the structures and suggest that the *p*-HMP would be selective in acidic and basic media and that the molecular interaction would be strong in an acidic solution. Since phloroglucinol has the largest HOMO coefficients, it would be selected in competing reactions. Studies into the potential transition states for these reactions would be useful in increasing the understanding of tannin-methylolphenol adhesive formation.

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REFERENCES

1. Pizzi, A. Wood Adhesives: Chemistry and Technology. Marcel Dekker, New York (1983).
2. MacLean, H.; Gardner, J.A.F. Bark extracts in adhesives. *Pulp and Paper Mag. of Canada* 53:111 (1952).
3. Herrick, F.S.; Boch, L.H. Adhesives from bark extracts. *For. Prod. J.* 8:269 (1958) and US Pat 2,819,295 (to Rayonier, Inc.) Jan 7 (1958).
4. McGraw, G.W.; Ohara, S.; Hemingway, R.W. Reactions of tannin model compounds with methylolphenols: competitive condensations of ortho- and para-hydroxylbenzyl alcohols with resorcinol, phloroglucinol or (+)-catechin. *In*: Hemingway, R.W., Conner, A.H., Branham, S.J. (eds.) Adhesives from Renewable Resources, American Chemical Society Symposium Series No. 385 pp. 185-202 (1989).
5. Hemingway, R.W.; McGraw, G.W.; Ohara, S. Further studies on the reactions of condensed tannin model compounds with methylolphenol. USDA-Forest Service, Study Plan FS-SO-4701-99 (1987).
6. Fleming, I. Frontier Orbitals and Organic Chemical Reactions. Wiley-Interscience, New York (1976).
7. Fukui, K. Theory of Orientation Stereoselection. Springer-Verlag, Berlin (1975).
8. Dewar, M.J.S.; Dougherty, R.C. The PMO Theory of Organic Chemistry. Plenum Publishing Company, New York (1975).
9. Sadlej, J. Semi-Emperical Methods in Quantum Chemistry. Cooper, I.L., transl. (ed.) Ellis Horward Ltd, Chichester (1985).
10. Konschin, H. An STO-3G orbital investigation of the molecular structure and internal rotation of phenol and catechol. *J. Mol. Struct.* 92:173 (1983).
11. Kudchadker, S.A.; Zwolinski, B.J. Conformational analysis of benzendiols: pyrocatechol, resorcinol and hydroquinone. *J. Mol. Struct.* 48:271 (1978).
12. Marriott, S.; Topsom, R.D. *Ab initio* calculations of the barriers in some monosubstituted benzenes. *Aust. J. Chem.* 39:1157 (1986).
13. Tylli, H.; Konschin, H. A Raman spectroscopic study of the OH and OD torsion in 1,2-dihydroxybenzene. *J. Mol. Struct.* 57:13 (1979).
14. Konschin, H. An STO-3G molecular orbital investigation of planar *m*- and *p*-dihydroxybenzene: fully optimized molecular structures. *J. Mol. Struct.* 110:267 (1984).
15. Marsili, G.; Cigniti, M. Theoretical study of diphenols and polyphenols. *Gass. Chim. Ital.* 109:553 (1979).
16. Elder, T.J.; Worley, S.D. The application of molecular orbital calculations to wood chemistry: IV. The formation of methylol derivatives. *J. Wood Chem. Technl.* 6:505 (1986).
17. Perrin, M.; Thozet, A.; Bertholon, G.; Decoret C.; Royer, J. Comparisons between crystallographic results and theoretical calculations on phenol molecules. *J. Mol. Struct.* 70:87 (1981).
18. Semenov, S.G.; Shevchenko, S.M. Electronic structure and reactivity of methylene- and benzyliceneanthrones. *Zhur. Organ. Khim.* 21:368 (1985).
19. Eck, V.; Schweig, A.; Vermeer, H. The ultraviolet photoelectron spectrum of *o*-benzoquinone. *Tetrahedron Letts.* 27:2433 (1978).

20. Musil, L.; Koutek, B.; Pisova, M.; Soucvek, M. Delocalization and stability of *o*- and *p*-quinone methides: an HMO study. *Coll. Czech. Chem. Commun.* 46:1148 (1981).

21. Remko, M.; Polcin, J. CNDO/CI investigation of the electronic spectra of quinone and quinonemethides. *Monatsh. Chem.* 108:1313 (1977).

22. Semenov, S.G.; Shevchenko, S.M. Electronic structure of *p*-quinonemethide. *Zhur. Struk. Khim.* 24:25 (1983).

23. Dewar, M.J.S.; Thiel, W. Ground states of molecules. 38. The MNDO method. Approximations and parameters. *J. Am. Chem. Soc.* 99:4899 (1977).

24. Dewar, M.J.S.; Thiel, W. Ground states of molecules. 39. MNDO results for molecules containing hydrogen, carbon, nitrogen, and oxygen. *J. Am. Chem. Soc.* 99:4907 (1977).

25. MOPAC MNDO, Quantum Chemistry Program Exchange #455, Version 4.0. Indiana University, Bloomington, Indiana.

26. Dewar, M.J.S.; Zoebisch, E.G.; Eamonn, F.H.; Stewart, J.J.P. AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* 107:3901 (1985).

27. Boyd, D.B.; Smith, D.W.; Stewart, J.J.P.; Wimmer, E. Numerical sensitivity of trajectories across conformational energy hypersurfaces from geometry optimized molecular orbital calculations: AM1, MNDO and MINDO/3. *J. Comp. Chem.* 9:387 (1988).

28. Wycoff, R.W.G. Crystal Structures. 2nd Ed. Wiley, New York p. 201, 306 (1975).

29. Wunderlich, H.; Mootz, D. The crystal structure of catechol. *Acta Cryst.* B27:1684 (1971).

30. Larsen, N.W. Microwave spectra of the six mono-^{13}C-substituted phenols and of some monodeuterated species of phenol. *J. Mol. Struct.* 51:175 (1979).

31. Onda, M.; Hasunma, K.; Hashimoto, T.; Hamaguchi, I. Microwave spectrum of catechol (1,2-dihydroxybenzene). *J. Mol. Struct.* 159:243 (1987).

32. Brown, C.J. The crystal structure of catechol. *Acta Cryst.* 21:170 (1966).

33. Fronczek, F.R.; Grannuch, G.; Mattice, W.L.; Tobiason, F.L.; Broeker, J.L.; Hemingway R.W. Dipole moment, solution conformation and solid state structure of (-)-epicatechin, a monomer of procyanidin polymers. *J. Chem. Soc. Perkin Trans. 2* :1611 (1984).

34. Minkin, V.I.; Osipov, O.A.; Zhdanov, Y.A. Dipole Moments in Organic Chemistry. Plenum Publishing Company, New York (1970).

35. Koopmans, T. Distribution of wave functions and characteristic values among the individual electrons of an atom. *Physica* 1:104 (1933).

36. McClellan, A.L. Tables of Experimental Dipole Moments. Vol. 2. Rahara Enterprises, El Cerrito, California (1974).

37. Cioslowski, J.; Baranski, A.; Juska, T. A simple algorithm for the calculation of the pi-ionization energies of substituted benzenes. *Tetrahedron* 42:4549 (1986).

38. Pedley, J.B.; Naylor, R.D.; Kirby, S.P. Thermochemical Data of Organic Compounds. Chapman and Hall, London p. 130 (1986).

39. Scheffer, J.R.; Wong, Y.G.; Patil A.O.; Curtin, D.Y.; Paul, I.C. CPMAS ^{13}C-NMR spectra of quinones, hydroquinones, and their complexes. *J. Am. Chem. Soc.* 107:4898 (1985).

40. Porter, L.J.; Newman, R.H.; Foo, Yeap; Wong, H.; Hemingway, R.W. Polymeric Proanthocyanidins. ^{13}C-NMR studies of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1217 (1982).

41. Johnson, L.F.; Jankowski, W.C. Carbon-13 NMR spectra. A Collection of Assigned, Coded, and Indexed Spectra. Wiley-Interscience, New York (1972).

42. Dunlap, B.I.; Cook, M. An NMR study of hydroxy pi-bonding and the conformations of benzyl alcohol and derivatives. *Inter. J. Quant. Chem.* 29:767 (1986).

43. Levy, G.C.; Lichter, R.L.; Nelson, G.L. Carbon-13 Nuclear Magnetic Resonance Spectroscopy. 2nd Ed. Wiley-Interscience, New York pp. 102-104 (1980).

44. Fukui, K.; Yonezawa, T.; Shingu, H. A molecular orbital theory of reactivity in aromatic hydrocarbons. *J. Chem. Phys.* 20:722 (1952).

45. Hemingway, R.W.; Karchesy, J.J.; McGraw, G.W. Phenolic resins, chemistry and applications. Weyerhaeuser Science Symposium 2. Weyerhaeuser Co: Tacoma, Washington pp. 33-69 (1981).

46. Hemingway, R.W. (private communication)

REACTIONS AT THE A-RING OF PROANTHOCYANIDINS

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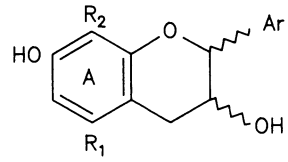
ABSTRACT

The most common reactions at the A-ring of proanthocyanidins (condensed tannins) are examples of electrophilic aromatic substitution where an electrophile displaces a hydrogen (i.e., at C-6 and/or C-8 of procyanidins and profisetinidins). Other types of displacements include dehalogenation, procyanidin degradation, and oxidative free-radical coupling. The hydroxylation pattern of the A-ring controls both reactivity and orientation of substitution, partially by steric effects, but electronic factors have more influence. Examples of A-ring reactions include those of commercial interest (methylolation, condensation) and of diagnostic interest (halogenation, alkylation, hydrogen/deuterium exchange). *C*- and *O*-glycosylation are further types of A-ring reactions leading to natural products.

INTRODUCTION

Condensed tannins are oligomeric or polymeric molecules based on flavan-3-ol units where the A-ring hydroxylation patterns, including the pyran ether oxygen, are generally derivatives of resorcinol (7-hydroxy), phloroglucinol (5,7-dihydroxy), or pyrogallol (7,8-dihydroxy) (Figure 1). These hydroxylation patterns play an important role in determining both the nature and rate of reactions that occur on the A-ring and B-ring as well as the interflavanoid bonds of condensed tannins. The reactions at the B-ring will be dealt with later by Laks (Chapter 15), and Hemingway will discuss the reactions of the interflavanoid bond (Chapter 16).



$R_1 = R_2 = H$; resorcinolic
$R_1 = OH$, $R_2 = H$; phloroglucinolic
$R_1 = H$, $R_2 = OH$; pyrogallic

Figure 1. *Common A-ring hydroxylation patterns.*

The presence of the hydroxy groups on the A-ring results in structures that are activated toward electrophilic aromatic substitution. The phenolic hydroxyl is a powerful electron donor, especially under basic conditions, increasing the electron density in the ring and thereby making it a better nucleophile as well as stabilizing carbocation intermediates that result from electrophilic attack on the positions *ortho* and *para* to the hydroxy groups. Thus, one observes reactions that are typical of activated ring systems, such as alkylation and condensation with aldehydes, among others. These electronic effects have been dealt with in much more detail by Tobiason (Chapter 13).

The hydroxy groups also exert some steric influence on the substitution into the A-ring, especially when the electrophiles are somewhat bulky. For resorcinol derivatives, there is evidence that the favored substitution site is C-6, thus avoiding substitution between the hydroxy and alkoxy groups.[1] For derivatives of phloroglucinol, numerous studies indicate that substitution occurs most readily at C-8.[1–4] The C-8 position is apparently sterically favored, since the alkoxy oxygen is tied back as part of the pyran ring and thus does not contribute any interference that would result from rotation about the C-O bond. In contrast, C-6 has hydroxyls on each side that may be free to rotate around the C-O bonds and thus partially block that position.

As mentioned above, basic conditions will increase the reactivity of these phenolic ring systems. The oxyanions generated under basic conditions are significantly better electron donors than the corresponding hydroxyl groups. This means that, with increased hydroxylation, the nucleophilicity of the ring systems will increase,[5,6] and the nucleophilicity should also vary directly with the degree of ionization of the phenolic hydroxyls.

Under acid conditions, rate increases are also observed for many of these electrophilic aromatic substitution reactions. Such rate increases are most likely a result of formation of a more reactive electrophile (e.g., a carbocation from a methylolphenol). Reaction rates are slowest at near neutral pH where there is limited ionization of the electrophile or nucleophile.

A-RING REACTIONS

Many different electrophiles react with the A-ring of condensed tannins. Some have been studied in depth, especially those expected to be of some significance with respect to commercial utilization of condensed tannins. Other electrophiles have been used diagnostically in the study of tannin structure and/or reactivity. A third category of reactions comprises those electrophiles that naturally occur and have been found condensed with the A-ring of the condensed tannins or their precursors.

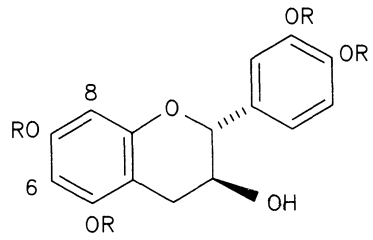
Halogenation

Both bromine and iodine have been used as electrophiles in reactions with flavanoids. The reactions have been used to study reactivity of the aromatic systems, to determine points of linkage between flavanoid units, and to introduce halogens to serve either as blocking groups or as reactive substitutents for flavanoid synthesis. The bromination of tetra-*O*-methyl-(+)-catechin with bromine in acetic acid was presumed by Weinges[7] to occur at the C-8 position. The 8-bromo derivative was then used for the synthesis of a 4,8-linked biflavanoid. Roux's group later proved that mono-bromination of tetra-*O*-methyl-(+)-catechin with pyridinium bromide-perbromide indeed occurs exclusively at C-8.[8]

Further studies by McGraw and Hemingway[2] confirmed Roux's work with tetra-*O*-methyl-(+)-catechin and pyridinium bromide-perbromide and expanded on it by looking at the reaction of (+)-catechin with pyridinium bromide-perbromide and bromine in aqueous ethanol. Their work illustrates the importance of steric factors in determining the site of substitution into the phloroglucinol A-ring. Treatment of (+)-catechin with one molar equivalent of pyridinium bromide-perbromide or bromine in aqueous ethanol gave similar product ratios of 8-bromocatechin: 6-bromocatechin: 6,8-dibromocatechin of 1.0:0.55:0.68 (Figure 2). Although the C-8 position is still the most reactive, the presence of hydroxy (rather than methoxy) groups at C-5 and C-7 allows for significant substitution at C-6. The bulkiness of the incoming electrophile is also of significance and will be illustrated later when the substitution by methylolphenols is discussed.

Bromination of catechin penta-acetate with N-bromosuccinimide (NBS) yields a 3:2 mixture of 6- and 8-bromocatechin penta-acetates.[9] The predominance of the 6-substituted isomer is attributed to the lone pairs of oxygen interacting with the electron-withdrawing carbonyl rather than the ring, thus decreasing the nucleophilicity of the aromatic system. This, along with the fact that the acetyl groups are not in the plane of the ring,[10] results in bromination at C-6 being slightly favored over bromination at C-8. The use of excess NBS does not lead to dibromination.

Another use of bromination has been reported by Hundt and Roux[11] who describe the degradative bromination and determination of point of linkage in 4-flavanyl-flavan-3,4-diols. The dimers had a resorcinolic upper unit and a phloroglucinolic lower unit. Bromination resulted in displacement of the upper unit with bromine entering the A-ring of the lower unit at the site to which the upper unit was attached. Nonaka and coworkers[12] used a similar approach to determine the



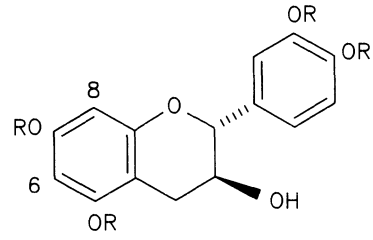
R	Reagent	Percent of Bromination Product		
		8	6	6,8-di-
Me	1	100	0	0
H	1,2	45	25	30
Ac	3	60	40	0

1 = Pyridinium bromide-perbromide
2 = Bromine in ethanol-water
3 = N-bromosuccinimide

Figure 2. *Regioselective bromination of (+)-catechin derivatives.*

point of linkage on a procyanidin trimer. Bromination with pyridinium bromide-perbromide in acetonitrile preceded acid degradation, so that bromine occupied the A-ring position not involved in interflavanoid linkages.

Iodination can be accomplished directly by I_2 in ethanol or more conveniently with N-iodosuccinimide (NIS) in dimethylformamide (DMF) or acetone. Kiehlmann and coworkers[9] have described the iodination of (+)-catechin and its derivatives using NIS (Figure 3). Tetra-*O*-methylcatechin is regiospecifically iodinated at C-8



R	Reagent	Percent of Iodination Product		
		8	6	6,8-di-
Me	NIS	100	0	0
H	NIS/Acetone	<5	>95	0
Ac	NIS/DMF	83	17	0
AC	NIS	0	0	0

Figure 3. *Iodination of (+)-catechin derivatives.*[9]

and, in contrast to bromination, excess NIS does not give di-iodination. Both of these results are attributed to the bulkiness of the electrophile. Acetylation of catechin reduces the nucleophilicity of the A-ring, resulting in no iodination of catechin penta-acetate by the weak iodine electrophile. (+)-Catechin undergoes regioselective iodination at C-6 with NIS in acetone but is preferentially substituted at C-8 with NIS in DMF. The use of 2 molar equivalents of NIS gives 6,8-diiodocatechin. The ease with which iodine is displaced by stronger electrophiles (e.g., Br^+ and H^+) makes its usefulness as a blocking group questionable. Such use has been reported in the synthesis of 4,8-coupled diflavanoids[13] and is brought into question by Kiehlmann in his study. Additional discussions of such dehalogenation reactions are presented below.

Reactions with Aldehydes

Formaldehyde. The effort to use tannins as a phenol replacement in phenol-formaldehyde resins naturally led to the investigation of the reaction of formaldehyde with condensed tannins. Some researchers have chosen to study this reaction directly,[14–19] whereas, others have opted for an investigation of the reaction of formaldehyde with model compounds or monomeric flavanoids.[3,20,21] Both approaches resulted in the same conclusions regarding the reaction of formaldehyde or formaldehyde donors with resorcinolic tannins (profisetinidins and prorobinetinidins) and phloroglucinolic tannins (procyanidins and prodelphinidins).

Studies with model compounds, especially (+)-catechin, indicate that under a variety of reaction conditions formaldehyde reacts rapidly with the phloroglucinolic ring system, giving 8-methylolcatechin, which further condenses to give the *bis*-(8-catechinyl)catechin dimer. Wellons and coworkers[3] report that at pH 9 and lower temperatures, the rate of methylolation was almost three times that of condensation, but as the temperature increased, the condensation rate increased more than the methylolation rate. The reaction of formaldehyde with (+)-catechin is slowest at pH 4.5, but even under those conditions, the catechin is highly condensed after 3 hours at high temperatures.[21] Hemingway and McGraw[20] determined that when reacted with formaldehyde even at mild pH and temperature conditions in alcohol solvents, (+)-catechin polymerized to high molecular weight products containing low proportions of methylol groups. This rapid condensation causes severe difficulties when attempts are made to use phloroglucinolic condensed tannins in adhesive formulations. Only Ayla[22] reports some success with the naturally occuring phloroglucinolic-type flavanoids of *Pinus brutia*, but this has been attributed to the co-occurence of resorcinolic A-rings in the *P. brutia* tannin.

In addition to the reactivity problem, as the size of the oligomeric tannin increases past about six units, the extent of cross-linking by formaldehyde decreases substantially, presumably because of steric factors.[19,23] Indeed, it is this factor to which Porter[19] attributes the suitability of the *P. brutia* tannins for use in adhesives, since they have a degree of polymerization of only 4-5.

The reaction of formaldehyde with resorcinolic-type phenols is more easily controlled due to the decreased nucleophilicity of the resorcinol ring compared to the phloroglucinol ring. Roux et al[16] reported that methylol-wattle tannin resols could be prepared by reacting wattle tannins with formaldehyde in the presence of high

proportions of alcohols. The methylols were then reacted with resorcinol to give methylene-linked tannin-resorcinol adducts that were suitable for use as cold-set adhesives. Wattle tannins have also been reacted with paraformaldehyde and phenol to give plywood and chip board adhesives.[16] Pizzi[23] reports several adhesive formulations for exterior-grade plywood based on combining resorcinolic tannin extracts with UF or PF resins. In some formulations, the UF or PF resin is condensed onto the tannin followed by reaction with formaldehyde; whereas, in others, there is no condensation prior to curing. In both situations, the curing occurs upon addition of paraformaldehyde and heating, resulting in cross-linking of the resins through the flavanoid A-rings. Pizzi also reports the use of phloroglucinolic tannins in an adhesive system where the tannin is reacted with a methylolphenol resin. No additional formaldehyde is added, and the slower reaction of the tannin with the resin provides better control of the reaction.

Other aldehydes. The difficulties associated with formaldehyde have led to the search for other aldehydes with cross-linking capabilities. In a kinetic study of a series of six aldehydes with resorcinolic and phloroglucinolic tannins, Pizzi[24] reported the rate constants for condensation of the aldehydes with the tannins ($k_{monomer}$) as well as the rate constants for polymerization ($k_{polymer}$) when methylol tannins react with another tannin molecule. For $k_{monomer}$ and wattle tannin, he reported HCHO $\gg$ *n*-butyraldehyde > acetaldehyde > furfuraldehyde > propionaldehyde, and for pine tannin, he found HCHO $\gg$ *n*-butyraldehyde > acetaldehyde > furfuraldehyde > propionaldehyde. The sequence for $k_{polymer}$ with wattle tannin was HCHO $\gg$ acetaldehyde > furfuraldehyde > *n*-butyraldehyde > propionaldehyde and for pine tannin HCHO $\gg$ *n*-butyraldehyde > acetaldehyde > furfuraldehyde > propionaldehyde. The higher than expected reactivity of *n*-butyraldehyde was unexplained.

In a later reference,[23] a somewhat different sequence was given for the speed of reaction of resorcinolic and phloroglucinolic tannins with these aldehydes. Furfuraldehyde was reported to be the slowest in reacting with phloroglucinolic tannins and second in reactivity only to formaldehyde with the resorcinolic tannins. This difference in reactivity of furfuraldehyde with the two types of tannins was attributed to steric factors relating to the less hindered C-8 position of resorcinolic tannins (primarily 4 $\rightarrow$ 6 interflavanoid linkages) compared to the C-6 position of phloroglucinolic tannins (primarily 4 $\rightarrow$ 8 interflavanoid linkages). Foo and Hemingway[25] have investigated the reaction of furfuraldehyde with (+)-catechin and phloroglucinol under mildly acidic conditions at ambient temperature. They were able to isolate and identify the disubstituted furfuryl derivatives but found the compounds to be rather unstable because of the reactive phloroglucinolic rings attached to a methine carbon.

Pizzi[26] has also investigated the replacement of formaldehyde in tannin-based wood adhesives with other aldehydes. He found that acetaldehyde, propionaldehyde, *n*-butyraldehyde, and furfuraldehyde could substitute for formaldehyde in these adhesives for wood beam laminating as long as formaldehyde was used as the

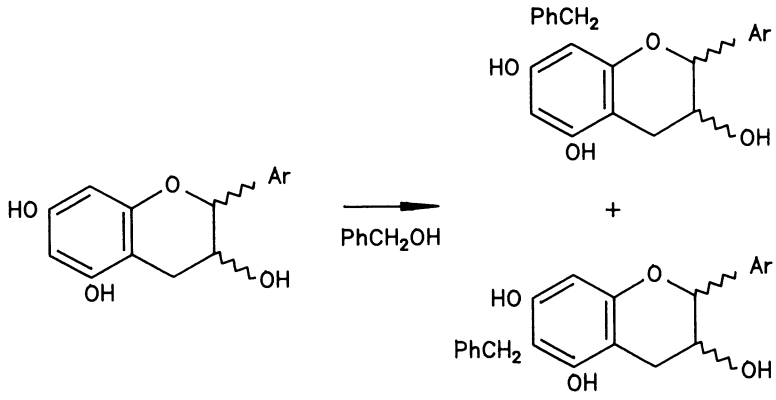
resin hardener. An interesting aspect of this study was the reported improvement in water resistance of these tannin adhesives with partial and progressive replacement of formaldehyde by *n*-butyraldehyde. The author refers to the improvement as slight but statistically significant. That conclusion is perhaps supported by the 6-hour boiling test results but not by the results on the 24-hour cold soak specimens.

Alkylation Reactions

Alkylation reactions are of some significance in any discussion of the A-ring reactions of condensed tannins. Some of these, such as the condensation reactions with methylolphenols and flavanyl groups, occur readily under acidic or basic conditions and take place on phenols with varying hydroxylation patterns. Others, like the base-catalyzed rearrangement to catechinic acid, are less general in nature.

Methylolphenols. Because of the reactivity and cross-linking problems encountered when condensed tannins are reacted with formaldehyde, methylolphenols have received attention for years as a key to the utilization of condensed tannins in adhesives. As early as 1952, MacLean and Gardner[27] advocated the use of methylolphenols as cross-linking agents. In 1958, Herrick and Bock[14] reported the development of exterior plywood glues based on western hemlock bark tannins that were cross-linked with methylolphenols. Pizzi's review[23] of tannin use in adhesives indicates that this is now the favored approach. He reports on formulations that have been used on the industrial level based on methylolphenols with resorcinolic tannins[17] as well as phloroglucinolic tannins.[28] A comparable approach for the preparation of cold-setting adhesives involved the use of ammonium hydroxide bark extracts of Douglas-fir reacted with resorcinol and paraformaldehyde.[26]

McGraw et al,[2,6,30] using hydroxybenzyl alcohols with model compounds, have studied the importance of steric factors and reactivity on the condensation of methylolphenols with condensed tannins. They found that in condensations with the catechin nucleus, significant regioselectivity for C-8 over C-6 was observed with *ortho*-hydroxybenzyl alcohol, but little regioselectivity was seen in condensations with *para*-hydroxybenzyl alcohol (Scheme 1). This difference in regioselectivity was attributed to the bulkiness of the electrophile and the additional steric hindrance at C-6 compared to C-8. The electrophile derived from *ortho*-hydroxybenzyl alcohol will be more affected by the increased steric hindrance at C-6. In a series of competitive reactions, it was determined that condensations were selective for the *para*-isomer over the *ortho*-isomer, especially at the more acidic and basic pH values. The selectivity for the *para*-isomer was observed for both resorcinolic and phloroglucinolic rings and suggests that improved reactivity will be obtained if methylolphenols with high *para*-methylol content are used as cross-linking agents with either type of condensed tannin. Similarities in condensation rates of *ortho*- and *para*-hydroxybenzyl alcohols with resorcinol, phloroglucinol, or (+)-catechin suggest that the stabilities of the carbocations/quinone methides rather than the nucleophilicity of the phenol are rate controlling. However, the nucleophilicity of the phenol was significant in determining product ratios when the phenols were competing for the same electrophile.



para-hydroxybenzyl alcohol– 1.2:1.0
ortho-hydroxybenzyl alcohol– 2.6:1.0

Scheme 1. *Hydroxybenzylation of (+)-catechin.*

It should be noted that the condensations of the methylolphenols with these phloroglucinol systems are significantly slower than the condensation of formaldehyde with such systems that yield methylolated phloroglucinol intermediates. Such methylolphloroglucinols should readily form stabilized carbocations/quinone methides.

Flavanyls. A second group of alkylation reactions that has proved to be of great importance in understanding the structures of the condensed tannins involves the generation of reactive flavanyl intermediates and their subsequent attack on the A-ring of monomeric or oligomeric flavanoids. Roux's group has especially made use of these biomimetic syntheses to prepare a number of oligomeric flavanoids, many occurring naturally among resorcinolic-type tannins.[31–33]

Roux's approach commonly involved the generation of flavanyl-4-carbocation electrophiles from flavan-3,4-diols [(+)-mollisacacidin, (+)-leucorobinetinidin, or (-)-leucofisetinidin] under acidic conditions at ambient temperatures. The nucleophiles were phloroglucinolic flavan-3-ols [(+)-catechin, (+)-gallocatechin, or (-)-epicatechin], resorcinolic flavan-3-ols [(-)-fisetinidol, (-)-robinetinidol], or dimers composed of the above units where trimers were being synthesized. Dimer formation with the phloroglucinolic flavan-3-ols was regioselective for the C-8 position of the nucleophile and generally stereoselective in the electrophile, favoring the all-*trans* isomer. Stereospecificity for the 3,4-*trans* isomer was observed when (-)-teracacidin, an all-*cis*-3,4-diol, was reacted with (+)-catechin. Condensation with resorcinolic nucleophiles was regiospecific for the C-6 position and stereoselective for the 3,4-*trans* isomers. These efforts and those described below illustrate the high degree of regio- and stereoselectivity in such biomimetic synthesis.

The trimers were synthesized using the condensation of flavanyl-4-carbocations onto dimers composed of a resorcinolic upper unit linked to a phloroglucinolic lower

unit or by having an excess of the electrophile react with the phloroglucinolic nucleophile. Although steric factors favored substitution onto a resorcinolic A-ring, substitution was predominant on the more nucleophilic phloroglucinolic A-ring.

The success of biomimetic syntheses of resorcinolic flavanoids has been paralleled, although with greater difficulty, in the synthesis of procyanidins. The syntheses have been more difficult because of the unavailability of the corresponding 3,4-diols with a phloroglucinol A-ring. These leucocyanidins have yet to be isolated from intact plant tissue, and only recently have Porter and Foo[34] reported the synthesis and isolation of (2**R**,3**S**,4**R**)-3,3′,4,4′,5,7-hexahydroxyflavan by the reduction of (2**R**,3**R**)-taxifolin [(+)-dihydroquercetin] with $NaBH_4$. The 3,4-diol produced by $NaBH_4$ reduction of (+)-dihydroquercetin is mostly the 2,3-*trans* isomer. More recently, Stafford determined that a reductase in extracts from cell suspension cultures of Douglas-fir needles will reduce (+)-dihydroquercetin to the 2,3-*trans*-3,4-*cis* diol.[35,36] The 3,4-*cis* isomer is more acid-labile and reacts more readily chemically with thiols and enzymatically with a reductase to give (+)-catechin. The benzylic 4-hydroxy group is axial in the 3,4-*cis* isomer, and the increased reactivity may account for the failure to isolate it from intact plant tissues to date.

Kolodziej[37] reports the condensation of (+)-leucocyanidin with (+)-catechin under acidic conditions to be regioselective for the C-8 position of (+)-catechin and stereoselective for the 3,4-*trans*-3,4-*cis*: 2,3-*trans* bi[(+)-catechin]. Similarly, Delcour et al[38] report that the reaction between 1.93 grams of (+)-leucocyanidin and a five molar excess of (-)-epicatechin gave predominantly [4,8]-linked procyanidins with predominantly 2,3-*trans*-3,4-*trans* stereochemistry in the upper units. Nevertheless, they did isolate 24 milligrams of the octamethyl diacetate of [4,8]-2,3-*trans*-3,4-*cis*:2′,3′-*cis*-(+)-catechin-(-)-epicatechin, the first procyanidin reported with 2,3-*trans*-3,4-*cis* stereochemistry.

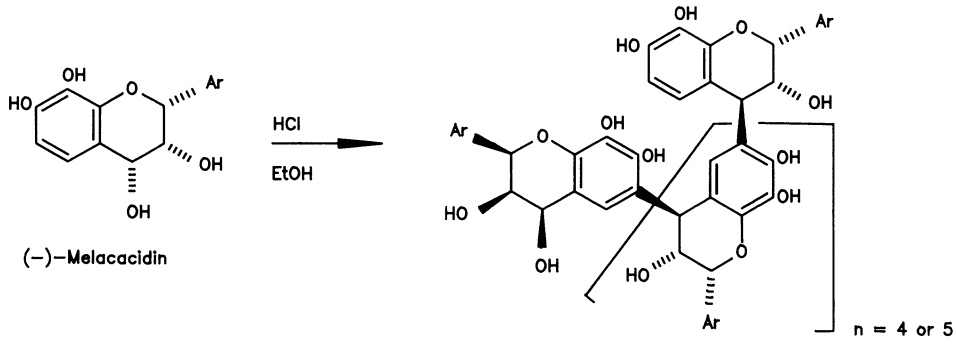
Another route for generation of the reactive flavanyl intermediates has been through the use of 4-thiosubstituted flavans, intermediates from the degradation of flavanoids in the presence of sulfur nucleophiles.[39–43] Haslam[4] reports the first use of these derivatives in the synthesis of procyanidins in the preparation of the procyanidin dimers B-1 and B-2 by the degradation of a 4-benzylthioflavan under acid conditions. Numerous researchers have since applied the acid-catalyzed degradation of the 4-thiosubstituted flavans to the synthesis of procyanidin dimers and trimers.

In a related but more direct route, Hemingway and co-workers[44] have used the acidic cleavage of condensed tannins to generate the flavanyl-4-carbocations followed by the *in situ* reaction with the nucleophile (-)-epicatechin to synthesize trimers as well as the epicatechin dimers B-2 and B-5. Similarly, Foo and Porter[45] used palm procyanidin tannin to produce *ent*-epicatechin-4-carbocations in reaction with (-)-epicatechin to prepare still other procyanidin dimers. In that same work, they reported the synthesis of catechin-(4α → 8)-*ent*-epicatechin by the sodium borohydride reduction of (+)-taxifolin followed by addition of *ent*-epicatechin and dilute HCl. This process closely approximates the reaction of resorcinolic 3,4-diols under acid conditions with phloroglucinolic nucleophiles utilized extensively by Roux's group and described earlier.

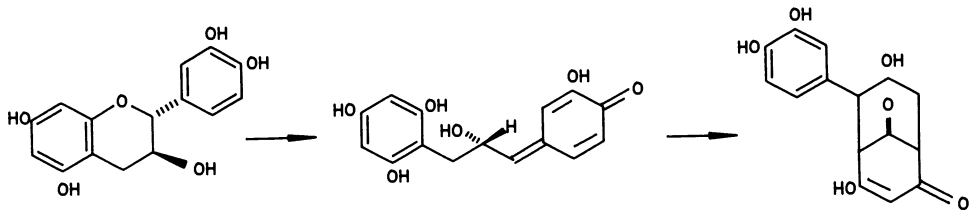
Foo[46] has treated (-)-melacacidin, a 3,4-diol with a pyrogallol A-ring, with 0.7 percent HCl in ethanol and upon concentration of the solvent obtained a polymer that resulted from attack of the flavan-4-carbocation on C-6 of the pyrogallol A-ring (Scheme 2). Shortly following this work, Foo[47] reported the isolation of a (4 → 6)-linked dimer with pyrogallol A-rings from the heartwood of *Acacia melanoxylon*. Prior to this work, it was felt that the hydroxylation pattern and the resulting electronic effects would make the A-ring of (-)-melacacidin too unreactive to form a polymer, and any naturally occurring oligomers with such rings were probably oxidation products.[48]

One other aspect of the alkylation of A-rings by flavanyl groups presently receiving attention is the generation of flavanyl electrophiles under basic conditions. Hemingway and Foo[49] compared acid- and base-catalyzed reactions of flavan derivatives containing good leaving groups at C-4. They found that mildly basic conditions led to more rapid condensations via a quinone-methide reacting with a phenolic anion than the corresponding acid-catalyzed condensations involving carbocations and phenols. The dimers generated were stable for hours at pH 9 and ambient temperatures. They then used this method to synthesize the first "branched" procyanidin trimer.[50]

Rearrangement alkylations. Under basic conditions, (+)-catechin undergoes rearrangement to form catechinic acid[51] (Scheme 3). Quinone methide formation in the B-ring results in the opening of the pyran ring followed by condensation of C-2 onto C-8 of the phloroglucinol ring to give an enolic form of 6-(3,4-dihydroxyphenyl)-7-hydroxy-2,4,9-bicyclo-[3.3.1]-nonatrione; i.e., catechinic acid. The authors suggested that a similar reaction may occur in polyflavanoids under basic conditions to yield the polymeric phenolic acids containing an enolic hydroxyl rather than a carboxyl group. Little evidence had been presented for such a rearrangement in polyflavanoids until Laks et al[52] studied the base-catalyzed reaction of polymeric procyanidins with phloroglucinol and isolated products shown to result from such a catechinic acid-type rearrangement. In further studies[53] on procyanidin-type condensed tannins under alkaline conditions, the condensed tannins rearranged in an analogous manner to give the class of procyanidins commonly



Scheme 2. *Acid-catalyzed condensation of melacacidin.*



Scheme 3. *Base-catalyzed rearrangement of (+)-catechin.*

called phenolic acids. The fact that phloroglucinol functionality is lost during these rearrangements is significant with regard to the utilization of polymeric procyanidins in adhesive formulations, especially cold-setting adhesives, since it is the A-ring that participates in the condensation reactions with methylolphenols, formaldehyde, or other electrophiles.

Related rearrangements occur in the resorcinolic condensed tannins under both acidic[54] and alkaline[55] conditions. Rearrangement occurs by the opening of the pyran ring, rotation around the interflavanoid bond, and condensation with the hydroxyl of the lower flavan unit. Ring opening in the lower phloroglucinolic unit followed by rotation and recondensation gives a product that is the equivalent of migration of the upper unit. These ring-isomerized condensed tannin units have been called "phlobatannins." In contrast to the catechinic acid-type rearrangements that result in the loss of A-ring reactivity, these rearrangements actually "liberate" the reactive resorcinol unit. These reactions are discussed in more detail by Ferreira (Chapter 17).

Displacement Reactions

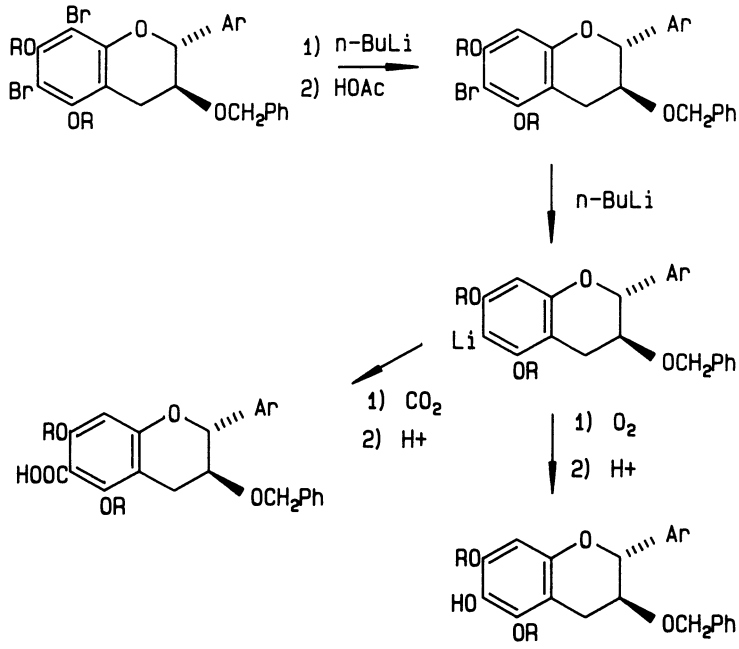
This discussion deals primarily with reactions where a substituent other than hydrogen is being displaced from the ring. An important reaction that meets this criterion but is purposely omitted is the displacement of a flavanyl group from the A-ring (i.e., cleavage of the interflavanoid bond of procyanidins). This reaction, which can occur under acidic or alkaline conditions, is described by Hemingway in Chapter 16.

A hydrogen-displacement reaction that needs to be noted here is hydrogen-deuterium exchange on the A-ring. The exchange, catalyzed by base and involving keto-enol tautomerism, occurs readily in resorcinol and phloroclucinol.[56] Kiehlmann[9] describes efforts to obtain regiospecific deuteration of (+)-catechin by recrystallization of catechin hydrate from D_2O. Substitution does occur regioselectively at C-8 but not without some substitution at C-6. None of the B-ring protons were replaced by deuterium. Even though the exchange is accelerated by base, it was determined that no exchange occurs upon acetylation with acetic anhydride and pyridine or upon transmethylation of catechin pentacetate with dimethyl sulfate and 50 percent aqueous potassium hydroxide in methanol.

Following a synthetic procedure utilized by Weinges et al[57] to prepare 6-deuteropentamethyl catechin, Hundt and Roux[58] used *n*-butyl lithium to debrominate

3-*O*-benzyl-6,8-dibromo-tetramethyl catechin to give the 6-bromo derivative in 55 percent yield. The intermediate 8-lithio derivative was decomposed with dilute acetic acid. From the pure 8- or 6-bromo- derivatives, the lithio compounds could be prepared and then treated with CO_2, which yielded the 8- or 6-carboxy derivatives following workup. These derivatives were methylated with diazomethane and analyzed as the 8- and 6-methoxycarbonyl compounds. When O_2 was bubbled through the reaction mixtures of the lithio derivatives, workup provided the 8- and 6-hydroxy-derivatives (Scheme 4). Direct carboxylation of (+)-catechin with CO_2 in sodium bicarbonate solution followed by methylation with dimethyl sulfate gave only the 6-methoxycarbonyl-tetra-*O*-methyl-(+)-catechin. It was believed that under the reaction conditions 8-carboxylation was reversible, leaving the more stable 6-isomer as the main product.

In that same study, the displacement of bromine by acid was observed. Treatment of 6-bromo-tetra-*O*-methyl-(+)-catechin with an isopropanol-concentrated HCl solution at 95 °C gave the 8-bromo derivative quantitatively. The 8-bromo derivative under identical conditions gave about 4 percent of the 6-isomer. Reaction of the 8-bromo derivative with radio-labelled tetra-*O*-methyl-(+)-catechin showed that intermolecular bromine exchange occurs.



Scheme 4. *Lithio derivatives as a route to 6-carboxy and 6-hydroxy substitution of (+)-catechin.*

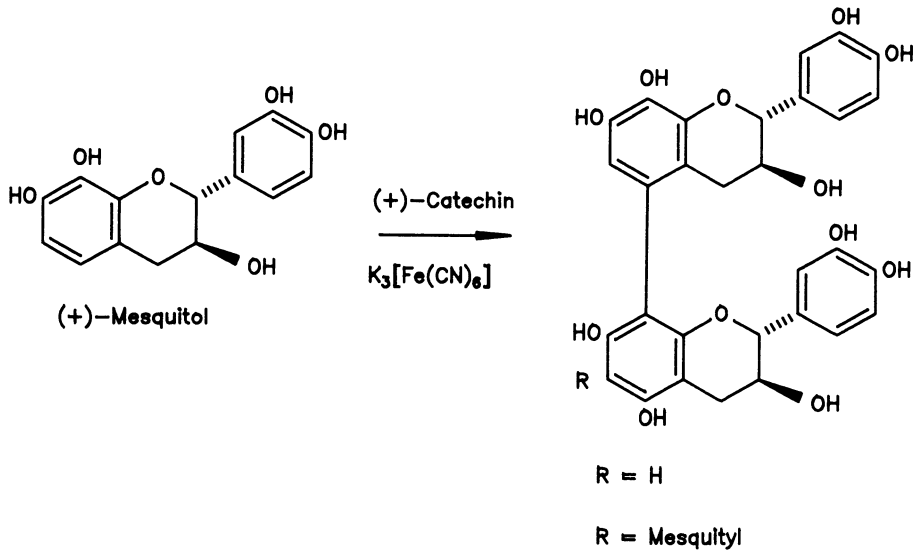
Kiehlmann has also looked at the dehalogenation of bromo- and iodocatechins and their derivatives. In contrast to the results of Hundt and Roux with brominated tetra-*O*-methyl-(+)-catechins under more vigorous conditions, Kiehlmann found that there was no debromination or bromine migration from the brominated catechins in phenolic form under conditions commonly used for interflavanoid coupling (0.1N HCl, ambient temperature, 29 hours).[59] 6,8-Dibromocatechin did undergo regiospecific debromination at C-8 on treatment with an equimolar amount of sodium sulfite in aqueous methanol in the presence of excess sodium bicarbonate. Sodium dithionite showed the same regiospecifity. The use of excess sodium sulfite gave complete debromination to catechin. All attempts to change the regioselectivity of the reaction to yield the 8-bromo isomer were unsuccessful.

Iodocatechins will undergo slow deiodination on standing in acetone, and in the presence of acid, deiodination occurs readily. Iodine is also easily displaced by bromine. In attempting to prepare 8-bromo-6-iodocatechin by the reaction of 6-iodocatechin with NBS, Kiehlmann[9] found that the major product was 6,8-dibromocatechin. The displacement of the iodine by both bromine and acid shows that iodine is only weakly bonded to carbon.

Oxidative Coupling

The use of oxidative coupling of flavones as a synthetic route to biflavonoids is well documented, and it is believed that it is this process that leads to naturally occurring biflavonoids.[60] The natural biflavones invariably possess an interflavone linkage at C-6 or C-8 of the A-ring. It has long been recognized that the autoxidation of (+)-catechin could result in either tail-to-tail or head-to-tail coupling.[61] Products resulting from head-to-tail coupling are not uncommon, and even though coupling can occur at C-6 or C-8, the C-8 linkages are preferred.[62] One proposed mechanism[63] involves oxidation of the catechol B-ring to the *ortho*-quinone, which can then undergo an ionic oxidative condensation with the highly nucleophilic A-ring of catechin. Coupling could occur between any of the electrophilic sites on the B-ring (2′, 5′, or 6′) and one of the nucleophilic sites on the A-ring (6 or 8). Young et al[62] indicate that the B-ring reacts electrophilically as a free radical with the nucleophilic A-ring. The condensation reaction is pH-dependent with increased condensation occurring on the alkaline side, a consequence of increased nucleophilicity of the catechin anion.[64] Other possible mechanisms include free radical coupling and a cationic mechanism where a phenolate ion is oxidized to an "inium" ion followed by electrophilic aromatic substitution.[65] The autoxidation of catechin yields polymeric material that is structurally similar to phlobatannins.[66]

The reaction of (+)-catechin with an excess of (+)-mesquitol in the presence of $K_3[Fe(CN)_6]$ has yielded a mixture of biflavanoids and triflavanoids oxidatively coupled through their A-rings (Scheme 5).[62] Self-condensation of the (+)-mesquitol gave biphenyl and *o*-terphenyl compounds that had been previously isolated from natural sources.[67] Crossed condensation products showed preference for coupling from C-5 of (+)-mesquitol to C-8 of (+)-catechin. This was attributed to the facile generation of a C-5 radical on the (+)-mesquitol A-ring and the increased nucleophilicity and lower degree of steric hindrance at C-8 compared to C-6 of



Scheme 5. *Oxidative coupling of (+)-mesquitol and (+)-catechin.*

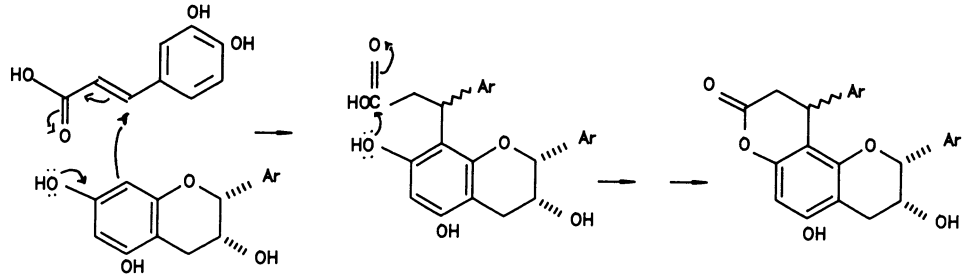
(+)-catechin. This reaction also produced a [5,6-5,8]-*m*-terphenyl with two (+)-mesquitol units condensed on the A-ring of (+)-catechin.

Natural Products

Many natural products result from condensation on flavanoid units, including the condensed tannins themselves. Reaction at the A-ring may occur on carbon or the phenolic oxygens. Most of the reactions are alkylations.

The two common methods of derivatization of flavanoids, esterification (acetylation) and etherification (methylation), find counterparts among natural products. Derivatives with gallic acid etherified to the A-ring oxygens have been reported for catechin,[68] epicatechin,[69] and epigallocatechin.[70] The cinchonains (Scheme 6) result from *C*-alkylation and *O*-acylation of the A-ring of epicatechin with a phenylpropanoid unit.[70] These derivatives could arise from a Michael addition of the nucleophilic A-ring onto caffeic acid followed by esterification. As has been observed in other instances, significantly more substitution occurs at C-8 than C-6 with the authors reporting extract yields of 3.39 grams of the C-8 isomers compared to 0.26 grams of the C-6 isomers from 2.75 kg of commercial red cinchona. A similarly substituted epicatechin dimer has also been identified.[71]

Ether derivatives of A-ring hydroxyls are relatively rare, but natural products with methylated A-ring hydroxyls as well as A-ring *O*-glycosides have been identified.[72] Naturally occurring methyl ethers have been found with phloroglucinolic,[73] resorcinolic,[74] and pyrogallolic[75] A-rings. A-ring *O*-glycosides vary with respect to the sugar unit and point of attachment. Sugars reported include glucose,[76]

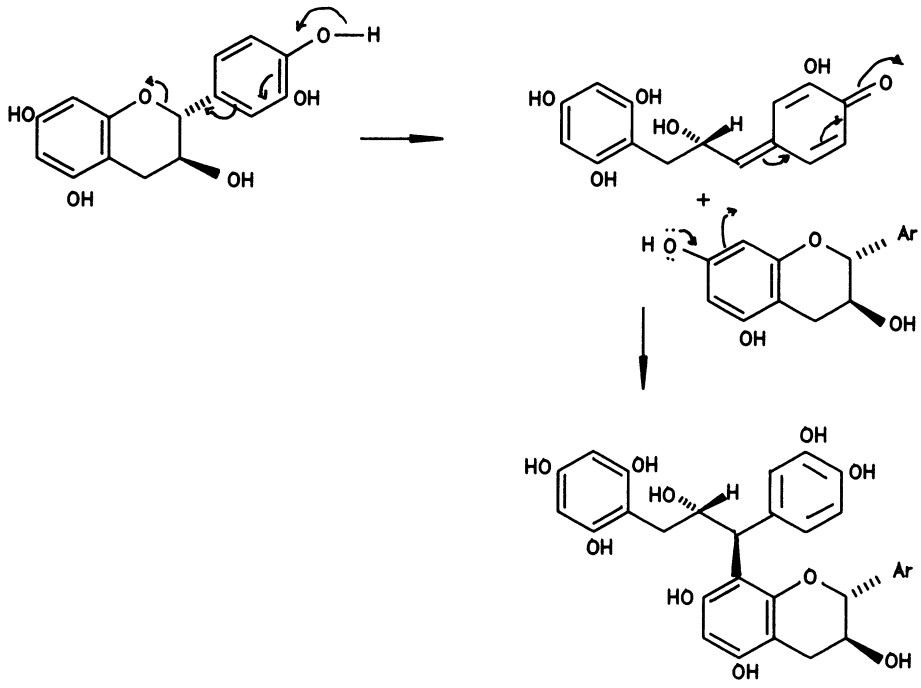


Scheme 6. *Formation of cinchonains.*

xylose,[77] arabinose,[78] and apiose[78] attached via an acetal to one of the aromatic hydroxyls. The most likely mode of condensation would involve nucleophilic attack by the phenolic hydroxyl on the hemiacetal carbon of the sugar. A-ring *C*-glycosides have also been identified, but to date only catechin and epicatechin derivatives have been reported. Kashiwada et al[79] isolated low yields of the *C*-glucosides of (+)-catechin and the procyanidin dimers B-1 and B-2 from rhubarb. The C-6 and C-8 isomers of each were isolated with C-8 substitution predominant by about 20 percent. The *C*-glucosides of (+)-catechin were also synthesized by the reaction of (+)-catechin with *D*-glucose in dioxane containing *p*-toluene sulfonic acid. The ratio of C-8 to C-6 β-*D*-glucosyl-(+)-catechin was 1.2:1. The 6-C- and 8-C-β-*D*-glucopyranosides of (-)-epicatechin were isolated from cassia bark.[80] This alkylation reaction is consistent with nucleophilic attack of the phloroglucinolic A-ring on the anomeric carbon of *D*-glucopyranose.

A group of novel biflavonoids has been isolated by Nonaka and Nishioka[81] from the tannins of gambir. These are formed by alkylation by a chalcan on the A-ring of (+)-catechin or (-)-epicatechin at the C-8 or C-6 positions to give chalcan-flavan dimers (Scheme 7). This chalcan-flavan dimer formation could come about by opening of the pyran ring of one flavan-3-ol followed by nucleophilic attack at C-2 by the C-6 or C-8 position of a second flavan-3-ol. This type of substitution process is comparable to that observed when catchin is reacted with phloroglucinol at pH 12.[52] The gambirin B group also includes a pentacyclic ring system resulting from *O*-alkylation on the A-ring. Nishioka and co-workers[82] have also isolated a group of complex flavanoid derivatives consisting of a gallic acid-ellagic acid-glucose moiety condensed onto the C-6 or C-8 position of catechin. The carbon-carbon linkage to the A-ring of catechin is from the aldehyde carbon of glucose, which is also attached to the aromatic ring in one of the ellagic acid units. Here again, one can envision electrophilic attack on the A-ring either by the aldehyde carbon of glucose before it reacts with ellagic acid or by the alcohol formed after glucose and ellagic acid condense. Interestingly, more of the C-6 isomer was isolated than of the C-8 isomer.

Several species of plants have been reported to contain prenylflavanoids. [83,84] Compounds have also been identified where the isoprene group is cyclized with the C-7 hydroxyl.[85] The prenyl group would presumably be attached via nucleophilic



Scheme 7. *Formation of the gambirins.*

attack by the A-ring on 3,3-dimethylallyl pyrophosphate, the likely precursor and an effective alkylating agent.[64] Homberger and Hesse[85] have isolated a new type of alkaloid, kopsirachine, where the alkaloid skytanthine is attached at the C-6 and C-8 positions of catechin (Figure 4). Skytanthine is not an obvious candidate for nucleophilic attack. Kopsirachine must arise by a mechanism not as readily related to in vitro chemistry as others we have seen. Larixinol, isolated from *Larix gmelini*, is still another example of an alkylated flavanoid whose biosynthesis would not seem to be straightforward (Figure 4). The authors,[87] however, do suggest a biosynthetic scheme that does indeed involve nucleophilic attack by the A-ring.

Recently, Ferreira and co-workers[88] have reported the isolation of [4,6]- and [4,8]-proguibourtinidin carboxylic acids. The guibourtacacidin group is attached either by 4 → 6 or 4 → 8 bonds to (+)-catechin or (-)-epicatechin with the other position of the phloroglucinolic A-ring occupied by a carboxyl group. The study reports that the 6-carboxylated biflavanoids predominated over the 8-carboxyl derivatives by a 7:5 ratio, but based upon the yields given, this appears to be in error, and the 8-isomers actually predominate. Although the above examples are by no means all inclusive, the variety of substitutents attached to the A-ring of the flavanoids and

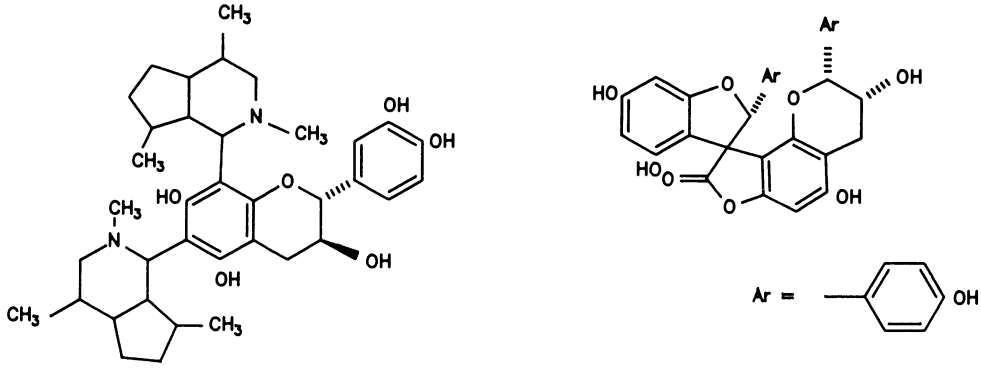


Figure 4. *Kopsirachine and larixinol, unusual examples of alkylation products.*

related compounds does seem to indicate that we have perhaps only scratched the surface. This area of natural products chemistry will probably experience significant expansion in the years ahead. The reactivity of the resorcinolic and phloroglucinolic A-rings will likely provide us with any number of combinations of substituents and flavanoid ring systems in the future.

CONCLUSIONS

Whether one is considering in vivo or in vitro reactions of the A-ring of the flavanoids, the obvious key is the increased nucleophilicity due to the hydroxylation pattern. The activation toward electrophilic aromatic substitution provided by the *meta*-hydroxyls appears to be both a blessing and a curse. For example, the extreme reactivity of the phloroglucinolic condensed tannins certainly limits their usefulness as phenol replacements in adhesive systems. The ease with which substituents may be added to and/or displaced from the A-ring may lead to errors in interpreting results, yields, and structures. Yet, this same reactivity has enabled us to utilize the resorcinolic tannins as phenol replacements. The activating effect of the A-ring makes possible cleavage of the interflavanoid bonds in the procyanidins and subsequent attachment of resorcinol so that they can be utilized in PRF resins.

Because the A-ring is so readily substituted by electrophiles, many new natural products based on A-ring substitution of flavan-3-ols and proanthocyanidins can be expected to be found. One anticipates continued interest and activity in this field of natural products chemistry.

The nucleophilicity of the A-ring makes possible electrophilic aromatic substitution reactions with weak electrophiles so that reactions that would not occur with most aromatic systems are commonplace; e.g., reactions with iodine, diazonnium salts, aldehydes, etc. As one considers utilization of the condensed tannins, surely this is one aspect of their chemistry that most often proves useful.

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REFERENCES

1. Roux, D.G.; Ferreira, D. The direct biomimetic synthesis, structure and absolute configuration of angular and condensed tannins. *Fortschr. Chem. Org. Naturst.* 41:47 (1982).
2. McGraw, G. W.; Hemingway, R.W. Electrophilic aromatic substitution of catechin: Bromination and benzylation. *J. Chem. Soc. Perkin Trans. 1* :974 (1982).
3. Kiatgrajai, P.; Wellons, J.D.; L. Gollob, L.; White, J.D. Kinetics of polymerizaiton of (+)-catechin with formaldehyde. *J. Org. Chem.* 47:2913 (1982).
4. Haslam, E. Biogenetically patterned synthesis of procyanidins. *J. Chem. Soc. Chem. Commun.* :594 (1974).
5. Botha, J.J.; Viviers, P.M.; Ferreira, D.; Roux, D.G. Condensed tannins: Competing nucleophilic centres in biomimetic condensation reactions. *Phytochemistry* 21:1289 (1982).
6. McGraw, G.W.; Hemingway, R.W.; Ohara, S. Reactions of tannin model compounds with methylolphenols. *In:* Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. Symposium Series No. 385, Amercian Chemical Society, Washington, DC. pp. 185-202 (1989).
7. Weinges, K.; Perner, J.; Marx, H.-D. Synthese des octamethyl-diacetyl-procyanidins B3, *Chem. Ber.* 103:2344 (1970).
8. Engel, D.W.; Hattingh, M.; Hundt, H.K.L.; Roux, D.G. X-ray structure, conformation, and absolute configuration of 8-bromotetra-*O*-methyl-(+)-catechin. *J. Chem. Soc. Chem. Commun.* :695 (1978).
9. Kiehlmann, E.; Lehto, N.; Cherniwchan, D. Iodination and deuteration of catechin derivatives. *Can. J. Chem.* 66:2431 (1988).
10. Fronczek, F.R.; Gannuch, G.; Mattice, W.L.; Hemingway, R.W.; Chiari, G.; Tobiason, F.L.; Houglum, K.; Shannafelt, A. Preference for occupancy of axial positions by substitutents bonded to the heterocyclic ring in penta-*O*-acetyl-(+)-catchin in the crystalline state. *J. Chem. Soc. Perkin Trans. 2* :1383 (1985).
11. Hundt, H.K.L.; Roux, D.G. Condensed tannins: Determination of the point of linkage in 'terminal' (+)-catechin units and degradative bromination of 4-flavanylflavan-3,4-diols. *J. Chem. Soc. Chem. Commun.* :696 (1978).
12. Nonaka, G.; Morimoto, S.; Nishioka, I. Tannins and related compounds. Part 13. Isolation and structures of trimeric, tetrameric, and pentameric proanthocyanidins from cinnamon. *J. Chem. Soc. Perkin Trans. 1* :2139 (1983).
13. Young, D.A.; Cronje, A.; Bates, A.L.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 14. Biflavanoid profisetinidins as synthons. The acid-induced 'phlobaphene' reaction. *J. Chem. Soc. Perkin Trans. 1* :2521 (1985).
14. Herrick, F.W.; Bock, L.J. Adhesives from bark extracts. *For. Prod. J.* 8:269 (1958).
15. Hillis, W.E.; Urbach, G. Reactions of polyphenols with formaldehyde. *J. Appl. Chem.* 9:665 (1959).
16. Roux, D.G.; Ferreira, D.; Hundt, H.K.L.; Malan, E. Structure, stereochemistry, and reactivity of natural condensed tannins as basis for their extended industrial applicaiton. *In:* Timell, T.E. (ed.) Proc. of the Eighth Cellulose Conf. Appl. Polym. Symp. No. 28. John Wiley and Sons, New York (1975).
17. Pizzi, A.; Scharfetter, H.O. The chemistry and development of tannin-based adhesives for exterior plywood. *J. Appl. Polym. Sci.* 22:1745 (1978).

18. Plomley, K.F. Tannin-formaldehyde adhesives for wood. CSIRO, Div. For. Prod. Technol. Paper No. 39 (1966).

19. Porter L.J. Viscosity and formaldehyde consumption of procyanidin solutions. *In:* Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. Symposium Series, No. 385, American Chemical Society, Washington, DC. pp. 172-184 (1989).

20. Hemingway, R.W.; McGraw, G.W. Formaldehyde condensation products of model phenols for conifer bark tannins. *J. Liq. Chromatog.* 1:163 (1978).

21. Hillis, W.E.; Urbach, G. The reaction of (+)-catechin with formaldehyde. *J. Appl. Chem.* 9:474 (1959).

22. Ayla, C. *Pinus brutia* tannin adhesives. *J. Appl. Polym. Sci., Appl. Polym. Symp.* 40:69 (1984).

23. Pizzi, A. Tannin-based wood adhesives. *In:* Pizzi, A. (ed.) Wood Adhesives: Chemistry and Technology. Marcel Dekker, Inc., New York, pp. 177-246 (1983).

24. Rossouw, D. du T.; Pizzi, A.; McGillvray, G. The kinetics of condensation of phenolic polyflavonoid tannins with aldehydes. *J. Polym. Sci. Polym. Chem. Ed.* 18:3323 (1980).

25. Foo, L.Y.; Hemingway, R.W. Condensed tannins: reactions of model compounds with furfuryl alcohol and furfuraldehyde. *J. Wood Chem. Technl.* 5:135 (1985).

26. Pizzi, A.; Rossouw, D.du T.; Daling, G.M.E. The role of aldehydes other than HCHO in tannin-based wood adhesives. *Holzforsch. Holzverwert* 32:101 (1980).

27. MacLean, H.; Gardner, J.A.F. Bark extracts in adhesives. *Pulp Paper Mag. Can.* 53:111 (1952).

28. Scharfetter, H.; Pizzi, A.; Roussouw, D.du T. IUFRO Conference on Wood Gluing, Merida, Venezuela, Oct. (1977).

29. Herrick, F.W.; Conca, R.J. The use of bark extracts in coldsetting waterproof adhesives. *For. Prod. J.* 10:361 (1960).

30. Hemingway, R.W.; McGraw, G.W.; Karchesy, J.J. Condensation of hydroxybenzyl alcohols with catechin: a model for methylolphenols in conifer-bark polyflavonoid adhesives. *In:* Phenolic Resins, Chemistry and Applications. Weyerhaeuser Science Symposium 2, Weyerhaeuser Co., Tacoma, Washington, pp. 33-67 (1981).

31. Botha, J.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 4. A direct biomimetic approach to [4,6]- and [4,8]-biflavanoids. *J. Chem. Soc. Perkin Trans. 1* :1235 (1981).

32. Botha, J.J.; Viviers, P.M.; Young, D.A.; duPreez, I.C.; Ferreira, D.; Roux, D.G.; Hull, W.E. Synthesis of condensed tannins. Part 5. The first angular [4,6;4,8]-triflavanoids and their natural counterparts. *J. Chem. Soc. Perkin Trans. 1* :527 (1982).

33. Viviers, P.M.; Botha, J.J.; Ferreira, D.; Roux, D.G.; Saayman, H.M. Synthesis of condensed tannins. Part 7. Angular [4,6;4,8]-prorobinetindin triflavanoids from black wattle ('mimosa') bark extract. *J. Chem. Soc. Perkin Trans. 1* :17 (1983).

34. Porter, L.J.; Foo, L.Y. Leucocyanidin: Synthesis and properties of (2**R**,3**S**,4**R**)-(+)-3,4,5,7,-3',4'-hexahydroxyflavan. *Phytochemistry* 21:2947 (1982).

35. Stafford, H.A.; Lester, H.H. Flavan-3-ol biosynthesis: The conversion of (+)-dihydroquercetin and flavan-3,4-*cis*-diol (leucocyanidin) to (+)-catechin by reductases extracted from cell suspension cultures of Douglas-fir. *Plant Physiol.* 76:184 (1984).

36. Stafford, H.A.; Lester, H.H.; Porter, L.J. Chemical and enzymatic synthesis of monomeric procyanidins (Leucocyanidins or 3',4',5,7-tetrahydroxy-flavan-3,4-diols) from (2**R**,3**R**)-dihydroquercetin. *Phytochemistry* 24:333 (1985).

37. Kolodziej, H. The first 2,3-*trans*-3,4-*cis*-procyanidin. *Phytochemistry* 24:2460 (1985).

38. Delcour, J.A.; Serneels, E.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 13. The first 2,3-*trans*-3,4-*cis*-procyanidins: sequence of units in a 'trimer' of mixed stereochemistry. *J. Chem. Soc. Perkin Trans. 1* :669 (1985).

39. Betts, M.J.; Brown, B.R.; Brown, P.E.; Pike, W.T. Degradation of condensed tannins: structure of the tannin from common heather. *J. Chem. Soc. Chem. Commun.* :110 (1967).

40. Sears, K.D.; Casebier, R.L. Cleavage of proanthocyanidins with thioglycollic acid. *J. Chem. Soc. Chem. Commun.* :1437 (1968).

41. Betts, M.J.; Brown, B.R.; Shaw, M.R. Reaction of flavanoids with mercaptoacetic acid. *J. Chem. Soc. (C)* :1178 (1969).

42. Sears, K.D.; Casebier, R.L. The reaction of thioglycollic acid with polyflavanoid bark fractions of *Tsuga heterophylla*. *Phytochemistry* 9:1589 (1970).

43. Thompson, R.S.; Jacques, D.; Haslam, E.; Tanner, R.J.N. Plant proanthocyanidins. Part 1. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1209 (1982).

44. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Linkage isomerism in trimeric and polymeric 2,3-*cis*-procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1209 (1982).

45. Foo, L.Y.; Porter, L.J. Synthesis and conformation of procyanidin diastereoisomers. *J. Chem. Soc. Perkin Trans. 1* :1535 (1983).

46. Foo, L.Y. Facile self-condensation of melacacidin: a demonstration of the reactivity of the pyrogallol A-ring. *J. Chem. Soc. Chem. Commun.* :1273 (1985).

47. Foo, L.Y. A novel pyrogallol A-ring proanthocyanidin dimer from *Acacia melanoxylon*. *J. Chem. Soc. Chem. Commun.* :236 (1986).

48. Malan, E.; Roux, D.G. Flavonoids and tannins of *Acacia* species. *Phytochemistry* 14:1835 (1975).

49. Hemingway, R.W.; Foo, L.Y. Condensed tannins: quinone methide intermediates in procyanidin synthesis. *J. Chem. Soc. Chem. Commun.* :1035 (1983).

50. Foo, L.Y.; Hemingway, R.W. Condensed tannins: synthesis of the first 'branched' procyanidin trimer. *J. Chem. Soc. Chem. Commun.* :85 (1984).

51. Sears, K.D.; Casebier, R.L.; Hergert, H.L.; Stout, G.H.; McCandlish, L.E. The structure of catechinic acid. A base rearrangement product of catechin. *J. Org. Chem.* 39:3244 (1975).

52. Laks, P.E.; Hemingway, R.W.; Conner, A.H. Condensed tannins. Base-catalyzed reactions of polymeric procyanidins with phloroglucinol: intramolecular rearrangements. *J. Chem. Soc. Perkin Trans. 1* :1875 (1987).

53. Laks, P.E.; Hemingway, R.W. Condensed tannins. Structure of the 'phenolic acids'. *Holzforschung* 41:287 (1987).

54. Steenkamp, J.A.; Steynberg, J.P.; Brandt, E.V.; Ferreira, D.; Roux, D.G. Phlobatannins, a novel class of ring-isomerized condensed tannins. *J. Chem. Soc. Chem. Commun.* :1678 (1985).

55. Steynberg, J.P.; Young, D.A.; Burger, J.F.W.; Ferreira, D.; Roux, D.G. Phlobatannins via facile ring isomerizations of profisetinidin and prorobinetinidin condensed tannin units. *J. Chem. Soc. Chem. Commun.* :1013 (1986).

56. Furlong, J.J.P.; Nudleman, N.S. Base-catalyzed aromatic hydrogen exchange in phenols. *Acta Sud Americana de Quimica* 3:129 (1983).

57. Weinges, K.; Ebert, W.; Huthwelker, D.; Mattauch, H.; Perner, J. Konstitution und Bildungsmechanismus des Dehydro-dicatechins. *Liebigs Ann. Chem.* 726:124 (1969).

58. Hundt, H.K.L.; Roux, D.G. Synthesis of condensed tannins. Part 3. Chemical shifts for determining the 6- and 8-bonding positions of 'terminal' (+)-catechin units. *J. Chem. Soc. Perkin Trans. 1* :1227 (1981).

59. Kiehlmann, E.; van der Merwe, P.J.; Hundt, H.K.L. Synthesis of 6-bromo-, 8-bromo-, and 6,8-dibromocatechin. *Org. Prep. Proc. Int.* 15:341 (1983).

60. Locksley, H.D. The chemistry of biflavonoid compounds. *Fortschr. Chem. Org. Naturstoffe* 30:208 (1973).

61. Hathway, D.E. The condensed tannins. *In:* Hillis, W.E. (ed.) Wood Extractives. Academic Press, New York (1962).

62. Young, D.A.; Young, E.; Roux, D.G.; Brandt, E.V.; Ferreira, D. Synthesis of condensed tannins. Part 19. Phenol oxidative coupling of (+)-catechin and (+)-mesquitol. Conformation of bis-(+)-catechins. *J. Chem. Soc. Perkin Trans. 1* :2345 (1987).

63. Hathway, D.E.; Seakins, J.W.T. Autoxidation of catechin. *Nature* 176:218 (1955).

64. Brown, B.R.; Whiteoak, R.J. Polymerisation of flavans. Part VII. Oxidative polymerisation of catechin. *J. Chem. Soc.* :6084 (1964).

65. Geismann, T.A.; Crout, D.H.G. Organic Chemistry of Secondary Plant Metabolism. Freeman, Cooper and Co., San Francisco. (1969).

66. Hathway, D.E. Autoxidation of polyphenols. Part IV. Oxidative degradation of the catechin-autoxidation polymer. *J. Chem. Soc.* :520 (1958).

67. Jacobs, E.; Ferreira, D.; Roux, D.G. Atropisomerism in a new class of condensed tannins based on biphenyl and *o*-terphenyl. *Tetrahedron Letters* 24:4627 (1983).

68. Tonaka, T.; Nonaka, G.; Nishioka, I. 7-*O*-Galloyl-(+)-catechin and 3-*O*-galloylprocyanidin B-3 from *Sanguisorba officinalis*. *Phytochemistry* 22:2575 (1983).

69. Coxon, D.T.; Holmes, A.; Ollis, W.D.; Vora, V.C.; Grant, M.S.; Tee, J.L. Flavanol digallates in green tea leaf. *Tetrahedron* 28:2819 (1972).

70. Nonaka, G.; Nishioka, I. Tannins and related compounds. VII. Phenylpropanoid-substituted epicatechins, cinchonains from *Cinchona siccirubra*. *Chem. Pharm. Bull.* 30:4268 (1982).

71. Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. VIII. A new type of proanthocyanidin, cinchonains 11a and 11b from *Cinchona succirubra*. *Chem. Pharm. Bull.* 30:4277 (1982).

72. Hemingway, R.W. Biflavonoids and proanthocyanidins. *In:* Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Wood Plants. Springer Verlag, New York (in press).

73. Miyamura, M.; Nohara, T.; Tomimatsu, T.; Nishioka, I. Seven aromatic compounds from the bark of *Cinnamomum cassia*. *Phytochemistry* 22:215 (1983).

74. Oberholzer, M.E.; Rall, G.J.H.; Roux, D.G. (2**R**,3**S**,4**S**)-3,4,7,3',4'-Pentamethoxy-2,3-*trans*-3,4-*cis*-flavan, a novel flavan from *Neorautanenia amboensis*. *Phytochemistry* 19:2503 (1980).

75. duPreez, I.C.; Roux, D.G. Novel flavan-3,4-diols from *Acacia culturiformis*. *J. Chem. Soc. (C)* :1800 (1970).

76. Nonaka, G.; Ezaki, E.; Hayashi, K.; Nishioka, I. Flavanol glucosides from rhubarb and *Raphiolepis imbellata*. *Phytochemistry* 22:1659 (1983).

77. Doskotch, R.W.; Mikhail, A.A.; Chatterjii, S.J. Structure of the water-soluble feeding stimulant for *Scolytus multistriatus*. A revision. *Phytochemistry* 12:1153 (1973).

78. Karl, C.; Muller, G.; Pederson, P.A. Ein neues Catechinglycosid aus *Polypodium vulgare*. *Z. Naturforsch.* 37c:148 (1982).

79. Kashiwada, Y.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XLV. Rhubarb (5). Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chem. Pharm. Bull.* 34:3208 (1986).

80. Morimoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XXXVIII. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark *Cinnamomum cassia* Blume). *Chem. Pharm. Bull.* 34:633 (1986).

81. Nonaka, G.; Nishioka, I. Novel biflavonoids, chalcan-flavan dimers from Gambir. *Chem. Pharm. Bull.* 28:3145 (1980).

82. Nonaka, G.; Nishimura, H.; Nishioka, I. Tannins and related compounds. Part 26. Isolation and structures of stenophyllanins A, B, and C, novel tannins from *Quercus stenophylla*. *J. Chem. Soc. Perkin Trans. 1* :163 (1985).

83. Bohlmann, F.; Zdero, C.; King, R.M.; Robinson, H. New prenylflavanoids from *Marshallia grandiflora*. *Phytochemistry* 18:1246 (1979).

84. Gomez, F.; Quijano, L.; Calderon, J.S.; Rodriquez, C.; Tios, T. Prenylflavans from *Tephrosia watsoniana*. *Phytochemistry* 24:1057 (1985).

85. Bohlmann, F.; Jakupovic, J.; King, R.M.; Robinson, H. Chromones and flavans from *Marshallia obovata*. *Phytochemistry* 19:1815 (1980).

86. Homberger, K.; Hesse, M. Kopsirachin, ein ungewohnliches Alkaloid aus der Apocynacea *Kopsia dasyrachis* Ridl. *Helv. Chim. Acta* 67:237 (1984).

87. Shen, Z.; Haslam, E.; Falshaw, C.P.; Begley, M.J. Procyanidins and polyphenols of *Larix gmelini* bark. *Phytochemistry* 25:2629 (1986).

88. Ferreira, D.; duPreez, I.C.; Wijnmaalen, J.C.; Roux, D.G. Biflavanoid proguibourtinidin carboxylic acids and the biflavanoid homologues from *Acacia luderitzii*. *Phytochemistry* 24:2415 (1985).

CHEMISTRY OF THE CONDENSED TANNIN B-RING

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ABSTRACT

The chemistry of the condensed tannin B-ring is closely related to that of the parent phenols – phenol, catechol, and pyrogallol. Many of the same reactions observed for the simple phenols can be reproduced on the tannin B-ring. These include esterification, etherification, and electrophilic addition for all the types, and metal chelation, oxidation, free-radical scavenging and ketal formation for the catechol and/or pyrogallol related B-rings. The latter four characteristics are particularly important, contributing to the biological activity and potential utilization of the more common types of condensed tannins. The B-ring also has an important effect on the C-2 in the pyran ring. The reactivity of this position is discussed. There is a considerable amount of literature available on the reactions of monomeric flavonoids. Inferences are drawn from this information about comparable reactions being possible for the condensed tannins.

INTRODUCTION

The chemistry of the condensed tannins is dominated by the properties of the A- and B-rings (see Figure 1). The reactivity of the A-ring is of primary importance to the lability of the interflavonoid bond and the use of condensed tannins as adhesives, whereas, the formation of metal complexes and the antioxidant characteristics of the tannins are associated with the B-ring.

Classes of condensed tannins are distinguished on the basis of the hydroxylation pattern in the A- and B-rings. There are three types of the latter – having functionality related to phenol, catechol, and pyrogallol (Figure 2). The chemistry

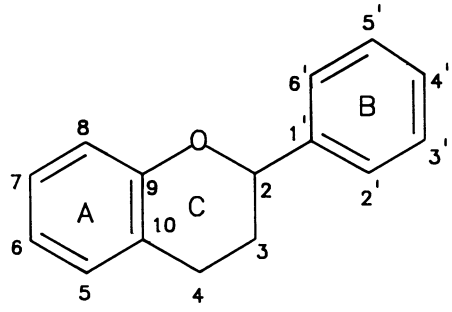
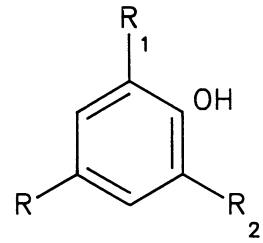


Figure 1. *Labelling and numbering system for a typical condensed tannin monomer unit. Catechin* (**1**) *is the 3,3',4',5,7-pentahydroxyflavan analog.*

of the B-rings reflects that of the parent simple phenols. In general, the degree of reactivity of the phenol and the complexity of its reactions increase with the addition of hydroxyl groups to the benzene nucleus. Comparing phenol to catechol to pyrogallol, there is an increase in acidity, reactivity to electrophiles in addition and substitution reactions, and reducing power. Pyrogallol is a strong reducing agent that can precipitate mercury, silver, and gold from salt solutions.[1] In fact, alkaline solutions of pyrogallol can be used to absorb oxygen from gas mixtures.[2] As will be seen later, the reducing power of the catechol and pyrogallol-derived B-rings is an important characteristic of flavonoids in general.

Monomeric flavonoids such as catechin (**1**) are often used as model compounds when investigating the chemistry of the polymeric condensed tannins. Similarly, the chemical investigation of a flavonoid in, for example, a pharmaceutical application often allows inferences to be drawn about the reactions of a polyflavonoid. An



Phenol related – $R_1 = R_2 = H$; (Propelargonidins, Proquibourtinidins)
Catechol related – $R_1 = H$, $R_2 = OH$; (Procyanidins, Profisetinidins)
Pyrogallol related – $R_1 = R_2 = OH$ (Prodelphinidins, Prorobinetinidins)
R = Remainder of flavanoid monomer unit

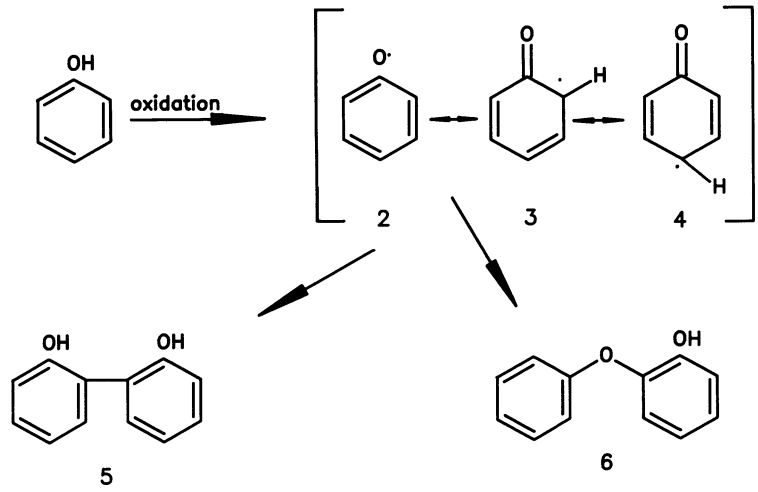
Figure 2. *Types of condensed tannin B-rings.*

important part of the following discussions is the application of information obtained with monomeric flavonoids to the reactivity of the B-ring in the condensed tannins.

OXIDATION

Oxidation is a very important aspect of the chemistry of all phenols, including polyflavonoids. The biosynthetic pathways in plants that include an oxidative coupling step are examples of this importance. Lignin is formed through oxidative coupling of relatively simple phenols. There was also early work proposing that the condensed tannins were formed by enzymatic oxidative coupling[3] or acid-catalyzed polymerization[4] of catechin. Of course, both of these mechanisms have been now shown to be incorrect. There are also many industrial applications for phenolics as antioxidants that are based on the relative ease with which some phenols can be oxidized.[5]

The products that are formed by the oxidation of phenols vary depending on the nature and pattern of substituents on the aromatic ring, and the type of oxidizing agent used. In general, however, phenols with free *ortho* and/or *para* positions react with one-electron oxidants to give aroxyl radicals, resonance stabilized through three major forms [Scheme 1, structures (**2**), (**3**) and (**4**)]. The oxidized phenol can then continue to react through a number of routes based on C-C coupling (e.g., (**5**) and/or ether formation [e.g., (**6**)].[6] Catechol and pyrogallol are oxidized in a similar manner, but with the additional property of being able to form an *ortho*-quinone (**7**) by two-electron oxidation (Figure 3). The quinone can then react with a nucleophile via a Michael reaction to give an addition product. The greater complexity of these di- and trihydroxy phenols results in a greater variety of products being formed. Some more complicated oxidation products from catechol and pyrogallol are shown by structures (**8**) and (**9**), and



Scheme 1. *Oxidation of phenol.*

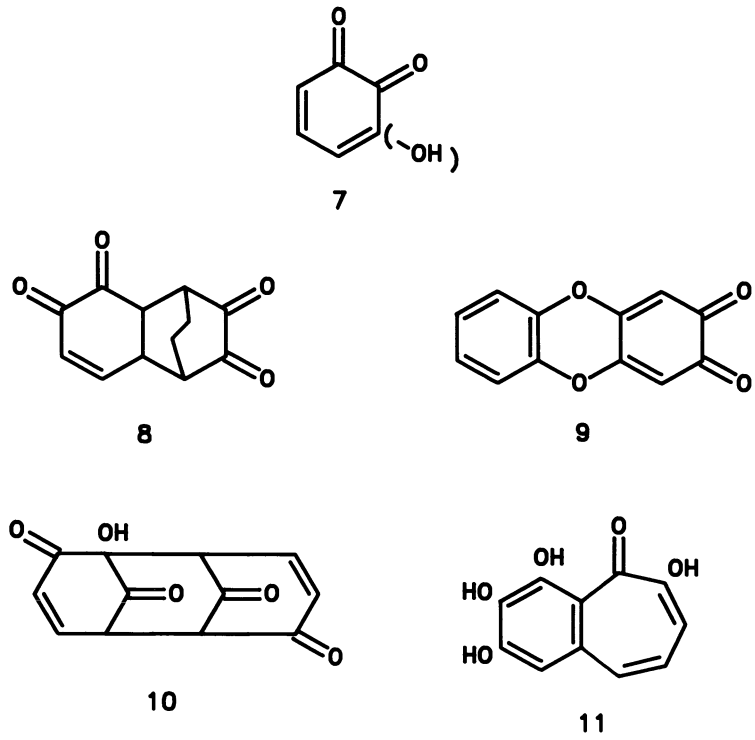


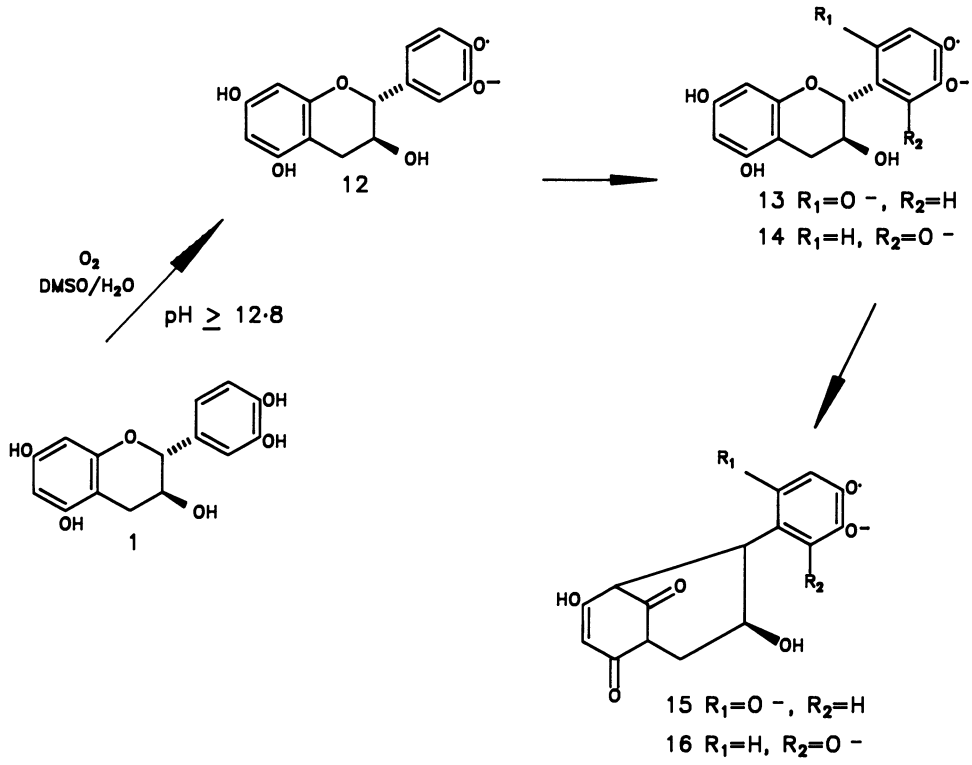
Figure 3. *Oxidative coupling products of catechol and pyrogallol.*

(**10**) and (**11**), respectively. Oxidation to give polymeric products can also occur with most phenols, particularly using metal oxides as the oxidizing agent (e.g., Ag_2O, MnO_2, and PbO_2).[7] As will be seen below, some reactions analogous to these with simple phenols occur with flavonoids.

Hydroxylation

Oxidation with O_2 in highly alkaline solution can lead to hydroxylation of C-4 substituted catechols to give 2,3,4- and 3,4,6-trihydroxy derivatives.[8,9] This same reaction has been shown by Jensen and Pedersen[10] to occur with catechin. The course of this reaction is summarized in Scheme 2. In a solution of DMSO/H_2O at pH 12.8 and saturated with O_2, catechin initially forms a radical anion through the B-ring (**12**), which can be hydroxylated at either C-2′ or C-6′ to give a hexahydroxy derivative of catechin, (**13**) or (**14**). At these high pH's, this compound then readily rearranges to catechinic acid derivatives as described by Sears et al,[11] (**15**) or (**16**).

Epicatechin undergoes a similar set of reactions, but the radical anions are more unstable than with catechin, rapidly rearranging to a catechinic acid derivative. In aqueous ethanol solvent, only C-2′ hydroxylation was observed. The course of these reactions was followed by ESR in this study. The B-ring hydroxylation



Scheme 2. *Oxidation and rearrangement of catechin in alkaline oxygen saturated solutions.*[10]

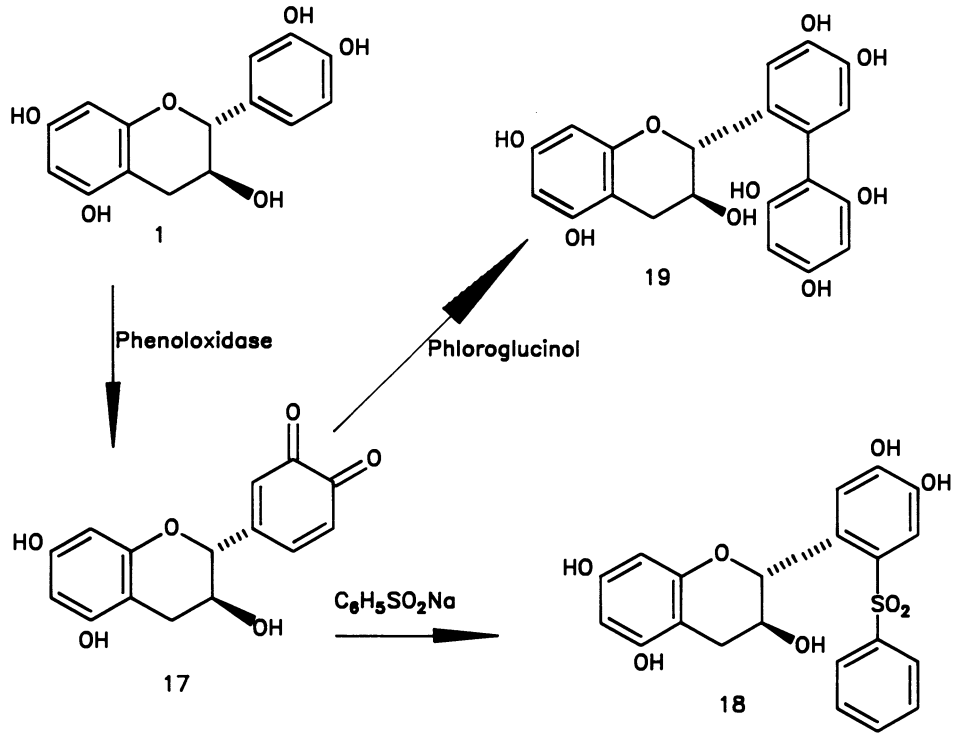
reaction was not observed by Sears et al[11] because they performed their work under oxygen-free conditions. In a related oxidation/hydroxylation reaction, Weinges and Ebert[12] reported the formation of 8-hydroxycatechin from the action of peroxidase and H_2O_2 on catechin. These studies indicate that the site and extent of hydroxylation appears to depend on the reagent, as well as the solvent and other reaction conditions.

There has been one report on the light-induced oxidation of polyflavonoids.[13] It was found that high molecular weight polyflavonoids from *Pinus radiata* bark were rapidly bleached by photolysis in the presence of oxygen. The oxidized product was found to have a high oxygen content (nominal elemental analysis of $C_{15}H_{15}O_{12}$), although the investigators did not indicate how much water was present in the oxidized tannin. The acidity of the oxidized product was attributed to 3.5 carboxylic acid groups per C_{15} unit, possibly formed by attack of singlet oxygen on the flavonoid aromatic rings to form carboxylic and aldehydic groups.

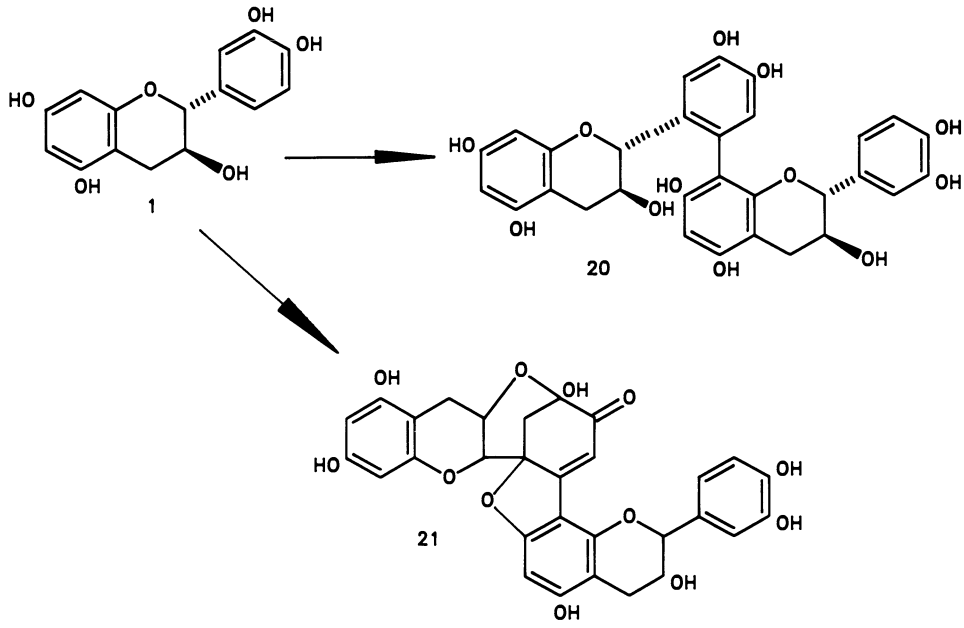
Oxidative Coupling

Under relatively mild oxidizing conditions, the catechin B-ring will form an *ortho*-quinone [(**17**), Figure 4], which can then react with nucleophiles in a similar way to the simple catechols mentioned above, to give an addition product. Only the product coupled to the 6′ position has been reported. In the presence of sodium benzenesulfinate and one of a variety of phenoloxidases, catechin forms a 6′-phenylsulfonyl derivative (**18**).[14,15] The rate of formation of this compound depends on the type of enzyme used (peroxidase, catalase, tyrosinase, or laccase) and the pH of the aqueous buffer reaction solution.[15] This reaction will also occur in the absence of enzyme at pH 6, although more slowly. A similar reaction in the presence of polyphenoloxidase and an excess of phloroglucinol gives the 6′-phloroglucinol derivative (**19**).[16] The latter reaction was performed as a model for the enzyme catalysed polymerization of catechin.

In the absence of any additional nucleophile, the phloroglucinolic A-ring of a second catechin molecule can react with a B-ring *ortho*-quinone to give polymers (Scheme 4). The expected dimer (**20**) has been reported,[16,17] as well as more complicated structures such as dehydrocatechin A (**21**).[18,19] Interestingly, the latter



Scheme 3. *Oxidative coupling of the catechin B-ring under mild conditions with added nucleophiles.*



Scheme 4. *Enzymatic oxidative coupling reactions of catechin without an additional nucleophile.*

compound was recently isolated from the plant, *Euonymus alatus*, as a natural product.[20] The presence of a B- to A-ring linkage in polymeric oxidized catechin has been indicated by the isolation of *meta*-hemipinic acid from the permanganate oxidation of the methylated hydrogenation product of the polymer.[21] More recently, the possibility of flavonoid monomers being incorporated into lignin formed in ray parenchyma at the sapwood/heartwood boundary has been investigated.[22] These workers found that catechin and coniferyl alcohol will copolymerize in the presence of a peroxidase and H_2O_2, apparently through the C-5′ of the catechin B-ring and C-5 of the coniferyl alcohol.

Practical Importance of Flavonoid B-Ring Oxidation

Oxidation with alkaline persulfate has been used as a way of introducing a *para*-hydroxyl group into the B-ring of monomeric flavonoids for synthetic purposes.[23] *Ortho*-hydroxyl groups can be synthesized by the introduction of a formaldehyde group and subsequent oxidation with alkaline hydrogen peroxide to give the phenol.[23]

A longstanding problem with western hemlock wood, especially pulp, has been the development of a brown stain. This is now attributed to oxidation of catechin, which is an extractive in this wood, to colored oxidized polymers.[24,25] As might be expected with this mechanism, antioxidants such as sulfur dioxide and thioglycolic acid are effective for controlling this color formation.[24]

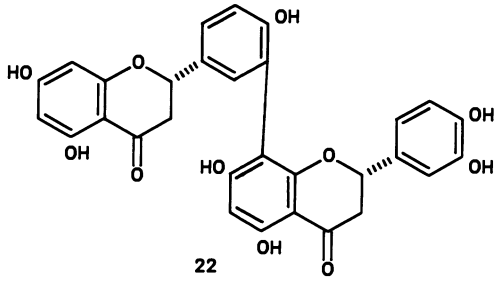
Extensive research has been performed in Europe on flavonoids for their use in pharmaceuticals, especially for treatment of liver disease.[26–29] They also protect against vascular disorders by reducing the fragility and permeability of capillaries. These properties may be related to the antioxidant, or free radical scavenging, properties of the flavonoids.[30] A recent paper determined the free radical scavenging ability of a number of flavonoids.[31] The scavenging ability decreased in the order: myricetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone > robinin > kaempferol > flavone. The activity increases as the reducibility of the B-ring increases, which is proportional to the number of hydroxyl substituents. A similar study was done comparing the antioxidant activity of flavonoids.[32] The antiviral activity of polyflavonoids has been reported to increase with the number of pyrogallol moieties in the molecule, as well as the degree of polymerization.[33] Catechin is marketed in Europe as a pharmaceutical by Zyma SA of Switzerland under the name (+)-Cyanidanol-3 for the treatment of liver diseases.

An excellent summary of the importance of oxidative coupling reactions in natural products has been prepared by Hemingway.[34] Mixed catechin and epicatechin dimers have been isolated from *Quercus robur* bark with C-8 to C-6′ oxidative linkages[35] as well as a very interesting trimer with a C-8 to C-6′ linkage between the upper units and a normal procyanidin C-8 to C-4 bond as the lower linkage.[36] Oxidatively coupled flavan-3-ols have also been found in mesquite (*Prosopis glandulosa*)[37] and later made synthetically by oxidative coupling using $K_3[Fe(CN)_6]$.[38] In *Semecarpus anacardium*, simple coupled flavanone dimers such as [(**22**), Figure 4] are present.[39] More complex catechin-gallocatechin dimers with purpurogallin groupings have been isolated from black tea (**23**).[39] The linkages in these compounds are analogous to the products observed from oxidative coupling of pyrogallol [e.g., (**11**)].

Oxidative coupling may have important consequences on the utilization of condensed tannins[34] (also Chapter 17 of this volume). The yields of acidic nucleophilic solvolysis products from condensed tannins are rarely quantitative. This might be explained by the formation of intra- and/or intermolecular oxidative bonds between polyflavonoid molecules. Oxidations of this type could occur within the plant tissue due to longterm exposure to air, or during the extraction procedure. The latter possibility needs to be taken into account during the commercial extraction of tannins, particularly those containing pyrogallol B-rings.

REDUCTION

Reduction of the flavonoid B-ring is relatively unimportant, except in the metabolism of these compounds. Groenewoud and Hundt[41] found that rat fecal microflora metabolized catechin through opening of the pyran ring and dehydroxylation of the C-4′ hydroxyl to two diarylpropanol compounds (**24**), (**25**). Metabolites from catechin isolated from man are broken down further, but also with *para*-dehydroxylation. Major compounds isolated were *m*-hydroxyphenylpropionic acid, δ-(3,4-dihydroxyphenyl)-γ-valerolactone and δ-3-(hydroxyphenyl)-γ-valero--lactone, as well as their glucuronides and etherial sulphates.[42] There is probably also acidic polymerization of catechin within the stomach.



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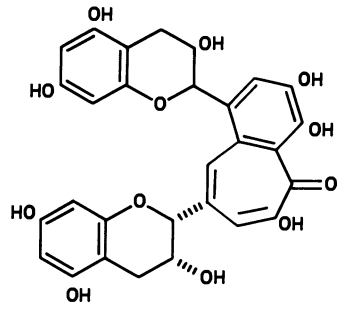

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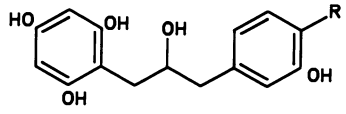
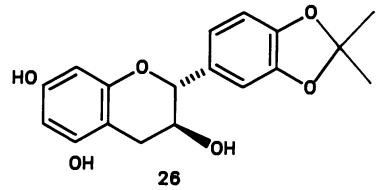


24 R=OH
25 R=H



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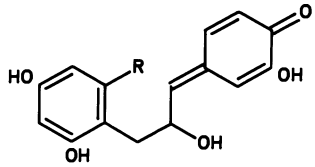
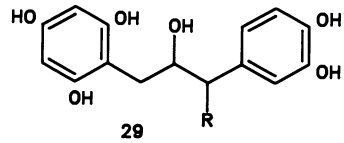


27 R= –O–
28 R= –O·



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METAL COMPLEXATION

A considerable amount of information is available in the literature on the complexing of monomeric flavonoids, particularly flavonols, with metal ions. The formation of these chelates is an important analytical technique used in the determination of the concentration of a variety of metals such as beryllium, magnesium, aluminum, boron, gallium, and uranium.[43] Metal complexation can also be used to determine the concentration of flavonoids.[44] Analysis of chelate formation can be used in structural characterization of flavonoids as well.[45]

There are three possible metal complexing sites within a flavonol containing hydroxyls at C-3,5,3′ and 4′. These are between the C-3 hydroxyl and the carbonyl, the C-5 hydroxyl and the carbonyl, and between the *ortho*-hydroxyls in the B-ring. It has been determined that the first site listed is normally the first occupied, whereas, the latter two sites have about the same complexing ability under neutral conditions.[46] An important conclusion that came out of this type of work is that the complexing ability of a catechol-type B-ring increases as the pH becomes more alkaline.[46,47]

Kennedy and Powell[48,49] have studied the interaction between polyphenols and Al(III) and Fe(III) in their research on the development of podzolic soils. This work was primarily concerned with the stability and solubility of these complexes. Three distinct complexes between catechin and Al(III) were observed – Al(catechin), Al(catechin)$_2$ and Al(catechin)$_3$ – depending on the pH of the solution and molar ratio of metal ion and ligand.[49] Interestingly, the catechin/Al complexes were more stable than the corresponding catechol complexes. This was attributed to the larger catechin molecule disrupting the secondary hydration sphere about the aluminium ion. Spectrophotometric data on the titration of the procyanidin dimer B2 or epicatechin with Fe(III) at neutral pH indicated that complexes with the formula, Fe(L)$_2$, are formed.[48] With a polymeric procyanidin (from quince, DP = 12), precipitates were evident in solutions when the molar ratio of Fe/B-rings exceeded 1:6. The equivalent molar ratios for Al(III), Fe(III) and Cu(II) in the precipitate formed from a solution at pH 6 containing 1:1 (metal/B-rings) stoichiometry were also determined and found to be one mole of metal to 1.9, 1.3, and 2.4 moles of B-rings, respectively.[48]

Practical Importance of Polyflavonoid-Metal Complexation

Even with the lack of research performed on the basic chemistry of the interaction of metal ions with polyflavonoids, quite a number of applications based on tannin chelation have been investigated and patented. This complexing ability was exploited by ITT-Rayonier in their western hemlock bark utilization program. A sulphited condensed tannin extract from western hemlock was prepared as a complex of various metal salts, evidently in the form of a chelate, and used as a source of mineral micronutrients in agricultural applications when applied as a foliar spray.[50] As an additive for boiler and cooling water, the sulphited extracts worked well as dispersants for scale-forming minerals and also as a corrosion inhibitor,[51] taking advantage of both the chelating and antioxidant properties of polyflavonoids.

Copper metal and ions are toxic to most lignocellulose-destroying organisms. However, to make the metal useful as a practical wood or fiber preservative, it is usually complexed with an organic ligand that also has some toxicity. Copper-flavonoid chelates are relatively insoluble in neutral aqueous solutions and have been evaluated as preservatives of various kinds. In one study, fishnets treated with tannins were exposed to Cu^{++} solutions, resulting in a copper-tannin complex having good preservative qualities.[52] The decay resistance of cotton fabrics treated with tannins and copper has also been investigated.[53] Wood preservatives based on copper(II) chelates of sulphited southern pine tannins have been developed[54,55] as well as metal complexes of wattle and quebracho tannins.[56,57] Polyflavonoids naturally found in some woods have been described as causing the poor performance of CCA in these species by complexing with the CCA components and not allowing complete penetration of the wood cell wall.[58]

The use of polyflavonoid-containing barks to remove heavy metals from mining and industrial waste waters has been investigated in North America by Randall.[59–62] The best tree species for this use was found to be coastal redwood, which bound up to 20 percent, by weight, of metal ion. The metal could be stripped, and the substrate regenerated, by treatment with a 0.1 N solution of a strong acid.[59] This is consistent with the observation that strength of a 3,4-diol complex with metals increases with the alkalinity, as described above for monomeric flavonoids. It was observed that color-bleed and loss of chelating capacity could be reduced by prereaction with formaldehyde in acid solution.[61] This would cross-link the polyflavonoid constituents and make them less prone to leaching. Similar results with some exotic species were reported by Kumar and Dara.[63–66] Nitric acid- and formaldehyde-treated, tannin-containing conifer barks have been reported to have absorption efficiencies for uranium in sea water comparable to synthetic agents.[67]

Complexation with metal ions has also been used as a purification method for a procyanidin/starch complex.[68] The organic components of the lead-precipitated complex are regenerated by treatment with EDTA. An aluminum hydroxide complex of (-)-epigallocatechin gallate has been patented as an antiulcer agent.[69] The flavonoid was prepared as a crude tea extract.

Miscellaneous Reactions of the B-Ring

One interesting reaction that can be done with catechol-derived compounds is the formation of ketals. The acetone ketals of catechin (**26**) and epicatechin have been patented as having choleretic, hypocholesterolimic, hypolipaemic, and hepatoprotective effects.[70] Catechin ketals of higher ketones have antibacterial and antifungal properties[71] (Chapter 32). The acetone ketal of red pine bark procyanidin has been prepared.[72]

An important reaction of flavonoids associated with the B-ring is nucleophilic addition to C-2, the carbon atom α to the aromatic ring. It was originally proposed[11] that under alkaline conditions, the pyran ring is opened through a reverse Michael addition to give a quinone methide intermediate (**27**). More recently, Kennedy et al[73] found that opening of the pyran ring for epimerization or nucleophilic addition is greatly slowed by the total exclusion of oxygen. They interpreted this by suggesting the ring-opening occurs through a radical mechanism. Oxidation of catechin leads to an intermediate (**12**), which can ring-open to give (**28**). In either case, the quinone methide formed from catechin can react with a variety of nucleophiles such as sulphite,[74], hydride,[75] resorcinol,[75] phloroglucinol,[76] thiols,[77] and catechin itself.[76] The basic structure of these adducts is shown as (**29**). A patent was taken out on some pharmaceutical properties of the sulfonic acid derivative (**29**), R = SO_3H, although the structure is shown incorrectly in the patent.[78] Sulfonation at C-2 is also important to profisetinidins. Viviers et al[79] have shown that fisetinidin → catechin dimers will react with sulfite to give a derivative with sulfonic acid

residues α to each B-ring. These results suggest that commercial sulfited quebracho tannin has sulfonic acid functionalities in similar positions.

CONCLUSIONS

The B-ring of the polyflavonoids is important to many of the fundamental chemical properties of these materials. The ability to complex metals and to be oxidized to reactive intermediates are perhaps the two most important characteristics that can be attributed to the B-rings that have di- or trihydroxy functionality. In North America, applied research on procyanidins has centered primarily on reactions of the A-ring, which has nucleophilic properties that allow the use of these tannins in adhesive applications. Interest in applications that utilize the metal chelating properties of the tannins is now growing. Further, more fundamental, research on the properties if the B-rings in the polyflavonoids is needed, however, before applications of this type can be fully developed.

REFERENCES

1. Forrester, A.R.; Wardell, J.L. Nuclear hydroxy derivatives of benzene and its homologues. *In*: Coffey, S. (ed.) Rodd's Chemistry of Carbon Compounds. IIIA:289 (1971).
2. Erdtman, H. Formation of complex oxidation and condensation products of phenols - origin and nature of humic acid. I. Reactivity of simple monocyclic quinones. *Proc. Roy. Soc.* 143A:196 (1933).
3. Hathway, D.E.; Seakins, J.W.T. Autoxidation of catechin. *Nature* 176:218 (1955).
4. Weinges, K.; Bahr, W.; Ebert, W.; Goritz, K.; Marx, H.-D. Konstitution, enstsehung, and bedeutung der flavonoid gerbstoffe. *Fortschr. Chem. Org. Naturst.* 17:158 (1969).
5. Scott, G. Atmospheric Oxidation and Antioxidants. Elsevier, Amsterdam (1965).
6. Musso, H. Phenol oxidation reactions. *Angew. Chem. Internat. Edit.* 2:723 (1963).
7. McNelis, E. Oxidative coupling reactions of 2,6 xylenol with activated manganese dioxide. *J. Org. Chem.* 31:1255 (1966).
8. Pedersen, J.A. Electron spin resonance studies of oxidative processes of quinones and hydroquinones in alkaline solution; formation of primary and secondary semiquinone radicals. *J. Chem. Soc., Perkin Trans. 2* :424 (1973).
9. Stone, T.J.; Waters, W.A. Aryloxy-radicals. Part IV. Electron spin resonance spectra of some *ortho*-monobenzosemiquinones and secondary radicals derived therefrom. *J. Chem. Soc.* :1488 (1965).
10. Jensen, O.H.; Pedersen, J.A. The oxidative transformations of (+)-catechin and (-)-epicatechin as studied by ESR. *Tetrahedron* 39:1609 (1983).
11. Sears, K.D.; Casebier, R.L.; Hergert, H.L.; Stout, G.H.; McCandlish, L.E. The structure of catechinic acid. A base rearrangement product of catechin. *J. Org. Chem.* 39:3244 (1974).
12. Weinges, K.; Ebert, W. Isolierung eines kristallisierten dehydrierungsdimeren aus (+)-catechin. *Phytochemistry* 7:153 (1968).
13. Duran, N.; Baeza, J.; Freer, J.; Rojas N. Biomass photochemistry: VI - Light-induced oxidation of phlobaphene from wood. *Polym. Photochem.* 6:393 (1985).
14. Brown, B.R.; Whiteoak, R.J. Polymerisation of flavans. Part VII. Oxidative polymerisation of catechin. *J. Chem. Soc.* :6084 (1964).
15. Piretti, M.V.; Serrazanetti, G.P.; Paglione, G. The enzymatic oxidation of (+)-catechin in the presence of sodium benzenesulphinate. *Ann. Chim.* 67:395 (1977).
16. Ahn, G.-Z; Gstirner, F. Enzymtische dimerisierung von (+)-catechin. *Arch. Pharm.* 303:925 (1970).

17. Weinges, K.; Huthwelker, D. Isolierung and konstitutionsbeweis eines 8,6'-verknupften dehydro-dicatechins (B4). *Liebigs Ann. Chem.* 731:161 (1970).

18. Weinges, K.; Mattauch, H.; Wilkins, C.; Frost, D. Spektorskopiche und chemische konstitutionsaufklarung des dehydro-dicatechins A. *Liebigs Ann. Chem.* 754:124 (1971).

19. Van Soest, T.C. Aufklarung der moleckularstruktur der dehydro-dicatechins A durch rontgenstrukturanalyse seines bromoheptamethylathers. *Liebigs Ann. Chem.* 754:137 (1971).

20. Chen, K.; Pan, D.; Xu, G. Fluvances from Guijianyu (*Euonymus alatus*). *Zhongcaoyao* 17:97 (1986).

21. Hathaway, D.E. Autoxidation of polyphenols. Part IV. Oxidative degradation of the catechin-autoxidation polymer. *J. Chem. Soc.* :520 (1958).

22. Kodera, M.; Tanahashi, M.; Higuchi, T. Dehydrogenative co-polymerization of d-catechin and coniferyl alcohol. *Wood Res.* 65:1 (1979).

23. Seshadri, T.R. Interconversions of flavonoid compounds. *In*: Geissman, T.A. (ed.) The Chemistry of Flavonoid Compounds. MacMillan Company, New York, p. 156 (1962).

24. Barton, G.M. Significance of western hemlock phenolic extractives in pulping and lumber. *For. Prod. J.* 18:76 (1968).

25. Hrutfiord, B.J.; Luthi, R.; Hanover, K.F. Color formation in western hemlock. *J. Wood Chem. Technol.* 5:451 (1985).

26. Slater, T.F.; Scott, R. The free-radical scavenging action of (+)-Cyanidanol-3 in relation to the toxicity of carbon tetrachloride. *In*: Conn, H.O. (ed.). International Workshop on (+)-Cyanidanol-3 in Diseases of the Liver. Royal Society of Medicine, International Congress and Symposium Series No. 47; Academic Press, London; pp. 33-39 (1981).

27. Hackett, A.M.; Shaw, I.C.; Griffiths, L.A. The prevention by (+)-Cyanidanol-3 of hepatitis-induced changes in the disposition of imipramine in the rat. *Biochem. Pharm.* 33:2179 (1984).

28. Baumann, J.; Wurm, G.; Bruchhausen, F.v. Hemmung der prostaglandinsynthetase durch flavonoide und phenolderivate im vergleich mit deren O_2-radikalfangereigenschaften. *Arch. Pharm. (Weinheim)* 313:330 (1980).

29. Younes, M.; Siegers, C.-P. Inhibitory action of some flavonoids on enhanced spontaneous lipid peroxidation following glutathione depletion. *Planta Med.* 43:240 (1981).

30. Sorata, Y.; Takahama, U.; Kimura, M. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Biochim. et Biophys. Acta* 799:313 (1984).

31. Husain, S.R.; Cillard, J.; Cillard, P. Hydroxy radical scavenging activity of flavonoids. *Phytochemistry* 26:2489 (1987).

32. Torel, J.; Cillard, J.; Cillard, P. Antioxidant activity of flavonoids and reactivity with peroxy radical. *Phytochemistry* 25:383 (1986).

33. Takechi, M.; Tanaka, Y.; Takehara, M.; Nonaka, G.-I.; Nishioka, I. Structure and antiherpetic activity among the tannins. *Phytochemistry* 24:2245 (1985).

34. Hemingway, R.W. Biflavonoids and proanthocyanidins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, New York, Chapter 6.6. (in press).

35. Ahn, B.-Z.; Gstirner, F. Uber catechin dimere der eichenrinde. *Arch. Pharmaz.* 304:666 (1971).

36. Ahn, B.-Z. Ein catehin trimer aus der eichenrinde. *Arch. Pharmaz.* 307:186 (1974).

37. Brandt, E.V.; Bezuidenhoudt B.C.B.; Roux, D.G. Direct synthesis of the first natural bi-isoflavonoid. *J. Chem. Soc. Chem. Commun.* :1409 (1982).

38. Young, D.A.; Young, E.; Roux, D.G.; Brandt, E.V.; Ferreira, D. Synthesis of condensed tannins. Part 19. Phenol oxidative coupling of (+)-catechin and (+)-mesquitol. Conformation of bis-(+)-catechins. *J. Chem. Soc. Perkin Trans. 1* :2354 (1987).

39. Muthy, S.S.N. Partial conversions in biflavonoids: Part 5. Confirmation of the structure of jeediflavonone, a biflavonone from *Semecarpus anacardium*. *Phytochemistry* 23:925 (1984).
40. Collier, P.D.; Bryce, T.; Mallows, R.; Thomas, P.E.; Frost, D.J.; Korver, O.; Wilkins, C.K. The theaflavins of black tea. *Tetrahedron* 29:125 (1973).
41. Groenewoud, G.; Hundt, H.K.L. The microbial metabolism of (+)-catechin to two novel diarylpropan-2-ol metabolites in vitro. *Xenobiotica* 14(9):711 (1984).
42. Das, N.P. Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. *Biochem. Pharm.* 20:3435 (1971).
43. Katyal, M. Analytical reactions of hydroxyflavones. *Talanta* 24:367 (1977).
44. Dowd, L.E. Spectrophotometric determination of quercetin. *Anal. Chem.* 31(7):1184 (1959).
45. Sekhon, B.S.; Kaushal, G.P.; Bhatia, I.S. Use of zirconium(IV) and antimony(III) for structural investigation of flavonoids. *Mikrochim. Acta (Wien)* II:421 (1983).
46. Porter, L.J.; Markham, K.R. The aluminium(III) complexes of hydroxyflavones in absolute methanol. Part II. Ligands containing more than one chelating site. *J. Chem. Soc. (C)* :1309 (1970).
47. Jurd, L.; Geissman, T.A. Absorption spectra of metal complexes of flavonoid compounds. *J. Org. Chem.* 21:1395 (1956).
48. Kennedy, J.A.; Powell, H.K.J. Polyphenol interactions with aluminium (III) and iron (III): Their possible involvement in the podzolization process. *Aust. J. Chem.* 38:879 (1985).
49. Kennedy, J.A.; Powell, H.K.J. Aluminium(III) and iron(III) 1,2-diphenolato complexes: a potentiometric study. *Aust. J. Chem.* 38:659 (1985).
50. Durkee, G.E. Micronutrient foliar sprays. *Agrichem. West* 1:17 (1965).
51. Herrick, F.W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food Chem.* 28:228 (1980).
52. Cecily, P.J.; Kunjappan, M.K. Preservation of cotton fish net twines by tanning: II. Fixation of tannin. *Fish. Technol.* 10:24 (1973).
53. Furry, M.S.; Humfield, H. Mildew-resistant treastment on fabrics. *Ind. Eng. Chem.* 33:538 (1941).
54. Laks, P.E.; McKaig, P.A.; Hemingway, R.W. Flavonoid biocides: Wood preservatives based on condensed tannins. *Holzforschung* 42:299 (1988).
55. Laks, P.E. Wood preservation as trees do it. Proceedings of the American Wood Preservers' Association. p. 147 (1988).
56. Lotz, W.R.; Holloway, D.F. Wood Preservation. U.S. 4,732,817 (1988).
57. Schmidt, E.L.; Lotz, W.R. Tropical wood extracts as preservatives for southern pine. Proceedings of the American Wood Preservers' Association. p. 173 (1988).
58. Pizzi, A.; Conradie, W.E.; Jansen, A. Polyflavonoid tannins - A main cause of soft-rot failure in CCA-treated timber. *Wood Sci. Technol.* 20:71 (1986).
59. Randall, J.M.; Bermann, R.L.; Garrett, V.; Waiss, A.C.Jr. Use of bark to remove heavy metal ions from waste solutions. *For. Prod. J.* 24(9):80 (1974).
60. Randall, J.M.; Hautala, E.; Waiss, A.C.Jr. Removal and recycling of heavy metal ions from mining and industrial waste streams with agricultural byproducts. Proceedings of the Fourth Mineral Waste Utilization Symposium. Chicago, Illinois, May 7-8 (1974).
61. Randall, J.M.; Jautala, E.; Waiss, A.C.Jr.; Tschernitz, J.L. Modified barks as scavengers for heavy metal ions. *For. Prod. J.* 26(8):46 (1976).
62. Randall, J.M. Variations in effectiveness of barks as scavengers for heavy metal ions. *For. Prod. J.* 27(11):51 (1977).
63. Kramer, P.; Dara, S.S. Utilisation of agricultural wastes for decontaminating industrial/domestic wastewaters from toxic metals. *Agric. Wastes* 4:213 (1982).

64. Kumar, P.; Dara, S.S. Studies on binding of copper ions by some natural polymeric materials. *Chemical Era* December:20 (1979).

65. Kumar, P.; Dara, S.S. Modified barks for scavenging toxic heavy metal ions. *Indian J. Environ. Health.* 22(3):196 (1980).

66. Kumar, V.; Sindhu, R.S. Removal of lead ions from its solution by the bark of *Adina cordifolia. Proc. Nat. Acad. Sci. India* 55(B), I:94 (1985).

67. Fujii, M.; Shioya, S.-I. Nitric acid-formaldehyde treated coniferous barks as recoverying agents of uranium from sea water. *ISWPC Posters* :97 (1987).

68. Rahman, M.D.; Richards, G.N. Interactions of starch and other polysaccharides with condensed tannins in hot water extracts of ponderosa pine bark. *J. Wood Chem. Technol.* 8:111 (1988).

69. Hara, M.; Asai, H.; Kitamikado, T.; Yamamoto, H.; Okushio, K.; Nakamura, K. Preparation of (-)-epigallocatechin gallate-aluminum hydroxide complex as antiulcer agent from tea extracts. Jpn. Kokai Tokkyo Koho JP 61,238,728 (1986).

70. Bonati, A.; Mustich, G. Pharmacologically Active Polyphenolic Substances. U.S. 4,166,861 (1979).

71. Laks, P.E.; Pruner, M.S. Flavonoid biocides: Structure/activity relations of flavonoid phytoalexin analogues. *Phytochemistry* 28:87 (1989).

72. Laks, P.E. (unpublished results 1987).

73. Kennedy, J.A.; Munro, M.H.G.; Powell, H.K.J.; Porter, L.J.; Foo, L.Y. The protonation reactions of catechin, epicatechin and related compounds. *Aust. J. Chem.* 37:885 (1984).

74. Yazaki, Y.; Hillis, W.E. Molecular size distribution of radiata pine bark extracts and its effect on properties. *Holzforschung* 34:125 (1980).

75. Weinges, K.; Toribio, F.; Paulus, E. Die konformation als ursache sterioselektiver reaktionen. *Ann.* 688:127 (1965).

76. Mayer, W.; Merger, F. Condensation of (+)-catechin with phloroglucinol: a model for the condensation of catechins and catechin tannins. *Chem. and Indust.* April:485 (1959).

77. Laks, P.E.; Hemingway, R.W. Condensed tannins: base-catalyzed reactions of polymeric procyanidins with toluene-α-thiol. Lability of the interflavanoid bond and pyran ring. *J. Chem. Soc. Perkin Trans. 1* :465 (1987).

78. Courbat, P.; Valenza, A. Medicaments containing epicatechin-2-sulfonic acids and salts thereof. U.S. 3,888,990 (1975).

79. Viviers, P.M.; Kolodziej, H.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 11. Intramolecular enantiomerism of the constituent units of tannins from the *Anacardiaceae*: stoichiometric control in direct synthesis: derivation of ^{1}H nuclear magnetic resonance parameters applicable to higher oligomers. *J. Chem. Soc. Perkin Trans. 1* :2555 (1983).

REACTIONS AT THE INTERFLAVANOID BOND OF PROANTHOCYANIDINS

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ABSTRACT

Condensed tannins with a 5,7-dihydroxy A-ring are particularly susceptible to interflavanoid bond cleavage under either acidic or basic conditions. The lability of the interflavanoid bond in this class of tannins has been important in development of analytical tools for determination of their structure and for their synthesis. The greatest hope for the use of condensed tannins of the 5,7-dihydroxy class as a renewable source of specialty chemicals lies in exploitation of the lability of the interflavanoid bond. Examples include the synthesis of tannin derivatives for use in cold-setting phenolic resins and biocides. Reductive cleavage offers the potential for production of significant yields of flavan-3-ols and low molecular weight proanthocyanidins. Cleavage of the interflavanoid bond with sulfite ion under mild acidic or alkaline conditions produces flavan- and oligomeric procyanidin-4-sulfonates that are useful intermediates for formulation of fast-setting adhesives. We are just beginning to learn how to use base-catalyzed cleavage reactions to our advantage. It can be anticipated that novel uses will be developed from both acid- and base-catalyzed cleavage products of condensed tannins as further understanding of these reactions is obtained.

INTRODUCTION

The facile cleavage of interflavanoid bonds in proanthocyandins with phloroglucinolic A-rings (i.e., procyanidins and prodelphinidins) is another important feature of this class of tannins that sets them distinctly apart from those with resorcinolic A-rings (i.e., profisetinidins and prorobinetinidins, Chapter 5). Acid-catalyzed cleavage of the interflavanoid bond in condensed tannins in alcohols with

HCl in the presense of oxygen leads to anthocyanidins.[1,2] Cleavage with weak acids in the presence of thiols,[3,4] gives flavan-4- or oligomeric proanthocyanidin-4-sulfide adducts. Both reactions have long been important analytical tools used in determining the structure of polymeric procyanidins. In the past 10 years, reactions involving cleavage of the interflavanoid bond have been studied further to define the structure of polymers, understand the reactions of condensed tannins under commercial processing conditions, and synthesize products with particular properties for commercial use as specialty chemicals. This chapter summarizes recent advances in these applications.

ACID-CATALYZED CLEAVAGE

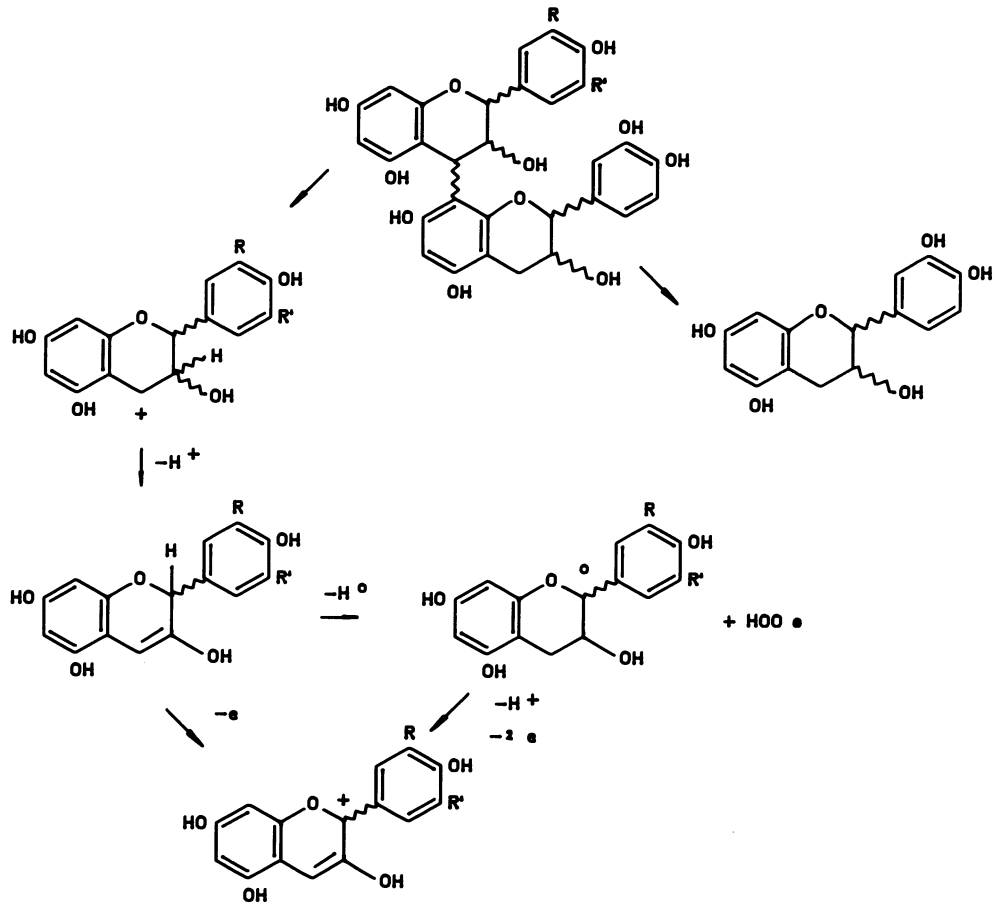
To date, most of the research on reactions at the interflavanoid bond has centered on acid-catalyzed cleavage as an analytical tool. However, use of acid-catalyzed cleavage reactions in the production of specialty chemicals from procyanindins and prodelphinidins holds considerable promise.

Anthocyanidin Formation

Acid-catalyzed cleavage of the interflavanoid bond with subsequent oxidation to yield anthocyanidins has historically[1] been one of the more important reactions of condensed tannin chemistry (Scheme 1). This reaction[2] is used as an indicator for the presence of condensed tannins in plant tissues and as a simple and effective way of estimating the proportions of propelargonidins, procyanidins, and prodelphinidins in tannins when coupled with paper or cellulose thin-layer chromatography of the resultant anthocyanidins using Forestal solvent (acetic acid-water-concentrated HCl, 30:10:3, v/v/v).

Typically, approximately 10 mg of tannin is dissolved in 20 ml of *n*-butanol-concentrated HCl (95:5, v/v) and heated for 40 minutes to produce intense red-colored solutions. Individual anthocyanidins can be isolated by preparative paper chromatography. For comparisons of relative yields, the absorbance is measured at 550 nm or the spectra are recorded with λ_{max} in ethanol-HCl for 530 nm for pelargonidin, 545 nm for cyanidin, and 557 nm for delphinidin.

The conversion of condensed tannins to anthocyanidins, although not quantitative, is still one of the most convenient and widely used analytical methods available for estimating the amounts of condensed tannins in plant tissues. Porter[5] has examined this reaction recently in an attempt to improve its use as a quantitative method and recommended the addition of ferric ion as a 2-percent solution of ammonium ferric sulfate to a methanolic solution of the tannin in the originally proposed *n*-butanol-HCl solution and heating at 90 °C for 40 minutes. This gave a substantial increase in anthocyanidin yield and improved the reproducibility of the results. The $E^{1\%}$ at 550 nm in this solvent ranged from 225 for epicatechin-(4β → 6)-catechin to 460 for **2R**,**3S**,**4R**-leucocyanidin and between 450-490 for various procyanidin polymers. Porter found that increased yields were obtained using



Scheme 1. *Anthocyanidin formation from condensed tannins as proposed by Porter.*[5]

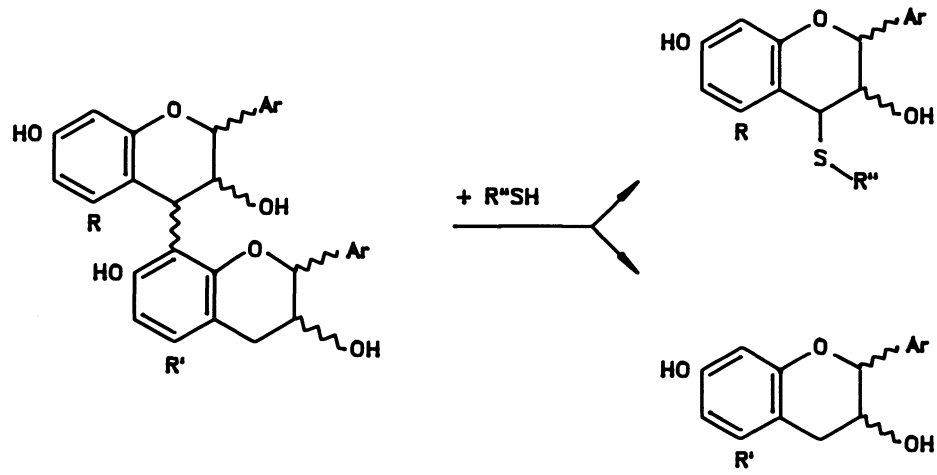
either Fe(II) or Fe(III) as a catalyst. Tiarks[6] found that Fe(II), added as ferrous sulfate, gave a much higher yield than was obtained using ferric sulfate as a catalyst in reactions that did not contain methanol. This suggests that the mechanism(s) involved in the oxidation to cyanidin are complex and that additional study of the mechanism of anthocyanidin formation could be fruitful.

Anthocyanidins are of considerable interest in the food industry. The excellent review of the chemistry of these compounds by Iacobucci and Sweeny[7] and recent U.S. Food and Drug Administration actions banning additional red dyes suggest that there is considerable potential for development of red colorants from condensed tannins if ways can be found to increase the yield and stability of the chromophores. Although Timberlake's reaction[8] with catechin and acetaldehyde does not appear

to have been pursued further, this work does suggest an approach to improving the stability of these chromophores that deserves more attention.

Thiolysis

Reactions of condensed tannins with weak acids in the presence of various nucleophiles became extremely important analytical tools after the late 1960's because the stereochemistry at C-2 and C-3 of the chain extender units is preserved (Scheme 2). Brown and coworkers[3] first used acid-catalyzed thiolysis in studies of the structure of a proanthocyanidin polymer from heather (*Calluna vulgaris*). Due to the ease with which flavan-4-thioethers were produced from this tannin, the interflavanoid bond was thought to be a benzyl ether linkage. Soon thereafter, Sears and Casebier[4] demonstrated that catechin-(4α → 2)-phloroglucinol was readily cleaved in reactions with thioglycollic acid, and similar reactions of isolated tannins or the bark of western hemlock (*Tsuga heterophylla*) followed by methylation gave both 2,3-*cis*- and 2,3-*trans* methyl (3-hydroxy-5,7,3′,4′-tetramethoxyflavan-4-yl-thio) acetates in approximately equal yield.[9] They postulated that the tannins in western hemlock bark were composed of approximately equal proportions of 2,3-*trans* (catechin) and 2,3-*cis* (epicatechin) chain extender units, a conclusion that has since been supported by ^{13}C-NMR studies.[10]



Where A-ring is:
R = H, R' = H; No Reaction
R = H, R' = OH; Low yield at 120 °C
R = OH, R' = H; Low yield at 120 °C
R = OH, R' = OH; 30% to quantitative yield at 105 °C

Where upper unit stereochemistry is:
2,3-*cis*; Stereospecific 3,4-*trans* adduct
2,3-*trans*; Stereoselective 3,4-*trans* > 3,4-*cis* adducts

Scheme 2. *Thiolytic cleavage of western hemlock bark tannins.*

Brown[11,12] continued to perfect thiolysis reactions, and acid-catalyzed cleavage using toluene-α-thiol or benzenethiol as nucleophiles became standard analytical practice through the 1970s to early 1980s.[13] Through use of partial thiolytic cleavage, it was possible to demonstrate heterogeneity of the interflavanoid bond location in oligomeric and polymeric procyanidins in *Pinus taeda*[14] and *Pinus palustris* bark tannins, *Photinia glaubrescens* leaf tannins,[15,16] and *Areca catechu* nut shell tannins.[17] While results obtained from thiolytic cleavage have been consistent with those obtained by ^{13}C-NMR analyses of procyanidins and prodelphinidins,[18,19] few examples[20] have been reported of recovery of thio-adducts from polymers in quantitative yields – a worrisome fact that deserves more attention.

In order to interpret the results of partial thiolytic cleavage, it is important to know what differences exist in the lability of the interflavanoid bonds of proanthocyanidins of different structures. Beart et al[21] showed that acid-catalyzed thiolytic cleavage was first order in hydrogen ion concentration and that the rate-determining step was protonation of the A-ring of the lower unit. These reactions have been employed to study the degradation of only one representative of the 5-deoxy oligomers. Epiafzelechin and guibourtacacidol-(4α)-benzyl sulfide were obtained from the dimer isolated from *Cassia fistula* by reactions catalyzed with acetic acid but only at 140 °C under pressure.[22] Likewise, epicatechin-(4$\beta \rightarrow$ 2)-resorcinol was essentially stable in reactions with benzene-thiol and acetic acid at 100 °C.[23] In comparison, the oligomeric procyanidins are completely cleaved in 8 hours or less at 90 °C.[24]

Comparisons of the rate of cleavage of epicatechin-(4$\beta \rightarrow$ 8)-catechin, epicatechin-(4$\beta \rightarrow$ 8)-epicatechin, catechin-(4$\alpha \rightarrow$ 8)-catechin, epicatechin-(4$\beta \rightarrow$ 6)-epicatechin, and epicatechin-(4$\beta \rightarrow$ 6)-catechin in acetic acid-catalyzed reactions with toluene-α-thiol showed large differences in the lability of the interflavanoid bonds associated with structure. Compounds with an axial flavan unit linked at C-8 (i.e., 4$\beta \rightarrow$ 8 bonds) were cleaved more rapidly than those with an equatorial flavan unit linked at C-8 (i.e., 4$\alpha \rightarrow$ 8) and much more rapidly than the compounds with axial flavan units linked at C-6 (i.e., 4$\beta \rightarrow$ 6) (Figure 1). Therefore, caution must be exercised in the interpretation of the ratio of (4$\beta \rightarrow$ 6) and (4$\beta \rightarrow$ 8) linked products from partial thiolysis. These results were used in conjunction with the equilibrium ratio of (4$\beta \rightarrow$ 8) and (4$\beta \rightarrow$ 6) linkages (1.3 to 1) obtained in a procyanidin polymer after a long period of rearrangement in acetic acid solutions to estimate the relative reactivity of the C-8 and C-6 positions of the A-ring with flavan-4-carbocations or quinone methides (Chapter 16). The ratio of 3.3 to 1 was very similar to the ratio of (4$\beta \rightarrow$ 8) and (4$\beta \rightarrow$ 6) found in oligomeric and polymeric procyanidins isolated from a variety of plants.[18,19] Since the interflavanoid bonds of procyanidins are labile at pH 3-4 and ambient temperature, some disproportionation of interflavanoid bond location could occur during isolation and purification of tannin extracts.[21,24] Where the interflavanoid bonds are regiospecific, as suggested in the tannins of sorghum,[20] enzymic control in the biogenesis of the polymer seems necessary (Chapter 2). As mentioned earlier, the comparatively low yield of flavan-4-thio adducts that is usually obtained from tannins (frequently only about 30 percent) should cause one to question the validity of the simple polymeric proanthocyanidin structures as suitable models for these polymers.

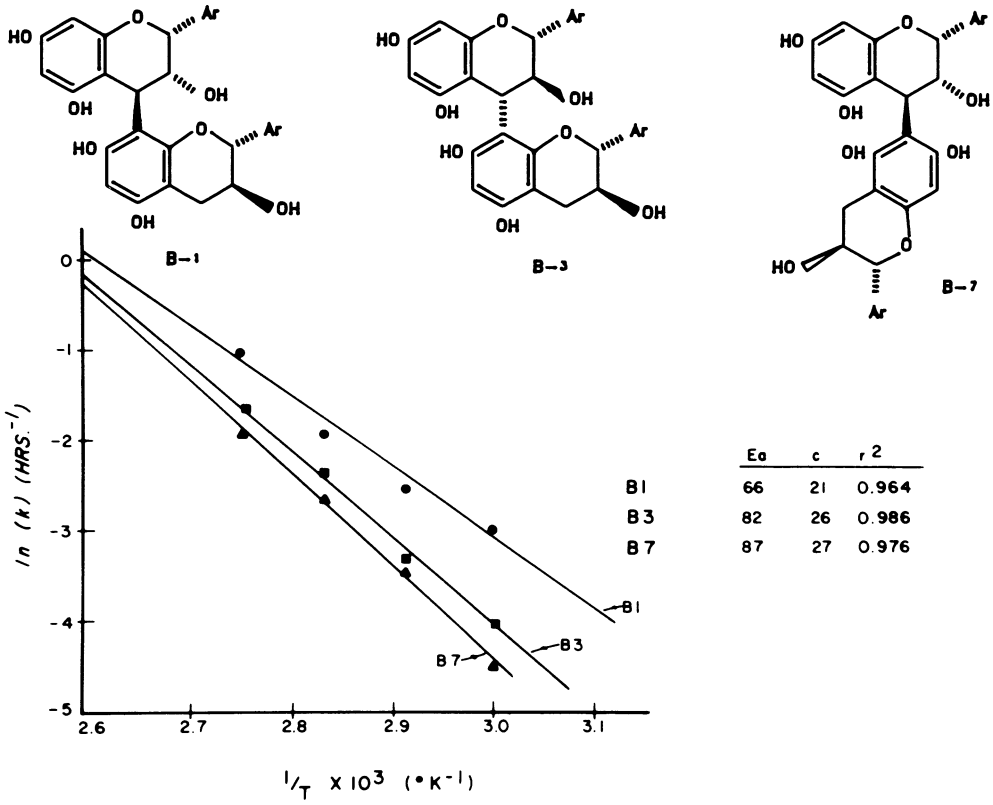


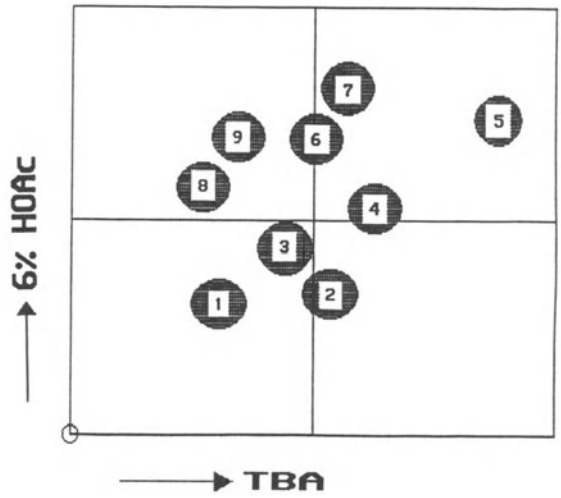
Figure 1. *Relative lability of the interflavanoid bonds of dimeric procyanidins of different bond configurations.*

Phloroglucinol Adducts

Use of phloroglucinol in place of thiols as the nucleophile offers many advantages, not the least of which is that the former compound is odorless.[25,26] In addition, there is much more selectivity in the formation of 3,4-*trans* adducts from the 2,3-*trans* proanthocyanidins and better separation of the products by two-dimensional cellulose thin-layer chromatography (Figure 2)[27] or by column chromatography, the latter providing good separations on Sephadex LH-20 when eluting with ethanol or ethanol-water mixtures. Finally, the phloroglucinol adducts serve as much better models for interpretation of ^{13}C-NMR spectra of related higher molecular weight oligomers.[18,19]

Synthesis of Oligomeric Procyanidins

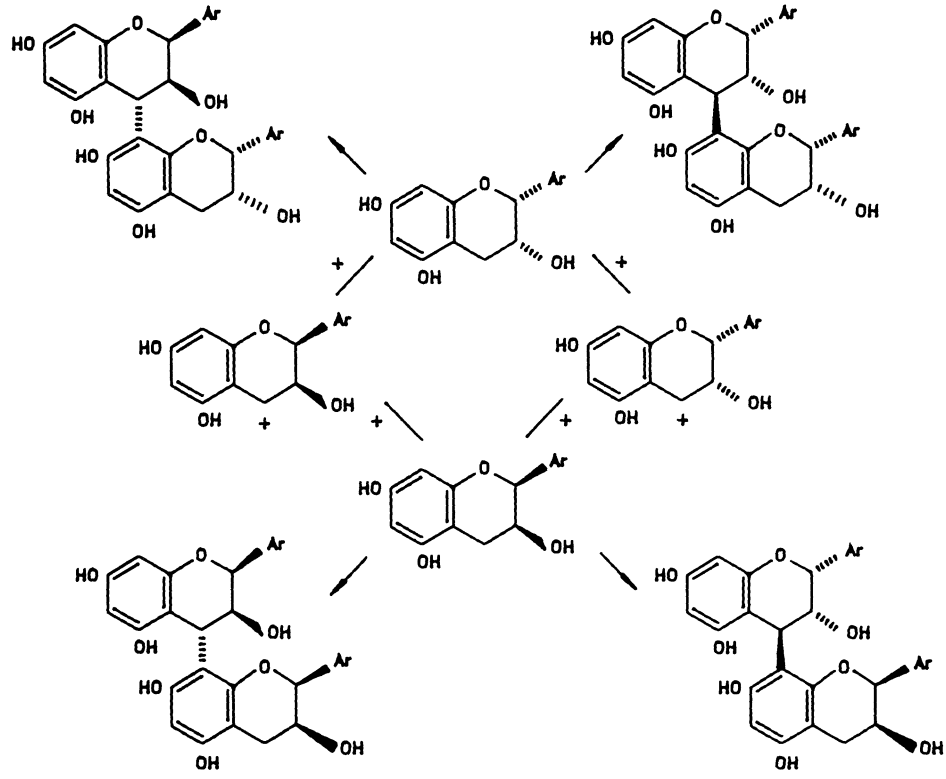
Most of the oligomeric procyanidins and prodelphinidins found as natural products have also been synthesized using similar reaction conditions, but with



1 = epigallocatechin; 2 = epicatechin; 3 = gallocatechin;
4 = catechin; 5 = phloroglucinol; 6 = epicatechin-4-phloroglucinol;
7 = catechin-4-phloroglucinol; 8 = epigallocatechin-4-phloroglucinol;
9 = gallocatechin-4-phloroglucinol.

Figure 2. *Two-dimensional cellulose TLC of cleavage products using phloroglucinol as a nucleophile.*[27]

flavan-3-ols as the nucleophiles.[14–16,28–30] The work of Foo and Porter[29] on the synthesis of procyanidin diastereoisomers is an interesting example of the use of interflavanoid bond cleavage to produce oligomeric proanthocyanidins. Although most plants contain proanthocyanidins with a (2**R**) configuration (Chapter 4), the procyanidins in the Palmaceae contain 2,3-*cis* procyanidin units with both 2**R** and 2**S** configuration. Acid-catalyzed cleavage of the palm tannins with either epicatechin or *ent*-epicatechin as nucleophiles afforded two pairs of enantiomers: one pair epicatechin-(4β → 8)-epicatechin and *ent*-epicatechin-(4β → 8)-*ent*-epicatechin, and the other *ent*-epicatechin-(4β → 8)-epicatechin and epicatechin-(4β → 8)-*ent*-epicatechin (Scheme 3). These compounds proved to be especially useful in studies of the conformation of dimeric procyanidins. For example, the ^{1}H-NMR spectrum of the peracetate of epicatechin-(4β → 8)- epicatechin shows the presence of two rotamers in relative proportions of about 3.3:1. The major rotamer has the upper unit projecting behind the plane of the lower unit, and the heterocyclic ring of the upper unit is a C(3) sofa (Figure 3). The predominant conformer in catechin-(4α → 8)-catechin and catechin-(4α → 8)-*ent*-catechin has the upper unit projecting out from the plane of the lower unit, and the heterocyclic rings are best described as half-chairs.



Scheme 3. *Synthesis of enantiomeric proanthocyanidins by acid-catalyzed cleavage of palm tannins in the presence of epicatechin and ent-epicatechin.*[29]

Application of Acid-Catalyzed Cleavage in Tannin-Based Adhesives

Most of the acid-catalyzed cleavage reactions of condensed tannins have been directed to the structural elucidation of these polymers. However, Kreibich and Hemingway[31,32] have studied the possibility of producing procyanidin-(4β → 4)-resorcinol adducts by reaction of tannins extracted from southern pine tree barks with acetic acid and resorcinol. Although resorcinol is not nearly as good a nucleophile as the phloroglucinolic A-ring of the tannins, epicatechin-(4β → 4)-resorcinol and procyanidin-(4β → 4)-resorcinol adducts accumulate over time because the interflavanoid bond in these compounds is essentially stable in comparison to the labile bonds in the polymeric procyanidins (Scheme 4).[32]

If co-extracted carbohydrate levels are not excessive, these products can be used directly as substitutes for resorcinol in cold-setting, wood-laminating adhesives. Where carbohydrate concentrations approach 20 percent of the tannin extract,

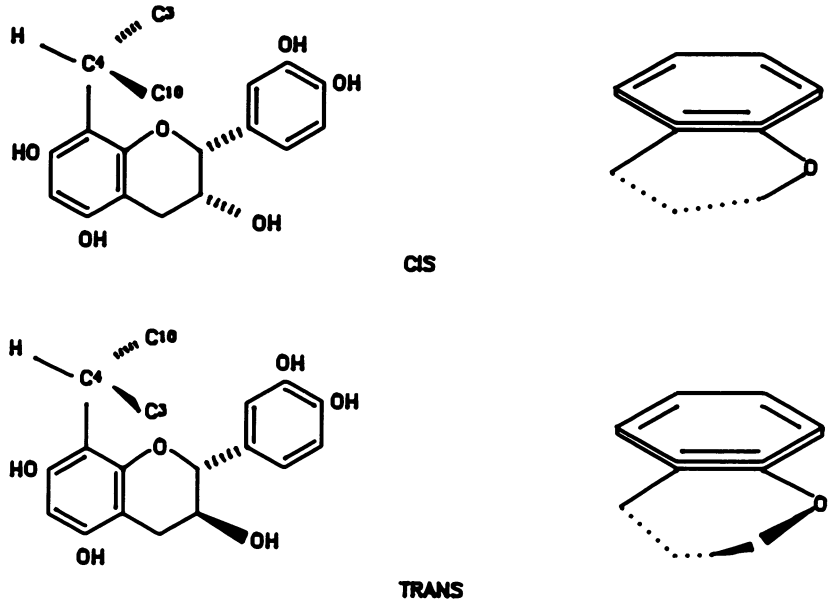
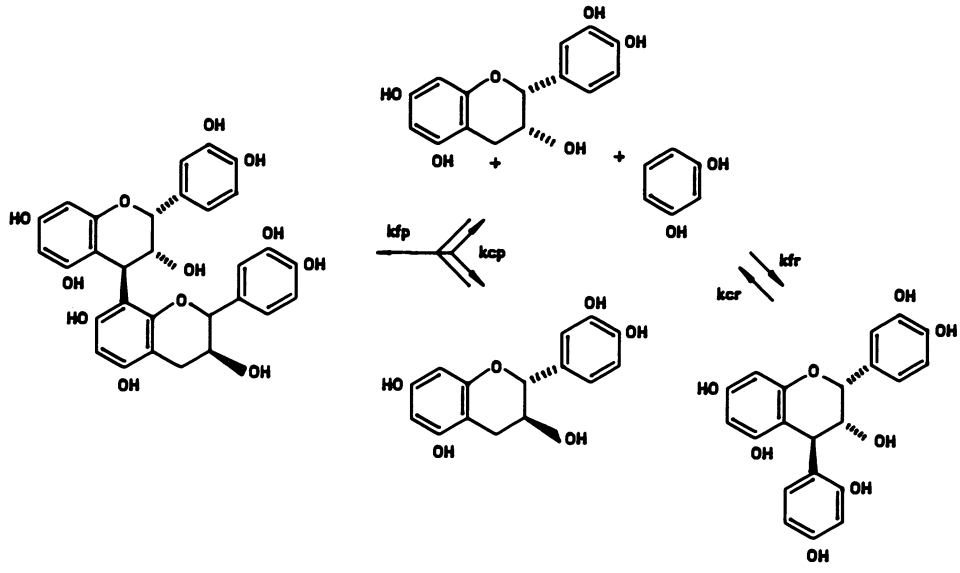


Figure 3. *Rotational isomer conformation for some dimeric procyanidins.*[29]



Scheme 4. *Formation of resorcinol adducts from condensed tannins by acid-catalyzed cleavage.*[32] *Resorcinol adducts will accumulate where kcp/kcr > kfp/kfr.*

partition of the reaction product with ethyl acetate to recover all unreacted resorcinol and oligomeric procyanidin-(4$\beta \rightarrow$ 4)-resorcinol adducts provides products that serve as direct substitutes for resorcinol in these types of adhesives.[31] Kreibich further discusses the use of these derivatives in wood adhesives later in Chapter 29.

Application of Acid-Catalyzed Cleavage to Synthesis of Biocides

Laks[33] made some interesting biocidal epicatechin-4-alkyl sulfides from the condensed tannins extracted from loblolly pine (*Pinus taeda*) L. and eastern hemlock (*Tsuga canadensis* (L.) Carr.) bark through acid-catalyzed cleavage in the presence of fatty thiols of chain length C_6 to C_{16}. The decyl sulfide was most effective against *Scytalidium lignicola*, *Phanerochaete chrysosporium*, *Coroiolus versicolor*, and *Lecythophora hoffmannii* with minimum inhibitory concentration limits of 8, 50, 200, and 300 ppm, respectively. The epicatechin-4-decyl sulfide was less effective than epicatechin-4-octyl sulfide against *Poria placenta* where the minimum inhibitory concentration was 250 ppm. These compounds did not inhibit the growth of the brown rot fungus *Goeophyllum trabeum*.

The same compounds were also tested against Gram-positive and Gram-negative bacteria. The decyl sulfide derivative was more toxic to Gram-positive bacteria than was Streptomycin used as a control. The bacteria tested within this group included *Streptococcus faciens*, *Bacillus cereus*, *Micrococcus lutens*, and *Staphylococus aureus*, and the minimum inhibitory concentrations for epicatechin-4-decyl sulfide were 30, 5, 5, and 40 ppm, respectively. None of the compounds was very effective against Gram-negative bacteria except the hexadecyl derivative against *Pseudomonas aeruginosa* where the minimum inhibitory concentration was 50 ppm, equivalent to that for Streptomycin. Laks provides additional information in Chapter 32 of this book.

HYDROGENOLYSIS

Hydrogenolysis Over Palladium

In comparison with lignins, for which extensive efforts have been directed toward producing low molecular weight phenolics through hydrogenolysis reactions,[34] the condensed tannins offer considerable advantages as a source of reactive phenols. Reports of facile cleavage of the interflavanoid bond under extremely mild hydrogenolysis conditions[35] sparked considerable attention in the United States as a reaction useful in producing reactive and low molecular weight phenols from renewable resources. Application of reaction conditions similar to those described by Jacques failed to produce low molecular weight cleavage products from the condensed tannins of southern pine barks.[36]

Foo[37] studied the hydrogenolysis of similar polymers obtained from *Photinia glabrescens* and showed that, with the use of high pressure and temperatures, low molecular weight procyanidins could be produced without opening the pyran ring. While these reaction conditions were far more severe than those described by Jacques,[35] should petroleum prices and scarcity reach the high level of the early 1970s, Foo's results[37] suggest that hydrogenolysis ought to be investigated further. This is particularly true for development of processes in which the condensed tan-

nins from conifer tree barks might be used as phenol or resorcinol substitutes in the formulation of durable thermosetting resins. Two of the most serious problems in using conifer tree bark extracts in these adhesives are the comparatively high viscosities of tannin solutions at usable solids contents (ca. 40+ percent) and the presence of co-extracted carbohydrates that seriously interfere with attaining good adhesion (wood failure) in tannin-based adhesives.[37] Hydrogenolysis or reductive cleavage reactions, as are attainable with sodium borohydride[39] (see the following section), serve to reduce the molecular weight of polymeric procyanidins and at the same time convert the aldehyde (reducing end) of carbohydrates to alcohols that do not interfere with adhesion in phenol-carbohydrate-based wood adhesives.[40]

Reductive Cleavage with Borohydride

(+)-Catechin is marketed as a pharmaceutical in Europe under the name (+)-Cyanidanol-3 for treatment of liver disease.[41–44] In addition, (-)-epicatechin has a cardiac stimulant activity and a hypoglycemic effect in diabetic animals.[45] One approach to production of these flavan-3-ols is to react condensed tannins under reductive cleavage conditions. Laks and Hemingway[39] have explored the application of mild alkaline cleavage in the presence of sodium borohydride and have obtained epicatechin and catechin in yields of approximately 35 percent of the tannin weight. The tannin employed was isolated from southern pine bark. Because this tannin is predominantly composed of 2,3-*cis* procyanidin units and a catechin terminal unit, the formation of a mixture of epicatechin and catechin was unavoidable. However, there are some excellent sources of homopolymers with either 2,3-*trans* or 2,3-*cis* stereochemistry throughout that could be used to obtain only (+)-catechin or (-)-epicatechin.[10,46] Reaction conditions must be carefully controlled to limit opening of the pyran ring with subsequent inversion of the catechol B-ring, which would result in formation of *ent*-catechin or *ent*-epicatechin[47] and/or related diarylpropanoid derivatives (Scheme 5).[35,47] Because approval for use of flavanoid compounds as pharmaceuticals was withdrawn by the U. S. Food and Drug Administration, interest in these applications is not as strong in North America as in Europe, where flavanoid compounds are widely used as pharmaceuticals.[49]

SULFONATION

Influence of Structure on the Course of Reaction

Here is a classic example of the errors that can be introduced in extending the reactions of flavan-3-ols as model compounds to the behavior of the condensed tannin polymers without due consideration of the reactions at the interflavanoid bond. The literature prior to 1983 suggested that sulfonation of condensed tannins proceeds by opening of the pyran ring with the formation of a sulfonic acid function on the carbon α to the B-ring. This was supported by the results obtained by Sears[50] on the sulfonation of (+)-catechin. However, it is now known[51] that reaction of oligomeric procyanidins (i.e., 5,7-dihydroxy proanthocyanidins) with sulfite ion under the usual pH conditions proceeds by cleavage of the interflavanoid bond with the formation of flavan-4- or proanthocyanidin-4-sulfonates (Scheme 6). Similar errors can be seen in projection of Freudenberg's early work on acid-catalyzed

reactions of (+)-catechin with phloroglucinol and resorcinol to the structure of condensed tannin polymers (see Weinges et al[52] for a thorough review). Even when epicatechin-4β-sulfonate is subjected to temperatures as high as 135 °C in the presence of excess sodium sulfite, no pyran ring cleavage with formation of diarylpropanoid sulfonates is detectable.[39]

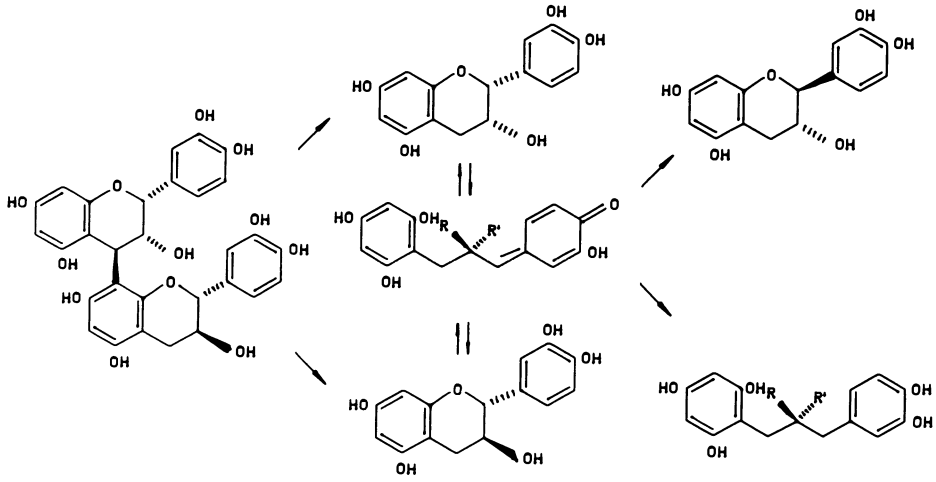
In contrast to the above reaction of procyanidins, reaction of ethyl acetate-soluble oligomers from a commercial quebracho tannin (*Schinopsis* spp. wood) that have been shown by Viviers et al[53] to be *ent*-fisetinidol → catechin oligomers did not result in the formation of an *ent*-fisetinidol-4-sulfonate derivative. Rather, a dimeric diarylpropanoid carrying sufonic acid functions at both carbons α to the catechol ring was isolated.[39] The basic structure of this compound was evident from negative ion FAB-MS and the ^{13}C-NMR spectrum where the carbons of the two propanoid chains were assigned as δ: 73.7 (C-2); 78.0 (C-3); 37.3 (C-4); 72.0 (C-2′); 74.3 (C-3′) and 30.1 (C-4′). The stereochemistry of this product has not yet been defined. Isolation of this compound as a major product of the reaction suggests that in sulfonation of the 5-deoxy proanthocyanidins, sulfonation does occur through cleavage of the pryan ring and that interflavanoid bond cleavage does not occur.

Application in Tannin-Based Adhesives

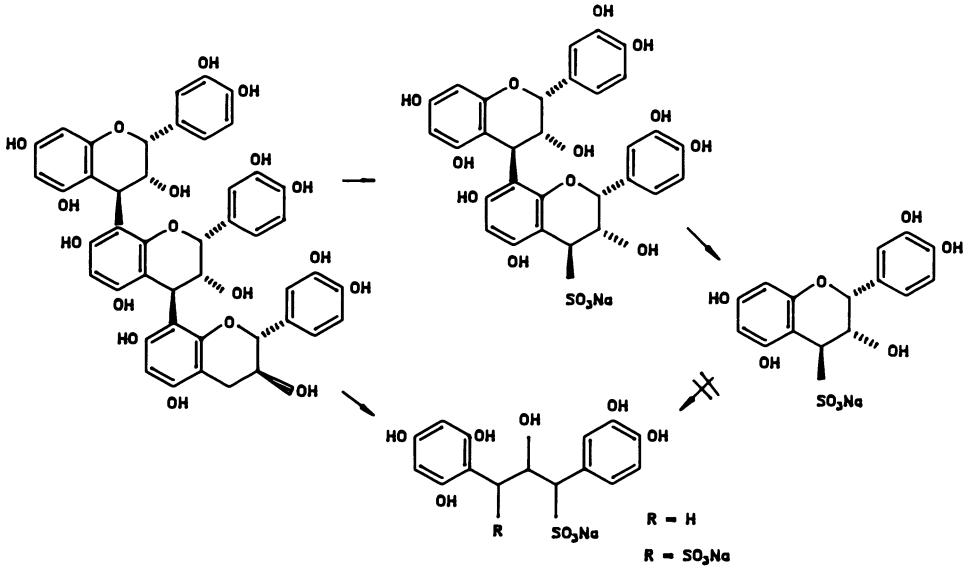
The fact that sulfonation of the 5,7-dihydroxy proanthocyanidins involves interflavanoid bond cleavage and not formation of polymeric sulfonates as had originally been thought has important implications for the utilization of this class of tannins. The molecular weight of tannins with 5,7-dihydroxy A-rings can be tailored to suit their use in applications such as wood adhesives through use of sulfonation reactions. In addition, desulfonation of 2,4,6-trihydroxybenzyl sulfonic acid and sodium epicatechin-(4β)-sulfonate is a very facile reaction under mild alkaline conditions (i.e., pH > 8.0 and ambient temperature) whereas, hydroxybenzylsulfonic acids with resorcinol or phenol functionality resist desulfonation even at pH 12 and 90 °C.[54] Therefore, not only is it possible to reduce the molecular weight with improvements in the viscosity and solubility of the product, it is also possible to remove the sulfonic acid function and obtain aldehyde condensation products that are insoluble in water.[55,56]

BASE-CATALYZED CLEAVAGE OF CONDENSED TANNINS

The interflavanoid bonds of the proanthocyanidins with phloroglucinolic A-rings are also extremely susceptible to cleavage under mild alkaline conditions. This was first recognized in use of the 4-phenylsulfide derivative of epicatechin to synthesize oligomeric procyanidins at pH 9.0 and ambient temperature to demonstrate that intermediates in procyanidin biogenesis were most likely quinone methides.[57] When reacted for several hours at ambient temperature under nitrogen, the interflavanoid bonds of the dimeric procyanidins epicatechin-(4β → 8)-catechin and epicatechin-(4β → 6)-catechin appeared to be stable because no significant regioisomerism could be detected in reaction products. This same reaction was used with epicatechin-



Scheme 5. *Reaction products of base-catalyzed reductive cleavage.*

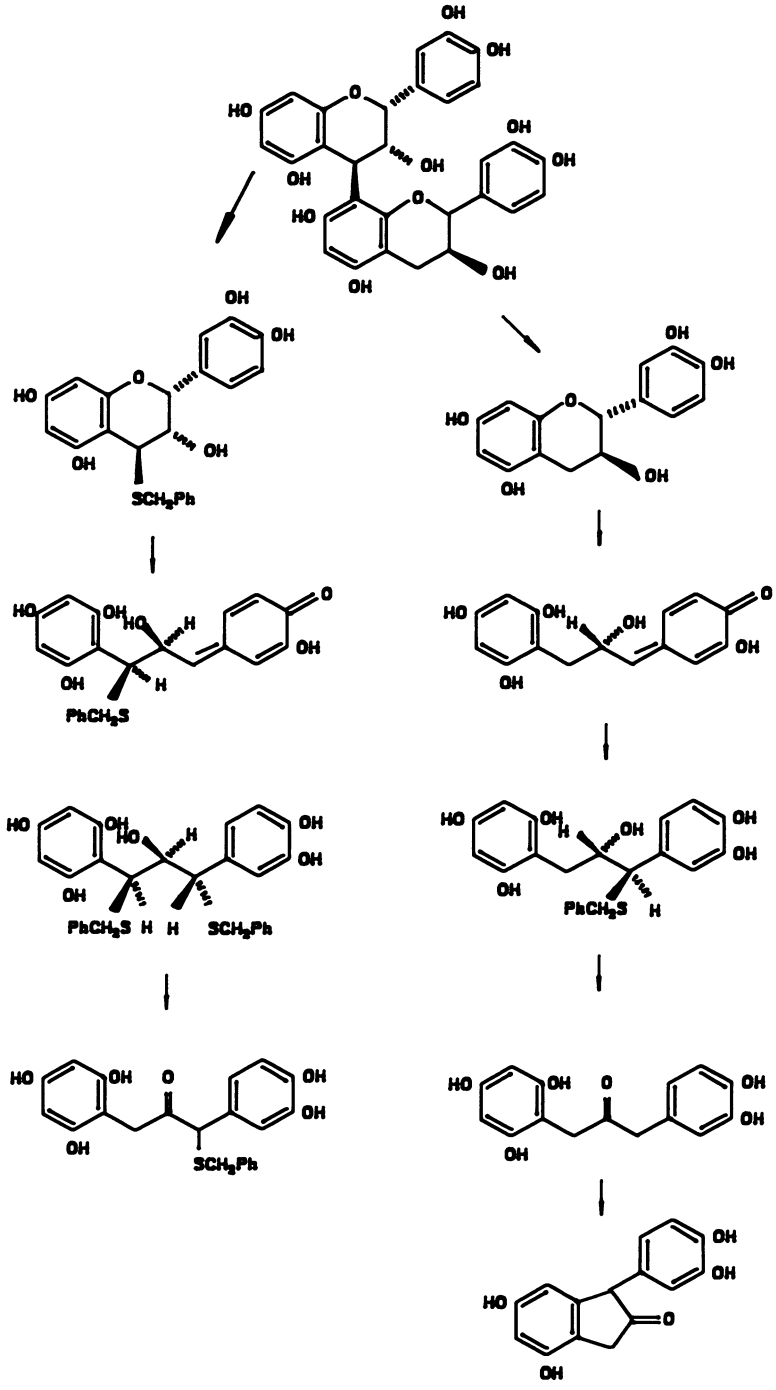


Scheme 6. *Interflavanoid bond cleavage in the sulfonation of polymeric procyanidins.*[51]

(4β → 6)-catechin as a nucleophile to obtain high yields of the trimer epicatechin-(4β → 8)-catechin-(6 → 4β)-epicatechin that is representative of a branch point in polymeric procyanidins.[30] When the pH is increased to about 12 at ambient temperature, both the interflavanoid bond and the pyran ether linkages suffer rapid cleavage.[48] Reaction of polymeric procyanidins isolated from southern pine bark with toluene-α-thiol at pH 12 and ambient temperature gave predominantly one stereoisomer of 1,3-dibenzylthio-1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl) propan-2-ol by stereoselective reactions at C-4 and C-2 of the quinone methides derived from the upper 2,3-*cis* procyanidin units of the polymer (Scheme 7).

The apparent stereoselectivity of this reaction indicated that the interflavanoid bond is more labile than the pyran ring under these reaction conditions. Reactions at ambient temperature for 48 hours gave a propanone derivative by loss of toluene-α-thiol selectively from C-1 and enol-ketol tautomerism. Loss of toluene-α-thiol from 1-benzylthio-1-(3,4-dihydroxyphenyl-3-(2,4,6-trihydroxyphenyl) propan-2-ol obtained from the terminal catechin unit by opening of the pyran ring and substitution at the C-2 carbon α to the catechol ring gave the diarylpropanone. At 100 °C, this compound rearranges to an indan-2-one derivative. The tautomeric rearrangement to the ketone is a chemical analogy[58] for the possible formation of a flav-3-en-3-ol suggested by Haslam as an intermediate permitting inversion of the 3-hydroxyl in reduction products of 2**R**,3**R**-(2,3-*trans*)-dihydroquercetin in the biogenesis of 2,3-*cis* procyanidins.[59] The means by which 2,3-*cis* procyanidins are produced in plants remains in some debate and is discussed by Stafford in Chapter 3. However, this experiment shows that the interflavanoid bond in polymeric procyanidins is extremely labile (even more so than the pyran ring) under comparatively mild alkaline conditions.

We are just beginning to understand base-catalyzed rearrangements that include rapid interflavanoid bond cleavage at pH conditions above about 10. Interflavanoid bond cleavage is generally accompanied by cleavage of the pyran ring and subsequent ring isomerizations to new pyran ring systems[60–62] or to the nonatrione ring system represented by catechinic acid.[47,61–63] Because most tannin-based adhesive formulations involve solution at high pH, these reactions are particularly important in this application. The most important aspect of these reactions for adhesive development involves the facile interflavanoid bond cleavage and recondensation that may result in changes in the dispersivity of the molecular weight distribution and in the procyanidins and prodelphinidins, loss of phloroglucinol functionality due to rearrangement of the phloroglucinol ring.[62] In the case of the profisetinidins and prorobinetinidins, ring isomerizations could lead to increased reactivity with aldehydes.[60] Interflavanoid bond cleavage has not been found when these polymers are reacted under comparatively mild alkaline conditions. It seems likely that base-catalyzed cleavage of the interflavanoid bonds in polymeric proanthocyanidins can become an effective route to novel specialty chemicals as our understanding of these reactions grows.



Scheme 7. *Base-catalyzed cleavage of polymeric procyanidins with toluene-α-thiol as a capture nucleophile.*

CONCLUSIONS

In summary, facile acid-catalyzed interflavanoid bond cleavage in condensed tannins with a 5,7-dihydroxy A-ring substitution has been one of the most important reactions used by tannin chemists for analytical purposes. Extension of these reactions to methods of production of a wide variety of specialty chemicals has recently received attention, and, if ways can be found to improve yields and simplify the purification of products, valuable chemicals could be derived from condensed tannins. Although hydrogenolysis or reductive cleavage reactions currently seem expensive, the products of the reactions are far more valuable than those that could be obtained from lignins, so further research on these reactions as a route to specialty chemicals seems warranted. Although base-catalyzed cleavage reactions have only recently received attention, increased understanding of these reactions has been particularly important to the development of tannin-based adhesives, and applications of these reactions to the development of specialty chemical uses for tannins seems a certainty.

REFERENCES

1. Swain, T.; Hillis, W.E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* 10:63 (1959).
2. Ribereau-Gayon, P. Plant Phenolics. Hafner Publishing Co. New York, pp. 135-168 (1972).
3. Betts, M.J.; Brown, B.R.; Brown, P.E.; Pike, W.T. Degradation of condensed tannins: structure of the tannin from common heather. *J. Chem. Soc. Chem. Commun.* :1110 (1967).
4. Sears, K.D.; Casebier, R.L. Cleavage of proanthocyanidins with thioglycollic acid. *J. Chem. Soc. Chem. Commun.* :1437 (1968).
5. Porter, L.J.; Hrstich, L.N.; Chan. B.G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223 (1986).
6. Tiarks, A.E. (unpublished results).
7. Iacobucci, G.A.; Sweeny, J.B. The chemistry of anthocyanins, anthocyanidins and related flavylium salts. *Tetrahedron* 39:3005 (1983).
8. Timberlake, C.R.; Bridle, P. Anthocyanidins: colour augumentation with catechin and acetaldehyde. *J. Sci. Food Agric.* 28:539 (1977).
9. Sears, K.D.; Casebier, R.L. The reaction of thioglycollic acid with polyflavonoid bark fractions for *Tsuga heterophylla*. *Phytochemistry* 9:1589 (1970).
10. Porter, L.J. Condensed tannins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer Verlag. Heidelberg, New York (in press).
11. Betts, M.J.; Brown, B.R.; Shaw, M.R. Reaction of flavonoids with mercaptoacetic acid. *J. Chem. Soc.* (C) :1178 (1969).
12. Brown, B.R.; Shaw, M.R. Reactions of flavanoids and condensed tannins with sulfur nucleophiles. *J. Chem. Soc. Perkin Trans. 1* :2036 (1974).
13. Samejima, M.; Yoshimoto, T. Systematic studies on the stereochemical composition of proanthocyanidins from coniferous bark. *Mokuzai Gakkashi.* 28:67 (1982).
14. Hemingway, R.W.; Karchesy, J.J.; McGraw, G.W.; Wielesek, R.A. Heterogeneity of interflavanoid bond location in loblolly pine procyanidins. *Phytochemistry* 22:275 (1983).
15. Hemingway, R.W.; Foo. L.Y.; Porter, L.J. Polymeric proanthocyanidins: Interflavanoid linkage isomerism in epicatechin-4)-epicatechin-4)-catechin procyanidins. *J. Chem. Soc. Chem. Commun.* :320 (1981).

16. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Linkage isomerism in trimeric and polymeric 2,3-*cis* procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1209 (1982).

17. Nonaka, G.; Hsu, F-L.; Nishioka, I. Structures of dimeric, trimeric and tetrameric procyanidins from *Areca catechu. J. Chem. Soc. Chem. Commun.* :781 (1981).

18. Porter, L.J.; Newman, R.H.; Foo, L.Y.; Wong, H.; Hemingway, R.W. Polymeric proanthocyanidins. ^{13}C-nmr studies of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1217 (1982).

19. Newman, R.H.; Porter, L.J.; Foo, L.Y.; Johns, S.R.; Willing, R.I. High resolution ^{13}C nmr studies of proanthocyanidin polymers. *Mag. Res. Chem.* 25:118 (1987).

20. Gupta, R.K.; Haslam, E. Plant proanthocyanidins. Part 5. Sorghum polyphenols. *J. Chem. Soc. Perkin Trans. 1* :892 (1978).

21. Beart, J.E.; Lilley, T.H.; Haslam, E. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations on some polymeric proanthocyanidins. *J. Chem. Soc. Perkin Trans. 2* :1439 (1985).

22. Patil, A.D.; Desphande, V.H. A new dimeric proanthocyanidin from *Cassia fistula* sapwood. *Indian J. Chem.* 21B:626 (1982).

23. Hemingway, R.W.; Kreibich, R.E. Condensed tannin-resorcinol adducts and their use in wood-laminating adhesives. An exploratory study. *J. Appl. Polym. Sci. Polym. Sci. Symp.* 40:79 (1984).

24. Hemingway, R.W.; McGraw, G.W. Kinetics of acid-catalyzed cleavage of procyanidins. *J. Wood Chem. and Technol.* 3:421 (1983).

25. Czochanska, Z.; Foo, L.Y.; Newman, R.H.; Porter, L.J. Polymeric proanthocyanidins. Stereochemistry, structural units and molecular weight. *J. Chem. Soc. Perkin Trans. 1* :2278 (1980).

26. Foo, L.Y.; Porter, L.J. Prodelphinidin polymers: Definition of structural units. *J. Chem. Soc. Perkin Trans. 1* :1186 (1978).

27. Foo, L.Y. (unpublished results). (1988).

28. Haslam, E. Biogenetically patterned synthesis of procyanidins. *J. Chem. Soc. Chem. Commun.* :594 (1974).

29. Foo, L.Y.; Porter, L.J. Synthesis and conformation of procyanidin diastereoisomers. *J. Chem. Soc. Perkin Trans. 1* :1535 (1983).

30. Foo, L.Y.; Hemingway, R.W. Condensed tannins: Synthesis of the first 'branched' procyanidin trimer. *J. Chem. Soc. Chem. Commun.* :85 (1984).

31. Kreibich, R.E.; Hemingway, R.W. Condensed tannin-resorcinol adducts in laminating adhesives. *For. Prod. J.* 35:23 (1985).

32. Hemingway, R.W.; Kreibich, R.E. Condensed tannins-resorcinol adducts and their use in wood laminating adhesives: an exploratory study. *J. Appl. Polym. Sci. Polym. Symp.* 40:79 (1984).

33. Laks, P.E. Flavonoid biocides: phytoalexin analogs from condensed tannins. *Phytochemistry* 26:1617 (1987).

34. Goheen, D.W. Chemicals from Lignin. *In*: Goldstein, I.S. (ed.) Organic Chemicals from Biomass. CRC Press, Boca Raton, Florida, pp. 143-161 (1981).

35. Jacques, D.; Haslam, E.; Bedford, G.R.; Greatbanks, D. Plant proanthocyanidins. Part II. Proanthocyanidin A2 and its derivatives. *J. Chem. Soc. Perkin Trans. 1* :2663 (1974).

36. Karchesy, J.J.; Hemingway, R.W. (unpublished results). (1978).

37. Foo, L.Y. Polymeric proanthocyanidins of *Photinia glaubrescens*, modification of molecular weight and nature of products from hydrogenolysis. *Phytochemistry* 21:1741 (1982).

38. Pizzi, A., Tannin-based wood adhesives. *In*: Pizzi, A. (ed.) Wood Adhesives: Chemistry and Technology. Marcel Dekker, New York, pp. 178-246 (1983).

39. Laks, P.E.; Hemingway, R.W. (unpublished results).

40. Conner, A.H.; Lorenz, L.F. Carbohydrate modified phenol- formaldehyde resins. *J. Wood Chem. Technol.* 6:591 (1986).

41. Hackett, A.M.; Shaw, I.C.; Griffiths, L.A. The prevention by (+)-cyanidanol-3 of hepatitis-induced changes in the disposition of imipramine in the rat. *Biochemical Pharm.* 33:2179 (1984).

42. Perissoud, D.; Weibel, I. Protective effect of (+)-cyanidanol-3 in acute liver injury induced by alactosamine or carbon tetrachloride in the rat. *Arch. Pharmacol.* 312:285 (1980).

43. Blum, A.L.; Doelle, W.; Kortum, K.; Peter, P.; Strohmeyer, G.; Berthet A.; Loebell, H.; Pelloni, S.; Poulsen, H.; Tygstrup, N. Treatment of acute viral hepatitis with (+)-cyanidanol-3. *The Lancet* 12:1153 (1977).

44. Laks, P.E. The chemistry and utilization of tree barks, *In*: Hon, D.N-S.; Shiraishi, N., (eds.), Handbook on Wood and Cellulose Materials, Marcel Dekker, New York. (in press).

45. Chakravarthy, B.K.; Gode, K.D. Isolation of (-)-epicatechin from *Pterocarpus marsupium* and its pharmacological actions. *Planta Med.* 51:56 (1985).

46. Porter, L.J. Flavans and proanthocyanidins. *In*: Harborne, J.B. (ed.) The Flavonoids. Advances in Research Since 1980. Chapman and Hall, Ltd. London, pp. 21-62, (1988).

47. Kiatragrajai, P.; Wellons, J.D.; Gollub, L.; White, J.D. Kinetics of epimerization of (+)-catechin and its rearrangement to catechinic acid. *J. Org. Chem.* 47:2910 (1982).

48. Laks, P.E.; Hemingway, R.W. Condensed tannins: base-catalyzed reactions of polymeric procyanidins with toluene-α-thiol. Lability of the interflavanoid bond and pyran ring. *J. Chem. Soc. Perkin Trans. 1* :465 (1987).

49. Farkas, L.; Gabor, M.; Kallay, F. Flavonoids and bioflavonoids. Current Research Trends. Elsevier Scientific, New York. (1977).

50. Sears, K.D. Sulfonation of catechin. *J. Org. Chem.* 37:3546 (1972).

51. Foo, L.Y.; McGraw, G.W.; Hemingway, R.W. Condensed tannins: preferential substitution at the interflavanoid bond by sulfite ion. *J. Chem. Soc. Chem. Commun.* :672 (1983).

52. Weinges, K.; Bahr, W.; Ebert, W.; Goritz, K.; Marx, H.-D. Konstitution, entstehung, und bedeutung der flavonoid gerbstoffe. *Fortschrt. Chem. Org. Naturst.* 27:158 (1969).

53. Laks, P.E.; Hemingway, R.W. (unpublished results).

54. Viviers, P.M.; Kolodziej, H.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 11. Intramolecular enantiomerism of the constituent units of tannins from the *Anacardiaceae*: stoichiometric control in direct synthesis: Derivation of ^{1}H nuclear magnetic resonance parameters applicable to higher oligomers. *J. Chem. Soc. Perkin Trans. 1* :2555 (1983).

55. McGraw, G.W.; Laks, P.E.; Hemingway, R.W. Condensed tannins: desulfonation of hydroxybenzylsulfonic acids related to proanthocyanidin derivatives. *J. Wood. Chem. Technol.* 8:91 (1988).

56. Kreibich, R.E.; Hemingway, R.W. Condensed tannin-sulfonates in cold-setting wood-laminating adhesives. *For. Prod. J.* 37:43 (1987).

57. Kreibich, R.E.; Hemingway, R.W. Tannin-based adhesives for finger-jointing wood. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385, American Chemical Society, Washington, DC. pp. 203-216 (1989).

58. Hemingway, R.W.; Foo, L.Y. Condensed tannins: quinone methide intermediates in procyanidin synthesis. *J. Chem. Soc. Chem. Commun.* :1035 (1983).

59. Hemingway, R.W.; Laks, P.E. Condensed tannins: a proposed route to 2**R**,3**R**-(2,3-*cis*)-proanthocyanidins. *J. Chem. Soc. Chem. Commun.* :746 (1985).

60. Jacques, D.; Opie, C.T.; Porter, L.J.; Haslam, E.J. Plant proanthocyanidins. Part 4. Biosynthesis of procyanidins and observations on the metabolism of cyanidin in plants. *J. Chem. Soc. Perkin Trans. 1* :1637 (1977).

61. Roux, D.G. Activation of some condensed tannins via facile ring isomerizations: potential adhesive applications. *In* Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources, ACS Symposium Series No. 385, American Chemical Society, Washington, DC. pp. 217-228 (1989).

62. Laks, P.E.; Hemingway, R.W.; Conner, A.H. Condensed tannins: Base-catalyzed reactions of polymeric procyanidins with phloroglucinol. Intramolecular rearrangements. *J. Chem. Soc. Perkin Trans. 1* :1875 (1987).

63. Laks, P.E.; Hemingway, R.W. Condensed tannins: structure of the 'phenolic acids'. *Holzforschung* 41:287 (1987).

64. Sears, K.D., Casebier, R.L., Hergert, H.L., Stoudt, G.H., McCandlish, L. E. The structure of catechinic acid, a base-rearrangement product of catechin. *J. Org. Chem.* 39:3244 (1975).

BASE-CATALYZED PYRAN REARRANGEMENTS OF PROFISETINIDINS

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ABSTRACT

Profisetinidins with 2,3-*trans*-3,4-*trans*-(-)-fisetinidol units are subject to stereospecific C-ring isomerization under mild basic conditions; whereas, those with 2,3-*trans*-3,4-*cis* "upper" units are susceptible to stereoselective pyran rearrangements. In the latter situation, the functionalized tetrahydropyranochromenes are accompanied by analogs in which the resorcinol A- and pyrocatechol B-ring have been interchanged. The formation of these ring interchanged compounds are explicable in terms of a unique 1,3-aryl migration in a quinone-methide intermediate, such a mechanism, however, requiring the inversion of the absolute configuration at the equivalent of 3-C(C) in the biflavanoid precursor. These base-catalyzed pyran rearrangements may have an important bearing on the industrial applications of condensed tannins.

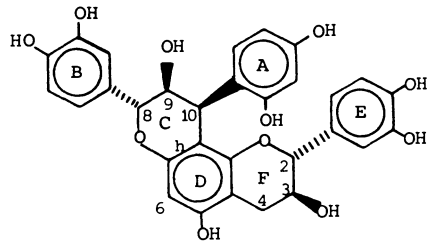
INTRODUCTION

The natural occurrence of a novel class of C-ring isomerized condensed tannins, termed phlobatannins, was recently demonstrated.[1] The structures of the functionalized 8,9-*trans*-9,10-*cis*-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-h]chromene (**1**), the homologous hexahydrodipyrano[2,3-f, 2′3′-h]chromene (**2**), and the "isomerization-intermediates" (**3**) and (**4**) were established by application of nuclear Overhauser effect (n.O.e.) difference spectroscopy to their phenolic methyl ether acetates. In each instance, n.O.e. associations of 2-OMe(A) with 3-H(A) and of 4-OMe(A) with both 3-H(A) and 5-H(A) indicated the "liberation" of resorcinol moieties from C-ring heterocycles, compared with invovement in the presumed (-)-fisetinidol-(4α,8)-(+)-catechin precursor (see below). In addition, the ^{1}H-NMR spectra of the deriva-

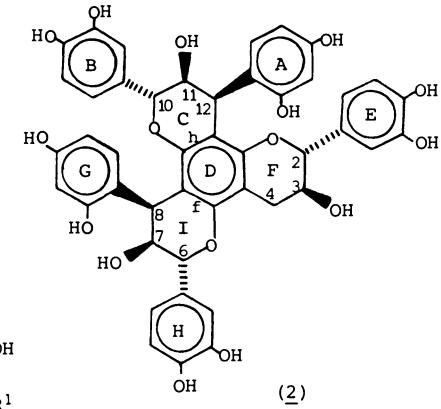
tives were characterized by the typical absence of the effects of dynamic rotational isomerism at ambient temperatures. Progress on definition of these pyran rearrangements in the profisetinidins is reviewed in this chapter.

REARRANGEMENT REACTIONS

Initial identification of these pyran-rearranged profisetinidins was succeeded by the recognition of additional novel members[2,3] of this class of condensed tannins,



(1)



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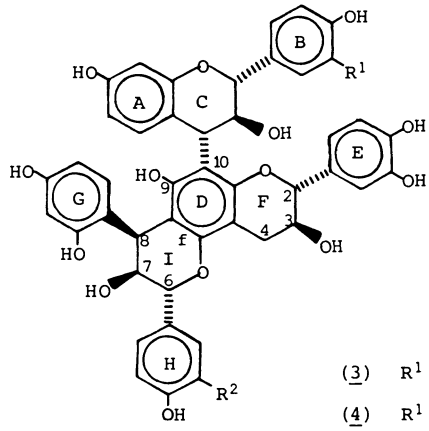
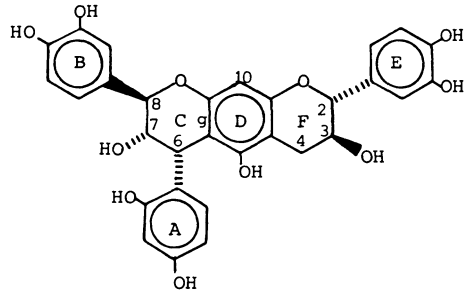


(3) R^1 = H, R^2 = OH

(4) R^1 = OH, R^2 = H



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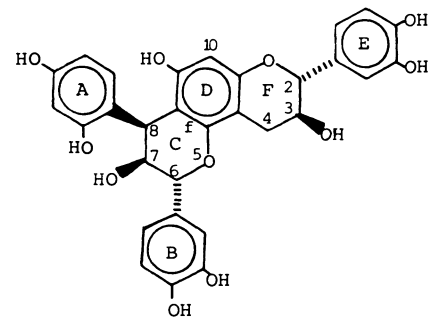


(6)

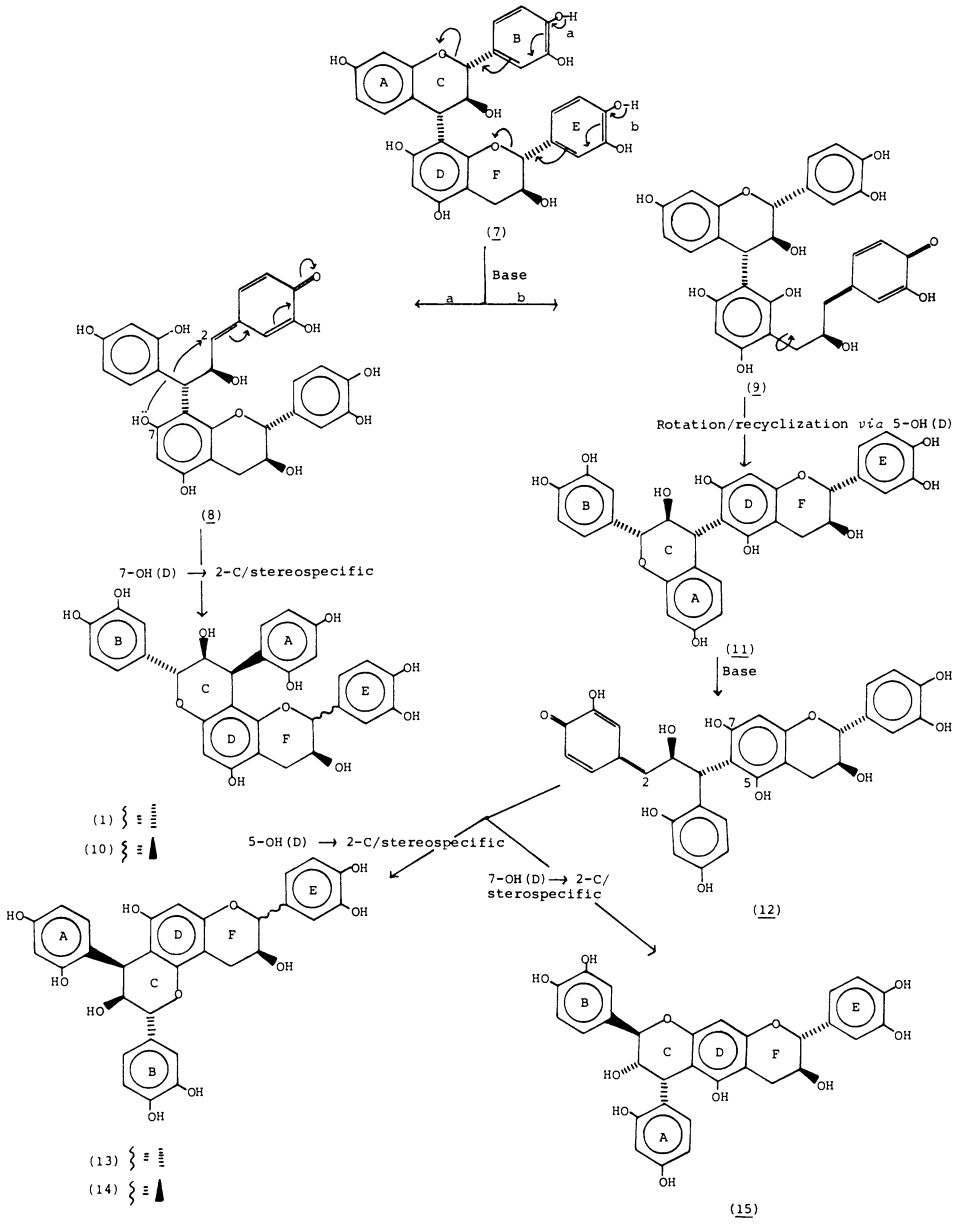
e.g., the tetrahydropyrano[2,3-g]- and [2,3-f]chromenes (**5**) and (**6**). The usual methods of differentiating regio-isomeric biflavanoids (i.e., those based on the absolute chemical shifts of "residual" D-ring singlets of methyl ether acetates in $CDCl_3$ at ambient temperatures)[4] and by observation of n.O.e. associations between the hydrogen- and methoxy protons of the D-ring[5] are less reliable for the methyl ether acetates of phlobatannins (**1**), (**5**), and (**6**). When taken in conjunction with the assignment of the absolute configuration of these novel condensed tannin analogs, the above difficulties prompted us to embark on a synthetic program[2,3,6] to unequivocally define the structures of the pyran-rearranged metabolites.

Thus, treatment of the (-)-fisetinidol-(4α,8)-(+)-catechin (**7**)[7] with 0.025 M Na_2CO_3 - 0.025 M $NaHCO_3$ buffer (pH 10) for 5 hours at 50 °C under nitrogen (i.e., conditions similar to those applied by Freudenberg[8] for epimerization at 2-C of (+)-catechin via intermediate fission of the heterocycle) gave significant (ca 75 percent) conversion into five products of stereospecific ring isomerization (**1**), (**10**), (**13**)-(**15**) (Scheme 1).[3,6] These comprise the anticipated tetrahydropyrano[2,3-h]chromene (**1**) ($J_{8,9}$ = 10.0, $J_{9,10}$ = 6.0 Hz) as a product of ring isomerization of the C-ring of biflavanoid (**7**); its C-2 (F-ring) epimer (**10**) representing conversion of the (+)-catechin moiety of (**7**) into (+)-epicatechin; the corresponding [2,3-f]-isomeric pair (**13**) and (**14**), their formation implying the equivalent of positional isomerization of the (-)-fisetinidol unit to the 6-position of the (+)-catechin moiety via the E-ring quinone-methide (**9**), ring isomerization with 5-OH(D), and also epimerization at C-2(F) as before; and finally the [2,3-g]-regiomer (**15**) indicative of the alternative mode of cyclization via 7-OH(D).

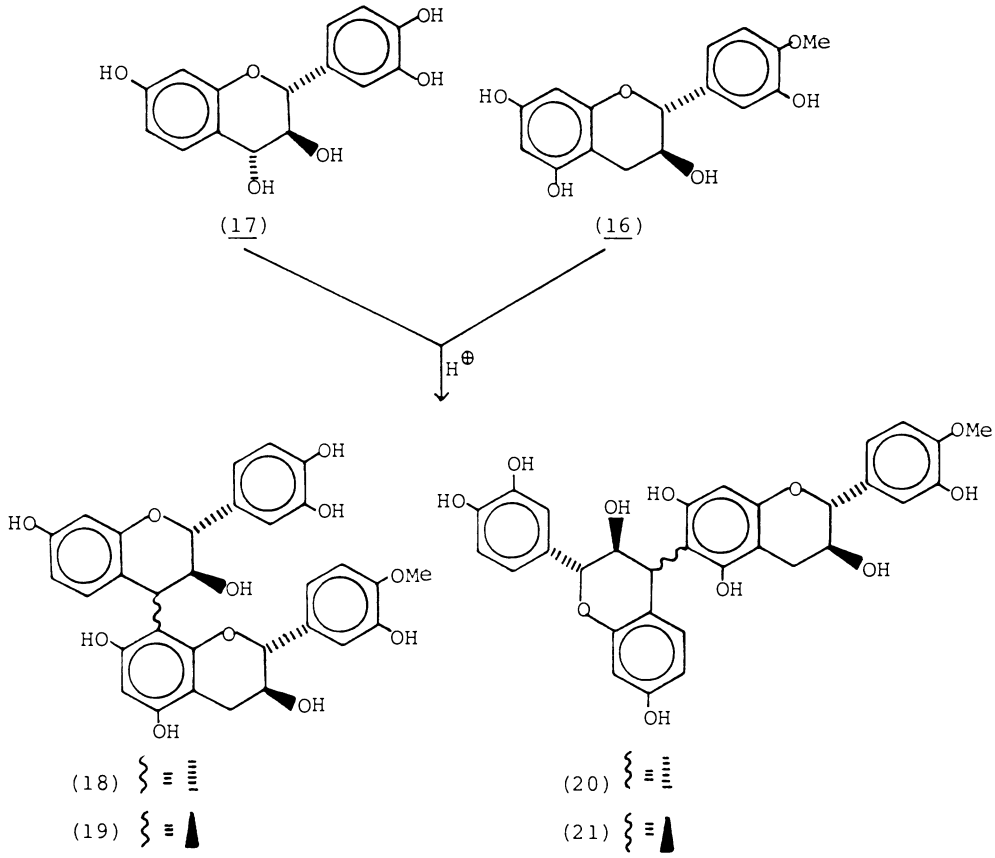
To prevent the side reactions associated with an E-ring quinone-methide[6] (**9**), biflavanoid precursors of type (**7**) had to be protected selectively at 4-OH(E). This was done by selective methylation[9] of (+)-catechin with molar quantities of methyl iodide in anhydrous K_2CO_3/acetone and subsequent acid-catalyzed coupling[7] of 4′-*O*-methyl-(+)-catechin (**16**) and (+)-mollisacacidin [(2**R**, 3**S**,4**R**)-2,3-*trans*-3,4-*trans*-flavan-3,3′,4,4′,7-pentaol] (**17**) to give the (4,8)- and (4,6)-(-)-fisetinidol-(+)-catechin mono-*O*-methyl ethers (**18**), (**19**), (**20**), and (**21**) (Scheme 2) following chromatography on Sephadex LH-20 and Fractogel TSK HW-40(S) in ethanol.

Treatment of the (-)-fisetinidol-(4α,8)-(+)-catechin-*O*-methyl ether (**18**) with base (pH 10) (Scheme 3) as above and subsequent chromatography on Sephadex LH-20 gave the 8,9-*trans*-9,10-*cis*-tetrahydropyrano[2,3-h]chromene (**22**) in 58 percent yield.[3] Phlobatannin (**22**), resulting from stereospecific ring isomerization with retention of the configuration at C-2 in (**18**), is accompanied by small amounts of a dehydro-(-)-fisetinidol-(+)-catechin (**23**) representing the alternative mode of cyclization via C-6(B) in a quinone-methide of type (**8**) followed by oxidative removal of hydride ion during workup. A computer simulated 3D perspective of (**23**) indicates a cuplike conformation involving the A-, B-, C-, D-, and eight-membered rings thus resembling those of the calixarenes,[10] which were recently established by X-ray analysis.

The presence of the dehydro analog (**23**) provides indirect evidence for the proposed quinone-methide mechanism. All efforts to trap an intermediate of type (**8**) intermolecularly with strong nucleophiles such as phenyl sulphide- and selenide ions invariably failed. Absence of products resulting from a migrating flavanyl moiety

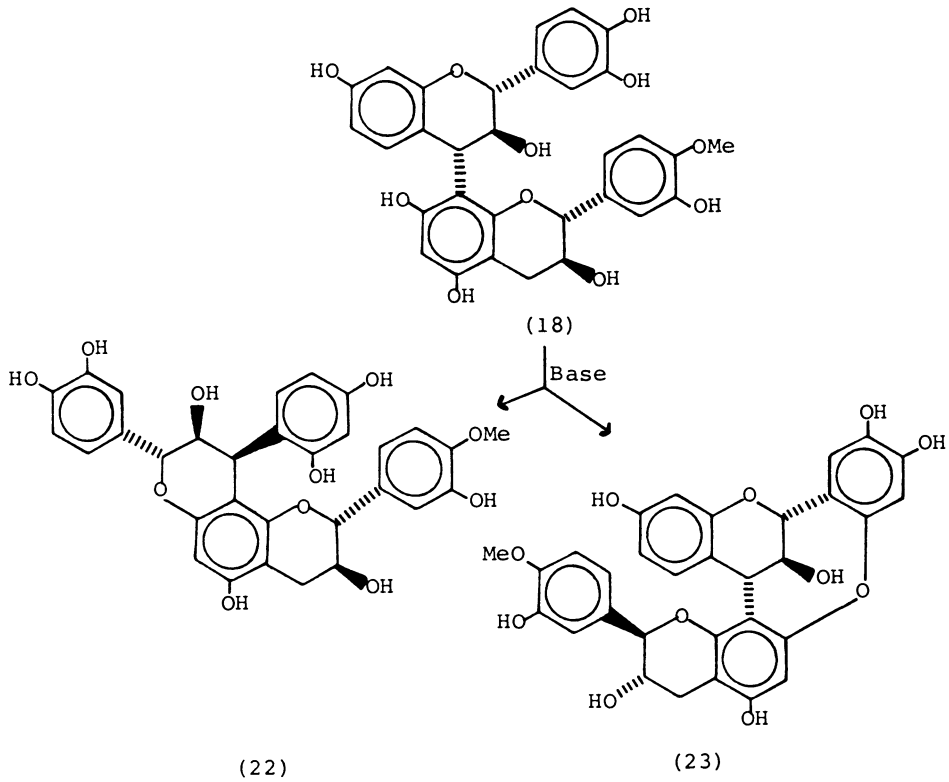


Scheme 1. *Base-catalyzed formation of tetrahydropyrano-[2,3-h]* (**1**), (**10**); *[2,3-f]* (**13**), (**14**), *and [2,3-g] chromenes from (-)-fisetinidol-(4α,8)-(+)-catechin* (**7**).



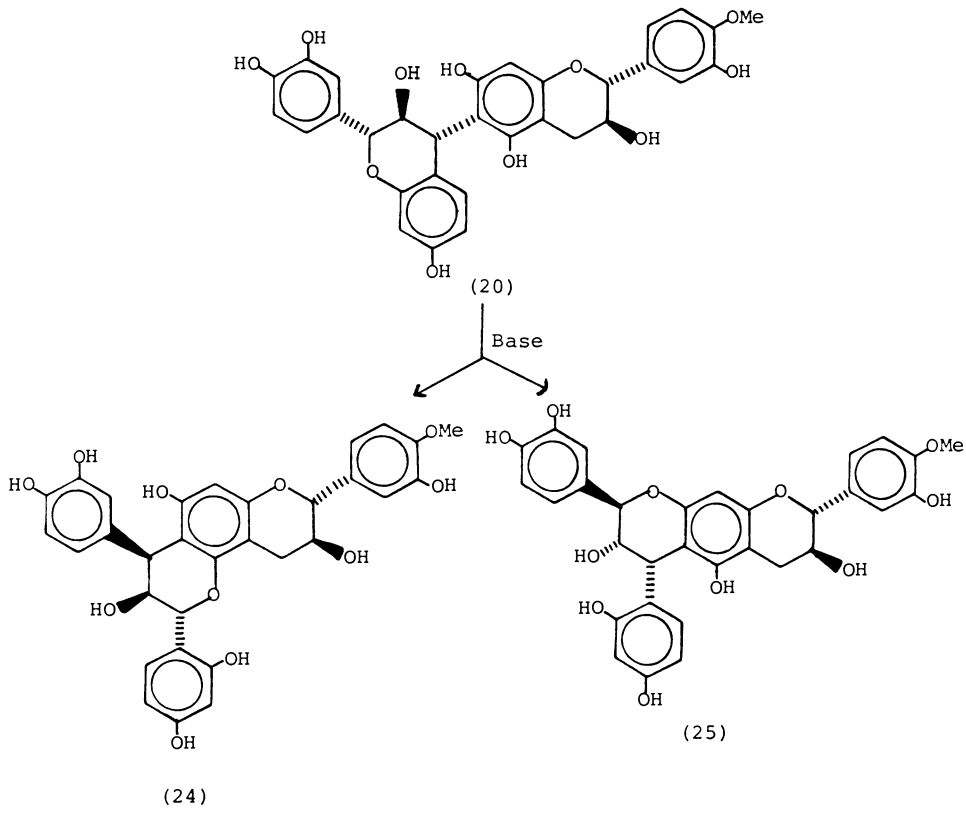
Scheme 2. *Synthesis of "protected" (4,8)- and (4,6)-(-)-fisetinidol-(+)-catechins* (**18**) – (**21**).

in the "protected" biflavanoid (**18**) confirms our conjecture regarding the mechanism of such a migration in the "uncontrolled" synthesis. Base treatment[3] of the (-)-fisetinidol-(4α,6)-(+)-catechin *O*-methyl ether (**20**) afforded the anticipated products of stereospecific C-ring pyran-rearrangement with retention of the absolute configuration at C-2, (i.e., the 6,7-*trans*-7,8-*cis*-tetrahydropyrano[2,3-f]chromene (**24**) [$J_{6,7}$ = 10.0, $J_{7,8}$ = 5.5 Hz for its heptamethyl ether diacetate] and the [2,3-g] regioisomer (**25**) [$J_{7,8}$ = 10.5, $J_{6,7}$ = 6.0 Hz for the phenolic methyl ether acetate] as a minor product) (Scheme 4) . The apparent preference for ring isomerization of biflavanoid (**20**) involving 5-OH(D) and 2-C in an intermediate quinone-methide of type (**12**) presumably reflects a preferred interflavanyl conformation favoring participation of 5-OH(D) in the cyclization step. Such an assumption is in line with the observation that the two rotational isomers at the interflavan bond are unevenly populated in the procyanidins.[11]



Scheme 3. *Base-catalyzed pyran rearrangement of the (-)-fisetinidol-(4α,8)-(+)-catechin mono-O-methyl ether* (**18**).

Treatment of the (-)-fisetinidol-(4β,8)-(+)-catechin-*O*-methyl ether (**19**) with the buffer solution (pH 10) for 3 hours at 50 °C under nitrogen led to complete conversion into a mixture from which four ring-isomerized products were obtained (Scheme 5). These included[2,3] the 8,9-*cis*-9,10-*trans*- and 8,9-*trans*-9,10-*trans*-tetrahydropyrano[2,3-h]chromenes (**26**) and (**27**) [$J_{8,9}$ = ca. 1.0, 7.0; $J_{9,10}$ = 2.0, 6.0 Hz for their heptamethyl ether diacetates] as well as an additional pair of *cis-trans*- and all-*trans* analogs (**28**) and (**29**) with ^{1}H-NMR characteristics [$J_{8,9}$ = ca. 1.0, 7.0; $J_{9,10}$ = 2.0, 6.0 Hz for the heptamethyl ether diacetates] closely resembling those of analogs (**26**) and (**27**). Prominent n.O.e. associations between 8-H(C) and 6-H(A) in the *cis-trans*- heptamethyl ether diacetate not only confirmed this configuration and thus differentiated it from a *cis-cis* arrangement but also indicated a preferred sofa conformation (C-ring) in which the resorcinol moiety at 10-C occupies a near-axial (α) orientation.

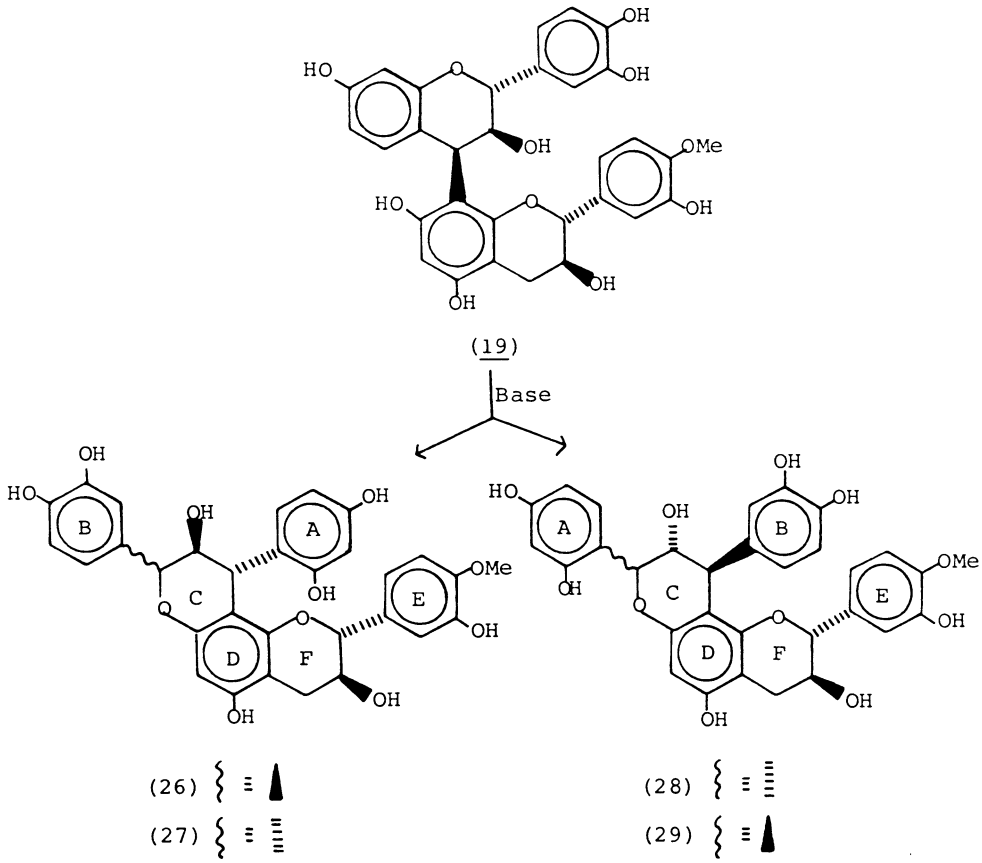


Scheme 4. *Base-catalyzed pyran rearrangement of the (-)-fisetinidol-(4α,6)-(+)-catechin mono-O-methyl ether* (**20**).

In the heptamethyl ether diacetates of the remaining pair of 8,9-*cis*-9,10-*trans*- and 8,9-*trans*-9,10-*trans*-tetrahydropyrano[2,3-h]chromenes (**28**) and (**29**), 8-H(C) exhibits prominent n.O.e. associations with 2- and 6-H of the pyrocatechol moiety in the *cis-trans* analog (**28**) only, whereas, 8- and 10-H(C) were correlated with, respectively, the resorinol- and pyrocatechol rings in both (**28**) and (**29**) by spin decoupling experiments using these protons as reference signals. Subject to the correct allocation of 8- and 10-H(C) resonances, these features collectively indicate an interchange of the resorcinol A- and pyrocatechol B-rings in (**28**) and (**29**) relative to their positions in the "normal" isomers (**26**) and (**27**). The chemical shifts of 8- and 10-H(C) (and thus unambiguous proof for such an A-/B-ring interchange) were confirmed by 2D-heteronuclear correlation of these protons with, respectively, 8- and 10-C. A similar strategy was also adopted to confirm the chemical shifts of 8- and 10-H(C) in (**26**) and (**27**). Also notable in the spectra of the groups (**26**), and (**27**), and (**28**), (**29**) is the conspicuous deshielding of 6-H(A) [$\Delta\delta$-0.74 and -0.80 for the heptamethyl ether diacetates of (**28**) and (**29**), respectively] in the

latter pair relative to its chemical shift in the *cis-trans-* and all-*trans* isomers (**26**) and (**27**). Such a feature is apparently characteristic of phlobatannins belonging to the classes (**28**) and (**29**) (see also below).

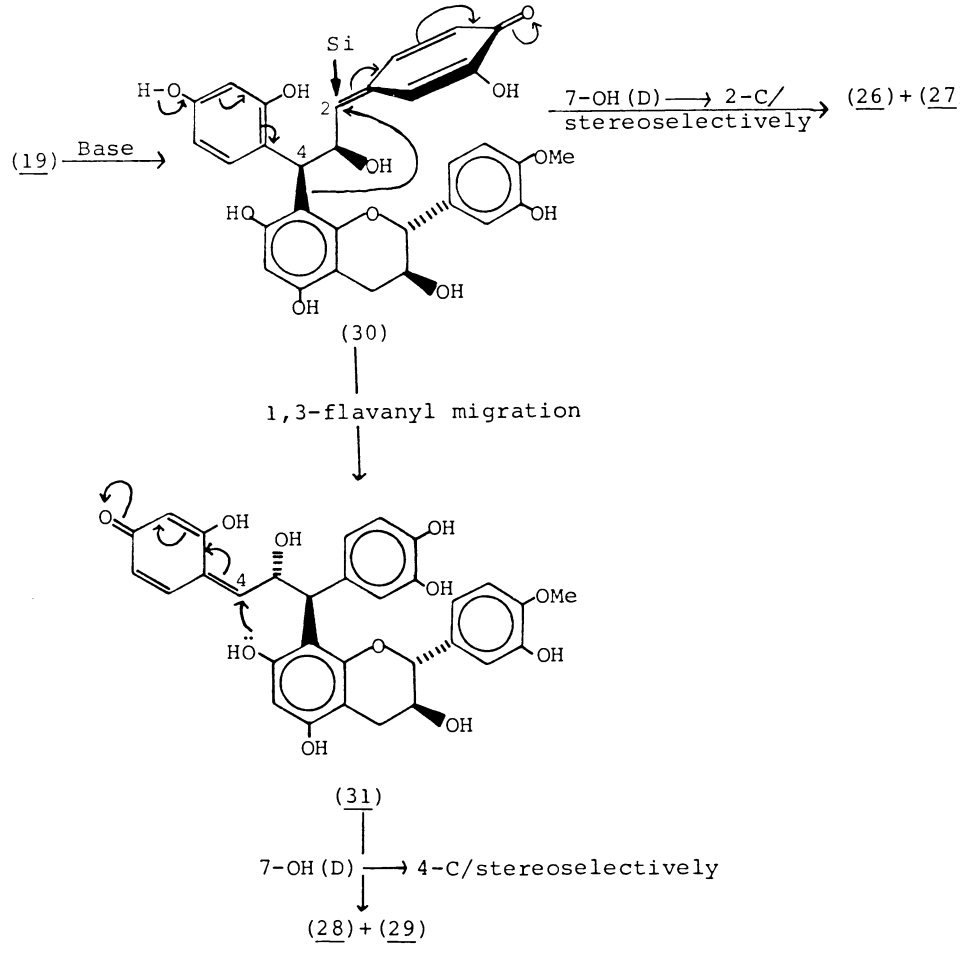
To establish the sequence of formation of tetrahydropyrano[2,3-h]chromenes (**26**) - (**29**) from biflavanoid (**19**), aliquots were taken at regular intervals and fully analyzed on a Buchi medium-pressure liquid chromatography (MPLC) system, using Sephadex LH-20/ethanol at 0.7-0.8 bar pressure. These results indicated that the (-)-fisetinidol-(+)-catechin (**19**) serves as direct precursor to both groups of phlobatannins. Formation of the pair (**26**), (**27**) may be rationalized (Scheme 6) by stereoselective recyclization involving 7-OH(D) and both Re- and Si-faces in quinone-methide (**30**). Treatment of the thermodynamically less stable 8,9-*cis*-9,10-*trans*-tetrahydropyrano[2,3-h]chromene (**26**) under conditions similar to those for its formation did not give equilibration with the all-*trans* isomer (**27**), thus proving their simultaneous genesis from the (4β,8)-biflavanoid (**19**).



Scheme 5. *Base-catalyzed conversion of the (-)-fisetinidol-(4β,8)-(+)-catechin mono-O-methyl ether* (**19**).

The novel conversion (**19**) → (**28**) + (**29**) is explicable in terms of initial migration of the (+)-catechin moiety to the Re-face at 2-C in quinone-methide (**30**). Stereoselective pyran recyclization of (**31**) via 7-OH(D) generates the tetrahydropyrano[2,3-h]chromenes (**28**) and (**29**), enantiomerically related to (**26**) and (**27**) with respect to their C-rings.

The heptamethyl ether diacetates of the "normal" analogs (**26**) and (**27**) exhibit intense negative Cotton effects in the 220-240 nm. region of their c.d. spectra.[12–15] These indicate a 10-C aryl substituent below the plane of the C/D-ring system and thus **R**-absolute configuration at this chiral center.[15] When taken in conjuntion with ^{1}H-NMR coupling constants, the c.d. data define the absolute configurations



Scheme 6. *Proposed route to the formation of phlobatannins with stereoselectivity in rearrangement.*

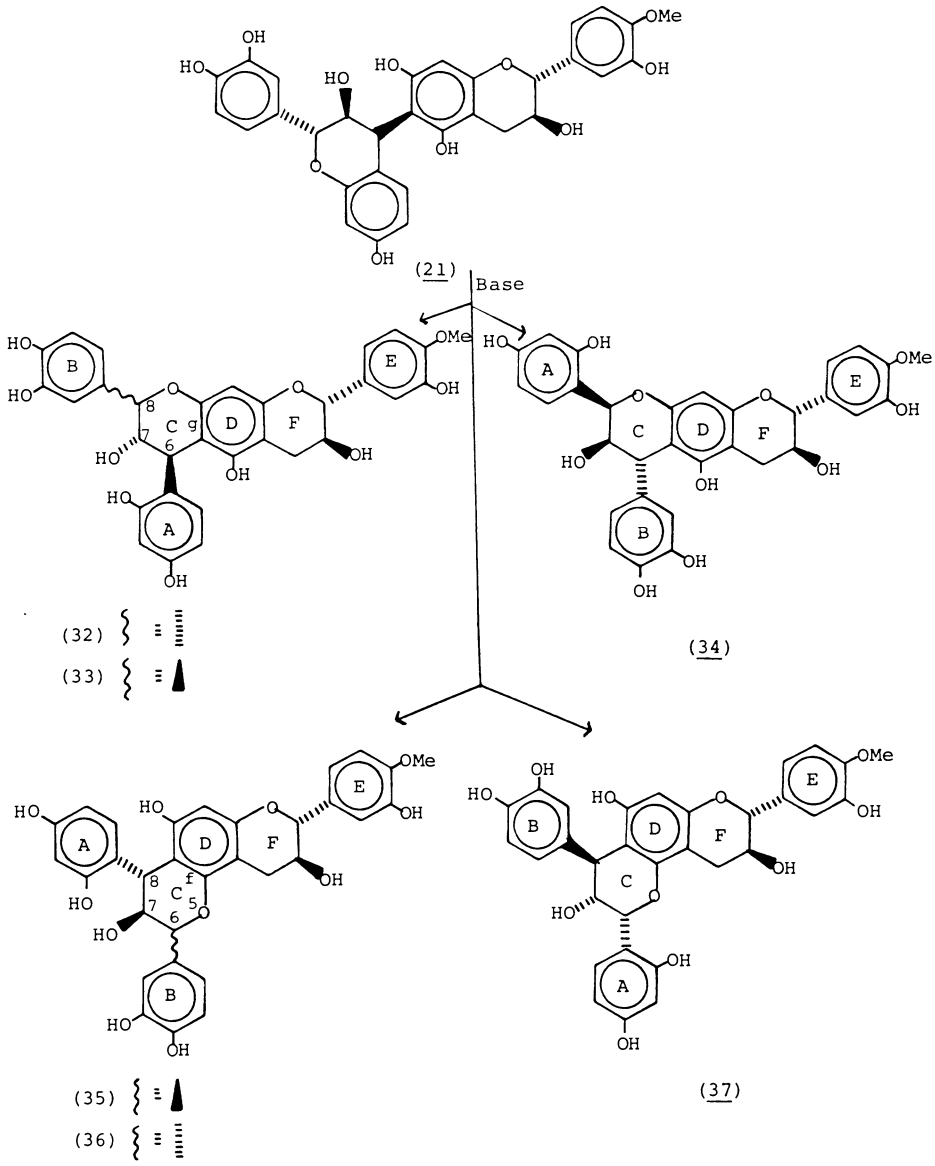
as 2**R**,3**S**,8**S**,9**S**,10**R** for (**26**) and 2**R**,3**S**,8**R**,9**S**,10**R** for (**27**). The same derivatives of the ring interchanged analogs (**28**) and (**29**) showed similar c.d. characteristics as those above, thus presumably reflecting similar 9**S**,10**R** absolute configuration for ring C. Such a contradiction may result from significant contributions of A-conformers (F-ring)[16] reversing the sign of the low-wavelength Cotton effect for 10β-aryl groups. The absolute configurations depicted in formulations (**28**) and (**29**) (i.e., 8**R**,9**R**,10**S** for (**28**) and 8**S**,9**R**,10**S** for (**29**), (and thus unambiguous proof for the inversion of absolute configuration at the chiral centers of ring C associated with the mechanism leading to the ring interchange) were confirmed by similar transformations on appropriate 4-arylflavan-3-ols as model compounds[17] where the structural features adversely affecting the sign of the low-wavelength Cotton effect are absent.

Base treatment of the (-)-fisetinidol-(4β,6)-(+)-catechin-*O*-methyl ether (**21**) afforded a mixture from which six ring-isomerized products (**32**) - (**37**) were obtained[3] (Scheme 7). Among these, the anticipated 6,7-*cis*-7,8-*trans*-tetrahydropyrano[2,3-f]chromene (**35**) [$J_{6,7}$ = ca. 1.0; $J_{7,8}$ = 2.0 Hz for its heptamethyl ether diacetate] and the 7,8-*cis*-6,7-*trans*-[2,3-g]regiomer (**32**) [$J_{7,8}$ = ca. 1.0; $J_{6,7}$ = 2.0 Hz for the heptamethyl ether diacetate] (formed in equal proportions), were differentiated by the selective n.O.e. association of 10-H(D) with 9-OMe(D) in the methyl ether diacetate of (**35**) but absence of association of the residual D-ring proton with methoxy hydrogens in the corresponding derivative of (**32**). The typical n.O.e. effect of 8-H(C) [for derivative of (**32**)] or 6-H(C) [for derivative of (**35**)] with 6-H(A), demonstrated above for *cis-trans* configurations, was again observed for both (**32**) and (**35**). Analogous n.O.e. associations between the D-ring singlet and methoxy protons of this ring also facilitated differentiation of the heptamethyl ether diacetates of the all-*trans* [2,3-f]-(**36**) [$J_{6,7}$ = 6.0; $J_{7,8}$ = 5.0 Hz for derivative of (**36**)] and [2,3-g]-(**33**) [$J_{7,8}$ = 8.0; $J_{6,7}$ = 7.0 Hz for derivatives of (**33**)] regiomers.

^{1}H-NMR data of the remaining pair of *cis-trans* tetrahydropyrano[2,3-f]-(**37**) ($J_{6,7}$ = ca. 1.0; $J_{7,8}$ = 2.0 Hz for its heptamethyl ether diacetate) and [2,3-g]-(**34**) ($J_{6,7}$ = 2.0; $J_{7,8}$ = ca. 1.0 Hz for the heptamethyl ether diacetate) chromenes, again differentiated by the appropriate n.O.e. effects, indicated the conspicuous deshielding of 6-H(A) [$\Delta\delta$-0.84, -0,71 for derivatives of (**37**) and (**34**) relative to those of the derivatives of the *cis-trans* pair (**35**) and (**32**)] associated with analogs where interchange of the resorcinol A- and pyrocatechol B-rings had occurred (see above). Such a ring interchange was again confirmed by the relevant spin decoupling- and HETCORR experiments. The anticipated all-*trans* regiomers with interchanged A- and B-rings, presumably formed as minor compounds, may have been overlooked due to the small quantities of available starting biflavanoid (**21**).

Negative Cotton effects in the 220-240 nm. region of the c.d. spectra of the heptamethyl ether diacetates of the tetrahydropyrano[2,3-g]-chromenes (**32**) and (**33**) are in accord with the 2**R**,3**S**,6**R**,7**S**,8**S** absolute configuration for (**32**) and 2**R**,3**S**,6**R**,7**S**,8**R** for (**33**).[12-15] Positive Cotton effects in the same region for the ring-interchanged [2,3-g] and [2,3-f] regiomers (**34**) and (**37**) similarly define their absolute configurations as 2**R**,3**S**,6**S**,7**R**,8**R** for (**34**) and 2**R**,3**S**,6**R**,7**R**,8**S** for (**37**), thus giving credence to the phenomenon of ring-interchange being associated with inversion of the absolute configuration at the equivalent of 3-C(C) of the start-

ing biflavanoid. C.d. data in the corresponding region for the derivatives of the tetrahydropyrano[2,3-f]chromenes (**35**) and (**36**) are, however, less reliable, presumably due to the proximity of the 8-C aryl substituent to the plane perpendicular to the D-ring through benzylic 8-C in conformations compatible with ^{1}H-NMR



Scheme 7. *Base-catalyzed conversion of the (-)-fisetinidol-(4β,6)-(+)-catechin* (**21**).

coupling constants. The proposed 2**R**,3**S**,6**S**,7**S**,8**R** absolute configuration for (**35**) and 2**R**,3**S**,6**R**,7**S**,8**R** for (**36**) are thus based on ^{1}H-NMR coupling constants and assumption of a mechanism for their formation prescribing retention of the configuration at 3-C(C) in biflavanoid (**21**).

Application[18] of the same protocol to the (+)-fisetinidol-(+)-catechins (2**S**,3**R** absolute configuration of their C-rings) revealed similar behavior to those described here for the (-)-fisetinidol-(+)-catechins. Thus, whereas, "upper" 2,3-*trans*-3,4-*trans*-flavan-3-ol units are susceptible to slower but stereospecific pyran rearrangement, those moieties with 2,3-*trans*-3,4-*cis* configuration react stereoslectively and are furthermore subject to interchange of resorcinol A- and pyrocatechol B-rings. It seems reasonable to suggest that the rate-determining step in these ring isomerizations involves reversible generation of the C-ring quinone-methide of type (**8**). In 3,4-*cis*-biflavanoids, e.g., (**19**), 7-OH(D) is favorably orientated to anchimerically assist cleavage of the O-C_2 bond, thus enhancing both the rate of quinone-methide formation and pyran rearrangement of 3,4-*cis*-flavan-3-ol units. Once formed, quinone-methides derived from 3,4-*trans*-flavan-3-ol moieties are favorably alligned for rapid and stereospecific recyclization via 7-OH(D). The near-axial (+)-catechin unit in 3,4-*cis* quinone methides, e.g., (**30**), would "ease" to a more equatorial orientation, thus facilitating stereoselective pyran recyclization with preference for attack of 7-OH(D) at the Si- and Re-faces in the 2**R**- and 2**S**-series of profisetinidins, respectively. This would presumably result in sufficient life-times to allow for secondary rearrangements to the A/B-ring interchanged products.

Among the analogs described above, the tetrahydropyrano[2,3-h]-chromenes (**1**), (**10**), (**27**), (**28**), (**29**), and (**35**), the latter four 4-O(E-ring) dimethyl ethers, have hitherto[3] been encountered in three species of the Caesalpiniodeae, *Guibourtia coleosperma* (false mopane), *Colophospermum mopane* (mopane), and *Baikiaea plurijuga* (Rhodesian teak).[19] Such natural occurrence presumably indicates mechanisms in nature similar to those proposed here.

Since many of the industrial applications of condensed tannins involve their dissolution and/or reaction at alkaline pH,[20,21] the base-catalyzed "liberation" of a resorcinol moiety from the C-ring of profisetinidin-type oligoflavanoids, e.g., transformation (**18**) → (**22**), should lead to enhanced reactivity of the pyran rearranged analogs with aldehydes. The fundamental principles outlined here may thus ultimately lead to the "activation" of the flavan-3-ol units present in commercially-available condensed tannin mixtures for use in "cold-set" adhesive applications through liberation of reactive nucleophilic resorcinol units.

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REFERENCES

1. Steenkamp, J.A.; Steynberg, J.P.; Brandt, E.V.; Ferreira, D.; Roux, D. Phlobatannins, a novel class of ring-isomerized condensed tannins. *J. Chem. Soc. Chem. Commun.* :1678 (1985).
2. Steynberg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V.; Steenkamp, J.A.; Ferreira, D. Novel base-catalysed rearrangements of (-)-fisetinidol-(+)-catechin profisetinidins with 2,3-*trans*-3,4-*cis*-flavan-3-ol constituent units. *J. Chem. Soc. Chem. Commun.* :1055 (1988).
3. Steynberg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V.; Steenkamp, J.A.; Ferreira, D. Oligomeric flavanoids. Part 3. Strucuture and synthesis of phlobatannins related to (-)-fisetinidol-(4α,6) and (4α,8)-(+)-catechin profisetinidins *J. Chem. Soc. Perkin Trans. 1* :3323 (1988). Part 4. Base-catalysed conversions of (-)-fisetinidol-(+)-catechin profisetinidins with 2,3-*trans*-3,4-it cis-flavan-3-ol constituent units *J. Chem. Soc. Perkin Trans. 1* :3331 (1988).
4. Hundt, H.K.L.; Roux, D.G. Synthesis of condensed tannins. Part 3. Chemical shifts for determining the 6- and 8-bonding positions of 'terminal' (+)-catechin units. *J. Chem. Soc. Perkin Trans. 1* :1227 (1981).
5. Young, E.; Brandt, E.V.; Young, D.A.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 17. Oligomeric (2R,3S)-3,3′,4′,7,8-pentahydroxyflavans: Atropisomerism and conformation of biphenyl and *m*-terphenyl analogues from *Prosopis glandulosa* ('Mesquite'). *J. Chem. Soc. Perkin Trans. 1* :1737 (1986).
6. Steynberg, J.P., Young, D.A.; Burger, J.F.W.; Ferreira, D.; Roux, D.G. Phlobatannins via facile ring isomerizations of profisetinidins and prorobinetinidin condensed tannin units. *J. Chem. Soc. Chem. Commun.* :1013 (1986).
7. Botha, J.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 4. Direct biomimetic approach to [4,6]- and [4,8]-biflavanoids. *J. Chem. Soc. Perkin Trans. 1* :1235 (1981).
8. Freudenberg, K.; Purrmann, L. Raumisomere catechine, III. (13. Mitteilung) Uber gerbstoffe und ahnliche verbindungen. *Chem. Ber.* 56:1185 (1923); IV. (16. Mitteilung) Uber gerbstoffe und ahnliche verbindungen. *Liebigs Ann. Chem.* 437:274 (1924).
9. Sweeny, G.J.; Iacobucci, G.A. Regiospecificity of (+)-catechin methylation. *J. Org. Chem.* 44:2298 (1979).
10. Ungaro, R.; Pochini, A.; Andreetti, G.D.; Domiano, P. Molecular inclusion in functionalized macrocycles. Part 9. The crystal and molecular structure of P-t-butylcalic[4]arene-anisole (2:1) complex: a new type of cage inclusion compound. *J. Chem. Soc. Perkin Trans. 2* :197 (1985).
11. Bergmann, W.R., Viswanadhan, V.N., Mattice, W.L. Conformations of polymeric proanthocyanidins composed of (+)-catechin or (-)-epicatechin joined by 4 $\rightarrow$ 6 interflavan bonds. *J. Chem. Soc. Perkin Trans. 2* :45 (1988).
12. Botha, J.J.; Ferreira, D.; Roux, D.G. Condensed tannins. Circular dichroism method of assessing the absolute configuration at C-4 of 4-arylflavan-3-ols, and stereochemistry of their formation from flavan3,4-diols. *J. Chem. Soc. Chem. Commun.* :699 (1979).
13. Barrett, M.W.; Klyne, W.; Scopes, P.M.; Fletcher, A.C.; Porter, L.J.; Haslam, E. Plant proanthocyanidins. Part 6. Chiroptical studies. Part 95. Circular dichroism of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :2375 (1979).
14. Botha, J.J.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 1. Stereoselective and stereospecific syntheses of optically pure 4-arylflavan-3-ols, and assessment of their absolute stereochemistry at C-4 by means of circular dichroism. *J. Chem. Soc. Perkin Trans. 1* :1213 (1981).
15. van der Westhuizen, J.H.; Ferreira, D.; Roux, D.G. Synthesis of condensed tannins. Part 2. Synthesis by photolytic rearrangement, stereochemistry and circular cichroism of the first 2,3-*cis*-3,4-*cis*-4-arylflavan-3-ols. *J. Chem. Soc. Perkin Trans. 1* :1220 (1981).

16. Porter, L.J.; Wong, R.Y.; Benson, M.; Chan, B.G. Conformational analysis of flavans: ^{1}H nmr and molecular mechanical (MM2) studies of the benzpyran ring of 3′,4′,5,7-tetrahydroxyflavan-3-ols: the crystal and molecular structure of the procyanidin: (2R,3S,4R)-3′, 4′,5,7-tetramethoxy-4-(2,4,6-trimethoxyphenyl)-flavan-3-ol. *J. Chem. Res. M.* :830 (1986).

17. Steynberg, J.P.; Burger, J.F.W.; Young, D.A.; Brandt, E.V.; Ferreira, D. Oligomeric flavanoids. Part 6. Evidence supporting the inversion of absolute configuration at 3-C associated with base-catalyzed A-/B-ring interchange of precursors having 2,3-*trans*-3,4-*cis* flavan-3-ol constituent units. *Heterocycles* COM-88-597 (1989).

18. Burger, J.F.W.; Steynberg, J.P.; Young, D.A.; Brandt, E.V.; Ferreira, D. Oligomeric flavanoids. Part 5. Base-catalyzed C-ring isomerization of (+)-fisetinidol-(+)-catechin profisetinidins. *J. Chem. Soc. Perkin Trans. 1* Paper 8/02280A (in press).

19. Palgrave, K.C. *In*: Moll, E.J., (ed.), Trees of Southern Africa. C. Struik Publishers, Cape Town p. 262 (1983).

20. Pizzi, A. Wood Adhesives: Chemistry and Technology. Marcel Dekker, New York (1983).

21. Kreibich, R.E.; Hemingway, R.W. The use of tannins in structural laminating adhesives. *In*: Proceedings of IUFRONTRI Symposium on Wood Adhesives 6:17-5 (1985).

KEY REACTIONS IN DEVELOPING USES FOR CONDENSED TANNINS: AN OVERVIEW

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ABSTRACT

Possibilities for the commercial use of condensed tannins as specialty chemicals are increasing rapidly with the increased focus of research on the reactions of polyflavanoid polymers. The past 5 years have seen considerable effort to define reactions of the A-ring primarily to assist in understanding the regioselectivity of interflavanoid bond locations in natural proanthocyanidins and to obtain more information on cross-linking reactions for use of tannins as adhesives. The interflavanoid bond has also received quite a lot of study, and some novel approaches to the use of tannins as adhesives and biocides have resulted from this work. Rearrangement reactions, both of the phloroglucinolic A-ring in procyanidins and of the pyran ring in profisetinidins, have been important in defining the properties of commericial tannin extracts. More interest is beginning to surface involving the B-ring; here, copper complexes of tannins have been shown to be effective fungicides. A summary of reactions that seem interesting from the author's perspective is presented.

INTRODUCTION

Over the past 5 years, the concentration of our research on the chemistry of condensed tannins has taken a distinct turn from heavy emphasis on the novelty of proanthocyanidin (polyflavanoid) structure, to focus on their reactions, a change that promises more rapid advance in the development of uses for these abundant renewable resources. This is not to imply that our earlier concentration on structure was wrong. Indeed, it was necessary to obtain definitive proof of the structures of the compounds we were trying to use. However, our understanding has now reached

such a level that, with the exception of the phlobaphenes (Chapter 6), enough is known to begin rationally exploiting our knowledge of polyflavanoid structure.

Much of the failure to develop commercially viable products from condensed tannins is due to the economics of cheap petroleum (Chapters 1 and 33). However, another significant deterrent to success has been our inadequate understanding of the reactions these compounds undergo both in the process of their isolation from plant tissues and during their formulation into specialty chemicals. In marked contrast to lignins, the polyflavanoids are exceptionally reactive compounds. Early work on the isolation and utilization of tannin extracts was perhaps unduly influenced by the experiences of lignin chemists. Therefore, emphasis tended to be on harsh reaction conditions. There are more than a few laboratories, including our own, that have converted these very reactive polymers into unreactive and unusable spray-dried powders by subjecting them to strong alkaline conditions. As shown in the preceding chapters, great progress has been made, and it is the author's opinion that we are on the verge of significant breakthroughs in the utilization of condensed tannins as specialty chemicals and that these are due in large measure to research on reactions of polyflavanoid polymers.

RECENT PROGRESS

McGraw (Chapter 14) has thoroughly reviewed the electrophilic aromatic substitution of the A-ring of proanthocyanidins that has been the focus of considerable attention over the past 10 years. The driving force for these studies was largely an attempt to understand the regioselectivity in substitution at the C-6 or C-8 positions of phloroglucinolic and resorcinolic rings of oligomeric proanthocyanidins. Less work was done in response to the more practical questions of how best to cross-link these polymers for the production of adhesive resins. Although the use of condensed tannins as partial replacements for phenol in wood adhesives has been the dominant thrust of tannin utilization since the early 1950's[1] (also Chapter 29) it has been only in the last year[2] that systematic study of the reactions of formaldehyde with purified proanthocyanidin oligomers of known molecular weight has been undertaken. Prior to Porter's work,[2] those attempting to make use of tannin-formaldehyde condensation products had little guidance – and that from studies of model phenols[3] and (+)-catechin – [4,5] on which to base adhesive formulations.

The interactive exchange involved in calculations of charge densities as are being undertaken by Tobiason (Chapter 13) with experimental observations, such as our studies on the reactions of tannins with hydroxybenzylalcohols[6] (also Chapter 14), is beginning to form a rational basis for understanding the behavior of tannins in phenolic resin systems. For example, although long advocated as a route to use of the tannins extracted from conifer tree barks,[7] we now know that cross-linking of tannins with polymethylolphenols has some very serious restrictions, both in terms of the rate of condensation to the nucleophilic A-rings and in limitations on the pH of these reactions because of facile A-ring rearrangement in the case of the phloroglucinolic procyandins and prodelphinidins.[8] The advances that have been made in using condensed tannins in wood adhesive systems, most notably use of

wattle tannins in a variety of bonding applications[9] and more recently Kreibich's work on cold-setting adhesives[10–12] (also Chapter 29), are remarkable because so much has been accomplished with our providing so little fundamental understanding of the chemistry of these reactions.

Base-catalyzed rearrangement of the phloroglucinolic ring, first demonstrated by Sears and coworkers[13] in rearrangement of (+)-catechin, has now been shown to be important reactions of the procyanidin polymers by Laks and Hemingway.[14,15] It is important to avoid these rearrangements if the products are to be used in applications where nucleophilicity of the A-ring is important such as in adhesive applications. The complexity involved in use of tannins as intermediates for specialty chemicals is highlighted by the contrasting result of base-catalyzed rearrangements of the 5-deoxy polymers such as profisetinidins and prorobinetinidins[16,17] (also Chapter 17). When working with the latter types of tannins, these rearrangement reactions may be advantageous.

We are just beginning to learn ways to exploit the lability of the interflavanoid bond in the procyanidin- and prodelphinidin-based tannins (Chapter 16). Demonstration of the ease with which it is possible to alter the viscosity and molecular weight distribution of polymeric procyanidins by reaction with sulfite[18] was essential to the success obtained in formulating cold-setting adhesives containing conifer tree bark or nutshell extracts as 50+ percent replacements for expensive phenol-resorcinol-formaldehyde resins used in wood-laminating industries[10,12] (also Chapter 29). These extracts also have potential as substitutes for significant amounts of the resorcinol used in tire cord adhesives[19] (also Chapter 30).

It is perhaps fortunate that the phenol glut during the early-to mid-1980's has caused a broadening of our perspectives for use of condensed tannins in specialty chemical markets. Earlier work at ITT-Rayonier (Chapter 1) had previously shown that extracts from western hemlock bark had excellent potential for applications ranging from soil grouting agents to heavy metal micronutrient preparations, and our research is moving from such intense focus on tannin-based adhesives to these and similar applications. Notable are the interesting reactions being studied by Laks[20,21] in his development of tannin-based biocides (Chapter 32). He has also taken advantage of the facile cleavage of the interflavanoid bond to produce fatty thiol adducts with high toxicity toward bacteria and fungi. That bacterial toxicity is closely correlated with lipophilicity was nicely demonstrated in his comparison of the fatty thiol adducts with ketals of various chain length through reactions at the catechol B-ring.

In addition, the great potential of metal complexes with the B-ring is once again being pursued. We can expect a number of specialty chemical applications such as copper(II)-tannin fungicide[22] (also Chapter 32) as our understanding of the reactions of the catechol and pyrogallol B-rings evolves.

OPPORTUNITIES FOR FUTURE RESEARCH

The unique structures of polyflavanoid polymers offer a wide range of avenues for commercially significant reactions of the A-ring, the B-ring, and by exploiting the lability of the interflavanoid bond. Some of the opportunities that seem apparent

now will require a long-term investment in research, but they do offer large potential paybacks.

Rearrangements of the structures of these compounds during their isolation or their formulation to specialty chemicals have received deserved attention over the past few years[14–17] (also Chapter 17), but research needs to be intensified. Far too much of our concept of polyflavanoid structure is based on small amounts of compounds isolated from plants under very carefully controlled conditions. Whether or not a polymeric proanthocyanidin structure serves as a suitable model for a commercial tannin extract from plants is a matter of some considerable debate.

The phloroglucinol or resorcinol A-rings are exceptionally good centers for electrophilic aromatic substitution reactions. Those studied to date have concentrated mainly on reactions with flavanyls, halogens, aldehydes, and methylolphenols, the former two mainly in an attempt to define the regioselectivity of interflavanoid bonds in natural proanthocyanidins and the latter two in attempts to use tannins in adhesive systems. Tobiason's work on analyses of charge distribution sets the stage for a deeper understanding of reactions at the A- and B-rings. Further study of the reactions of flavanoid compounds with furans[23] could lead to adhesive polymers based totally on renewable resources. Other modification reactions at the C-6 or C-8 positions to impart special properties to the polyphenol core could prove useful.

The catechol or pyrogallol B-rings offer special opportunities for the formation of metal complexes. The ability of tannins to form metal complexes has been utilized in leather manufacture, oil-well drilling mud additives, water treatment chemicals, agricultural micronutrients (Chapter 1), and more recently as Cu(II) complexes for fungicides with far less environmental impact than currently used wood preservatives (Chapter 32). Complexes with aluminum form the basis for a vegetable – aluminum tannage that would seem to have the best possibility of displacing chromium tannage should supply limitations or environmental restrictions occur (Chapter 31). Tannin-micronutrient complexes are also important in terms of soil productivity (Chapter 23). It is surprising that so little research has been devoted to this broad topic with such a widely based practical significance.

The extreme lability of the interflavanoid bond in either mild acidic[24] or alkaline[25] conditions offers the opportunity to produce low-molecular-weight oligomers with specially selected functional groups at the C-4 position. Laks[20,21] has taken advantage of this in the preparation of biocides based on fatty thiol adducts, and cold-setting phenolic resins have been made using either resorcinol or sulfite ions as nucleophiles (Chapter 29). The quinone methide (carbocation) generated at C-4 is especially reactive because of the phloroglucinol hydroxylation of the A-ring. Further opportunities exist to produce products with unusual properties by exploiting the lability of the interflavanoid bond.

The abundance of phenolic hydroxyls on each flavanoid unit offers a wide range of possible modification or cross-linking options. These would include the usual esterification or etherification reactions. For applications such as adhesives, tannins can be useful polyols for reactions with diisocyanates.[26] Although this mode of cross-linking appears to be the best way to produce plywood or particleboard

adhesives using tannins extracted from conifer tree barks, more information on these reactions is needed.

The complexation of tannins with proteins needs further attention from a number of different viewpoints. Most significant from the standpoint of the world economy is the effect of tannins on nutrition and health (Chapters 24 and 25). The leather industry also needs more fundamental research undertaken on the mechanisms of tanning (Chapter 31). Much of the biological significance of condensed tannins seems to be couched in terms of their possible complexation and inactivation of enzymes (Chapters 20 and 21). All of these areas will be assisted by increasing the level of support for fundamental studies of tannin – protein interactions such as is being done by Haslam, Haggerman, and Mattice.

Porter's[27] recent work on the reactions involved in the conversion of condensed tannins to anthocyanidins emphasizes the fact that, although this reaction has been used for decades both as a qualitative and quantitative analytical tool, much more work could be done on this topic. Not only is it important that we continue to improve on this reaction for use as a method of tannin assay, excellent commercial opportunities might appear if we were successful in learning to stabilize these chromophores.

The list of exciting directions for our research is no doubt endless, and the reactions given priority would obviously vary, depending on the particular interests of the author. We are just now beginning to know enough about these compounds to make research on the chemistry and utilization of condensed tannins both fun and productive. It will be most interesting to see how much our understanding of the reactions of condensed tannins can be advanced before the next North American Tannin Conference takes place.

REFERENCES

1. Hergert, H.L. Condensed tannins in adhesives, introduction and historical perspectives. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385, American Chemical Society, Washington, DC, pp. 155-171 (1989).
2. Porter, L. Viscosity and formaldehyde consumption of procyanidin solutions. *In*: Adhesives from Renewable Resources. Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) ACS Symposium Series No. 385, American Chemical Society, Washington, DC, pp. 172-184 (1989).
3. Hemingway, R.W., McGraw, G.W. Formaldehyde condensation products of model phenols for conifer bark tannins. *J. Liquid Chromtog.* 1:163 (1978)
4. Hillis, W.E., Urbach, G. The reaction of (+)-catechin with formaldehyde. *J. Appl. Chem.* 9:474 (1957)
5. Kiatgrajai, P., Wellons, J.D., Gollub, L., White, J.D. Kinetics of the polymerization of (+)-catechin with formaldehyde. *J. Org. Chem.* 47:2913 (1982).
6. McGraw, G.W., Ohara, S., Hemingway, R.W. Reactions of tannin model compounds with methylolphenols. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No.385, American Chemical Society, Washington, DC. pp. 185-202 (1989).
7. MacLean, H., Gardner, J.A.F. Bark extracts in adhesives. *Pulp Paper Mag. of Can.* 53:111 (1952).

8. Laks, P.E., Hemingway, R.W. Condensed tannins. Structure of the "phenolic acids". *Holzforschung* 41:287 (1987).
9. Pizzi, A. Tannin-based adhesives. *In*: Pizzi, A. (ed.) Wood Adhesives: Chemistry and Technology. Marcel Dekker, New York. pp. 177-246 (1983).
10. Kreibich, R.E., Hemingway, R.W. Condensed tannin-sulfonate derivatives in cold-setting wood-laminating adhesives. *For. Prod. J.* 37:43 (1987)
11. Kreibich, R.E., Hemingway, R.W. Condensed tannins: resorcinol adducts in laminating adhesives. *For. Prod. J.* 35:23 (1985)
12. Kreibich, R.E., Hemingway, R.W. Tannin-based adhesives for finger-jointing wood. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385, American Chemical Society, Washington DC. pp. 203-216 (1989)
13. Sears, K.D., Casebier, R.L., Hergert, H.L., Stout, G.H., McCandlish, L.E. The structure of catechinic acid a base rearrangement product of catechin. *J. Org. Chem.* 39:3244 (1975).
14. Laks, P.E., Hemingway, R.W., Conner, A.H. Condensed tannins: base-catalyzed reactions of polymeric procyanidins with phloroglucinol. Intramolecular rearrangements. *J. Chem. Soc. Perkin Trans. 1* :1875 (1987)
15. Laks, P.E., Hemingway, R.W. Condensed tannins: based-catalyzed reactions of polymeric procyanidins with toluene-α-thiol. Lability of the interflavanoid bond and pyran ring. *J. Chem. Soc. Perkin Trans. 1* :465 (1987).
16. Steynberg, J.P., Burger, J.R.W., Young, D.A., Brandt, E.V., Steenkamp, J.A., Ferreira, D. Novel base-catalysed rearrangements of (-)-fisetinidol-(+)-catechin profisetinidins with 2,3-*trans*-3,4-*cis*-flavan-3-ol costituent units. *J. Chem. Soc. Chem. Commun.* :1055 (1988).
17. Steenkamp, J.A., Steynberg, J.P., Brandt, E.V., Ferreira, D., Roux, D.G. Phlobatannins, a novel class of ring-isomerized condensed tannins. *J. Chem. Soc. Chem. Commun.* :1678 (1985).
18. Foo, L.Y., McGraw, G.W., Hemingway, R.W. Condensed tannins: preferential substitution at the interflavanoid bond by sulfite ion. *J. Chem. Soc. Chem. Commun.* :672 (1983)
19. Hamed, G.R., Chung, K.H., Hemingway, R.W. Condensed tannins as substitutes for resorcinol in bonding polyester and nylon cord to rubber. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385, American Chemical Society, Washington, DC. pp. 242-253 (1989).
20. Laks, P.E. Flavonoid biocides: phytoalexin analogues from condensed tannins. *Phytochemistry* 26:1617 (1987).
21. Laks, P.E., Pruner, M.S. Flavonoid biocides: structure activity relations of flavonoid phytoalexin analogues. *Phytochemistry* (in press).
22. Laks, P.E., McKaig, P.A., Hemingway, R.W. Flavonoid biocides: wood preservatives based on condensed tannins. *Holzforschung* 42:299 (1988).
23. Foo, L.Y., Hemingway, R.W. Condensed tannins: reactions of model compounds with furfuryl alcohol and furfuraldehyde. *J. Wood Chem. Technl.* 5:135 (1985).
24. Hemingway, R.W., McGraw, G.W. Kinetics of acid-catalyzed cleavage of procyanidins. *J. Wood Chem. Technl.* 3:421 (1983).
25. Hemingway, R.W., Laks, P.E. Condensed tannins: a proposed route to 2**R**,3**R**-(2,3-*cis*-proanthocyanidins. *J. Chem. Soc. Chem. Commun.* :746 (1985).
26. Dix, B., Marutzky, R. Modification of diisocyanate-based particle and plywood glues with natural polymers, polyphenols, carbohydrates, and proteins. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385, American Chemical Society, Washington, DC. pp. 229-241 (1989).
27. Porter, L.J., Hrstich, L.N., Chan, B.G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223 (1986).

Complexation

CARBOHYDRATE - POLYPHENOL COMPLEXATION

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ABSTRACT

Comprehensive studies of the complexation of polyphenols (vegetable tannins) with other substrates are of great practical significance and utility. Fundamental studies of these phenomena form part of the strategy adopted in Sheffield to pursue an understanding of the possible function and metabolic role of this distinctive group of natural products. With regard to polyphenols, molecular size, conformational mobility and shape, and water solubility are the three principal critera that most strongly influence association with polysaccharides. The differing affinities of polyphenols for polysaccharides result from a balance between a variety of effects– adsorption, sequestration, and solvation. The importance of polyphenol sequestration into "pores" in the polysaccharide structure has been demonstrated by model studies with Schardinger dextrans or cyclodextrins.

INTRODUCTION

Secondary metabolism concerns the biosynthesis and transformation of a wide array of "natural products" – alkaloids, terpenes, polyacetylenes, phenols, mycotoxins – in plants and microorganisms.[1,2] Although secondary metabolite production is often correlated with cellular and morphological differentiation, a unified theory to rationalize the function of secondary metabolism in the host organism has not yet been put forward and accepted. Theories that have been canvassed fall into four broad categories:[2] (a) At some point in the life of the organism, the secondary metabolites had (or have) a functional (metabolic) role; (b) the secondary metabolites are a measure of the organism's fitness to survive; (c) secondary metabolism provides organisms with a means of adjustment to changing circumstances;[3] (d) secondary metabolites are waste or detoxification products.

Polyphenols (M_R 500-20,000), the vegetable tannins described in the earlier chemical and botanical literature, constitute a unique group of phytochemicals and as such a distinctive class of secondary metabolites. They show great structural diversity, based principally on the two themes of gallic acid and flavan-3-ol metabolism,[4] and wide phylogenetic distribution.[5] Their uniqueness as secondary metabolites lies not only in the extensive molecular weight range they encompass but also in their polyphenolic nature. Both characteristics endow polyphenols with the ability to complex strongly with metabolites like proteins and carbohydrates. In this respect, plant polyphenols resemble many antibiotics that are more toxic to the producing organism than their metabolic precursors. However, it should be noted that in *Pinus elliotti* cells, tannin toxicity appears to occur only after a large concentration of material accumulates in the cell, or membrane integrity is lost.[6]

One theory of secondary metabolism (b, *vide supra*) suggests that secondary substances such as plant polyphenols are intimately concerned in the complex interactions between organisms and their environment and that this is their *raison d'etre*. Various workers have propounded the view that secondary substances play the leading role in the determination of patterns of plant utilization by herbivores. The idea has an immediate intuitive appeal, and Feeny's early seminal work[7] led to the view that vegetable tannins constitute a unique quantitative defense for plants. The relevant physiological effects of polyphenols derive from their ability to interact with proteinaceous and carbohydrate materials, thereby rendering plant tissues repellent to potential predators and, once ingested, decreasing their intrinsic nutritional value.

It was envisaged that a comprehensive study of the complexation properties of polyphenols, in particular to discover if real specificity exists in the interactions with proteins and carbohydrates, would ultimately throw light on the possible role (if any) of polyphenols in higher plant metabolism. Such studies are also, parenthetically, of great practical utility and significance. Polyphenolic containing plant materials are remarkable for their astringent taste. The acceptability and nutritional value of foodstuffs, of beverages such as ciders, wines, and teas, the efficacy of many herbal remedies, and the ability of plant extracts to execute the age-old process of vegetable tannage of animal skins all devolve to a greater or lesser extent on the astringency of plant polyphenols. Plant tannins likewise play a central role in the formation of soil humus and the development of soil profiles. They thus influence the retention of organic matter in the soil by producing complexes with proteins and polysaccharides that are resistant to microbial decay.[8]

Studies of protein-polyphenol complexation have, to date, commanded major interest and attention. Various mechanisms have been suggested, and complexation is believed to be mediated by a combination of hydrogen bonding, hydrophobic interactions, and differential solvation.[9] Studies of the association of polyphenols with carbohydrates were commenced not only to throw light on the unexplored nature of these interactions themselves but also to seek further evidence concerning the general importance of hydrophobic forces in polyphenol complexation. A third, more immediate, factor that prompted studies was the need to understand in greater detail the structure of the "insoluble" polymeric proanthyocyanidins.[10–12] It has been suggested that these insoluble forms are oligomeric proanthocyanidins, which, very

simply, are strongly complexed to carbohydrate structures in the cell. Porter[13] and Haslam[14] have however, likened the general structure of these polymers to that of lignin. Porter[13] suggested it was possible that condensed proanthocyanidins are glycosidically bound, via the C-3 hydroxyl group, to cell wall hemicelluloses. Haslam and Shen,[14] using arguments based on biosynthetic considerations, proposed an alternative structural model in which condensed proanthocyanidin fragments were linked terminally at C-4 to hemicellulose units. Proanthocyanidins and phenolic flavan-3-ols invariably occur free or occasionally as gallate esters in plants. Sporadic reports of glycosylated flavan-3-ols have been made[13] following the discovery of (+)-catechin 7-*O*-arabinoside in *Polypodum vulgare* by Herout[15] in 1967. Porter and his colleagues[13] have reported the spectroscopic identification of three *O*-β-glucopyranoside procyanidin polymers from *Cydonia oblonga* (quince) fruit and the barks of *Pinus brutia* and *Picea abies*. Nishioka and his collaborators have described the characterization[16–18] of several flavan-3-ol and procyanidin *C*- and *O*-glucosides from the bark of *Cinnamomum* species and rhubarb. Yields are almost invariably low. Fifteen *C*- and *O*-glucosides of (+)-catechin and various procyanidins were thus isolated by Nishioka, Nonaka, and Kashiwada[18] from commercial rhubarb (choukichio). The maximum isolated yield was that of (+)-catechin 5-*O*-β-*D*-glucopyranoside (0.01 percent); the total yield of glucosides was 0.03 percent. It was, therefore, also as an attempt to resolve the important structural problem of the nature of the "insoluble" condensed proanthocyanidins[13,14] that studies of polyphenol-carbohydrate complexation were undertaken.

CARBOHYDRATE COMPLEXATION

Sephadex Gels

Studies of the reversible association of polyphenols with proteins have a long history, which, in the last decade, has been considerably facilitated by the availability of both classes of substrate in pure, structurally defined forms. Similar comparative studies of polyphenol-carbohydrate complexation have to some extent been hampered by the absence of water-soluble polysaccharides with clearly defined structures and molecular weights.[19] The most satisfactory quantitative data have been obtained using polysaccharides in the solid state – the various chromatographic gels Sephadex G-25, G-50, and LH-20 and cellulose triacetate in membrane form. Dextran is a polymeric glucan derived microbiologically by the action of *Leuconstoi mesenteroides* on sucrose and consists of α-*D*-(1-6)-linked units to an extent of 90-95 percent. Cross-linking by epichlorhydrin yields gels such as Sephadex. The affinity of aromatic compounds for dextran-gels is well documented, and it is noteworthy that the addition of hydroxy groups – particularly in the 1,3 and 1,3,5 orientation – enhances that affinity.[20] The origins of this affinity are uncertain. Several suggestions have been proposed: interaction between the phenolic hydroxy groups and the ether oxygen atoms of the cross-linking chains, the phenyl rings acting as electron donors to the hydroxy groups of the Sephadex, and inclusion of the substrate within the pores of the Sephadex gel.[21–23]

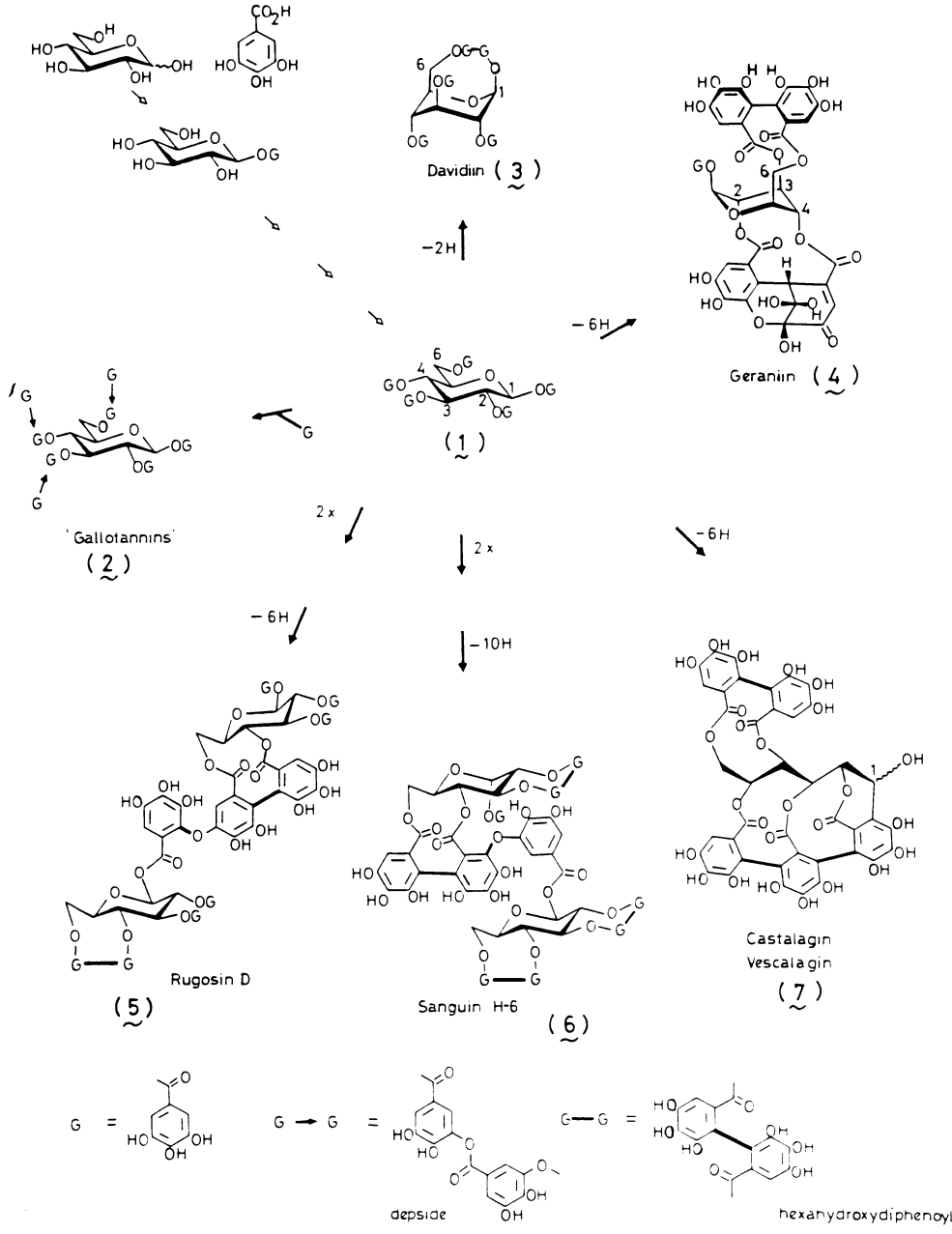
The rank order of binding of a range of naturally occuring phenols to Sephadex gels has been determined by using the apparent partition coefficient, K_{AV}, which is proportional to the more frequently employed distribution coefficient K_D.[24] Both Sephadex G-25 and G-50 were employed with aqueous buffers and Sephadex LH-20 with methanol-water (19:1, v/v) as eluant. As with proteins, subtle changes in polyphenol structure result in marked changes in affinity for the polysaccharide gel (Table 1). Molecular size, conformational flexibility, and water solubility appear to be the three principal criteria that most strongly influence the retention of polyphenols by the polysaccharide matrix.

The highly condensed rigid polyphenolic structures of vescalagin (Scheme 1) and its C-1 enantiomer, castalagin, (**7**) are bound weakly to Sephadex. Hexahydroxydiphenyl-bridged polyphenols based on the $^4C_1$-*D*-glucopyranose conformation [e.g., eugeniin (**9**) and casuarictin (**10**) (Figure 1)] are comparatively much more strongly bound to Sephadex than analogous phenolic metabolites, such as geraniin (**4**) and its dihydro-derivative (**8**), which are based on the thermodynamically less favorable $^1C_4$ conformation and which have a much more compact molecular structure. Two other shape-related observations are also significant. The introduction of a second intramolecular hexahydroxydiphenoyl linkage in the relatively compact $^1C_4$-*D*-glucopyranose metabolites produces a comparatively small additional decrease in affinity for the gel and hence K_{AV} value [cf. davidiin (**3**) and dihydrogeraniin (**8**)], whereas, the same change in the more open $^4C_1$-*D*-glucopyranose metabolites results in a substantial change in K_{AV} [cf eugeniin or tellimagrandin-2 (**9**) and casuarictin (**10**)]. Secondly, the relatively small value, K_{AV}, between geraniin (**4**)

Table 1. Chromatography of Polyphenols on Sephadex Gels: K_{AV} Values at 298 °K

Polyphenol	(a)[a]	(b)[b]
Phenol	1.1	1.13
(-)-Epicatechin (**15**)	1.83	1.96
Procyanidin B-3 (**18**)	2.36	3.04
β-1,3,6-Trigalloyl-*D*-glucose	4.43	2.49
β-1,2,4,6-Tetragalloyl-*D*-glucose	16.25	6.17
β-Pentagalloyl-*D*-glucose (**1**)	–	8.56
Eugeniin (**9**)	53.9	6.2
Casuarictin (**10**)	7.57	-
Davidiin (**3**)	7.54	-
Geraniin (**4**)	5.89	-
Dihydrogeraniin (**8**)	6.18	-
Vescalagin (**7**)	2.5	2.28
Sanguin H-6 (**6**)	-	24.8
Rugosin D (**5**)	-	56.7

[a]Sephadex G-25, Cl^- buffer
[b]Sephadex LH-20, MeOH-H_2O (9:1, v/v)



Scheme 1. *Relationships among different hydrolyzable tannins.*

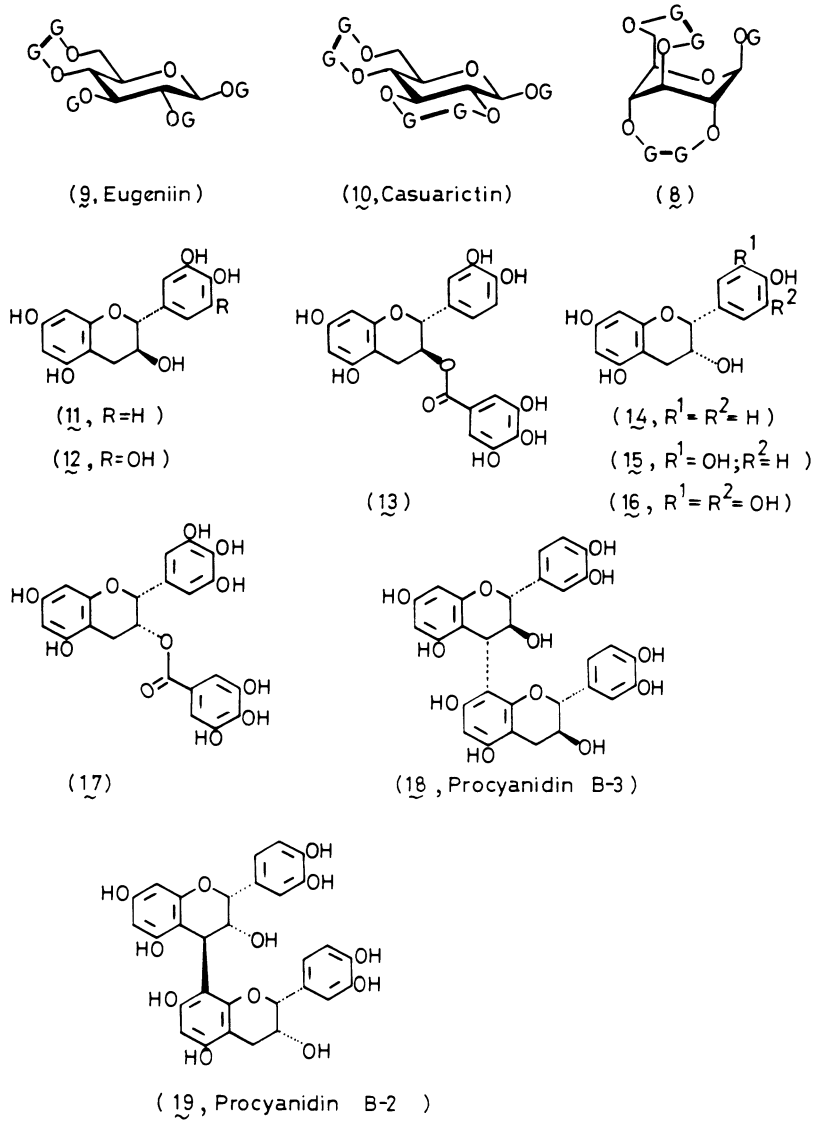


Figure 1. *Structures of hydrolyzable and condensed tannins and their derivatives.*

and its dihydro derivative (**8**) indicates that the overall shape is probably of as much significance in determining the strength of association with dextran gel as the number of phenolic groups. The "dimers" rugosin D (**5**) and sanguin H-6 (**6**) were not eluted from Sephadex G-25 and G-50. In order for a relationship between these metabolites, their presumed biosynthetic precursor (**1**), and the simpler phe-

nolic substrates to be established, comparisons were made using the more lipophilic Sephadex LH-20 as the adsorbent and 5 percent aqueous methanol as the eluant. Determination of K_{AV} (Table 1) gave a relative order of affinities in broad agreement with the data from G-25 and G-50 gels. Nevertheless, both "dimers" (**5** and **6**) were bound substantially more strongly to the polysaccharide gel than the other phenolic metabolites, including (**1**). This observation confirms the view that the molecular size of the polyphenol, with the possibility of enhanced cooperative effects in association, may well be a factor of paramount importance in polyphenol-polysaccharide (gel) complexation.

The differing affinities of the polyphenolic metabolites for the dextran gels may be ascribed to the balance between a variety of effects – adsorption, sequestration, and solvation. Metabolites may thus be simply adsorbed to different degrees on the surface of the gels. However, the importance of shape of the polyphenol (*vide infra*) appears to point to other influences. Sephadex G-25 has an exclusion limit at M_R 5,000; hydroxypropylation (to give LH-20) does not alter the number of free hydroxyl groups but increases the carbon/hydroxyl ratio and reduces, somewhat, the exclusion limit. If it is assumed that all the metabolites described have the capacity to penetrate the pores of the gels, then, the differential effects may well arise from interactions between the phenolic aroyl ester groups of the substrates and the *interior* of the pores of the gels. Substrates once encapsulated may not readily dissociate. The question remains an intriguing one to seek to resolve.

One of the key factors in this whole problem is the role of solvent in the complexation – particularly that of water in aqueous media. Its presence and influence are accepted but invariably tacitly ignored. The structure of aqueous solvent surrounding a polysaccharide molecule or within a gel is nevertheless one of the most important facets of its structure. The degree of order of the solvent externally is generally proportional to its proximity to the polysaccharide surface where water molecules are anchored by hydrogen bonding to acceptor and donor groups. Solvation is likewise a key factor that determines the properties of natural polyphenols. Various observations suggest that individual phenolic groups and the associated aromatic nuclei may be solvated by water to quite different degrees. If, for example, a titration of proton chemical shift (δ) versus solvent composition is made for the aroyl protons of (**1**), in 100 percent D_6 acetone (or D_4 methanol), progressively changing the solvent until it reaches 100 percent D_2O, marked changes are observed (Figure 2). Presumably, these effects are in large part due to preferential solvation. It is not yet clear how all these factors are related to polysaccharide-polyphenol complexation. Presumably, displacement and reorganization of solvation shells are inevitable consequences of intermolecular association, and if there is a relationship to polyphenol-protein association, then polyphenols (which are freely soluble in water) would be expected to have a relatively poor affinity for polysaccharides. Where the association is primarily a surface effect, then, broad similarities may be noted with the patterns of polyphenol-protein complexation although molecular size appears to be a more dominant factor. Significant departures from these patterns may be predicted also where the polysaccharide is capable of forming inclusion complexes by sequestration of aromatic nuclei.

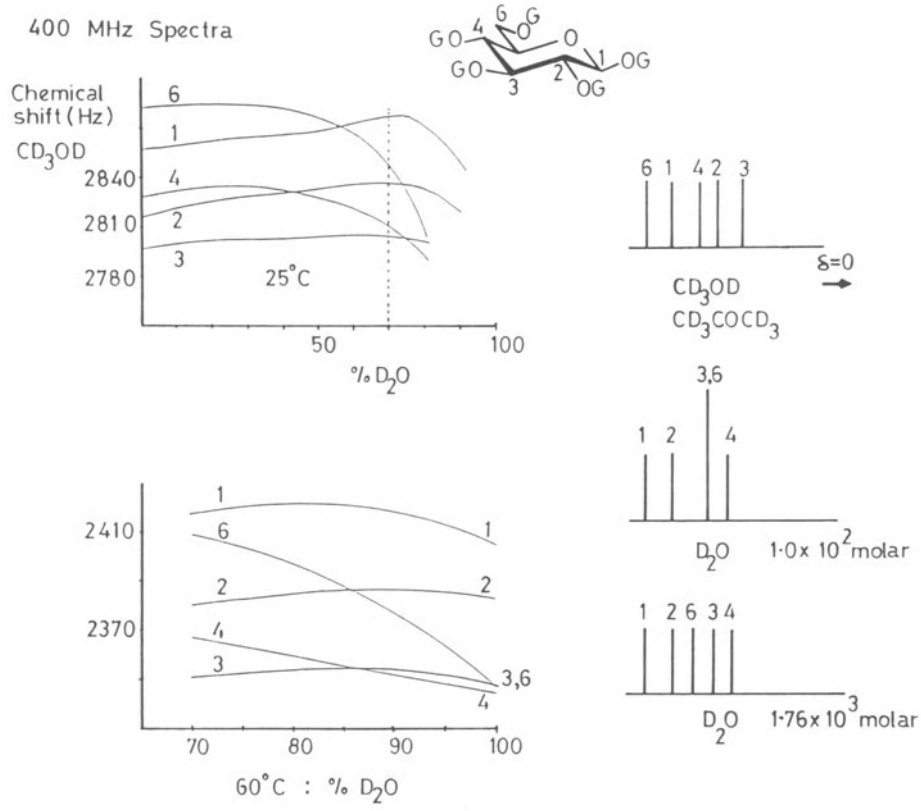


Figure 2. *Chemical shift changes with change in solvent composition: aroyl protons of β-1,2,3,4,6-penta-O-galloyl-D-glucose* (**1**).

Cyclodextrins

The association of polyphenols with macromolecules such as proteins and polysaccharides is a specific example of the biological phenomenon of molecular recognition. The principles that underly molecule recognition may be analyzed not only in terms of the composition, structure, and conformational flexibility of both acceptor (host) and donor (guest) molecules but also in terms of three idealized concepts.[25] "Dye-mold" ("jig-saw") matching is essentially static with an exact fit of donor and acceptor molecules. "Key-lock" matching is time dependent, since the key (donor) invariably has to be maneuvered into the lock (acceptor) to achieve the necessary precise fit. Finally, "hand-in-glove" matching of donor and acceptor is both time dependent and dynamic. Donor and acceptor molecules are mobile and flexible and may assume a variety of shapes as complexation proceeds. In such situations, to bring about strong and effective binding, it is imperative that many strong contact points are ultimately made between donor and acceptor species. Such associative processes frequently exhibit strong cooperative effects. Present evidence suggests

that polyphenol complexation is largely of the "hand-in-glove" type. The critical questions that remain to be answered concern the nature and the identity of the points of matching and recognition. Attention has focused on (i) hydrogen bonding and (ii) hydrophobic interactions as the vehicles for complexation. The relative significance of these two types of non-covalent interaction, however, remains a subject for speculation and debate. Studies of polyphenol-polysaccharide association indicate (*vide supra*) that the ability of the polysaccharide host to generate shapes that are able to encapsulate, either wholly or in part, the polyphenol guest is often a critical feature promoting strong binding. In order for this form of hydrophobic interaction to be studied and its significance comprehended fully, attention has been directed toward the interaction of polyphenols with α- and β-cyclodextrins.

Cyclodextrins, first isolated in 1891, are cyclic oligosaccharides composed of (1-α-4) linked *D*-glucosyl residues; α- and β-cyclodextrins possess, respectively, 6 and 7 glucose units. They have the shape of a doughnut with all the *D*-glucopyranose units in substantially undistorted ($^4C_1$, C-1) conformations. The cavities are slightly "V"-shaped; the secondary hydroxyl groups (at C-2 and C-3) on the upper side of the torus and primary hydroxyl groups (at C-6) on the lower face. The interior of the torus consists of the glycosidic oxygen atoms and two concentric rings of C-H groups (at C-3 and C-5, Figure 3). The cavity is generally thought to be more accessible from the face bearing the secondary hydroxyl groups and, compared to an aqueous environment, is apolar and relatively hydrophobic.[26]

One of the most important properties of the cyclodextrins is their ability to sequester substrates in the hydrophobic cavity. The nature of the binding and the driving forces that lead to inclusion remain uncertain although several

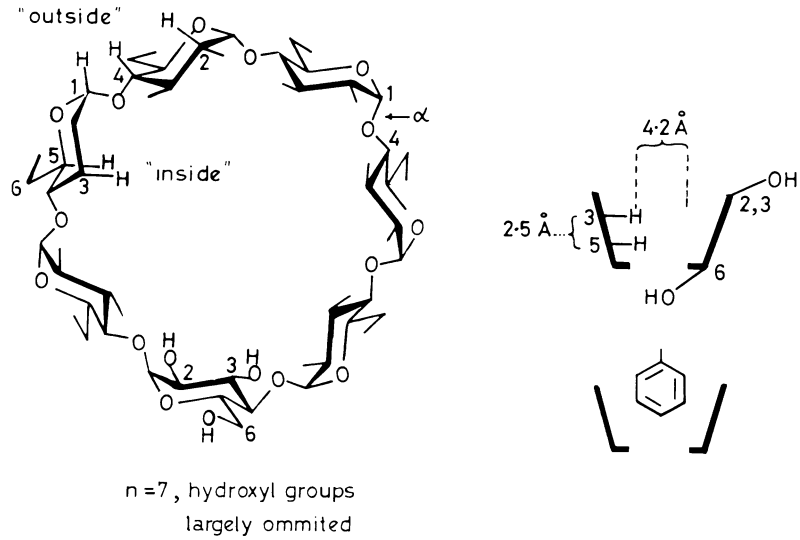
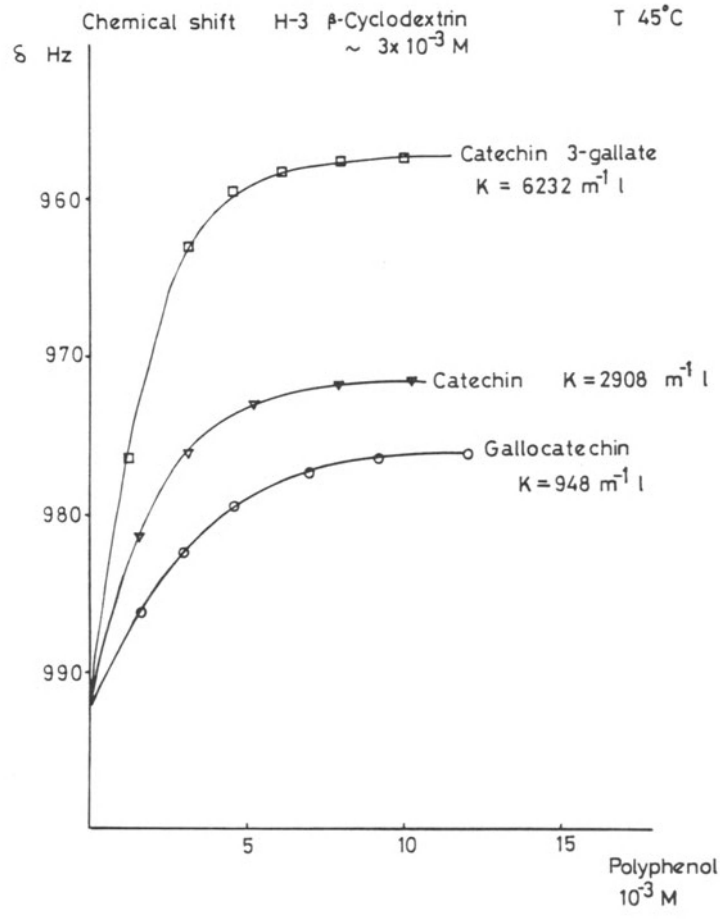


Figure 3. *β-Cyclodextrin*

suggestions have been put forward[26] including (i) hydrogen bonding, (ii) van der Waals interactions, (iii) release of strain energy in the cyclodextrin, and (iv) release of solvent water molecule(s) from the cavity. In order for the importance of "cavity sequestration" to be ascertained in relation to polyphenol complexation overall, the mechanism of the formation of cyclodextrin inclusion complexes with phenolic substrates has been investigated by high-resolution ^{1}H- and ^{13}C-NMR spectroscopy. The resonances of H-3 and H-5, located *inside* the cavity of the cyclodextrins, show substantial changes of chemical shift (δ) upon complexation with aromatic compounds, including polyphenols (Figure 4). This provides direct and compelling evidence of insertion of the aromatic nucleus of the phenolic substrate into the cyclodextrin cavity in aqueous media. The ^{1}H- and ^{13}C-NMR signals of the phenolic guest also display characteristic chemical shift changes, and measurement of these various complexation-induced shifts and application of variations of the Benesi-Hildebrand equation lead to the association constant for the formation of the inclusion complex. Measurements were made for various phenols with α- and β-cyclodextrin (Table 2). Several features command immediate attention. Compared to galloyl esters, the binding of phenolic flavan-3-ols is quite strong. It is dependent on the stereochemistry of the hydroxyl group at C-3 in the flavan and is diminished by hydroxyl substitution in the B-ring. Complexation is enhanced by galloylation at the 3 hydroxyl group: modestly (2x) in the case of (+)-catechin (**11**), but substantially (9x) with (-)-epigallocatechin (**16**). Significantly, substitution at C-4 by additional flavan-3-ol molecules to give the typical proanthocyanidin structures (**18,19**) virtually suppresses completely the ability to complex with the cyclodextrins.

The question of the orientation and location of the aromatic nuclei of the phenolic substrates, particularly the flavan-3-ols, within the cyclodextrin cavity has proved more difficult to elucidate. In earlier work, the geometry of cycloamylose inclusion complexes with a series of substituted phenols was determined.[27,28] In all cases examined, the phenyl rings were inserted into the cavity from the secondary hydroxyl group side with the *para* substituent in the lead and with the phenolic group remaining in the aqueous media. ^{1}H-NMR data were interpreted to show that the guest phenols were more deeply inserted into the cavity of β-cyclodextrin (cyclomaltoheptaose) than into that of α-cyclodextrin (cyclomaltohexaose). For the α-cyclodextrin complexes, these structures agree well with those determined by X-ray crystallography for the two crystalline complexes formed between the cycloamylose and *p*-nitrophenol and *p*-hydroxybenzoic acid, respectively.[29]

In the case of polyphenol cyclodextrin complexation, a wide range of parameters has been probed and measured in an attempt to throw light on the manner in which the phenolic substrates associate with the oligosaccharide host. These parameters include the measurement of the proton chemical shift changes (δ) in both guest (polyphenol) and host (cyclodextrin), which occur on complexation. For the cyclodextrin host, attention has focused particularly on the chemical shift changes observed for the "inner-protons" H-3 and H-5 in the cyclodextrin cavity (Figure 2). For very simple phenolic substrates, the most probable (time-averaged) position of the phenolic guest in the cyclodextrin cavity was determined by fitting the intrinsic chemical shifts of H-3 and H-5 to those calculated using the Johnson-Bovey



$$\frac{1}{\Delta\delta} = \frac{1}{K.\Delta\delta_o}\cdot\frac{1}{[P]} + \frac{1}{\Delta\delta_o}$$

$\Delta\delta$ = Chemical shift change induced by polyphenol at concentration [P].

$\Delta\delta_o$ = Chemical shift difference between the H-3 Cyclodextrin resonance in the unbound state and that in which it is totally as a 1:1 complex.

Figure 4. *Flavan-3-ol-β-cyclodextrin complexation. H-3 chemical shift changes (250 MHz, 318 °K, β-cyclodextrin ~ 3mM).*

Table 2. Polyphenol-Cyclodextrin Complexation Association Constants[a] (K_{cp} molar^{-1} litre) in Deuterium Oxide

Compound	β	α
Flavan-3-ols[b]		
(+)-Catechin (**11**)	2908	–
(+)-Catechin-3-gallate (**12**)	6232	–
(+)-Gallocatechin (**13**)	948	–
(-)-Epiafzelechin (**14**)	792	–
(-)-Epicatechin (**15**)	464	–
(-)-Epigallocatechin (**16**)	208	–
(-)-Epigallocatechin-3-gallate (**17**)	1889	–
Procyanidin B-3 (**18**)	101	–
Procyanidin B-2 (**19**)	63	-
Miscellaneous phenols[b,c]		
p-Hydroxybenzoic acid	652[b]	472[c]
3,4-Dihydroxybenzoic acid	459[b]	702[c]
Methyl-3,4-dihydroxybenzoate	280[b]	138[b]
Gallic acid	114[c]	215[c]
Methyl gallate	147[c]	–
Methyl-2,3,4-trihydroxybenzoate	212[b]	–
Resorcinol	117[c]	–
Phloroglucinol	110[c]	–
β-1,2,3,4,6-Penta-*O*-galloyl-*D*-glucose (**1**)	340[d]	–

[a]Association constants quoted as average of several measurements calculated from the chemical shift changes ($\Delta\ \delta$) of H-3 and H-5 of the cyclodextrin.
[b]318 °K
[c]298 °K
[d]333 °K

equation reflecting the magnetic anisotropy of the aromatic ring of the guest.[30,31] The shielding and/or deshielding effects of the phenolic groups were ignored. Although measurements of H-3 and H-5 chemical shifts can give valuable information on the position of the center of the phenolic nucleus in the cyclodextrin cavity, they provide no information on the host-guest orientation in the complex. In seeking to determine the orientation and location of the phenolic molecule in the cavity, ^{1}H-nuclear Overhauser (n.O.e.) measurements and ^{13}C-NMR studies have been made. Investigations of α-cyclodextrin inclusion complexes of benzene derivatives have shown, not infrequently, that the lead carbon atom of the guest molecule is largely shielded (high-field shift) compared to the corresponding *para* carbon atoms,

which generally exhibit a low-field shift. These various methods may be applied with facility to simple phenolic substrates such as the various hydroxybenzoic acids and their esters, resorcinol, catechol, and phloroglucinol, and phenolic glucosides such as arbutin.

For more complex phenolic substrates, as for example the phenolic flavan-3-ols and their derivatives (**11-19**), the expectation is that several modes of association are probably in operation involving the insertion of both rings A and B into the cyclodextrin cavity. (Figure 5,*a,b,c*). That association occurs in part by method (*c*) – insertion of the B-ring – is indicated by comparison of the association constants (Table 2) for (-)-epiafzelechin (**14**), (-)-epicatechin (**15**) and (-)-epigallocatechin (**16**). Increasing hydroxylation in ring "B" (increasing size and hydrophilic character) steadily reduces the affinity of the phenolic flavan-3-ol substrate for β-cyclodextrin. Molecular model-building points to possible explanations for the influence of stereochemistry at the C-3 hydroxyl group in the heterocyclic ring upon complexation and its enhancement by gallate ester formation. This latter effect is envisaged as being due to the pendant 3-gallate ester acting as an anchor (by hydrogen bonding) to the peripheral hydroxyl groups at C-2 and C-3 on the top edge of the cavity of the cyclodextrin. Likewise, the hydroxyl group at C-3 in the catechin series (**3-5**) is more able to assist complexation by hydrogen bonding in a similar fashion than is that in the epicatechin series (3**R**).

Clearly, although a great deal more work remains to be carried out to produce a wholly satisfying rationale of the manner of polyphenolic complexation with α- and β-cyclodextrins, several important features have emerged. Polyphenolic substrates have a strong affinity for preformed oligosaccharide "holes" such as are provided by cyclodextrins. Complexation is probably largely of the "lock and key" type and is critically dependent on cavity size and structural and stereochemical features of the polyphenolic substrate. It is, nevertheless, evident that natural polyphenols have a peculiar affinity for "holes, pores or crevices" in polysaccharide structures whether these are preformed (cyclodextrins and Sephadex gels) or whether they develop as the polyphenol and oligosaccharide undergo molecular recognition (amyloses). Broad features of the structure-activity relationships that define (poly) saccharide-

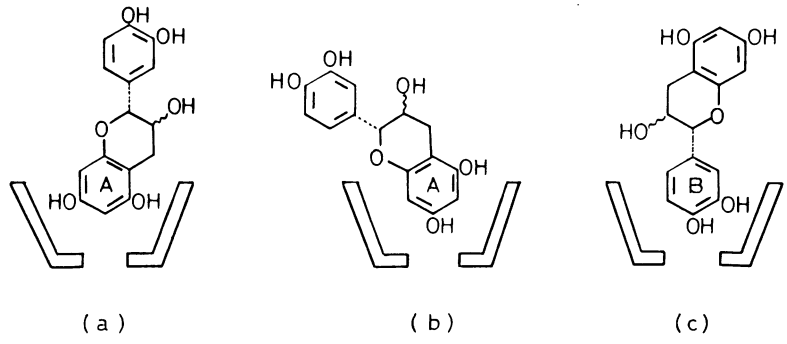


Figure 5. *Molecular recognition: flavan-3-ol-β-cyclodextrin complexation.*

polyphenol interactions are summarized below. Their significance in terms of the delineation of the possible role of plant polyphenols in plant metabolism has yet to be determined. In relation to the question of the nature of the so-called "insoluble proanthocyanidins" referred to earlier,[10-12] this work demonstrates that polymeric proanthocyanidins, if they become entrapped within the pores of a polysaccharide structure such as pectin or the crevices within a cellulose matrix, may well become very firmly bound by non-covalent interactions. Whether the proanthocyanidins then subsequently resist all attempts at solubilization appears doubtful. The balance of evidence still points to some form of chemical linkage of the oligomeric proanthyocyanidins to the cellular matrix of the plant.[14]

CONCLUSIONS

The results of this work can be summarized as follows:

Complexation is dependent on polysaccharide type but is broadly independent of pH (4-7). Open filamentous polysaccharides (1-α-6-dextrans) bind polyphenols weakly. Polysaccharides that contain crevices, pores, or holes in their three-dimensional structure or polysaccharides that assemble in solution to loosely form pores or holes within the structure bind polyphenols strongly. Association is principally mediated probably by sequestration of the polyphenol substrate into the pores.

Molecular size of the polyphenol: increased molecular size leads to significantly enhanced affinity for polysaccharide.

Conformational flexibility of the polyphenol: when conformational restraints are placed on the polyphenol substrate, then its capacity to complex is reduced. Overall shape and mobility are probably of as much significance in the polyphenol structure as the number of phenolic groups.

Water solubility of the polyphenol: a broadly inverse relationship exists between the strength of association and the solubility of the polyphenol in water.

ACKNOWLEDGMENTS

The authors wish to thank the Agriculture and Food Research Council (S.H.G.) and the Government of the Peoples Republic of China (Y.C.) for financial support and Professor I Nishioka (Kyushu University, Japan) for the gift of a sample of (-)-epiafzelechin.

REFERENCES

1. Bu'Lock, J.D. Intermediary metabolism and antiobiotic synthesis. *Adv. Appl. Microbiol.* 3:293 (1961).
2. Haslam, E. Secondary metabolism - fact and fiction. *Natural Products Reports Chem. Soc.* :217 (1986).
3. Bu'Lock, J.D. Mycotoxins as secondary metabolites. *In:* Steyn, P.S. (ed.) The Biosynthesis of Mycotoxins. Academic Press, London and New York, p. 1 (1980).
4. Haslam, E. Vegetable tannins. *In:* Conn, E.E. (ed.) The Biochemistry of Plants. Academic Press, New York. 7:527 (1981).
5. Bate-Smith, E.C. The phenolic constituents of plants and their taxonomic significance. 1. Dicotyledons. *J. Linn. Soc. (London) Bot.* 58:95 (1962).

6. Baur, P.S.; Walkinshaw, C.H. Fine structure of tannin accumulations in cells of *Pinus elliotti* (slash pine). *Can. J. Bot.* 52:615 (1974).
7. Feeny, P.P. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565 (1970).
8. Bloomfield, C. Polyphenols in soil formation. *J. Sci. Food Agric.* 8:389 (1957).
9. Spencer, C.M.; Cai, Y.; Martin, R.; Gaffney, S.H.; Goulding, P.N.; Magnolato, D.; Lilley T.H.; Haslam, E. Polyphenol complexation – some comments and observations. *Phytochemistry* 27:2397 (1988).
10. Hillis W.E.; Swain, T. The phenolic constituents of *Prunus domestica*. II. The analysis of tissues of the victoria plum tree. *J. Sci. Food Agric.* 10:135 (1959).
11. Robinson R.; Robinson, G.M. Leuco-anthocyanidins and leuco-anthocyanidins. Part 1. The isolation of peltogynol and its molecular structure. *J. Chem. Soc.* :744 (1935).
12. Bate-Smith, E.C. Tannins of the herbaceous leguminosae. *Phytochemistry* 122:1809 (1973).
13. Porter, L.J.; Foo L.Y.; Furneaux, R.H. Isolation of three naturally occuring *O*-β-**D**-glucopyranosides of procyanidin polymers. *Phytochemistry* 24:567 (1985).
14. Haslam, E.; Shen, Z.; Falshaw, C.P.; Begley, M.J. Procyanidins and polyphenols of *Larix gmelini* bark. *Phytochemistry* 25: 2629 (1986).
15. Herout, V.; N.J. Uvarova N.J.; Jizba, J. Plant Substances XXVII. The proof of structure of polydine-aglycone from *Polypodium vulgare L. Coll. Czech Chem. Comm.* 32:3075 (1967).
16. Morimnoto, S.; Nonaka, G.; Nishioka, I. Tannins and related compounds XXXVIII. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamonum cassia* BLUME). *Chem. Pharm. Bull.* 34:633 (1986).
17. Morimoto, S.; G. Nonaka G.; Nishioka, I. Tannins and related compounds XXXIX. Procyanidin *C*-glucosides and an acylated flavan-3-ol glucoside from the barks of *Cinnamonum cassia* BLUME and *C. obtusifolium* NEES. *Chem. Pharm. Bull.* 34:643 (1986).
18. Kashiwada, Y.; Nonaka G.; Nishioka, I. Tannins and related compounds XLV. Rhubarb (5). Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chem. Pharm. Bull.* 34:3208 (1986).
19. McManus, J.P.; Davis, K.G.; Beart, J.E.; Gaffney, S.H.; Lilley, T.H.; Haslam, E. Polyphenol interactions. Part 1. Introduction. Some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc. Perkin Trans. 2* :1429 (1985).
20. Haglund, A.C.; Marsden, N.V.B. Hydrophobic and polar contributions to solute affinity for a highly cross-linked water-swollen (Sephadex) gel. *J. Polym. Sci. Polym. Lett.* 18:271 (1980).
21. Brook, A.J.W.; Munday, K.C. Interactions of phenols, anilines and benzoic acids with Sephadex gels. *J. Chromatogr.* 47:19 (1970).
22. Haglund, A.C. Adsorption of monosubstituted phenols on Sephadex G-15. *J. Chromatogr.* 156:317 (1978).
23. deLigny, C.L., Adsorption of monosubstituted phenols on Sephadex G-15. *J. Chromatogr.* 172:397 (1979).
24. Laurent T.C.; Killander, J. A theory of gel filtration and its experimental verification. *J. Chromatogr.* 14:317 (1964).
25. Williams, R.J.P. Lecture, Biochemical Society Annual Meeting, Sheffield. 15 April (1988).
26. Bender; M.; Komiyama, K. Cyclodextrin Chemistry. Springer-Verlag, Basel (1978).
27. Inoue, Y.; Okuda, T.; Miyata Y.; Chujo, R. NMR studies of cycloamylose-inclusion complexes with substituted phenols. *Carbohydrate Res.* 125:65 (1984).
28. Inoue, Y.; Hoshi, H.; Sakurai, M.; Chujo, R. Geometry of cycloamylose inclusion complexes with some substituted benzenes in aqueous solution based on Carbon-13 NMR chemical shifts. *J. Amer. Chem. Soc.* 107:2319 (1985).

29. Harata, K. The structure of the cyclodextrin complex. V. Crystal structures of α-cyclodextrin complexes with p-nitrophenol and p-hydroxybenzoic acid. *Bull. Chem. Soc. Japan* 50:1416 (1977).

30. Wood, D.J.; Hruska, F.E.; Saenger, W. ^{1}H NMR study of the inclusion of aromatic molecules in -cyclodextrin. *J. Amer. Chem. Soc.* 99:1735 (1977).

31. Johnson C.E.; Bovey, F.A. Calculation of nuclear magnetic resonance spectra of aromatic hydrocarbons. *J. Chem. Phys.* 29:1012 (1958).

CHEMISTRY OF TANNIN-PROTEIN COMPLEXATION

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ABSTRACT

The importance of tannin-protein interactions in leathermaking and in plant-animal interactions has long been recognized, but detailed knowledge of the chemistry of the interaction has only recently become available. Tannin-protein interactions are influenced by characteristics of the protein (including size, amino acid composition, pI, and extent of post translational modification), characteristics of the tannin (size, structure, heterogeneity of the preparation), and conditions of the reaction (pH, temperature, solvent composition, time). The most familiar tannin-protein interactions result in precipitation of the complex, but soluble complexes also form under certain conditions. Both soluble and insoluble complexes are stabilized by reversible, noncovalent bonds between tannin and protein.

INTRODUCTION

The role of tannin in diverse processes, including ecological interactions, manufacturing, and food processing, is at least in part a consequence of the tendency of tannins "to precipitate alkaloids, gelatin and other proteins".[1] Historically, leather manufacture depended upon treatment of animal skins with tannin-containing plant preparations to form insoluble, microbe-resistant products. Interactions of collagen and gelatin with plant tannins during leathermaking have been investigated,[2] as have interactions of proteins with phenolics during beverage preparation from fruits and grains.[3] The suggestion that tannins protect plants from herbivores by complexing with protein and thus reducing the digestibility of the tannin-containing plants[4,5] has influenced ecologists for a decade and has stimulated many investigations of tannin-protein interactions.[6] Similar ideas have been pursued by nutritionists and agronomists interested in the effects of tannin on humans and

domestic animals.[7] The efforts of plant biochemists to overcome enzyme inhibition by phenolics in plant extracts have yielded substantial insight into tannin-protein interactions.[8,9]

For the earliest investigations of tannin-protein interactions, pure tannins and proteins of defined structure were unavailable, but those problems have been resolved by newer methods of purification and structure determination. No attempt has been made in this review to summarize the older literature. Instead, only investigations of the interactions of condensed tannins (proanthocyanidins) with proteins have been included. Detailed characterization of the interactions between hydrolyzable tannins and protein can be found in recent publications from Haslam's laboratory.[10,11] The formation of insoluble proanthocyanidin-protein complexes will be the primary topic of this review, since descriptions of insoluble complexes dominate the literature.

NATURE OF THE TANNIN-PROTEIN INTERACTION

Tannin-protein complexes are normally established by hydrogen bonds and hydrophobic interactions, without contribution from covalent or ionic bonds. It has long been believed that hydrogen bonds are involved in formation of tannin-protein complexes,[8,12,13] and the importance of those bonds is demonstrated by the ability of hydrogen-bonding solvents such as formamide and its N-substituted derivatives to disrupt the tannin-protein interaction.[14] The pH dependence of protein precipitation by condensed tannin suggests that the phenolic hydroxyl acts as a hydrogen bond donor, since at pH values above the pKa of the phenolic hydroxyl group ($\sim$ pH 10), proteins are not precipitated by condensed tannin.[15,16] The carbonyl oxygen of the peptide bond presumably serves as the hydrogen bond acceptor. Hydrogen bonding is augmented by hydrophobic interactions between nonpolar domains of certain proteins and the nonpolar regions of the tannin molecule,[14,17] particularly at acidic pH.[10] The only ionizable group in condensed tannins is the phenolic group, but tannin-protein interactions at pH values above the pKa of that moiety have not been documented,[15,16] leading to the conclusion that ionic interactions between tannin and protein are not important. Under oxidizing conditions, phenolics react covalently with nucleophilic amino acid side chains such as lysine or cysteine.[18] However, tannin-protein complexes can be dissociated with a variety of mild treatments that do not disrupt covalent bonds, including treatment with detergents, phenol, some organic solvents, or with excess caffeine.[19–23] The ease of disruption of the complexes suggests that covalent bonds do not normally form between condensed tannin and protein.

The complexes that form between tannin and protein can be either soluble or insoluble. Tannin and protein are both multivalent binding agents, with many sites for hydrogen bond formation or hydrophobic interaction. At optimum ratios of tannin to protein and at optimum pH values, the cross-linked complexes that form are macromolecular and insoluble in aqueous solution.[10,24] The optimum pH for formation of insoluble complexes is usually around the isoelectric pH of the protein.[15] At that pH, electrostatic replusions that prevent aggregation of protein are minimized.

If tannin is present in excess, all of the protein available is precipitated.[24] Haslam has suggested that precipitation when tannin is in excess is due to formation of hydrophobic tannin-coated proteins, rather than to the formation of cross-linked tannin-protein complexes.[25] When protein is present in excess, the complexes remain soluble,[10,24,26] because each molecule of protein is bound by only a few phenolic ligands. Soluble complexes between condensed or hydrolyzable tannin and protein have been detected by measuring molecular weights or light scattering properties of mixtures of tannin and protein.[26–29]

Although condensed tannin and protein can be released from complexes by a variety of nondestructive treatments,[19–23] complex formation is not readily reversible. If the reaction were reversible, insoluble complexes would dissociate, and soluble complexes would form when a precipitate was suspended in a solution containing excess protein.[10] However, the insoluble complexes formed between condensed tannin and serum albumin are not solubilized by the addition of excess protein (Table 1).[31] A more thorough examination of the reversibility of the reaction between tannin and protein would be fruitful, particularly since the use of many common methods of analyzing binding data (e.g., Scatchard plots) are dependent on an understanding of the reversibility of the reaction.

SPECIFICITY OF PROTEIN INTERACTION WITH TANNIN

Tannin-protein interactions are characterized by a specificity that was not anticipated by early workers. The high affinity of tannin for some proteins was exploited in the brewing and leathermaking industry long before it was recognized in the laboratory, since tannin binds especially tightly to proline-rich proteins such as collagen and the prolamins.[13,14,20] Use of polyvinylpyrrolidone to remove tannin

Table 1. Reversibility of Protein Precipitation by Tannins.[a]

Resuspension	BSA Precipitated (mg)
buffer	1.36
0.17 mg BSA	1.83
1.67 mg BSA	1.41
6.68 mg BSA	1.44
16.7 mg BSA	1.31

[a]Blue dye labeled BSA (6.7 mg) was mixed with 0.50 mg sorghum tannin. After 15 minutes, the mixtures were centrifuged and the supernatants discarded. The precipitates were suspended in buffer, or in buffer containing the indicated amount of blue BSA. After 15 minutes, the samples were centrifuged and the amount of BSA in the precipitate determined spectrophotometrically.[30] Numbers are the averages of duplicate samples.

from plant extracts by plant biochemists[8] also exploits the high affinity of tannin for proline-rich polymers, since vinylpyrrolidone is structurally reminiscent of proline.

The specificity of interaction between tannin and protein is in part due to the pH dependence of aggregation, which was described above. For example, in a solution at pH 5, bovine serum albumin (BSA) (pI = 4.9) would be selectively precipitated from a mixture containing BSA and lysozyme (pI = 11). The pH-dependence of tannin-protein interaction may be especially important in consideration of the role of tannin in digestibility and nutrition, since various regions of the gastrointestinal tract have rather different pH values.[32]

Hagerman and Butler devised a method for directly measuring the relative affinities of proteins for tannins.[14] The method measures the tendency to form either soluble or insoluble complexes and has been used to examine the interaction between condensed tannin and a variety of proteins and other materials. Affinities range over four orders of magnitude (Figure 1), and materials can be divided into those with high affinity, those with moderate affinity, and those with low affinity for tannin. The high affinity proteins and polymers, including gelatin, polyproline, and polyvinylpyrrolidone, are conformationally open and readily accessible for hydrogen bonding with tannin. The high proline content of gelatin and polyproline is responsible for their open structures, since proline obstructs the formation of hydrogen-bonded structures such as alpha helices. Polysarcosine (poly N-methyl glycine), with hydrogen-bonding characteristics similar to polyproline, also has a high affinity for tannin. Polyhydroxyproline and poly(pro-gly-pro) have low affinities for tannin because they form interchain hydrogen bonds in solution rather than remaining accessible to the tannin.[14] The low affinity proteins, including ribonuclease, cytochrome c, and lysozyme, have compact globular structures, so hydrogen bonds with tannin are not readily formed.

Polymer size is an additional determinant of tannin-binding affinity. Larger proteins and polymers tend to bind tannin more tightly, although small proline-rich polymers are preferred over large proline-poor molecules. Comparing the affinities of a series of polyprolines with various molecular weights established that molecules with molecular weights below 600 do not bind tannin very efficiently.[14]

Post-translational modifications, such as glycosylation, can influence the affinity of a protein for tannin. Although it has been suggested that glycoproteins do not bind tannin,[33] some proline-rich glycoproteins have very high affinity for tannin.[34] When affinities were determined using a competitive binding method, so that both soluble and insoluble tannin-protein complexes could be detected, a native glycoprotein had about a fourfold higher affinity for condensed tannin than did the deglycosylated protein.[34] The carbohydrate may enhance the binding of tannin by converting the protein to a more open conformation. The glycoprotein-tannin complexes are more soluble than the complexes of deglycosylated protein with tannin, suggesting that these complexes could not be detected using precipitation methods.

Three classes of proline-rich proteins that bind tightly to tannin are of particular interest. Collagen and its derivatives (such as gelatin) are of obvious importance in leathermaking. The prolamins are alcohol soluble, proline-rich proteins of certain

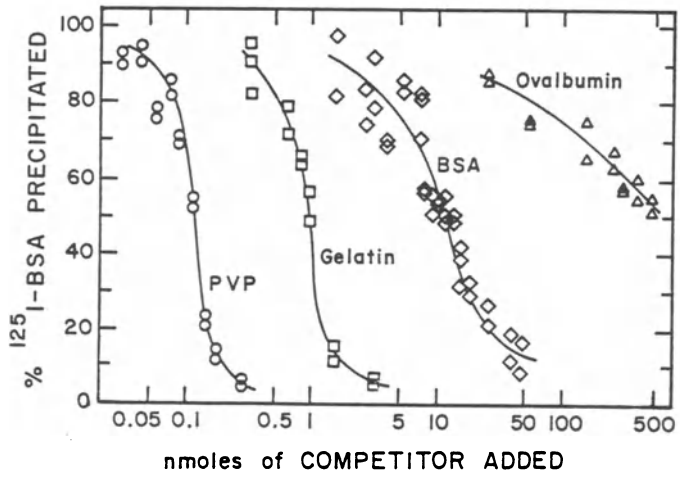

Figure 1. *Inhibition of tannin-BSA precipitation by proteins and polymers at pH 4.9. The amount of labeled BSA precipitated by sorghum procyanidin in the absence of competitor was set at 100 percent. Precipitation of the labeled BSA is inhibited by small amounts of competitors such as polyvinylpyrrolidone (PVP) or gelatin; these materials have high affinity for tannin. Inhibition occurs only with large amounts of competitors with low affinity for tannin, such as ovalbumin. (Figure reproduced from J. Biol. Chem. 256:495 (1981)).*

cereal grains including sorghum and barley. These grains contain tannins that under some conditions bind to the proline-rich seed proteins.[20,35] A third interesting group of proline-rich proteins is the salivary proline-rich proteins. The high affinity of tannin for these proteins may be important to mammals that consume tannin-containing plants.[7] The potential ecological significance of highly specific tannin-protein interactions has been discussed.[36]

Tannin binds several nonpeptides, including alkaloids[37] and some polysaccharides. The affinity of dextran gels such as Sephadex (Pharmacia Chemical Co., Uppsula, Sweden) for tannin has been exploited in a variety of tannin purification schemes,[20,38] although the nature of the interaction has not been established. Condensed tannin does not bind soluble dextran,[39] the oligosaccharides released from a tannin binding salivary protein,[34] or agarose.[40] Interaction between hydrolyzable tannins and several carbohydrates, including semipermeable membranes made of cellulose triacetate, has been reported.[10,37,41]

SPECIFICITY FOR TANNIN

Although the composition and structure of the protein are important determinants of the tannin-protein interaction, the structure of the tannin also influences the reaction. Many early studies of tannin-protein interactions were carried out

with poorly defined tannin preparations, but recent advances in structural chemistry and methods of purification have made it feasible to use well-defined tannins in binding studies.

One major structural feature that might influence tannin-protein interactions is the three-dimensional shape of the tannin. Although both condensed and hydrolyzable tannins bear *ortho*-dihydroxy phenolic groups that readily hydrogen bond to proteins,[10,25] gallotannins are flat, disclike molecules with the phenolic groups on the outside of the disc, and condensed tannins are helical chains.[42] Despite these differences, some investigators have found that similar amounts of condensed and hydrolyzable tannins are required to precipitate hemoglobin or BSA.[43,44] However, a procyanidin trimer was sixfold less efficient as a precipitant of β-glucosidase than was pentagalloyl glucose.[45] Further study of the role of the three-dimensional shape of the tannin molecule is required to clarify its importance.

The size of the condensed tannin molecule is a major determinant of its ability to interact with protein. Unpolymerized flavanols do not precipitate protein,[46,47] and the oligomers must contain at least three flavanol subunits to be effective tanning agents.[48] Dimers do precipitate protein under some conditions, but are less effective precipitating agents than trimers and larger oligomers.[49,50] The importance of chain length was clearly demonstrated by Porter and Woodruffe,[43] who compared the ability of eight proanthocyanidins of defined composition to precipitate hemoglobin (Figure 2). Stereochemistry and number of hydroxy groups on the B-ring of the proanthocyanidin had less effect on the ability of the polymer to precipitate protein than molecular weight. Similarly, the pattern of linkage between the flavanol subunits has less impact on affinity of tannin for protein than chain length.[16]

Although structure of the flavanol subunits of the condensed tannin is not as important as chain length in determining interaction with protein, some dependence on subunit structure has been found. Prodelphinidins (three *ortho*-hydroxy groups on the B-ring) bind protein more tightly than procyanidins (two *ortho*-hydroxy groups on the B-ring).[47,51] It is surprising that a condensed tannin containing propelargonidin, with only a single hydroxy group on the B-ring, precipitated protein as efficiently as polymers containing only procyanidin or prodelphinidin subunits,[43] since in general *ortho* dihydroxy groups are required for strong interaction of phenolics with protein.[10,25] The propelargonidin monomers comprised only 25 percent of the oligomer tested, and may thus have had little effect on binding capacity. Tests with condensed tannins containing more of the monohydroxy flavanol subunit would be informative.

Substitution on the condensed tannin in areas other than the B-ring may also affect affinity for protein. Two profisetinidins, which have *ortho*-dihydroxy groups on the B-ring but only one hydroxyl group on the A-ring, were less efficient protein-precipitating agents than a procyanidin of approximately the same chain length.[39] Addition of galloyl groups as esters at C-3 of the heterocyclic ring increased the cytotoxicity and antiviral activity of various condensed tannins,[52] suggesting that interaction with proteins may also be increased by the addition of galloyl groups (Figure 3).

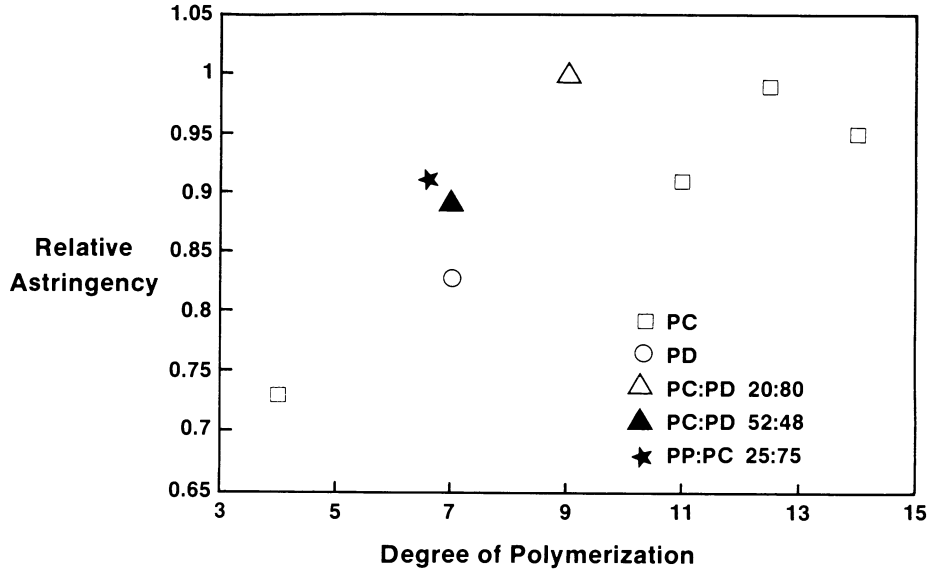

Figure 2. *Relative astringency of proanthocyanidins of defined composition and degree of polymerization. The ability of the tannins to precipitate hemoglobin at pH 5.0 was determined. The relative astringency of a standard tannic acid solution was defined as 1.00. The number average molecular weight obtained from ^{13}C-NMR studies was used to calculate the degree of polymerization for each proanthocyanidin. The symbols indicate the composition of the polymers, where PC is procyanidin, PD is prodelphinidin, PP is propelargonidin. These data were published in tabular form by Porter and Woodruffe, 1984.*[43]

Haslam's group has emphasized that tannin and protein must have complementary structures to form complexes.[10] The importance of complementarity has been evaluated in just a few studies. A salivary proline-rich protein had far higher affinity for the profisetinidin from quebracho than for other proanthocyanidins.[39]

A procyanidin tetramer and a pentamer precipitated proline-rich seed proteins more effectively than did the dimer; the dimer precipitated polyproline more effectively.[47] In contrast to these findings, the relative affinities of several proteins for a procyanidin and a gallotannin were compared,[44] and the two tannins were found to be very similar: gelatin had a very high affinity for both tannins, BSA had lower affinity, and ovalbumin had very low affinity for both tannins.

TANNIN-PROTEIN INTERACTIONS IN COMPLEX SYSTEMS

Examination of tannin-protein interactions with purified proteins and with purified tannins has provided insight into the mechanism of interaction and the parameters that influence the reaction. However, for many situations, these laboratory

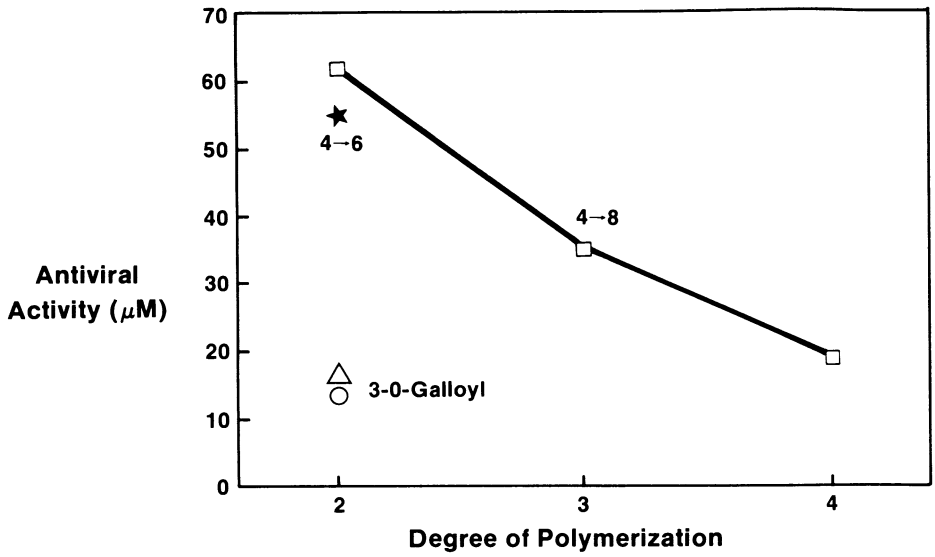

Figure 3. *Antiviral activities of proanthocyanidins of defined structure and degree of polymerization. Antiviral activity was measured as the amount of tannin required to reduce the number of plaques to 50 percent of those found in control viral cultures. The symbols indicate the structures of the polymers;* (□), *4 → 8 linked procyanidin;* (⋆), *4 → 6 linked procyanidin;* (○), *4 → 8 linked 3-O-galloyl procyanidin or 4 → 6 linked 3-O-galloyl prodelphinidin* (△). *These data were published in tabular form by Takechi et al, 1985.*[52]

studies are not satisfactory. The leather chemist, ecologist, or beverage producer needs to understand interactions that occur when complex natural mixtures of tannin and other phenolics are mixed with complex mixtures of protein. The possible synergistic consequences or antagonistic effects of introducing mixtures of phenolics into mixtures of proteins must be considered.

One element of the tannin-protein interaction that is influenced by heterogeneity of the tannin preparation is the rate of the reaction. For both condensed and hydrolyzable tannin, the reaction between tannin and protein is complete within 15 minute of mixing the reactants.[15] The rate reaction is faster for some proteins, such as ovalbumin, than for other proteins, such as BSA,[14] but the reaction is complete within 15 minute for all proteins and polymers examined. However, plant extracts containing mixtures of phenolics and tannins react much more slowly with BSA than do the purified tannins, with complete precipitation not observed until 14 hours after mixing the reactants.[24]

The reaction conditions used in laboratory studies of tannin-protein interactions may be inadequate for complete understanding of tannin-protein interactions

in important systems. For example, in beverage manufacture, the phenolics oxidatively polymerize during the manufacturing process, so that the types of phenolics present are constantly changing.[47] In ecological systems, if tannin is to act as a digestibility-reducing substance,[4,5] it must interact with protein during transit of the gastrointestinal tract. A wide range of pH values and substances such as detergents that inhibit tannin-protein interactions are present in the gastrointestinal tract, so that studies of simple tannin-protein interactions in buffered solutions are probably ecologically inappropriate.[53]

In most studies of tannin-protein interactions, insoluble complexes have been emphasized, because these complexes are analytically easy to handle. However, soluble complexes may be important in ecological and industrial settings. More work is needed to develop methods for examining soluble complexes. An electrophoretic method that has recently been developed offers promise.[54]

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REFERENCES

1. Bate-Smith, E.C.; Swain, T. *In*: Mason, H.S.; Florkin, A.M. (eds.) Comparative Biochemistry, Academic Press, New York (1962)
2. Gustavson, K.H. The Chemistry of Tanning Processes. Academic Press, New York. (1956)
3. Lea, A.G.H. The phenolics of ciders: Oligomeric and polymeric procyanidins. *Phytochemistry* 29:471 (1978).
4. Rhoades, D.F.; Cates, R.G. Toward a general theory of plant antiherbivore chemistry. *In*: Wallace, J.W., Mansell, R.L. (eds.) Biochemical Interaction Between Plants and Insects. (Recent Advances in Phytochemistry) Plenum Publishing Company, New York (1976)
5. Feeny, P. Plant apparency and chemical defense. *In*: Wallace, J.S.; Mansell, R.L. (eds.) Biochemical Interaction Between Plants and Insects. (Recent Advances in Phytochemistry). Plenum Publishing Company, New York (1976).
6. Mole, S.; Waterman, P.G. A critical analysis of techniques for measuring tannins in ecological studies. II. Techniques for biochemically defining tannins. *Oecologia* 72:148 (1987).
7. Mehansho, H.; Butler, L.G.; Carlson, D.M. Dietary tannins and salivary proline-rich proteins: Interactractions, induction and defense mechanisms. *Ann. Rev. of Nutr.* 7:423 (1987).
8. Loomis, W.D.; Battaile, J. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5:423 (1966).
9. Anderson, J.S. Extraction of enzymes and subcellular organelles from plant tissues. *Phytochemistry* 7:1973 (1968).
10. McManus, J.P.; Davis, K.G.; Beart, J.E.; Gaffney, S.H.; Lilley, T.H.; Haslam, E. Polyphenol interactions. Part 1. Introduction; Some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc. Perkin Trans. 2* :1429 (1985).

11. Beart, J.E.; Lilley, T.H.; Haslam, E. Plant polyphenols–secondary metabolism and chemical defense: Some observations. *Phytochemistry* 24:33 (1985).

12. Gustavson, K.H. Interaction of vegetable tannins with polyamides as proof of the dominant function of the peptide bond of collagen for its binding of tannins. *J. Polym. Sci.* 12:317 (1954).

13. Russell, A.E.; Shuttleworth, S.G.; Williams-Wynn, D.A. Further studies on the mechanism of vegetable tannage: Part V: Chromatography of vegetable tannins on collagen and cellulose. *Soc. Leather Trades Chemists J.* 52:459 (1968).

14. Hagerman, A.E.; Butler, L.G. Specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* 256:494 (1981).

15. Hagerman, A.E.; Butler, L.G. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* 26:809 (1978).

16. Ezaki-Furuichi, E.; Nonaka, G.; Nishioka, I.; Hayashi, K. Affinity of procyanidins (condensed tannins) from the bark of *Rhaphiolepis umbellata* for proteins. *Agric. Biol. Chem.* 51:115 (1987).

17. Oh, H.I.; Hoff, J.E.; Armstrong, G.S.; Haff, L.A. Hydrophobic interaction in tannin-protein complexes. *J. Agric. Food Chem.* 28:394 (1980).

18. Pierpoint, W.S. O-quinones formed in plant extracts. Their reaction with bovine serum albumin. *Biochem. J.* 112:619 (1969).

19. Hagerman, A.E.; Butler, L.G. Determination of protein in tannin-protein precipitates. *J. Agric. Food Chem.* 28:944 (1980).

20. Hagerman, A.E.; Butler, L.G. Condensed tannin purification and characterization of tannin-associated proteins. *J. Agric. Food Chem.* 28:947 (1980).

21. Hoff, J.E.; Singleton, K.I. A method for determination of tannins in foods by means of immobilized protein. *J. Food Sci.* 42:1566 (1977).

22. Merrill, H.B.; Cameron, D.H.; Ellison, H.L.: Hall, C.P. The stripping of vegetable tannins from leather by aqueous organic solvents. *J. Am. Leather Chemists Assoc.* 42:536 (1947).

23. Katzenellenbogen, W.; Dobryszycka, W.M. Immunochemical properties of serum proteins after regeneration from protein-tannin complexes. *Nature* 193:1888 (1962).

24. Hagerman, A.E.; Robbins, C.T. Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. *J. Chem. Ecol.* 13:1243 (1987).

25. McManus, J.P.; Davis, K.G.; Lilly, T.H.; Haslam, E. The association of proteins with polyphenols. *J. Chem. Soc. Chem. Comm.* :309 (1981).

26. Calderon, P.; Van Buren, J.; Robinson, W.B. Factors influencing the formation of precipitates and hazes by gelatin and condensed and hydrolyzable tannins. *J. Agric. Food Chem.* 16:479 (1968).

27. Fishman, M.L.; Neucere, N.J. Partial characterization of tannin-protein complexes in five varieties of grain sorghum by automated gel filtration chromatography. *J. Agric. Food Chem.* 28:477 (1980).

28. Takechi, M.; Tanaka, Y. Binding of 1,2,3,4,6-pentagalloylglucose to proteins, lipids, nucleic acids and sugars. *Phytochemistry* 26:95 (1987).

29. Van Buren, J.P.; Robinson, W.B. Formation of complexes between protein and tannic acid. *J. Agric. Food Chem.* 17:772 (1969).

30. Asquith, T.N.; Butler, L.G. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. *J. Chem. Ecol.* 22:1535 (1985).

31. Hagerman, A.E. Condensed tannin of sorghum grain: Purification and interactions with proteins. Ph.D. Dissertation. Purdue University, W. Lafayette, Indiana, 1980.

32. Jones, W.W.; Mangan, J.L. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction I leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agric.* 28:126 (1977).

33. Strumeyer, D.H.; Malin, M.J. Resistance of extracellular yeast invertase and other glycoproteins to denaturation by tannins. *Biochem. J.* 118:899 (1970).

34. Asquith, T.N.; Uhlig, J.; Mehansho, H.; Putnam, L.; Carlson, D.M.; Butler, L. Binding of condensed tannins to salivary proline-rich glycoproteins: The role of carbohydrate. *J. Agric. Food Chem.* 35:331 (1987).

35. Asano, K.; Shinagawa, K.; Hashimoto, N. Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J. Amer. Soc. of Brewing Chemists* 40:147 (1982).

36. Zucker, W.V. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335 (1983).

37. Gaffney, S.H.; Martin, R.; Lilley, T.H.; Haslam, E.; Magnolato, D. The association of polyphenols with caffeine and α- and β-cyclodextrin in aqueous media. *J. Chem. Soc. Chem. Commun.* :107 (1986).

38. Strumeyer, D.H.; Malin, M.J. Condensed tannins in grain sorghum: Isolation, fractionation, and characterization. *J. Agric. Food Chem.* 23:909 (1975).

39. Asquith, T.N.; Butler, L.G. Interactions of condensed tannins with selected proteins. *Phytochemistry* 25:1591 (1986).

40. Hagerman, A.E. Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* 13:437 (1987).

41. Ozawa, T.; Lilley, T.H.; Haslam, E. Polyphenol interactions: Astringency and the loss of astringency in ripening fruit. *Phytochemistry* 26:2937 (1987).

42. Haslam, E. Vegetable tannins. *In*: Swain, T.; Harborne, J.B.; VanSumere, C.F. (eds.) Biochemistry of Plant Phenolics (Recent Advances in Phytochemistry). Plenum Publishing Company, New York, pp. 475-523 (1979).

43. Porter, L.J.; Woodruffe, J. Haemanalysis: The relative astringency of proanthocyanidin polymers. *Phytochemistry* 23:1255 (1984).

44. Hagerman, A.E.; Klucher, K.M. Tannin-protein interactions. *In*: Cody, V.; Middleton, E.; Harborne, J. (eds.) Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure Activity Relationships. Alan R. Liss, Inc., New York, pp. 67-76 (1986).

45. Haslam, E. Polyphenol-protein interactions. *Biochem. J.* 139:285 (1974).

46. Butler, L.G. Relative degree of polymerization of sorghum tannin during seed development and maturation. *J. Agric. Food Chem.* 30:1090 (1982).

47. Asano, K.; Ohtsu, K.; Shinagawa, K.; Hashimoto, N. Affinity of proanthocyanidins and their oxidation products for haze-forming proteins of beer and the formation of chill haze. *Agric. Biol. Chem.* 48:1139 (1984).

48. Roux, D.G. Recent advances in the chemistry and chemical utilization of the natural condensed tannins. *Phytochemistry* 11:1219 (1972).

49. Bate-Smith, E.C. Haemanalysis of tannins: The concept of relative astringency. *Phytochemistry* 12:907 (1973).

50. Artz, W.E.; Bishop, P.D.; Dunker A.K.; Schanus, E.G.; Swanson, B.G. Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumin and purified bean globulin fraction G-1. *J. Agric. Food Chem.* 35:417 (1987).

51. Bate-Smith, E.C. Phytochemistry of proanthocyanidins. *Phytochemistry* 14:1107 (1975).

52. Takechi, M.; Tanaka, Y.; Takehara, M.; Nonaka, G.I.; Nishioka, I. Structure and antiherpetic activity among the tannins. *Phytochemistry* 24:2245 (1985).

53. Martin, M.M.; Rockholm, D.C.; Martin, J.S. Effects of surfactants, pH and certain cations on precipitation of proteins by tannins. *J. Chem. Ecol.* 11:485 (1985).

54. Austin, P.J.; Suchar, L.A.; Robbins, C.T.; Hagerman, A.E. Tannin binding proteins in the saliva of deer and their absence in the saliva of sheep and cattle. *J. Chem. Ecol.* 15:1335 (1989).

INTERACTION OF CONDENSED TANNINS WITH BIOPOLYMERS

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ABSTRACT

Condensed tannins form complexes with a multitude of species, ranging in size from divalent and trivalent metal ions to macromolecules, both naturally occurring and synthetic. Many of these complexes are of low solubility, permitting detection by techniques as simple as precipitation. Determination of the interactions responsible for the initial events in complex formation is facilitated by the use of techniques that can detect the interaction in extremely dilute solution, thereby suppressing the influence of aggregation.

INTRODUCTION

One of the more noteworthy properties of the condensed tannins is their propensity for the formation of complexes with other molecules. Many of these complexes, including those responsible for the name "tannin," have very low solubility (Chapters 19 and 20).[1-3] The types of molecules that are involved in these complexes include many (like proteins and carbohydrates) that are essential components of any living system.[1-6] The condensed tannins were exposed to these molecules long before mankind began using them. Condensed tannins also form complexes with other materials, such as poly(vinylpyrrolidone), only through the intervention of man (Chapter 20).[2,7] The study of both types of complexes has been prompted by

several motivations. The pure chemist seeks to identify the specific interactions responsible for the stabilization of the complexes of condensed tannins with molecules of all types and to determine the structures of the complexes. A biochemist would be inclined to emphasize the complexes of condensed tannins with other biological macromolecules, notably proteins, carbohydrates, and nucleic acids. In addition to learning about their stabilities and structures, the biochemist would seek to understand how these complexes might be related to the role that nature intended for the condensed tannins. A materials scientist would investigate whether the ability of condensed tannins to form complexes with other molecules might be useful in articles of commerce, perhaps as a means of discouraging microbial attack, as in the case of the complexes with divalent and trivalent metal ions (Chapter 32). The contributions in this section speak to all of these motivations.

The insolubility of many of the complexes has advantages in that very simple techniques, such as the precipitation of the red color from a solution of hemoglobin,[3] can be used to demonstrate the existence of a strong intermolecular interaction. Of course, the insolubility can be a problem if the objective is the study of the initial events in the formation of the complexes. Minimization of the influence of aggregation requires the utilization of sensitive techniques that can detect the formation of a complex in very dilute solution. Among the techniques that can be used are circular dichroism and fluorescence. The fluorescence quantum yield, denoted by Q, of catechin, epicatechin, and their oligomers, is environmentally sensitive in a way that permits detection of complex formation in dilute aqueous solution.[7,8] Circular dichroism is a powerful technique for the identification of cases where the binding of a small amount of procyanidin produces a large change in the conformation of an optically active ligand, such as a poly(amino acid).[9] The specificity with regard to a macromolecular ligand can be explored by using water-soluble polymers of different types, with emphasis on synthetic poly(amino acids), that adopt a variety of well-known conformations in dilute solution. The following paragraphs indicate how synthetic poly(amino acids) can be chosen so that they include samples with a variety of conformational properties: flexible random coils, locally stiff random coils, helix-coil transitions, and sheet-coil transitions. The study also includes four water-soluble synthetic polymers that are not poly(amino acids). The work described here includes the use of Q to determine the optimal size for the binding of an oligomer of epicatechin to poly(vinylpyrrolidone) and the use of Q to document a much different interaction of catechin with poly(*L*-proline),[7] as well as the use of circular dichroism to detect the interaction of catechin with the first strand of a β sheet.[9]

CONFORMATIONS OF THE POLYMERS AND POLY(AMINO ACIDS) USED

What follows is a brief description of the conformational properties of the several poly(amino acids) and polymers under conditions where they can be studied in the presence of condensed tannins. A concise summary is presented in Table 1. Poly(vinylpyrrolidone), poly(oxyethylene), and poly(acrylamide) are flexible, disordered polymers in water. Poly(hydroxypropylcellulose) is a much stiffer polymer. The concentrations of poly(acrylamide) and poly(hydroxypropylcellulose) never

Table 1. Summary of the Polymers and Poly(amino acids) Conformations

Polymer	Conformational Behavior (under conditions used)
Poly(acrylamide)	Flexible nonionic random coil
Poly(*L*-arginine)	Cationic random coil
Poly(S-carboxymethyl-*L*-cysteine)	Anionic random coil → β sheet
Poly(*D*-glutamic acid)	Anionic random coil → α helix
Poly(*L*-glutamic acid)	Anionic random coil → α helix
Poly(*L*-hydroxyproline)	Locally stiff nonionic random coil
Poly(hydroxypropylcellulose)	Stiff chain, nonionic
Poly(hydroxypropyl-*L*-glutamine)	Flexible nonionic random coil
Poly(*L*-lysine)	Cationic random coil
Poly(oxyethylene)	Flexible nonionic random coil
Poly(*L*-proline)	Locally stiff nonionic random coil
Poly(sarcosine)	Flexible nonionic random coil
Poly(vinylpyrrolidone)	Flexible nonionic random coil

exceeded 1.6 and 0.8 mg/ml, respectively, in the experiments summarized here, due to gelation of the former and the limited solubility of the latter. Concentrations of poly(vinylpyrrolidone) and poly(oxyethylene) extended up to 8 mg/ml.

At a pH near 7, poly(*L*-arginine), poly(*L*-lysine), poly(S-carboxymethyl-*L*-cysteine), poly(*D*-glutamic acid), and poly(*L*-glutamic acid) are fully ionized random coils. As the pH is lowered into the region where protonation of the carboxyl groups occurs, the latter three poly(amino acids) change conformation. Poly(*D*-glutamic acid) forms a left-handed α helix, and poly(*L*-glutamic acid) forms a right-handed α helix, and poly(S-carboxymethyl-*L*-cysteine forms a β sheet structure.[10,11] Poly(sarcosine)[12] and poly(hydroxypropyl-*L*-glutamine)[13] are nonionic random coils at ambient temperature in water. Poly(*L*-proline) and poly(*L*-hydroxyproline) are locally stiff, but they have the overall architecture of a random coil when their degrees of polymerization are several hundred.[14–16]

DETECTION BY FLUORESCENCE OF INTERACTIONS OF THE POLYMERS WITH CATECHIN OR EPICATECHIN

Q for catechin and epicatechin is larger in organic media than in water.[7,8] Complexation by a polymer will increase Q if the complex simply transfers the catechin or epicatechin from an aqueous to a nonpolar environment. Fluorescence methods were used to search for this effect at low concentrations of the chromophore. The studies summarized here were performed with concentrations on the order of 10^{-5} molar in catechin or epicatechin units.

Of the four polymers that do not contain amino acid residues, by far the largest effect on Q of catechin or epicatechin was seen with poly(vinylpyrrolidone).[17] The fluorescence enhancement is demonstrated by the lower curve in Figure 1. The fluorescence intensity of epicatechin increases in the presence of poly(vinylpyrrolidone), which is the expected result if the chromophore is shielded from water by the polymer. Collapse of the poly(vinylpyrrolidone) chain in the presence of a low concentration of catechin was verified by a marked reduction in the intrinsic viscosity of

the polymer.[7] The other three polymers, poly(oxyethylene), poly(acrylamide), and poly(hydroxypropylcellulose), also caused an increase in Q, but the magnitude of the increase was much less than that seen with poly(vinylpyrrolidone).

Several disordered poly(amino acids) had little effect on the intensity of the fluorescence by catechin. Included in this list were poly(*L*-arginine) at pH 8.2, poly(*L*-glutamic acid) and poly(*D*-glutamic acid) at pH 6.7, poly(hydroxypropyl-*L*-glutamine), poly(*L*-lysine) at pH 7.8, and poly(sarcosine).[17,18] Neither poly(hydroxypropyl-*L*-glutamine) nor poly(sarcosine) contain ionizable groups, other than those at the chain ends, and consequently, their conformational properties are independent of pH. Special care must be exercised in studying the effects of poly(hydroxyalkyl-*L*-glutamines) on the fluorescence of procyanidins because the hydroyalkylamines are strong quenchers of this fluorescence.[17] A slight degree of hydrolysis of the amide group in the side chain of a poly(hydroxyalkyl-*L*-glutamine) would, therefore, produce a strong quencher. If its presence were unsuspected, the quenching of the fluorescence by the product of the hydrolysis might erroneously be interpreted as arising from the interaction of the procyanidin with the poly(hydroxyalkyl-*L*-glutamine).

Poly(*L*-proline) was a strong quencher of the fluorescence of catechin.[7,18] The tripeptide, *L*-Pro-*L*-Pro-*L*-Pro, was nearly as effective as a sample of poly(*L*-proline) with a degree of polymerization of several hundred,[18] showing that catechin interacts predominantly with a local portion of the poly(amino acid) chain. This result was not unexpected, in view of the well-known fact that poly(*L*-proline) is locally stiff, even though samples with degrees of polymerization of several hundred have the overall architecture of a random coil.[14,16] In contrast, poly(*L*-hydroxyproline) had very little effect on the fluorescence of catechin.[18] Its behavior in this regard is indistinguishable from that of poly(sarcosine). This result is particularly noteworthy because the conformational properties of the homopolymers of *L*-proline and *L*-hydroxyproline in dilute aqueous solution have much in common, with poly(*L*-hydroxyproline) being a slightly stiffer random coil than poly(*L*-proline),[14,15] presumably because of a decrease in the flexibility of the pyrrolidine ring when the hydroxyl group is appended. The detailed molecular origin of the selectivity between poly(*L*-proline) and poly(*L*-hydroxyproline) has not yet been identified.

The critical minimum size for an oligomeric procyanidin in its formation of the initial complex with poly(vinylpyrrolidone) was apparent from the results reported in Figure 1. This study utilized six monodisperse oligomers of epicatechin, ranging in degree of polymerization from the monomer through the hexamer. When the concentration of poly(vinylpyrrolidone) was 5 mg/ml, the fluorescence enhancement seen with epicatechin was smaller than that seen with the higher oligomers, but there was little difference in the results with the dimer through hexamer. When the poly(vinylpyrrolidone) concentration fell below 1 mg/ml, the dimer showed significantly less fluorescence enhancement than the higher oligomers, although its enhancement remained higher than that seen with the monomer. On this basis, the detailed modeling of the initial formation of a complex between a single oligomer of epicatechin and a single molecule of poly(vinylpyrrolidone) should seek to explain why the trimer of epicatechin is the smallest oligomer that shows optimal binding.

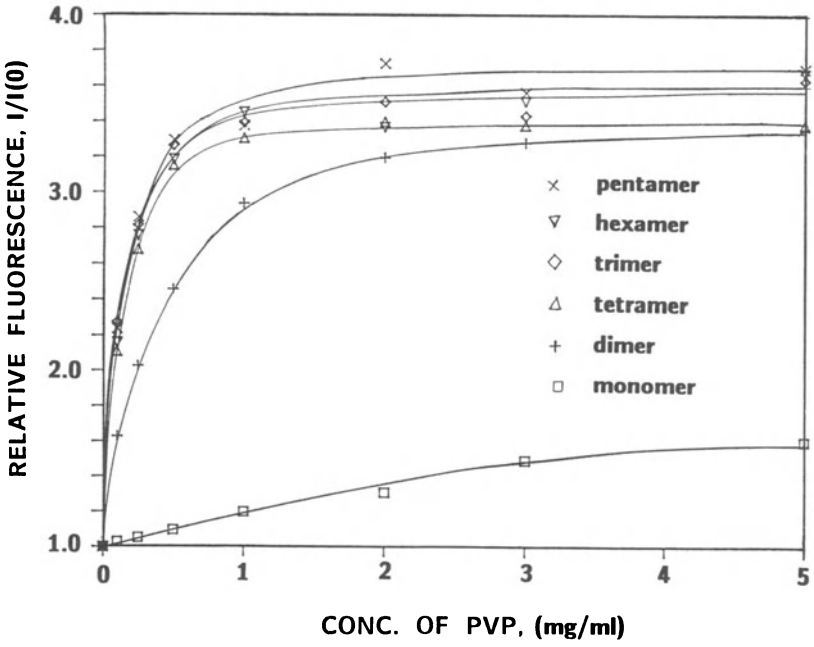

Figure 1. *Influence of poly(vinylpyrrolidone), denoted by PVP, on the fluorescence intensity of monodisperse oligomers of epicatechin in aqueous solution. I/I(O) is the ratio if the fluorescence intensity in the presence of the poly(vinylpyrrolidone) to that in its absence. The concentration of the oligomers was 0.03 mg/ml. The supplier reported a molecular weight of 3.6 x 10^5 for the poly(vinylpyrrolidone).*

DETECTION BY CIRCULAR DICHROISM OF THE INTERACTION OF CATECHIN WITH THE INITIAL STRAND OF A β SHEET

Poly(S-carboxymethyl-*L*-cysteine) undergoes a transition from a random coil to a β sheet as the degree of ionization of the carboxyl groups in the side chains is decreased.[11] This change in conformation can be monitored by measurement of the circular dichroism spectra as a function of pH. The conformational transition takes place over a very narrow range of pH in the vicinity of pH 5. This transition was spread over a much broader range of pH when catechin was present at a concentration of 3 x 10^{-5} M.[9] Transition curves measured in the presence and absence of catechin crossed one another when the content of β sheet was slightly less than 50 percent. The significance of this behavior was obtained with the use of the statistical mechanical treatment for the transition from a random coil to an intramolecular antiparallel β sheet.[19,20] This approach showed that the observed influence of catechin on the transition curves was that expected if the catechin bound in a manner that stabilized the initial strand of the β sheet.[9] Any interaction of

catechin with the internal strands of the β sheet must be substantially weaker than the interaction with the initial strand.

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REFERENCES

1. Haslam, E. Polyphenol-protein interactions. *Biochem. J.* 139:285 (1974).
2. Hagerman, A.E.; Butler, L.G. Specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* 256:4494 (1981).
3. Porter, L.J.; Woodruffe, J. Haemanalysis: the relative astringency of proanthocyanidin polymers. *Phytochemistry* 23:1255 (1984).
4. Oh, H.I.; Hoff, J.E.; Armstrong, G.S.; Haff, L.A. Hydrophobic interaction in tannin-protein complexes. *J. Agric Food Chem.* 28:394 (1980).
5. McManus, J.P.; Davis, K.G.; Beart, J.E.; Gaffney, S.H.; Lilley, T.H.; Haslam, E. Polyphenol interactions: Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc. Perkin Trans 2* :1429 (1985).
6. Beart, J.E.; Lilley, T.H.; Haslam, E. Plant polyphenols — secondary metabolism and chemical defense: some observations. *Phytochemistry* 24:33 (1985).
7. Bergmann, W.R.; Mattice, W.L. Specific interactions of (+)-catechin and (-)-epicatechin with polymers that contain the *L*-prolyl residue. *ACS Symp. Ser.* 358:162 (1987).
8. Bergmann, W.R.; Barkley, M.D.; Hemingway, R.W.; Mattice, W.L. Heterogeneous fluorescence decay of 4 → 6 and 4 → 8 linked dimers of (+)-catechin and (-)-epicatechin with axial or equatorial dihydroxyphenyl rings at C(2). *J. Amer. Chem. Soc.* 109:6614 (1987).
9. Tilstra, L.F.; Maeda, H.; Mattice, W.L. Interaction of (+)-catechin with the edge of the β sheet formed by poly(S-carboxymethyl-L-cysteine). *J. Chem. Soc., Perkin Trans. 2* :1613 (1988).
10. Nagasawa, M.; Holtzer, A. The helix-coil transition in solutions of polyglutamic acid. *J. Amer. Chem. Soc.* 86:538 (1964).
11. Maeda, H.; Gatto, Y.; Ikeda, S. Effects of chain length and concentration on the β-coil conversion of poly[S-(carboxymethyl)-L-cysteine] in 50 mM NaCl solutions. *Macromolecules* 17:2031 (1984).
12. Fessler, J.H.; Ogston, A.G. Studies of the sedimentation, diffusion, and viscosity of some sarcosine polymers in aqueous solution. *Trans. Faraday Soc.* 47:667 (1951).
13. Lupu-Lotan, N.; Yaron, A.; Berger, A.; Sela, M. Conformation changes in the nonionizable water-soluble synthetic polypeptide poly-N^5-(3-hydroxypropyl)-L-glutamine. *Biopolymers* 3:625 (1965).
14. Mattice, W.L.; Mandelkern, L. Conformational properties of poly-L-proline form II in dilute solution. *J. Amer. Chem. Soc.* 93:1769 (1971).
15. Clark, D.S.; Mattice, W.L. Hydrodynamic properties and unperturbed dimensions of poly(γ-hydroxyl-L-proline) in aqueous solution. *Macromolecules* 10:369 (1977).
16. Darsey, J.A.; Mattice, W.L. Local configuration of poly(L-proline) in dilute solution. *Macromolecules* 15:1626 (1982).

17. Tilstra, L.F. Polypeptides: conformational transition and complex formation with catechins and procyanidins. Ph.D. Dissertation, Louisiana State University, Baton Rouge (1987).

18. Bergmann, W.R. Fluorescence of proanthocyanidin polymers. Ph.D. Dissertation, Louisiana State University, Baton Rouge (1986).

19. Mattice, W.L.; Scheraga, H.A. Matrix formulation of the transition from a statistical coil to an intramolecular antiparalled β sheet. *Biopolymers* 23:1701 (1984).

20. Mattice, W.L.; Tilstra, L.F. Basis for large differences in the cooperativity of the formation of antiparallel β-sheets and clusters of interacting α-helices in isolated chains. *Biopolymers* 26:203 (1987).

Biological Significance

MICROSCOPIC STUDIES OF TANNIN FORMATION AND DISTRIBUTION IN PLANT TISSUES

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ABSTRACT

The functions and localization of condensed tannins and their catechin precursors, together with the microscopic techniques for observing them, are discussed in this chapter. Unlike previous reviews,[1] this chapter does not focus on heartwood formation, but rather on the presence of condensed tannins in living tissues. The occurrence and role of condensed tannins in buds, leaves, roots, seeds, and stems are discussed, as is the historical development of the techniques used to identify and quantitate condensed tannins in vivo.

INTRODUCTION

Throughout this chapter, the original usage of chemical names has been followed as closely as possible. This of course will lead to confusion, as the proanthocyanidins and condensed tannins have had a myriad of names during their historical development. Therefore, the names tannin, leucoanthocyanin, leucoanthocyanidin, proanthocyanidin, and condensed tannin may all pertain to the same compound. Haslam's excellent review[2] discourages the use of the terms leucoanthocyanin and leucoanthocyanidin when describing catechin, its cohorts, and the oligomers and polymers they form. Instead, the terms proanthocyanidin and condensed tannin are used when possible in this chapter. The reader may also be confused by the numerous references to "phenolics". In many instances, specific phenolic compounds, such as flavanoids, are not identified or isolated by the researchers. However, because of the importance of the cited works to the thesis of this chapter, the information is included for reasons that should become clear.

The development of phenolic compounds may have occurred in response to the lack of motility in plants. These compounds may have offered protection from

predators and from ultraviolet radiation, which was much more intense when higher plants were evolving.[3] Phenolics in particular absorb far (40-290 nm) and midrange (290-320 nm) UV, which are damaging wavelengths to proteins and nucleic acids. They also act as radical quenchers of stress-created singlet oxygen.[4] It seems likely that any new secondary product that did not confer some kind of advantage to the plant would eventually have been lost through natural selection, unless it was closely associated on a chromosome with a highly favorable characteristic. Therefore, most of the so-called secondary products found in present-day plants probably have functions associated with increasing survival, either by coping with unfavorable environmental conditions, including predation, or by regulating metabolic processes.

Phenolics are ubiquitous in higher plants and occur in some lower plants as well. Many phenolics are esterified with sugar moieties or are likewise detoxified in the intact plant; most of them are bound to the cell wall or are stored in the vacuole. However, disruption of the cell by disease, environmental stress, or laboratory tissue homogenization releases these compounds, which then dissociate enzymatically from their ester linkages and quickly modify cell components such as enzymes and proteins. By various chemical modifications, phenolics and their oxidation products can effectively tie up proteins, modify enzymatic active sites and thus decrease activity, and even create artifactual enzymes by blocking ionic amino acid residues such as lysine.[5]

Condensed tannins and their catechin precursors comprise one of the largest phenolic subgroups. In an early review, Nierenstein[6] claimed tannins occur in every part of the seed plant, especially in regions of active growth (i.e., the buds and junctions of side roots and shoots). Although tannin nomenclature and division have become more sophisticated since that time, catechin and its cohorts are still known to occur in many plants, but primarily in woody tissue and leaves. Because their potentially reactive *o*-dihydroxy groups are not associated with sugar moieties, the condensed tannins are thought to have significant biological activity.[7] Hillis[8] suggested the leucoanthocyanins produced in the leaves of woody plants are then transported to the bark and heartwood, where they form tannin extractives. Catechin and related flavanoid oligomers may be easily transported within the cell, but through condensation reactions they form condensed tannins, which (because of their high molecular weight) are not transported within the cell. Condensed tannins are, however, chemically reactive and will readily "tan" proteins when cell tissues are disrupted, lending astringency to unripe fruits and acting as an intrinsic plant defense against herbivores.

In contrast, catechin itself is thought to have antioxidant and free-radical scavenging properties,[9,10] and may even stabilize cytoplasmic and other cell membranes in animal systems.[11] It would seem likely that a similar protective function could be found in plant tissues.

Cell Wall Associations

Although lignin is a part of secondary thickening of cells, it is the catechins and proanthocyanidins that contribute to the "woodiness" of a plant.[2] Bate-Smith and Lerner[12] found close correlations between lignification of plant tissues and

leaf leucoanthocyanin content. Similarly, Reeve[13] saw an increase in catechol derivatives in peach fruit associated with maturation and lignification. Rubin and Artsikhovskaya[14] suggested that phenolic compounds function as proton donors or acceptors in oxidation-reduction reactions and thus play a role in lignification. Recent research suggests condensed tannins have a structural role, much like that of lignin;[15] they have been found on the wall surfaces of Douglas-fir sclereids.[16] Catechin and leucoanthocyanidins were seen to form a "lignin-like" material in casava roots.[17] Cottle and Kolattukudy[18] as well as Simonds[19] suggested condensed tannins may be involved in suberin structure also. Because catechins are structurally similar to lignin and suberin phenolic precursors, it seems likely they could comprise part of the cell wall by oxidative polymerization.

Like lignin, catechins and their products are synthesized in response to biotic and abiotic stresses[20] such as cold temperature,[5] drought,[15] and high-intensity sunlight.[21] Disease-resistant tomato varieties have been shown to contain more tannins than susceptible types.[22]

It is quite likely that the outer layers of the cell are not as distinct as once was assumed. The primary cell wall, consisting of cellulose and other sugars, has been recently found to contain cinnamate,[23] ferulic acid,[24] and *p*-coumaric acid,[25] which are normally associated with the secondary cell wall. They could serve as insoluble linkages between cellulose and secondary cell wall materials. Zucker[26] hypothesized that some tannins specifically complex with pectins and cellulose to form cell walls. Beardmore et al[27] suggested wall-associated phenolic acid-protein or -carbohydrate complexes could act as the initial matrix for lignin deposition. Suberin, which may contain condensed tannins,[18] is already known to be deposited between the cell wall and the plasma membrane.[28] Suberin could possibly provide phenolic attachments toward the outer layer, and lipophilic attachments toward the membrane, which could increase and stabilize the membrane-cell wall interface. This would increase the resistance of the plant to various environmental stresses, perhaps by decreasing stress-induced cell wall cracking and collapse.[15]

FUNCTIONS OF CONDENSED TANNINS

Early work reviewed by Nierenstein[6] found tannins to serve as defense mechanisms against environmental stress. As biotic defenses, tannins were seen to be fungitoxic and antipredatory, and could protect the plant against abiotic factors due to their water binding and antioxidant abilities.[6] More recent research has expanded the roles of condensed tannins in environmental stress responses and in growth regulation and are reviewed below.

Predator, Parasite, and Competitor Inhibitors

Antipredation mechanisms may be active, such as the responsive increase in phenolic synthesis in lettuce resistant to root aphids,[29] and the increase in catechol tannins in wasp-resistant chestnut[30] and *Salix*[31] leaves, or passive, as in the normal storage of phenolics within the vacuole.[32]

High phenolic content in plant tissues susceptible to grazing can often act as a deterrent to herbivores. Because many phenolics readily react with functional groups of salivary proteins and enzymes, they provide a general chemical defense against predation; this may be a result of coevolution of plant and animal relationships.[33] Increases in condensed tannins reduced the palatability of *Barteria*,[21] *Machaerium*,[34–36] and *Quercus*[37,38] leaves to their parasites and predators. The mechanism by which condensed tannins inhibit predation is unknown, although theories include reduced ingestion[38–40] or assimilation[37] of plant tissues. Although some researchers have seen little or no evidence of reduced assimilation as a result of condensed tannins,[39–41] Feeny[37,42–43] emphasized the quantitative nature of this defense mechanism, maintaining that increased condensed tannins lead to decreased protein availability in leaves, and thus decreased assimilation by the predator. This ongoing controversy has been recently reviewed by Zucker,[26] who suggested condensed tannins have specific protein-binding properties. Interestingly, there is no known process in animals for detoxification of tannins,[7] although some foraging herbivores apparently produce salivary proteins that bind tannins, thereby increasing the plant protein availability.[44–45] The controversy surrounding the relationships of tannins and insect predation of plant tissues is discussed more in Chapter 26 by Schultz.

Not only can condensed tannins act as herbivore deterrents, they may also function as allelopathic agents;[46] catechin and epicatechin inhibit seed germination.[47]

Growth Regulation

Phenolic compounds appear to be involved in the growth status of plants intrinsically as well. Seeds may be protected from premature germination by the phenolic growth inhibitors in their outer layers,[48–50] thus reducing the possibility of germination during environmentally unfavorable conditions. The mechanism by which germination is inhibited may involve the enzymatic formation of impermeable suberin,[51] condensed tannins,[52–53] and lignins within the coat.[54–55] This would not only make the coat impervious to water, but would also restrict gas exchange through the seed and protect the embryo from environmental stresses and disease.

In contrast, the presence of phenolics in vegetative and reproductive plant parts may actively inhibit or stimulate growth and development. Depending on their potential reactivity with proteins and enzymes, phenolics are classified as either "growth inhibitory" or "growth stimulatory". The stimulatory *o*-dihydroxy and trihydroxyphenols such as caffeic acid have highly reactive hydroxyl groups in a conformation that can inactivate indole acetic acid oxidase (IAA-oxidase), thus inhibiting IAA decarboxylation. In addition, Beckman et al[56] claimed the oxidation of dihydroxyphenolics leads to the formation of tryptophan and thus to IAA. Conversely, the monohydroxyphenolics including *p*-coumaric acid and the nonhydroxylated cinnamic acids do not have the obligatory adjacent hydroxyl groups and are therefore not inimical to IAA-oxidase activity; hence, they are considered to be inhibitory to growth. Because phenolics can regulate IAA-oxidase activity, they can presumably regulate other enzymes as well, which may or may not inhibit growth.

Studies conducted on buds or other vegetative plant parts generally support the idea of phenolic participation in plant growth.[57] Flavans and catechins were positively correlated with growth in *Prunus*,[58] and high levels of proanthcyanidins were found in easily rooted *Castanea* cuttings.[59] Endogenous phenolics were found to be major determinants of IAA-oxidase activity in barley[60] and Douglas-fir seedlings,[61] tomato callus,[62] and *Lens* sp.[63] It is likely the disappearance of growth inhibition in cold-stored chestnut cuttings[64] is due to either degradation of IAA-oxidase inhibitory phenolics or the biosynthesis of growth stimulatory phenolic compounds.

The contradictory information surrounding the regulatory action of phenolic compounds upon growth was reviewed by Kefeli and Dashek.[65] They concluded that although endogenous phenolic regulators are found outside the vacuole in compartments where they may act upon metabolic pathways, it is questionable whether the in vivo concentration of these compounds can become sufficiently elevated to function in a regulatory manner. Nevertheless, given the amount of data compiled through in vitro and in vivo experimentation, it seems likely that phenolics act as nonhormonal stimulators and inhibitors of plant growth.

Disease Resistance

The general nature of phytoalexin induction is suggested by the appearance of "reaction zones" in tree sapwood subjected to wounding injury or fungal attack.[66] Similar barriers of disease-resistant, phenolic-impregnated cell walls have been found in bean,[67] soybean,[68] cucumber,[69]radish,[70] tomato,[71] tobacco,[72] cotton,[73–74] wheat,[27,75] beech,[76] *Prunus*,[77] banana root,[56] and peach trees[78–79] as a result of wounding and/or fungal infection. Walkinshaw discusses the relationships of tannins specifically with fusiform rust disease in southern pine trees in Chapter 27 of this volume.

In some cases, there appears to be an "immunization" against disease by a previous infection or wounding.[56,80] This concept is illustrated in the work by Ostrofsky et al.[76] Wound-altered bark of fungus-resistant beech trees was found to have a higher phenolic content than that of susceptible beech; the healthy barks of both trees were equal in their phenolic content. Similarly, peach trees that had been wounded and then subjected to fungal infection showed an increase in phenolics over and above that due to the wounding response.[79] An increase in proanthocyanidins, especially catechin, was seen in *Salix* tissues that had been infected with *Erwinia*.[81] Increases of these compounds may exemplify what Mace et al[73] called "agents of induced systemic resistance."

Naturally resistant plants or specific tissues may have inherently higher phenolic contents. For instance, the roots of rot-resistant species including pecan, persimmon, and passionfruit have higher phenolic levels than susceptible species such as apple, almond, and peach.[82] More tannins and their precursors are found in disease-resistant tomatoes,[22] roses[83] and cotton[73–74] compared to susceptible varieties. Younger cotton leaves are thought to be more resistant to verticillium wilt due to high endogenous concentrations of catechin, gallocatechin, and condensed tannins.[84] Similarly, the polymerization of flavanols in tea shoots is speculated to protect the tissues from infection and decay.[85] Condensed tannins, such as those found in clover[86] and cotton,[35,73,87] also show antibiotic properties.

The basis for resistance may be the activity of enzymes responsible for phenolic synthesis; the acceleration of enzymatic activity is often greater in resistant than in susceptible hosts.[70] Phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), diphenyl oxidase (DPO), and peroxidase activities have been implicated in disease resistance.[70,87–88] Through these enzymatic activities, biologically reactive phenolic compounds, such as oxidized flavonols, are formed and can inhibit hydrolytic enzymes and pectinases[73] or render the plant tissues impenetrable.[74,85] Additionally, treatments that reduce or inhibit PAL synthesis or activity will reduce resistance to pathogens.[89]

General Stress Response

In addition to acting as protective agents against fungal stress, phenolic quantity and quality are affected by other types of stresses as well. In response to stress, oxidation of preformed phenolic compounds to quinones or other polymers may occur; in other cases, monomeric phenols are synthesized and accumulated in greater proportion than in undamaged tissues.[90] The end result is the formation of either a physical barrier or antibiotically active compounds.

In general, any kind of mechanical wounding will lead to increased phenolic synthesis[91–93] and accumulation,[77,94–99] especially in the cell walls.[17,91,97,100–101] Disruption of cell wall carbohydrates with such enzymes as cellulase[102] and chitosan[68] also results in increased phenolic production and lignification.

Nutrient stress also has a marked effect on phenolic levels in plant tissues. Deficiencies in nitrogen and phosphate lead to accumulation of phenolics[103–104] and lignification.[105] Tiarks reviews work on the relationships of leaf tannin content with nutrient availability in southern pines in Chapter 23 of this volume. Shortages of potassium and sulfur are reported to increase phenolic concentrations.[106] Magnesium deficiency is also reported to increase suberization,[107] whereas, iron deficiency inhibits the process.[98] Interestingly enough, high concentrations of glucose[108] and sucrose[109] will also stimulate phenolic production.

The synthesis of PAL is seen to increase following chilling of barley seedlings,[60] apple fruit[110], and potatoes,[110] leading to an increase in flavonoids and other phenolics. A similar accumulation was noted in chestnut cuttings stored at -10 °C.[64] The quality and quantity of proanthocyanidins increased in several varieties of *Rhododendron* following low temperature stress.[5] Xerophytic species (growing in dry conditions) often have a high tannin content in their leaves, perhaps as a protective screen against UV.[111] Caldwell et al[112] specifically mentioned flavonoids as powerful UV-B absorbers, whose synthesis in response to enhanced irradiation could protect proteins and nucleic acids from possible damage.

Other stresses found to increase phenolic accumulation include cadmium toxicity,[113] heat shock,[99,114] drought,[15] and enhanced UV-irradiation.[115–116] Because of their antioxidant and free-radical scavenging properties,[4,9–10] catechin and related oligomers could serve as generalized plant stress metabolites and stabilize cytoplasmic and other cell membranes.

HISTOCHEMICAL METHODOLOGY

To identify further the roles condensed tannins play in living plants, their localization within the tissues must be determined. An assortment of histological stains has been used to identify condensed tannin-containing areas of living tissues (Table 1). The historical development of these stains and their relative selectivity and sensitivity are discussed below.

Iron-based Reagents

The histochemical localization of tannins in plant tissues has interested botanists since 1807, when Link first described the blue-black reaction complex of tannins and iron.[117] Reichard[118] later noted the blue-black complex in barley grains treated with ferrous sulfate. Since then, relatively few staining processes were developed for observing tannins in living tissues until the advent of Johansen's landmark histochemical work.[119] In this text, a ferrous sulfate-formalin-water fixative was outlined for staining tannin-containing vacuoles; similarly, a mixture of ferric chloride and sodium carbonate turns blue-green in the presence of tannins,[119] primarily due to the reactivity of *o*-dihydroxy phenols.[120] During this period in history, no distinctions were made between condensed and hydrolyzable tannins; in fact, Johansen[119] stated "the word tannin does not denote a single substance but is a generic name covering a whole group of substances having certain characteristics in common". Nevertheless, Johansen's iron-based reagents, among others (see Rawlins and Takahashi[121]), have been widely used for localizing tannins in a variety of tissues, including fruit,[13] roots,[119–120] stems and bark,[16,122] and galls.[30] Interestingly, the color reactions for tannin in these tissues have ranged from the typical blue or blue-green through green[120] and even yellow-green.[122]

Nitroso Reagent

The first reagent used "specifically" for staining condensed tannins in living tissues was reported by Reeve.[123] Dissatisfied with the nonspecificity of Johansen's ferric chloride stain, he modified Hoepfener's[124] nitroso reagent for use in fresh tissues. (A similar reaction involving nitrous ethers was first reported by Vinson[125] in 1910). The stain imparts a cherry-red color to catechol-type compounds and varies from orange-red to yellow when used with other phenolic substances.[123] Further refinement of this technique[13,126] led to widespread use of the stain in locating catechin-type compounds in apple and peach fruit, juniper and pine leaves,[13,123] banana[120] and casava roots,[17] cotton[53] and barley seed,[127] vegetables,[126] and various cell cultures.[128] Although Halloin[53] reported the reagent was more specific for large condensed tannins than was the dimethoxybenzaldehyde reagent of Mace and Howell[74] mentioned below, Stafford et al[128] cautioned against using the reagent as a specific proanthocyanidin test. Although the reagent does impart a red color to *o*-dihydroxy-containing compounds including catechins, it also turns red in the presence of caffeic and chlorogenic acid, yellow or gold when added to trihydroxy phenols, and brown with prodelphinidins.[128] This helps explain the range of color products described for the various plant sources listed in Table 1.

Table 1. Stain Reagents used for Detection of Tannins in Plant Tissues

Reagent	Color Reaction	Material	Reference
DMACA	not described	*Prunus* tissues	130
	blue	*Prunus* shoots	131
	blue	*Prunus* cambium, leaves	77
DMB	red	cotton roots	74
	red	cotton seedling stems	78
	red	cotton seeds	53
	red	casava root	17
	bright purplish-red	*Vitis, Calycopteris*	129
$FeCl_3$	blue-green	fresh tissues	119
	greenish	peach fruit	13
	green	banana root	120
	not described	Douglas-fir	16
	not described	chestnut galls	30
$FeSO_4$	blue-black	barley grains	118
	not described	*Pinus* root tips	119
	blue	peach fruit	13
	yellow-green	soybean stems	122
Lugol's I_2 and NH_4OH	bright red	*Calycopterus, Vitis*	129
Nitroso	bright red	dates, persimmons	125
	cherry red	juniper, pine leaves, peach and apple fruit	123
	cherry red	peach fruit	13
	cherry red	banana root	120
	intense red	vegetable tissues	126
	brown	cotton seeds	53
	light orange	casava root	17
	red	barley seeds	127
	brown red and bright red	Douglas-fir, *Gingko*, and *Ribes* cell cultures	128
OsO_4/Sudan Black B	brownish orange	*Pseudotsuga* and *Pinus* cell cultures	136,137
TBO	blue-green	*Magnolia*	134
	blue-green to yellow-green	barley seeds	127
Vanillin-HCl	red	*Salix* leaves and galls	31
	bright red	ovarian tissues	133
	red	casava root	17
	red	barley seeds	52,132
Vanillin-*p*-toluene sulfonic acid	red	ovarian tissue	133

Dimethoxybenzaldehyde Reagent

Mace and Howell[74] developed the dimethoxybenzaldehyde (DMB) reagent for use in two-dimensional thin layer chromatography (TLC) of cotton seedling extracts. The compound forms a bright red reaction product in the presence of catechin monomers and oligomers. Apparently, it cannot be used with materials that have been preserved or fixed in formaldehyde, as the aldehyde group of DMB seems to be required for the formation of the red catechin-DMB compound.[74] The staining technique was later adapted for localization of condensed tannins in cotton stems[73] and cotton seeds;[53] in the cotton seeds, the reagent was reported to be less specific than the nitroso reagent[123] for large condensed tannins. It has also been used to localize condensed tannins in casava root xylem occlusions[17] and in leaves of *Calycopteris* and *Vitis*.[129] The latter tissues were described as giving a purplish-red color in the presence of the reagent,[129] an interesting departure from the more commonly reported red reaction product.

p-Dimethylaminocinnamaldehyde Reagent

Another reagent that may react similarly to the DMB stain is the *p*-dimethylaminocinnamaldehyde (DMACA) reagent mentioned by Tanrisever.[130] The translated abstract from the original Turkish article indicated the author used DMACA to localize condensed tannins in *Prunus* tissues. Perhaps independently, Feucht[77,131] reported the use of this same compound for staining condensed tannins in *Prunus* shoots; the reaction product was blue and could be measured spectrophotometrically.[77] The authors also suggested incubating freshly cut sections in a 1 percent caffeine-sodium benzoate solution, which coagulated flavanols and prevented leakage during subsequent staining.[77]

Vanillin-Based Reagents

Also developed originally for use with paper chromatography, the vanillin-HCl test has been successfully used in locating proanthocyanidins in a variety of plant tissues. Barley seeds,[52,132] various ovarian tissues,[133] casava roots,[17] and *Salix* leaves and galls[31] all turn a distinctive red in the presence of condensed tannins. Gardner[133] also reported the use of vanillin-*p*-toluene sulfonic acid in staining ovarian tissues, but found the reaction product less intense than the HCl-based reagent.

Toluidine Blue O Reagent

Originally, Toluidine Blue O (TBO) was used with plant tissues primarily to identify lignified cell walls with a blue or blue-green reaction product. Sakai[134] reported that the stain will also react with some tannins in fixed *Magnolia* tissues, turning them blue-green, as well as the lignified and suberized tissues. Barley seeds also turn blue-green to yellow-green after TBO is added in areas previously determined to contain condensed tannins.[127]

Lugol's Iodine and Ammonium Hydroxide

In an apparently isolated instance, Lugol's iodine and ammonium hydroxide (NH_4OH) was used to characterize the presence of tannins in a variety of leaf tissues.[129] A bright red color is indicative of the presence of tanniniferous cells in *Calycopteris, Loranthus, and Vitis*.[129]

Transmission Electron Microscopy Methods

During preparation of plant tissues for transmission electron microscopy (TEM), osmium tetroxide (OsO_4) is commonly added to fix and stain phenolic-containing cells. Combining OsO_4 with pure solutions of *o*-dihydroxy containing phenols, Nielson and Griffith[135] reported a blue reaction using aqueous solvents, and a red color using buffer solutions; these colors, however, would not be visible using TEM. Parham and Kaustinen[136–137] used this fixation method combined with Sudan Black B to stain differentially the tannins in Douglas-fir cell cultures. Although Sudan Black B is used primarily as a lipid stain, when combined with the osmium tetroxide, it imparts a brownish orange color to tannin globules as compared to the blue-black color given to lipids. Under TEM, the two bodies are seen as black and dark gray, so differentiation can be seen under both light microscopy and TEM.

LOCALIZATION OF CONDENSED TANNIS IN PLANT TISSUES

Bud Tissues

There are apparently few published reports on the presence of condensed tannins in either floral or vegetative buds. Lynch and Rivera[138] described the occurrence of tannin-containing vacuoles in the pith, cortex, and rib meristem of dormant *Rhododendron* vegetative buds. Unpublished results by Chalker-Scott show an interesting localization in dormant *Azalea* floral buds of compounds that react positively with ferric chloride, vanillin, and the nitroso reagent (Figures 1-3). Under all three staining regimes, red-colored compounds, presumably proanthocyanidin in nature, appear within the bud scales and periderm, and beneath the bud axis (Figure 4). Previous microscopy work[5] shows these same areas also tested positively for suberin and lignin. The hypothesized role of these phenolic-rich areas is to retard the formation of ice within the tender bud tissues during freezing stress, perhaps by acting as a physical barrier to ice nucleation.[5]

Leaf Tissues

The leaves of many plant species of commercial or ornamental value are rich in proanthocyanidin compounds. Crop plants such as cocoa,[139] cotton,[35,73–74] tomato,[22] and tea[140–141] are especially rich in condensed tannins; the presence of catechins in the latter crop accounts for the astringency of strongly brewed tea. Woody shrubs such as rhododendron[2,5] and deciduous trees including oak,[37] willow,[142] and *Prunus*[130] also have leaves rich in condensed tannins, which may undergo quantitative and qualitative seasonal changes.[5,37,142] Typically, these changes

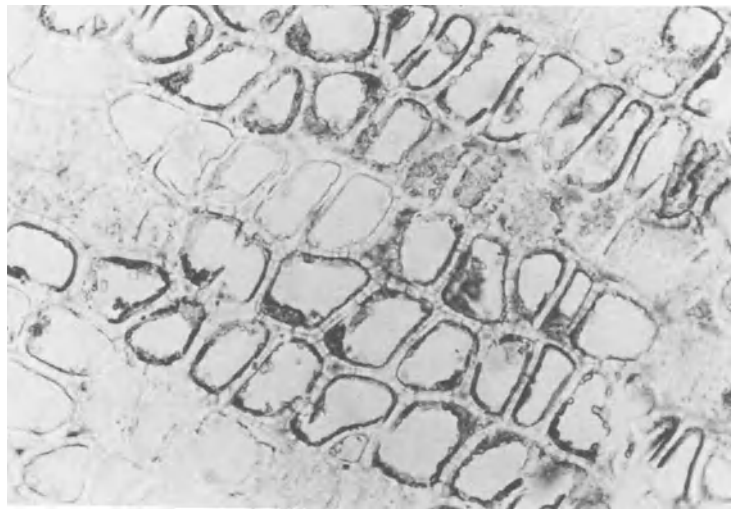

Figure 1. *Photomicrograph of dormant Azalea floral bud tissue showing localization of dark-stained condensed tannins in bud base using ferric chloride stain* (250x).

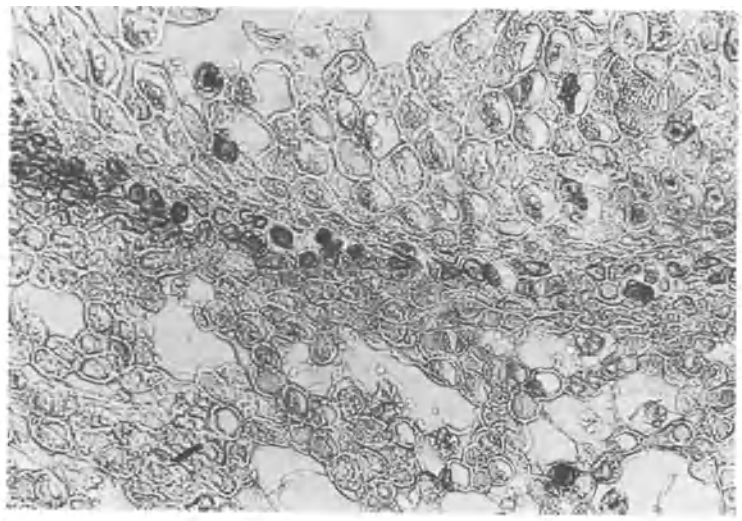

Figure 2. *Photomicrograph of dormant Azalea floral bud tissue showing localization of dark-stained condensed tannins in the base of the bud scale using vanillin reagent* (160x).

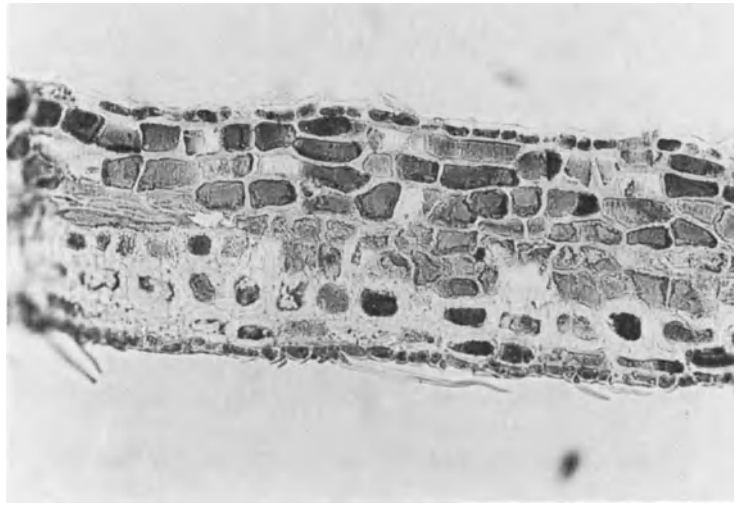

Figure 3. *Photomicrograph of dormant Azalea floral bud tissue showing localization of dark-stained condensed tannins in the bud scale using nitroso reagent* (160x).

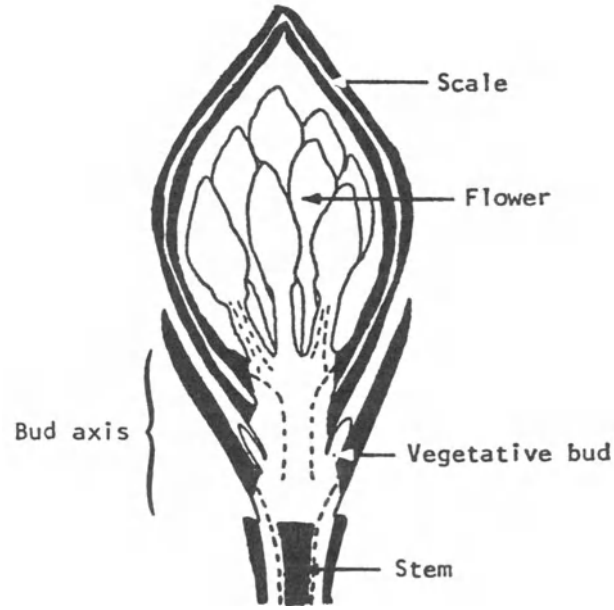

Figure 4. *Localization of proanthocyanidins in bud tissues. Areas shaded black indicate reported presence of condensed tannins.*

include the appearance of catechin and condensed tannins in older leaves, coincident with a decrease in insect infestation.[37] Such increases may also be associated with growth inhibition,[142] UV-radiation resistance,[111] and increased cold hardiness.[5]

The high tannin content in plant tissues susceptible to grazing can often act as a deterrent to herbivores. Increases in condensed tannins reduced the palatability of *Barteria*,[21] *Machaerium*,[35] and *Quercus*[37–38] leaves to their predators. Condensed tannins, which rapidly polymerize proteins, may inhibit predator ingestion[40] or assimilation of plant tissues.[37]

Disease-resistant tomato varieties have been shown to contain more tannins than susceptible types;[22] likewise, the condensed tannins in cotton[35,73] show antibiotic properties. Young leaves of some species such as cotton[84] may be more resistant to verticillium wilt than older leaves due to their high concentrations of catechin, gallocatechin, and condensed tannins. Interestingly, Chiang and Norris[122] reported the leaves of resistant soybean did not contain any condensed tannins, although phenolic content was higher than that of stems.

The cellular localization of condensed tannins has not been well documented; Figure 5 illustrates the leaf areas where condensed tannins are generally seen. Early work reviewed by Nierenstein[6] reported the occurrence of tannins in the epiderm, periderm, companion cells, and rays of tannin-rich species such as *Rhododendron, Ribes*, and *Rosa*. However, other plants, including evergreen trees, apparently have tannins evenly distributed throughout their tissues. More recently, Blunden et al[31] reported the appearance of catechins and leucoanthocyanidins in the mesophyll of healthy and gall-infected *Salix* leaves; infected leaves had two to eight times more condensed tannins than the control tissues. Mikeladze and Shalamberidze[140] found most tannins in the intercellular spaces of the parenchyma in the mesophyllum of fresh tea leaves; rare occurrences were found in the transport system and epidermis cells. When leaves were extracted in hot water, only the tannins in the transport system and epidermis were left. *Prunus* condensed tannins were located in entire young leaf blades; in older leaves, they were localized in vascular tissue, especially phloem.[130] The leaves of *Calycopteris* and *Vitis* contain condensed tannins primarily in the epidermal cells and palisade mesophyll;[129] these locations lend credence to the theory advanced by Caldwell et al[112] that flavonoid substances located in the epidermis and mesophyll may act as UV-protectants.

Root Tissues

Apparently, few histochemical studies on the location of tannins in root tissues have been reported. Those that have been published, however, are fairly recent and have used specific techniques to identify the tannins. Using cotton roots, Mace and Howell[74] reported locating catechin and gallocatechin in the hypodermis, endodermis, xylem parenchyma, and proximal root cap. *Rheum* roots have tannins concentrated in cork cells, with more occurring at the tops and outside of roots.[142] The latter investigators warn against using high drying temperatures (60 °C), as this caused artifacts in tannin measurement.[143] The widespread distribution of condensed tannins in the roots (Figure 6) may serve as a chemical barrier to penetration and colonization of roots by plant pathogens.[74]

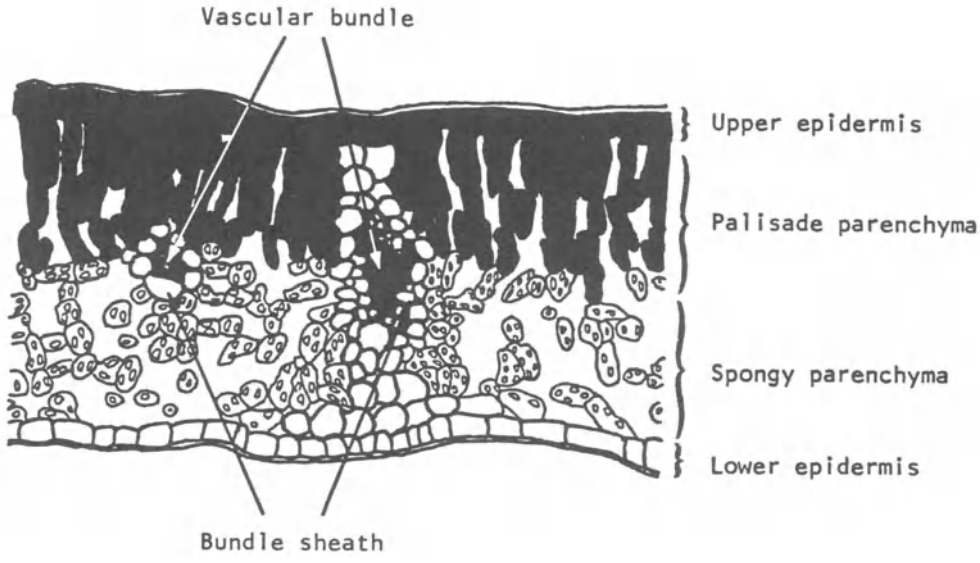

Figure 5. *Localization of proanthocyanidins in leaf tissues. Areas shaded black indicate reported presence of condensed tannins.*

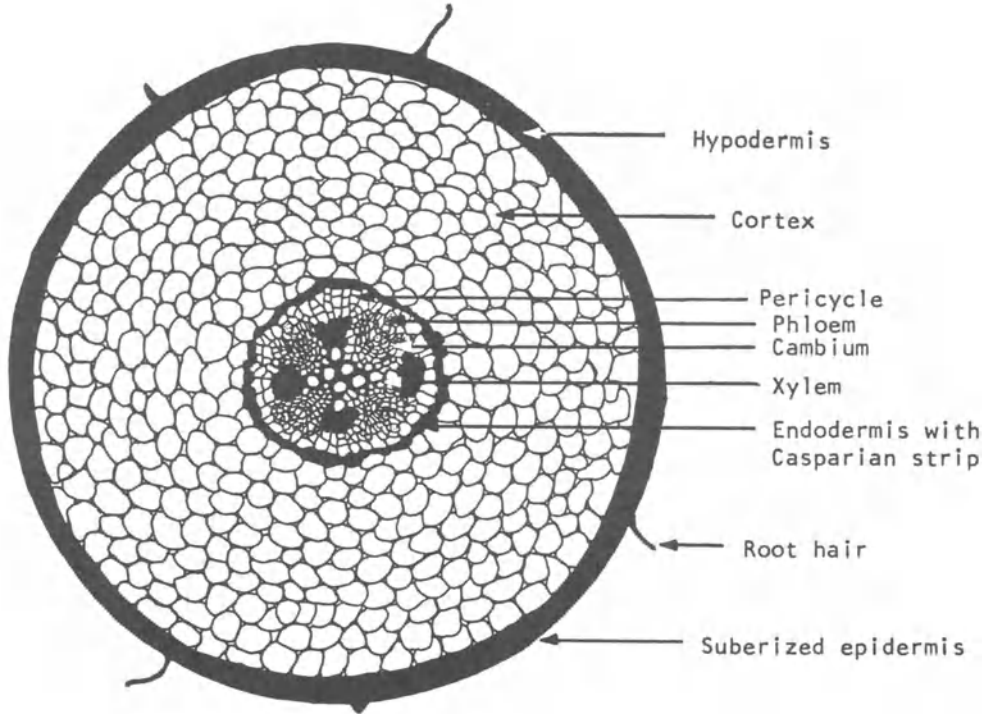

Figure 6. *Localization of proanthocyanidins in root tissues. Areas shaded black indicate reported presence of condensed tannins.*

Seed Tissues

Seed coat tannins, which play a role in the growth status and disease resistance of the seeds, have long been regarded as nuisances by the beer industry. The presence of condensed tannins in barley seeds leads to the formation of colloidal hazes in beer,[52] which decrease its esthetic value to the consumer. Histological work with barley grain has determined that (+)-catechin and other proanthocyanidins occur as early as 4 days after flowering.[132] Having shown previously that these proanthocyanidins are located in the seed coat just outside the aleurone layer,[52] Aastrup and Outtrup[131] later determined the testa is either the site of synthesis or the site of embedding of the condensed tannins in barley. In an independent study, Felker et al[127] described the presence of proanthocyanidins in vacuoles of the chalaza and seed coats. Figure 7 depicts the seed coat areas that commonly contain condensed tannins.

Little other historical work has been done with condensed tannin development in seeds, although (+)-catechin was also found to be the primary tannin precursor in cottonseed,[53] and high concentrations of epicatechin and leucocyanidins are found in cocoa cotyledons.[139] Condensed tannins in seeds appear to be associated with maintaining dormancy and acting as allelopathic[144] or bacteriocidal[86] agents.

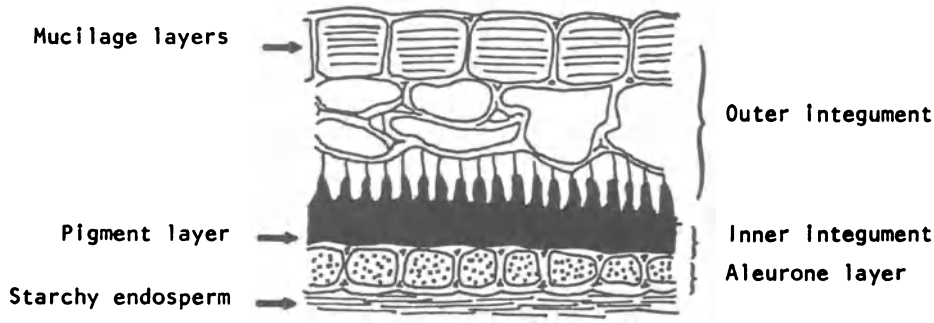

Figure 7. *Localization of proanthocyanidins in seed coats. Areas shaded black indicate reported presence of condensed tannins.*

Stem Tissues

The presence of condensed tannins as heartwood extractives in conifers is well known to researchers in forest products.[1] Their location in living tissues of stems and trunks, however, is less well understood, although both angiosperm and gymnosperm species have been studied.

Increased synthesis of condensed tannins as a result of environmental stress has been observed in the stem tissues of several economically important crops. Catechins were found in young floral and vegetative shoots of tea, and flavanols were the only phenolics found in the older stems.[85] Catechin, gallocatechin, and other fungitoxic condensed tannins were isolated from the stems of wilt-resistant cotton.[73] Specifically, these strains produced more flavanols in the ray and axial

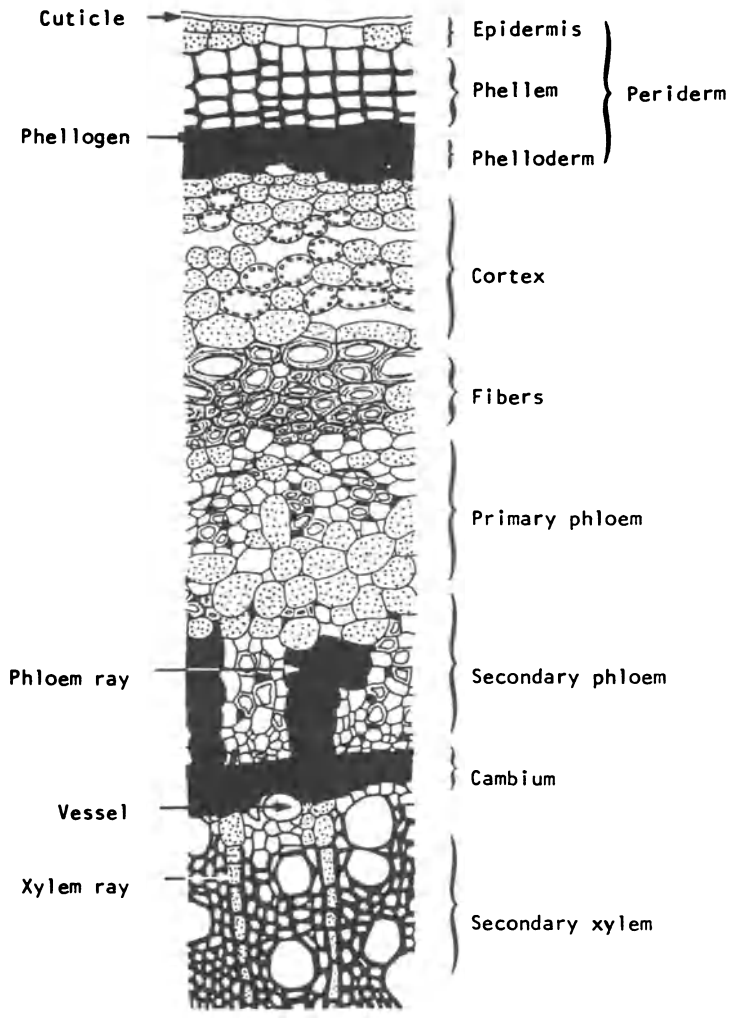

Figure 8. *Localization of proanthocyanidins in stem tissues. Areas shaded black indicate reported presence of condensed tannins.*

parenchyma between the cambium and newly formed xylem vessels, and in the pith adjacent to xylem vessels that had been infected with fungus.[73] Such localization of the tannins is thought to create a toxic environment throughout the stem stele, and thus confine the fungi to the vessel lumens.[73] Similarly, the presence of catechin in the intercellular spaces of blight-resistant cotton is hypothesized to restrict the multiplication of the bacteria.[87] Cotton stem condensed tannins, including catechin, are also phytotoxic to tobacco budworm and corn earworm, apparently by inhibiting the intestinal flora of the insects.[35] In contrast, Chiang and Norris[122] stated that no condensed tannins were detected in the stems of any soybean varieties tested, conluding bean fly-resistant strains. In view of the contradictory data,

and considering the ubiquity of condensed tannins, it seems likely the latter results could be questioned.

Condensed tannins of *Prunus* were located in the vascular and cork cambium[130] and phloem rays, ray initials, and phelloderm of young, wounded internodes.[77] The area between the cork cells and secondary phloem of *Acacia*, which consists of the periderm, stone cells, phloem parenchyma, and rays, was found to contain over 60 percent of the total bark condensed tannins.[145] A developmental study by Ross and Corden[16] of Douglas-fir periderm showed the non-lignified parenchyma cells of the inner bark collect condensed tannins as they lose storage materials and increase in size. Given their general location in active growth areas in these trees (Figure 8), it seems likely that condensed tannins could indeed have a role in growth regulation, as has been suggested previously.

CONCLUSIONS

Rather than existing merely as waste products, the proanthocyanidins and condensed tannins are thought to play major roles within the living plant in growth regulation and in defense against environmental stresses. Their relative importance in these functions is supported by their respective localization within regions of active growth and outer layers of the plant. Although it is expanding, the body of histochemical knowledge regarding condensed tannins is historically young and will be vastly improved by the development of more specific microscopic examination techniques and histological stains.

REFERENCES

1. Hillis, W.E. Secondary changes in wood. *In*: Loewus, F.A.; Runeckles, V.C. (eds.) Recent Advances in Phytochemistry. Plenum Publishing Company, New York; pp.247-309 (1977).
2. Haslam, E. Proanthocyanidins. *In*: Harborne, J.B.; Mabry, T.J. (eds.) The Flavonoids: Advances in Research. Chapman and Hall, London; pp. 419-447 (1982).
3. Lowry, B.; Lee, D.; Hebant, C. The origin of land plants: a new look at an old problem. *Taxon* 29:183 (1980).
4. Knox, J.P.; Dodge, A.D. Singlet oxygen and plants. *Phytochemistry* 24:889 (1985).
5. Chalker-Scott, L. Relationships between Endogenous Phenolic Compounds of Rhododendron Tissues and Organs and Cold Hardiness Development. PhD. thesis, Oregon State University, Corvallis, 269 p. (1988).
6. Nierenstein, M. The Natural Organic Tannins. History: Chemistry: Distribution. J. & A. Churchill Ltd., London; 319 p. (1934).
7. Harborne, J.B. Plant phenolics. *In*: Bell, E.A.; Charlwood, B.V. (eds.) Secondary Plant Products. Springer-Verlag, Berlin; pp. 329-402 (1980).
8. Hillis, W.E. Leucoanthocyanins as the possible precursors of extractives in woody tissues. *Aust. J. Biol. Sci.* 9:263 (1956).
9. Akaranta, O.; Odozi, T.O. Antioxidant properties of red onion skin tannin extract. *Agric. Wastes* 18:299 (1986).
10. Slater, T.F.; Scott, R. The free-radical scavenging action of (+)-cyanidanol-3 in relation to the toxicity of carbon tetrachloride. *In*: Conn, H.O. (ed.) International Workshop on (+)-Cyanidanol-3 in Diseases of the Liver. The Royal Society of Medicine, International Congress and Symposium Series No. 47; Academic Press, London; pp. 33-39 (1981).

11. Perissoud, D.; Maigram, M.F.; Anderset, G. Morphological and biochemical study of the interaction of (+)-cyanidanol-3 with the plasma membrane and protection against phalloidin and carbon tetrachloride toxicity in rat hepatocytes. *In*: Conn, H.O. (ed.) International Workshop on (+)-Cyanidanol-3 in Diseases of the Liver. The Royal Society of Medicine, International Congress and Symposium Series No. 47; Academic Press, London; pp. 21-26 (1981).

12. Bate-Smith, E.C.; Lerner, N.H. Leuco-anthocyanins. 2. Systematic distribution of leucoanthocyanins in leaves. *Biochem. J.* 58:126 (1954).

13. Reeve, R.M. Histological and histochemical changes in developing and ripening peaches. 1. The catechol tannins. *Am. J. Bot.* 46:210 (1959).

14. Rubin, B.A. Artsikhovskaya, E.W. Biochemistry of pathological darkening of plant tissues. *Ann. Rev. Phytopathol.* 2:157 (1964).

15. Pizzi, A.; Cameron, F.A. Flavonoid tannins – structural wood components for drought-resistance mechanisms of plants. *Wood Sci. Technol.* 20:119 (1986).

16. Ross, W.D.; Corden, M.E. Microscopic and histochemical changes in Douglas-fir bark accompanying fungal invasion. *Wood and Fiber* 5:129 (1973).

17. Rickard, J.E.; Gahan, P.B. The development of occlusions in cassava (*Manihot esculenta* Crantz) root xylem vessels. *Ann. Bot.* 52:811 (1983).

18. Cottle, W.; Kolattukudy, P.E. Biosynthesis, deposition, and partial characterization of potato suberin phenolics. *Plant Physiol.* 69:393 (1982).

19. Simonds, A.O.; Johnson, G.; Schaal, L.A. Comparative effects of catechol, some related compounds and other chemicals on suberization of cut potato tubers. *Bot. Gaz.* 115:190 (1953).

20. Hoque, E. Biochemical aspects of stress physiology of plants and some considerations of defense mechanisms in conifers. *Eur. J. For. Pathol.* 12:280 (1982).

21. Waterman, P.G.; Ross, J.A.M.; McKey, D.B. Factors affecting levels of some phenolic compounds, digestibility, and nitrogen content of the mature leaves of *Barteria fistulosa* (Passifloraceae). *J. Chem. Ecol.* 10:387 (1984).

22. Bhatia, I.S.; Uppal, D.S.; Bajaj, K.L. Study of phenolic contents of resistant and susceptible varieties of tomato (*Lycopersicon esculentrum*) in relation to early blight disease. *Indian Phytopathol.* 25:231 (1972).

23. Fry, S.C. Incorporation of [^{14}C]cinnamate into hydrolase-resistant components of the primary cell wall of spinach. *Phytochemistry* 23:59 (1984).

24. Carpita, N.C. Incorporation of proline and aromatic amino acids into cell walls of maize coleoptiles. *Plant Physiol.* 80:660 (1986).

25. Taiz, L. Plant cell expansion: regulation of cell wall mechanical properties. *Ann. Rev. Plant Physiol.* 35:585 (1984).

26. Zucker, W.V. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335 (1983).

27. Beardmore, J.; Ride, J.P.; Granger, J.W. Cellular lignification as a factor in the hypersensitive resistance of wheat to stem rust. *Physiol. Plant Pathol.* 22:209 (1983).

28. Kolattukudy, P.E. Biopolyester membranes of plants: cutin and suberin. *Science* 208:990 (1980).

29. Cole, R.A. Phenolic acids associated with the resistance of lettuce cultivars to the lettuce root aphid. *Ann. Appl. Biol.* 105:129 (1984).

30. Matsui, S.; Torikata, H. The resistance of chestnut trees (*Castanea* spp.) to chestnut gall wasps (*Dryocosmus kuriphilus* Yasumatsu). X. Formation of necroses and histochemical distribution of polyphenolic substances in bud tissues. *Engei Gakkai Zasshi* 45:107 (1976).

31. Blunden, G.; Challen, S.B.; Jaques, B. Accumulation of anthoxanthins and imino-acids in leaf galls of *Salix fragilis* L. *Nature* 212:514 (1966).

32. Hendry, G. Why do plants have cytochrome P-450? Detoxification versus defence. *New Phytol.* 102:239 (1986).

33. Beart, J.E.; Lilley, T.H.; Haslam, E. Plant polyphenols - secondary metabolism and chemical defence: some observations. *Phytochemistry* 24:33 (1985).

34. Lane, H.C.; Schuster, M.F. Condensed tannins of cotton leaves. *Phytochemistry* 20:425 (1981).

35. Waage, S.K.; Hedin, P.A.; Grimley, E. A biologically-active procyanidin from *Machaerium floribundum*. *Phytochemistry* 23:2785 (1984).

36. Yokoyama, V.Y.; Mackey, B.E. Protein and tannin in upper, middle, and lower cotton plant strata and cigarette beetle (Coleoptera: Anobiidae) growth on foliage. *J. Econ. Entomol.* 80:843 (1987).

37. Feeny, P.P.; Bostock, H. Seasonal changes in the tannin content of oak leaves. *Phytochemistry* 7:871 (1968).

38. Scalbert, A.; Haslam, E. Polyphenols and chemical defence of the leaves of *Quercus robur*. *Phytochemistry* 26:3191 (1987).

39. Klocke, J.A.; Chan, B.G. Effects of cotton condensed tannin on feeding and digestion in the cotton pest, *Heliothis zea*. *J. Insect Physiol.* 28:911 (1982).

40. Reese, J.C.; Chan, B.G.; Waiss, A.C., Jr. Effects of cotton condensed tannin, maysin (corn), and pinitol (soybeans) on *Heliothis zea* growth and development. *J. Chem. Ecol.* 8:1429 (1982).

41. Martin, M.M.; Martin, J.S. Surfactants: their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia* 61:342 (1984).

42. Feeny, P.P. Effect of oak leaf tannins on larval growth of the winter moth *Operophtera brumata*. *J. Insect Physiol.* 14:805 (1968).

43. Feeny, P.P. Plant apparency and chemical defense. *In*: Wallace, J.W.; Mansell, R.L. (eds.) Recent Advances in Phytochemistry - Biochemical Interactions between Plants and Insects. Vol. 10. Plenum Publishing Company, New York; pp. 1-40 (1976).

44. Robbins, C.T.; Hanley, T.A.; Hagerman, A.E.; Hjeljord, O.; Baker, D.L.; Schwartz, C.C.; Mautz, W.W. Role of tannins in defending plants against ruminants: reduction in protein availability. *Ecology* 68:98 (1987).

45. Robbins, C.T.; Mole, S.; Hagerman, A.E.; Hanley, T.A. Role of tannins in defending plants against ruminants: reduction in dry matter digestion? *Ecology* 68:1606 (1987).

46. Kubo, I.; Matsumoto, T.; Hanke, F.J.; Taniguchi, M.; Hayashi, Y. Epicatechin can cause the seedling growth inhibitor, nagilactone E, to induce growth stimulation. *Experientia* 41:1462 (1985).

47. Buta, J.G.; Lusby, W.R. Catechins as germination and growth inhibitors in *Lespedeza* seeds. *Phytochemistry* 25:93 (1986).

48. Cezard, R. Orobanchaceae. II. Breaking dormancy in orobanchaceous seeds by removing an endogenous inhibition. *Bull. Acad. Soc. Lorraine Sci.* 12:97 (1973).

49. Fadl, M.S.; Baz, A.G.I.O.; Tayel, S. Dormancy termination in apricot seeds. II. Interaction between endogenous phenols and germination. *Egypt. J. Physiol. Sci.* 5:303 (1980).

50. Naqvi, H.H.; Hanson, G.P. Germination and growth inhibitors in guayule (*Parthenium argentatum* Gray) chaff and their possible influences in seed dormancy. *Amer. J. Bot.* 69:985 (1982).

51. Espelie, K.E.; Davis, R.W.; Kolattukudy, P.E. Composition, ultrastructure and function of the cutin- and suberin-containing layers in the leaf, fruit peel, juice-sac and inner seed coat of grapefruit (*Citrus paradisi* Macfed.). *Planta* 149:498 (1980).

52. Aastrup, S.; Outtrup, H.; Erdal, K. Location of the proanthocyanidins in the barley grain. *Carlsberg Res. Commun.* 49:105 (1984).

53. Halloin, J.M. Localization and changes in catechin and tannins during development and ripening of cottonseed. *New Phytol.* 90:651.

54. Aparicio-Tejo, P.M.; Pena, J.I. Sanchez-Diaz, M.; Becana, M. Coat phenolic compounds inhibit germination of *Halimium halimifolium*. *Plant Physiol. Suppl.* 80:128 (1986).

55. Egley, G.H.; Paul, R.N., Jr.; Duke, S.O.; Vaughn, K.C. Peroxidase involvement in lignification in water-impermeable seed coats of weedy leguminous and malvaceous species. *Plant. Cell and Environ.* 8:253 (1985).

56. Beckman, C.H.; Mueller, W.C.; Mace, M.E. The stabilization of artificial and natural cell wall membranes by phenolic infusion and its relation to wilt disease resistance. *Phytopathology* 64:1214 (1974).

57. Kefeli, V.I.; Kutacek, M. Phenolic substances and their possible role in plant growth regulation. *In*: Pilet, P.E. (ed.), Plant Growth Regulation. Springer-Verlag, Berlin pp. 181-188, (1977).

58. Nachit, M.; Feucht, W. Inheritance of phenolic compounds, indoles and growth vigour in *Prunus* crosses (cherries). *Z. Pflanzenzuchtg.* 90:166 (1983).

59. Vaquez, A.; Gesto, D.V. Rooting, endogenous root-inducing cofactors and proanthocyanidins in chestnut. *Biol. Plant.* 28:303 (1986).

60. Podstolski, A.; Sznajder, J.; Wichowska, G. Accumulation of phenolics and growth rate of barley seedlings (*Hordeum vulgare* L.) *Biol. Plant.* (Praha) 23:120 (1981).

61. Johnson, M.A.; Carlson, J.A. Indoleacetic acid oxidase and related enzymes in cultured and seedling Douglas fir. *Biochem. Physiol. Pflanzen.* 174:115 (1979).

62. Ranade, S.; David, S.B. Quinones as plant growth regulators. *Plant Growth Reg.* 3:3 (1985).

63. Dangar, T.K.; Basu, P.S. Seasonal changes and metabolism of plant harmones in root nodules of *Lens* sp. *Biol. Plant.* 26:253 (1984).

64. Vaquez, A.; Gesto, M.D.V. Effect of cold storage on endogenous growth substances in cuttings of *Castanea sativa* Mill. *Phyton* 42:171 (1982).

65. Kefeli, V.I.; Dashek, W.V. Non-hormonal stimulators and inhibitors of plant growth and development. *Biol. Rev. Cambridge Philos. Soc.* 59:273 (1984).

66. Kemp, M.S.; Burden, R.S. Phytoalexins and stress metabolites in the sapwood of trees. *Phytochemistry* 25:1261 (1986).

67. Stumpf, M.A.; Heath, M.C. Cytological studies of the interaction between the cowpea rust fungus and silicon-depleted French bean plants. *Physiol. Plant Pathol.* 27:369 (1985).

68. Kohle, H.; Young, D.H.; Kauss, H. Physiological changes in suspension-cultured soybean cells elicited by treatment with chitosan. *Plant Sci. Lett.* 33:221 (1984).

69. Hammerschmidt, R.; Bonnen, A.M.; Bergstrom, G.C.; Baker, K.K. Association of epidermal lignification with nonhost resistance of cucurbits to fungi. *Can. J. Bot.* 63:2393 (1985).

70. Friend, J. Plant phenolics, lignification and plant disease. *In*: Reinhold, L.; Harborne, J.B.; Swain, T. (eds) Progress in Phytochemistry. Pergamon Press, New York; pp. 197-261 (1981).

71. DeLeeuw, G.T.N. Deposition of lignin, suberin and callose in relation to the restriction of infection by *Botrytis cinerea* in ghost spots of tomato fruits. *Phytopath. Z.* 112:143 (1985).

72. Zhou, J.P.; Wang, B.C. Field observation on the epiphytology of tobacco black shank (*Phytophthora parasitica* f. *nicotianae* [Breda de Haan] Tucker). *Acta Phytopathol. Sin.* 14:39 (1984).

73. Mace, M.E. Bell, A.A.; Stipanovic, R.D. Histochemistry and identification of flavanols in *Verticillium* wilt-resistant and -suseptible cottons. *Physiol. Plant Pathol.* 13:143 (1978).

74. Mace, M.E.; Howell, C.R. Histochemistry and identification of condensed tannin precursors in roots of cotton seedlings. *Can. J. Bot.* 52:2423 (1974).

75. Ride, J.P. Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. *Physiol. Plant Pathol.* 5:125 (1975).

76. Ostrofsky, W.D.; Shortle, W.C.; Blanchard, R.O. Bark phenolics of American beech (*Fagus grandifolia*) in relation to the beech bark disease. *Eur. J. For. Pathol.* 14:52 (1984).

77. Feucht, W.; Schmid, P.P.S.; Christ, E. Distribution of flavanols in meristematic and mature tissues of *Prunus avium* shoots. *J. Plant Physiol.* 125:1 (1986).

78. Biggs, A.R. Wound age and infection of peach bark by *Cytospora leucostoma*. *Can. J. Bot.* 64:2319 (1986).

79. Wisniewski, M.; Bogle, A.L.; Shortle, W.C.; Wilson, C.L. Interaction between *Cystospora leucostoma* and host-phenolic compounds in dormant peach trees. *J. Amer. Soc. Hort. Sci.* 109:563 (1984).

80. Kuc, J. Induced immunity to plant disease. *BioScience* 32:854 (1982).

81. Wong, W.C.; Preece, T.F. *Erwinia salicis* in cricket bat willows: phenolic constituents in healthy and diseased wood. *Physiol. Plant Pathol.* 12:349 (1978).

82. Sztejnberg, A.; Azaiza, H.; Chet, I. The possible role of phenolic compounds in resistance of horticultural crops to *Dematophora necatrix* Hartig. *Phytopathol. Z.* 107:318 (1983).

83. Conti, G.G.; Bassi, M.; Maffi, D.; Bocci, A.M. Host-parasite relationship in a susceptible and a resistant rose cultivar inoculated with *Sphaerotheca pannosa*. *J. Phytopathol.* 117:312 (1986).

84. Howell, C.R.; Bell, A.A.; Stipanovic, R.D. Effect of aging on flavonoid content and resistance of cotton leaves to verticillium wilt. *Physiol. Plant Pathol.* 8:181 (1976).

85. Forrest, G.I.; Bendall, D.S. The distribution of polyphenols in the tea plant (*Camellia sinensis* L.). *Biochem. J.* 113:741 (1969).

86. Young, H.; Paterson, V.J. Condensed tannins from white clover seed diffusate. *Phytochemistry* 19:159 (1980).

87. Venere, R.J. Role of peroxidase in cotton resistant to bacterial blight. *Plant Sci. Lett.* 20:47 (1980).

88. Bell, A.A. Biochemical mechanisms of disease resistance. *Ann. Rev. Plant Physiol.* 32:21 (1981).

89. Darvill, A.G.; Albersheim, P. Phytoalexins and their elicitors - a defense against microbial infection in plants. *Ann. Rev. Plant Physiol.* 35:243 (1984).

90. Rhodes, J.M.; Wooltorton, L.S.C. The biosynthesis of phenolic compounds in wounded plant storage tissues. *In*: Kahl, G. (ed.) Biochemistry of Wounded Plant Tissues. Walter de Gruyter, Berlin; pp. 243-286 (1978).

91. de Jaegher, G.; Boyer, N.; Gaspar, T. Thigmomorphogenesis in *Bryonia dioica*: changes in soluble and wall peroxidases, phenylalanine ammonia-lyase activity, cellulose, lignin content and monomeric constituents. *Plant Growth Reg.* 3:133 (1985).

92. Thorpe, J.R.; Hall, J.L. Chronology and elicitation of changes in peroxidase and phenylalanine ammonia-lyase activities in wounded wheat leaves in response to inoculation by *Botrytis cinerea*. *Physiol. Plant Pathol.* 25:363 (1984).

93. van Loon, L.C.; Gerritsen, Y.A.M. Ethylene and a product of aromatic biosynthesis induce pathogenesis-related proteins in hypersensitively reacting tobacco. *Plant Physiol. Suppl.* 80:145 (1986).

94. Baldwin, I.T.; Schultz, J.C. Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants. *Science* 221:22 (1983).

95. Barker, W.G.; Peterson, R.L. Wound responses of *Impatiens balsamina* seedlings after collet excision. *Scanning Electron Micros.* 1984:1001 (1984).

96. Biggs, A.R. Boundary-zone formation in peach bark in response to wounds and *Cytospora leucostoma* infection. *Can. J. Bot.* 62:2814 (1984).

97. Biggs, A.R. Phellogen regeneration in injured peach tree bark. *Ann. Bot.* 57:463 (1986).

98. Kolattukudy, P.E.; Soliday, C.L. Effects of stress on the defensive barriers of plants. *In*: Key, J.L.; Kosuge, T. (eds.) Cellular and Molecular Biology of Plant Stress. Alan R. Liss, Inc., New York; pp. 381-400 (1985).

99. Tronchet, J. Reactions of the flavonoid substances from aerial parts of *Coleus blumei* Benth. var Colour Pride (Lamiacees) to various injuries: bruises, thermal shock, water deficiency. *Ann. Sci. Univ. Besancon. Bot.* 16:17 (1975).

100. Biddington, N.L. A review of mechanically induced stress in plants. *Sci. Hortic.* 36:12 (1985).

101. Rittinger, P.A.; Biggs, A.R.; Peirson, D.R. Histochemistry of lignin and suberin deposition in boundary layers formed after wounding in various plant species and organs. *Can. J. Bot.* 65:1886 (1987).

102. Geballe, G.T.; Galston, A.W. Wound-induced resistance to cellulase in oat leaves. *Plant Physiol.* 70:781 (1982).

103. DiCosmo, F.; Towers, G.H.N. Stress and secondary metabolism in cultured plant cells. *In*: Timmermann, B.N.; Steelink, C.; Loewus, F.A. (eds.) Phytochemical Adaptations to Stress. Plenum Publishing Company, New York; pp. 97-175 (1983).

104. Nobloch, K.H.; Berlin, J. Influence of phosphate on the formation of the indole alkaloid and phenolic compounds in cell suspension cultures of *Catharanthus roseus*: 1. Comparison of enzyme activities and product accumulation. *Plant Cell Tissue Organ Culture* 2:333 (1983).

105. Farmer, E.E. Effects of fungal elicitor on lignin biosynthesis in cell suspension cultures of soybean. *Plant Physiol.* 78:338 (1985).

106. Gershenzon, J. Changes in the levels of plant secondary metabolites under water and nutrient stress. *In*: Timmermann, B.N.; Steelink, C.; Loewus, F.A. (eds.) Phytochemical Adaptations to Stress. Plenum Publishing Company, New York; pp. 273-321 (1983).

107. Kolattukudy, P.E. Biochemistry and function of cutin and suberin. *Can. J. Bot.* 62:2918 (1984).

108. Amorim, H.V.; Dougall, D.K.; Sharp, W.R. The effect of carbohydrate and nitrogen concentration on phenol synthesis in Paul's scarlet rose cells grown in tissue culture. *Physiol. Plant.* 39:91 (1977).

109. Phillips, R.; Henshaw, G.G. The regulation of synthesis in stationary phase cell cultures of *Acer pseudoplatanus* L. *J. Exp. Bot.* 28:785 (1977).

110. Graham, D.; Patterson, B.D. Responses of plants to low, non-freezing temperatures: proteins, metabolism, and acclimation. *Ann. Rev. Plant Physiol.* 33:347 (1982).

111. Hallam, N.D.; Luff, S.E. Fine structural changes in the leaves of the desiccation-tolerant plant *Talbotia elegans* during extreme water stress. *Bot. Gaz.* 141:180 (1980).

112. Caldwell, M.M.; Robberecht, R.; Flint, S.D. Internal filters: prospects for UV-acclimation in higher plants. *Physiol. Plant.* 58:445 (1983).

113. Fuhrer, J. Early effects of excess cadmium uptake in *Phaseolus vulgaris*. *Plant, Cell and Environ.* 5:263 (1982).

114. Stermer, B.A.; Hammerschmidt, R. Heat shock induces resistance to *Cladosporium cucumerinum* and enhances peroxidase activity in cucumbers. *Physiol. Plant Pathol.* 25:239 (1984).

115. Duell-Pfaff, N.; Wellmann, E. Involvement of phytochrome and a blue light photoreceptor in UV-B induced flavonoid synthesis in parsley (*Petroselinum hortense* Hoffm.) cell suspension cultures. *Planta* 156:213 (1982).

116. Zhakote, A.G.; Negru, F.V.; Gaugash, M.V. The effect of the duration and spectral composition of natural light on grapevine photosynthesis and frost resistance. *Fiziol. Biokhim.* 1979:55 (1979).

117. Lillie, R.D. A historical reaction from 1807: iron tannin. *J. Histochem. Cytochem.* 20:295 (1972).

118. Reichard, A. Zur Kenntnis der Gerbstoffgehaltes der Gerste, des Maltzes and ungehopfler Wurzen. *Zeitschrfit fur das gesamte Brauwesen* pp. 27, 229-235, 253-258, 271-275 (1904).

119. Johansen, D.A. Plant Microtechnique. McGraw-Hill Book Co., Inc., New York; 523 p. (1940).

120. Mace, M.E. Histochemical localization of phenols in healthy and diseased banana roots. *Physiol. Plant.* 16:915 (1963).

121. Rawlins, T.E.; Takahashi, W.N. Technics of Plant Histochemistry and Virology. National Press, California; 125 p. (1952).

122. Chiang, H.; Norris, D.M. Phenolic and tannin contents as related to anatomical parameters of soybean resistance to agromyzid bean flies. *J. Agric. Food Chem.* 31:726 (1983).

123. Reeve, R.M. Histochemical tests of polyphenols in plant tissues. *Stain Technol.* 26:91 (1951).

124. Hoepfener, W. Zwei neue Reaktionen fur Koffeesaure and Chlorogensaure. *Chem. Ztg.* 56:991.

125. Vinson, A.E. Fixing and staining tannin in plant tissues with nitrous ethers. *Bot. Gaz.* 49:222 (1910).

126. Reeve, R.M. Histochemical differentiation between tyrosine and chlorogenic acid in plant tissues. I. Nitrous acid reactions and methal chelation of nitrosotyrosine. *J. Histochem. Cytochem.* 16:191 (1968).

127. Felker, F.C.; Peterson, D.M.; Nelson, O.E. Development of tannin vacuoles in chalaza and seed coat of barley in relation to early chalazal necrosis in the seg1 mutant. *Planta* 161:540 (1984).

128. Stafford, H.A.; Lester, H.H.; Weider, R.M. Histochemical assay of proanthocyanidin heterogeneity in cell cultures. *Plant Sci.* 52:99 (1987).

129. Gopinathan, K.; Ananthakrishnan, T.N. Morphogenesis and histochemistry of some thrips (Thysanoptera: Insecta)-induced galls. *Proc. Indian Natn. Sci. Acad.* B51:413 (1985).

130. Tanrisever, A. New method for the histochemical analysis of condensed tannins and their physiological significance. *Ege Univ. Ziraat Fak. Derg.* 19:27 (1982).

131. Feucht, W.; Schmid, P.P.S. Selektiver histochemischer Nachweis von Flavanen (Catechinen) mit *p*-Dimethylaminozimaldehyd in Sprossen einiger Obstgeholze. *Gartenbauwiss.* 48:119 (1983).

132. Aastrup, S.; Outtrup, H. Location and characterization of barley flavonoids during grain filling. *Proc. Congr. Eur. Brew. Conv.* 20th, 667 (1985).

133. Gardner, R.O. Vanillin-hydrochloric acid as a histochemical test for tannin. *Stain Technol.* 50:315 (1975).

134. Sakai, W.S. Simple method for differential staining of paraffin embedded plant material using Toluidine Blue O. *Stain Technol.* 48:247 (1973).

135. Nielson, A.J.; Griffith, W.P. Tissue fixation and staining with osmium tetroxide: the role of phenolic compounds. *J. Histochem. Cytochem.* 26:138 (1978).

136. Parham, R.A.; Kaustinen, H.M. Differential staining of tannin in sections of epoxy-embedded plant cells. *Stain Technol.* 51:237 (1976).

137. Parham, R.A.; Kaustinen, H.M. On the site of tannin synthesis in plant cells. *Bot. Gaz.* 138:465 (1977).

138. Lynch, D.V.; Rivera, E.R. Ultrastructure of cells in the overwintering dormant shoot apex of *Rhododendron maximum* L. *Bot. Gaz.* 142:63 (1981).

139. Jalal, M.A.F.; Collins, H.A. Polyphenols of mature plant, seedling and tissue cultures of *Theobroma cacao*. *Phytochemistry* 16:1377 (1977).

140. Mikeladze, R.M.; Shalamberidze, S.M. Histochemical investigation of the kinetics of tannin and caffeine extraction from tea leaves. *Soobsch. Akad. Nauk Gruz. SSR* 46:645 (1967).

141. Shipilova, S.V.; Zaprometov, M.N. Phenylalanine ammonia-lyase and synthesis of catechins in the tea plant. *Sov. Plant Physiol.* (Engl. Transl.) 24:657 (1978).

142. Kefeli, V.I.; Turetskaya, R.K. Localization of natural phenolic growth inhibitors in cells of willow leaves. *Dokl. Akad. Nauk SSSR* 170:472 (1966).

143. Hubik, J.; Starhova, H.; Housova, B. Study of the drug *Radix rhei* of domestic origin. II. Localization of anthracene derivatives and tannins in the plant. *Cesk. Farm.* 28:234 (1979).

144. Kubo, I.; Hanke, F.J. A possible new role for the flavan-3-ol (-)-epicatechin in plants. *In*: Cody, V.; Middleton, E., Jr.; Harborne, J.B. (eds.) Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships. Alan R. Liss, Inc., New York; pp. 101-112 (1986).

145. Mahadeswaraswamy, V.P.; Theresa, Y.M.; Nayudamma, Y. Histochemical studies on the distribution of tannins in Indian and African babul barks (*Acacia nilotica* ssp. *indica* and *A. nilotica* ssp. *nilotica*). *Leather Sci.* 27:157 (1980).

CONDENSED TANNINS IN SOUTHERN PINES AND THEIR INTERACTIONS WITH THE ECOSYSTEM

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ABSTRACT

Condensed tannins extracted from the foliage of selected southern pine species were found to be predominately prodelphinidin polymers of 14 to 18 flavanoid units, whereas, tannins from phloem were mostly procyanidins of 8 to 11 units. These chemical characteristics indicate that the tannins from the different plant parts may have different biological activities in the ecosystem. Slash pine (*Pinus elliottii* Engelmann var. *elliottii*) foliage contained an average of 49 percent more tannin than loblolly (*Pinus taeda* L.) or longleaf (*Pinus palustris* Miller) pine foliage. Tannin concentration in the phloem did not seem to be related to site factors that control the rate of pine growth, but tannin concentration in the foliage increased by 30 percent as the site quality decreased from good to poor. Application of phosphorus fertilizer decreased the concentration of tannin in the foliage of loblolly pine from 110 to 85 g/kg. Concentrations of tannins in the foliage increased in the spring of the first year of the lifespan of needles, followed by a period of stability, and then another increase in concentration the second spring. In the months prior to senescence, tannins were lost from the foliage, either by leaching or by chemical transformations. The importance of tannins in nutrient cycling suggests that research on the fate of tannins released into the ecosystem needs to be intensified.

INTRODUCTION

The southern pine ecosystem is characterized by highly weathered, nutrient deficient soils. As in other nutrient-poor ecosystems,[1] much of the nutrient supply is derived from litter decomposition. For example, in a 20-year-old loblolly pine plantation, litter decomposition is the source of 65 percent of the nitrogen and 57 percent of the phosphorus taken up by pines.[2] Southern pines produce condensed tannins as secondary metabolites that are attributed with the roles of slowing litter decomposition and repelling foragers, thereby increasing retention of nutrients in the system. Condensed tannins have been shown to reduce decomposition rates[3] and have been associated with differential rates of decomposition.[4] On nutrient-poor sites in Africa, where the cost of replacing nutrients lost to herbivores is high, increased concentrations of condensed tannins were found to reduce the amount of feeding by monkeys.[5] Condensed tannins have also been reported to affect the soil chemistry including rates of nitrification,[6,7] aluminum and iron mobility,[8] and cation exchange capacity.[9]

Although the importance of tannins to ecosystems has been recognized and studied for many years, recent development of tannin purification methods[10] and advances in instrumentation for characterizing chemistry such as ^{13}C-NMR[11,12] allow new insights into the interaction of tannins with the environment. Also, the rapidly increasing intensive management of the southern pine resource requires an understanding of how tannins interact with other components of the environment. For example, fertilization may decrease tannin concentrations,[13] which could increase the susceptibility to insect attack or the rate of nutrient leaching.

This chapter examines the tannins of the southern pines, the quantities of tannin present under different management and environmental regimes, and the fate of the tannins after entering the ecosystem as litterfall or leachates. Also reviewed is the literature on the effect that tannins released from the pines have on the ecosystem in order to gain insight into the type of research needed to increase the understanding of this relationship.

METHODS

The materials for the tannin analyses reported on here were collected from pine plantation study plots established over a period of 30 years for various research objectives. Initially, large samples of foliage and phloem were collected for purification of sufficient tannin to be used in characterization of the chemistry and as standards for quantification in subsequent analyses. Smaller samples were collected to elucidate the effects of site or treatment factors on tannin concentrations.

Field Plots

Samples for tannin characterization and for comparison of the effect of site and species on tannin concentrations were collected from plots that had been established in 1955 as a species comparison trial.[14] At one of the locations, large samples (>1 kg) of foliage and phloem were collected from a tree 30 cm d.b.h. (diameter at breast height) of each of four species (slash, loblolly, longleaf, and shortleaf). At this location and at two other locations chosen to give a range in site index,

smaller needle and phloem samples were collected from 10 trees each of slash, loblolly, and longleaf. Differences between species, location, and the interaction were tested by analysis of variance. When the F-test indicated significance, means were separated by Duncan's multiple range test since insufficient information was available to design preplanned comparisons.

Phloem samples were also collected from 15 stands in Texas, Louisiana, and Mississippi ranging in age from 37 to 117 years. Two phloem samples (5 x 5 cm) were collected at d.b.h. on opposite sides of each of five trees in each stand. The ring width of the previous seasons growth was measured on wood cores collected at the same time. Linear regression was used to measure the relationship between growth rate and tannin concentration.

To follow the concentration of tannin in needles with time, samples were collected from loblolly pine in a fertilizer study that had 12 plots consisting of four levels of phosphorus fertilizer and three replicates.[15] In December after the third growing season, a tree limb was tagged in each plot. Needle samples were collected monthly from the previous year's growth and from the subsequent first flush of new growth. Regression was used to fit a fifth power polynomial relating tannin concentration to month, phosphorus rate, and the time by phosphorus interaction.

To relate the effect of phosphorus fertilization to tannin concentration, samples were collected from two sites that had received 11 rates of phosphorus and 11 rates of nitrogen fertilizer applied in as continuously increasing function.[16] Regression was used to compare the tannin concentration to the amount of phosphorus and nitrogen applied.

Sample Collection and Preparation

Trees were felled for the initial sampling for characterization of the tannins. Smaller samples were also collected for the quantification work. Unless otherwise noted, the samples were collected in the winter months when the trees were dormant. Needle samples were collected from the top third of the crown and placed on ice. Phloem was collected from the main stem at 1.5 m above the ground and placed on dry-ice for transportation to the laboratory. All samples were kept frozen until they were freeze-dried and ground, and they remained in a cold-room until analyzed.

Purification and Characterization of Tannins

The foliage and phloem tissues, collected from the same trees as described above, were extracted at ambient temperature with acetone-water (1:1; v/v). After evaporation of the acetone, the aqueous solutions were repeatedly extracted with chloroform and then ethyl acetate. The ethyl acetate-soluble fraction was evaporated to dryness on a rotary evaporator, and the water-soluble fraction was freeze-dried.

The ethyl acetate-soluble fractions were examined by two-dimensional thin layer chromatography (2D-TLC, Baker-flex 1F, developed in the first dimension with t-BuOH-acetic acid-water; 3:1:1; v/v/v and in the second dimension with 6 percent acetic acid). Plates were sprayed with vanillin-HCl, diazotized sulfanilic acid, or ferric chloride-potassium ferricyanide. These chromatograms indicated the same

composition for the phloem samples as has been reported previously[17–19] except that shortleaf pine phloem contained more epicatechin than the other samples.

The 2D-TLC of foliage extracts showed that they differed in composition from the phloem samples by having a strong spot at the same Rf value as gallocatechin. A sample of the ethyl acetate-soluble fraction from the loblolly pine foliage was separated by chromatography on Sephadex LH-20 by elution with ethanol, and the two predominant flavan-3-ols, catechin and gallocatechin, were isolated in relative yields of 2.9 to 1. These compounds were acetylated and their composition verified by ^{1}H-NMR.

The water-soluble fractions remaining after extraction with ethyl acetate were purified by chromatography on Sephadex LH-20 by elution with methanol-water (1:1; v/v) followed by acetone-water (1:1; v/v).[10] The acetone-water elutate was collected, the acetone removed, and the product freeze-dried. Yields of purified tannins were calculated on the basis of the dry weight of the unextracted tissue.

For each tannin fraction, ^{13}C-NMR spectra were recorded at 20 MHz in d_6-acetone-H_2O (1:1; v/v), and the spectra were interpreted from results reported earlier.[11,12] Anthocyanidins were produced from each sample by refluxing 15 mg of tannin in 10 mL of 2-BuOH containing 5 percent of concentrated HCl for 1 hour. Aliquots (1 mL) of the products were applied to Whatmann No. 3 papers, which were developed in Forestal (HCl-HOAC-H_2O; 3:30:10; v/v/v) solvent. Bands with cyanidin chloride and delphinidin chloride and a blank were cut from the chromatographs and extracted with 20 mL of 1 percent HCl in ethanol. The absorbance at 547 nm and 557 nm was measured for the cyanidin and delphinidin chlorides, respectively.

A 10 mg sample of each tannin was combined with 10 mg of phloroglucinol and dissolved in 2 mL of ethanol containing 3 drops of HOAc. The vials were sealed and heated in an oven at 105 °C for 24 hours. Aliquots of the reaction products were examined by 2D-TLC as described above. Interpretation of the chromatograms was made by reference to authentic compounds that had been made previously except for epigallocatechin-(4β → 2)-phloroglucinol. Assignment of that compound was made by reference to data by Foo and Porter.[20] A 1 g sample of the tannin from loblolly pine foliage was reacted under similar conditions and the product separated by chromatography on Sephadex LH-20. Fractions containing the epicatechin-(4β → 2)- and epigallocatechin-(4β → 2)-phloroglucinol were collected and acetylated. Their identity was verified by ^{1}H-NMR spectral analysis.

In addition, 5 mg of each of the tannin isolates were dissolved in 1 mL of ethanol, two drops of toluene-α-thiol, and two drops of acetic acid. The mixture was heated at 105 °C overnight in sealed vials and the reaction products analyzed by 2D-TLC. The ratios of products were estimated by visual comparison of relative spot intensity of the thiol and phloroglucinol adducts.

To obtain estimates of the molecular weight of each tannin isolate, approximately 200 mg of each sample was acetylated with acetic anhydride in pyridine for 2 days at ambient temperature. The acetate was extracted into dichloromethane and precipitated from *n*-hexane. The number average molecular weight was determined by vapor pressure osmometry in chloroform. Both number- and weight-average

molecular weights were estimated by gel permeation chromatography on a series of Microstyragel columns as described by Williams et al.[21]

Quantification of Condensed Tannins

Tannin was extracted from the samples by shaking 0.15 g of sample with 30 mL of methanol at 20 °C for 24 hours. After filtering, a modification of the vanillin method[22,23] was used to measure the amount of tannin present. The final solution was 2 percent vanillin and 12.5 percent concentrated HCl in methanol. The purified condensed tannin sample collected from loblolly pine phloem described above was used as a standard so the results could be expressed as concentration by mass of dry plant material. As a check of the vanillin method, the anthocyanidin method was used on a variety of samples. The two methods gave comparable results.

CHEMICAL CHARACTERISTICS OF TANNINS

Before tannin interactions with the ecosystem can be studied effectively, the chemical structure of the tannins should be known because the types of reactions involved depend upon that structure and the structure of the other ecosytem constituents.[24] Also, the chemistry of the tannins will affect the method most appropriate for their extraction and quantification.

The tannin chemistry of southern pine bark has been studied extensively because of its availability as a waste product. The oligomeric procyanidins in the phloem of loblolly and longleaf pine have been described previously.[17–19] The dimers epicatechin-(4β → 8)-catechin, epicatechin-(4β → 6)-catechin and catechin-(4α → 8)-catechin, and the trimers epicatechin-4β → 8)-epicatechin-(4β → 8)-catechin, epicatechin-(4β → 8)-epicatechin-(4β → 6)-catechin, and epicatechin-(4β → 6)-epicatechin-(4β → 8)-catechin have been isolated and their structures proven.[18] Studies of the oligomeric proanthocyanidins in the foliage of the southern pines have not yet been done. However, the methods used to study bark tannins are easily adapted to the other plant parts that have not been studied as extensively. These methods were used to extract and characterize the tannins from the foliage and phloem of four southern pine species.

Tannins extracted from the phloem and foliage of the four pine species are quite distinct, whereas, the differences between species is of lesser degree (Tables 1 and 2). Acid-catalyzed cleavage of the foliage tannin in the presence of either phenylmethane thiol or phloroglucinol showed that the four species examined are similar, with the chain extender units being made up of approximately 65 percent epigallocatechin, 20 percent epicatechin, and 13 percent gallotannin units (Table 1). The terminal units are made up of about 52 percent catechin, 43 percent gallocatechin, and 5 percent epicatechin units. Phloem tannin is made up predominately of 2,3-*cis* procyanidin chain extender units with (+)-catechin terminal units (Table 1). Lebreton et al[25] also reported that the tannins from the foliage of several pine species, including loblolly pine, are made up predominately of prodelphinidins. Prodelphinidins have been reported to be twice as astringent as procyanidins,[26] and when the two occur in the same plant part, the increase in astringency is proportional to the prodelphinidin:procyanidin ratio.[27]

Table 1. Constitution of Foliage and Phloem Tannins of Four Southern Pines.

Species	Terminal Units				Extender Units				Anthocyanidins	
	C[a]	EC	GC	EGC	C	EC	GC	EGC	Cyan.	Del.
					Foliage					
Loblolly	50	3	47	0	0	15	10	75	17	83
Longleaf	50	5	45	0	0	30	10	60	30	70
Shortleaf	50	8	42	0	0	25	15	60	16	84
Slash	55	5	40	0	0	20	15	65	22	78
					Phloem					
Loblolly	97	3	0	0	0	100	0	0	100	0
Longleaf	97	3	0	0	2	98	0	0	96	4
Shortleaf	75	25	0	0	5	95	0	0	94	6
Slash	97	3	0	0	2	98	0	0	100	0

[a]C = catechin
EC = epicatechin
GC = gallocatechin
ECG = epigallocatechin
Cyan. = cyanidin
Del. = delphinidin

The yield of purified condensed tannins is not an accurate measurement of the amount of tannin present, but the threefold difference between the amount extracted from the phloem and foliage indicates that the foliage contains much less tannin (Table 2). The amounts extracted agree with other reports in the literature. In a survey of 140 species, *Pinus taeda* foliage was found to contain 6.3 percent proanthocyanidin or condensed tannins.[25] The proanthocyanidin content of bark from 20 species was reported by Samejima and Yoshimoto.[28] The monomeric flavan-3-ols ranged from trace to 3.6 percent of the inner barks and consisted mainly

Table 2. Yield and Molecular Weight Distributions of Foliage and Phloem Tannins of Four Southern Pine Species.

Species	Tannin Yield g/kg	Molecular Weight			
		VPO	GPC		
		Mn	Mn	Mw	Dispersivity
		Foliage			
Loblolly	26	3500	4400	12400	3.1
Longleaf	24	4700	5400	13800	2.7
Shortleaf	13	3700	5400	14400	3.2
Slash	13	3600	4100	9800	2.6
		Phloem			
Loblolly	68	3700	3100	6200	1.8
Longleaf	48	3200	3300	7400	2.3
Shortleaf	56	3400	2400	4800	1.7
Slash	100	3300	2900	6800	2.2

of catechin and epicatechin. Dimeric proanthocyanidin concentrations were less than 2 percent except in two species, whereas, polymerized proanthocyanidins comprised 50 to 80 percent of the total proanthocyanidins. One difficulty with reports on contents and chemistry of tannins in bark is that the material analyzed is not well defined, even though the location and age of the bark have been reported to affect the tannin.[29] The concentration of acetone-water soluble tannins in the phloem of *Pinus palustris* decreased from 6.4 percent in the phloem to 1.1 percent in the new rhytidome and to 0.3 percent in the old bark. However, the outer bark of southern pine contains approximately 40 percent by weight of total polyflavanoid based polymers.[30]

The weight-average molecular weight of the tannin extracted from the foliage ranges from 4,100 to 5,400 indicating that the polymers consist of 14-18 units (Table 2). The weight-average molecular weight of the tannin from the phloem is lower with the chains containing 8-11 units. The molecular weight distributions are broad with dispersivities ranging from 1.8 to 2.3 for phloem and 2.6 to 3.2 for foliage tannin. The molecular weight is important as it is an indicator of the reactivity of tannins with enzymes[31] and proteins.[32] Maximum binding efficiency evidently occurs when the molecular weight is in the range of 2,000 for condensed tannins.[33] Williams[21] found the number-average molecular weight of condensed tannins extracted from loblolly bark was about 1,560 with a low dispersivity of 1.26. Molecular weight was found to be very species-specific and varied little with season or tissue maturity. However, Karchesy and Hemingway[17] found the molecular weight of condensed tannins from the phloem of loblolly pine affected the solubility. The ethyl acetate soluble material had the lowest molecular weight (1,200) but it made up only 0.2 percent of the phloem. The hot-water soluble material had a number-average molecular weight of 2,100 and the number-average molecular weight of the acetone-water soluble material was 2,900. The last two fractions made up 6.3 and 3.7 percent of the phloem, respectively.

Differences in chemistry of the tannins originating from the phloem and needles are of possible importance to tannin interactions with soil microorganisms. Tannins dominated by prodelphidins from the foliage probably have greater capability to precipitate protein and inactivate enzymes. Thus, foliage tannin might resist degradation by fungi better than the bark tannins. The differences in structure may also affect the relative reactivity of the tannins with metals, clays, and other soil components.

EFFECTS OF SITE QUALITY ON TANNINS

The rate of tree growth varies within a climatic area because of differences in site, with soil factors usually dominating once the trees shade out other vegetation. Three sites where loblolly, longleaf, and slash pine had been planted in the same year were sampled to find whether site differences affected the amount of tannin in the phloem and foliage. The height of loblolly pine (dominant and codominant trees) at age 25 years was used to differentiate the site quality as poor, medium, or good.

The amount of tannin in the phloem averaged 146 g/kg or about twice the 72 g/kg in the needles (Table 3). Bartlett's test showed the variances of the tannin in

phloem and needles to differ, thus separate analyses of variance were used for the two parts to test the effect of species and site on the tannin concentration. Both analyses of variance showed no significant interaction between species and site. The phloem and foliar samples were collected and analyzed from individual trees to find the amount of variability caused by genetics and other uncontrolled variation. About 47 percent of the variation between both phloem and foliar samples was accounted for by the site and species variation. The coefficient of variation was 20 percent for the phloem and 26 percent for the foliage. Thus, phloem samples from 15 trees per experimental unit are needed to keep the 95 percent confidence limits within 20 percent of the mean. For foliar samples, 28 trees are required per experimental unit to obtain the same confidence interval. For comparison, the coefficient of variations for nitrogen and phosphorus concentrations in the foliage are 16 and 21 percent, respectively.

In the foliage, the only species difference in tannin concentration was a significantly higher level in slash where the concentration of 91 g/kg was 49 percent more than the average of longleaf and loblolly. Averaged across the species, the foliar tannin concentration increased significantly as the site quality decreased. The increase in tannin concentration was approximately proportional to the decrease in the site quality as expressed by the height of the loblolly pine. While

Table 3. Relationship of Southern Pine Site Quality to the Concentration of Condensed Tannin in Phloem and Foliage and of the Nitrogen and Phosphorus in Foliage.

Site Quality	Height at 25 years	Tannin in		Foliage	
		Phloem	Foliage	N	P
	m	g/kg			
		Loblolly			
Poor	13	140	74	9.7	0.67
Medium	18	160	62	11.6	0.82
Good	22	119	46	13.9	1.01
		Longleaf			
Poor	14	129	69	9.4	0.57
Medium	18	165	63	8.3	0.58
Good	19	103	55	10.7	0.81
		Slash			
Poor	16	171	107	8.2	0.54
Medium	19	186	92	9.0	0.58
Good	21	142	74	9.9	0.71
LSD(p=0.05)		15	10	0.8	0.08

factors affecting soil water are associated with differences in site quality,[14] increasing concentrations of both nitrogen and phosphorus in the foliage as tree heights increase indicate that nutrient deficiency is probably a concurrent factor limiting pine tree height on these sites by age 25.

The concentration of tannin in the phloem from slash pine averaged across the three sites was 166 g/kg, significantly more than found in the other two species. The concentration in loblolly was an insignificant 8 g/kg more than the 132 g/kg found in longleaf. The tannin concentration in the phloem was significantly different among all three sites, but the medium site had the highest concentration. No explanation can be given for this lack of consistency.

Although the site quality does not appear to affect the tannin concentration in the phloem, other factors including the basal area and age of the stand influence the diameter growth rate. To evaluate the possibility that these factors also affect the tannin production, the diameter growth rate and tannin concentration in the phloem were measured in 15 stands of loblolly pine representing a range in basal area and age. In these stands, no relationship existed between tannin concentration and diameter growth rate (Figure 1). Thus, the tannin concentration in the phloem does not seem to be affected by factors that control growth rates of the pines. The physiological factors that determine tannin production in the phloem may be independent of those that determine rate of wood production. Or the variables that do control both were not measured.

The effect of site on condensed tannins or the broader grouping of polyphenols has been reported previously and has been used to develop ecological hypotheses on food preference by mammals[5] and the distribution of litter decomposing species.[34] However, as in our site comparison, the factor or factors affecting the tannin production cannot be determined with certainty.

Many environmental factors that could affect the amount of tannins in plants have been studied including nutrient status, light intensity, water deficit, and temperature. In the first paper of a series, Coulson et al[13] speculated that the base status of the soil, meaning the availability of nutrients such as magnesium and calcium, determined the yield of polyphenols in a beech forest. Sites on soils with a low pH were expected to have higher polyphenol concentrations in the leaves. Later, deficiencies of nitrogen and phosphorus were found to be more important in controlling polyphenol production, whereas, a calcium deficiency seemed to decrease polyphenol contents.[35] In Sarawak, Malaysia, polyphenol concentrations in litterfall varied from 2.49 mg per 100 g tannic acid equivalents on a heathy site to 1.19 mg per 100 g on an alluvial site.[36] The site with the lowest concentration of nitrogen in the foliage was also the one with the highest concentration of polyphenols.[36,37] Contrary results were reported in a comparison of Douglas-fir stands where the stand with the highest foliar nitrogen also had significantly higher astringency.[38] However, in the Douglas-fir comparison, the light intensity within the canopy also varied as a function of the amount of canopy closure.[38] The stand with an open canopy in which the needles would receive more sunlight had the greater astringency. The reasons for the effect of light intensity have not been explained, but light-induced changes can be significant. Leaves receiving direct sunlight were found to contain 60 percent more condensed tannins than shade leaves.[39] Fronds of the fern

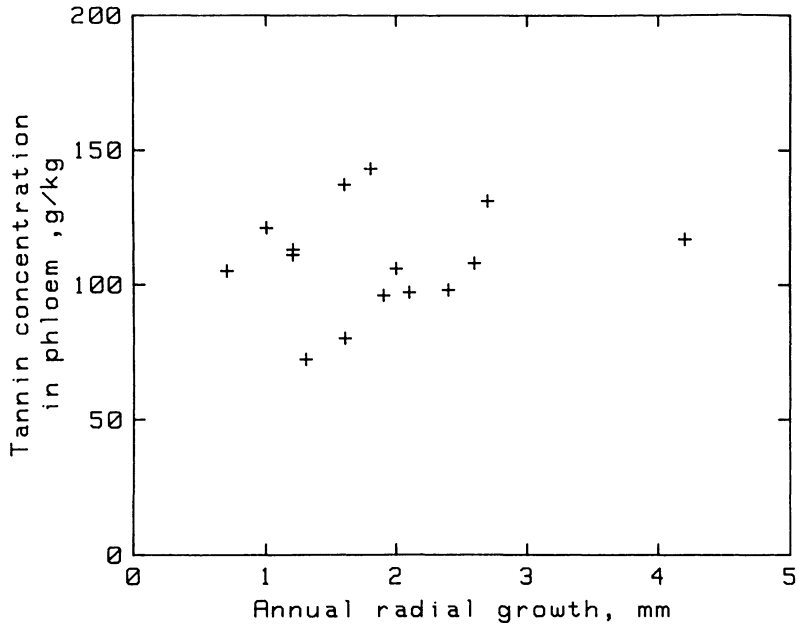

Figure 1. *The relationship between the annual stem growth of loblolly pine and the concentration of tannins in the phloem.*

Pteridium aquilinum growing in the open sunlight contained about 150 g/kg of condensed tannins in August compared to 50 g/kg in shaded fronds.[40] Also, the light intensity and nutrient availability apparently interact in affecting tannin production. In an artificial environment, high light intensity increased the relative tannin concentration in willow leaves only if nutrients were in less than optimal supply.[41,42] In some rainforest species, increasing light intensity from 2 to 25 percent of sunlight doubled the polyphenol content.[43] Of the four rainforest species studied, only one showed decreased polyphenol contents in response to additions of nitrogen and phosphorus, and only at higher light intensities. In field-grown lespedesa, the amount of tannin increased as the temperature increased and rainfall decreased, but this response could not be isolated from the normal seasonal fluctuations. In a controlled environment experiment, higher temperatures did increase the tannin content of lespedesa compared to a lower temperature regime.[45] However, the amount of the increase, which ranged from 24 to 78 percent, depended on the cultivar used and was greater in the leaves than in the stems. The effect of water stress on tannin production is perplexing because tannins are secondary compounds that are expected to increase when growth is reduced without a corresponding reduction in photosynthesis.[44] Other secondary compounds such as resins in pines have been observed to increase when the plant is under water stress.[46] However, Tempel[47] concluded that it is unlikely that a relationship exists between tannin production and water stress. In cotton, no relationship between water deficits and tannin content of leaves could be found.[48]

EFFECTS OF NUTRIENT SUPPLY ON TANNINS

In a study where loblolly pine had been treated with phosphorus fertilizer at planting, the concentration of condensed tannin in the foliage at age 3 decreased as the amount of phosphorus fertilizer increased (Figure 2). The tannin concentration decreased from 110 g/kg when only 5 kg/ha of phosphorus had been applied to a minimum of 85 g/kg in the pines receiving more than about 200 kg/ha of phosphorus. The response was similar for the two sites included in the study. Interestingly, the response in height growth to the fertilizer followed a similar but opposite pattern (Figure 2), except that height response peaked at a slightly lower rate of phosphorus fertilizer. The best fit regression equation between fertilizer applied and height at age 3 accounted for 76 percent of the variation, whereas, the best relationship between phosphorus applied and tannin concentration accounted for only 62 percent of the variation. Thus, the tannin concentration seems to be an indicator of the growth potential when phosphorus is the limiting factor.

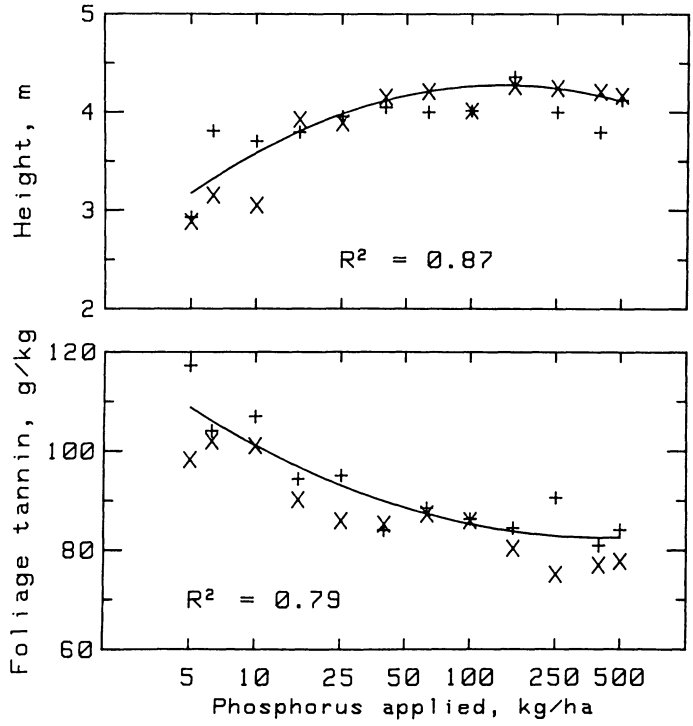

Figure 2. *The effect of the rate of fertilizer phosphorus on the height and tannin concentration in the foliage of 3-year-old loblolly pines at two locations (+ and X).*

Nitrogen had also been applied to the site as a separate treatment at rates ranging from 10 to 1,000 kg/ha. The nitrogen had no significant effect on the tannin concentration in the foliage of these pines. The effect of the nitrogen on the height of the pines was negative when the rate of nitrogen exceeded 100 kg/ha.[16] Because the height response was negative, this was not be a valid test of the effect of nitrogen on tannin concentrations in loblolly pine foliage.

There are many reports that tannin production is affected by nutrient supply, but in most cases the nutrient supply was not a controlled variable; concentrations of nutrients in the soil or plant were related to tannin concentration.[4,5,13,43,49,50] In a few studies, the tannins were measured in plants grown under conditions of controlled nutrient supply. In a pot experiment where all nutrients but one were supplied, nitrogen and phosphorus deficiencies in *Pinus sylvestris* increased leaf polyphenols.[35] Potassium and calcium deficiencies seemed to decrease polyphenol contents. In *Lespedeza sericea*, potassium applications decreased tannin concentrations only when potassium deficiencies severely limited plant growth.[51] Applications of phosphorus and sulfur to *Lotus pedunculatus* reduced tannin concentrations by 50 percent compared to unfertilized plants.[52] Of four rainforest species studied, additions of nitrogen and phosphorus decreased polyphenol in only one.[43] In barley, the relationship between added nitrogen showed no clear trend.[53] Although increased nitrogen reduced tannin in one variety, the opposite response was noted in other varieties. Negative correlations have been reported between the manganese and phenolic contents of tea[54] and wheat[55] plants. In both instances, additions of nitrogen decreased tannin contents. A manganese deficiency may disrupt phenylalanine and tyrosine production, which are prerequisites for phenol production.[55] High nitrogen levels may drain carbon from the Krebs cycle and increase production of other amino acids. However, the reason that deficiencies of nutrients such as phosphorus can increase tannin concentrations is still obscure.

SEASONAL EFFECTS ON TANNINS

The concentration of tannin in loblolly pine foliage through a life cycle can be modeled with a fifth power polynomial (Figure 3). In unfertilized pines (Figure 3A), the tannin concentration increased rapidly after needle initiation. The concentration leveled off by August of the first year and remained stable through the dormant season. As growth resumed in the second year, tannin concentration again increased, maximizing in July. The tannin measured in the needles then declined until senescence. The data points, which represent samples taken monthly from the same limbs, show the variation was consistent throughout the season.

The tannin in pines fertilized with phosphorus was not statistically different from that in unfertilized pines in the first year (Figure 3B). In the old needles, the tannin concentration in fertilized pine increased less rapidly with the change proportional to the amount of phosphorus applied. The greatest effect of phosphorus on tannin concentration occurred in July when tannin concentration maximized. The phosphorus treatment had little effect on the decline of measured tannin as the needles senesced.

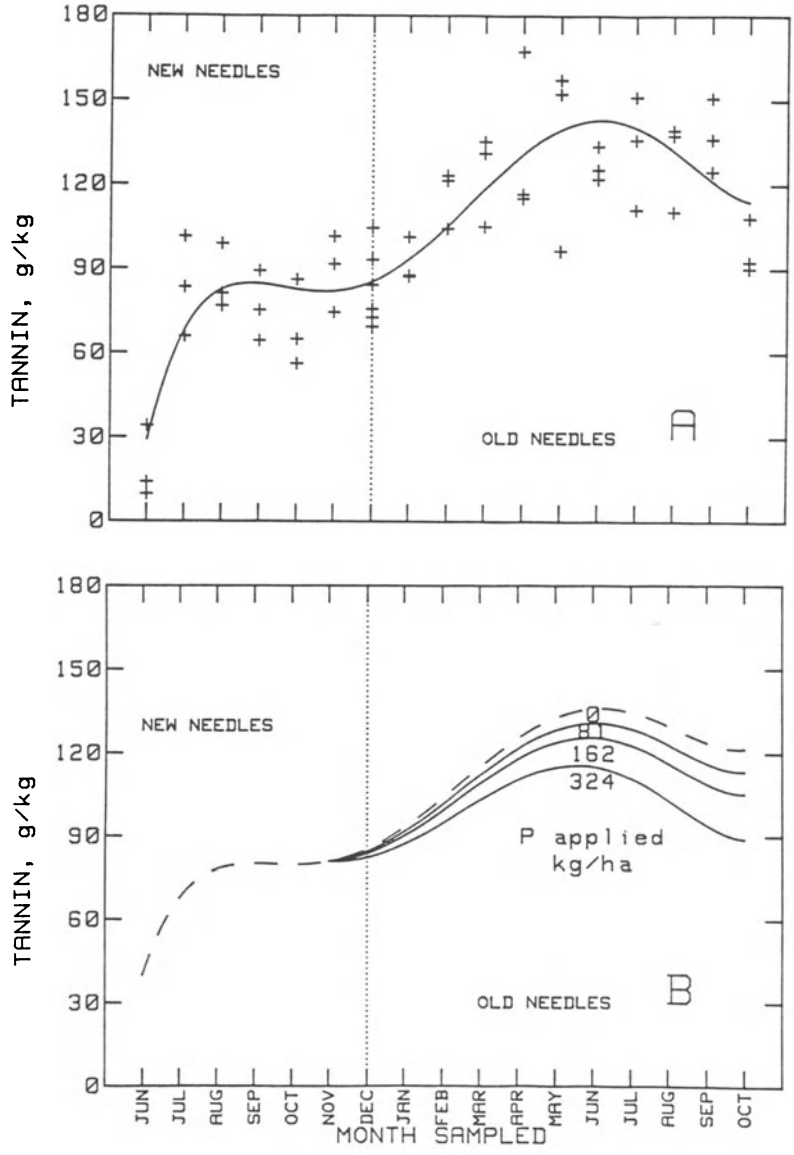

Figure 3. *The change of tannin concentration in loblolly pine foliage with time without fertilizer (A) and the effect of phosphorous fertilizer on seasonal changes in tannin content (B).*

The change in measurable tannin concentration with season in loblolly pine follows a pattern similar to that in ferns.[40] In both cases, the tannin increased rapidly as new leaf material was formed and declined prior to senescence. In primitive cotton, the tannin concentration rises rapidly in leaves formed in the spring and then stabilizes until early fall.[56] As we repeatedly sampled the same limbs to reduce variability, the increase in tannin concentration could be caused by a defensive response to mechanical defoliation.[57] However, the concentrations in both old and new needles sampled in December were the same. As the December sampling represented the first defoliation for old needles and the seventh sampling of the limb for the new needles, the defoliation probably had no effect on tannin concentrations.

The decline in tannin concentration as the needles approach senescence could be due to leaching of tannins from the needles,[8] increased polymerization making the tannins more difficult to extract,[58] the degradation of the tannins,[59] or the deposition of the tannins into vacuoles.[60] The relative importance of these processes is the subject of the next section.

RELEASE OF TANNINS FROM PINES AND EFFECTS ON ENVIRONMENT

Tannins occur in three major pools in the pine ecosystem: the living pines themselves, the litter layer, and the soil (Figure 4). In general, tannins move from the pines to the litter layer, and then to the soil by physical movement including leaching and litterfall. Degradation of the tannins into other forms occurs in all three of the pools. While in each of the respective pools, the tannins can have various influences on the other components that comprise the pools.

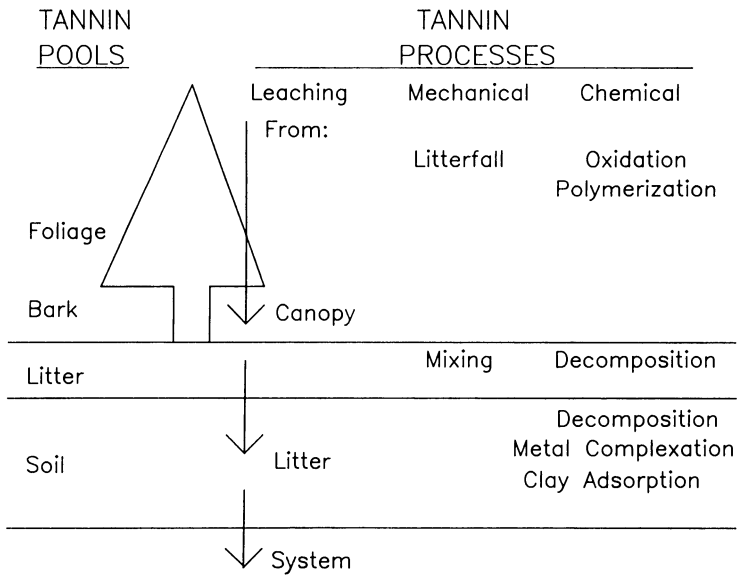

Figure 4. *Tannin processes and pathways in the southern pine ecosystem.*

Release from Pines

While the plant part containing tannin is vigorous, the tannins seem to be stable because of antioxidants[61] or because the tannin is isolated in vacuoles.[62] However, once senescence is initiated, the tannins begin a complicated transformation of oxidation, polymerization, and degradation. As shown in Figure 3, approximately 20 percent of the tannins in pine foliage in July have disappeared by the time of litterfall. The tannins may be present but are not as easily extracted because of increased polymerization, or they may be in a form not detected by the analytical method. The processes of degradation of the tannins have been studied in some detail in bark and to a lesser degree in foliage, but the chemistry is still poorly understood.[29] A polymerization to a higher molecular weight would make the tannins more difficult to extract[17,27] and is the process thought to occur in ferns.[40] However, the chemistry involved in the further polymerization of previously stable chains has not been elucidated. Cleavage of the polymer into its anthocyanidin components is another possibility,[63] but in our laboratory, we have not been able to extract anthocyanidin with acidified methanol without breaking down existing polymers as well. During senescence, tannins released from the organelles where they were stored may combine with proteins, reducing the extractability of both the tannins and proteins.[64] Tannin leaching from living and dead plant material is the most direct pathway to other parts of the ecosystem such as the soil, but because of rapid oxidation[65] in environments such as throughfall containers, there is no good documentation of the amount of tannin leached from pine canopies. Polyphenols, probably including tannins, made up a large share of the organic compounds found in the throughfall of lodgepole pine.[66] Under simulated leaching conditions, foliage of longleaf pine released the equivalent of 1 kg/ha of polyphenols annually,[8] but this is probably a severe underestimate. Leaching of condensed tannins from bark by stem flow and from roots by soil water movement probably occurs but has not been studied.

Tannins in Litter

Litterfall accounts for the bulk of tannin released from the pines into the ecosystem. The annual amount of litterfall in a typical loblolly pine stand is about 7,180 kg/ha.[67] If the average tannin concentration in the foliage at abscission is 100 g/kg (Figure 3), then the annual input of tannin within litterfall is about 718 kg/ha. We measured only foliage exposed to sunlight, but because the litter contains foliage formed under shaded conditions as well, its tannin concentration should be less. The average tannin concentration of fresh loblolly litter collected from 10 stands in Louisiana was 22 g/kg,[68] which would be equivalent to about 158 kg/ha annual input of tannins. Leaching, decomposition, and degradation processes are rapid because measureable quantities of tannins are not detected after 1 year.[69,70] Leaching of tannins from litter has been studied extensively but the reports are contradictory. The amounts of water soluble polyphenols in *Pinus sylvestris* L. litter were less than 2 g/kg tannic acid equivalents for three sampling periods during the initial stages of decomposition.[71] The indications were that the needles had a low permeability for water and retained most of the phenolics. During snowmelt periods, the leachate

of lodgepole pine contained 16 kg/ha of polyphenols or about 50 percent of the polyphenols lost annually.[69] The early snowmelt periods contained three times as much polyphenolic materials compared to later periods, indicating that water can leach considerable amounts of polyphenols if it stays in contact with the litter for any length of time.

Tannins are known to act as inhibitors of decomposition by reducing palatability, inactivating enzymes, and precipitating proteins. Soil animals contribute to the decomposition process by their physical action on the litter. The presence of polyphenols affects taste and palatability of the litter to soil animals.[72] Earthworms attacked only 44 percent of the pine needles containing 18 g/kg of condensed tannins but fed on 74 percent of weathered needles that had no condensed tannins remaining.[73] Although many microbes are inhibited by condensed tannins, a strain of *Penicillium adametzi* Zaleski was able to utilize low-molecular-weight condensed tannins as its sole carbon source.[74] However, even with this fungus, the tannins bound to the mycelial filaments reduced the amount of substrate broken down.

The ability of tannins to precipitate proteins plays a major part in their ability to retard decomposition, since tannins react with proteins to form resistant complexes.[75] The precipitation of protein by condensed tannins is rapid,[76] but soluble tannin-protein complexes may form in the presence of an excess of protein. The ability of tannin to precipitate protein seems to depend on the structure of both compounds.[77] Tannin protein complexes are difficult to extract and are probably insoluble in acid. The formation of an insoluble tannin-protein complex may explain the increase in Klason lignin that occurs as decomposition initiates,[78,79] especially since the nitrogen content of the lignin increases at the same time. Definitive work needs to be done using methods for measuring both tannins and proteins[80,81] to explain the importance or chemistry of this reaction.

Tannins and tannin degradation products affect the soil chemistry by complexing metals and influencing the exchange capacity. The polyphenol content in soils under pines has been found to be low,[82] but polyphenols last longer in acid soil compared to soils with neutral pH.[13] The amount of aluminum removed from soil by litter leachate was highly correlated to the polyphenolic content of the leachate.[83] Leachate from longleaf pine was effective in mobilizing aluminum, whereas, leachate from oaks was more effective on iron,[8] partially explaining the different profile development under pines and hardwoods.[84] The soil mineralogy may also affect the amount of polyphenols leached through the soil, since allophanic soils absorb more polyphenols, contributing to humus formation.[85,86] Humic substances formed from tannin-rich plant residues were found to have a higher exchange capacity compared to soil organic matter formed from plants low in tannins.[9]

As in litter, condensed tannins have an important inhibitory effect on soil biology, reducing decomposition and affecting different parts of the nitrogen cycle. When condensed tannins were mixed with tannin-free plant material, decomposition was reduced by 50 percent.[87] The condensed tannins markedly reduced the decomposition of hemicellulose and cellulose.[88] The mechanism by which tannins reduce decomposition could be incrustation of plant materials with tannins or

inactivation of microbial enzymes.[87] The supply of plant available phosphorus mineralized from spruce litter was related to the concentrations of phosphorus, lignin, and tannin in the forest floor.[1] Additions of tannins to proteins reduced the rate of decomposition and the amount of ammonification.[3,89]

As almost all of the nitrogen utilized by plants comes from decomposition of nitrogen-containing compounds in the litter and soil, tannins released by the plants can have a direct influence on the amount of nitrogen available. Besides the effect of ammonification, tannins prevent the conversion of urea to ammonia by reducing the activity of urease.[87,90] The effects of condensed tannins on nitrification are not as clear. Extracts from the bark, needles, and soil of ponderosa pine were toxic to *Nitrosomonas* bacteria, reducing the conversion of ammonia to nitrate by more than 68 percent.[6] Extracts of the balsam fir forest floor also reduced the amount of nitrification in the soil.[7] Toxins did not inhibit nitrification in a rainforest environment, but the condensed tannin concentrations were probably low because of the high soil fertility and the types of plants in the community.[91] The additions of condensed tannins from quebracho did not inhibit nitrification even when as much as 500 g/kg or the equivalent of 1,000 kg/ha was added to the soil.[92] Although this seems to be a definitive demonstration that condensed tannins do not affect the rate of nitrification, the tannin used was predominately a profisetinidin, whereas, the foliage of most pines contain tannins that are predominately prodelphinidin. The type and quality of the tannins may be of greater importance than the quantity.[93]

RESEARCH NEEDS

The literature indicates that many researchers have attempted to investigate the effects of tannin on ecosystems without heeding the advice of Coulson et al.[13] who commented that "anomalous results that puzzled earlier workers could be resolved if due regard were paid to changes consequent on this lack of stability of the polyphenols, e.g., changes that might be imposed by methods of drying for storage, by methods of extraction, and by methods of isolation". Proper attention to these details and adoption of new methods of purification and analyses as they are developed will speed the understanding of tannins in the environment.

The leaching of tannins from plants is a difficult process to measure and needs to be studied using innovative techniques. Tannin leaching from roots has important implications in soil chemistry, plant physiology, genetics, and ecology and should be explored. Phosphorus seems to have a direct effect on tannin production but the physiological basis has not been elucidated. Although the effects of tannins on the rate of litter decomposition have been studied and somewhat discredited, the topic deserves further study now that pure tannin polymers can be isolated and new methods are available to examine the chemistry of the reactions. The effects of tannins on the aluminum and iron in the soil need to be better understood because of the reactions of these soil constituents with phosphorus. As phosphorus is often the single factor limiting southern pine growth, this research could be of great economic importance.

REFERENCES

1. Van Cleve, K.; Harrison, A.F. Bioassay of forest floor phosphorus supply for plant growth. *Can. J. For. Res.* 15:156 (1985).
2. Switzer, G.L.; Nelson, L.E. Nutrient accumulation and cycling in loblolly pine (*Pinus taeda* L.) plantation ecosystems: The first twenty years. *Soil Sci. Soc. Amer. P.* 36:143 (1972).
3. Basaraba, J.; Starkey, R.L. Effect of plant tannins on decomposition of organic substances. *Soil Sci.* 101:17 (1966).
4. Lamb. D. Decomposition of organic matter on the forest floor of *Pinus radiata* plantations. *J. Soil Sci.* 27:208 (1976).
5. McKey, D.; Waterman, P.G.; Mbi, C.N.; Gartlan, J.S.; Struhsaker, T.T. Phenolic content of vegetation in two African rain forests: ecological implications. *Science* 202:61 (1978).
6. Lodhi, M.A.K.; Killingbeck, K. T. Allelopathic inhibition of nitrification and nitrifying bacteria in a ponderosa pine (*Pinus ponderosa* Dougl.) community. *Amer. J. Bot.* 67:1423 (1980).
7. Baldwin, I.T.; Schultz, J.C. Tannins lost from sugar maple (*Acer saccharum* Marsh) and yellow birch (*Betula allegheniensis* Britt.) leaf litter. *Soil Biol. Biochem.* 16:421 (1984).
8. Malcolm, R.L.; McCracken, R.J. Canopy drip: A source of mobile soil organic matter for mobilization of iron and aluminum. *Soil Sci. Soc. Amer. P.* 32:834 (1968).
9. Sivapalan, K. Phenolics and the exchange capacity of humic materials. *Soil Biol. Biochem.* 13:331 (1981).
10. Czochanska, Z.; Foo, L.Y.; Newman, R.H.; Porter, L.J. Polymeric proanthocyanidins. Sterochemistry, structural units, and molecular weight. *J. Chem. Soc. Perkin Trans. 1* :2278 (1980).
11. Porter, L.J.; Newman, R.H.; Foo. L.Y.; Wong, H.; Hemingway, R.W. Polymeric proanthocyanidins. ^{13}C N.M.R. studies of procyanidins. *J. Chem. Soc. Perkin Trans. 1* :1217 (1982).
12. Newman, R.H.; Porter, L.J.; Foo, L.Y.; Johns, S.R.; Willing, R.I. High resolution ^{13}C-nmr studies of proanthocyanidin polymers. *Mag. Res. Chem.* 25:118 (1987).
13. Coulson, C.B.; Davies, R.I.; Lewis, D.A. Polyphenols in plant, humus, and soil I. Polyphenols of leaves, litter, and superficial humus from mull and mor sites. *J. Soil Sci.* 11:20 (1960).
14. Shoulders, E.; Tiarks, A.E. Predicting height and relative performance of major southern pines from rainfall, slope, and available soil moisture. *Forest Science* 26:437 (1980).
15. Tiarks, A. E. Phosphorus requirements for establishment of dual-cropped loblolly pine. *In*: Shoulders. E. (ed.) Proceedings of the third biennial southern silvicultural research conference, November 7-8, 1984, Atlanta, Georgia; Gen. Tech. Rep. SO-54, pp. 390-394 (1985).
16. Shoulders, E.; Tiarks, A.E. A continuous function design for fertilizer rate trials in young pine plantations. *In*: Jones, E.P., Jr. (ed.) Preceedings of the second biennial southern silvicultural research conference, November 4-5, 1982. Atlanta, GA. Gen. Tech. Rep. SE-24. pp. 352-356 (1983).
17. Karchesy, J.J.; Hemingway, R.W. Loblolly pine bark polyflavanoids. *J. Agric. Food Chem.* 28:222 (1980).
18. Hemingway, R.W.; Foo, L.Y.; Porter, L.J. Linkage isomerism in trimeric and ploymeric 2,3-cis-procyanidins. *J. Chem. Soc., Perkin Trans. 1* :1209 (1982).
19. Hemingway, R.W.; Karchesy, J.J.; McGraw, G.W.; Wielesek, R.A. Heterogeneity of interflavanoid bond location in loblolly pine bark procyanidins. *Phytochemistry* 22:275 (1983).
20. Foo, L.Y.; Porter, L.J. Prodelphinidin polymers: Definition of structural units. *J. Chem. Soc., Perkin Trans. 1* :1186 (1978).

21. Williams, V.M.; Porter, L.J.; Hemingway, R.W. Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry* 22:569 (1983).

22. Burns, R.E. Method for estimation of tannin in grain sorghum. *Agron. J.* 63:511 (1971).

23. Broadhurst, R.B.; Jones, W.T. Analysis of condensed tannins using acidified vanillin. *J. Sci. Fd. Agric.* 29:788 (1978).

24. Zucker, W.V. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335 (1983).

25. Lebreton, Ph.; Thivend, S.; Boutard, B. Distribution des proanthocyanidins chez les gymnospermes. *Plantes Medicinales et Phytotherapie* 14:105 (1980).

26. Bate-Smith, E.C. Phytochemistry of proanthocyanidins. *Phytochemistry* 14:1107 (1975).

27. Jones, W.T.; Broadhurst, R.B.; Lyttleton, J.W. The condensed tannins of pasture legume species. *Phytochemistry* 15:1407 (1976).

28. Samejima, M.; Yoshimoto,T. Systematic studies of the stereochemical compositon of proanthocyanidins from coniferous bark. *Mokuzai Gakkaishi* 28:67 (1982).

29. Porter, L.J.; Foo, L.Y.; Hemingway, R.W. Differences in condensed tannin structures in pine bark as a function of tree age. Proceedings FRI Symposium No 23 page 10-11 (1980).

30. Hemingway, R.W. Bark: Its chemistry and prospects for chemical utilization. *In*: Goldstein, I.S. (ed.) Organic Chemicals from Biomass. CRC Press, Inc. Boca Raton, Florida. pp. 189-248 (1981).

31. Williams, A.H. Enzyme inhibition by phenolic compounds. *In*: Pridham, J.B. (ed.) Enzyme Chemistry of Phenolic Compounds. pp. 87-96. Macmillan, New York (1963).

32. Bate-Smith, E.C. Haemanalysis of tannins: the concept of relative astringency. *Phytochemistry* 12:907 (1973).

33. Porter, L.J.; Woodruffe, J. Haemanalysis: The relative astringency of proanthocyanidin polymers. *Phytochemistry* 23:1255 (1984).

34. Nicolia, V. Phenolic and mineral content of leaves influences decomposition in European forest ecosystems. *Oecologia* 75:575 (1988).

35. Davies, R.I.; Coulson, C.B.; Lewis, D.A. Polyphenols in plant, humus, and soil IV. Factors leading to increase in biosynthesis of polyphenol in leaves and their relationship to mull and mor formation. *J. Soil Sci.* 15:310 (1964).

36. Procter, J.; Anderson, J.M.; Fogden, S.C.L.; Vallack, H.W. Ecological studies in four contrasting lowland rain forests in Gunung Mulu National Park, Sarawak. II. Litterfall, litter standing crop and preliminary observations on herbivory. *J. Ecol.* 71:261 (1983).

37. Anderson, J.M.; Procter, J.; Vallack, H.W. Ecological studies in four contrasting lowland rain forests in Gunung Mulu National Park, Sarawak. III. Decomposition processes and nutrient losses from leaf litter. *J. Ecol.* 71:503 (1983).

38. Horner, J.D.; Cates, R.G.; Gosz, J.R. Tannin, nitrogen, and cell wall composition of green vs. senescent Douglas-fir foliage: Within- and between-stand differences in stands of unequal density. *Oecologia* 72:515 (1987).

39. Waterman, P.G.; Ross, J.A.M.; McKey, D.B. Factors affecting levels of some phenolic compounds, digestibility, and nitrogen content of the mature leaves of *Barteria fistulosa* (*Passifloraceae*). *J. of Chem. Ecol.* 10:387 (1984).

40. Cooper-Driver, G.; Finch, S.; Swain, T. Seasonal variation in secondary plant compounds in relation to the palatability of *Pteridium aquilinum*. *Biochemical Systematics and Ecology* 5:177 (1977).

41. Waring, R.H.; McDonald, A.J.S.; Larsson, S.; Ericsson, T.; Wiren, A.; Arwidsson, E. Differences in chemical composition of plants grown at constant relative growth rates with stable mineral nutrition. *Oecologia* 66:157 (1985).

42. Larsson, S.; Wiren, A.; Lundgren, L.; Ericsson, T. Effects of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (*Coleoptera*). *Oikos* 47:205 (1986).

43. Chandler, G.; Goosem, S. Aspects of rainforest regeneration. III. The interaction of phenols, light and nutrients. *New Phytol.* 92:369 (1982).

44. Donnelly, E.D. The effect of season, plant maturity, and height on the tannin content of *Sericea lespedeza*, L. cuneata. *Agron. J.* 51:71 (1959).

45. Fales, S.L. Influence of temperature on chemical composition and in vitro matter disappearance of normal- and low-tannin *Sericea lespedeza*. *Can. J. Plant Sci.* 64:637 (1984).

46. Lorio, P.J. Jr. Growth-differentiation balance: a basis for understanding southern pine beetle-tree interactions. *Forest Ecology and Management* 14:259 (1986).

47. Tempel, A.S. Field studies of the relationship between herbivore damage and tannin concentration in bracken (*Pteridium aquilinum* Kuhn). *Oecologia* 51:97 (1981).

48. Guinn,G.; Eidenbock, M.P. Catechin and condensed tannin contents of leaves and bolls of cotton in relation to irrigation and boll load. *Crop Sci.* 22:614 (1982).

49. Jonasson, S.; Bryant, J.P.; Chapin, F.S. III; Andersson, M. Plant phenols and nutrients in relation to variations in climate and rodent grazing. *Amer. Nat.* 128:394 (1986).

50. Haukioja, E.; Niemela, P.; Siren, S. Foliage phenols and nitrogen in relation to growth, insect damage, and ability to recover after defoliation, in the mountain birch *Betula pubescens* ssp *tortuosa*. *Oecologia* 65:214 (1985).

51. Wilson, C.M. The effect of soil treatments on the tannin content of *Lespedeza sericea*. *Agronomy J.* 47:83 (1955).

52. Barry,T.N.; Forss, D.A. The condensed tannin content of vegetative *Lotus pedunculatus*, its regulation by fertilizer application, and effect upon protein solubility. *J. Sci. Food Agric.* 34:1047 (1983).

53. Sorensen, C.; Truelsen, E. Chemical composition of barley varieties with different nutrient supplies. I. Concentration of nitrogen, tannins, phytate, beta-glucans, and minerals. *Tidsskr. Planteavl* 89:253 (1985).

54. Sarzhveladze, A. G. Manganese content of tea leaves and its effect on the quality of prepared tea. (abstr.) Chemical Abstracts 101:564 (1984).

55. Brown,P.H.; Graham, R.D.; Nicholas, D.J.D. The effects of manganese and nitrate supply on the levels of phenolics and lignin in young wheat plants. *Plant and Soil* 81:437 (1984).

56. Lane, H.C.; Schuster, M.F. Condensed tannins of cotton leaves. *Phytochemistry* 20:425 (1981).

57. Walters, T. ; Stafford, H.A. Variability in accumulation of proanthocyanidins (condensed tannins) in needles of Douglas-fir (*Pseudotsuga menziesii*) following long-term budworm defoliation. *J. of Chemical Ecology* 10:1469 (1984).

58. Goldstein, J.L.; Swain, T. Changes in tannins in ripening fruits. *Phytochemistry* 2:371 (1963).

59. Foo, L.Y.; Porter, L.P. The phytochemistry of proanthocyanidin polymers. *Phytochemistry* 19:1747 (1980).

60. Doherty, C.A.; Waniska, R.D.; Rooney, L.W.; Earp, C.F.; Poe, J.H. Free phenolic compounds and tannins in sorghum caryopsis and glumes during development. *Cereal Chem.* 64:42 (1987).

61. Sunlin, M. Naturally occurring antioxidants of plant origin as potential pharmaceuticals. *American Laboratory* February 1988 news edition (1988).

62. Baur,P.S.; Walkinshaw, C.H. Fine structure of tannin accumulations in callus cultures of *Pinus elliotti* (slash pine). *Can. J. Bot.* 52:615 (1974).

63. Pettipher, G.L. An improved method for the extraction and quantification of anthocyanins in cocoa beans and its use as an index of the degree of fermentation. *J. Sci. Food Agric.* 37:289 (1986).
64. Nyman, B.F. Protein-proanthocyanidin interactions during extraction of Scots pine needles. *Phytochemistry* 24:2939 (1985).
65. Lindroth, R.L.; Pajutee, M.S. Chemical analysis of phenolic glycosides: art, facts, and artifacts. *Oecologia* 74:144 (1987).
66. Fahey,T.J.; Yavitt, J.B.; Joyce, G. Precipitation and throughfall chemistry in *Pinus contorta* ssp *latifolia* ecosystems, southeastern Wyoming. *Can. J. For. Res* 18:337 (1988).
67. Lockaby, B.G.; Taylor-Boyd, J.L. Nutrient dynamics in the litterfall and forest floor of an 18-year-old loblolly pine plantation. *Can. J. For. Res.* 16:1109 (1986).
68. Tiarks, A.E.; Meier, C.E. (unpublished data, 1988).
69. Yavitt, J.B.; Fahey, T.J. Litter decay and leaching from the forest floor in *Pinus contorta* (lodgepole pine) ecosystems. *Journal of Ecology* 74:525 (1986).
70. Suberkropp, K.; Godshalk, G.L.; Klug, M.J. Changes in the chemical composition of leaves during processing in a woodland stream. *Ecology* 57:720 (1976).
71. Kuiters, A.T.; Sarink, H.M. Leaching of phenolic compounds from leaf and needle litter of several deciduous and coniferous trees. *Soil Biol. Biochem.* 18:475 (1986).
72. King, H.G.C.; Heath, G.W. The chemical analysis of small samples of leaf material and the relationship between the disappearance and composition of leaves. *Pedobiologia* 7:192 (1967).
73. Swift, M.J.;Heal, O.W.; Anderson, J.M.. Decomposition in Terrestrial Ecosystems. Blackwell Scientific Publications, Oxford (1979).
74. Grant, W.D. Microbial degradation of condensed tannins. *Science* 193:137 (1976).
75. Benoit, R.E.; Starkey, R.L.; Basaraba, J. Effect of purified plant tannin on decomposition of some organic compounds and plant materials. *Soil Sci.* 105:153 (1968).
76. Hagerman, A.E.; Robbins, C.T. Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. *J. Chem. Ecol.* 3:1243 (1987).
77. Asquith, T.N.; Butler, L.G. Interactions of condensed tannins with selected proteins. *Phytochemistry* 25:1591 (1986).
78. Theander, O. Leaf litter of some forest trees. Chemical composition and microbial activity. *Tappi* 61:69 (1978).
79. Berg, B. Dynamics of nitrogen (^{15}N) in decomposing Scots pine (*Pinus silvestris* L.) needle litter. Long-term decomposition in a Scots pine forest VI. *Can. J. Bot.* (in press)
80. Marks, D.L.; Buchsbaum, R.; Swain, T. Measurement of total protein in plant samples in the presence of tannins. *Anal. Biochem.* 147:136 (1985).
81. Makkar, H.P.S.; Dawra, R.K.; Singh, B. Determination of both tannin and protein in a tannin-protein complex. *J. Agric. Food Chem.* 36:523 (1988).
82. Kuiters, A.T.; Denneman, C.A.J. Water-soluble phenolic substances in soils under several coniferous and deciduous tree species. *Soil Biol. Biochem.* 19:756 (1987).
83. Pohlman, A.A.; McColl, J.G. Soluble organics from forest litter and their role in metal dissolution. *Soil Sci. Soc. Am. J.* 52:265 (1988).
84. Gast, P.R. Contrasts between the soil profiles developed under pines and hardwoods. *J. Forestry* 35:11 (1937).
85. Shindo, H.; Kuwatsuka, S. Behavior of phenolic substances in the decaying process of plants IV. Adsorption and movement of phenolic acids in soils. *Soil Sci. Plant Nutr.* 22:23 (1976).
86. Huang, P.M.; Wang, T.S.C.; Wang, M.K.; Wu, M.H.; Hsu, N.W. Retention of phenolic acids by noncrystalline hydroxy-aluminum and -iron compounds and clay minerals of soils. *Soil Sci.* 123:213 (1977).

87. Benoit, R.E. ; Starkey, R.L. Enzyme inactivation as a factor in the inhibition of decomposition of organic matter by tannins. *Soil Sci.* 105:203 (1968).

88. Benoit, R.E.; Starkey, R.L.; Basaraba, J. Effect of purified plant tannin on decomposition of some organic compounds and plant materials. *Soil Sci.* 105:153 (1968).

89. Heng, S.; Goh, K.M. Organic matter in forest soils and the mineralization of soil carbon and nitrogen. *Soil Biol. Biochem.* 16:201 (1984).

90. Sivapalan, K.; Fernando, V.; Thenabadu, M.W. Humified phenol-rich plant residues and soil urease activity. *Plant and Soil* 70:143 (1983).

91. Lamb, D. Soil nitrogen mineralization in a secondary rainforest succession. *Oecologia* 47:257 (1980).

92. McCarty, G.W.; Bremner, J.M. Effects of phenolic compounds on nitrification in soil. *Soil Sci. Soc. Am. J.* 50:920 (1986).

93. Sivapalan, K.; Fernando, V. N-mineralization in polyphenol-rich plant residues and their effect on nitrification of applied ammonium sulphate. *Soil Biol. Biochem.* 17:547 (1985).

EFFECTS OF CONDENSED TANNIN ON ANIMAL NUTRITION

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ABSTRACT

Addition of condensed tannins to diets of experimental animals usually results in diminished weight gains and lowered efficiency of feed utilization, as well as increased fecal nitrogen. These effects have been interpreted in terms of inhibition by tannin of the digestion of dietary protein. Recent studies suggest that inhibition of digestion is much less significant than inhibition of the utilization of digested and absorbed nutrients. Some component(s) of condensed tannin appear to be absorbed from the digestive tract and produce systemic effects. Herbivorous mammals partially protect themselves against dietary tannin by producing specific proline-rich salivary proteins that strongly and selectively bind the tannins, thus sparing dietary proteins. Without these defensive proteins, which may be the source of the increased fecal nitrogen, the antinutritional effects of tannin would be much more severe.

INTRODUCTION

Although the conception of tannins as plant defense chemicals has been questioned,[1] condensed tannins are widely recognized as deterrents to herbivory.[2] Moreover, condensed tannins are also widely recognized as antinutritional factors.[3–6] Given these prevailing views, it is perhaps surprising to note that condensed tannins are so frequently present in the diets of livestock, wild animals, and humans. The reasons are complex and incompletely understood. Traditional legume and cereal crop cultivars containing tannins are somewhat easier to produce than "improved" tannin-free cultivars because of their superior resistance to pests such as grain-eating birds[7] or fungal pathogens.[8] This is particularly true in developing countries

where technological refinements such as pesticides are unavailable for crop protection, and where tannin-containing sorghum or legumes are major crops. Wild animals generally avoid tannin-containing plants as foods when other suitable plants are available.[9–11] But when food choices are limited, some herbivores adapt to diets high in tannin, and even develop a preference for them.[12–13]

In human diets, tannins and related phenolics are present in beverages, legumes, some cereals, fruits and berries, condiments, and the betel quid so commonly utilized in the Eastern world.[4] Even in societies in which tannin-free foodstuffs are plentiful, tannin-containing foods such as tea or red wine remain popular and contribute nutritionally significant amounts of tannins.[3] Dr. J. Morton[14] has suggested that humans have a unique "taste" for tannins (Chapter 25). Although the astringency associated with dietary tannins can be harsh and unpleasant, modest astringency levels have a pleasurable effect on the human palate.[15] It is otherwise difficult to understand the continued popularity of tannin-rich but nutrient-poor foodstuffs, especially decaffeinated tea.[16]

Whether by choice or by necessity, tannins are common dietary components. This discussion will address the nutritional consequences of the consumption of condensed tannins, the biochemical basis for these effects, the physiological mechanisms by which herbivorous animals are protected against potentially harmful effects, and processing technologies that minimize these effects. Other aspects (e.g., carcinogenicity in Chapter 25) of tannin consumption are addressed by others at this symposium. Because condensed tannins are far more abundant in food than are hydrolyzable tannins, only condensed tannins are considered in this discussion unless hydrolyzable tannins are explicitly mentioned.

NUTRITIONAL EFFECTS OF TANNINS

Dietary tannins have been reported to be responsible for a wide range of nutritional problems including mortality.[2–6] Many of the effects of tannins are narrowly specific;[17,18] closely related animal species may respond to tannins quite differently.[19] Different effects may vary independently with the concentration of tannin; in no case has a definite threshold level been established. Hydrolyzable and condensed tannins produce somewhat different effects.[18,20] The nutritional effects of tannic acid have been more thoroughly investigated than those of condensed tannins, probably because tannic acid is more readily available in purified form than are condensed tannins, which are present in more foods. It is likely that different sources of condensed tannins will produce different effects unless the tannins are purified and added to identical basal diets.

Food Consumption

Inhibited food consumption is expected for substances such as tannins, which are effective deterrents to herbivory. Accurate measurement of food consumption requires that animals be confined and placed on more restricted diets than those of wild herbivores. Animals choose only the amount of diet eaten, with different sets of animals fed the same diets ± tannin. Mole and Waterman[2] have summarized numerous tannin feeding trials of this type. Although some studies found decreased

food consumption, in a few cases, an increase in consumption due to tannin was reported. In most cases, little effect is observed on food consumption; the effects of tannin are even less significant if consumption is calculated on an animal weight basis rather than a per-animal basis. Tannin-consuming animals are likely to be smaller than their counterparts, and therefore tend to consume less food.

Growth Rate and Feed Efficiency

Growth rate and feed utilization efficiency (growth rate/feed consumption) of immature non-ruminant vertebrates like rats,[17,20–23] meadow voles,[19] and swine[24,25] are usually decreased by dietary condensed tannins at the levels (1-3%) found in the seed of tannin-rich cultivars of crops such as sorghum or beans. More mature animals are less sensitive to dietary tannin.[20]

The nutritional effects of tannin on ruminants appear to be complex but generally less severe than on non-ruminants.[26,27] Their growth rates can be diminished by sorghums rich in tannin.[28,29] It has been suggested that dietary tannins may have a beneficial effect on ruminants by preventing bloat[30] and by binding and protecting dietary protein from digestion and loss within the rumen.[31,32]

The growth rate and feed utilization of poultry are also significantly diminished by dietary condensed tannins.[17,33–39] Chicks on diets containing high tannin sorghums develop leg abnormalities.[35,38] Sorghum tannins can diminish egg production and feed utilization of mature hens on diets with suboptimal levels of protein.[40]

The extensive investigations of the effects of tannins on insects will not be considered here. Bernays[41] has provided a thoughtful appraisal of that aspect. The nutritional effects of tannins in human diets have received little attention, despite their importance, possibly because of the experimental limitations.[42]

BIOCHEMICAL BASIS FOR TANNIN'S NUTRITIONAL EFFECTS

The most prominent physiochemical property of tannins is their capacity for strongly binding to proteins (Hagerman, Chapter 19). As a consequence, tannins inhibit almost[43] all enzymes when tested in vitro under conditions where the enzyme is the only protein available for binding to tannin. This propensity for protein binding and enzyme inhibition has long been invoked as being responsible for the nutritional effects of tannins.[44] Harborne,[45] for example, suggested that deterrence of herbivory by tannins is partially due to unpleasant astringent sensations (resulting from tannin binding of proteins lining the oral cavity) and partially due to unconscious recognition of reduced digestibility of dietary protein caused by its complexation with tannins.

Digestibility

In most studies in which the effect of dietary tannin on fecal material was examined, tannins were found to increase the level of fecal nitrogen and dry matter. This results in a decrease in protein and dry matter digestibility when calculated from the proportion not recovered in the feces. A reduction in protein digestibility can be rationalized on the basis of tannin binding either to digestive enzymes, resulting in inhibition of their digestive activity, or to dietary protein, producing a

less-digestible protein-tannin complex. In this way, tannins have come to be known primarily as "digestibility-reducing substances". Several types of evidence suggest, however, that tannin's nutritional effects are not due to a reduction in digestibility of dietary proteins or other components. At the risk of overkill, it seems necessary to document the evidence in order to set to rest this widespread misconception.

Digestive enzymes isolated from animals fed condensed tannins show levels of activity equal to or higher than those obtained from animals fed tannin-free diets.[21] In vitro assays of protein digestion may show a stimulation, rather than inhibition, by tannins due to partial denaturation of the protein substrate, making it more digestible.[46,47] Under in vivo conditions, digestive enzymes are much less susceptible to inhibition by tannins because many other proteins are available to compete for binding, including specific salivary tannin-binding proteins, which are the first endogenous proteins to be exposed to the tannins.[4,48] The digestive tract also contains phospholipids, bile acids, and other natural detergents that efficiently disrupt protein binding by tannins.[49,50]

Supplementation with isolated soybean protein of a high tannin sorghum diet overcame the inhibition of chick growth by tannin, but supplementation with the equivalent amino acids (predigested protein) did not suppress the growth effect of tannin.[51] Supplementation of hamster and rat diets with gelatin, a protein deficient in essential amino acids, effectively overcame growth inhibition by tannin; and again, supplementation with the equivalent amino acids to gelatin was ineffective.[52] These studies strongly indicate that a reduction in protein digestibility is not the major reason for the diminished growth rate caused by tannin.

Comparison of the amino acid profile of fecal material and dietary protein shows large differences induced by tannin for rats[53,54] and sheep[32] but not for chicks,[55] suggesting that for some animals the increased fecal nitrogen in response to tannin is due to endogenous, rather than dietary, protein. For chicks, the increased fecal nitrogen could be dietary protein as a byproduct of increased demand for non-protein nutrients as sources of metabolic energy or cofactors for phenol detoxification.[56,57]

Systemic Effects

Metabolic responses induced by dietary condensed tannins include secretion of salivary tannin-binding proteins,[58] and elevated levels of growth hormone in plasma.[61] Voles[62] and hamsters[63] show high mortality rates on tannin-containing diets. For these animals, death is too rapid to be explainable by starvation due to inhibition of digestion; the mechanism for this effect is unknown. Martin-Tanguy et al[64] observed that condensed tannins seem to reduce the metabolic utilization of absorbed amino acids. We have recently made similar observations (unpublished), indicating that inhibition of metabolism of digested and absorbed metabolites is more significant than inhibition of digestion in understanding the nutritional effects of tannins.

These and other toxic effects seem to require that tannins be absorbed out of the digestive tract into the animal's body. Given the macromolecular size of tannins and their affinity for proteins, absorption seems unlikely, but preliminary studies have shown significant uptake of radio-labeled tannin from rat diets.[65,66] Tannin-induced erosion of the intestinal epithelium[67] might enhance uptake, but

such erosion is characteristic of tannic acid, not condensed tannins.[18] Perhaps more likely than tannin uptake is uptake of metabolically associated low MW phenolics, which occur in the same plant tissue as tannins.[68] Future investigations should be directed toward identification of the toxic materials and their metabolic targets and toward possible biotransformations of tannins by gut microflora or various mammalian enzyme systems.

METABOLIC DEFENSES AGAINST DIETARY TANNINS

Animals that normally consume tannin-containing plant materials presumably have survived by developing defenses against these potentially harmful components of their diet. Observed antinutritional effects do not necessarily reflect the total toxic capacity of the plant material, but only the part that is not neutralized by the animal's defenses. The most readily detected defenses are those that do not develop until after introduction of tannin into a previously tannin-free diet, because the resulting short-term effects are acute until the defensive response is induced. Generalized metabolic reactions that detoxify absorbed phenols are not considered here.

Salivary Proline-Rich Proteins

When condensed or hydrolyzable tannins are introduced into the diets of rats[20,58] or mice,[60,69] particularly when the dietary protein level is suboptimal, the animals experience a weight loss for a few days, then recover rapidly. During this adaptation period, the parotid glands increase severalfold in size[58,67] and produce unique proline-rich (up to 45%) salivary proteins hardly detectable before the animals began eating tannin.[58,69] As predicted from their high proline content,[70] these proteins have a very high affinity for tannin.[58,59] The proline-rich, tannin-binding salivary proteins apparently function as a primary defense against dietary tannins by strongly complexing with the tannins immediately on their introduction to the digestive tract. The resulting tannin-protein complex may pass intact through the digestive tract. The fecal proteins from animals fed tannin-containing diets have an altered amino acid profile that reflects that of these salivary proteins[24,54,71] in which proline, glycine, and glutamate can comprise almost 90 percent of the amino acids.[58] These endogenous proteins are presumably responsible for the increase in fecal nitrogen in tannin-containing diets.[53]

The importance of the protection provided by these proline-rich salivary proteins is illustrated by the effects of dietary tannin on hamsters, which have the genes coding for these proteins but do not express them in response to dietary tannin.[63] Hamsters cannot adapt to dietary tannin and die after a few days on diets containing levels of tannins to which rats readily adapt.[63] Propranolol, a β-adrenergic antagonist drug, blocks tannin induction of the proline-rich proteins in rats and renders them much more vulnerable to tannins, much like hamsters.[72] In other animals examined including humans[73] and ruminants,[48] these unique proteins appear to be constitutive and present at levels that reflect the level of tannins and similar polyphenols in their normal diets. Without the defense provided by

proline-rich salivary proteins, the antinutritional effects of consuming tannin would undoubtedly be much more severe for many animals.

There is no evidence that birds produce proline-rich, tannin-binding proteins. Methionine supplementation of a high tannin sorghum-soybean meal diet can overcome the depression of growth rate caused by tannin[56] without affecting protein digestibility.[74] Rather than proline-rich proteins, insects apparently protect themselves against dietary tannin with other materials that prevent tannin binding to dietary proteins and to digestive enzymes and by maintaining high gut pH, which disrupts tannin-protein complexes.[49] In contrast, a pathogenic fungus that causes the cereal disease anthracnose protects its spores against toxic phenolics such as tannins produced by the host plant by making a somewhat proline-rich glycoprotein that has an extremely high affinity for tannins.[75]

METHODS FOR ALLEVIATING TANNIN'S ANTINUTRITIONAL EFFECTS

Supplementation with Tannin-Binding Materials

Antinutritional effects of tannins are excerbated by low levels of dietary protein, so supplementary protein, when available, diminishes or eliminates the harmful effects.[23,51,76] The supplementary protein is apparently not utilized as a source of amino acids, but as a tannin binding agent; proteins low in essential amino acids are effective but equivalent free amino acids are not.[51,52] This effect may be the basis for the common practice of adding milk, with its tannin-binding casein, to strong tea. Supplementation with non-nutritive, tannin-binding polymers such as polyvinylpyrrolidone (PVP)[56,57] or polyethyleneglycol (PEG)[61,78] is similarly effective. Freeland et al[60] reported that mice are capable of selecting diets containing saponins and tannins at levels that neutralize the antinutritional effects of both, presumably by forming tannin-saponin complexes.

Tannin Removal by Dehulling

In cereal and legume seeds, tannins occur largely in the testa layer under the seed coat, which can be removed to provide a nutritionally improved product.[6,79] Traditional methods of preparing sorghum for food in many cultures began with pounding in a crude mortar and pestle to decorticate the grain.[80] The mechanized milling methods being introduced to replace this laborious process do not work efficiently with most high tannin sorghums, which have a soft endosperm that tends to shatter, giving poor recoveries.[81]

Reducing Tannin Levels by Cooking

Cooking diminishes the levels of assayable tannin of beans[82] and sorghum,[83] presumably by enhancing tannin binding to seed components from which it cannot be extracted. But the cooked grain still exhibits the antinutritional effects; the slow

growth rates of rats on high tannin sorghum were not improved by cooking, and in some cases were worsened.[24,85,86]

Chemical Detoxification

Traditional methods of processing tannin-rich oak acorns or sorghums[87] included soaking with aqueous alkaline solutions obtained from lye or wood ashes. Calcium hydroxide reduced tannin levels in a tannin-rich seed meal[88] and served as an effective antidote for toxic tannins in oak buds and leaves.[89] Recent investigations have demonstrated that high tannin sorghums soaked with dilute alkaline solutions not only have much lower levels of assayable tannins but are dramatically improved in nutritional value.[84,90–93] Dilute (0.1 N) aqueous ammonia is especially effective as a soak because it penetrates the grain so rapidly, and is not detectable in the treated dried grain.[94] In contrast to treatment with harsher alkalis,[17,95,96] this mild ammonia treatment does not remove protein, tannin, or any other component from the grain. Presumably, the tannin is converted to forms that do not extract and therefore do not respond to assays or affect nutritional value. Treatment of whole grain is much more effective than treatment of ground grain; grinding expedites interaction of tannins with sensitive seed components.[94] "Reconstitution" of high tannin sorghum grain by storage under high moisture conditions effectively reduced the level of assayable tannins and improved the nutritional value,[55,92,97] but this treatment takes several days during which growth of molds must be controlled. Treatment with dilute solutions of formaldehyde effectively inactivates tannins in sorghum grain[98] in a manner that may be similar to the natural process by which acetaldehyde diminishes the astringency of tannins in persimmon fruit.[99]

Supplementation to Promote Metabolic Detoxification

Finally, in some cases, the effects of dietary condensed tannins can be overcome by supplementation of the diet with methionine, which may be involved in metabolic detoxification of tannins. Supplementation with L-methionine or its hydroxy analog overcame the detrimental effect of condensed tannins of sorghum in chicks, but not in rats.[17] Although methionine supplementation was effective against sorghum tannin in chick diets, it was ineffective against tannic acid.[56] This beneficial effect of methionine was not mediated via detoxification by the methyl group as evidenced by a lack of effect of supplemental choline, another methyl donor.[56] Although methionine overcame the growth rate depression of tannin in chicks fed high tannin sorghum-soybean meal diets, it had no effect in improving the tannin-induced reduction in apparent protein digestibility.[74] Methionine supplementation greatly improved rat weight gains on diets containing bean condensed tannins, and the effect was greatest on the diets containing the most tannin.[100] The basis for the beneficial effect of methionine has not been established.

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REFERENCES

1. Beart, J.E.; Lilley, T.H.; Haslam, E. Plant polyphenols–secondary metabolism and chemical defense: Some observations. *Phytochemistry* 24:33 (1985).
2. Mole, S.; Waterman, P.G. Tannins as antifeedants to mammalian herbivores–still an open question? *In*: Waller, G.R. (ed.) Allelochemicals in Agriculture, Forestry, and Ecology. *Am. Chem. Soc. Symp. Series*, Washington, DC. (1987).
3. Deshpande, S.S.; Sathe, S.K.; Salunkhe, D.K. Chemistry and safety of plant polyphenols. *In*: Freidman, M. (ed.) Nutritional and Toxicological Aspects of Food Safety. Plenum Press, New York (1984).
4. Mehansho, H.; Butler, L.G.; Carlson, D.M. Dietary tannins and salivary proline-rich proteins: Interactions, induction, and defense mechanisms. *Ann. Rev. Nutr.* 7:423 (1987).
5. Salunkhe, D.K.; Jadhav, S.J.; Kadam, S.S.; Chavan, J.K. Chemical, biochemical and biological significance of polyphenols in cereals and legumes. CRC Crit. Rev. *Food Sci. Nutr.* 17:277 (1982).
6. Reddy, N.R.; Pierson, M.D.; Sathe, S.K.; Salunkhe, D.K. Dry bean tannins: A review of nutritional implications. *J. Am. Chem. Soc.* 62:541 (1985).
7. McMillian, W.W.; Wiseman, B.R.; Burns, R.E.; Harris, H.B.; Greene, L. Bird resistance in diverse germplasm of sorghum. *Agron. J.* 64:821 (1972).
8. Harris, H.B.; Burns, R.E. Relationship between tannin content of sorghum grain and preharvest seed molding. *Agron. J.* 65:957 (1973).
9. Wranghan, R.W.; Waterman, P.G. Feeding behaviour of vervet monkeys on *Acacia tortilis* and *Acacia xanthophloea*: With special reference to reproductive strategies and tannin production. *J. Animal Ecol.* 50:715 (1981).
10. Cooper, S.M.; Owen-Smith, N. Condensed tannins deter feeding by browsing ruminants in a South African savanna. *Oecologia* 67:142 (1985).
11. Smallwood, P.D.; Peters, W.D. Grey squirrel food preferences: The effects of tannin and fat concentration. *Ecology* 67:168 (1986).
12. Martin, R.W. Overbrowsing and decline of a population of the koala, *Phascolactor cinereus*, in Victoria. I. Food Preference and Food Tree Defoliation. *Aust. J. Wildlife Res.* 12:355 (1985).
13. Atsatt, P.R.; Ingram, T. Adaptation to oak and other fibrous phenolic-rich foliage by a small mammal, *Neotoma fuscipes*. *Oecologia* 60:135 (1983).
14. Morton, J.F. Further associations of plant tannins and human cancer. *Quarterly J. Crude Drug Res.* 12:1829 (1972).
15. Pierpoint, W.S. Phenolics in food and feedstuffs: The pleasures and perils of vegetarianism. *In*: van Sumere, C.F.; Lea, P.J. (eds.) Biochemistry of Plant Phenolics. Oxford Academic Press, London (1985).
16. Stagg, G.V.; Millin, D.J. The nutritional and therapeutic value of tea–a review. *J. Sci. Food Agric.* 26:1439 (1975).
17. Featherston, W.R.; Rogler, J.C. Influence of tannins on the utilization of sorghum grain by rats and chicks. *Nutr. Rep. Intl.* 11:491 (1975).
18. Sell, D.R.; Reed, W.M.; Chrisman, C.L.; Rogler, J.C. Mucin excretion and morphology of the digestive tract as influenced by sorghum tannins. *Nutr. Rep. Intl.* 31:1369 (1985).
19. Lindroth, R.L.; Batzli, G.O.; Avildsen, S.I. Lespedeza phenolics and penstemon alkaloids: Effects on digestion efficiencies and growth of voles. *J. Chem. Ecol.* 12:713 (1986).
20. Joslyn, M.A.; Glick, Z. Comparative effects of gallotannic acid and related phenolics on the growth of rats. *J. Nutr.* 98:119 (1969).
21. Tamir, M.; Alumot, E. Carob tannins–growth depression and levels of insoluble nitrogen in the digestive tract of rats. *J. Nutr.* 100:573 (1970).

22. Jambunathan, R.; Mertz, E.T. Relationship between tannin levels, rat growth, and distribution of protein in sorghum. *J. Agric. Food Chem.* 21:692 (1973).

23. Schaffert, R.E.; Oswalt, D.L.; Axtell, J.D. Effect of supplemental protein on the nutritive value of high and low tannin sorghum bicolor (L.) Moench grain for the growing rat. *J. Animal Sci.* 39:500 (1974).

24. Mitaru, B.N.; Reichert, R.D.; Blair, R. The binding of dietary protein by sorghum tannins in the digestive tract of pigs. *J. Nutr.* 114:1787 (1984).

25. Myer, R.O.; Gorbet, D.W. Waxy and normal grain sorghums with varying tannin contents in diets for young pigs. *Animal Feed Sci. Tech.* 12:179 (1985).

26. Kumar, R.; Singh, M. Tannins: Their adverse role in ruminant nutrition. *J. Agric. Food Chem.* 32:447 (1984).

27. Barry, T.N.; Manley, T.R. Interrelationships between the concentrations of total condensed tannin, free condensed tannin and lignin in lotus sp. and their possible consequences in ruminant nutrition. *J. Sci. Food Agric.* 37:248 (1986).

28. Fox, D.G.; Klosterman, EW.; Newland, H.W.; Johnson, R.R. Net energy of corn and bird resistant grain sorghum rations for steers when fed as grain or silage. *J. Animal Sci.* 30:303 (1970).

29. Maxson, W.E.; Shirley, R.L.; Bertrand, J.E.; Palmer, A.Z. Energy values of corn, bird-resistant and non-bird-resistant sorghum grain in rations fed to steers. *J. Animal Sci.* 37:1451 (1973).

30. Reid, C.S.W.; Ulyatt, M.J.; Wilson, J.M. Plant tannins, bloat, and nutritive values. Proc. *New Zealand Soc. Animal Prod.* 34:82 (1974).

31. Dreidger, A.; Hatfield, E.E. Influence of tannins on the nutritive value of soybean meal for ruminant. *J. Animal Sci.* 34:465 (1972).

32. Waghorn, G.C.; Ulyatt, M.J.; John, A. Fisher, M.T. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *British J. Nutr.* 57:115 (1987).

33. Chang, S.I.; Fuller, H.L. Effect of tannin content of grain sorghums on their feeding value for growing chicks. *Poultry Sci.* 43:30 (1964).

34. Vohra, P.; Kratzer, F.H.; Joslyn, M.A. The growth depressing and toxic effects of tannins to chicks. *Poultry Sci.* 45:135 (1966).

35. Rostagno, H.S.; Featherston, W.R.; Rogler, J.C. Studies on the nutritional value of sorghum grains with varying tannin contents for chicks. I. Growth Studies *Poultry Sci.* 52:765 (1973).

36. Kratzer, F.H.; Singleton, V.L.; Kadirvel, R.; Rayudu, G.V. Characterization and growth-depressing activity for chickens of several natural phenolic materials. *Poultry Sci.* 54:2124 (1975).

37. Marquardt, R.R.; Ward, A.T.; Campbell, L.D.; Cansfield, P.E. Purification, identification and characterization of a growth inhibitor in faba beans (*Vicia faba* L. var. *minor*. *J. Nutr.* 107:1313 (1977).

38. Blair, R.; Mitaru, B.N. New information on the role of tannins in the utilization of feedstuffs by growing birds. Proc. *Florida Nutr. Conf.* :139 (1983).

39. Garwood, V.A.; Rogler, J.C. Response of growth-selected Japanese quail lines to tannin levels in grain sorghum diets with suboptimal protein. *Poultry Sci.* 66:1095 (1987).

40. Sell, D.R.; Rogler, J.C. The effects of sorghum tannin and methionine level on the performance of laying hens in two temperature environments. *Poultry Sci.* 63:109 (1984).

41. Bernays, E.A. Plant tannins and insect herbivores: an appraisal. *Ecolog. Entomol.* 6:353 (1981).

42. Hussein, L.; Abbas, H. Nitrogen balance studies among boys fed combinations of faba beans and wheat differing in polyphenolic contents. *Nutr. Rep. Intl.* 31:67 (1985).

43. Strumeyer, D.H.; Malin, M.J. Resistance of extracellular yeast invertase and other glycoproteins to denaturation by tannins. *Biochem J.* 118 (1970).

44. Zucker, W.V. Tannins: Does structure determine function? An ecological perspective. *Amer. Naturalist* 121:335 (1983).

45. Harborne, J.B. Introduction to Ecological Biochemistry. Academic Press, London. p. 164 (1982).

46. Oh, H.I.; Hoff, J.E. Effect of condensed grape tannins on the in vitro activity of digestive proteases and activation of their zymogens. *J. Food Sci.* 51:577 (1986).

47. Mole, S.; Waterman, P.G. Stimulatory effects of tannin and cholic acid on tryptic hydrolysis of proteins: Ecological implications. *J. Chem. Ecol.* 11:1323 (1985).

48. Robbins, C.T.; Mole, S.; Hagerman, A.E.; Hanley, T.A. Role of tannins in defending plants against ruminants: reduction in dry matter digestion? *Ecology* 68:1606 (1987).

49. Martin, J.S.; Martin, M.M.; Bernays, E.A. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores: implications for theories of plant defense. *J. Chem. Ecol.* 13:605 (1987).

50. Blytt, H.J.; Guscar, T.K.; Butler, L.G. Antinutritional effects and ecological significance of dietary condensed tannins may not be due to binding and inhibiting digestive enzymes. *J. Chem. Ecol.* 14:1455 (1988).

51. Rogler, J.C.; Ganduglia, H.R.R.; Elkin, R.G. Effects of nitrogen source and level on the performance of chicks and rats fed low and high tannin sorghum. *Nutr. Res.* 5:1143 (1985).

52. Mehansho, H.; Rogler, J.C., Butler, L.G.; Carlson, D.M. An unusual growth inhibiting effect of tannins on hamsters. *Fed. Proc.* 44:1860 (1985).

53. Glick, Z.; Joslyn, M.A. Effect of tannic acid and related compounds on the absorption and utilization of proteins in the rat. *J. Nutr.* 100:516 (1970).

54. Eggum, B.O.; Christensen, K.D. Influence of tannins on protein utilization in feedstuffs with special reference to barley. *In:* Breeding for Seed Protein Improvement Using Nuclear Techniques. International Atomic Energy Agency, Vienna. p. 135 (1972).

55. Mitaru, B.N.; Reichert, R.D.; Blair, R. Protein and amino acid digestibilities for chickens of reconstituted and boiled sorghum grains varying in tannin contents. *Poultry Sci.* 64: 101 (1985).

56. Armstrong W.D.; Featherston, W.R.; Rogler, J.C. Influence of methionine and other dietary additions on the performance of chicks fed bird resistant sorghum grain diets. *Poultry Sci.* 52: 1592 (1973).

57. Wang, R.S.; Kies, C.; Sullivan, T. Niacin availability by mice as affected by tannin from sorghum. *FASEB J.* 2:A442 (1988).

58. Mehansho, H.; Hagerman, A.E.; Clements, S.; Butler, L.G.; Rogler, J.C.; Carlson, D.M. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc. *Natl. Acad. Sci.* 80:3948 (1983).

59. Sell, D.R.; Rogler, J.C. Effects of sorghum grain tannins and dietary protein on the activity of UDP-glucuronyltransferase. Proc. *Soc. Exper. Biol. Med.* 174:93 (1983).

60. Freeland, W.J.; Calcott, P.H.; Anderson, L.R. Tannins and saponin: Interaction in herbivore diets. *Biochem. System. Ecology* 13:189 (1985).

61. Barry, T.N.; Allsop, T.F.; Redekopp, C. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 5. *British J. Nutr.* 56:607 (1986).

62. Lindroth, R.L.; Batzli, G.O. Plant phenolics as chemical defenses: effects of natural phenolics on survival and growth of prairie voles. *J. Chem. Ecol.* 10:229 (1984).

63. Mehanso, H.; Ann, D.K.; Butler, L.G.; Rogler, J.C.; Carlson, D.M. Induction of proline-rich proteins in hamster salivary glands by isoproterenol treatment and an unusual growth inhibition by tannins. *J. Biol. Chem.* 262:12344 (1987.

64. Martin-Tanguy, J.; Vermorel, M.; Lenoble, M.; Martin, C. Les tanins des graines de sorgho. Importance dans l'utilisation digestive de l'azote ches le rat en croissance. *Ann. Biol. Anim. Bioch. Biophys.* 16:879 (1976).

65. Holzl, J. cited in Wagner, H. Phenolic compounds in plants of pharceutical interest. *In*: Swain, T.; Harborne, J.; van Sumere, C.F. (eds.) Recent Advances in Phytochemistry and Biochemistry of Plant Phenolics. Plenum Press, New York. p. 589 (1979).

66. Butler, L.G.; Rogler, J.C.; Mehansho, H.; Carlson, D.M. Dietary effects of tannins. *In*: Cody, V.; Harborne, J.B.; Middleton, E. (eds.) Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships. Alan R. Liss, New York (1986).

67. Freeland, W.J.; Calcott, P.H.; Geiss, D.P. Allelochemicals, minerals, and herbivore population size. *Biochem. System. Ecology* 13:195 (1985).

68. Robbins, C.T.; Hanley, T.A.; Hagerman, A.E.; Hjeljord, O.; Baker, D.L.; Schwartz, C.C.; Mautz, W.W. Role of tannins in defending plants against ruminants: reduction in protein availability. *Ecology* 68:98 (1987b).

69. Mehansho, H.; Clements, S.; Sheares, B.T.; Smith, S.; Carlson, D.M. Induction of proline-rich gycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. *J. Biol. Chem.* 260:4418 (1985).

70. Hagerman, A.E.; Butler, L.G. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* 256:4494 (1981).

71. Knudsen, K.E.B.; Kirleis, A.W.; Eggum, B.O.; Munck, L. Carbohydrate composition and nutritional quality for rats of sorghum to prepared from decorticated white and whole grain red flour. *J. Nutr.* 118:588 (1988).

72. Subramanian, V.N.; Mehansho, H.; Carlson, D.M. Inhibition of proline-rich protein synthesis and induction of tissue-specific proteins by Beta-1 Adrenergic Blockers. *FASEB J.* 2:A779 (1988).

73. Mole, S.; Glander, K.E.; Butler, L.G. Studies of proline-rich proteins in the ecological context: techniques and preliminary data. *J. Chem. Ecol.* In press.

74. Elkin, R.G.; Rogler, J.C.; Featherston, W.R. Influence of sorghum grain tannins on methionine utilization in chicks. *Poultry Sci.* 57:704 (1978).

75. Nicholson, R.L.; Butler, L.G.; Asquith, T.N. Glycoproteins from *Colletotrichum graminicola* that bind phenols: implications for survival and virulence of phytopathogenic fungi. *Phytopathol.* 76:1315 (1986).

76. Hill, G.M.; Utley, P.R.; Newton, G.L. Influence of crude dietary protein on peanut skin digestibility and utilization by feedlot steers. *J. Animal Sci.* 62:887 (1986).

77. Rayudu, G.V.N.; Kadirvel, R.; Vohra, P.; Kratzer, F.H. Effect of various agents in alleviating the toxicity of tannic acid for chickens. *Poultry Sci.* 49:1323 (1970).

78. Ford, J.E.; Hewitt, D. Protein quality in cereals and pulses. 3. Bioassays with rats and chickens on sorghum (*Sorghum vulgare* Pers.), barley, and field beans (*Vicia faba* L.). Influence of Polyethylene glycol on digestibility of the protein in high-tannin grain. *British J. Nutr.* 42:325 (1979).

79. Chibber, B.A.K.; Mertz, E.T.; Axtell, J.D. Effects of dehulling on tannin content, protein distribution, and quality of high and low tannin sorghum. *J. Agric. Food Chem.* 26:679 (1978).

80. Whitby, G. Successfully processing sorghum. *Appropriate Techn.* 12:11 (1985).

81. Reichert, R.D.; Mwasaru, M.A.; Mukuru, S.Z. Characterization of colored-grain sorghum lines and identification of high-tannin lines with good dehulling characteristics. *Cereal Chem.* 65:165 (1988).

82. Bressani, R.; Elias, L.G.; Braham, J.E. Reduction of digestibility of legume proteins by tannin. *J. Plant Foods* 4:43 (1982).

83. Glennie, C.W.; Daiber, K.H.; Taylor, J.R.N. Reducing the tannin content in sorghum grain by heat. *South African Food Review* 9:51 (1982).

84. Price, M.L.; Butler, L.G.; Featherstone, W.R.; Rogler, J.C. Detoxification of high tannin sorghum grain. *Nutr. Rep. Intl.* 17:229 (1978).

85. Price, M.L.; Hagerman, A.E.; Butler, L.G. Tannin in sorghum grain: effect of cooking on chemical assays and on antinutritional properties in rats. *Nutr. Rep. Intl.* 21:761 (1980).

86. Eggum, B.O.; Monoway, L.; Knudsen, K.E. Bach; Munck, L.; Axtell, J.D. Nutritional quality of sorghum and sorghum foods from Sudan. *J. Cereal Sci.* 1:127 (1983).

87. Sehene, C.; Guiragossian, V. La fabrication de la biere locale de sorgho au rwanda. Proc. 5th Regional Workshop on Sorghum & Millet Improvement in Eastern Africa, SAFGRAD/ICRISAT, Nairobi. p. 259 (1986).

88. Wah, C.S.; Sharma, K.; Jackson, M.G. Studies of various chemical treatments of sal-seed-meal to remove or inactivate tannins. *Indian J. Animal Sci.* 47:8 (1977).

89. Dollahite, J.W.; Housholder, G.T.; Camp, B.J. Effect of calcium hydroxide on the toxicity of post oak (*Quercus stellata*) in calves. *J. Amer. Vet. Med. Assoc.* 148:908 (1966).

90. Price, M.L.; Butler, L.G.; Rogler, J.C.; Featherston, W.R. Overcoming the nutritionally harmful effects of tannin in sorghum grain by treatment with inexpensive chemicals. *J. Agric. Food Chem.* 27:441 (1979).

91. Ford, J.E.; Hewitt, D. Protein quality in cereals and pulses. 2. Influence of polyethyleneglycol on the nutritional availability of methionine in sorghum (*Sorghum vulgare pers.*), field beans (*Vicia faba* L.), and barley. *British J. Nutr.* 42:317 (1979).

92. Reichert, R.D.; Fleming, S.E.; Schwab, D.J. Tannin deactivation and nutritional improvement of sorghum by anaerobic storage of H_2O-, HCl-, or NaOH-treated grain. *J. Agric. Food Chem.* 28:824 (1980).

93. Muindi, P.J.; Thomke, S. The nutritive value for rats of high and low-tannin sorghums treated with magadi soda. *J. Sci. Food Agric.* 32:139 (1981).

94. Butler, L.G. Polyphenols and their effects on sorghum quality. Proc. Intl. Symp. on Sorghum Grain Quality, ICRISAT, Patancheru, India. p. 294 (1982).

95. Chavan, J.K.; Kadam, S.S.; Ghonsikar, C.P.; Salunkhe, D.K. Removal of tannins and improvement of in vitro protein digestibility of sorghum seeds by soaking in alkali. *J. Food Sci.* 44:1319 (1979).

96. Kock, J.L.F.; Groenwald, E.G.; Kruger, G.H.J.; Eloff, J.N.; Lategan, P.M. Extraction of polyphenols and hydrolysis of birdproof sorghum starch. *J. Sci. Food Agric.* 36:1140 (1985).

97. Teeter, R.G.; Sarani, S.; Smith, M.O.; Hibberd, C.A. Detoxification of high tannin sorghum grain. *Poultry Sci.* 65:67 (1986).

98. Daiber, K.H.; Taylor, J.R.N. Effects of formaldehyde on protein extraction and quality of high- and low-tannin sorghum. *J. Agric. Food Chem.* 30:70 (1982).

99. Pesis, E.; Ben-Arie, R. Involvement of acetaldehyde and ethanol accumulation during induced deastringency of persimmon fruits. *J. Food Sci.* 49:896 (1984).

100. Bressani, R.; Elias, L.G.; Wolzak, A.; Hagerman, A.E.; Butler, L.G. Tannin in common beans: methods of analysis and effects on protein quality. *J. Food Sci.* 48:1000 (1983).

TANNIN AS A CARCINOGEN IN BUSH-TEA: TEA, MATE', AND KHAT

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ABSTRACT

Field investigations of high-risk areas for esophageal cancer have revealed significant correlations between the disease and excessive intake of condensed tannin in bush-tea (plant decoctions taken as cold remedies) and have led to increasing awareness of the carcinogenicity of high-tannin foods, beverages, and masticatories taken in quantity frequently and over a long period of time. These include ordinary tea (*Camellia sinensis*), mate' (*Ilex paraguariensis*), and khat (*Catha edulis*), the use of which is expanding with cultural migrations and air transport supply.

INTRODUCTION

I have focused on tannin as a cause of esophageal cancer in widely separated high-risk areas of the world because of the results of early field work undertaken at the request of the National Cancer Institute. Under its auspices, I began, in 1963, an investigation of the prevalence of the disease on the island of Curacao in the Netherlands Antilles and all possible contributing factors. I gathered data, learned about the local plants and their uses, interviewed patients and survivors of the deceased, gathered in quantity the plants most used in bush-tea by victims of the disease, lyophilized and supplied them to Dr. Roger O'Gara, Laboratory of Pathology, National Cancer Institute, for bioassay. After 6 years of such activity, 3 popular folk-medicine plants of Curacao out of 23 being tested produced tumors in 100 percent of the experimental animals. I determined that these, *Acacia glauca, Melochia tomentosa*, and *Krameria ixina*, were unrelated botanically but had in common a high level of condensed tannin (proanthocyanidin). *Krameria* induced the earliest tumors[1-5] and is the most significant source of bush-tea in the closely

allied State of Falcon, Northern Venezuela, where I found a previously unrecognized pocket of esophageal cancer, later confirmed by Venezuelan cancer authorities.[1]

At this point, I analyzed 53 epidemiological reports covering esophageal cancer in various parts of the world, tabulating suspected factors. Using the statistics from these reports, Dr. Frank Venning prepared for me a world bar-graph that made it easy to match up plant uses with high-risk areas for esophageal cancer – tea in the cancer belt of northern Iran, southern Russia, and Turkey; dark sorghum in the Honan Province of northern China; dark sorghum and tea in the Transkei; dark sorghum and tannin-rich folk remedies in Curacao; and dry red wine in western Europe generally, but high-tannin apple cider in northern France.[6]

As a result, the National Cancer Institute asked me to pick a high-risk area in the United States and start over again to see what might develop. Therefore, over a period of 3 years, I made an intimate study of the habits of the people of the Low Country of South Carolina, swampy in contrast to arid Curacao, and found a traditional intake of bush-tea from high-tannin plants such as the cherrybark oak, sweet gum, wax myrtle, blackberry root, marsh rosemary (*Limonium carolinianum* and *L. nashii*), sweet pond lily root, etc., plus indulgence in homemade wine from muscadine grapes and elderberries, both noted for their tannin content. Further, among poor people, tea is boiled to "stretch it," and it is given to infants to control diarrhea. I tabulated data from 32 years of cancer cases involving the upper digestive tract in the files of the Cancer Clinic of the Medical University of South Carolina and mapped the data, showing that esophageal cancer was common in the eight Low Country counties in which these bush-tea uses prevailed.[7,8]

Dr. Robert Zaldivar's report on esophageal cancer in Chile intrigued me because of the peculiar localization, the disease ranging from 6.7 to 11.1 per 100,000 in the five northern provinces and amounting to 15.1 in the distant southern province of Aysen, whereas, in the rest of the country, the range is from 1.2 to 4.8 except for Maule (central), where the rate is 5.6.[9] Among traditional Chilean folk-medicine plants, two of the most popular are *Krameria cistoidea* and *Limonium guaicuru*, both strong astringents.[10] Now, in addition to these tannin sources and the uniquely tannin-rich white wines of Chile,[5] we have to consider tea.

TEA (*Camellia sinensis* L.)

Dr. Patricio Castro, a chemical engineer, originally from Chile but currently active in Lima, Peru, has told me that in northern Chile the copper miners receive a free ration on the night shift – a packet of black tea and a packet of sugar – no milk; a packet of crackers and a tin of sardines. There are 7,000 workers in one company, and this figure should be multiplied by five to include the family, for the miners take the rations home. In the nitrate mines, there are 12,000 people who receive similar rations and this figure, too, should be multiplied by five to include the family consumers.[11]

There seems to be an interesting contrast between Chile and Peru. Peru is not on the Kurihara et al chart showing the rates of esophageal cancer in 39 countries.[12] I have not found published data on the incidence of esophageal cancer in Peru, but Dr. Castro very helpfully obtained statistics, for Lima only, from Dr. Laura Olivares, Head, Departmento de Estadisticas, Instituto Nacional de Enfermedades

Neoplasticas: 1978 – cases per 100,000 – 1.7 for men and 0.5 for women. They have no funds for updating or for surveying the entire country. Dr. Castro also gleaned information on the beverage habits of the Lima population from Encuesta Nacional de Consumo de Alimentos, Comision Multisectorial, D.S. 59-70 AG, Ministerio de Agricultura (Peru), August 16, 1971-August 16, 1972.

Other data from the same source covering eastern Peru (one overall economic level) show similar low wine and tea consumption rates relative to beer intake rates.

Dr. Castro has consulted with the assistant manager of an important group of mining companies in Peru as well as an engineer at the main mine-smelter complex at La Croya. They assured him that the workers do not receive a free ration on the night shifts. In fact, they refuse to eat on the premises, preferring to go home for every meal. As for hard liquor, Scotch whisky is the luxury of a very few Peruvians. Beer from barley imported mostly from the United States is, as the figures show, the beverage of the populus at large (Table 1).[11]

In 1966, Kaiser and Bartone demonstrated co-carcinogenicity of tea by painting the neck area of 15 young Swiss mice with a 1 percent acetone solution of 3,4 benzo[a]pyrene followed by painting with black tea on alternate days for a total of 55 applications. A control group of 15 mice was given only the single benzo[a]pyrene painting, and they developed no lesions, but all the others evidenced various stages of cancer.[13,14]

In 1973, Dr. Mitsuo Segi requested reprints of my papers on my field work and, some months later, wrote that he had applied my tannin/cancer theory to the high-risk provinces of Japan and made a nearly perfect correlation between the incidence of esophageal cancer and the custom of eating tea-rice-gruel. The tea is boiled, rice added; it is boiled again and eaten.[15] After Dr. Segi's demise, a team of Japanese investigators made a 10-year followup study, confirmed his findings, and found the highest risk in those who ate the gruel and also drank tea with the meal.[16]

Table 1. Family Beverage Consumption in Lima, Peru.

	Consumption/Year (kilos)		
Beverage	Stratum I	Stratum II	Stratum III
Beer (black beer insignificant)	27.252	35.502	5.743
Wine	0.443	0.878	2.697
Red wine	0.444	–	0.165
Herbal teas	1.069	0.802	0.242
Instant coffee	0.516	0.556	0.611
Roasted, ground coffee	5.603	6.357	4.359
Filtered coffee (liquid)	0.326	0.494	–
Canned cocoa powder	1.157	0.688	0.450
Tea	1.190	0.820	0.395
Family members	6.63	5.49	4.42
Proportion in stratum (%)	49.46	33.60	16.94

Dr. Govind Kapadia and colleagues at Howard University obtained, by subcutaneous injection of the tannin fraction of tea from Assam, tumors at the injection site in 26 of 30 NIH black rats.[17]

I have previously recorded the circumstances in Holland in the mid-18th century (reported to me by Dr. Gerhard Haneveld of Utrecht) when alarm arose over the increasing incidence of esophageal cancer. At that time, tea without milk was the national beverage. When the Dutch coffee production in Java reached a level that brought the price within the means of all (about 1825), coffee replaced tea, and the disease became quite rare.[18] I have also reported elsewhere firsthand knowledge of esophageal cancer cases in this country among heavy consumers of iced or hot tea without milk.[18,19] And I receive letters containing statements such as: "I had an aunt who died of esophageal cancer. She was a non-smoker and (non-)drinker of booze but drank lots of hot tea".

Chinese tea is usually relatively low in tannin, as the Chinese do not like an astringent tea. The total amount of polyphenols in tea (as much as 30 percent by dry weight)[20] varies not only with the type of tea grown but also with the soil and other environmental factors, the state of growth, the blend, and the brand. The bud and first young leaf (the highest grade) contain the most tannin and caffeine. The tannin and caffeine content declines as the leaves are plucked lower on the stem. Chemical constituents vary, too, with the time of plucking, being at a peak in the early morning, declining progressively throughout the day.[21] Kakicha (twigs), drunk in Japan only by those of the lowest economic level, contains little tannin and caffeine.[22] It is simmered for 10 minutes to extract enough flavor. Coarse Bancha, or Banjhi (mature leaves and stems), is one grade higher.[22,23]

There are seasonal changes in tea. In Japan, the spring crop is the highest in quality.[24] The strongest and most richly colored teas are produced in Assam in June and early July when the polyphenol content is high. During overcast weather at other seasons, the teas are weak.[20] Tea grown in the shade is high in chlorophyll and caffeine but lower in tannin than tea grown in the usual way. The deluxe Gyokuro tea of Japan is shade-grown and hand-plucked and very expensive.[23,25] It behooves tea drinkers to choose low-tannin tea and, even then, either add milk (as the British wisely do) or avoid excessive consumption. Anyone can judge the relative tannin content by making a strong brew and sipping it to determine the degree of "furriness" (astringency) on the tongue.

MATE′ (*Ilex paraguariensis*)

The case against tea is strengthened by a seeming cancer link with another heavily-consumed botanical beverage. Mate′, often called South American tea, Paraguay tea, or, locally, *congonha*, *yerba mate′*, *yerva mate′*, or spelled *matte* for the American market,[26] is native to a limited area of South America – the region bounded by the Parana′, Paraguay, and Uruguay Rivers (Figures 1 and 2),[27,28] in Paraguay, southern Brazil, and Uruguay. The leaves were chewed by primitive Guarani′ Indians who lived where the tree grew wild,[29] and some say they also made a "tea" of the leaves. The Spaniards who occupied Paraguay created, in 1554, 1557, and 1576, three colonies for collection and distribution of mate′ leaves, using the Indians as laborers. In 1578, Jesuit missionaries undertook the systematic

Figure 1. *Ilex paraguariensis as pictured by Dr. Victor Ferreira do Amaral on the cover of a booklet prepared for the Sociedade Nacional de Agricultura do Rio de Janeiro in 1903.*

culture of mate′, selected and planted superior types, and improved its processing and handling, and they spread the word that the infusion was health-promoting. Bandits from Sao Paulo, Brazil, caused them to move with their laborers to Paraguay in 1610.[30] The aggressors returned to Brazil with Indian prisoners and made the reputation of mate′ known elsewhere in Brazil. By this time, some Jesuits were voicing disapproval of mate′ because its effect on the natives was too exhilarating. One padre called it the "Devil's Herb".[28,30] In Brazil, the state of Parana′ is the leading producer of mate′, followed by Rio Grande do Sul and, on a much lower scale, Santa Catarina and Mato Grosso. However, in 1950, Parana′ had only 24 mate′ factories, Rio Grande do Sul 43,[28] and the latter state also imports mate′. Parana′ exports mainly to Argentina and Paraguay.

Since 1935, mate′ has been cultivated in plantations covering millions of acres in the Province of Misiones, also in a part of Corrientes in northern Argentina[30–32] – the cattle country or "gaucho" territory.

Both Brazil and Argentina export to Chile. Today, Argentina is the world's leading producer and consumer of mate′,[29] and the beverage is promoted as a substitute for alcohol. Annual consumption of mate′ in Argentina and Uruguay was calculated in 1950 to be 10 kg per capita.[28]

Figure 2. *Mate′ is native to a limited area of South America bounded by the Parana′, Paraguay, and Uruguay Rivers in Paraguay, southern Brazil, and Uruguay, but northern Argentina leads in production and consumption. (Map reproduced from the bulletin Yerba Mate published by the Pan American Union, Washington, DC, in 1946).*

Leafy branches of mate′ are heat-treated to preserve the green color, and then the threshed leaves are transported by boat or railway to mills for grinding, sifting, and grading, and the mate′ may be sold green or toasted.[26,30,33,35,36] Green mate′ and green mate′ powder (called *chimarrao* in Brazil) are quite popular in Argentina and Chile where mate′ is drunk hot, sipped through a reed, bone, or silver tube (*bombilla*) from a decoratively carved and often silver-rimmed gourd, calabash, or coconut shell (*cuia*), or a cow's horn or elaborately fashioned metal cup.[26,30,34] For North American markets, mate′ is double-toasted to give it an amber-brown color and more tangy flavor.[26] Also for export, Brazil developed "instant mate′" in 1955.[37]

Chemistry and Alleged Effects of Mate′

Mate′ is more than a beverage to its South American consumers. With sugar, it is taken as a bitter tonic, astringent, stomachic, laxative, sudorific, and febrifuge. It is given to expectant mothers, recommended for women, children, and other ner-

vous persons. It does not produce insomnia. The gauchos value it as a diuretic, benefitting the kidneys.[38] It allegedly improves digestion, eliminates fatigue, stimulates the muscles and nerves, and clarifies the mind. Externally, it is used to treat conjunctivitis, various ulcers of the skin and mucosa, and serves as a cicatrizant on gangrenous wounds.[35,39]

The chemical components of mate′ vary with the region, the type grown, the presence or absence of twigs,[35] and the mode of processing. 'Gaucho' brands are the strongest.[40] Analyses have revealed: 9.05 to 12.40 percent tannin, 1.17 to 1.5 percent caffeine.[28,29] Argentine assays show the following food values per 100 g: carotene, 1.23 mg, or, expressed in international units as vitamin A, 2.095 I.U.; vitamin B_1, 222.7 I.U.; riboflavin, 404.3 I.U.; nicotinic acid, 0.92 mg.; ascorbic acid, 11.9 mg.[28] Chlorogenic acid content is much higher than in tea.[20] French chemists report also vitamin E, chlorophyll, and pantothenic acid.[38] It is distressing to Argentina that emphasis on medicinal virtues of mate′ has caused it to be sold mainly by pharmacies in Europe.[41] It is handled mainly by "health food" dealers in the United States, and it is not-so-subtly promoted by mail as an aphrodisiac.

Theodore Roosevelt, in his book *Through the Brazilian Wilderness*, related that he included mate′ in the supplies for his *Rio da Duvida* (River of Doubt) expedition, for, "with it a native can do a wonderful amount of work on little food."[38,40,42] Workers in a mate′ mill in Asuncion, Paraguay, in 1946, were viewed as physically active and strong, doing heavy work, despite having little for breakfast and laboring all day with no food or drink except Mate′.[28] In World War I, mate′ was furnished to British, French, and German troops.[26] The gauchos of the pampas spend many days in the interior with no solid food but with an infusion of mate′ in a saddlebag. Mate′ is to the gaucho what coffee is to most other Brazilians. They drink mate′ before breakfast and many times during the day and when relaxing in the evening. They are "almost magically sustained" as were Brazilian soldiers for 22 days without food in the war against Paraguay.[27]

The highest consumption of mate′ in Brazil is in the State of Rio Grande do Sul (famed for its wine industry), with lesser intake in southern Parana′, Santa Catarina, and Mato Grosso.[28] In northern Parana′, Sao Paulo, and Minas Gerais, coffee is the main beverage. According to Prudente, the highest incidence of esophageal cancer in Brazil is among the gauchos of Rio Grande do Sul – 24.3 percent in males, 2.1 percent in females.[43]

The Kurihara et al table of death rates for malignant neoplasms of the esophagus in 39 countries (1978-1979) gives top billing to males in Uruguay (15.5 per 100,000), 6th place to Argentina, 7th to Chile, 13th to Paraguay. The rates for females are much lower but higher than for those in non-mate′ consuming countries.[14]

Dr. Patricio Castro states that Aysen in southern Chile is unique in that the people there consider themselves more Argentinian than Chilean – "live more in Argentina than in Chile, and so are bound to drink more mate′ than the rest of Chile."[13] It is possible that some change took place in the importation of mate′, for Zaldivar reported that the high rate of esophageal cancer for that Province in 1960 (15.1/100,000) disappeared. No deaths from esophageal cancer were recorded in 1962, and the Province exhibited very low death rates from the disease in subsequent years.[11]

I have found in my files a note that an investigator by the name of Rossi, in a letter to Dr. Lucia Dunham, NCI Laboratory of Pathology, in 1955, suggested hot mate′ as a cause of the high rate of esophageal cancer on the northeast coast of Argentina, adding that tobacco and alcohol should also be considered.

KHAT (*Catha edulis* Forsk.)

In 1978, the Hopitaux des Armees, Djibouti, French Somaliland, East Africa, reported seeing 36 patients with advanced esophageal cancer over a 2-year period, and all were chewers of the leaves of the tree, *Catha edulis*[44] known commonly as khat, kat, qat, gat, chat, or Abyssinian tea. The leaves contain from 7 to 14.9 percent condensed catechin tannin (Figure 3).[45]

The United Nation's *Bulletin on Narcotics* devoted a special issue to khat in 1980. It is stated therein that "gastrointestinal tract disturbances are most often described in chronic khat users. The astringent characteristics of the tannins appear to account for reports of periodontal disease, stomatitis, esophagitis, and gastritis."[46]

In mid-January of this year, I received a letter from Dr. Iain Murray-Lyon, Charing Cross Hospital, London, saying: "I have just come back from a medical trip to Yemen where I was impressed by the high prevalence of carcinoma of the oesophagus. A high proportion of the population now chew qat regularly."[47] And, in the April 1988 issue of the journal, *HortScience*, there is a brief item from the

Figure 3. *Khat (Catha edulis) in bloom in southern Florida. (Photo by Julia Morton).*

1987 Yearbook, *Arab Agriculture*, reading, in part, "Historically, Yemen's most reknowned agricultural export, coffee, has suffered a sad decline in the modern-day Yemen Arab Republic. A major reason for the stagnation or decline in the production of coffee and food crops over the past decade has been the increasing cultivation of gat, the leaves of which contain a stimulant drug, and around which much Yemeni social life and expenditure revolve."[48]

In June, 1986, Dr. Dennis Johnson, who was in Kenya as a consultant for the United States Agency for International Development, sent me a full-page news story from Nairobi on the local 'booming business' in khat leaves (there called *miraa*).[49] I sent a copy to Dr. Clarence Muir, of the International Agency for Research on Cancer at Lyon, and urged that an epidemiological study be made, comparing the incidence of esophageal cancer in Nairobi with that of Djibouti. In such a study, it is necessary to determine the manner of khat use in each case (Figure 4).

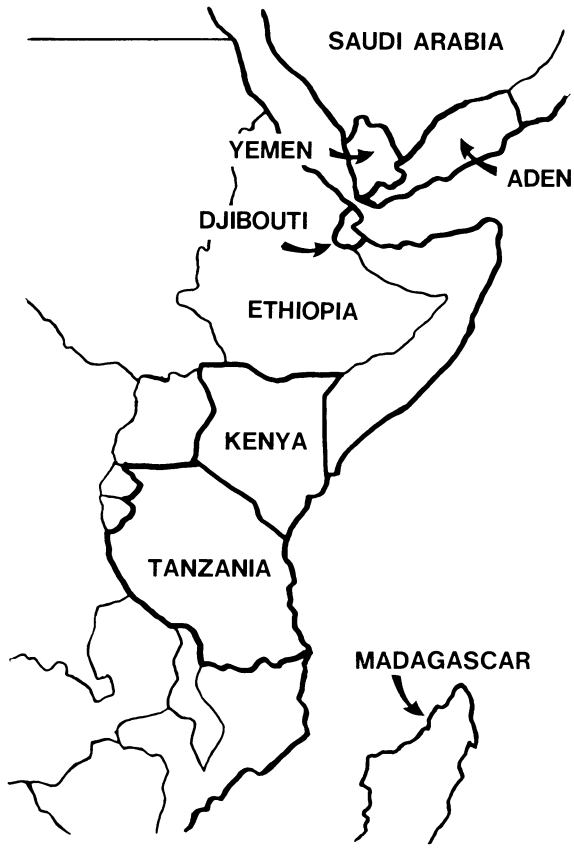

Figure 4. *Khat leaves are chewed in vast amounts in Yemen, Aden, Djibouti, Ethiopia, Kenya, and to a considerable extent in Tanzania and Madagascar. (Art by William May, University of Miami)*

Khat is least commonly consumed as a beverage. Arabs in the interior of southern Arabia make tea of the leaves. In southern Turkestan and in parts of Afghanistan east of Kabul, dried leaves are added to boiling water and boiled for 15 minutes. In Yemen, Muslem priests drink an infusion or decoction of the leaves sweetened with honey. A bitter decoction in water or milk, with honey added, has been given to halt dysentery.[50] In South Africa, khat is used mostly by bushmen who make a tea of the leaves and also chew the young shoots.[51] Ethiopian Christians have often made khat into beer (*talla*) or wine (*tedji*).[52,53]

Khat is primarily a masticatory, and vast amounts of the leaves (actually a young twig with 10 leaves, weighing about 1 g) are so employed in the Province of Harar, Ethiopa, the center of origin and culture; in Yemen, southern Arabia, where it was in use in 1429;[54,55] and in Djibouti; and by Muslems, especially in the Meru District of Kenya. There is also considerable use in Tanzania[45] and Madagascar.[56,57]

In Yemen, rich and poor alike conduct business from 9 a.m. until noon, go home, have a meal followed by coffee, and then chew khat 'til 3 p.m. They return to work, and then, 2 hours after sunset, resume chewing khat until dawn, sleeping only 3 or 4 hours.[50] Moser, in 1917, found that in the cool village of Sanaa, merchants chewed khat all afternoon without closing up shop.[55] In other areas, it is said that Muslems, reading the Koran, indulge in khat from 9 a.m. til noon, then eat and drink local beer. Around Harar, farmers have breakfast, chew khat for 2 to 3 hours (1/4 to 3/4 kg), work for 2 to 3 hours, eat lunch, and chew khat again.[58] In Ethiopia and Somaliland, a "fistful" of leaves at a time (with or without the twig[59]) is chewed for up to 10 or 15 minutes[53,54] and then swallowed with the aid of water (the poor) or milk (the rich). Some Ethiopians chew as much as 2,500 g of khat per day.[58] The toothless and elderly and those who can obtain only overmature or dry leaves pound the leaves in a mortar, add sugar and spices and water, to make a pasty ball, which is then chewed and swallowed.[53] On the other hand, some people chew the leaves, gargle with water, and then spit the saliva, water, and leaf wad into a cuspidor repeatedly.[50] In recent years, khat has been adopted by students facing scholastic examinations and by truck drivers and other motorists to avoid drowsiness on long distance trips.[60]

Physiological Effects of Khat

Khat contains several alkaloids of the phenylalkylamine group. The plant is highly variable physically,[61] and the content of the main stimulant, cathinone (-) α-aminopropiophenone)[45,62] differs with the locale, the type, age of the plant, and the plant part.[63] Young shoots, very fresh, are richest in cathinone, also cathine, the secondary intoxicant, and in tannin. The stimulant properties decline quickly, and khat leaves are regarded as fresh only for 4 days and are sold as rapidly as possible. In Ethiopia, the highest grade, called "Kudda", is believed to be extra-potent because of attack by a leafhopper, the damage resembling a virus disease.[58] When transported to markets (formerly by camel caravan or railway), the leafy shoots are first wrapped in fresh grass or leaves and then covered with dry banana or palm leaves, burlap, or plastic bags to delay dehydration,[50,54,57,58,59] and they may be occasionally sprinkled with water.[54] In Madagascar, the shoots are usually sold unwrapped.[57] When Muslems travel through regions where no khat is available,

they carry dried khat, but this produces only a milder intoxication.[50] Ethiopia exports khat mainly to Aden and Djibouti, since 1942 mostly by air.[58]

It is claimed that most novice users feel no reaction (or only headache[59] and/or insomnia[55]) the first time khat is chewed. The second and subsequent experiences, often accompanied by smoking tobacco (which enhances the effects[64]), produce dilated pupils, initial excitement, euphoria, intense thirst (alleviated by water, milk, or beer), elevated temperature and blood pressure, profuse perspiration, loss of appetite, insomnia, eagerness for physical activity (couriers and soldiers run or march all night). Afterwards, there may be chills, somnolence or stupor, and dreams.[50,64,65] Long-range effects include constipation (due to tannin), blackening and deterioration of teeth, pink, "furry" tongue, impotence, emaciation, loss of memory, and general mental degeneration, and khat has been blamed for the frequency of cardiac complaints in Harar, as well as digestive disorders, esophagitis, and liver cirrhosis in Djibouti.[44,45,64,65,66] Large doses may cause vomiting, incoordination, collapse, even death.[65] Women may never indulge in the substance or may be allowed only the lower grades of khat not favored by men.[55,67]

In spite of its negative aspects, khat still has its advocates because it gives energy to the worker (though this is offset by tardiness and absenteeism[46]), and analyses show that it has appreciable levels of ascorbic acid, niacin, β-carotene, calcium, and iron.[63] These advocates do not take into account that the tannin content is an antinutrient interfering with the body's utilization of protein and also affects iron absorption[68] in addition to being increasingly implicated as a carcinogen.

Because khat is addictive, undeniably harmful, and absorbs a large part of a worker's income,[64] it has been banned a number of times in Ethiopia, Djibouti, Kenya, and Yemen. High taxes on imports have been imposed, but these are not fully effective due to smuggling from Ethiopia. In Yemen today, khat is legally consumed only 2 days a week, but illicit trade continues. With the increase of migration of East African people to other countries, who bring their customs with them, khat is surely going to become a widespread health problem. Dr. Abraham Krikorian reports that the substance is being exported to Britain, where it was first introduced in the mid-1800's,[50] and there is an active demand for it among the many Arabs living in Brooklyn, New York, who hope to be able to import supplies by air. It is certainly time to make its hazards known to governments and the general public.

REFERENCES

1. Morton, J.F. Plant tannins and esophageal cancer, *In*: Deichmann, W. (ed.) Toxicology and Occupational Medicine (Proc. 10th Inter-Amer. Conf. on Toxicology and Occupational Medicine), Elsevier, North Holland, 129-137 (1979).
2. O'Gara, R. Biologic screening of selected plant material for carcinogens. *Cancer Res.* 28:2272 (1968).
3. O'Gara, R.; Lee, C.; Morton, J.F. Carcinogenicity of extracts of selected plants from Curacao after oral and subcutaneous administration to rodents. *J. Natl. Cancer Inst.* 46:1131 (1971).
4. O'Gara, R.; Lee, C.; Morton, J.F.; Kapadia, G.J.; Dunham., L.J. Sarcoma in rats from extracts of whole plants and from fractionated extracts of *Krameria ixina*. *J. Natl. Cancer Inst.* 52:445 (1974).

5. Morton, J.F. Further associations of plant tannins and human cancer. *Quart. J. Crude Drug Res.* 12:1829 (1971).
6. Morton, J.F. Tentative correlations of plant usage and esophageal cancer zones. *Econ Bot.* 24:217 (1970).
7. Morton, J.F. Folk Remedies of the Low Country. E.A. Seemann, Miami, Florida (1974).
8. Morton, J.F. Plant products and occupational materials ingested by esophageal cancer victims in South Carolina. *Quart. J. Crude Drug Res.* 13:2005 (1973).
9. Zaldivar, R. Geographic pathology of oral, esophageal, gastric, and intestinal cancer in Chile. *Z. Krebsforsch.* 75:1 (1970).
10. Murillo, A. *Plantes medicinales du Chili.* Sec. Chilienne, Exposition Universelle de Paris, Paris, France (1889).
11. Castro, P.; Gerente Tecnico, Quimica ITD, La Victoria, Lima, Peru, (personal communication 1987).
12. Kurihara, M.; Aoki, K.; Tominaga, S. (eds.) Cancer mortality statistics in the world. Univ. Nagoya, Nagoya, Japan (1984).
13. Kaiser, H.E. Cancer-promoting effects of phenols in tea. *Cancer* 40:614 (1967).
14. Kaiser, H.E.; Bartone, J.C. The carcinogenic activity of ordinary tea. *J. Natl. Med. Assn.* 58:361 (1966).
15. Segi, M. Tea-gruel as a possible factor for cancer of the esophagus. *Gann* 66:199 (1975).
16. Shimizu, T.; Kinki, I.; Masuda, C. A ten-year follow-up study of the relationship between tea gruel and gastric cancer. *Nippon Koshu Eisei Zasshi* 27:237 (1980).
17. Kapadia, G.J.; Paul, B.D.; Chung, E.B.; Ghosh, B.; Pradhan, S.N. Carcinogenicity of *Camellia sinensis* (Tea) and some tannin-containing folk medicinal herbs administered subcutaneously in rats. *J. Natl. Cancer Inst.* 57:207 (1976).
18. Morton, J.F. Search for carcinogenic principles. *In*: Swain, T.; Kleiman, R. (eds.) The Resource Potential in Phytochemistry, Plenum Publishing Company, New York (1980).
19. Morton, J. The potential carcinogenicity of herbal tea. *Envir. Carcino. Revs.* (*J. Envir. Sci. Health*) C4:203 (1986).
20. Roberts, E.A.H. The chlorogenic acids of tea and mate'. *Chem. and Indus.* 37:985 (1956).
21. Ullah, M.R. Time of plucking and tea quality. *Two-and-a-bud* 14(1):14-15 (1967).
22. Werkhoven, *J. Tea processing.* Food and Agriculture Orginization U.N., Rome, Italy (1974).
23. Joy, T.A. Green tea manufacture – Japanese style. *Planters' Chron.* 80:205 (1986).
24. Nakagawa, M. Constituents in tea leaf and their contribution to the taste of green tea liquor. *Jap. Agr. Res. Quart.* 5:43 (1970).
25. Hilton, P.J. The effect of shade upon the chemical composition of the flush of tea (*Camellia sinensis* L.). *Trop. Sci.* 16:15 (1974).
26. Cabral, O. South American tea. *Agr. in the Americas* 2:11 (1942).
27. Anon. 'Gaucholand' (Rio Grande do Sul) attracts Americans. *Brazil. Bull.* 26(492):8 (1968).
28. Porter, R.H. Mate – South American or Paraguay Tea. *Econ. Bot.* 4:37 (1950).
29. Anon. Take your choice – tea or yerba mate'. *Investors Guardian.* Aug. 13, p. 440 (1971).
30. Anon. *Yerba mate'.* Pan Amer. Union, Washington, DC (1945).
31. Laserre, S.R.; Galharretborde, J.O. *Reordenamiento de la economia yerbatera. Plan integral.* Informe Tecn. Estac. Exper. Agrop. Misiones, Argentina (1966).
32. Anon. En tierra fertil de Misiones – la yerba mate. *La Nacion* (newsp.) Mar. 27, Sec. 4, Buenos Aires (1938).

33. Anon. Latin America's yerva – twenty million people drink it. *For. Agr.* 26:11 (1962).
34. Farnsworth, C.H. Gifts of the Americas – yerba mate'. *Agr. in the Americas* 7: 3 (1947).
35. Correa Filho, V. *Ervais do Brasil e ervateiros.* Serv. de Inform. Agricola, Rio de Janeiro (1957).
36. Ferreira Filho, J.C. *Cultura e preparo da erva-mate'.* Serv. de Inform. Agricola, Rio de Janeiro (1957).
37. Anon. US $3,000,000 firm formed to promote matte here. *Brazil. Bull.* Nov. 1, p. 3, 6 (1958).
38. Anon. Mate was discovered in a dream, so the Indians say. *Brazil. Bull.* Apr. 1, p. 7 (1962).
39. do Amaral, V.F. *Herva matte o cha' do Parana'.* Curytiba, Parana', Brazil (1903).
40. Anon. Mate'. *Fortune*, p. 36 April 15, (1937).
41. Eyer, E. Yerba mate' – su pasado y su futuro. *Anales Soc. Rural Argentina* 105:49 (1971).
42. Roosevelt, T. Through the Brazilian Wilderness. Chas. Scribner's Sons, New York (1914).
43. Prudente, A. Patologia geographica do cancer no Brasil. *Bol. de Oncologia* 46:281 (1963).
44. Gendron, Y.; Ardouin, C.; Lassalle, Y.; Sirol, J. Cancer of the esophagus in Djibouti: report of 36 cases collected in two years. *Bull. Soc. Pathol. Exot.* 70:74 (1977) (Abs. in ICRDB *Cancergram* Ser. CK03 No. 4 (Apr.).
45. Abebe, W. Khat: its medical importance. *SINET Newsletter* 6:1 (1983).
46. WHO Advisory Group. Review of the pharmacology of khat. *Bull. on Narcotics* 32:83 (1980).
47. Murray-Lyon, Ian, Consultant Physician and Gastroenterologist, Charing Cross Hospital, London, (personal communication 1988).
48. Anon. Coffee loses ground in Yemeni horticulture. *Arab Agr. 1987 Yearbook. In: Hort. Sci.* 23:247 (1988).
49. Anon. Miraa can take you to 'heaven' and back. *The Standard* (newsp.) June 30, p. 15, Nairobi, Kenya (1986).
50. Krikorian, A.D. Kat and its use: an historical perspective. *J. Ethnopharmacol.* 12:115 (1984).
51. Watt, J.M.; Breyer-Brandwijk, M.G. Medicinal and Poisonous Plants of Southern and Eastern Africa. E. and S. Livingstone, Ltd., Edinburgh (1962).
52. Trellu, M. The pharmacodynamics of khat. *Bull. on Narcotics* 11:43 (1959).
53. Anon. Khat. *Bull. on Narcotics* 8:6 (1956).
54. Peters, D.W.A. Khat: its history, botany, chemistry and toxicology. *Pharm. J.* 169:17, 36 (1952).
55. Moser, C. 'The flower of Paradise' – the part which khat plays in the life of the Yemen Arab. *Natl. Geog. Mag.* 32:173 (1917).
56. Anon. *Catha edulis* (khat): some introductory remarks. *Bull. on Narcotics* 32:1 (1980).
57. Anon. Conclusions and recommendations of the Expert Group on the botany and chemistry of khat. *Bull on Narcotics* 32:65-66 and 7 pls.; also Selected Bib. on khat, pp. 95-99 (1980).
58. Hill, B.G. Cat (*Catha edulis* Forsk.). *J. Ethiopian Stud.* 3:13-23 (1965).
59. Getahun, A.; Krikorian, A.D. Chat: Coffee's rival from Harar, Ethiopia. I. Botany, cultivation and use. *Econ. Bot.* 27:353 (1973).
60. Asuni, T.; Pala, O.A. Drug abuse in Africa. *Bull on Narcotics* 38 (1/2):55 (1986).
61. Nordal, A. Khat: pharmacological aspects. *Bull on Narcotics* 32:51 (1980).
62. Klein, M. Review – analysis of khat constituents by the U.N. Lab., Geneva, Switzerland. *Microgram* 12:186 (1979).

63. Krikorian, A.D.; Getahun, A. Chat: Coffee's rival from Harar, Ethiopia. II. Chemical composition. *Econ. Bot.* 27:378 (1973).

64. Anon. The medical and social problems of khat in Djibouti. *Bull. on Narcotics* 9:34 (1957).

65. Margetts, E.L. Miraa and myrrh in East Africa – clinical notes about *Catha edulis*. *Econ. Bot.* 21:358 (1967).

66. Kennedy, J.G. The Flower of Paradise. D. Riedel, Dordrecht, Neth. (1987).

67. Kervingant (Dr.). The consumption of khat in French Somaliland. *Bull. on Narcotics* 11:42.

68. Halbberg, L. Bioavailability of dietary iron in man. *Ann. Rev. Nutr.* 1:123 (1981).

TANNIN-INSECT INTERACTIONS

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ABSTRACT

Significant evidence exists that dietary tannin can reduce growth and fecundity of some insect species. However, few studies have identified clear physiological or toxicological impacts of tannins on insect herbivores; some have suggested that tannins are positive nutritional factors. Insect species that feed on tanniniferous plant tissues often appear able to tolerate dietary tannins and may even be stimulated to feed by their presence. Alkaline midgut pH, surfactants, and the peritrophic membrane all may help these species tolerate moderate tannin concentrations in the diet. Reduced growth of adapted species at high tannin concentration may represent metabolic costs, not direct tannin effects. New evidence suggests an important impact of dietary tannin on microbial enemies of insects. The study of tannin-insect interactions has been hampered by poor communication between chemists and biologists.

INTRODUCTION

Ecologists, entomologists, biologists, and the few chemists interested in tannin-insect interactions have generally addressed two broad questions:

1. Is there a significant impact of plant tannins on insects?
2. Do tannins protect plants?

The first of these areas can be broken down further into related questions:

1a. If there is an impact, what is its form?

1b. How are any potential impacts mediated by insect traits (e.g., adaptations)?

The second broad question is really beyond the scope of this review, but experiments designed to answer it have provided inferences about tannin impacts on insects. Hence, these studies cannot be ignored here.

Researchers interested in any of the above questions generally have taken one of three approaches. The largest group of studies employs correlations between measures of leaf chemistry and growth, final mass, fecundity, mortality, or food choice of some experimental insect to infer a functional role for dietary tannin. In many studies, correlations between measures of leaf chemistry and damage to the plant have been used to infer impact of that chemistry on insects. This is a very weak experimental approach, since no study can measure all leaf variables (including non-tannins) or other factors working to protect plants from attack, and the action of any one leaf trait may be more complex than damage estimates indicate. Finally, there is a limited number of amended-diet experiments in which the investigator has attempted to influence the various insect parameters listed above directly by adding materials to artificial or more natural diets.

This selective review summarizes the findings of these kinds of studies as they relate to the functional questions posed above. The study of tannin-insect interactions is only about 20 years old, and the number of published studies is relatively small. Moreover, progress has been slowed by confusion about the identities and characteristics of the very diverse plant phenolics and by lack of agreement about analytical methods. Some of these difficulties are identified below, and their impact on the state of our current understanding is discussed. Finally, some of the more profitable and interesting directions that research on tannin-insect interactions might take are suggested.

POTENTIAL AND REALIZED TANNIN IMPACTS

There is widespread agreement that great potential exists for physiological and behavioral impacts of tannins on herbivorous insects.[1] A fact most frequently cited is that tannins bind with proteins.[2] In theory, this could result in reduced digestibility of food protein,[3,4] inhibition of digestive enzymes,[5] and a possibly repellent taste sensation, astringency.[6] Paul Feeny's[3,4] seminal investigation of the relationship between feeding by the winter moth (*Operophtera brumata*) and tannin concentrations in oak leaves introduced these possibilities to ecologists. Since then, most studies have been aimed at identifying some negative impact of plant tannins on insect growth, fecundity, or feeding.

The vertebrate toxicology literature suggests that tannins may have diverse toxic effects on herbivores. A variety of toxic and dietary deficiency symptoms can be related to tannin consumption in chickens,[7,8] rats,[9] and even humans.[10] Nonetheless, there are no true toxicological studies of tannins in insects.

Dietary tannins can be implicated as growth-reducing agents for some insect species. Feeny showed that growth rates of winter moth larvae were negatively correlated with condensed tannin concentrations in *Quercus robur* leaves.[4] Gypsy moth (*Lymantria dispar*) growth, fecundity, and individual egg weight are all reduced as much as 30 percent as a function of hydrolyzable tannin (gallotannin) concentrations and protein-binding capacity in red oak (*Quercus rubra*) leaves.[11,12]

Similar results have been obtained with a variety of insect species, especially Lepidoptera larvae (Table 1).

In a few cases, these correlation studies have been backed up by experiments employing tannin-amended diets. For example, Feeny[3,4] was able to reduce winter moth larval growth by adding leaf powder and purified tannins to artificial diets, thus isolating the effects of tannins from those of leaf toughness and nutrient concentrations. Growth of several Lepidoptera species, including *Heliothis zea* and *Earias vitella* larvae, can be reduced by incorporating cotton condensed tannins in laboratory diets.[13,14,15] Several other studies have shown similar effects (Table 1). The impression that dietary tannin reduces insect growth and fecundity is by no means universal. Indeed, there are about as many correlation and amended diet studies finding no effect (or even positive effects) of dietary tannin as there are findings of negative impact (Tables 2 and 3). Very few tannin-damage correlations have shown tannins to be effective in preventing defoliation (Tables 1 and 2).

Overall, there are about as many studies finding no net negative tannin impact on insects as there are showing a negative effect. Although tannins can affect insect survival, most studies have focused on growth rates and final weights. This situation probably represents a kind of "sampling bias," since few workers have treated tannins as toxins.

Most of the negative effects, especially those obtained at relatively low tannin concentrations, involve species that do not ordinarily feed on tannin-containing diets and/or have no evolutionary history of feeding on such diets. The best examples involve insects fed cotton condensed tannin. All of the insect species studied feed commonly on other plants that do not produce condensed tannins; most are pests on various field crops. There is evidence[15] that these species focus their feeding activity on plant tissues low in tannin concentration; they are actually tannin-avoiders (see below). It is no surprise that one can demonstrate negative consequences for these insects when they are forced to ingest tannins.

It is far more unusual to find studies yielding a negative impact of dietary tannin on insect species that commonly feed on tannin-containing diets. Those that have found negative effects have done so at quite high tannin concentrations (e.g., 12-20 percent dry weight or higher). Apparently, some insect species possess traits that make them tolerant of dietary tannin, although these traits can be overcome (or overwhelmed) at high concentrations. These potential adaptations are discussed below.

MODE OF TANNIN ACTION IN INSECTS

Several assumptions made by Feeny[3,4] or arising from his work, have shaped studies of tannin-insect interactions. Most investigators have focused on the potential antidigestive or antiassimilation activity of dietary tannin. Usually, it has been assumed that a negative association should be found between tannin content of the diet and insect growth, assimilation efficiency, and/or fecundity (which is positively correlated with mass in many insect species). Limited reviews[1,16,17] of these studies have failed to find the evidence for antidigestive effects compelling. Indeed, there is virtually no evidence that digestive processes are inhibited directly by dietary tannin (Tables 1-3). In most studies that have included detailed

Table 1. Negative Effects of Tannins on Insects.

Insect Studied	Factor Reduced	Tannin Source	Study Type[a]	Reference
Winter Moth	Growth	*Quercus robur*	PC	3
Winter Moth	Growth	*Quercus robur*	AD	3
Winter Moth	Growth, Assimilation	Six tree spp	PC	64
Gypsy Moth	Growth, Fecundity	*Quercus rubra*	PC	11
Spotted Bollworm	Growth, Survival	Cotton	AD	14
Cotton Bollworm	Growth, Survival	Cotton	PC	65
Cotton Bollworm	Growth, Digestion	Cotton	AD	13
Corn Earworm	Growth, Consumption	Cotton	AD	14
Pink Bollworm	Growth, Consumption	Cotton	AD	66
Tobacco Budworm	Growth, Consumption	Cotton	AD	15
Tobacco Budworm	Growth	Cotton	PC	15
Two-Spotted Spider Mite	Growth, Fecundity	Cotton	PC	15
Southern Armyworm	Survival, Growth	*Liriodendron tulipifera*	AD	22
Black Swallowtail	Survival	*Liriodendron tulipifera*	AD	21
Bruchid Beetle	Growth, Survival	*Vicia faba*	AD	67
Aspen Tortrix	Growth	*Populus tremuloides*	AD	68
Grasshoppers (8 spp.)	Conversion Efficiency	Quebracho, Tannic Acid	AD	25
Grasshopper	Consumption	*Pteridium aquilinum*	PC	69
Tobacco Budworm	Growth, Conversion Efficiency	*Geranium*	AD	70
Birch Casebearer	Growth	*Betula pendula*	PC	71
Oak Leafminer	Survival	*Quercus emoryi*	PC	37
Autumnal Moth	Growth	*Betula pubescens*	PC	72
Leafcutter Ants	Consumption	*Caryocar microcarpum*	PC	73
Leafcutter Ants	Consumption, Utility	*Inga edulis*, Tannic Acid	PC, AC	74, 75
Cereal Aphid	Consumption	Winter Wheat	PC	76
Migratory Locust	Consumption	Various	AD	31
Cabbage Butterfly	Consumption	Quebracho	AD	77
Alfalfa-Pea Aphid	Consumption	Tannic acid	AD	78, 79
Greenbug	Consumption	Tannic acid	AD	80

[a]Study types: AD = amended diet, PC = correlation between insect performance and diet chemistry, DC = correlation between damage to plant and plant chemistry.

Table 2. Studies Showing Lack of Tannin Effect on Insects.

Insect Studied	Factors Studied	Tannin Source	Study Type[a]	Reference
California Oakworm	Growth Fecundity Survival	*Quercus lobata* *Q. agrifolia*	PC	81
Gypsy Moth	Growth Fecundity	*Quercus nigra* *Q. rubra* *Q. prinus*	PC	12
Striped Oakworm	Growth Assimilation Nitrogen Use	*Quercus* (5 Spp.)	PC	49
Fall Cankerworm	Growth Assimilation Nitrogen Use	*Quercus* (5 Spp.)	PC	49
Corn earworm	Digestion	Cotton	AD	82
Heliconius 2 spp.	Growth Survival	*Passiflora*	PC	83
Spodoptera littoralis	Growth Survival	Fern (6 spp)	PC	84
Paropsis atomaria	Consumption Nitrogen Use	*Eucalyptus* (13 spp.)	DC	85
Cigarette Beetle	Survival	Cotton	PC	86
Tobacco Budworm	Growth	Cotton	PC	15
Pine Beauty Moth	Oviposition Growth Survival	*Pinus contorta*	PC	87
Spruce Budworm	Growth	*Abies balsamea* *Picea glauca* *P. mariana*	PC	88
Western Spruce Budworm	Growth	*Pseudostuga menziesii* *Abies spp.* *Picea spp.*	PC	89 90
Grasshoppers (2 spp.)	Growth	Quebracho Tannic Acid	AD	25
Unknown	Consumption	Various trees	DC	91

[a]Study types as in Table 1.

measures of digestive activity (e.g., the "Waldbauer measures"[18,19]), tannins have not been shown to reduce approximate digestibility of the ingested food.[20,21,22] Instead, the most frequently influenced digestive/assimilative measure is "efficiency of conversion of digested food" (ECD). This is a measure of the efficiency with which an insect converts digested food material into insect mass. A reduction in this measure generally suggests increased metabolic expenditures or respiration. The most reasonable cause for decreased ECD would be that some metabolic cost is involved in dealing with tannin, as in a detoxification system.[23] These results are not in agreement with the standard view that digestibility or assimilation ought to be inhibited by tannin.[16,17,21,22]

Little attention has been given to the possibility that more specific tannin-protein interactions might have other, more subtle, effects on insects.[1] For example, any particular enzyme with which condensed tannins have contact in the insect gut

Table 3. Positive Effects of Tannins on Insects.

Insect Studied	Factor Increased	Tannin Source	Study Type[a]	Reference
Gypsy Moth	Growth	Tannic acid	AD	33
Gypsy Moth	Growth	*Quercus rubra* *Q. primus*	PC	62
Gypsy Moth	Consumption	Tannic acid	AD	34
Winter Moth	Consumption	Tannic acid	AD	33
Euproctis chrysorrhoea	Consumption	Tannic acid	AD	33
Tree Locust	Consumption Growth	Tannic acid	AD	25
Oak Leaf Miner	Survival	*Quercus emoryi*	AD	37
Gall Wasp	Survival[b]	Oak Tannin	PC	39
Gypsy Moth	Survival[b]	*Quercus rubra* *Q. nigra*	PC AD	41
Gypsy Moth	Survival[b]	Tannic Acid	AD	42
Cabbage Butterfly	Survival[b]	Terminalia Chebula	AD	40

[a]Study types as in Table 1.
[b]Effect in presence of pathogen.

could be bound and inactivated, even under the alkaline conditions of some midguts.[1,23] Hence, specific functions, such as lipid or carbohydrate digestion and absorption, or water recovery, could be inhibited by tannins. No studies have addressed these possibilities.

Dietary tannin has been shown to cause midgut lesions in several insect species,[24,25] resulting in mortality probably due to septicemia.[24] Lesions are usually seen in insect species that do not encounter tannins in their normal diet and should not to be adapted to tannin.[16,24,25] Hydrolyzable, not condensed, tannins yield toxic and gut lesions. This is possibly due to the relative insolubility of the latter in the aqueous gut medium and their inability to pass through the peritrophic membrane in insects having one. This membrane, actually a loose protein meshwork, envelops the food bolus as it passes through the midgut of insects in the Lepidoptera and Orthoptera, among other taxa.[26] Presumably, initial stages of digestion occur inside this 'membrane'; products such as peptides then pass through pores for additional digestion in the gut lumen and absorption through the gut wall.[26] Feeny[14] found no evidence that condensed tannins ever left the peritrophic membrane of winter moth larvae, and Bernays found that tannic acid and gallic acid (but not condensed tannins) passed into the midgut lumen of certain grasshopper species.[16,25] Hence, two consequences appear likely. First, inhibitory activity by any tannin based on protein binding is likely to occur inside the peritrophic membrane; indeed, it would seem that tannins ought to bind to the membrane and could even occlude its pores. Second, hydrolysis products of hydrolyzable tannins may have toxic or other effects in the gut lumen, perhaps by interacting with the gut wall.

The impact on insects of phenolics modified by digestive conditions has not been investigated. The redox conditions of the lepidopteran midgut (below) should result in the production of modified phenolic structures and highly reactive quinones. Conditions may then favor the formation of amino, thioether, ether, and other linkages between quinones and proteins, with consequent inactivation (or even denat-

uration) of the proteins. These effects have been shown for viral proteins exposed to plant phenolics[27,28] but are uninvestigated for insect proteins (e.g., digestive enzymes).

Because tannins produce a taste sensation called astringency,[6] which has been shown to be repellent to vertebrates,[29,30] many investigators have sought deterrent activity in plant tannins (Tables 1-3). There is some evidence for this, including one study of structure-function relationships.[31] However, as is the case for many insect species apparently adapted to particular host plants, tannins also appear to serve as feeding cues and phagostimulants (Table 3). Again, researchers have rarely sought such an effect, so there is experimental bias against obtaining this result. Nonetheless, species like the gypsy moth focus their feeding activities primarily on plant species having secondary chemistries characterized by phenolics[32] and can be stimulated to eat more as a function of tannin concentration.[16,33,34] Whether such species are compensating for low food quality or merely recognizing "correct" or "superior" food based on their adaptive traits is unknown. In the few cases studied in any detail, there appears to be a threshold tannin concentration beyond which consumption, growth (or both) decline.[11,12,35]

A new area of investigation focuses on the indirect impacts of tannins on insects via their natural enemies.[36] Feeny[4] suggested that if tannins slowed insect growth and increased longevity, this ought to widen the time interval during which the insect is exposed to predators and parasites. Hence, feeding on tannin-rich diets would result in greater insect mortality from these sources. In the only reasonably detailed study of such interactions, Faeth and Bultman[37] found that adding oak tannin extracts to leaves in which leafminer (Lepidoptera) larvae were developing increased mortality due to parasitism. Their results suggested that the extracts may have attracted parasitoids to these sites, rather than having a reduced-growth influence on the host insects. Perrins[38] found that young birds ingesting tannin with (in) their insect food might experience reduced growth, although the effects did not appear great, and the consequences for the insects remain obscure.

More recently, several studies have identified significant positive tannin effects on insects, mainly in reduced mortality due to pathogens. It has been known for many years that woody plant galls are often rich sources of tannin. Taper et al[39] have shown that this accumulation of tannin – perhaps stimulated by the insect – appears to reduce growth of a fungus that is the major source of mortality for a gall-inhabiting wasp.

Strong circumstantial and limited experimental evidence[40] identifies tannins as responsible for reducing effectiveness of the bacterial pesticide, *Bacillus thuringiensis*. Both correlative and amended-diet studies have shown that hydrolyzable tannins can reduce the pathogenicity of the gypsy moth nuclear polyhedrosis virus fiftyfold.[41,42] In both of these examples, protein-binding is implicated as at least one mechanism involved. Other studies[43] suggest that viral inhibition could come about via the oxidative formation of quinones and ester-bonding with viral proteins. In any event, these tannin-pathogen interactions have the potential to yield much stronger impacts on insects at the population level than do any of the direct impacts described above.

In the cases of a positive tannin impact on insect growth (Table 3), two potential mechanisms may be involved. Bernays[44] postulated that digestive hydrolysis of tannic acid in certain grasshoppers may "spare" investment of substrates (mainly phenylalanine) in constructing the integument. Radiolabelled gallic acid from consumed tannic acid was found in the insects' integument, possibly reducing the insect's need to synthesize this or similar integumentary phenolics. There would also appear to be the potential for some insects to recover sugars hydrolyzed from ingested tannins as an energy source.

As Zucker[1] pointed out, tannins may influence insects in a variety of ways via a diverse set of mechanisms. As he also noted, it remains true that "...the discussion (of tannin effects) occurs in the virtual absence of... explicit chemical and biochemical arguments."

INSECT ADAPTATIONS TO TANNIN

It is clear that dietary tannin can have an effect on some herbivorous insects and that the most frequently documented effect is reduced growth. However, it is equally clear that many insect species are not affected, and negative impacts have been obtained with relatively high tannin concentrations. Some insect species are stimulated to feed by the presence of tannins, and some even appear to benefit from dietary tannin in various ways. Apparently, many insect species are adapted to dietary tannin and may use its presence to identify appropriate food, as predicted by coevolutionary theory.[45]

Several insect traits that would reduce the potential physiological impact of dietary tannin have been identified. Perhaps the most widely recognized is the ability of many insect species, especially Lepidoptera, to produce strongly alkaline conditions in the midgut, where most digestion occurs.[46] Indeed, the highest naturally occurring biogenic pH values known have been recorded from caterpillar guts.[47] At pH values above 9, a large fraction of tannin-protein complexes cannot be maintained.[48] Several Lepidoptera species that typically consume very high tannin concentrations with no evident ill effects have been shown to produce very high midgut pH values.[46,47,49] A review of the literature suggested that the midgut pH values of Lepidoptera species feeding on tanniniferous plant species were slightly, but significantly, higher than those feeding on non-tannin plant species.[50] However, virtually every study surveyed suffered from one or more very serious technical flaws, so that the value of this comparison is dubious. In addition, alkaline earth cation concentration counters the pH effect,[51] and very little is known about the distribution of these concentrations in nature. In addition, many tannin-bearing leaves are so strongly buffered that they can slow or eliminate the insect's ability to raise midgut pH.[46] Nonetheless, it is likely that many Lepidoptera larvae may gain some advantage from an alkaline midgut when consuming tannins.

There are problems with treating elevated pH as an adaptation to dietary tannins. First, many insects (e.g., grasshoppers) that regularly consume tannins do not have elevated gut pH; perhaps some other trait is equally effective. Second, Lepidoptera are virtually the only taxa producing such high pH values, and the evidence that differences among species relate to diet is very weak; essentially, all Lepidoptera have alkaline guts. Although this may explain the dominance of this

group in feeding on woody plants, the trait may be phylogenetically constrained, making its adaptive significance difficult to infer.[52] Third, the ability of condensed tannins to form complexes with proteins is a function of molecular structure and the protein's isolectric point.[23] As a result, at least some ingested condensed tannins ought to be able to bind under the alkaline conditions of any midgut, restricting the value of alkalinity to the insect. Finally, there are several advantages to alkalinity in an insect midgut that provide suitable alternative explanations for its existence. These include favoring the activity of epoxide hydrolases, alkaline phosphate, and arginase, aiding cleavage of lipids into triglycerides, and maintaining surfactant conditions necessary for lipid uptake. Hence, it is difficult to assert that alkaline midgut pH has evolved specifically as a response to dietary tannin, although it is likely to be beneficial in that regard.

A second potential insect adaptation to tannins is the existence of surfactants, mainly lysophosphatidylcholine (lysolecithin) and related compounds, in the midgut.[53,54] These detergents tend to dissociate tannin-protein complexes, and thus would seem useful if such complexes are important. It is clear that these compounds inhibit formation of tannin-protein complexes under simulated gut conditions.[53,54] Unfortunately, their occurrence and activity have only been studied in a few Lepidoptera species. Again, the question of their "adaptive" nature – whether they are evolutionary responses to dietary tannin – is unsettled. Because insects have no organ analagous to the vertebrate gall bladder, they do not produce bile, a steroidal detergent that creates the "critical micelle concentration" required for solubility and absorption of triglycerides. Lysophospholipid surfactants would appear to fill this role; without them, insects would not be able to obtain lipids from their diets.[55] Hence, there are several equally important "advantages" to the existence of surfactants in the midguts of insects. Indeed, all insects surveyed thus far possess such detergents.

A third mechanism by which insects could deal with dietary tannin comprises what might be called "trap" proteins. There is evidence that vertebrates may produce proline-rich salivary proteins that are competitively superior in binding tannins;[56] these may "trap" incoming tannins and bind them safely before they enter the digestive portion of the alimentary tract. Such proteins are unstudied in insects, but the several reports of heavy accumulation of tannins in and on the peritrophic membrane suggest that they may act in a similar fashion.[4,17]

Many insect species avoid tannin in selecting plant species and tissues to feed on. Among the cotton pests, most do poorly when force-fed cotton tannin, but tend to feed on low-tannin cotton tissues.[15] Some species exhibit rapid gut passage on tannin-containing food,[57] suggesting "avoidance" of tannin effects by accepting food quality losses and maximizing throughput. Although literally hundreds of insect species may feed on a given species of oak with little apparent consequence (for the insect), a very much greater number cannot feed on this genus, presumably in part because of the presence of tannins. There can be little doubt that tannins are a significant barrier to feeding by many insects, which recognize their presence and avoid plants containing them. Some apparently tannin-adapted species (e.g., the gypsy moth) do not appear capable of distinguishing among tissues that differ in tannin levels, at least not to the extent that they avoid high concentrations.[57] This

is one of several reasons why investigators rarely find damage-tannin correlations in nature (Table 2); physiologically adapted insects do not avoid high-tannin plants or tissues.

Unexplored (but likely) means for dealing with tannin should include associations with microbial symbionts capable of interfering with tannin effects, production of tannase enzymes by symbionts or the insect itself, and the existence of digestive or other enzymes conformationally protected from tannin binding.

PROBLEMS IN THE STUDY OF TANNIN-INSECT INTERACTIONS

The study of tannin-insect interactions by ecologists has suffered from three classes of serious flaws. These problems prevent us from drawing many serious general conclusions and have led to several misleading and incorrect views.

First, chemical analysis methods have often been weak, incorrect, or too broad. There is a lamentable tendency to use one or another Folin reagent to "quantify" phenolics.[58] Because the intensity of the blue color produced with this reagent varies with molecular structure as well as concentrations of phenolic groups,[58,59] it does not yield quantitative results unless the exact same structures are present in each sample. This is unlikely to be the case when comparing different species, tissues, individuals, or samples collected at different times. Moreover, Folin-type reagents do not identify "tannins," defined as phenolics having a binding affinity for proteins. Hence, many ecological papers are misleading (or incorrect) in claiming to have studied "tannins" when the assay was more broadly for "phenolics".

When tannins have been detected using an astringency (protein precipitation) assay, each investigator has tended to use a different protein as a binding substrate. Hence, most studies cannot be compared. Recent work[59] suggests that soluble tannin-protein complexes may be common; these are not detected with most astringency assays. Worse, the relationship between the assay and the phenomenon under study is obscure at best.[60] The only totally correct astringency assay would employ the actual proteins involved in the system under study. Of course, this is rarely possible because of the difficulty in identifying and purifying these compounds and obtaining quantitative yields.

Similarly, most investigators use purchased phenolics of variable quality and composition as standards for quantitative assays. This means that all results are expressed in relative units (e.g., percent tannic acid equivalents), the meaning of which is unclear. Again, studies cannot be compared when standards differ. Worse, the sources of standards are not always provided, their purity is uncertain, and availability is limited. The only useful approach is to purify the phenolics of the plant under study to some degree and develop one's own standards.[59,60,61]

There is little information available in the literature about the details of analytical procedures. For example, air- or oven-drying leaf samples before extracting phenolics seriously compromises quantitative analyses.[60,61,62] In my estimation, this means that 25-50 percent or more of published ecological "tannin" studies may be meaningless for this reason alone. The need to keep reagents or samples cool during analyses, precise timing needed for certain colorimetric reactions, and the presence of interfering compounds are all issues that the literature does not address.

Ecologists and physiologists do have some valid reasons for the lack of precision in phenolic analyses. Foremost among these is the tradeoff between precision and the number of samples that can be done. Many ecological studies focus at the population level and must account for natural variation among samples. As a result, the typical study involves dozens, or even hundreds, of samples. Most detailed, precise analyses take far too long to permit such studies, so ecologists have adopted methods from chemical taxonomy. Careful modification of some of these methods can produce replicable, quantitative results.[65] However, these methods have shortcomings, and individual investigators must overcome them independently, often for each plant species or sample type they work with. Add to this the observation that most ecologists are unfamiliar with chemical methods and are poorly equipped to do quantitative analysis, and it is not surprising that the literature is rife with incorrect methods and results.

The second shortcoming in studies of tannin-insect interactions is a failure to realize that insects may adapt to tannins. This error probably arises from uncritical acceptance of a statement by Feeny,[4] who claimed that the "broad, general" protein binding activity of tannins is a phenomenon that insects are unlikely to be able to overcome evolutionarily. We now realize that this guess was not correct,[1,23] but many investigators carry on as though it were. The most important consequence of this is that many investigators work under unrealistic conditions with unrealistic tannin concentrations. The insect gut has many characteristics that profoundly influence tannin and phenolic conformation and activity, so in vitro assays must be done in media that simulate these conditions. Insects that possess traits reducing the impact of tannins (above) are influenced only by higher tannin concentrations.[11,17] Investigators have rarely been careful to assure that their studies span the range of naturally-occurring tannin concentrations and have often worked with levels below those that can influence their insects. This yields an unrealistic estimate of tannin's influence in nature and provides another reason why tannin-damage correlations are rarely significant (Table 2). Only some fraction of leaves sampled in any survey many have tannin concentrations high enough to deter insects, and none may deter adapted herbivores.

The third problem with ecological tannin studies is common to all ecological studies: a failure to account for interacting factors. "Interacting factors" are of two types: chemical and ecological. Interacting factors of the first type include gut conditions, like cation and surfactant concentrations, pH, rate of passage, etc. For example, we have found[62] that tannins bind quantitatively to lysophosphatidylcholine, the most common midgut surfactant in Lepidoptera guts. The consequence for tannin activity is that there is a threshold amount of ingested tannin above which inhibitory surfactants are lost and tannin-protein binding can proceed. Moreover, loss of detergency in the gut should severely inhibit lipid absorption across the gut wall, producing a potentially serious nutritional deficit for the insect.

Most ecological studies are plagued with conflicting, interacting factors of the second type. Integrating potential tannin impacts, such as reduced growth and fecundity, increased survival when pathogens are present, reduced survival when predators are present, etc., is an enormous problem that may only be handled by modelling efforts. These effects are missed in tannin-damage correlation studies and

explicitly ignored in controlled, mechanistic, or laboratory, studies. The question, "what is (are) the ecological impact (s) of tannins on insects?" will never have a simple answer.

FUTURE DIRECTIONS AND NEEDS

Obviously, Question 1 (posed early in this chapter), "Is there a significant impact of tannins on insects?", is physiological and mechanistic in nature. Preliminary results suggest that its answer is "yes", and that tannins might be accorded a role in plants similar to that of other secondary metabolites that have negative influences on herbivores. However, confirmatory results have been slow in coming because the question is mainly of interest to ecologists who have no training or background in plant chemistry or physiology, and whose focus may not be closely mechanistic. Few chemists have addressed questions of phenolic function, and there has been little interaction between chemists and biologists on this issue. As a result, some biologists have forged ahead, adopting chemical methods that may not be useful or appropriate, and we are left with a misleading literature.

Upon reviewing the evidence, I cannot (as do some authors) conclude that tannins have no defensive function in plants, or that they are ineffective against insects. They may be no more effective than are other secondary compounds, and some of the classification schemes separating them functionally, ecologically, or evolutionarily from other compounds may be incorrect. However, their impact on nonadapted insects, dose-dependent effects on apparently "adapted" insects, high concentrations in many plants, significant synthesis costs, structural diversity, and widespread taxonomic distribution all suggest that they deserve serious study.

"Serious study" demands more interaction between chemists and biologists, so that chemically accurate and statistically rigorous characterizations of tannin extracts become possible. There is a clear need to consider the potentially diverse physiological activities, including those other than protein binding, of which tannins are capable. Attention must be focused on modifications tannins may undergo in the insect gut and on the impact of gut conditions on the activity of hydrolysis and other products. The complicated interplay of gut pH, buffering capacity of ingested foliage, redox potential, cation concentrations, passage rates, gut volume, and phenolic constituents of the food must be studied in a way that integrates these factors to understand how insects digest leaves.

Finally, the diverse ecological roles of tannins – those including insect and plant pathogens – need considerably more study. It is time to accept chemistry as a part of natural history, to acquire the means for its study under realistic conditions, and to integrate it with the other parts of complex ecological interactions for an understanding of the interactions between plant tannins and insects.

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REFERENCES

1. Zucker, W.V. Tannins: does structure determine function? An ecological perspective. *Amer. Nat.* 121:335 (1983).
2. Bate-Smith, E.D.; Swain, T. Flavonoid compounds. *In*: Florkin, M.; Mason, H.C. (eds.) Comparative Biochemistry, Vol. III, Academic Press, New York, (1962).
3. Feeny, P. Effect of oak leaf tannins on larval growth of the winter moth *Operopthera brumata. J. Insect Physiol.* 14:805 (1968).
4. Feeny, P. Seasonal changes in the oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565 (1970).
5. Feeny, P. Inhibitory effect of oak leaf tannins on the hydrolysis of proteins by trypsin. *Phytochemistry* 8:2119 (1969).
6. Bate-Smith, E.C. Haemanalysis of tannins: the concept of relative astringency. *Phytochemistry* 12:907 (1973).
7. Chang, S.I.; Fuller, H.L. Effect of tannin content of grain sorghums on their feeding value for growing chicks. *Poultry Sci.* 43:39 (1964).
8. Potter, D.K.; Fuller, H.L. Metabolic fate of dietary tannins in chickens. *J. Nutrition* 96:187 (1968).
9. Butler, L.G.; Rogler, J.C.; Mehansho, H.; Carlson, D.M. Dietary effects of tannins. *In*: Cody, V.; Harborne, J.B.; Middleton, E. (eds.) Biochemical Pharamocological, and Structure-Activity Relationships, A. R. Liss Inc., New York, (1986).
10. Singleton, V.L.; Kratzer, F.H. Plant phenolics. *In*: "Toxicants Occurring Naturally in Foods". Nat. Acad. Sci., Washington, DC (1973).
11. Rossiter, M.C.; Schultz, J.C.; Baldwin, I.T. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. *Ecology* 69:267 (1988).
12. Rossiter, M.C.; Schultz, J.C. (unpublished results).
13. Klocke, J.A.; Chan, B.G. Effects of cotton condensed tannin on feeding and digestion in the cotton pest. *Heliothis zea, J. Insect Physiol.* 28:911 (1982).
14. Sharma, H.C.; Agarwal, R.A. Effect of some antibiotic compounds in *Gossypiumon* the post-embryonic development of spotted bollworm (*Earias vittella*). *Ent. Exp. and Appl.* 31:255 (1982).
15. Hedin, P.A.; Jenkins, J.N.; Collum, D.H.; White, W.H.; Parrott, W.L. Multiple factors in cotton contributing to resistance to the tobacco budworm. *Heliothis virescens* F. *ACS Symp.* 208:347 (1983).
16. Bernays, E.A. Plant tannins and insect herbivores: an appraisal. *Ecol. Entomol.* 6:353 (1981).
17. Bernays, E.A. Tannins: an alternative viewpoint. *Ent. Exp. and Appl.* 24:44 (1978).
18. Waldbauer, G.P. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229 (1968).
19. Scriber, J.M.; Slansky, F. The nutritional ecology of immature insects. *Ann. Rev. Entomol.* 26:183 (1981).
20. Bernays, E.A.; Chamberlain, D.J.; Leather, E.M. Tolerance of acridids to ingested condensed tannin. *J. Chem. Ecol.* 7:247 (1981).
21. Berenbaum, M.R. Effects of tannins on growth and digestion in two species of papilionoids. *Ent. Exp. and Appl.* 34:245 (1983).

22. Manuwoto, S.; Scriber, J.M.; Hsia, M.T.; Sunarjo, P. Antibiosis/antixenosis in tulip tree and quaking aspen leaves against the polyphagous southern armyworm. *Spodoptera eridania, Oecologia.* 67:1 (1985).

23. Hagerman, A.E.; Butler, L.G. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* 256:4494 (1981).

24. Steinly, B.A.; Berenbaum, M. Histopathological effects of tannins on the midgut epithelium of *Papilio polyxenes* and *Papilio glaucus. Ent. Exp. and Appl.* 39:3 (1985).

25. Bernays, E.A.; Chamberlain, D.J.; McCarthy, P. The differential effects of ingested tannic acid on different species of Acridoidea. *Ent. Exp. and Appl.* 28:158 (1980).

26. Brandt, C.R.; Adang, M.J.; Spence, K.D. The periotrophic membrane: ultrastructural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata. J. Inv. Path.* 32:12 (1978).

27. Cadman, C.H. Inhibition of plant virus infection by tannins. *In*: Pridham, J.D. (ed.) Phenolics in Plants in Health and Disease, Pergamon Press, New York (1960).

28. Mink, G.I.; Huisman, O.; Saksena, K.N. Oxidative inactivation of tulare apple mosaic virus. *Virology* 29:437 (1966).

29. Mole, S.; Waterman, P.G. Tannins as antifeedants to mammalian herbivores - still an open question? *ACS Symp.* 250:572 (1987).

30. Tipton, K.W.; Floyd, E.H.; Marshall, J.C.; McDevitt, J.B. Resistance of certain grain sorghum hybrids to bird damage in Louisiana. *Agron. J.* 62:211 (1970).

31. Bettolo, G.B.M.; Marta, M.; Pomponi, M.; Bernays, E.A. Flavan oxygenation pattern and insect feeding deterrence. *Biochem. Syst. Ecol.* 14:249 (1986).

32. Barbosa, P.; Krischik, V.A. Influence of alkaloids on feeding preference of eastern deciduous trees by the gypsy moth. *Lymantria dispar* L. *Amer. Nat.* 130:53 (1987).

33. van Gornitz, K. Frassauslosende stoffe fur polyphagen holzgewachsen fressenden raupen. *Verhandl. Deutchen Gesell. fur Ange. Entomol.* 6:38 (1954).

34. Grevillius, A.Y. Zur Kenntnis der Biologie des goldafters (*Euproctis chrysorrhoea* L.) und der durch denselben verusachten Beshadigungen. *Botan. Zentral.* 18:222 (1905).

35. Glyphis, J.P. "Herbivory and Tannin Polyphenols in Mediterranean Ecosystems", PhD. Dissertation, Faculty of Science, University of Cape Town, South Africa, (1985).

36. Schultz, J.C. Impact of invariable plant chemical defenses on insect susceptibility to parasites predators, and diseases. *ACS Symp.* 208:267 (1983).

37. Faeth, S.H.; Bultman. Interacting effects of increased tannin levels on leaf-mining insects. *Ent. Exp. and Appl.* 40:297 (1986).

38. Perrins, C.M. Possible effects of qualitative changes in the insect diet of avian predators. *Ibis* 118:580 (1976).

39. Taper, M.L.; Zimmerman, E.M.; Case, T.J. Sources of mortality for a cynipid gall-wasp (*Dryocosmus dubiosus* (Hymentoptera: Cynipidae)): The importance of the tannin/fungus interaction. *Oecologia* 68:437 (1986).

40. Luthy, P.; Hofmann, C.; Jaquet, F. Inactivation of delta-endotoxin of *Bacillus thuringiensis* by tannin. *FEMS Microbiol. Letters* 28:31 (1985).

41. Keating, S.T.; Yendol, W.G. Influence of selected host plants on gypsy moth (Ledidoptera: Lymantriidae) larval mortality caused by a baculovirus. *Environ. Entomol.* 16:459 (1987).

42. Keating, S.T.; Yendol, W.G.; Schultz, J.C. Relationship between susceptibiliity of gypsy moth larvae (Lepidoptera: Lymantriidae) to baculovirus and host plant foliage constituents. *Environ. Entomol.* (in press).

43. Felton, G.W.; Duffey, S.S.; Vail, P.V.; Kaya, H.K.; Manning, J. Interaction of nuclear polyhedrosis virus with catechols: potential incompatibility for host-plant resistance against noctuid larvae. *J. Chem. Ecol.* 13:947 (1987).

44. Bernays, E.A.; Woodhead, S. Plant phenols utilized as nutrients by a phytophagous insect. *Science* 216:201 (1982).

45. Ehrlich, P.R.; Raven, P.H. Butterflies and plants: a study in coevolution. *Evolution* 18:586 (1964).

46. Schultz, J.C.; Lechowicz. Host plant, larval age and feeding behavior influence midgut pH in the gypsy moth (*Lymantria dispar* L.), *Oecologia* 71:133 (1986).

47. Dow, J.A.T. Insect midgut function. *Adv. Insect Physiol.* 19:187 (1986).

48. Schultz, J.C.; Baldwin, I.T.; Nothnagle, P.J. Hemoglobin as a binding substrate in quantitative analysis of plant tannins. *J. Agric. Food Chem.* 29:823 (1981).

49. Lawson, D.L.; Merritt, R.W.; Martin, M.M.; Martin, J.S.; and Kukor, J.J. The nutritional ecology of larvae of *Alsophila pometaria* and *Anisota senatoria* feeding on early- and late-season oak foliage. *Ent. Exp. and Appl.* 35:105 (1984).

50. Berenbaum, M. Adaptive significance of midgut pH in larval Lepidoptera. *Amer. Nat.* 115:138 (1980).

51. Martin, M.M.; Rockholm, D.C.; Martin, J.S. Effects of surfactants, pH, and certain cations on preciptation of proteins by tannins. *J. Chem. Ecol.* 11:485 (1985).

52. Gould, S.J.; Lewontin, R.C. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. Proc. R. Soc. Lond. B. 205:581 (1979).

53. Martin, M.M.; Martin, J.S. Surfactants: their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia* 61:342 (1984).

54. Martin, J.S.; Martin, M.M.; Bernays, E.A. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores: implications for theories of plant defense. *J. Chem. Ecol.* 13:605 (1987).

55. Turunen, S. Digestion and absorption of lipids in insects. *Comp. Biochem. Physiol.* 63A:455 (1979).

56. Asquith, T.; Mehansho, H.; Rogler, J.; Butler, L.G.; Carlson, D.M. Induction of proline-rich protein biosynthesis in salivary glands by tannins. *Fed. Proc.* 44:1097 (1985).

57. Schultz, J.C.; Keating, S.T. (unpublished results).

58. Swain, T.; Goldstein, J.L. The quantitative analysis of phenolic compounds. *In*: Pridham, J.B. (ed.) Methods in Polyphenol Chemistry, Pergamon Press, New York (1964).

59. Hagerman, A.E.; Butler, L.G. Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* (in press).

60. Martin, J.S.; Martin, M.M. Tannin assays in ecological studies: lack of correlation between phenolics, proanthocyanidins and protein- precipitating constituents in mature foliage of six oak species. *Oecologia* 54:205 (1982).

61. Hagerman, A.E. Extraction of tannin from fresh and preserved leaves. *J. Chem. Ecol.* 14:453 (1988).

62. Schultz, J.C. (unpublished results).

63. Baldwin, I.T.; Schultz, J.C. Rapid damage-induced changes in tree leaf chemistry and evidence of communication between plants. *Science* 221:277 (1984).

64. Wint, G.R.W. The effect of foliar nutrient upon the growth and feeding of lepidopteran larva. *In:* Lee, J.A.; McNeill, S.; Rorison, I.H. (eds.) Nitrogen as an Ecological Factor, Blackwell, London (1983).

65. Zummo, G.R.; Benedict, J.H.; Segers, J.C. No-choice study of plant-insect interactions for *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) on selected cottons. *Environ. Entomol.* 12:1833 (1983).

66. Elliger, C.A.; Chan, B.G.; Waiss, Jr., A.C. Relative toxicity of minor cotton terpenoids compared to gossypol. *J. Econ. Entomol.* 71:161 (1978).

67. Boughdad, A.; Gillon, Y.; Gagnepain, C. Influence des tanins condenses du tegument de feves (*Vicia faba*) sur le developpement larvaire de *Callosobruchus maculatus*. *Ent. Exp. and Appl.* 42:125 (1986).

68. Bryant, J.P.; Clausen, T.B.; Reichardt, P.B.; McCarthy, M.C.; Werner, R.A. Effect of nitogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix (*Choristoneura conflictana* (Walker)), *Oecologia* 73:513 (1987).

69. Cooper-Driver, G.; Finch, S.; Swain, T.; Bernays, E.A. Seasonal variation in secondary plant compounds in relation to the palatability of *Pteridium aquilinum*. *Biochem. Syst. Ecol.* 5:177 (1977).

70. Klocke, J.A.; Van Wagenen, B.; Balandrin, M.F. The ellagitannin geraniin and its hydrolysis products isolated as insect growth inhibitors from semi-arid land plants. *Phytochemistry* 25:85 (1986).

71. Bergelson, J.; Fowler, S.; Hartley, S. The effects of foliage damage on casebearing moth larvae. *Coleophora serratella,* feeding on birch. *Ecol. Entomol.* 11:241 (1986).

72. Haukioja, E.; Niemela, P.; Siren, S. Foliage phenols and nitrogen in relation to growth, insect damage, and ability to recover after defoliation in the mountain birch *Betula pubescens* ssp. *tortuosa, Oecologia* 65:214 (1985).

73. Kawanishi, K.; Raffauf, R.F. *Caryocar microcarpum:* an ant repellent and fish poison of the Northwest Amazon. *J. Nat. Prod.* 49:1167 (1986).

74. Nichols-Orians, C.; Schultz, J.C. (unpublished results).

75. Seaman, F.C. The effects of tannin acid and other phenolics on the growth of the fungus cultivated by the leafcutting ant. *Myrmicocrypta buenzlii. Biochem. Syst. Ecol.* 12:155 (1984).

76. Leszczynski, B.; Warchol, J.; Niraz, S. The influence of phenolic compounds on the preference of winter wheat cultivars by cereal aphids. *Insect Sci. Applic.* 6:157 (1985).

77. Jones, C.G.; Firn, R.D. Some allelochemicals of *Pteridium aquilinum* and their involvement in resistance to *Pieris brassicae. Biochem. Syst. Ecol.* 8:187 (1979).

78. Bennett, S.E. Tannic acid as a repellent and toxicant to alfalfa weevil larvzae. *J. Econ. Entomol.* 58:372 (1965).

79. Schoonhoven, L.M.; Dersken-Koppers, I. Effects of secondary plant substances on drinking behaviour in some Heteroptera. *Ent. Exp. and Appl.* 16:141 (1973).

80. Todd, G.W.; Getchum, A.; Cress, D.C. Resistance in barley to greenbug. *Schizaphis graminum* L. Toxicity of the phenolic and flavonoid compounds and related substances. *Ann. Entomol. Soc. Am.* 64:718 (1971).

81. Puttick, G.M. Utilization of evergreen and deciduous oaks by the California oak moth. *Phryganidia californica, Oecologia* 68:589 (1986).

82. Chan, B.G.; Waiss, A.C.; Lukefahr, M. Condensed tannin, an antibiotic chemical from *Gossypium hirsutum. J. Insect Physiol.* 24:113 (1978).

83. Smiley, J.T.; Wisdom, C.S. Determinants of growth rate on chemically heterogeneous host plants by specialist insects. *Biochem. Syst. Ecol.* 13:305 (1985).

84. Ottosson, J.H.; Anderson, J.M. Seasonal and interspecific variation in the biochemical composition of some British fern species and their effects on *Spodoptera littoralis* larvae. *Biol. J. Linn. Soc.* 19:305 (1983).

85. Fox, L.R.; Macauley, B.J. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia* 29:145 (1977).

86. Yokoyama, V.Y.; Mackey, B.E. Protein and tannin in upper, middle, and lower cotton plant strata and cigarette beetle (Coleoptera:Anobiidae) growth on the foliage. *J. Econ. Entomol.* 80:843 (1987).

87. Leather, S.R.; Watt, A.D.; Forrest, G.I. Insect-induced chemical changes in young lodgepole pine (*Pinus contorta*): the effect of previous defoliation on oviposition, growth and survival of the pine beauty moth. *Panolis flammea*. *Ecol. Entomol.* 12:275 (1987).

88. Mattson, W.J.; Slocum, S.S.; Koller, C.N. Spruce budworm (*Choristoneura fumiferana*) performance in relation to foliar chemistry of its host plants. *U.S. For. Serv. Tech. Rpt.* 85:55 (1983).

89. Wagner, M.R.; Blake, E.A. Western spruce budworm consumption - effects of host species and foliage chemistry. *U.S. For. Serv. Tech. Rpt.* 85:49 (1983).

90. Cates, R.G.; Redak, R.A.; Henderson, C.B. Patterns in defensive natural product chemistry: interactions. *ACS Symp.* 208:3 (1983).

91. Coley, P.D. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 53:209 (1983).

92. Schmitt, M.D.C.; Czapowskyj, M.M.; Allen, D.C.; White, E.H.; Montgomery, M.E. Spruce budworm fecundity and foliar chemistry: influence of site. *U.S. For. Serv. Tech. Rpt.* 85:97 (1983).

ARE TANNINS RESISTANCE FACTORS AGAINST RUST FUNGI?

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ABSTRACT

Histological observations were made of pine callus cultures and seedlings inoculated with *Cronartium quercuum* f. sp. *fusiforme*. Growth of fungus in susceptible tissues caused cellular disruption, which was severe during sporulation of the fungus. Tannin deposition appeared closely related to invasion of host cells by fungus. Tannin synthesis was associated with membrane activity and increased starch accumulation. Although tannin accumulated in resistant tissues, initial events of resistance were changes in cell volume, decreases in stain affinity for host nuclei, and a deterioration of fungal hyphae. Thus, tannin accumulation was not found to play a primary role in resistance.

INTRODUCTION

Forest management practices and spring weather patterns that disseminate *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* in certain years in the South shift the ecological balance in favor of the rust fungus. The result is devastating outbreaks of fusiform rust in yellow pines in certain areas.[1,2] High early mortality and stem distortion reduce yields and lower wood quality.[3,4] Progress has been made in controlling disease in nurseries with fungicides; however, control of fusiform rust in the millions of acres of loblolly (*Pinus taeda* L.) and slash (*P. elliottii* Engelm. var. *elliottii*) pine plantations is feasible only by gradual replacement of susceptible pines with those having one or more types of resistance. To meet this goal, the forest pathologist and other specialists must identify trees with natural resistance, combine different types of resistance through natural or artificial breeding, and propagate large numbers of these trees that possess adequate resistance.

Trees resistant to fusiform rust can be selected in highly infected plantings and may have one or several genes for resistance. Such genes have been difficult to identify because virulence of the pathogen varies, causing trees resistant in one location to be susceptible in another. Thus, markers of genetic resistance are needed.

One possible marker is the accumulation of tannin during active resistance to infection. In this study, tannin was examined in a large number of pine families inoculated with different fungus isolates. The study was warranted because tannins and other phenols have been found in many host:fungus pathogen relationships.[5,6,7,8] Scientists have reported that deposition of tannins appears to parallel limitation of pathogen development,[9,10,11] suggesting that tannins may play a role in resistance to pathogenic fungi. In this study, however, tannin accumulation was found to be greatest in pine cells colonized by the fungus rather than in cells resisting the fungus.

MATERIALS AND METHODS

Initially, over 10,000 loblolly and slash pine seedlings with rust symptoms were cut at the Resistance Screening Center near Asheville, North Carolina. These were inoculated as previously described.[12,13] All seedlings with symptoms were sliced four to eight times in transverse section to detect tannins in the pith zone. Tannin identity was verified by light microscopy of random specimens.

Propagation of tissue cultures, seedlings, and fungus for experiments at Gulfport has been described previously.[14,15] Inoculations were made according to methods given in Griggs and Walkinshaw[16] and Walkinshaw and Bey.[15]

The experimental design for microscopical studies was eight seedlings per family, eight families per replication, and three replications per experiment. Nine isolates of the fungus were used in the first six experiments; only two were used in the other four. Specimens for histological observations were fixed, dehydrated, embedded, and sectioned as reported elsewhere.[11,14,17] Tissues were fixed at 21 and 60 days in all experiments. Additional specimens were fixed at 9, 14, 30, 45, and 180 days in three of the experiments. Sporulating host tissues were fixed 9 months after inoculation for spermagonia and at 2 years for aeciospore production. Uninoculated tissue cultures were fixed 7 to 21 days after subculturing. Cultures with fungus were fixed 30 days after inoculation.

Specimens for histology were stained according to schedules that have been published.[11,18] Additionally, a new stain schedule was used for rapid differentiation of pathogen and host. Sections were placed in xylene for 5 minutes or longer to remove paraffin; dipped 15 times each in 100, 95, and 50 percent ethyl alcohol; and stained 1 minute in a 50-percent solution of ethyl alcohol containing 0.5 g of fast green (C.I. No. 42053), rose bengal (C.I. No. 45440), and acridine red (C.I. No. 45000). Then, sections were washed for two changes in absolute ethyl alcohol, dipped in xylene, and mounted with Pro-Texx (Lerner Laboratories, Inc.). This stain was suitable for both fresh (not fixed) and fixed tissues. Histochemical stains for tannin precursors and tannins were reported previously.[11,19] The Papanicolaou stain[20] was used to distinguish tannin from starch, primary, and secondary pine cell walls.

RESULTS

Deposition of tannin in infected loblolly and slash pines was not related to resistance of pine families in field trials (Figures 1 and 2). However, tannin was more prevalent in certain families of inoculated pines. Disease-related and normal tannin accumulation in the cortex could not be distinguished, therefore, tannin incidence in pith and inner xylem ray cells was chosen as a response variable.

Tannin was rare in the densely packed cells of the tissue cultures (Figure 3). Inoculated and uninoculated callus cultures of slash pines exhibited tannin in loosely connected cells in the periphery of callus (Figure 4). Infected callus had significantly more tannin in outer cells (Figure 5).

Tannin accumulated in slash pines inoculated by either technique.[12,15] Marked variation occurred in tannin deposited at the site of the stem infection and in invasion zones in the cortex, xylem, and pith. In susceptible pines, tannin accumulated with growth of the fungus (Figure 6) and increased with higher infection, an effect most apparent in pith tissues 60 days after inoculation. By 21 days after inoculation, the fungus was in the pith. By 60 days, it was throughout the pith. Tannin was low at 21 days and high at 60 days. In short, the fungus moved in and then the tannin. Had tannin been a resistance factor, the opposite condition would have occurred.

Highest tannin content in inoculated seedlings occurred in cortical cells supporting spore productions (Figures 7 and 8). Tannin cells surrounded sporulating hyphae in spermogonia. These hyphae and spores stained identically to hyphae in intact cortex tissue. Haustoria were abundant in cortical cells filled with tannin (Figure 9). After tannin accumulations in susceptible and resistant pines were

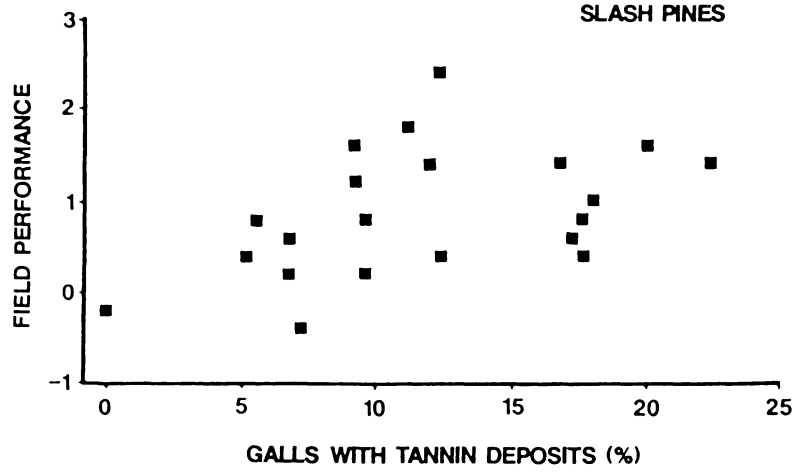

Figure 1. *Incidence of tannin in pith cells and field resistance indices for slash pine families. An index above 0.75 indicates useful resistance.*

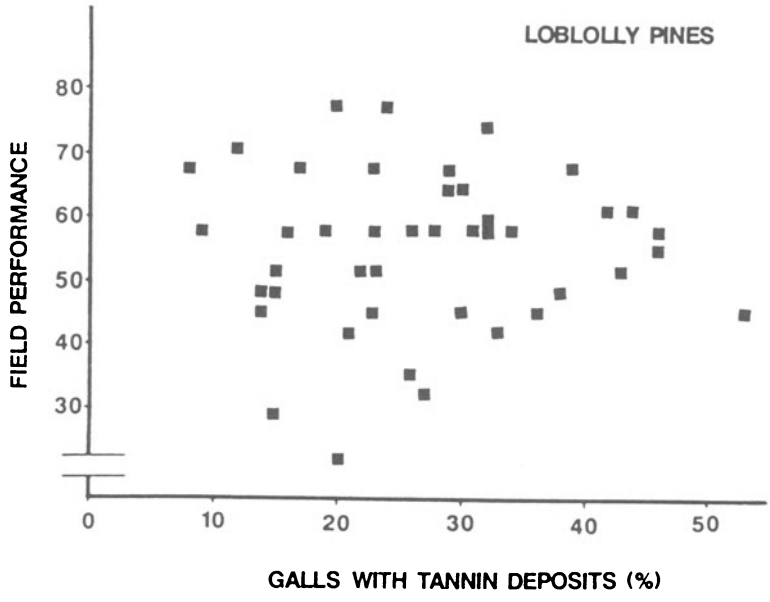

Figure 2. *Zero correlation between tannin deposition and field resistance for loblolly pines. Peformance above 60 indicates resistance.*

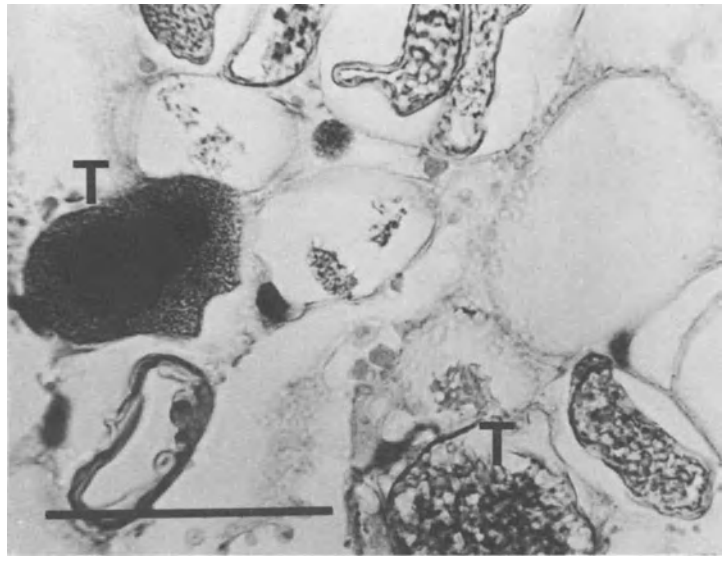

Figure 3. *Pine cells with nuceli (N) within callus. In Figures 3-12, scale bar is 50 microns, except for Figure 9.*

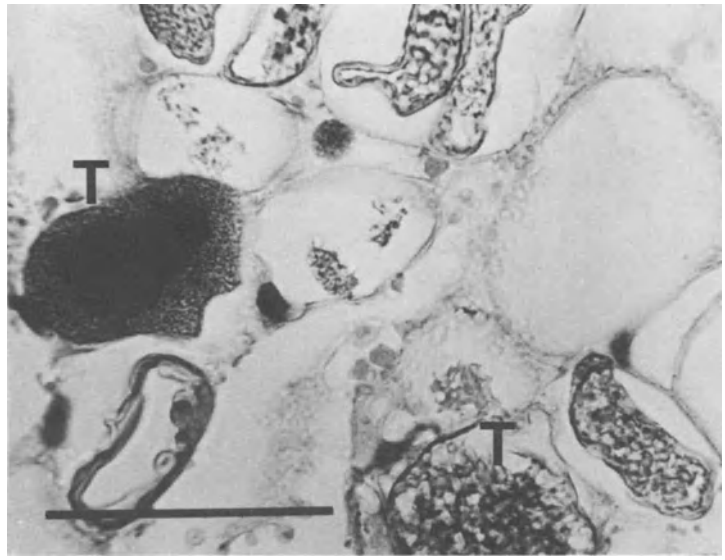

Figure 4. *Tannin cells (T) in periphery of slash pine callus culture.*

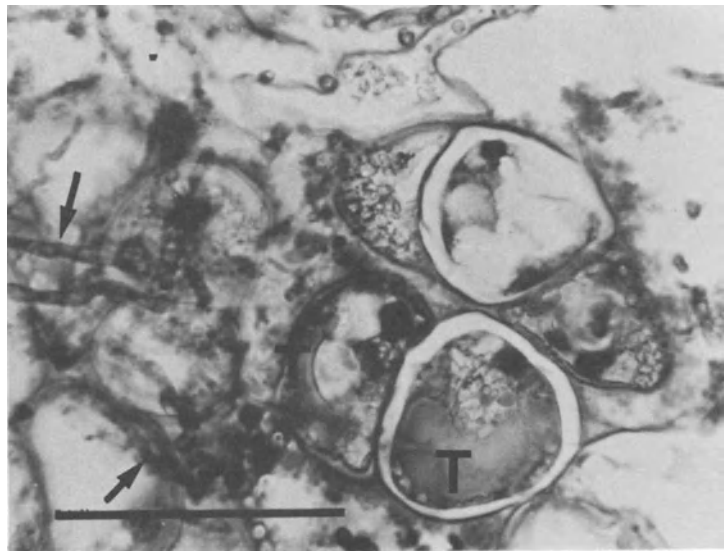

Figure 5. *Tannin cells (T) in slash pine callus inoculated with the rust fungus (arrows).*

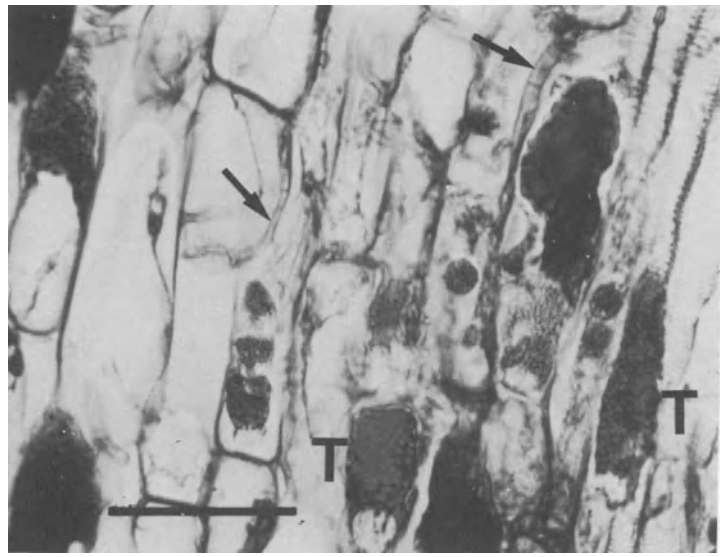

Figure 6. *Early infection in a susceptible pine stem. Fungus (arrows) and tannin (T) are prominent.*

identified, different stages of tissue reactions were observed. Growth of fungus in susceptible seedlings caused cell distortion, particularly in rays (Figure 10). The large rust hyphae (3 to 4 micrometers in diameter) appeared to separate many host cells, and tannin accumulation was maximum. At the site of invasion, the stem increased 400 percent in area 6 months after inoculation. After 1 year, the accelerated growth rate and tannin deposition decreased. Thereafter, tannin accumulation was low, except during bark formation and fungus sporulation.

Families with moderate field resistance reacted to fungal inoculation by excessive division of cambial or ray cells to form pockets of disoriented cells (Figure 11). Such abnormal growth, if extensive, caused girdling of stems, accumulation of starch above the girdle, and tannin accumulation in constricted tissues below the girdle.

Invading hyphae in highly resistant pine families[17] such as LA-11 grew to the cambium and stopped (Table 1). Few haustoria were seen, and tannin cells were rare except at the site of penetration. After 14 to 21 days, fungal hyphae appeared as a gel. Fungal cell walls appeared dissolved, and nuclei did not stain. Dying parenchyma cells in these resistant zones were enlarged. The basophilia of their chromatin was faded and stained abnormally (Figure 12). Pyknosis, nuclear vacuolization, and swelling of nuclei were common conditions. Nucleus and nucleoli often could not be observed. Necrotic cells stained acidophilic and anucleate. A variety of pigments was in these cells. Such cellular changes were seen frequently in cortical parenchyma cells next to the cambium 9 to 21 days after inoculation.

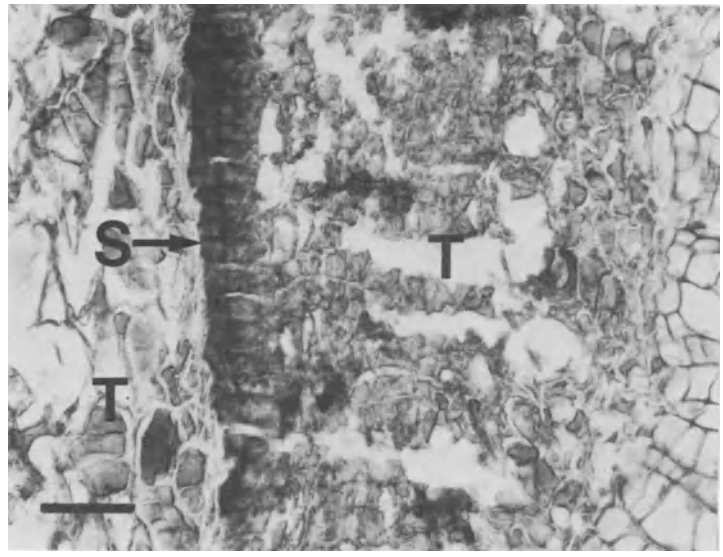

Figure 7. *Numerous tannin cells (T) surrounding a spermogonium (S) formed in the outer cortex.*

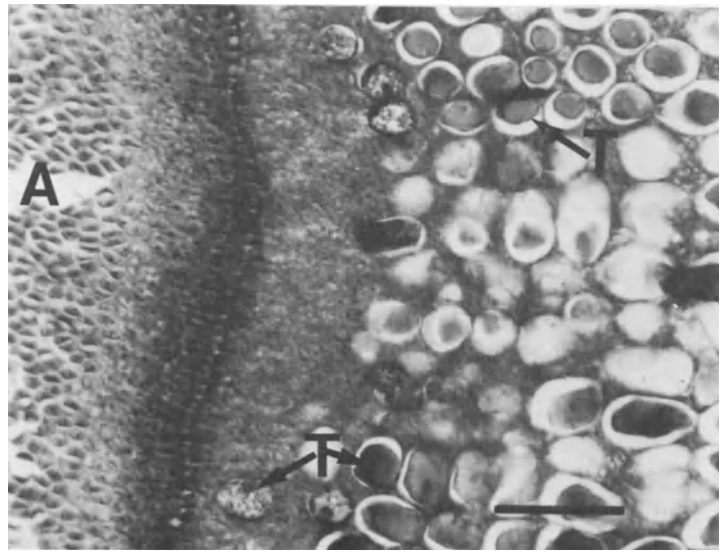

Figure 8. *Aeciospores (A) forming from fungus intermingled with tannin cells (T).*

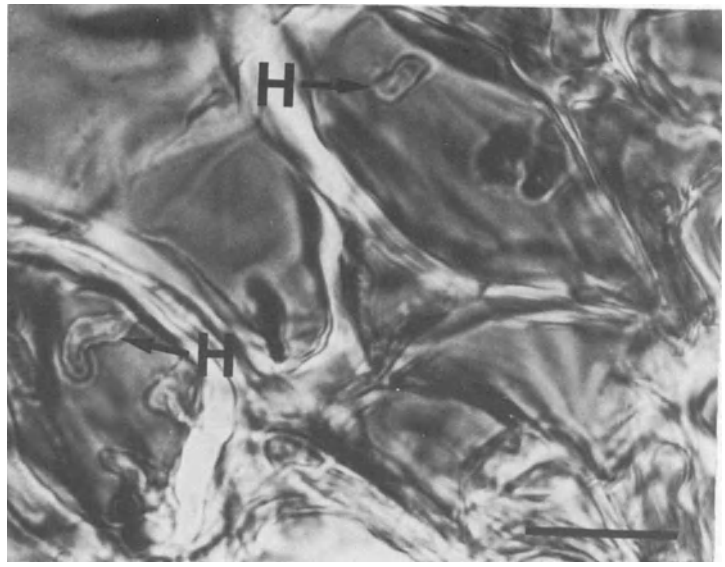

Figure 9. *Abundance of fungal haustoria (H) in tannin cells. Bar equals 10 microns.*

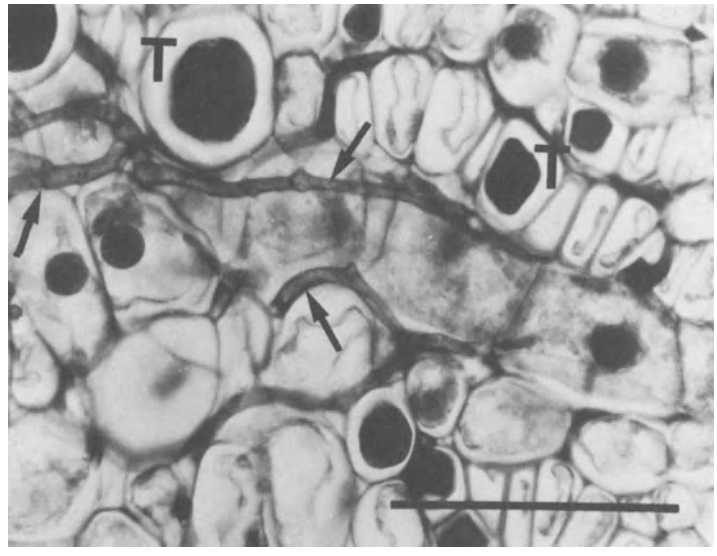

Figure 10. *Fungus (arrows), cell distortion, and tannin (T) in 21-day-old infection of a susceptible pine.*

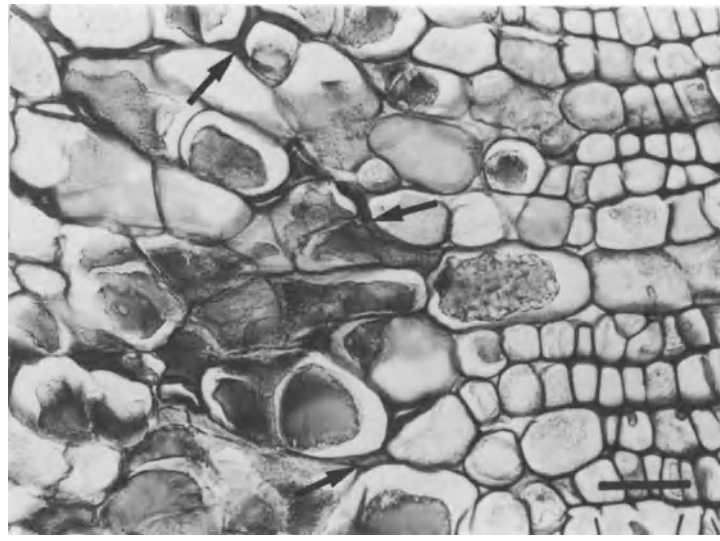

Figure 11. *Rust hyphae (arrows) and dying host cells without tannin 21 days in resistant pine tissue.*

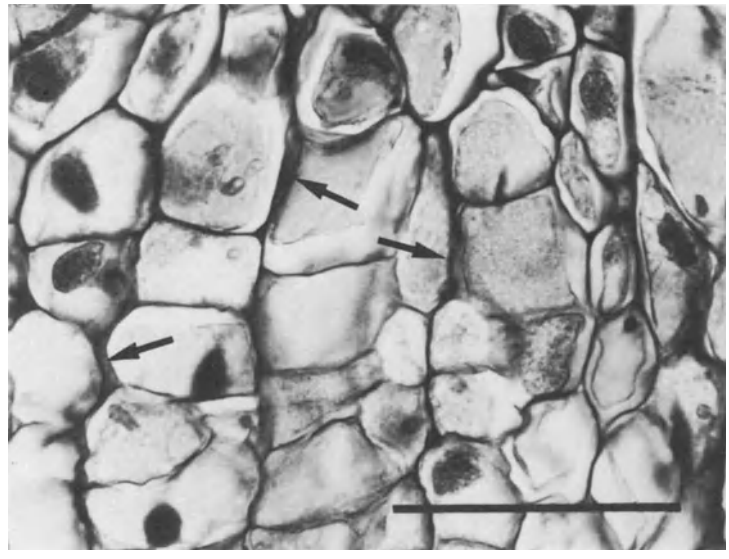

Figure 12. *Reduced nuclear staining, increased cell volume, and dying of rust hyphae (arrow) in early resistance reaction.*

Table 1. Variation in Growth of the Rust Fungus in the Cambium of Slash Pines 21 Days after Inoculation.

Female Parent	N[a]	Number with Fungus in Cambium	Percent with Fungus Growing in Cambium
18-62	28	28	75
8-7	80	29	59
LA-11	50	15	0

[a]Number of fixed sections observed.

Conclusions from fixed tissues were verified by histochemical tests on fresh specimens. The 2,4-dimethyoxybenzaldehyde test for tannin precursors was strongly positive for cells in the infection zone 9 days after inoculation. The pattern of reddish color included the fungus. Catechol tannins (nitroso reaction) were positive in epidermal and periderm cells with fungus. High metabolic activity (peroxidase, phenolase, and esterase) occurred in growing fungus, including those hyphae adjacent to tannin cells. Fungus haustoria had peroxidase and phenolase activities in cells, which were positive for tannin precursors.

DISCUSSION

Accumulation of tannin in pine cells and tissues examined in this study was not a marker for rust resistance. Tannin was easily observed as a polymerized, ergastic substance in fixed and fresh pine tissues. It occurred more frequently in susceptible than in resistant seedlings. A close association between tannin accumulation and growth of fungus was found during ray cell proliferation, invasion of cortical parenchyma, and fungus sporulation. As the fungus hyphae grew between cells, they broke the continuity of the cells. A similar breakage of cell bonds occurred in peripheral cells of infected and healthy callus cultures.

Many studies[21–26] support the view that tannin is synthesized and packaged in membrane units. In an earlier study, such units appeared intact after isolation by cell homogenization techniques.[14] When the isolated tannin globules were used in measuring respiration of pine cells, tannin was not toxic to pine cell metabolism.[14,25] The packaging of tannin closely parallels the compartmentalizing of enzymes in cell lysosomes.

Living fungal hyphae occurred in clumps of tannin cells, offering further evidence that tannins are bound within units. A variety of stains and peroxidase tests showed the hyphae in these clumps were alive. This lack of tannin toxicity was most evident in spermogonia, where living spores were surrounded by tannin cells. If the tannins are toxic to the rust fungus, they must be unable to pass through the cell walls or haustoria of the fungus. Tannin appears to be insoluble in the cell sap of pines, and cellular pH does not favor the formation of soluble complexes.[27–29] Even if host protein became denatured by tannins, it might still be a substrate for the fungus to use.

Fungi stimulate host respiration and synthetic pathways to accommodate their needs.[5,6,9,30] This was evident by high stain affinity and intense enzymic activity in tissues with hyphae. Storing carbon as starch or tannin accumulated in infected tissues would reduce tissue osmolarity. Excess aromatic compounds, which are pro-

duced by infected pines, could be stored as tannin after removal of any nitrogen for recycling. Observations of reaction zones in highly resistant pines (Figure 12) suggest that changes in host cell nuclei are among the first recognizable signs of resistance. Concurrent lysis of fungal hyphae probably resulted from chitinase[31] and other hydrolytic enzymes of the host.[32,33] These specific changes, rather than accumulations of secondary plant products, are more likely to explain the specific rust resistance among host strains.[15,16,34,35,36] Compatibility between invading hyphae and host cambium is essential for chronic infection.[16,18] Failure of the fungus to spread in the cambium is one marker of incompatibility (Table 1). The relationship between host and fungus in the cambium is delicate.

Besides cambial interactions, alterations in plasmodesmata number and distribution might be studied for effect on resistance and tannin accumulation. Plasmodesmata of ray parenchyma cells in pines regulate the flow of sugars and nutrients.[37] Any alteration of plasmodesmata by invading rust fungi might affect resistance and tannin accumulation.

REFERENCES

1. Miller, T.; Schmidt, R.A. A new approach to forest pest management. *Plant Disease* 71:204 (1987).
2. Phelps, W.R. Evaluation of fusiform rust incidence on loblolly and slash pine in the south. *Plant Disease Reporter* 58:1137 (1974).
3. Froelich, R.C. Sawtimber as an alternative forest management strategy for sites with a high fusiform rust hazard. *South. J. Appl. For.* 11:228 (1987).
4. Powers, H.R., Jr.; McClure, J.P.; Knight, H.A.; Dutrow, G.F. Incidence and financial impact of fusiform rust in the south. *J. For.* 72:398 (1974).
5. Arora, Y.K.; Wagle, D.S. Interrelationship between peroxidase, polyphenol oxidase activities, and phenolic content of wheat for resistance to loose smut. *Biochem. Phys. Pflanzen* 180:75 (1985).
6. Friend, J. Phenolic substances and plant disease. *In*: Swain, T.; Harborne, J.B.; Van Sumere, C.F. (eds.) Biochemistry of Plant Phenolics. Plenum Publishing Company, New York, pp. 557-588 (1979).
7. Kemp, M.S.; Burden, R.S. Phytoalexins and stress metabolites in the sapwood of trees. *Phytochemistry* 25:1261 (1986).
8. Misaghi, I.J. Physiology and Biochemistry of Plant-Pathogen Interactions. Plenum Publishing Company, New York, 287 pp. (1982).
9. Goodman, R.N.; Kiraly Z.; Wood, K.R. The Biochemistry and Physiology of Plant Disease. University of Missouri Press, Columbia. 433 p.. (1986).
10. Robb, J.; Harvey, A.E.; Shaw, M. Ultrastructure of tissue cultures of *Pinus monticola* infected by *Cronartium ribicola*. I. Prepentration host changes. *Physiol. Plant Pathol.* 5:1 (1975).
11. Walkinshaw, C.H. Cell necrosis and fungus content in fusiform rust-infected loblolly, longleaf, and slash pine seedlings. *Phytopathology* 68:1705 (1978).
12. Laird, P.P.; Phelps, W.R. A rapid method for mass screening of loblolly and slash pine seedlings for resistance to fusiform rust. *Plant Dis. Report.* 59:238 (1975).
13. Walkinshaw, C.H.; Dell, T.R.; Hubbard, S.D. Predicting field performance of slash pine families from inoculated greenhouse seedlings. USDA Forest Service Research Paper SO-160; Southern Forest Experiment Station, New Orleans; 6 pp. (1980).
14. Hall, R.H.; Baur, P.S.; Walkinshaw, C.H. Variability in oxygen consumption and cell morphology in slash pine tissue cultures. *Forest Sci.* 18:298 (1972).

15. Walkinshaw, C.H.; Bey, C.F. Reaction of field-resistant slash pines to selected isolates of *Cronartium quercuum* f. sp. *fusiforme*. *Phytopathology* 71:1090 (1981).
16. Griggs, M.M.; Walkinshaw, C.H. Diallel analysis of genetic resistance to *Cronartium quercuum* f. sp. *fusiforme* in slash pine. *Phytopathology* 72:816 (1982).
17. Walkinshaw, C.H.; Ammon, V.D.; Jewell, F.F. Sr. Comparison of slash pine seedlings of varying fusiform rust resistance. *In*: Barrows-Broaddus, J; Powers, H.R. (eds.) Proceedings of the Rusts of Hard Pines Working Party Conference, S2.06-10. pp. 67-87 (1984).
18. Jewell, F.F.; True, R.P.; Mallett, S.L. Histology of *Cronartium fusiforme* in slash pine seedlings. *Phytopathology* 52:850 (1962).
19. Mace, M.E.; Howell, C.R. Histochemistry and identification of condensed tannin precursors in roots of cotton seedlings. *Can. J. Bot.* 52:2423 (1974).
20. Croley, T.E.; Walkinshaw, C.H.; Baur, P.S.; Scholes, V.E. Papanicolaou staining technique for plant cells. *Stain Technol.* 48:259 (1973).
21. Hillis, W.E. Biosynthesis of Tannins. *In*: Higuchi, T. (ed.) Biosynthesis and Biodegradation of Wood Components. Academic Press, Inc.; New York, pp. 325-347 (1985).
22. Baur, P.S.; Walkinshaw, C.H. Fine structure of tannin accumulations in callus cultures of *Pinus elliottii* (slash pine). *Can. J. Bot.* 52:615 (1974).
23. Parham, R.A.; Kaustinen, H.M. On the site of tannin synthesis in plant cells. *Bot. Gaz.* 138:465 (1977).
24. Robinson, D.G. Plant Membranes. John Wiley and Sons, New York; 305 pp. (1985).
25. Cowles, J.R.; Fowler, J.A.; Walkinshaw, C.H. Comparison of isocitrate dehydrogenase activity, pyruvate kinase activity, and polyphenol content in physiologically different pine callus tissue. *Physiol. Plant.* 33:177 (1975).
26. Stafford, H.A. Compartmentation in natural product biosynthesis by multienzyme complexes. *In*: Conn, E. (ed.) The Biochemistry of Plants, Academic Press. New York, pp. 117-137 (1981).
27. Anderson, R.G.W.; Orci, L. A view of acidic intracellular compartments. *J. Cell Biol.* 106:539 (1988).
28. Hagerman, A.E.; Robbins, C.T. Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. *J. Chem. Ecol.* 13:1243 (1987).
29. Beart, J.E.; Lilley, T.H.; Haslam, E. Plant polyphenols–Secondary metabolism and chemical defense: some observations. *Phytochemistry* 24:33 (1985).
30. Pascholati, S.F.; Heim, D; Nicholson, R.L. Phenylalanine ammonia-lyase and susceptibility of the maize mesocotyl to *Helminthosporium maydis*. *Physiol. Plant Pathol.* 27:345 (1985).
31. Hedrick, S.A.; Bell, J.N.; Boller, T.; Lamb, C.J. Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *Plant Physiol.* 86:182 (1988).
32. Harvey, A.E.; Chakravorty, A.K.; Shaw, M.; Scrubb, L.A. Changes in ribonuclease activity in *Ribes* leaves and pine tissue culture infected with blister rust, *Cronartium ribicola*. *Physiol. Plant Pathol.* 4:359 (1974).
33. Gahan, P.B. Cell senescence and death in plants. *In*: Bowen, I.D.; Lockshin, R.A. (eds.) Cell Death in Biology and Pathology. Chapman and Hall, New York, pp. 145-169 (1981).
34. Shaw, M. Cell biological aspects of host-parasite relations of obligate fungal parasites. *Can. J. Bot.* 45:1205 (1967).
35. Abu-Zinada; A. -A.H.; Cobb, A.; Boulter, D. An electron-microscopic study of the effects of parasite interaction between *Vicia faba* L. and *Uromyces fabae*. *Physiol. Plant Pathol.* 5:113 (1975).
36. Gray, D.J.; Amerson, H.V. In vitro resistance of embryos of *Pinus taeda* to *Cronartium quercuum* f. sp. *fusiforme*: ultrastructure and histology. *Phytopathology* 73:1492 (1983).
37. Barnett, J.R. Changes in the distribution of plasmodesmata in developing fibre-tracheid pit membranes of *Sorbus aucuparia* L. *Ann. Bot.* 59:269 (1987).

THE BIOLOGICAL SIGNIFICANCE OF TANNINS: AN OVERVIEW

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ABSTRACT

This chapter provides an overview of the author's perceptions on the biological significance of tannins both with respect to a) their functions as specialized metabolites within plants and b) the consequences of the properties of these compounds with regard to their use as food or renewable materials by man. The former is, as shown in the preceding chapters, particularly undefined. Clarification of the role of polyflavanoids in plants must largely await the results of research yet to be performed. All chemists studying the hydrolyzable or condensed tannins no doubt share Edwin Haslam's "serious and nagging fear that a part at least of (their) scientific career(s) has been spent inspecting the loot in the garbage bin of plant metabolism".[1] With few exceptions, this same concern must be expressed about what we know about their significance to man. However, considering the advances in our knowledge of tannins as summarized in this volume, we all have reason to be confident that there are good reasons for the the large amount of photosynthetic energy that is expended in biosynthesis of these compounds and with some ingenuity man can take advantage of that synthesis in improving his well-being.

INTRODUCTION

The broad subject of the *biological significance of tannins* is naturally divided into two general areas: a) the function of tannins while in the plant, and b) their real or potential effect after the plant has been harvested and is subject to being eaten or processed in some way. Of the two, we probably have a greater understanding of the latter, although relatively little is known about either dimension.

Our understanding of the post-harvesting effects is greater because practically all of man's early medicine came from plant extracts, and the bulk of these extracts were what we are now calling tannins. These early extracts, largely made with hot water or alcohol, have been used with varying degrees of success to treat all kinds of maladies that bother man and animal. Headaches, fleas, snakebites, constipation, fever, and stomach problems are at least typical of the target of these extracted tannins.[2] Even the wine from the grape has for centuries been praised for its healing qualities. The Roman soldiers used to pour wine into wounds, and ancient Egyptian warriors mixed wine with unfamiliar waters of countries they invaded. We can cite at least one industrial use for tannins at a very early age. Leather production used tannins and no doubt gave us the term (see Chapters 1 and 31).

Learning about the effect of tannins within plants has had to wait until the sciences of chemistry and plant physiology developed. Much has been learned, but much remains to be learned. There is much disagreement among authorities as we become more specific about the substances in question. References are made at a later place in this review about some of the types of research that have been done in this area. We certainly know enough today to say that tannins are not inert substances in plants, but are very active in the life and well-being of plants.

We can also get general agreement about the thought that the use of the term tannins will be replaced as our knowledge grows: probably with several terms. The dictionary points out the problem as it tries to define the term. Webster says the following about tannins, "1. a yellowish, astringent substance, $C_{14}H_{10}C_9$, derived from oak bark, gallnuts, etc., and used in tanning, medicine, etc., 2. any number of similar substances." Hardly satisfying and certainly not suitable for our purpose in this book.

In discussing the biological significance of tannins, we are speaking about phenolic plant products based on gallic acid (the hydrolyzable tannins) and/or polyflavonoids (the condensed tannins).

What, then, do we know about tannins and plants? We know that plants have the ability to defend themselves against their enemies; those plants that could not meet this challenge no longer exist. This gives us a good Darwinian base to build on. We know that much of a plant's ability to defend itself is chemical in nature. We know that much of this chemistry can be extracted, that much of this activity survives the extraction process, and that much of it comes from tannins.

Linking the tannins to plant defense has not been tied down to everyone's satisfaction, but there is increasing evidence that this will be done. Phytochemists and plant pathologists have published widely on the chemistry and mode of action of plant defenses both in this country and across the world. Some of the literature is very specific about certain compounds and the enemy involved. Here are some typical examples:

1. A phosphorus-containing fraction associated with antiviral activity in *Nicotiana* spp. carrying the gene for location of TMV infection.[3]

2. Plant protection by activation of latent mechanisms for resistance.[4]

3. Allelochemicals: chemical interactions between species.[5,6]

One of the most interesting and encouraging things about the biological significance of tannins is breadth of the fields of study involved with the subject. Here is a list of some of the research fields who have published materials related to tannins:

- Phytochemistry
- Phytopathology
- Plant Physiology
- Animal and Human Nutrition
- Animal and Human Physiology
- Soil Chemistry
- Animal and Human Pathology
- Pharmacy
- Plant Genetics
- Entomology
- Organic Chemistry
- Plant Nutrition
- Mariculture
- Ecology
- Microbiology
- Fermentology

This list gives vivid testimony to the significance of the subject of this section. Tannins affect animal and human nutrition, hence, nutritionists must study tannins. Insects are affected by tannins, so entomologists must get involved. Tannins affect availability of plant nutrients, so soil chemists must get into the field. In a variety of ways, marine life is affected by nutrient availability, and this brings mariculture into the area. The opportunity for exchange of useful information is obvious as one looks at this list, and this must be encouraged and fostered as we move ahead.

A review of the literature reveals frequent references to the hazards and dangers that accompany tannins. These should come as no surprise, since in at least some instances we have good evidence that tannins were created to defend (hence cause) damage to an enemy. Very early, man learned of ample reasons to respect plant extracts. The American Indian knew that abortions could be caused by drinking a hot water extract from ponderosa pine needles. They knew that parts of the May apple could cause death; and, after all, it was Socrates who immortalized the extract from the hemlock plant.

It does not follow that panic and placards are called for as we learn that tannins can cause harm; after all, water can cause drowning if not treated with respect. We do know that man has lived in the presence of tannins since the beginning of time. Further, it is essentially impossible to eat any plant matter without ingesting tannins. This is particularly true of the fibrous foods that recent research has shown to be so beneficial. Actually, there is good reason to believe that the chemically beneficial part of fibrous foods are tannins. So, we are working here with a plant complex that is very active chemically and biologically. As in the case with most active chemicals, this activity will be heard from whether it is within a plant cell wall or within someone's stomach.

Before going further, we need to stress the biological effect of tannins in ways the plant never intended – that is, the residual effect outside the plant. We know

that an extract from the bark of a willow tree was used to ease the toothache of an early American, but the willow surely did not have this in mind when it formed salicylic acid in its bark. It is amazing that man learned this fact, amazing that this chemistry survived the extraction, and amazing that this taught us to produce aspirin! Here are some references that explore tannin chemistry and suggest productive areas for further effort:

- **Inhibition of urease activity in soils.**[7]
  Control of urease activity in the soil would help to control the level of available soil nitrogen.

- **Influence of dietary fiber on trace element balance.**[8]
  Dietary fiber in human diets has been shown to affect retention of zinc, iron, and copper.

- **Stimulation of plant growth by humic substances.**[9]
  Applications of humic acid to soils low in organic matter stimulates plant growth. Flavanoid compounds such as (+)-catechin can retard the growth of plants. The whole area of plant phenolic compounds relative to soil formation (see Chapter 23) deserves increased emphasis.

- **Influence of phenolic compounds on the growth of** *Goniotrichum elegans.*[10]
  The growth of this red algae was stimulated by coumarin. The area of plant phenolic's effects on aquatic biosystems needs further attention.

- **Nematicidal activity of aqueous extracts from raspberry canes and roots.**[11]
  Cane extracts inhibited nematodes – this was followed by addition of low levels of polyphenols that were also inhibitory.

- **Phenolic plant compounds function as reproductive inhibitors of** *Microtus montanus.*[12]
  Cinnamic acid levels in grass were shown to effectively inhibit reproduction in mice. What do we know of tannin's effects in this regard?

- **Role and function of humus in soil with emphasis on absorption of herbicides and chelation of micronutrients.**[13]
  Availability of both herbicides and nutrients is controlled by humus formed largely from litterfall (see Chapter 23).

- **Prevention of fungal infection of plants by specific inhibition of cutinase.**[14]
  Is protection against fungal infection by cutinase inhibition associated with tannin-protein-like complexation?

- **Influence of various purified and isolated cell wall fibers on the utilization of certain nutrients by swine and hampsters.**[15]
  Animal performance improved when purified diets were fortified with lignin-hemicellulose. Do tannin-carbohydrate complexes have beneficial or harmful effects (see Chapter 24)?

Except for man's early use of tannins in pharmacy, this useful area of the plant has been largely ignored, particularly during the last 100 years. We eat from a plant its seeds and fruits and stems, we use it for building, and we heat with it, make paper with it, and use it as fabric; but, we have been ignoring the useful tannin chemistry. Tannins are either ignored or destroyed in most modern industrial plants that process organic matter.

It is ironic to find that the impetus that exists today to exploit this area is coming not from an industrial desire to take advantage of a new profit opportunity, but from governmental pressure to protect the environment. Costs of destruction of agricultural residue are becoming a more significant factor and, when added to recovery value of the tannins, this is an increasingly attractive profit opportunity.

In considering the industry that processes plant matter, it is easy to think of it as being monolithic, and it is not. The problem in processes and profits for a paper company are quite different from that of a company that processes almonds, that produces rolled oats, or that produces flakeboard. It follows that changes will vary as we move from today's accepted methods to a system that includes tannin recovery.

Companies that process organic matter are going to accept the pattern of processing that we find in the oil-refining industry. A logical sequence of processes will develop in which the feedstock is partitioned into many useful products. Each step will take advantage of previous process inputs. Tannins will be an integral part of this new scheme.

As we move in the general direction of more completely harvesting the useful parts of plants, we need to keep our eyes on the prize. We periodically read where someone is developing a way to extract a biocide from a plant that can be applied to effect a natural control. This has happened, for rotenone and strychnine are good examples. It seems reasonable, however, to assume that the odds are overwhelming against finding any major tonnage of tannins moving in this direction.

A much better bet is to again use the refining industry as a model and search for intermediates and building blocks. Laks' work on the development of a wide range of biocides based on tannins (Chapter 32) is a fine example of such a process. This aim of looking beyond the biological significance of tannins as they exist in the plant or (even as they exist in an extract) opens up a new and fascinatingly fertile field as we consider useful building blocks for biologically active specialty chemicals from tannin sources. With this in mind, we need to support basic research in the following areas: 1) Chemistry of plant defenses. 2) Modes of action of plant defenses. 3) How plants produce tannins. 4) How tannin chemistry changes as extractions are made and stored. 5) Comparative chemistry of tannins from various species, from various plant parts, and from stages of plant maturity. 6) Screening of tannins for activity in various physiological uses. 7) Modification reactions that will impart additional biological activity.

Our objective in this fundamental work must be to match the chemical and biological opportunities that we find with needs and problems in the various disciplines. Learning (as the Utah researchers did[6]) that there is a relationship between the ingestion of cinnamic acid and mouse reproduction opens a potential field for a number of applications. Physiologists certainly must learn why this happens and see if they can use this information. Nutritionists for both humans and animals need to look for implications here. Pharmacologists certainly should want to probe this area further. The use of (+)-cyanidanol-3 for treatment of liver diseases in Europe is an example of potential high-value markets for tannin derivatives based on their biological activity.[16] This implies that heralding these findings is important in fully exploiting this area.

This field of the biological significance of tannins is amply demonstrated by the excellent papers that make up this section. These chapters will serve well those who are seeking to build upon their knowledge of tannins and biology. In this author's view, an exciting future lies ahead of us both in understanding the biological properties of these compounds and in finding ways to use this biological activity to better our lives.

REFERENCES

1. Haslam, E. Symmetry and promiscuity in procyanidin biochemistry. *Phytochemistry* 16:1625 (1977)
2. Kansas City Star 3/4/77.
3. Antignus, Y.; Sela, I.; Harpaz, I. A phosphorus containing fraction associated with antiviral activity in *Nicotiana* spp. carrying the gene for location of TMV infection. *Physiol. Plant Pathol.* 6:159 (1975).
4. Neth, J.K. Plant protection by activation of latent mechanisms for resistance. *J. Plant Pathol.* 83 (Suppl. 1) 463.
5. Whittaker, R.H.; Feeny, P.P. Allelochemics: chemical interactions between species. *Science* 171:757 (1977).
6. Waller, G. R. (ed.) Allelochemicals: Role in agriculture and forestry. ACS Symposium Series 330, American Chemical Society, Washington, DC. 606 p (1987).
7. Bremner, J.; Douglas, L. Inhibition of urease activity in soils. *Soil Biol. Biochem.* 3:297 (1971).

8. Sandstead, H. Influence of dietary fiber on trace element balance. *Am. J. Clin. Nutr.* 31:5180 (1978).
9. Bartlett, L. Stimulation of plant growth by humic substances. *Soil Sc. Soc. Am. J.* 40:6 (1976).
10. Fries, L. The influence of phenolic compounds on the growth of *Goniotrichum elegans.* Proceedings 7th Seaweed Symposium (1971).
11. Taylor, C.; Murant, A. Nematicidal activity of aqueous extracts from raspberry canes and roots. *Nematologica* 12:488 (1966).
12. Berger, S.; Gardner, N.. Phenolic plant compounds functioning as reproductive inhibitors of *Microtus montanus. Science* 195:575 (1977).
13. Stevenson, F.J. Role and function of humus in soil with emphasis on absorption of herbicides and chelation of micronutrients. *BioScience.* 22:643 (1972).
14. Maiti, I.B.; Kolattukudy, P.E. Prevention of fungal infection of plants by specific inhibition of cutinase. *Science* 205:507 (1979).
15. Fakey, F., Jensen, M. Influence of various purified and isolated cell wall fibers on the utilization of certain nutrients by swine and hamsters. *J. Food Sci.* 45:1675.
16. Conn, H.O. (ed.) International Workshop on (+)-Cyanidanol-3 in Diseases of The Liver, The Royal Society of Medicine, International Congress and Symposium Series No. 47. Academic Press, London (1981).

Specialty Chemicals

TANNIN-BASED WOOD ADHESIVES

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ABSTRACT

Past efforts aimed at replacing the phenolic material in wood adhesives by tannins of various origins have been extensive throughout the world, with research activity normally paralleling the price of phenol. With the exception of the use of "wattle" tannins for both thermosetting and cold-setting wood adhesives primarily in South Africa, success has been limited. More recent attempts (during the past 5 years) carried out in the United States to develop cold-setting wood adhesives for end-jointing and laminating lumber for structural applications requiring exterior glueline quality indicate substantial promise for the use of conifer tree barks as partial substitutes for resorcinol. These tannin-based, cold-setting adhesive formulations include an adhesive blend to be applied as a one-component system and a two-component "Honeymoon" system, each of which has been formulated either as face-laminating or end-jointing adhesives. In most formulations, half the resorcinol has been replaced by tannins extracted from the bark of southern pine trees.

INTRODUCTION

Because of their phenolic character, tannins from various sources such as tree barks or nutshells have stimulated interest as possible replacements for phenolic substances in adhesive resins. Although an in-depth study has led to publications by a large number of very excellent contributors, this chapter must make reference only to a small number of workers and summarize only the major steps in development. The excellent reviews by Pizzi,[1,2] Ayla and Weissman,[3] Hall,[4] Hemingway,[5]

Herrick,[6] and Roux and co-workers[7] quickly resulted in hundreds of publications describing the structure, reactions, and potential uses of tannins from different sources. Extensive research directed to the use of tannins as substitutes for phenol in wood adhesive formulations has been done in Australia, South Africa, New Zealand, Europe, and North America.

Even though research has been conducted worldwide, South Africa, largely through the efforts of Pizzi and his team, remains the only part of the world where tannin or its derivatives (in this case, tannin extracted from the bark of black wattle) is used as major component of wood adhesives in daily production in commercial operations. The South African research on wattle tannins has resulted in the replacement of phenol as well as resorcinol in wood adhesives for bonding plywood, composites such as particleboard, and fully structural exterior adhesives for the manufacture of laminated timbers.[1,2]

Efforts in Australia and New Zealand have pursued similar targets, in this case, using extracts from *Pinus radiata* bark. Compared to wattle tannin, the tannins from radiata pine bark react much more rapidly with formaldehyde, and such extracts tend to have disadvantageously high viscosities. At one time during the recent past, New Zealand Forest Products, Ltd., produced tannins from radiata pine bark in commercial quantities. Recently, this production was discontinued.

North American commercial wood bonding processes have involved the use of tannin rich fillers or extenders, such as ground bark fractions from Douglas-fir and alder. In addition, ground pecan shells and peanut shells are or have been used at some time. Major attempts were made by several northwestern wood products corporations to use alkaline bark extracts for the replacement of either phenol during resin preparation or of phenolic resin in the adhesive formulation. None of these projects has led to sustained commercial application (See Chapter 1 by Hergert for a review of the efforts of ITT-Rayonier to develop uses for tannins from western hemlock bark). The relatively low price of phenol in North America has discouraged investment of research funds directed to the use of tannins as substitutes for phenol. However, prices for resorcinol have remained at or above $1.80/lb. during the past 5 years, so costs for PRF and RF adhesives have remained high. For economic and technical reasons, research in North America has concentrated on the use of tannins as substitutes for resorcinol used in wood laminating and finger-jointing adhesives.

Work by Pizzi[8] and coworkers opened the door to tannin-based cold-setting adhesive application when the concept of "Honeymoon" adhesives[9,10] was applied to wattle tannin-based laminating and end-jointing adhesives. Pizzi found that good end-joints could be made with one of the adhesive components being a modified PRF resin and with the other component exclusively wattle tannin. We have pursued a similar application for the use of tannins extracted from conifer tree barks and various nutshells because of the very high formaldehyde reactivity of the 5,7-dihydroxy A-ring classes (i.e., procyanidin and prodelphinidin) of tannins (See Chapter 14 by McGraw for a review of the reactions of various tannins with formaldehyde). This chapter reviews progress that has been made over the past 5 years on the use of tannins (primarily those extractable from southern pine bark) in structural wood-laminating adhesives.

COLD-SETTING ADHESIVES FOR FACE LAMINATION

Tannin-Resorcinol Adducts

In our first attempt to formulate wood adhesives with tannins, extracts from southern pine bark were reacted with resorcinol under mild acidic conditions.[11] The objective of this reaction was to reduce the molecular weight of the tannin and at the same time provide a stable linkage between the procyanidin and resorcinol moieties (see Chapter 17 by Hemingway). A product in which 50 to 60 percent by weight of resorcinol was replaced by tannin with low viscosity and high reactivity with formaldehyde was obtained. Adhesive bond durability was good when the tannin extracts contained comparatively low levels of co-extracted carbohydrates. However, wood failure after vacuum-pressure water soak tests was low when carbohydrate contents were much greater than 25 percent.[12] In an attempt to solve this problem, the reaction products were purified of carbohydrates by dilution with water and liquid extraction with ethyl acetate, giving a mixture of predominantly tannin-resorcinol adduct plus unreacted resorcinol.[13]

This tannin-resorcinol product was used to replace all the resorcinol in a (more or less) "standard" phenol-resorcinol-formaldehyde resin preparation[13]. The resin was formulated for thermosetting adhesive use by addition of paraformaldehyde and walnut shell flour. One lay-up (2-ply, Douglas-fir, 6 x 8 x 1/2") was pressed under standard conditions, allowing a long room temperature cure time. Shear blocks (AITC/ANSI A 190.1-1983) were cut, then sheared either dry, after the AITC vacuum-pressure cycle, or after submersion in boiling water for 2 hours. The results in Table 1 showed that the bond quality was not high enough to pass AITC standards; however, these results were good enough to give encouragement for further exploration.

Table 1. Glue-Bond Quality of Douglas-fir Face Laminations Made with Tannin-Resorcinol Based Adhesives[a].

		Resin		
		1	2	3
Dry Shear (AITC-T107)				
Shear Strength	(kPa)	9100	7830	9690
	(psi)	1320	1140	1400
Wood Failure	(%)	0	80	95
Vacuum Pressure (AITC-T110)				
Shear Strength	(kPa)	2610	3390	110
	(psi)	870	490	17
Wood Failure	(%)	12	18	0
2-Hour Boil				
Shear Strength	(kPa)	10,420	5500	5340
	(psi)	1510	800	780
Wood Failure	(%)	85	78	85

[a]All values are averages of only two specimens.

For the second experiment, larger quantities of the tannin raw material were available. This made it easier to formulate the resins under better controlled conditions. Three resins, differing in viscosity and sodium hydroxide content, were prepared and formulated into adhesives. Table 2 summarizes the results that show much improved bond durability.

It became obvious that viscosity, sodium hydroxide content, degree and condition of advancement, and possibly the addition of a small amount of organic solvent, as practiced freely in this art, are of great influence for gluing capability, also for this type of wood adhesive, and will have to be adjusted to the appropriate levels in order to achieve consistently good results. Nevertheless, good gluing results again were obtained with normal spread levels, especially with Resin No. 8 at a spread level of 70 lbs. per 1,000 ft^2, as is normally used commercially for laminated structures when spreading adhesive film.

Due to the good performance of tannins as a replacement for resorcinol in PRF resins when such resins were formulated to be wood adhesives designed for timber laminations, other means were explored to obtain tannin or its usable derivatives, hopefully without the use of (expensive) organic solvents.

Table 2. Gluebond Quality from Tannin-Resorcinol Adduct Resins.[a]

		Resin			
		6	7	8	8
Spread Rate	lb.	70	70	70	40
	Dry Shear (AITC-T107)				
Shear Strength	(kPa)				
	(psi)	1680	1890	1800	1600
Wood Failure	(%)	62	82	92	97
	Vacuum Pressure (AITC-T110)				
Shear Strength	(kPa)				
	(psi)	510	610	780	1020
Wood Failure	(%)	52	88	70	35
	2-Hour Boil				
Shear Strength	(kPa)				
	(psi)	990	780	1020	1080
Wood Failure	(%)	88	95	87	72

[a] All values are averages of two specimens.

Tannin-Sulfonates

Tannin-sulfonates can be extracted from the ground bark of southern pine in yields of about 20 percent by using an aqueous solution of sodium sulfite and sodium carbonate.[14] Reaction of tannins of the procyanidin class with sulfite ion at 100 to 130 °C cleaves the interflavanoid bond with the formation of epicatechin-(4β)-sulfonate and oligomeric procyanidin-4-sulfonate derivatives.[15] Thus, the molecular weight was reduced while the phloroglucinol functionality has been retained, and

a product with excellent water solubility has been produced. Further reaction of epicatechin-(4β)-sulfonate with additional sufite ion at 130 °C did not result in cleavage of the pyran ring with sulfonation at the carbon α to the catechol ring. These reactions are discussed in more detail in Chapter 16. Studies of the desulfonation of various hydroxybenzyl sulfonic acids in alkaline solution showed that compounds with a phloroglucinol hydroxylation analogous to and including epicatechin-(4β)-sulfonate rapidly lose the sulfonic acid function at ambient temperature in base.[16] The quinone methide obtained from epicatechin could be captured by other strong nucleophiles or could prepolymerize to tannin type derivatives. Therefore, the initial excellent water solubility of the tannin-sulfonates is not a great concern when aiming to formulate resins that must, after the final stage of cure, result in glue lines with excellent water and boil resistance. The exact extraction conditions and resin formulations have been described before.[17]

An attempt was made to assess the potential reactivity of this tannin sulfonate product: to an aqueous 41 percent solids solution (0.5 g), 50 percent sodium hydroxide (0.04 g), and 0.5 g of a modified commercial PRF adhesive (PRF resin plus 10 percent paraform) were added. After this mixture was allowed to harden at room temperature for 2 days, it was ground and suspended in water. Observation of the aqueous suspension for several weeks showed that the solid material appeared unchanged. The aqueous phase had only a slight beige to light brown discoloration.

Following a further series of preliminary testing, Douglas-fir wood was parallel laminated using a number of different conditions and proportions:

Douglas-fir, 6 x 8 x 3/4 inch
Conditioned at 70 °F, 65% relative humidity
Pressing: 150 psi at 72 °F overnight (16+ hrs.)
Adhesive spread: not measured, squeeze-out limited

At this point, although very early in the game, we decided to explore – again on a very preliminary basis – the possibility of using the tannin extracts from southern pine as one of the two components in a "Honeymoon"[9,10] adhesive system as well as a mixed adhesive formulation. Results of these preliminary trials are summarized in Tables 3 and 4.

It should be pointed out that the experiments reported in Tables 1 through 4 were carried out for the purpose of orientation, and the data reported are based on an extremely small number of specimens. Nevertheless, the results do establish that good bonds can be made with tannin replacing approximately half of the PRF adhesive.

As a result of the encouraging results achieved with small laboratory tannin samples and, therefore, a very small number of glued wood specimens (Tables 1 – 4), Hemingway at the Southern Forest Experiment Station in Pineville, Louisiana, carried out a larger extraction yielding approximately 15 pounds of dry tannin sulfonate extract. The extract was designated 5-X. Despite many modifications of extract preparation conditions used subsequently, the gluing performance of this 5-X extract still ranks among the best of all extracts examined.

Table 3. Effect of PRF to Tannin Ratio on Gluebond Quality of Douglas-fir Laminates Bonded with a Mixed Adhesive.

PRF/Tannin Ratio		AITC-T107	AITC-T110	2 Hr. Boil
50/50				
	Shear Strength[a]	2,113	1,615	800
	Wood Failure	82	82	50
40/60				
	Shear Strength	1,567	267	1,953
	Wood Failure	70	40	100
30/70				
	Shear Strength	1,915	100	445
	Wood Failure	80	5	30
23/77				
	Shear Strength	1,672	0	370
	Wood Failure	80	2	30

[a]Shear strength values are expressed in psi and wood failure in %.

Table 4. Bond Quality of Laminates Made Using Sulfonated Tannins and PRF Resins in "Honeymoon" Systems.

		Extract A	Extract B
	Dry Shear (AITC-T107)		
Shear Strength	(psi)	1,288	1,509
Wood Failure	(%)	97	97
	Vacuum Pressure (AITC-T110)		
Shear Strength	(psi)	748	695
Wood Failure	(%)	97	90
	2-Hour Boil		
Shear Strength	(psi)	822	626
Wood Failure	(%)	88	88

The adhesive formulations and gluing conditions used in evaluation of the large 5-X extract as a substitute for PRF resins in face-laminating adhesives can be represented by the following examples. The PRF component of the adhesives A, B, and C were prepared as follows:

Borden's LT-75	50 g
FM-260 Hardener	10 g
Paraformaldehyde	5 g

This resin mix was aged for 10 minutes.
It was then blended with the following tannin solutions:

	Adhesive		
	A	B	C
41% Tannin 5-X Solution	50 g	50 g	50 g
50% Sodium Hydroxide	3.5 g	2.8 g	2.0 g

The billets glued were 6 x 8 inches, so the major determining factor for the effective spread rate was the squeeze-out. Results of the gluing tests with adhesive A are summarized in Table 5.

The pot life of the adhesive A mix was only 27 minutes, thus, too short for most existing plant conditions. A second gluing experiment was carried out with the adhesive B mixture aiming at a longer pot life. The pot life of adhesive B was still short, only 40 minutes. The adhesive B was used to bond both Douglas-fir and hemlock laminates. Pressing and test conditions were the same as before. The results summarized in Tables 6 and 7 showed that good bonds could be obtained on both Douglas-fir and hemlock laminations even though the curing speed of the adhesives was reduced by addition of less caustic.

To provide a control reference, a series of laminates was bonded with the Borden LT-75/FM-260 mix. Results summarized in Tables 8 and 9 show that bond qualities obtained using the PRF resin are similar to those obtained when half of the PRF resin was replaced with the 5-X tannin sulfonate extract (Tables 5 – 7).

A third adhesive mix (adhesive C), prepared as described above but with only 2 g of a 50 percent sodium hydroxide solution, was evaluated to lengthen the pot life still further. Results shown in Table 10 are the averages of data obtained from four laminations of Douglas-fir. The results of this experiment were not quite up to expectations in terms of dry shear strength and the marginal wood failure average of 82 percent after the vacuum-pressure soak treatment.

The last two adhesive formulations studied were prepared as described above but with 2.5 g of a 50-percent sodium hydroxide solution added to the 5-X tannin sulfonate solution. Adhesive D was made by combining 50 g of the PRF-hardener mix with 50 g of the 5-X tannin sulfonate-caustic component. Adhesive E was made by combining 40 g of the PRF-hardener mix with 50 g of the 5-X tannin sulfonate-caustic component. Both adhesives had a pot life of 90 minutes. Results

Table 5. Effect of Spread Rate on Bond Quality Using Mixed PRF-Tannin Glue A.

Sample No.	Spread Rate	Test Condition	Shear Strength kPa	Shear Strength psi	Wood Failure (%)
1	79	Dry	7274	1053	98
		VPS	3940	570	87
		Boil	4741	686	88
2	77	Dry	6924	1003	98
		VPS	4771	691	88
		Boil	4339	628	82
3	61	Dry	6461	936	98
		VPS	4711	682	88
		Boil	4090	592	86
4	83	Dry	9032	1308	99
		VPS	4788	693	96
		Boil	4495	651	97
5	55	Dry	7819	1132	100
		VPS	5081	736	79
		Boil	4618	669	96
6	49	Dry	7857	1138	95
		VPS	4905	710	95
		Boil	4655	674	86
7	67	Dry	8090	1172	100
		VPS	4800	695	97
		Boil	3966	574	84
Average		Dry	7637	1106	98
		VPS	4714	683	90
		Boil	4415	639	89

obtained from bonding of four Douglas-fir laminates each with adhesives D and E are shown in Table 11. Results very similar to those reported in Tables 5 – 11 have been achieved with bark extracts from juvenile trees, from fully matured trees (plywood logs), and from mixtures of these extracts.

The amount of sulfite used during the extraction has an effect on the viscosity of the resulting aqueous tannin solutions. In the author's opinion, based on experience with a fair number of such extracts, concentrations up to about 45 percent solids can be handled under existing mill conditions with conventional equipment. Pot life, and with it cure speed, can be adjusted over a wide range with the quantity of sodium hydroxide in the adhesive.

Table 6. Bond Quality of Douglas-Fir Laminations Using PRF-Tannin 5-X Glue B.

Sample No.	Spread Rate	Test Condition	Specimen kPa	Strength psi	Wood Failure (%)
11	57	Dry	9387	1360	100
		VPS	4948	716	85
		Boil	4558	661	96
12	66	Dry	10142	1470	97
		VPS	5081	736	87
		Boil	5151	747	79
13		Dry	9967	1444	99
		VPS	5730	830	92
		Boil	4823	699	100
14	60	Dry	9251	1340	99
		VPS	4950	716	92
		Boil	4934	714	95
15	60	Dry	9998	1448	100
		VPS	5609	812	97
		Boil	5784	838	98
Average		Dry	9749	1413	99
		VPS	5264	762	91
		Boil	5050	731	94

Table 7. Bond Quality of Hemlock Laminations Using PRF-Tannin 5-X Glue B.

Sample No.	Spread Rate	Test Condition	Specimen kPa	Strength psi	Wood Failure (%)
16	62	Dry	6938	1005	99
		VPS	5266	762	85
		Boil	5057	732	84
17	74	Dry	8499	1231	96
		VPS	4922	712	71
		Boil	4534	656	75
Average		Dry	7719	1118	98
		VPS	5094	738	78
		Boil	4796	695	80

Table 8. Bond Quality of Douglas-Fir Laminations with an Unmodified PRF Adhesive (Borden's LT-75).

Sample No.	Spread Rate	Test Condition	Specimen kPa	Strength psi	Wood Failure (%)
21	64	Dry	9600	1390	98
		VPS	5610	812	93
		Boil	4878	706	94
22	61	Dry	9861	1428	99
		VPS	5574	807	94
		Boil	5268	763	97
25	93	Dry	8890	1288	99
		VPS	5525	800	90
		Boil	5555	805	91
Average		Dry	9450	1370	99
		VPS	5570	807	92
		Boil	5234	758	94

Table 9. Bond Quality of Hemlock Laminations with an Unmodified PRF Adhesive (Borden's LT-75).

Sample No.	Spread Rate	Test Condition	Specimen kPa	Strength psi	Wood Failure (%)
23	77	Dry	7873	1140	98
		VPS	5011	725	85
		Boil	5545	803	95
24	75	Dry	6866	995	100
		VPS	5228	757	90
		Boil	5007	725	89
Average		Dry	7370	1068	98
		VPS	5120	742	88
		Boil	5276	764	92

Table 10. Bond Quality of Douglas-Fir Laminations with a Tannin-Modified PRF Resin at Low Caustic Content Adhesive A.

Test Condition	Specimen Strength kPa	psi	Wood Failure (%)
Dry	7077	1025	93
VPS	5612	813	82
Boil	5261	761	90

Table 11. Bond Quality of Douglas-Fir Laminations with a Tannin-Modified PRF Resin at Low Caustic Content Adhesives B and C.

Sample No.	Dry		VPS		2-Hour Boil	
	Shear psi	WF[a] (%)	Shear psi	WF (%)	Shear psi	WF (%)
B-1	1359	95	970	93	831	100
B-2	1179	90	1005	90	735	98
B-3	928	85	930	98	729	100
B-4	1052	90	958	98	734	98
Avg.	1130	90	966	95	757	99
C-5	1119	93	936	90	748	98
C-6	1111	93	934	88	722	100
C-7	1328	78	947	80	774	95
C-8	1024	95	941	98	770	95
Avg.	1146	90	940	89	754	97

[a]WF = wood failure.

With respect to use of tannin extracts from southern pine tree bark as a 50 percent replacement for PRF resins used in a cold-setting face-laminating application it can be concluded that:

- About half of the conventional PRF adhesive used for laminating structural beams requiring exterior durability glue lines can be replaced by tannin derived from natural sources, thus considerably reducing glue line costs.

- Adhesive properties, such as pot life, cure time, viscosity (penetration) can be adjusted over a wide range. This should make such adhesives effective with many species used for laminating purposes.

- Individual adhesive formulations aimed at meeting special individual plant operating conditions can be developed with modest efforts. The same applies to plants located in areas where temperature fluctuations between winter and summer are extreme.

END-JOINTING OF STRUCTURAL LUMBER WITH TANNIN-MODIFIED ADHESIVES.

Ever since glued laminated beams have been made, there has been the need to secure dried, high-quality, dimension lumber of long lengths to be used for the individual plies. As defect-free lumber of this type is not obtainable from normal sawmill operations, it has been necessary to end-glue good shorter pieces. In order to secure end-joints with acceptable durability, resorcinol formaldehyde (RF), phenol-resorcinol-formaldehyde, and (more recently) melamine-formaldehyde (MF) or melamine-urea-formaldehyde (MUF) resins have been used.

PRF resins are chiefly used in countries where it is customary to mate the end joints under high pressure, off-bear gently, and allow the end-joint adhesive to cure at room temperature overnight. The U.S. industry prefers to use RF, MF, or MUF resins in combination with radio-frequency heating in order to accomplish very rapid (10-20 seconds) cure of the polymer system. PRF resins do not lend themselves to radio-frequency curing; their high caustic content, necessary to accomplish solubility of the resin, causes arcing when radio frequency energy is applied. MF and MUF resins have shown questionable durability under truly exterior exposure conditions. RF resins are very expensive.

The modification of phenolic type end-joint adhesives (such as PRF) was therefore thought to bring a number of possible benefits: if joints were mated at high pressure and allowed to 'cure overnight', then, the modification with tannin could possibly shorten cure times drastically, either by meter/mixing the two components just before application or by using the "Honeymoon" system as practiced in South Africa. So far, all indications have been that the tannin modification, to about 50 percent replacement, does not at all degrade the durability of the underlying phenolic system. Therefore, it was decided to address the possibility of using tannins in end-jointing adhesives.

End-Joints with Mixed Adhesives

The end-joints were made with 2" x 2" (1 1/2 x 1 1/2) lodgepole pine or Douglas-fir using a specially designed end-joint test unit that applied a designated pressure to form the end-joint and then pulled the joint in tension after a specified time period.[18] Some of the end-joints were pulled in tension in the test machine immediately after the prescribed press time had elapsed to provide strength buildup curves, and others were removed from the test unit without joint destruction. The results of the measurements of strength buildup showed that there was no significant difference in the rate of strength development between RF, PRF, or PRF-tannin adhesive formulations. Approximately 10 minutes at ambient temperature was required to reach 500-800 psi (a strength judged sufficient to permit gentle handling), and the end-joints reached a tensile strength of 50 percent of ultimate in about 20 minutes.

The end-joints selected for test of ultimate bond strength were allowed to cure for several days at room temperature and were then edge-trimmed to 1-inch width in order to remove at least some of the edge effect. Following edge-trimming, they were cut from the other face into strips of 1/4 inch thickness and tested to destruction in tension. Each such end-joint resulted in four specimens for tension

testing after full cure. In each case, one of these four strips was tested dry, two were tested after exposure to the normal vacuum-pressure water immersion cycle, and one strip was tested in tension after having been submersed in boiling water for 2 hours.

The tests after submersing specimens in boiling water for 2 hours are not tests accepted or specified by any regulatory or testing society or agency. Gluing and test experience over many years, however, has convinced this investigator that important conclusions with respect to reasons for adhesive failure as well as important pointers for adhesive development can be obtained by careful interpretation of the comparison of the test results after boil with those from dry and vacuum-pressure treatment.

Two types of tannin extracts were used for this work, extract 5-X (bark from peeler logs) and extract 2-X (from bark of pulpwood juvenile trees). The adhesive formulated for end-joints G-1 to G-10 was prepared using the 5-X extract as follows:

Component A	Borden's LT-75	50 g
	FM-260 Hardener	10 g
	Paraformaldehyde	5 g
Component B	45% Tannin 5-X Solution	50 g
	50% Sodium Hydroxide	2.5 g

The two components were blended just prior to application, both ends of the joint were spread with adhesive, and samples were pressed for 1 minute except for samples G-6 and G-10, which were pressed for 5 minutes. The ultimate bond strengths of end-joints made with this adhesive are shown in Table 12.

Another series of end-joints was bonded with an adhesive formulated from an extract obtained from the bark of pulpwood aged (young) trees. This adhesive was formulated with a PRF resin mix the same as combining the PRF mix with a tannin solution containing 50 g of tannin 2-X solution at 45 percent solids and 2.67 g of a 50-percent sodium hydroxide solution. This adhesive had a high viscosity – the mix was puttylike, and for that reason, the gel time was difficult to measure, but appeared to be about 1 hour. The end-joints G-11 to G-20 were bonded with this adhesive and tested as described above. All end-joints were pressed for 1 minute except for sample G-20, which was pressed for 5 minutes. The results, given in Table 13, again exceeded commercial (AITC) strength criteria.

End-Joint Experiments with the Tannin-PRF Honeymoon System

Due to the negligible difference between a PRF and an RF resin when modified for use under "Honeymoon" conditions,[18] all subsequent experimental work was done using the PRF resin as one of the components. This decision was based on the substantial cost advantage for PRF resins as compared to pure resorcinol polymers. The following pages give data for the preparation of the adhesive components and for the gluing conditions. The phenol-resorcinol-formaldehyde resin chosen as the

Table 12. Tensile Strength of End-Joints Glued with Mixed Tannin-PRF Adhesive Made From Tannin Extract 5-X from Bark of Old Trees.

Specimen No.	Tensile Strength psi	kPa	Wood Failure (%)
		Dry	
G-1	6515	44953	95
G-2	6151	42442	100
G-3	7446	51375	65
G-4	6554	45224	100
G-5	5157	35585	100
G-6	5306	36609	100
G-7	6900	47607	75
G-8	9677	66768	90
G-9	4762	32856	100
G-10	5477	37788	100
Ave.	6395	44121	93
		Vacuum Pressure	
G-1	5607	38691	95
	5831	40237	60
G-2	5821	40163	90
	4394	30319	98
G-3	4598	31723	95
	2494	17209	75
G-4	5588	38558	85
	5120	35326	85
G-5	5023	34662	90
	6621	45688	98
G-6	3375	23286	95
	4949	34147	95
G-7	4542	31341	75
	4014	27694	80
G-8	5597	38622	65
	3607	24892	45
G-9	5297	36551	75
	6400	44160	80
G-10	5599	38636	90
	6284	43360	80
Ave.	5038	34763	83
		2-Hour Boil	
G-1	5801	40025	98
G-2	4796	33092	90
G-3	6360	43883	70
G-4	5202	35892	80
G-5	4841	33403	90
G-6	4126	28468	75
G-7	4061	28024	70
G-8	5101	35194	65
G-9	5938	40969	70
G-10	5573	38456	95
Ave.	5180	35741	80

Table 13. Tensile Strength of End-Joints Glued with Mixed Tannin-PRF Adhesive Made From Tannin Extract 2-X from Bark from Juvenile Trees.

Specimen No.	Tensile Strength psi	kPa	Wood Failure (%)
		Dry	
G-11	6064	41842	50
G-12	6269	48349	60
G-13	6249	43118	65
G-14	5102	35204	98
G-15	5127	35376	98
G-16	5216	35990	100
G-17	6350	43815	85
G-18	8031	55414	80
G-19	3819	26351	75
G-20	6151	42442	100
Ave.	5838	38790	81
		Vacuum Pressure	
G-11	5428	37454	80
	6078	41940	85
G-12	6067	41865	75
	3837	26472	95
G-13	6199	42775	95
	6613	45629	95
G-14	6239	43048	90
	6593	45492	85
G-15	5415	37361	95
	6557	45240	90
G-16	4107	28335	95
	5093	35144	60
G-17	4409	30422	70
	4597	31716	98
G-18	6195	42743	50
	6633	45770	98
G-19	2867	19781	100
	1712	11810	100
G-20	5414	37357	90
	4432	30584	90
Ave.	5224	36047	87
		2-Hour Boil	
G-11	5237	36132	75
G-12	5555	38327	70
G-13	6460	44573	55
G-14	5968	41181	65
G-15	5869	40499	65
G-16	5064	34939	85
G-17	5283	36450	85
G-18	6882	47488	85
G-19	2114	14590	100
G-20	4601	31745	85
Ave.	5303	36592	77

PRF component of the system was Borden's LT-75 resin with Borden's FM-260 hardener. As the formulations show (Table 14), this system was substantially modified for the work reported here. It was necessary to supply the entire amount of paraformaldehyde required for both components through the resin side only. Otherwise, the tannin component, under highly alkaline conditions, would have reacted with the paraformaldehyde very quickly and rendered the tannin component unusable within just a few seconds. There is no question that other PRF adhesive resins by other manufacturers can equally well be adapted to this system. Strength buildup curves as well as ultimate bond line performance may vary initially, but, by proper formulation, all such resins with medium or high solids content (40 - 60 percent) can be made to perform satisfactorily under current U.S. criteria for the manufacture of structural wooden members.

The expression "conventional Honeymoon system" designates the use of the type of Honeymoon system as described in the literature covering this gluing system.[9,10] For the formulations reported here, it means that the tannin component will carry the sodium hydroxide needed to catalyze the reaction, whereas, the PRF component will carry double the formaldehyde donor (in our case, paraformaldehyde). The tannin-based adhesive formulations examined are summarized in Table 14. The tannin material used (5-X) was part of the first large batch that became available. The data showing ultimate bond strength and wood failure of fully cured end-joints tested dry, following vacuum-pressure water soak, and after 2-hour boil tests for each of the adhesive compositions examined are summarized in Tables 15 and 16.

The rate of mixing and paraformaldehyde dissociation would be expected to be increased by increasing the amount of sodium hydroxide in the adhesive. However, strength values in the early stages of cure were too variable to establish this relationship. Addition of small amounts of Methocel did not alter the rate of strength development.

In adhesives formulated in the F-Series, approximately 5 to 10 minutes at ambient temperatures was required to reach an end-joint tensile strength of 500 psi, a value deemed adequate to permit gentle handling of the end-jointed members. Between 15 and 20 minutes at ambient temperature was required to reach a tensile strength of 1,500 psi.[18]

Table 14. "Honeymoon" Adhesive Formulations Using Tannins in Component A.

Experiment	Component A				Component B		
	Tannin	Water	NaOH	Methocel	LT-75	FM-260	Para
D	45.0	55.0	10.59	0.00	40.0	4.0	8.0
F-A	42.0	58.0	10.64	0.05	40.0	4.0	8.0
F-B	42.0	58.0	10.50	0.10	40.0	4.0	8.0
F-C	42.0	58.0	10.50	0.20	40.0	4.0	8.0
F-D	44.0	56.6	7.58	0.23	40.0	4.0	8.0
F-E	44.0	56.3	5.00	0.21	40.0	4.0	8.0
F-F	44.0	56.0	2.26	0.21	40.0	4.0	8.0
F-G	44.0	56.0	1.27	0.05	40.0	4.0	8.0
M-A	44.1	56.3	7.54	0.25	40.0	4.0	8.0
M-B	44.0	56.0	1.25	0.05	40.0	4.0	8.0

Table 15. Gluebond Quality of Lodgepole Pine End-Joints Obtained with the F-Series Adhesive Formulations.

Adhesive Formulation	Tensile Strength or Wood Failure	Test Condition Dry	VPS	Boil
F-A	psi	4830	3390	3371
	kPa x 10^3	33.3	26.8	23.3
	(%) WF	97	74	84
F-B	psi	6051	5107	4730
	kPa x 10^3	41.8	35.2	32.6
	(%) WF	93	79	86
F-C	psi	5841	4569	4301
	kPa x 10^3	40.3	31.5	29.7
	(%) WF	100	87	76
F-D	psi	6878	5750	5353
	kPa x 10^3	47.5	39.7	36.9
	(%) WF	90	81	55
F-E	psi	4942	4663	4032
	kPa x 103	34.1	32.2	28.3
	(%) WF	100	89	78
F-F	psi	5265	4384	4152
	kPa x 10^3	36.3	30.3	28.6
	(%) WF	98	81	87
F-G	psi	5609	5054	5469
	kPa x 10^3	38.7	34.9	37.7
	(%) WF	93	77	93

The tensile strengths of fully cured end-joints in lodgepole pine or Douglas-fir when tested dry, following vacuum-pressure water soak, and after 2 hours in boiling water were very high, equal to or exceeding those commonly accepted by industry. Many of the adhesives examined gave bonds that had tensile strength values in excess of 5,000 psi even after vacuum-pressure water soak or immersion in boiling water for 2 hours.

Ultimate bond strength of the end-joints was not influenced by press time providing that a) full joint consolidation was achieved and b) the joint was not disturbed by transport of the assembly during the gel and cure period. For plant applications of this system, press times of less than 1 minute should be explored, including the use of several levels of end pressure. At this time, it appears that the press time can be limited to the minimum time necessary for full joint mating. In bonding Douglas-fir end-joints with the M-Series adhesives, an increase in mating pressure from 440 to 660 psi did not change the ultimate tensile strength values.

Table 16. Gluebond Quality of Douglas-Fir End-Joints obtained with the M-Series "Honeymoon" Adhesives.

Adhesive Formulation	Tensile Strength or Wood Failure	Test Condition Dry	VPS	Boil
M-A-1	psi	6342	5174	4768
	kPa x 10^3	43.7	35.7	32.9
	(%) WF	96	57	84
M-A-2	psi	7127	5268	5466
	kPa x 10^3	49.2	36.4	37.7
	(%) WF	92	37	74
M-B-1	psi	5425	4145	4336
	kPa x 10^3	37.4	28.6	29.9
	(%) WF	90	79	82
M-B-2	psi	5704	4698	4616
	kPa x 10^3	39.4	32.4	31.9
	(%) WF	90	84	81

Considering all the data obtained from the F-Series formulations, there was not a consistent change in ultimate tensile strength or resistance to degradation by exposure to water when the sodium hydroxide content of the component A was varied over wide ranges. The adhesive formulated with 7.5 parts of sodium hydroxide in the component A gave higher tensile strength values than that with 1.25 parts of sodium hydroxide in bonding of Douglas-fir end-joints (Table 16). Some caution must be exercised in interpreting this result, since a high proportion of these end-joints had wood failure values in the 70- to 90-percent range. Like the results of the studies of lodgepole pine, bond strength values were primarily related to differences in substrate strength rather than the adhesive bonding capability.

Given the success of end-jointing either lodgepole pine or Douglas-fir with adhesives containing 50 percent of tannin sulfonate extracts, these adhesives were then evaluated for end-jointing southern pine lumber. One set of southern pine lumber without any impregnation and an end-matched set that had been impregnated with a conventional CCA preservative by Mississippi State Forest Products Laboratory personnel were received from the Southern Forest Experiment Station. This material was used for end gluing with a tannin/PRF "Honeymoon" system. The adhesive used for this trial was formulated as follows:

Borden's LT-75	100 g
FM-260 Hardener	20 g
Paraformaldehyde	10 g
45% Tannin 2-X Solution	100 g
Sodium Hydroxide Pellet	5 g

Joints were made with 440 psi end pressure. Both adhesive components were very viscous, the viscosity judged too high, especially for impregnated lumber. However, gluing results given in Table 17 indicate that it will be possible to glue non-impregnated southern pine with this adhesive system to achieve end-joints of good quality, meeting AITC standards. This was the first attempt on southern pine. Further adhesive development should lead to excellent results.

Glue lines on CCA-impregnated lumber do not achieve water resistance (Table 18). The dry bond strength and wood failure were respectable, but the strength and wood failure values for all water-immersed specimens of the CCA-treated substrate were low.

CONCLUSIONS

It has been clearly demonstrated that tannin material extracted from the bark of southern pine trees can be used to replace about half of the phenol-resorcinol-formaldehyde resin in conventional parallel laminating operations, such as practiced in the United States for the manufacture of laminated beams. The same extracted tannin lends itself for the gluing of structural end-joints when tannin solution and PRF resins are used in blended adhesive with slow room temperature cure, such as practiced by a few U.S. plants and most plants in Europe. Such tannin solutions can be used as one of the adhesive components of "Honeymoon" systems for the production of structural end-joints.

It has also been shown that tannin-sulfonate extracts from the bark of any age trees can be used; that product uniformity can be achieved by small variations in sulfite extraction process; that pot lives and cure times can be adjusted over wide limits by proper adhesive formulation.

ACKNOWLEDGMENTS

The financial support of RWU-4701, Southern Forest Experiment Station, USDA Forest Service, Pineville, Louisiana; the USDA-Forest Service Competitive Grants Program through Grant No. 85-FSTY-9-0127; and the USDA Small Business Innovation Research Program through Grant No. 86-SBIR-8-0126 is gratefully acknowledged. Dr. Richard W. Hemingway, Mr. Elmer H. Eskola, and Mr. Gary W. Smith of the Southern Forest Experiment Station, Pineville, Louisiana, assisted in preparation of tannin samples and testing bond quality in this study.

Table 17. End-Joint Bonding of Southern Pine with a Tannin-Modified "Honeymoon" System.

Specimen No.	Tensile Strength psi	kPa	Wood Failure (%)
		Dry	
1	7154	49363	100
1	6439	44430	100
3	6656	45927	100
3	6375	43988	100
5	6162	42518	100
5	5103	35211	100
7	6155	42467	100
7	6510	44920	100
9	7343	50666	100
9	5431	37475	100
Average	6333	43697	100
		Vacuum Pressure	
1	4467	30820	90
1	5160	35606	90
3	3648	25173	80
3	4244	29282	80
5	3795	26186	50
5	3052	21057	40
7	3960	27321	85
7	4860	33536	80
9	5123	35350	70
9	4209	29043	75
Average	4250	29337	74
		2-Hour Boil	
1	4260	29396	85
3	3237	22334	80
5	3348	23098	75
7	4369	30144	40
9	4545	31360	80
Average	3950	27266	72

Table 18. End-Joint Bonding of CCA-Treated Southern Pine with a Tannin-Modified "Honeymoon" System.

Specimen No.	Tensile Strength psi	Tensile Strength kPa	Wood Failure (%)
		Dry	
2	8274	57090	90
2	7338	50634	80
4	8079	55742	90
4	7676	52964	95
6	4441	30644	90
6	5476	37782	100
8	6289	43397	80
8	7564	52195	90
10	9437	65114	75
10	7765	53580	95
Average	7234	49914	89
		Vacuum Pressure	
2	4902	33824	2
2	4004	27626	4
4	4681	32299	2
4	4393	30309	8
6	3017	20818	8
6	2915	20116	20
8	2948	20344	4
8	3422	23609	0
10	4286	29576	4
10	3429	23659	2
Average	3800	26218	5
		2-Hour Boil	
2	3033	20928	5
4	5415	37366	2
6	3190	22008	2
8	3658	25241	10
10	3800	26217	5
Average	3820	26352	5

REFERENCES

1. Pizzi, A. Tannin-based adhesives. *In*: Pizzi, A. (ed). Wood Adhesives: Chemistry and Technology. Marcel Dekker, New York pp. 177-246 (1983).
2. Pizzi, A. Tannin-based adhesives. *J. Macromol. Sci. Rev. Macromol. Chem.* C18:247 (1980).
3. Ayla, C.; Weissmann, G. New developments in the use of tannin formaldehyde resins for the manufacture of particleboard. *Plant Res. Develop.* 19:107 (1984).
4. Hall, J.A. Utilization of Douglas-fir bark. Pacific Northwest Forest and Range Experiment Station, USDA Forest Service, Portland, Oregon. Misc. Rept. 138 p. (1971).
5. Hemingway, R.W. Significance of Condensed Tannins. *In*: Rowe, J.W. (ed.). Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer Verlag, New York (in press).
6. Herrick, F.W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food Chem.* 28:228 (1980).
7. Roux, D.G.; Ferreira, D.; Hundt, H.K.L.; Malan, E. Structure, stereochemistry, and reactivity of natural condensed tannins as basis for their extended industrial application. *J. Appl. Polym. Sci. Polym. Symp.* 28:335 (1975).
8. Pizzi, A.; Rossouw, D. duT.; Knuffel, W.E.; Singmin, M. 'Honeymoon' phenolic and tannin-based fast-setting adhesive systems for exterior grade finger joints. *Holzforsch. und Holzverwer.* 32:140 (1980).
9. Baxter, G.F.; Kreibich, R.E. A fast-curing phenolic adhesive system. *For. Prod. J.* 23:17 (1973).
10. Kreibich, R.E. High-speed adhesives for the wood gluing industry. *Adhesives Age* 17:26 (1974).
11. Hemingway, R.W.; Kreibich, R.E. Condensed tannin-resorcinol adducts and their use in wood-laminating adhesives: an exploratory study. *J. Appl. Polym. Sci. Polym. Symp.* 40:79 (1984).
12. Hemingway, R.W.; McGraw, G.W.; Laks, P.E.; Kreibich, R.E. Condensed tannins. Problems and prospects for their extended use in wood adhesives. *In*: Christiansen, A.W.; Gillespie, R.H.; Meyers, G.E.; River, B.H. (eds.) Wood Adhesives in 1985: Status and Needs. Forest Products Research Society, Madison, Wisconsin, p. 156-166 (1986).
13. Kreibich, R.E.; Hemingway, R.W. Condensed tannins: resorcinol adducts in laminating adhesives. *For. Prod. J.* 35:23 (1985).
14. Hemingway, R.W.; Lloyd, R.C. Economic prospects for conifer bark extracts. Southern Forest Experiment Station, USDA Forest Service, New Orleans, Louisiana. Final Report No. FS-SO-3201-47. (1983).
15. Foo, L.Y.; McGraw, G.W.; Hemingway, R.W. Condensed tannins: preferential substitution at the interflavanoid bond by sulfite ion. *J. Chem. Soc. Chem. Commun.* 12:672 (1983).
16. McGraw, G.W.; Laks, P.E.; Hemingway, R.W. Condensed tannins: desulfonation of hydroxybenzylsulfonic acids related to proanthocyanidin derivatives. *J. Wood Chem. Technl.* 8:91 (1988).
17. Kreibich, R.E.; Hemingway, R.W. Condensed tannin-sulfonate derivatives in cold-setting wood-laminating adhesives. *For. Prod. J.* 37:43 (1987).
18. Kreibich, R.E.; Hemingway, R.W. Tannin-based adhesives for finger-jointing wood. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. American Chemical Society, Washington DC, pp. 203-216 (1989).

ADHESIVES CONTAINING PINE BARK TANNIN FOR BONDING NYLON CORD TO RUBBER

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ABSTRACT

Purified loblolly pine bark tannin was studied as an additive in resorcinol-formaldehyde latex (RFL) adhesive dips for bonding nylon to a styrene-butadiene-rubber (SBR) vulcanizate. The viscosity of the adhesive and its bonding characteristics depended on the order in which ingredients were combined in adhesive preparation. Pre-dilution *before* combining reactive components led to increased viscosity, higher pullout forces, and prevention of precipitate formation when compared to the addition of water after mixing resorcinol, tannin, formalin, and NaOH (post-dilution). Viscosity increased as the resin solids content of the adhesive increased. Stress-strain measurements indicated an initial toughening of the adhesive as resorcinol was replaced by tannin, but at sufficiently high substitutions, a marked softening and reduction in toughness occurred. Pullout forces with nylon 66 cord were increased if the adhesive was applied without prior aging. Pullout forces could be optimized by varying the formaldehyde concentration and latex-to-resin ratio in the tannin-containing adhesives.

INTRODUCTION

A previous publication[1] explored the use of four types of condensed tannins in resorcinol-formaldehyde-latex (RFL) adhesives for bonding nylon and polyester tire cord to a styrene butadiene rubber (SBR) vulcanizate. Tests showed that, in some cases, high pullout forces could be obtained with adhesives containing tannin as a partial replacement for resorcinol. This report examines in more depth the properties of RFL-type adhesives containing purified loblolly pine bark tannin. Not

only were pullout data determined for various adhesive compositions, but viscosity and stress-strain properties were evaluated as well.

EXPERIMENTAL METHODOLOGY

Materials

The elastomer used in this investigation was SBR 1502, a cold emulsion random copolymer of styrene and butadiene containing 23.5 percent bound styrene. It was supplied by Polysar and compounded as indicated in Table 1. Masterbatches were mixed 7 minutes at 60 rpm in a 350 mL Brabender Plasticorder, and curatives were added on a two-roll mill. An oscillating disc rheometer (ASTM D 2084) was employed to determine the cure characteristics, which are given in Table 2. The Shore A hardness of the final vulcanizate was about 60.

Purified pine bark tannin was obtained using the following procedure: the phloem of freshly felled loblolly pine trees was removed by carefully peeling the outer bark away at the cork cambium and then peeling the white phloem from the xylem cambium. Phloem strips were cut into about 2 to 5 in. sections and immediately immersed in acetone-water (70:30, v/v). The extraction flasks were kept at ambient temperature and protected from exposure to light for 48 hours, after which the solvent was recovered by filtration. The acetone was removed under vacuum on a rotary evaporator, and the aqueous solution was extracted four times with an approximately equal volume of ethyl acetate to remove low-molecular-weight

Table 1. Composition of Rubber Used in This Study.

Ingredients	Parts by Weight
SBR 1502	100.0
N330 carbon black	50.0
Zinc oxide	5.0
Steric acid	0.5
Sulfur	1.7
2-Morpholino-thiobenzothiazole	2.0

Table 2. Cure Characteristic of the Rubber at 155 °C.

Test	Result
Maximum Torque (Nm)	15.6
Minimum Torque (Nm)	5.0
Scorch Time (min.)	7.5
90% Cure Time (min.)	22.5

phenolics. The remaining water-soluble extract was freeze-dried. Aliquots (about 50 gm) were redissolved in methanol-water (1:1, v/v), and the solutions were applied to 2.4 X 90 cm Sephadex LH-20 columns packed in this same solvent. The columns were eluted with methanol-water until no more colored material was eluted. The condensed tannin polymers absorbed on the column packing were then eluted with acetone-water (50:50, v/v). The acetone was removed by evaporation under vacuum on a rotary evaporator, and the aqueous solution was freeze-dried.

Three types of cord were employed in the adhesion testing: two typical tire cords – a 1260/2 construction nylon 66 and a 1000/3 polyethylene terephthalate cord – as well as a nylon 610 monofilament.

The latex contained in the adhesive dips was Gentac 118 (General Corporation), a styrene-butadiene-vinyl pyridine polymeric emulsion with 41 percent total solids.

Adhesive Preparation

In adhesive preparation, all ingredients except the latex were mixed and allowed to react at room temperature for 2 hours. Any insolubles (from the tannin) were then filtered out, and the appropriate quantity of latex was added. Generally, a further reaction time of 24 hours was allowed before the adhesive was applied. The cord was dipped into the adhesive for 30 seconds, then allowed to dry 5 hours before being embedded into a rubber test block.

Viscosity Determination

The viscosity of a dip was determined with a Brookfield viscometer in a co-axial cylinder geometry. The shear rate was about 265 sec^{-1}. Because some of the (high-viscosity) adhesives were thixotropic, viscosity readings were taken after a fixed time of 30 seconds after shearing commenced.

Tensile Tests

Tensile tests of films of adhesive were performed on an Instron mechanical tester according to ASTM D412. Films were prepared by casting the wet adhesive, which had been aged for 1 day, onto Teflon and allowing the water to evaporate. Dried sheets were then molded at 155 °C for 50 minutes in order to simulate cure.

Pullout Specimen

Rubber that had been sheeted on the mill was cut into strips 1.3 cm wide and 7.6 cm long. Two pieces were plied-up to half fill the mold (preheated to 155 °C) cavity, then a cord was centered on the rubber so that its embedment depth was 1 cm. Two rubber strips were placed over the cord, followed by the top plate of the mold. Vulcanization was carried out in a compression mold for 50 minutes at 155 °C. The cured TCAT (tire cord adhesion test) specimens (Figure 1) were trimmed with a razor blade to remove mold flash and allowed to rest for 24 hrs before testing. Pullout values were determined by clamping the emergent cords in an Instron and pulling at the rate of 5 cm/min. Initially, the force increased but then levelled out at the onset of fracture and decreased sharply as one of the cords was pulled out of the rubber block. The maximum force is termed the pullout force, F, whereas,

the area under the force-deflection curve is the pullout energy. Additionally, the fracture energy, G, can be calculated by equation 1:

$$G = \frac{F}{4\pi \cdot A \cdot a \cdot E} \tag{1}$$

where A = undeformed cross-sectional area of the TCAT block; a = the effective radius of the cord; and E = modulus of the rubber.

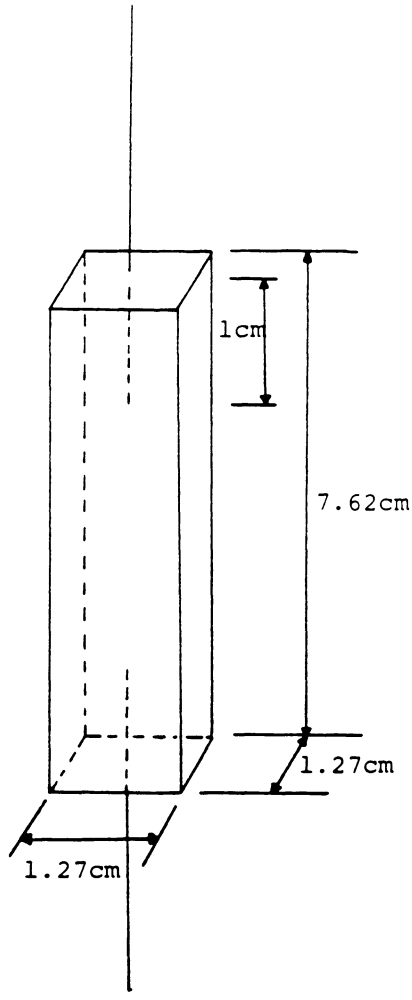

Figure 1. *TCAT pullout geometry*

Table 3. Composition of the Control RFL Adhesive Dip.

Ingredients	Amount	Dry Weight (g)
Resorcinol	11.0 g	11.00
Formalin (37% Formaldehyde)	16.4 ml	6.57
10% Aqueous NaOH	3.0 g	0.30
Distilled Water	236 g	0.00
Gentac 118 Latex	244 g	100.00

Microscopy

To study the failure locus of TCAT specimens, optical microscopy was employed to examine the pulled-out cord ends and the cavity left in the rubber block after pullout.

RESULTS AND DISCUSSION

The composition of the control dip in this investigation is given in Table 3. With other ingredients held constant, portions of resorcinol were replaced by tannin to give various dip compositions. It was found that the mixing order of ingredients in preparing the adhesive affected the viscosity of the dip and its adhesion.

If the adhesive was prepared by mixing together resorcinol, tannin, NaOH solution, and the formalin solution, there was an immediate precipitate formed that did not dissolve upon adding the remaining water (post-dilution method). However, no precipitate formed, and all remained soluble if the NaOH solution and formalin were mixed and diluted with the water first before adding the tannin and resorcinol with vigorous stirring (pre-dilution method). The viscosities after latex addition and 1 day of aging for the two mixing procedures are compared in Table 4. (With the former method, the precipitate was filtered out before viscosity was determined.) The pre-dilution method gave higher viscosities, especially when the ratio of resorcinol to tannin was 30/70 or less. This was expected, since with this procedure, the reaction products remained dissolved, thereby impeding the ease of viscous flow. In both cases, the viscosity increased with tannin content, reflecting its relatively high initial molecular weight. Compositions with less than about 70 percent tannin (based on resorcinol plus tannin amount) showed little viscosity change upon adding the polymeric latex with the pre-dilution procedure. However, dips with high tannin levels had marked viscosity increases upon latex addition, although the viscosity remained stable thereafter (Table 5).

In one case (resorcinol/tannin = 60/40), the viscosity was examined as a function of formaldehyde concentration in the dip. Other additions were fixed. Results are shown in Table 6. There is only a modest increase in viscosity as the formaldehyde/resorcinol mole ratio was increased from 0.44 to 7.12.

Additionally, while holding the resorcinol/tannin/formalin proportions at 3/2/3, the latex solid to resin solid ratio (i.e., tannin plus resorcinol plus formaldehyde) was varied from 1 to 15 and the viscosities determined. Results are given in Table 7. At equal portions of each, the adhesive gelled, and the viscosity could not be determined. The viscosity decreased as the latex solid content was increased.

Table 4. Viscosity[a] of Adhesive Preparations.

Resorcinol/Tannin Ratio	Viscosity (cp) Post-dilution	Viscosity (cp) Pre-dilution
100/0 Control	23.7	39.5
90/10	45.8	43.4
80/20	50.8	59.2
70/30	50.8	57.4
60/40	69.0	63.2
50/50	71.1	94.8
40/60	94.8	300.0
30/70	103.0	1340.0
20/80	118.0	2760.0
10/90	190.0	2710.0
0/100	356.0	2765.0

[a]Viscosity measured one day after preparation and 30 seconds after start of test; Temperature = 22 °C; Shear rate = 265 sec^{-1}.

Table 5. Viscosity Change of Adhesive with Latex Addition and Aging Time.

Resorcinol/Tannin Ratio	Viscosity (cp) 20/80	0/100
Before adding latex	89.0	111
After adding latex:		
Initial	2530	2510
After 1 hour	2530	2500
After 6 hours	2560	2580
After 1 day	2760	2760

Table 6. Viscosity Change of Adhesive with Formaldehyde-to-Resorcinol Ratio.

Formalin (mL)	Formaldehyde/Resorcinol (mole ratio)	Viscosity (cp)
2	0.44	62
4	0.89	65
8	1.78	65
16	3.50	65
32	7.12	68

Table 7. Viscosity of Adhesives Formulated with Various Rubbery Latex Contents.

Latex/Resin (solids ratio)	Viscosity (cp)
1	Gelled
3	350.0
6	63.2
9	45.0
12	45.0
15	45.0

Tensile Test

Stress-strain responses of cast adhesive films are shown in Figures 2-4. In some cases, tensile samples could not be easily prepared because when the dip dried, it was hard, brittle, and easily cracked. A cracked film could not be molded into a coherent sheet even by heating under pressure, indicating the films were highly cross-linked. Figure 2 gives results for three compositions like the control (Table 3), but with varying resorcinol/tannin ratios. All materials exhibited a yield point and some cold drawing. At higher resorcinol contents, the yield stress and breaking stress increased, and strain-hardening was sufficient to result in a breaking stress that exceeded the yield stress. As shown in Figure 3, stress-strain curves were dependent on the mole ratio of formaldehyde to resorcinol, while keeping the resorcinol to tannin weight ratio fixed at 3/2. At a mole ratio of 0.44 or 0.89, the adhesive was soft and highly extensible, with little difference in stress-strain response. However, upon an increase in the ratio to 1.78, the adhesive stiffened and strengthened markedly, as well as developing a yield point. Increased formaldehyde leads to an increased concentration of active methylol groups that then enter into cross-linking reactions.

In Figure 4, deformation curves are given for comparisons in which the latex/resin solid content was varied (while resorcinol/tannin = 3/2). There was a steady softening and loss of tensile strength as the latex content was increased. The latex alone is a poor adhesive, both because of a lack of reactive groups for the cord and the weak cohesive strength it possesses. On the other hand, too much resin is detrimental to adhesive performance due to brittleness and lack of sufficient reactivity with the rubber.

Pullout Testing

Previous work has shown that high pullout forces of nylon and polyester tire cords from an SBR vulcanizate were obtainable when tannins were used as a partial replacement for resorcinol in a typical RFL adhesive dip. Purified loblolly pine bark tannin for use in RFL-type adhesives was selected for further investigation

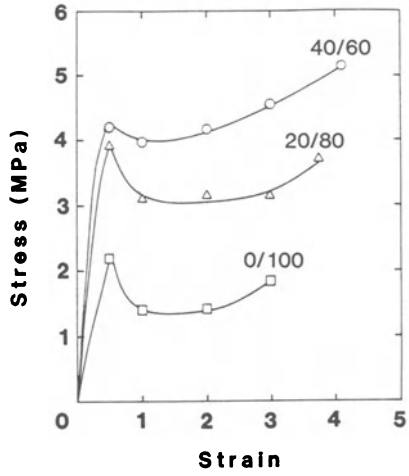

Figure 2. *Stress-strain response of cast adhesive films at three different ratios of resorcinol to tannin.*

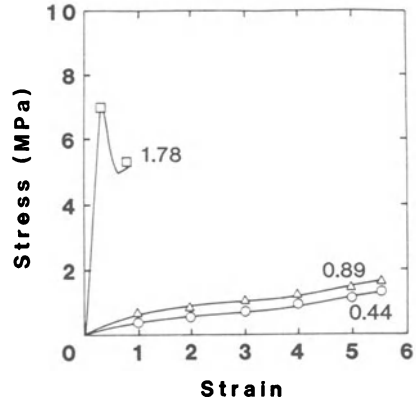

Figure 3. *Stress-strain curves at three different mole ratios of formaldehyde to resorcinol for cast adhesive films.*

in this study because it contains the least amount of impurities among the tannins previously tested.

Mixing Order

In the previous investigation, the post-dilution mixing procedure was used in preparing adhesive dips. This resulted in some precipitation from solution as noted

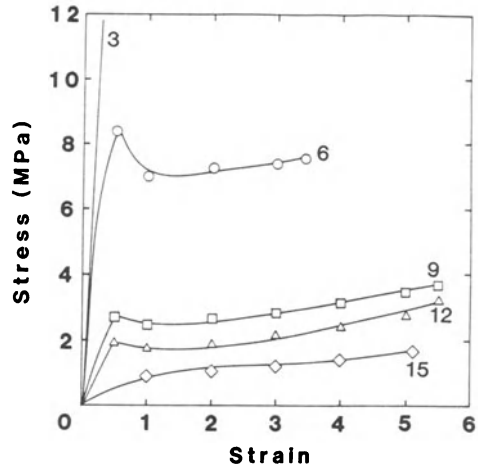

Figure 4. *Stress-strain curves for five different latex-solid to resin-solid ratios for adhesive cast films.*

earlier. When the pre-dilution method was employed, higher pullout forces generally were obtained, as shown in Figure 5 with the nylon-66 cord. Since the adhesion to the cord is dominated by the interaction with the resorcinol-tannin adducts, keeping these materials in solution is expected to improve bonding. With pre-dilution, pullout forces comparable to the control were obtained at either low or high replacement levels of tannin for resorcinol.

An important consideration after testing of an adhesive joint is determination of the fracture locus (i.e., the crack path during fiber pullout). The general types of fracture, identified by direct microscopic examination of the pulled-out fibers and the cavity left in the rubber block after pullout, are given schematically in Figure 6. The solid lines show cords embedded in rubber blocks with adhesive interlayers (which are exaggerated). Although the adhesive-rubber and adhesive-cord boundaries are shown as sharp interfaces (lines), it is expected that there will be some intermingling of material across the interface to form interphases, especially for the former boundary. The dotted lines indicate the three fracture patterns encountered.

With type I (Figure 6), tearing occurs within both the adhesive and the rubber – propagating across the rubber – adhesive boundary region. There is no failure between the adhesive and the cord; the pulled-out cord is completely covered with rubber or adhesive. To further characterize the failure, the percent of the surface area of the pulled-out cord that is covered with rubber has been estimated. Thus, the designation I-50 signifies type I fracture with 50 percent rubber coverage. I-0 indicates complete cohesive failure within the adhesive and/or bond failure between the adhesive and rubber.

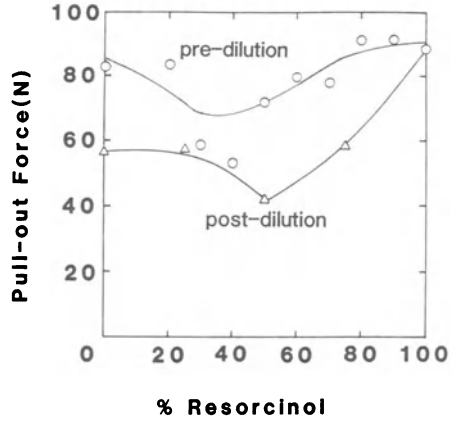

Figure 5. *Comparison of pullout forces of nylon 66 cord at various resorcinol contents for adhesives prepared by pre-dilution (upper curve) and post-dilution (lower curve) methods.*

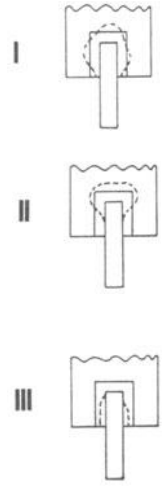

Figure 6. *Schematic diagram showing the general types of fracture paths during pullout.*

In type II failure (Figure 6), there is again tearing in both the adhesive and rubber, but this usually occurs at the portion of the cord most deeply embedded. Apparent interfacial failure between the cord and adhesive occurs at the other portion of the embedded cord. Again, an estimate has been made of percent rubber coverage of pulled-out cords – II-25 denotes type II fracture with 25 percent rubber coverage. In the final fracture mode (III), there is mixed cohesive rupture of the adhesive (typically near the emergent region) and interfacial detachment of the adhesive and cord (region near the embedded end). There is no rubber coverage on the pulled-out cords.

The control dip and those containing up to (and including) 50 percent tannin replacement for resorcinol exhibited fracture pattern I-0 (i.e., cohesive tearing within the adhesive dip). For 50 percent, 60 percent, and 70 percent replacement by tannin, the failure was I-25, I-50, and I-75 –an increasing tendency toward more rubbery tearing. However, the pullout force is lowest for these samples among all those tested for the upper curve in Figure 5. This suggests that there is a weakening of the rubber near the adhesive due to interdiffusion of tannin components or rubber additives, which may affect vulcanization. For complete replacement of resorcinol with tannin, failure becomes Class III–combined adhesive-cord detachment and tearing of the adhesive.

Aging of Adhesive

A parameter expected to influence the adhesion performance is the time that the adhesive is allowed to age (react) prior to being applied to the cord. Pullout results are shown in Table 8 for the case in which the adhesive is applied to the nylon 66 cord immediately after adding the latex to the adhesive mixture (rather than waiting 24 hours as done previously). The control dip and those containing up to 40 percent tannin replacement for resorcinol resulted in fiber breakage rather than pullout upon testing. This obviously represents the upper limit of the pullout force. At 60 percent or more tannin replacement, failure occurred by pullout at a reduced force. Similar to previous results, at those intermediate levels of tannin replacement (60 and 80 percent), failure was type I with moderate rubber coverage. Upon complete resorcinol replacement, pullout occurred with some clean separation of cord and adhesive, and by some adhesive rupture. Except at the two highest tannin concentrations, failure forces were higher than when the dip had been aged before application (compare to upper curve in Figure 5).

Nylon Monofilament

With tire cord, the adhesive can "penetrate" between the yarn strands, complicating detemination of the true bonded surface area. In order to remove the complexity of a multi-strand cord in the pullout tests, some experiments were done with a nylon 610 monofilament. Results are presented in Table 9. There is

Table 8. TCAT Results for Nylon 66 Cord after Application of Unaged Adhesives.

Resorcinol/Tannin Ratio	Failure Locus	Pullout Force (N)
100/0	fiber	140
80/20	fiber	132
60/40	fiber	133
40/60	I-25	85.4
20/80	I-50	72.4
0/100	III	84.4

a steady decline in pullout force with increasing tannin levels. This occurs in spite of an increase in rubber coverage at intermediate ratios of resorcinol to tannin. Once again, this suggests a weakening of the rubber near to the dip at these proportions. The pullout forces are dependent on the formaldehyde concentration. This is illustrated in Figure 7, where pullout forces are plotted versus formalin concentration for a dip with a ratio of resorcinol/tannin of 3/2. At the highest formaldehyde concentration, the pullout force is similar to the all-resorcinol control.

The effect of rubber to resin solids ratio on the pullout of the nylon 610 monofilament was carried out while holding the resorcinol/tannin ratio at 3/2. The results shown in Figure 8 indicate there is an optimum in the pullout force at a rubber/resin ratio of about 3.

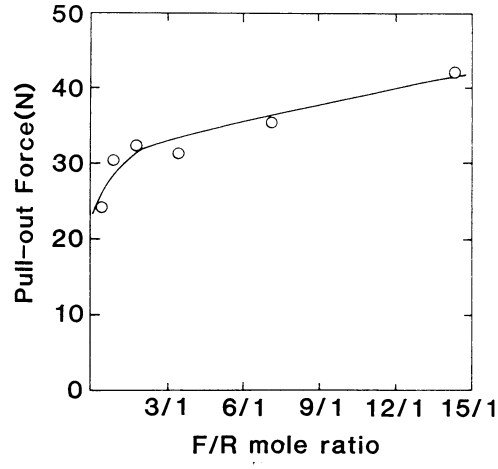

Figure 7. *Effect of formaldehyde to resorcinol ratio on the pullout force of nylon 610 monofilament. Resorcinol to tannin ratio is 60/40.*

Table 9. TCAT Results for Bonding to Nylon 66 Monofilament.

Resorcinol/Tannin Ratio	Failure Locus	Pullout Force (N)
100/0	I-0	42.8
80/20	I-25	35.9
60/40	I-25	31.4
40/60	I-50	19.6
20/80	I-0	12.3
0/100	I-0	10.3

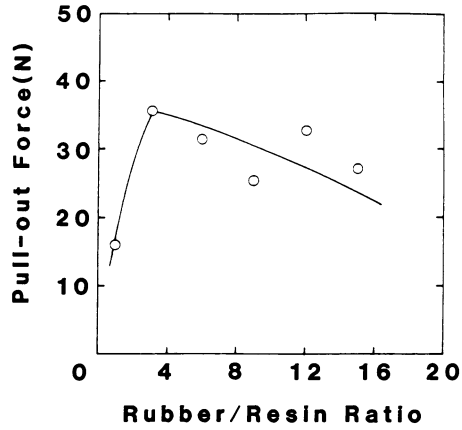

Figure 8. *Pullout forces of nylon 610 monofilament for various latex solid to resin solid ratios. Resorcinol/tannin = 60/40.*

CONCLUSIONS

Nylon cord can be firmly bonded to an SBR vulcanizate with an adhesive made of resorcinol, formaldehyde, and a styrene-betadiene-vinyl pyridine terpolymer latex. When part of the resorcinol in the adhesive is replaced with purified loblolly pine bark tannin, pullout forces similar to the control (without tannin) can be maintained if the portion of ingredients is modified. In particular, to optimize pull-out forces with tannin-containing adhesives, relatively high levels of formaldehyde are required, and the pre-dilution method of preparing the adhesive is preferred. Also, higher pullout forces are obtained if the adhesive is applied to the cord immediately after preparation with no "maturing" time – chemical reaction and cross-linking within the adhesive after application are advantageous for developing a strong bond to the nylon cord.

ACKNOWLEDGMENTS

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REFERENCES

1. Hamed, G.R.; Chung, K.H.; Hemingway, R.W. Condensed tannins as substitutes for resorcinol in bonding polyester and nylon cord to rubber. *In:* Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385. American Chemical Society, Washington, DC. p. 242-253 (1989).

USING TANNINS TO PRODUCE LEATHER

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ABSTRACT

This chapter reviews some history of leathermaking prior to 1930 and the methods and materials changes that have been made over the past 50 years. An overview of the relationships between different types of tannins and the properties of the resulting finished leather is presented. Causes of the decline of the leather industry in the United States and Canada and future prospects are discussed. Problems that need special attention are new manufacturing methods and overcoming governmental ignorance about raw material supply and the impacts of government regulations on processes. An analysis of the competition and where leathermaking is headed suggests changes that are needed in the North American industry.

INTRODUCTION

The tanning profession is a disease that, once contracted, stays with you until death. My involvement with vegetable tanning remains because leathermaking with vegetable tannins offers a challenging blend of chemistry and art. We talk of the "art" of tanning because a tanner must have a natural awareness to cope with the vast variations in different hides and tannages. Never does one find a hide exactly like the one beside it. Therefore, a tannery cannot simply follow a formula without producing a large proportion of low-grade product. The tanner must be able to sense the changes in hides and tanning liquors and then correctly vary processing conditions as the hides progress to the finished leather. When tanning with vegetable tannins, every hour of the day is a new challenge.

It is important to remember that in vegetable vat tannages, the work cannot be seen for many days, since the product is covered with tanning liquor for the length of the process. During this period, it is necessary to rely mainly on judgment based on long experience and to be especially sensitive to abnormal changes. Until the

late 1960's, vat tanning required 28 working days — today, the period remains a lengthy 7+ days in most tanneries making vegetable-tanned leather. This is a long time to protect your investment without seeing it.

Each year, progress is made in our knowledge because of research such as that presented in this volume. The great progess that has been made becomes evident by making comparisons with our state of understanding of vegetable tannins in the mid-1950's summarized by White.[1] As this knowledge grows and chemicals (especially the vegetable tannin preparations) are upgraded and standardized, the problems of the tanner decrease. Much of the burden on individual tanneries was removed when the extraction, bisulfiting, and spray-drying of tannin extracts were done at the source. Consistency in the properties of these tannins and adherence to quality guidelines are now realities instead of possibilities. The emergence of synthetic tannins has also played a major role in our ability to produce quality leather. Changes in market appeal, modes of transportation, sharing of research, and updated industrial practice have all contributed to vast changes in the manufacture of vegetable leather even though these changes have come only slowly in comparison with those of other industries.

VEGETABLE TANNING PROCESSES

For a recorded period of over 10,000 years, man has used tannin to convert hides into leather. A part of the reason for the slow progress in this industry is that in the past leather tanneries were small, family-oriented businesses that tended to pass from generation to generation. "One must not disturb the process that grandfather perfected." Being unable to exchange ideas has no doubt played a major role in the lack of progress, and this problem remains even today.

The earliest methods of tanning were simple "pits of liquors" with the hides being "laid in" by alternating layers of hides and plant materials containing high proportions of tannins such as bark. After the tannin was exhausted, the hides were pulled and turned. A fresh layer of bark was placed between the hides as the hides were "laid in" again. Another old method, still used today in some remote areas, is to sew the hide into a bag, fill the bag with tannin material, and hang it on a tree limb. The interaction between the wind's movement and the natural tannins does make leather. Both of these methods required long time periods, up to a year.

An early tannage was accomplished in the following way. Hair was removed by soaking hides in solutions of wood ashes or a more common method of "sweat pits." The "sweat pit" was a building where hides were hung on racks over a floor covered with chicken manure. The rising gases would loosen the hair roots quite effectively. Needless to say, the operators of sweat pits were either not allowed to go to church or had to sit in special areas! After a thorough washing, the hides were "machined" to remove all the hair possible before a "hand beamer" took over. The hand beamer actually scraped the hair and flesh off with a broad curved knife. The "bating" of the hide (to clear away surface oils and fats) was accomplished by immersing the hides in solutions containing dog dung. Although this all sounds very crude, the enzymes associated with this treatment did assist in preparing the hide for tannage.

Since vegetable tannins are quite astringent, it was critical that the initial tanning liquor of the first bath carry some buffering material. Therefore, the hide was placed in a vat containing very old liquor. This liquor would normally have a specific gravity of 1.010, a purity of 0.25 percent, and a pH of 5.0. Liquors with standards lower than these would permit growth of bacteria and cause rotting. Liquors with higher standards would cause a drawing of the hide surface or shrunken grain.

From the first day forward, the strength of the tannin solution was gradually increased, or the hides were moved to stronger solutions. Progression through liquors of 1.025 specific gravity, purity of 0.30 percent, and a pH value of 4.0 to 4.5 up to a final solution of 1.040 specific gravity, 0.40 percent purity, and a pH of 3.5 to 4.2 was common. The hides were then rinsed in a bath of low specific gravity, high tannin purity and low pH. Not only did this wash the leather, but it also contributed to fixing of the surface tannins to the hide. Again, we must remember that laboratory equipment was not available to the tanners of that day, so each had his own method of checking the properties of the liquors. Astringency was normally checked by placing liquor in the mouth. The tanner soon knew whether he should hang his hides in that liquor or make an adjustment.

Unfortunately, little has been written in English about vegetable tanning processes since the book, "The Chemistry and Technology of Leather," edited by O'Flaherty, Roddy, and Loller[2] was published. Kay[3] reviewed vegetable tanning processes used in the United States up to about 1978 in that book.

Pirich[4] has compared vegetable tanning practices used in Italy with those used in the United States. The Italian industry uses blends of chestnut, quebracho, and wattle tannins for the manufacture of shoe sole leathers. The quebracho tannins are particularly useful because of their fast penetration and good capacity to fix the fiber. The main advantage of the wattle tannin is the light color of leather that can be obtained, although this tannin also penetrates very well. The chestnut tannin has a particularly high fixation level and good color characteristics that are stable to light. In Italy, the skins are first treated with a light syntan pretannage followed by a pit tannage in which the tannin blend is composed of about 50 percent chestnut, 15 percent of a sulfited chestnut tannin, 18 percent quebracho, and 17 percent wattle tannin. The pit tannage is followed by a drum tannage with a blend of 60 percent chestnut tannin, 30 percent of a sulfited chestnut tannin, and 10 percent quebracho. Pirich also describes a ultra-rapid drum tanning process in which mixtures of these same tannins are used.[4]

Research on vegetable tanning remains active in India at the Central Leather Research Institute in Madras.[5–7] Wattle – myrobalan tannin mixtures are commonly used to manufacture a wide variety of leathers.[8–10] Wattle, myrobalan, chestnut, cutch, and quebracho mixtures were used in conjunction with an aluminum sulfate secondary treatment to produce shoe sole leathers with particularly low water absorbency.[10] Chrome tannage is commonly followed by a secondary wattle tannage and a final aluminum sulfate treatment for making water-resistant shoe uppers.[11] The Leather Industries Research Institute in Grahamstown, South Africa, has contributed greatly to our understanding of the vegetable tanning processes, particularly with regard to the use of wattle tannins. Williams-Wynn[12] has summarized the Institute's research on the use of wattle retannage of chromium-tanned leathers

for the production of shoe uppers. To reduce hydrolytic degradation of the collagen caused by increases in acidity resulting from longterm reactions involved with the chromium-tannin complexes, Williams-Wynn recommended reducing the degree of chromium pretannage and/or the use of anionic syntans prior to the vegetable retannage. Formate masking was effective in reducing the loss of chromium in the retannage, in reducing the amount of vegetable tannin fixed in the retannage, and in reducing the acidity of the resulting leather. Wattle tannins are more effective than quebracho or hydrolyzable tannins as a retannage for these leathers, the wattle retannage providing the most stable leather and the least amount of chromium stripped off in the retannage.[13]

The LIRITAN system developed by Shuttleworth and coworkers has been widely adopted by the South African tanning industry because of environmental pressures against the disposal of tanning liquors.[14] This "no-effluent" system requires some changes from the traditional pit tannin processes, but it is readily employed in traditionally designed plants. The skins are pickled with a 2.5 percent Calgon solution that needs to be discarded only about once a year. The pickled skins are then washed in two color vats one day each and then tanned in a 6 to 8 hot pit system. For manufacture of sole leather, the strength of the tanning liquor is kept high and at 95 °C. The tanning liquor is circulated through a series of vats for 8 days. The liquor does not need to be discarded as an equilibrium in the proportion of tannins to non-tans is established. A rapid-tannage combination pit-drum process has also been developed using the same basic process.

The most recent major advance in vegetable tanning processes is the development of a combination vegetable-aluminum tannage.[15–21] Leathers produced with this type of tannage have exceptionally high heat stability. Sykes and coworkers[17,18] showed that aluminum forms complexes with the phenolics with two adjacent hydroxyls (catechols), and the presence of a third adjacent hydroxyl (pyrogallols) increases the stability of the complex. Aluminum also can complex with carbonyl and amino functions, so that effective cross-linking of tannin – aluminium – collagen occurs. Coordination of tannin – aluminum complexes permits the formation of extended tannin complexes that account for the substantial increase in shrinkage temperatures above those obtained with conventional vegetable-tanned leathers. Of all the processes developed for the use of vegetable tannins, the vegetable-aluminum tannage shows the most promise of displacing chromium tanning processes should that ever be required.[19,20]

I use a 48-hour paddle method that allows 6 hours soaking to bring the water content up and remove the preserving salt. Lime, enzymes, sulfide, and sulfhydrates are used to "burn" the hair during one day. The second day is a lime cold-water bath to swell the hides and open the fibers to make room for the tannage. The lime is removed in a bath of ammonium sulfate or ammonium chloride. Pancreatic enzymes are added to remove oils and greases. A fast vegetable tannage is used in our tannery. Day one is a pretan with Calgon or hexametaphosphate. Day two is a color bath in a weak liquor. This is followed by 5 days in strong, hot liquors. Rinsing is done in a weak, high-purity, low-pH bath.

Although much has been written on the merits of "no effluent" tannages, I have yet to see one that works. Some balancing does occur, but in actual practice, one is left with two basic choices: remove a little daily or remove larger amounts of spent materials later on. We also find that, although the modern processes work very well, they also require strict guidelines as to phosphates, acids, tannins, motion, and temperatures. Should any of these parameters get out of line, you will not make good leather. To be compatible with today's rapid tannage, the tannin extracts must be highly bisulfited. Not only does this give smaller molecules, permitting better penetration, but this type of tannin also enhances the final color of the leather. Temperatures are held at the highest safe range to keep the hide more open and the liquor more fluid. Higher temperatures should also accelerate any chemical reactions between the salts and hide collagen. Phenolic syntans are a must for most rapid tannages. The dispersion of non-tans and the interaction between syntans and natural tannins tend to speed-up penetration, even out the colors, and retard bacterial growth. The constant heating and entrainment of air in tannin liquors tend to oxidize the tannin liquors, and the addition of syntans helps to reduce this problem.

LEATHER PROPERTIES RELATED TO TANNIN TYPE

Tannins from different sources, with their own particular makeup, will determine the properties of the resulting leather. Most North American tanners (like those in most other parts of the world) prefer to use blends of different tannins, but as described above, the blends used vary considerably in different countries. The marketplace determines the properties required, and each tanner must adjust the composition of the tannins used accordingly. The U.S. market requires heavy, tight, light-colored leathers in comparison to the market demands in England or Italy, for example. Each type of leather will be produced using different tannin sources. It is only recently that other countries have really tried to match the properties of North American leathers. Recent import figures indicate their great success.

When comparing the properties of leathers made with different tannin sources, I would make the personal observations summarized in Table 1.

STATUS OF THE U.S. LEATHER INDUSTRY

The United States is not a major producer of leather goods, with about 21,000 tons of tannin imported annually. The major sources of tannins imported into the United States are wattle from South Africa and Brazil and quebracho from South America (primarily Argentina and Paraguay). Smaller amounts of chestnut tannin are imported from France and Italy, and some specialty tannins are imported from Peru, Turkey, and India. Actually, Turkey exports large tonnages of sumac leaves to the United States, most of which are used for medicines and the beer industry. The United States government stockpiles various tannins in the event of war. Although a portion is released for sale each year, there still should be a tannin stockpile of approximately 100,000 tons.

Table 1. Properties of Different Types of Vegetable Tannages

Tannin Source	Properties
Quebracho	good rapid penetration mild acid produces good roundness especially good for leathers of medium thickness leaves some areas loose fiber bond is excellent
Wattle	good rapid penetration mild acid produces flat, thin leather good tight surface areas fiber bond is good
Chestnut	slow penetration strong natural acid produces round, thick leather very tight surface areas fiber bond is good
Eucalyptus	well-balanced, catecholic and pyrogallolic penetrates well carries good acid produces a round leather of medium thickness fiber bond is good
Myrobalans	very good natural acid value poor color slow penetration good surface tightness fiber bond is good
Valonia	very good soft tannage penetration good acid value good appears to have especially fine molecular structure does not break at low temperatures fiber bond is fair
Sumac	good for light fastness flat, soft tannage
Tara	clear color good light fastness low temperature leaching ability

At present, only slightly over 20,000 hides per day are tanned in the United States. Of this total, about 4,700 hides are tanned with vegetable tannins, and 15,300 hides are tanned with chromium. Much of the chrome-tanned leather is retanned with vegetable tannin blends, so the current U.S. use of vegetable tannins is approximately as follows:

4,700 hides	x	(13 lbs. pure tannin x 1.5)	=	91,650 lbs.[a]
15,300 hides	x	1.3[b]	=	19,890 lbs.[a]
Total			=	111,540 lbs./day
			=	13,365 tons/year

[a]Extract at 66% tannin content
[b]Based on 5% of hide weight

These figures may underestimate the amount of vegetable tannin used in retannage but not by more than 10 percent of the total volume. Therefore, the U.S. use of vegetable tannins for leather manufacture is about 15,000 tons per year. The leather goods industry has steadily declined in North America since the end of World War II. Many reasons for this drop toward extinction are suggested:

- With the modernization of industry and revolution wrought by the automobile, no one walks anymore, and the need for good quality shoes has declined.
- Foreign styling has excelled over North American styling, resulting in importation of footwear from abroad.
- The lack of capital expenditure has resulted in U.S. tanneries becoming outdated and uneconomical.
- Research funding has not been made available either by industry or government to stimulate modernization of tanning processes.
- The environment has become a concern. All available research money has been funneled into waste management.
- Packing houses have gladly allowed brokers to handle their byproducts, so it became easy to export hides.
- Our government chooses to assist in exportation of hides off the continent to provide industrial income for Third World countries.
- No formal training program has been established to educate young people in the science and art of leather manufacture.

- Tanners have clung to protection of their processing knowledge.
- Public opinion of the industry has become negatively slanted.
- Labor unions have gained a large voice in the day-to-day operations of tanning facilities.

Despite these problems, there will always be some leather produced in North America. Vegetable-tanned leather will only share in the high-quality markets such as briefcases, luggage, pet equipment, harnesses, waist belts, purses and, yes, quality leather shoes will always be produced and sold in North America.

The next 10 years will see a worldwide shortage of raw hides. With the decrease in consumption of red meat, we are already seeing times when hides are in short supply. The United States alone has seen a drop in beef consumption of 9 percent in the last 2 years. How far this drop will continue and what the government might do about it are only guesswork at this time. The current controversy between the United States and EEC countries on steroid-fed U.S. cattle is a good example of the problems we will face in the future. Renewed emphasis on environmental quality, if allowed to get out of hand, could also stifle the leather industry as well as many other industries in the United States.

The competition all over the world is intense. Recently, I had a supplier of chemicals at my business place for 2 days. He services over 100 tanneries in Taiwan, not a country well-known to the layman as a producer of leather goods. He feels he has the opportunity to sell more products in Taiwan than I have in the United States and Canada combined. Brazilian production has caused the closing of several of our plants but, before long, countries such as Brazil may begin consuming most of the leather they produce. They are on the move today, but how long will it be before tanners in countries such as Taiwan and Brazil face government regulations to match those in the United States? Are our shoe companies going to stay in Brazil or the Far East when they can do better on this continent? All things run in cycles and, with research done now, we could be ready to pick up our production when other countries are forced to slow down. We badly need the cooperation of our governments and our research groups and all the good public relations we can muster.

CONCLUSIONS

Today, the use of vegetable tannins in leather manufacture needs a boost. Research such as that presented in the other chapters of this volume is one of the most effective ways of changing our current situation and needs strong support by both government and industry. The research needs to be sponsored by funding sources that are not responsible to any one supply group as is often the case today. Not only do we need more and different sources of supply of tannins, we also need new avenues for using tannins to produce leather goods. If new materials for tanning can be produced from agricultural wastes that are already a problem, possibly regulators will become more understanding. The recent work of Dale Galloway and his associates provides just such an example in the eyes of many. Here, possibilities exist for production of a wide range of specialty chemicals from a

very troublesome agricultural residue. Development of tannin extracts suitable for leather manufacture from domestically produced residues is particularly attractive in North America because our ordinary sources of tannins are strictly controlled by foreign cartels. Why should we as taxpayers support the huge stockpile of tannin held by the U.S. government? The highest quality leather is still produced with vegetable tannage, and it is not entirely unrealistic to think that, with streamlined processing, we could eliminate the importation of chrome for leather manufacture.

Our tanning processes badly need upgrading, and much of the negative treatment handed us by our own citizens is brought about by poor process procedures and outdated facilities. These problems must be corrected, not ignored, if our industry is to survive. A valid school for proper training of young people in the science and art of leather manufacture needs to be established. The training provided should span basic research on tannin chemistry and on proteins and the mechanism of tannin-protein complexation through actual practice in existing tanneries and this background combined with good business and marketing training.

REFERENCES

1. White, T. The chemical principles of vegetable tannage. *J. Soc. Leather Trades Chem.* 40:77 (1956).
2. O'Flaherty, F.; Roddy, W.T.; Loller, R.M. (eds.) The Chemistry and Techonology of Leather. Reinhold Publishing Company, New York. Vols. 1-4 (1958).
3. Kay, A.N. The process of vegetable tannage. *In*: O'Flaherty, F.; Roddy, W.T.; Loller, R. M. (eds.) Chemistry and Technology of Leather. Vol. 2, Reinhold Publishing Company, New York, pp. 165-200 (1958).
4. Pirich, E.P. Experiences with vegetable tanning systems and the extract blends used. *J. Am. Leather Chem. Assoc.* 68:209 (1984).
5. Rageeb, H.A. Towards pragmatism in tanning systems. *Tanner* 32:467 (1978).
6. Santappa, M.; Rao, V.S.S. Vegetable tannins – a review. *J. Sci. Ind. Res.* 41:705 (1982).
7. Gosh, D.; Vijayalakshimi, K.; Nayudamma, Y. Studies on the effect of non-tans in vegetable tannins. *Leather Sci. Madras* 14:154 (1967).
8. Deb, J.C.; Prasad, L.M.; Hore, G.N.; De, J.M. Manufacture of rapid vegetable tanned sheep and goat skin for leather goods. *Leather Sci. Madras* 20:284 (1973).
9. Ganesan, A.; Rao, M.V. Vegetable tanning of Punjab wool sheep skin. *Leather Sci. Madras* 24:144 (1977).
10. Olivannan, M.S.; Ramaswany, T. Waterproofing of sole leather with basic aluminum sulphate dipping. Effect of pretanning with various vegetable tanning materials. *Leather Sci. Madras* 18:186 (1971).
11. Rao, M.S.; Kamat, D.H.; Selvarangan R. Upper leather from raw sheep skins (red hair). *Leather Sci. Madras* 27:263 (1980).
12. Williams-Wynn, D.A. Consideration of the vegetable tannin/chromium-collagen system. *J. Soc. Leather Trades Chem.* 54:27 (1970).
13. Atkinson, J.H. Modern methods for producing vegetable tanned leather. *In*: Radnotti L. (ed.) Proc. 6th Cong. Leather Ind. OMIKK-Technoinform. Budapest, Hungary, Vol. 1 pp. 1-13 (1978).
14. Shuttleworth, N.P. The LIRITAN minimum effluent vegetable tanning system. *J. Soc. Leather Chem. Assoc.* 71:336 (1976).
15. Slabbert, N.P. Metal complexes and mineral tanning. *Das Leder* 31:79 (1980).

16. Slabbert, N.P. Mimosa-Al tannages – an alternative to chrome tanning. *J. Am. Leather Chem. Assoc.* 76:231 (1981).

17. Sykes, R.L.; Carter, C.W. Tannage with aluminum salts. Part 1. Reactions involving simple polyphenolic compounds. *J. Soc. Leather Tech. Chem.* 64:29 (1980).

18. Sykes, R.L.; Hancock, R.A.; Orsulick S.T. Tannage with aluminum salts. Part II. Chemical basis for the reactions with polyphenols. *J. Soc. Leather Tech. Chem.* 64:32 (1980).

19. Tonigold, L.; Heidemann, E. Limitations and possibilities of chromium free tanning. *Das Leder* 33:131 (1982).

20. Tonigold, L.; Heidemann, E. Tannage with a combination of vegetable tanning agents, syntans, and alumninum salts. *In*: Radnotti, L. (ed.) Proc. 7th Congress Leather Industry. OMIKK-Technoinform. Budapest, Hungary, pp. 428-434 (1982).

21. Kallenberger, W.E.; Hernadez J.F. Preliminary experiments in the tanning action of vegetable tannins combined with metal complexes. *J. Am. Leather Chem. Assoc.* 78:217 (1983).

CONDENSED TANNINS AS A SOURCE OF NOVEL BIOCIDES

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ABSTRACT

The condensed tannins are natural preservatives of lignocellulosic materials. They are commonly found in high concentrations in many external protective plant tissues like seedcoats and bark as well as in the heartwood of some tree species. Condensed tannins have traditionally been used in many areas of the world as preservatives for fishing nets, twines, and fabrics. More modern applications that have been investigated are food and wood preservatives, with especially good results achieved for the latter. Treatment with a procyanidin/copper(II) chelate yielded wood blocks with greater resistance to decay by *Coriolus versicolor* than pentachlorophenol when evaluated in a standard ASTM soil-block test. Condensed tannins can also be reacted directly with the wood to give it decay resistance and dimensional stability similar to a naturally durable wood. It is also possible to synthesize flavonoids with antimicrobial activity by thiolysis of procyanidins and ketalization of catechin. The structure-activity relations of these semisynthetic flavonoids have provided information on the mode of action of flavonoid phytoalexins.

INTRODUCTION

Flavonoids have a broad spectrum of functions in plants ranging from biocides to pigments. Although there has been some controversy over the role of polymeric flavonoids, it is now generally accepted that they have a protective function based on their protein complexing ability.[1] Many plant pathogenic fungi – for example, the wood-destroying white-rot fungi – excrete extracellular enzymes that break down cellulose to glucose, which is subsequently absorbed and metabolized by the fungal mycelium. Condensed tannins may act as growth inhibitors of these fungi

by complexing with their extracelular enzymes to block (or at least slow down) their action. Inhibition of mycelial growth by polyflavonoids has been reported for a number of fungal species.[2–4] This protective activity of condensed tannins is consistent with their localization in the outer tissues of plants (e.g., bark, seedcoats, foliage, etc.)

Monomeric flavonoids are also important natural antifungal and antibacterial agents. When a plant suffers some type of damage, either biological or mechanical, a common response is the production of a toxic chemical by the tissues immediately surrounding the trauma site to help restrict the spread of the invading pathogen. These induced natural biocides are called phytoalexins. Although a wide variety of chemicals is classified as phytoalexins, perhaps the most commonly reported ones are flavonoids.[5] Of course, non-phytoalexin flavonoid biocides have been reported as well.[6,7] In this chapter, some work we have performed on the pesticidal properties of polyflavonoids and monomeric flavonoids will be discussed.

MONOMERIC FLAVONOID BIOCIDES FROM POLYFLAVONOIDS

Naturally occurring flavonoid biocides have a wide variety of structures and functional groups (Figure 1). There is very little information available, however,

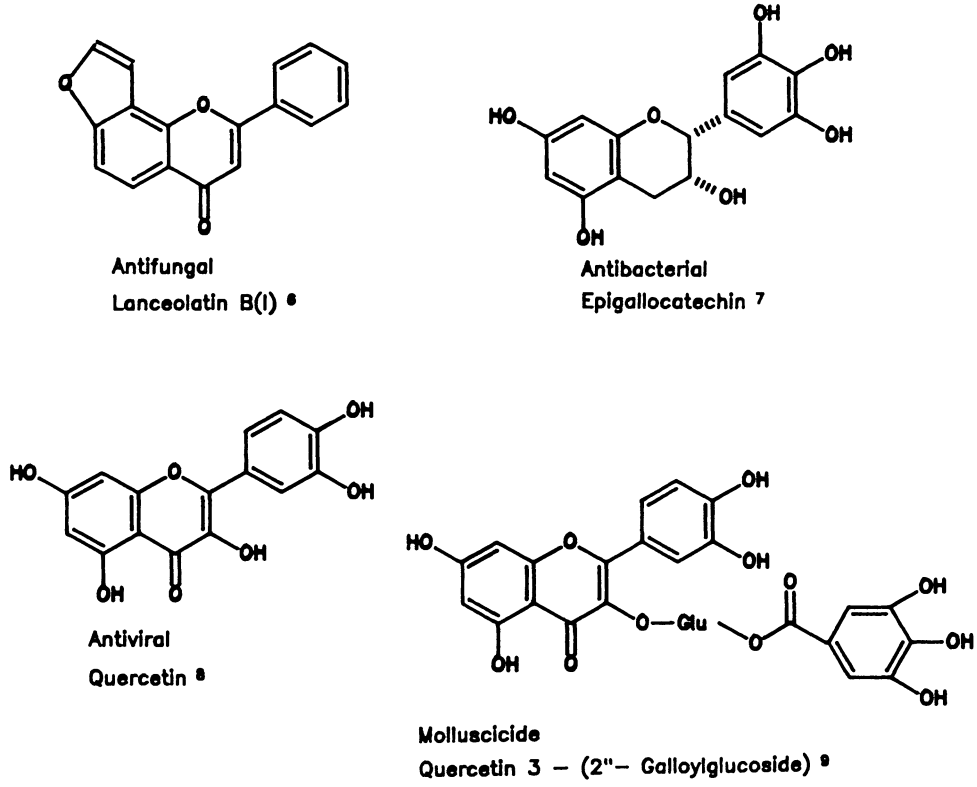


Figure 1. *Some naturally occurring flavonoid biocides.*

on the mode of action of flavonoids or on what structural features are required for biological activity. Such information is critical for the commercial development of flavonoid biocides. We have an ongoing interest in trying to define the structure-activity relationships of flavonoid phytoalexins, particularly those with fungicide properties, by using semisynthetic analogues.[10,11]

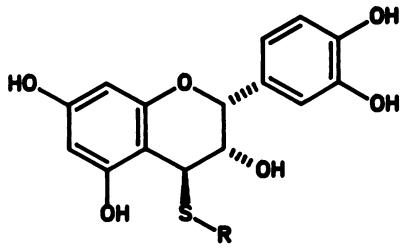
Research into the chemistry and biochemistry of phytoalexins has become one of the dominant themes of plant science. An important objective of this work is to explore the application of phytoalexins for commercial crop protection. One way this could be done is to apply "elicitors" to crop plants to make them produce phytoalexins prior to infection and, therefore, make them more resistant to fungal attack.[12,13] This concept, however, is not practical due to the phytotoxicity and possible mammalian toxicity of the induced phytoalexins. Another approach is to tailor phytoalexin structure to be effective against specific pathogens. Plant pathogenic fungi have evolved mechanisms to detoxify phytoalexins. It might be possible to biologically engineer the phytoalexin biosynthetic mechanism of the plant to produce a different phytoalexin that the fungal pathogens cannot detoxify.[14] Again, information on the structure/activity relationships and mode of action of the phytoalexins would be valuable in developing this type of approach. There is also the possibility that optimized flavonoid biocides will have sufficient activity in themselves to warrant use as conventional commercial pesticides, or act as models for synthetic analogues.

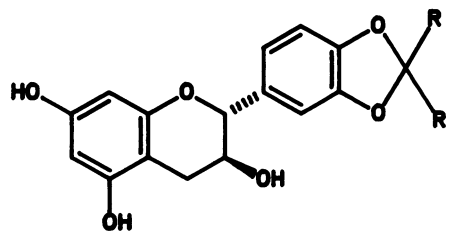
For a biologically active compound like a fungicide to exhibit activity, it must first diffuse from its site of application (usually the exterior of the cell) to its site of action (often within the cell), then partition itself onto the active site.[15] The rate of both these events will depend on the lipophilicity of the compound. Once at the active site, the compound exerts some chemical or physical effect that accounts for its activity. It is well known that, within a set of related fungicides or other biologically active compounds, the most active chemicals will often have a similar lipophilicity.[15] This might be expected since a particular lipophilicity will allow the most efficient transportation of a chemical to an active site within the target cell. Biologically active natural products, such as the flavonoids discussed here, would have similar polarity requirements.

A standard measure of lipophilicity is P, the octanol/water partition coefficient. It is often experimentally difficult to measure P directly, however. In order to compare the lipophilicity of different compounds, a relative measure, R_M, is often used.[16] An R_M can be calculated using a number of techniques, but most commonly by TLC using the following equation:

$$R_M = \log(1/R_f - 1) \tag{1}$$

Our initial work involved investigating the toxicity characteristics of a series of flavonoid derivatives made by acidic thiolysis of procyanidins with alkylthiols [epicatechin-4-alkylsulphides, (**1**) - (**6**)].[10] The overall lipophilicity of these compounds, as well as their antifungal and antibacterial properties, varied with the length of the alkyl chain. Preliminary work with epicatechin derivatives contain-





(1) R = -$(CH_2)_5$-CH_3
(2) R = -$(CH_2)_7$-CH_3
(3) R = -$(CH_2)_8$-CH_3
(4) R = -$(CH_2)_9$-CH_3
(5) R = -$(CH_2)_{11}$-CH_3
(6) R = -$(CH_2)_{15}$-CH_3

(7) R = -CH_2-CH_3
(8) R = -$(CH_2)_2$-CH_3
(9) R = -$(CH_2)_3$-CH_3
(10) R = -$(CH_2)_4$-CH_3
(11) R = -$(CH_2)_5$-CH_3
(12) R = -$(CH_2)_7$-CH_3

ing an even number of carbon atoms in the alkyl substituent indicated maximum activity occurred with the decyl derivative (**4**),[10] whereas, later research showed epicatechin-4-nonylsulphide (**3**) to be the most active compound of this type.[11] In order to obtain further information on the relationship between lipophilicity and biocidal activity for flavonoids, a different set of derivatives, catechin dialkyl ketals (**7**) - (**12**), was prepared by the reaction of catechin with the appropriate ketone at 0 °C with HCl as catalyst.[11] To allow comparison between the lipophilicity requirements of the two types of analogues, the R_M's of both sets of flavonoid derivatives were determined.

The relationships between R_M and activity for the two series of semisynthetic flavonoids against *Aphanomyces euteiches*, *Fusarium solani*, and *Streptococcus faecium* are shown in Figures 2, 3, and 4. Radial growth inhibition was determined for the first two organisms on agar plates doped with the test compound at 0.1 or 0.05 mM. For the bacterium, the minimum inhibitory concentrations (MIC's) of the flavonoids were determined. The antibacterial activity of the most effective ketal, catechin 7-tridecanone ketal (**11**), was compared with that of conventional antibiotics against some Gram-positive bacteria (Table 1) and found to be similar in some cases.

Table 1. Antibacterial Properties (Minimum Inhibitory Concentration, MIC) of Catechin 7-Tridecanone Ketal (CTK) Compared to Some Antibiotics.

Bacteria	CTK	Streptomycin	Penicillin G	Tetracycline
Streptococcus faecium	2	75	0.5	0.25
Bacillus cereus	5	5	1.0	<1.0
Micrococcus luteus	2	5	0.75	0.75
Staphylococcus aureus	10	4	<0.1	0.25

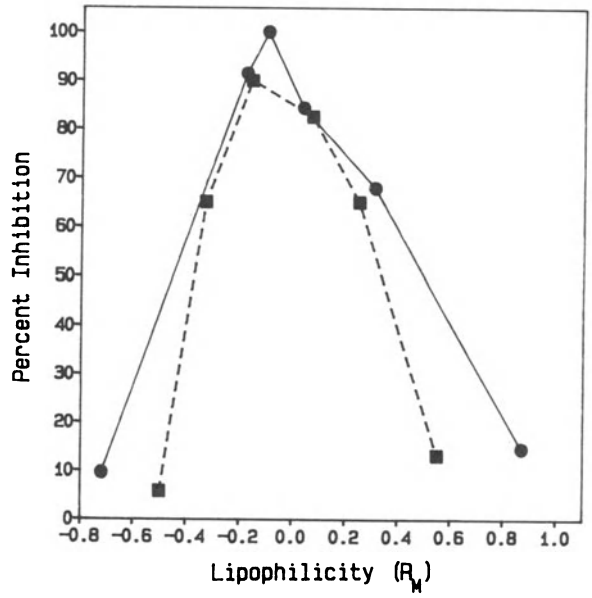


Figure 2. *Sensitivity of Aphanomyces euteiches to epicatechin-alkylsulfides* (O, (**1**) [R_m = -0.72], (**2**) [-0.18], (**3**) [-0.10], (**4**) [0.035], (**5**) [0.31], (**6**) [0.87]) *and catechin dialkyl ketals* (□, (**7**) [R_m = -0.50], (**8**) [-0.33], (**9**) [-0.16], (**10**) [0.07], (**11**) [0.25], (**12**) [0.55]) *of differing lipophilicities at a concenteration of 0.05 mM.*

Naturally occurring flavonoids with pesticidal properties have a wide variety of structures (Figure 1, also see[17]). The two semisynthetic flavonoids described here are also different in functionality and three-dimensional shape. It is unlikely that both could have the same mechanism of action if a specific molecular shape is required for activity. As shown in Figures 1 and 2, the most active members of the two sets of flavonoid derivatives have a very similar R_M, approximately -0.1, and a level of activity within a factor of 2 against both fungi. The situation with the baterium, *Streptococcus faecium*, is quite different, however. The most active epicatechin-4-alkylsulphide and catechin dialkylketal differed in R_M by more than 0.2 units and have a difference in activity greater than an order of magnitude. These observations are consistent with the two types of derivatives having similar modes of action against fungi, but a different mechanism on the bacterium.

There is a growing consensus that, in most systems, flavonoids exert their toxicity by some membrane-associated phenomenon[18–20] again indicating the possible importance of lipophilicity in their activity. The relative lipophilicities of flavonoid phytoalexins have been qualitatively compared,[18,21] but no clear trends have been reported. Similarly, the structural requirements for activity have not been well defined. It is generally agreed, however, that at least one hydroxyl group and a certain degree of lipophilicity are required for biocidal activity.

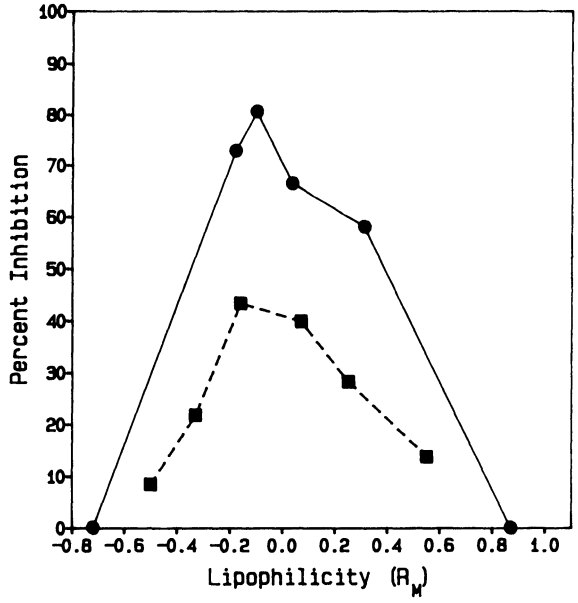


Figure 3. *Sensitivity of Fusarium solani to epicatechin-alkylsulfides. (See caption to Figure 2 for legend.)*

These same requirements for a hydroxyl group and a degree of lipophilicity are also found in the simpler commercial phenolic fungicides such as *p*-pentyl phenol, dinitrophenol, and pentachlorophenol. These compounds exert their toxicity through the acidity of the hydroxyl group by uncoupling oxidative phosphorylation. Protons are conducted across the lipid-containing mitochondrial membrane, thereby destroying the proton differential produced by electron transport that is required for the formation of ATP.[18] We hypothesized that the flavonoid phytoalexins exert their fungitoxicity through a similar mechanism.[11]

So that more information could be obtained on the structural requirements for the antifungal activity of flavonoids, the lipophilicity and activity of two naturally occurring phytoalexins, pisatin (**13**), and maackiain (**14**), were compared to those of the alkylsulfide and ketal compounds described above (Table 2). Pisatin and maackiain have a similar structure, both being based on the pterocarpan skeleton with a dioxole ring fused to the flavonoid B-ring, and they contain a single hydroxyl group, aliphatic in the former and phenolic in the latter. With these structural similarities, it is not surprising that they have similar R_M's, -0.14 for pisatin and -0.18 for maackiain (Table 2).

During the evolution of the phytoalexin system in the plant, the more fungitoxic phytoalexins would be expected to be selected for, as this would help maximize a plant's reproductive potential. Overall lipophilicity of the phytoalexin can be readily changed by the plant during the biosynthetic process through hydroxylase and dehydroxylase enzymes and by methylation and addition of isoprenyl

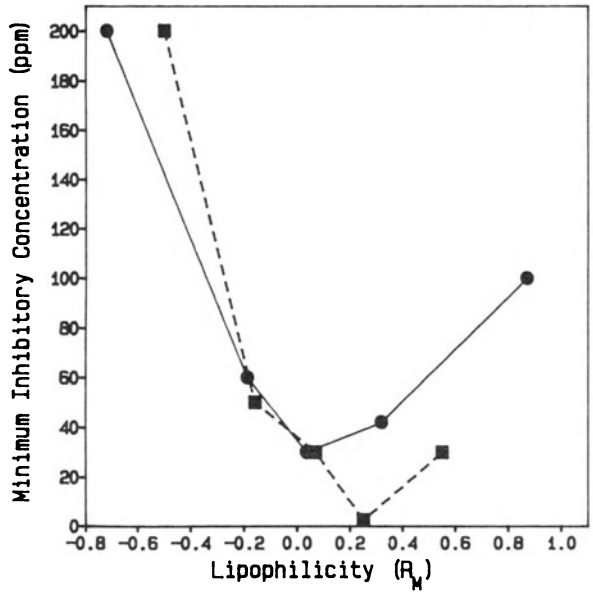
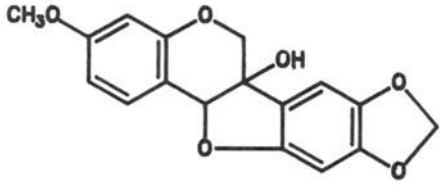


Figure 4. *Minimum inhibitory concentrations of epicatechin-alkylsulfides of differing lipophilicities toward Streptococcus faecium. See caption to Figure 2 for legend.*

substituents. If the analogues described here have the same fungitoxic mechanism as the phytoalexins, one might expect the R_M's of the most active derivatives to be similar to that of the evolution-optimized flavonoid phytoalexins. This is what is observed. The R_M's for the most active flavonoid analogues, -0.10 for epicatechin-4-nonylsulphide (**3**) and -0.16 for catechin-nonanone ketal (**9**), are very close to values for the phytoalexins (Table 2).

The effects of pisatin and maackiain on the growth of the two fungi tested are quite different. Maackiain is a significantly better inhibitor of radial fungal growth than pisatin. If these compounds act as uncouplers, this difference could be due to



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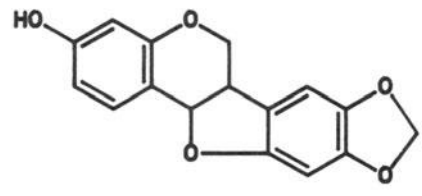


(14)

Table 2. R_M, Fungal Radial Growth Inhibition and Bacterial MIC of Pisatin (13) and Maackiain (14) Compared to Epicatechin-4-Nonylsulphide (3), Catechin 5-Nonanone Ketal (10), and Catechin-Tridecanone Ketal (11). The MIC for the Latter is Estimated from Figure 4.

		Percent Inhibition		MIC (ppm)
	R_M	*A. euteiches*[a]	*F. solani*[b]	*S. faecium*
Epicatechin-4-nonylsulphide	-0.010	100	80.6	45 (est.)
Catechin 5-nonanone ketal	-0.16	89.9	43.3	50
Catechin 7-tridecanone ketal	0.25	65.0	28.2	2.5
Pisatin	-0.14	6.2	9.4	>200
Maackiain	-0.18	34.5	39.2	>200

[a] Concentration of test compound - 0.05 mM
[b] Concentration of test compound - 0.1 mM

the relative acidity of the hydroxyl groups of the two compounds. Within certain limits, stronger acids are better uncouplers than weaker acids. Since maackiain has a phenolic hydroxyl, it will be more acidic than the aliphatic hydroxyl of pisatin and thus be expected to be the more fungitoxic of the two compounds. This relative fungitoxicity is what was observed.

Even with having essentially the same R_M's, the phytoalexins have lower antifungal activity than epicatechin-4-nonylsulphide (**3**) and catechin 5-nonanone ketal (**9**) (see Table 2). This can be explained in terms of the number of hydroxyl groups per molecule. It might be expected that for phenolics of a given lipophilicity, compounds with a greater number of hydroxyl groups will be more efficient uncouplers on a molar basis since they will be able to transfer more protons per molecule. The most active analogue (**3**) has five hydroxyl groups, whereas, the less active (**9**) has three. Both pisatin and maackiain are less active still, and have only one hydroxyl group.

The observed structure/activity relationships suggest that the epicatechin alkylsulfides and catechin ketals, as well as the natural phytoalexins, pisatin and maackiain, function primarily as uncouplers of oxidative phosphorylation against fungi. The relative acidity and number of hydroxyl groups per molecule appear to be the main factors affecting the antifungal activity of these flavonoids. The situation with the bacteria is more complex. In the latter case, there is no simple correlation between lipophilicity and antibacterial activity for both analogues, indicating they have different modes of action. This may also be true for the natural phytoalexins.

WOOD PRESERVATIVES BASED ON POLYFLAVONOIDS

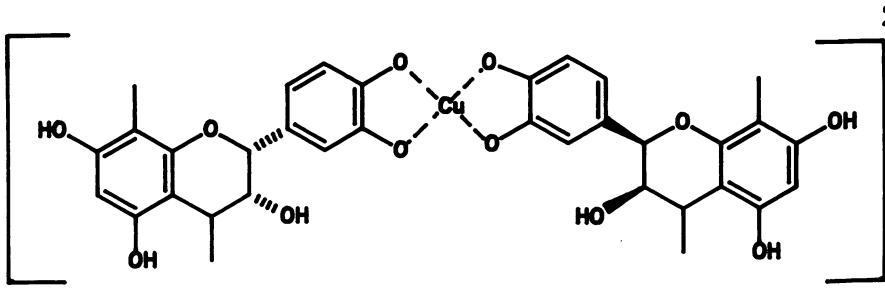
A tree has to protect itself from many of the same wood decay hazards that wood-in-use is exposed to. It is interesting that there are close parallels between how the wood treatment industry protects wood from degradation and how trees do it. Just as conventional wood-treating technology impregnates the outer layer of a nondurable piece of wood with a material (e.g., oil-borne pentachlorophenol)

that either kills or retards the growth of wood-decaying organisms, a live tree such as a southern pine will have chemicals with similar properties (polyflavonoids) in its outer layer, the bark. Trees that grow in particularly hazardous environments or have long lifetimes (e.g., baldcypress and the redwoods, respectively) also tend to have a large proportion of the stem composed of heartwood that contains extractives to make it decay resistant. The coastal redwood is a particularly vigorous species and can have a lifetime as long as 3,500 years.[22] Interestingly, polyflavonoids appear to be responsible for the decay resistance of both the bark and wood of this species.[23] Of course, trees also have active defence mechanisms, such as the phytoalexins described above, that contribute to tree survivability.[24]

As mentioned previously, it is thought that polyflavonoids exert their pesticidal effects through their ability to complex with proteins. Most wood-destroying fungi grow by excreting extra-cellular enzymes that break down the wood constituents so they can be absorbed and metabolized by the fungus. If polyflavonoids are present, they form a complex with the enzyme, rendering the latter insoluble and inactive. The same chemical functionality that allows this protein complexation, the *ortho*-3,4-dihydroxy aromatic ring of the monomers, is also capable of complexing with metals (Chapter 15 of this volume).

Copper(II) salts and complexes are effective and environmentally benign fungicides. This metal forms the basis for the preservative efficacy of CCA, copper naphthenates and copper quinolinolate, as well as other fungicidal systems. We have investigated the use of condensed tannins complexed with copper ions as wood preservatives for both ground-contact and above ground applications. A representation of the type of complex that could form between a procyanidin (as is found in southern pine bark) and copper(II) is shown in structure (**15**).

Laboratory testing has shown that polyflavonoid/copper(II) complexes are effective wood preservatives (Table 3).[24,25] Condensed tannin-containing bark extracts from loblolly pine (*Pinus taeda*) were evaluated in laboratory soil block tests (ASTM D1413-81) in both complexed and uncomplexed forms. Bark extracts by themselves did not cause any reduction in weight loss of pressure-treated wood blocks at the retentions tested. They do have efficacy as wood preservatives, however, when complexed with copper(II) ions. The best experimental wood preservative



(15)

formulation shown in Table 3 is a dual treatment entailing initial impregnation with an aqueous sulphited southern pine bark extract, followed by treatment with an aqueous solution of $CuCl_2$. At some retentions, this method yielded birch wood blocks with greater resistance to decay by *Coriolus versicolor*, a standard white-rot fungus, than pentachlorophenol. At a total retention of 10 kg/m,[3] wood samples treated with the sulphited tannin extract/$CuCl_2$ combination had about a third the weight loss of pentachlorophenol-treated blocks at approximately the same loading.

The commercial preservative system most similar to a copper/polyflavonoid system is probably copper naphthenate. Both systems rely on copper to be the primary fungicidal component. The naphthenic acid and polyflavonoid components are mainly there to provide fixation sites for the copper and some supplementary

Table 3. Soil Block (Birch) Tests Using Copper-Complexed, Loblolly Pine Bark Sulfite Extract (LPBSE), and Loblolly Pine Bark Acetone/Water Extract (LPBAWE) Treated With a Two-Stage Procedure and LPBSE/$CuSO_4$ in Aqueous Ammonia, Compared to Pentachlorophenol, $CuCl_2$ and Untreated Controls with the White Rot Fungus *C. versicolor*.

Treatment	Conc. in Soln.(%)		Mean Retention[a]		Mean	(S.D.)[1]
	Organic Component	Copper Salt	Organic Component	Copper Salt	Wt. Loss (%)	
LPBSE II + $CuCl_2$	0.5	0.25	3.09	1.53	15.3	(3.8)
	1.0	0.5	6.26	3.14	2.6	(1.2)
	2.0	1.0	12.34	6.27	1.5	(1.2)
	4.0	2.0	22.93	12.22	0.3	(0.4)
LPBAWE + $CuCl_2$	0.5	0.25	3.53	1.77	49.0	(10.2)
	1.0	0.5	7.13	3.58	32.7	(9.0)
	2.0	1.0	14.23	7.07	18.5	(3.8)
	4.0	2.0	29.38	13.75	11.5	(2.2)
1:1 LPBSE/$CuSO_4$	1.0		6.09		32.1	(6.2)
	2.0		12.47		13.6	(2.5)
	3.0		18.60		8.1	(2.1)
	4.0		24.87		3.1	(0.8)
	5.0		31.46		1.0	(0.6)
Pentachlorophenol (Ethanol Solvent)	0.5		2.52		62.3	(6.9)
	1.0		5.05		33.6	(4.0)
	2.0		10.27		7.0	(1.8)
	3.0		15.19		0.9	(1.5)
$CuCl_2$ Only	0.25			1.49	35.3	(6.7)
	0.5			2.97	15.5	(5.9)
	1.0			6.11	11.4	(1.8)
	2.0			12.18	12.1	(3.3)
Untreated Controls					>50[b]	

[a]Average of five replicates

[b]Blocks in these sets could not be separated from the soil.

fungicidal activity. Comparison of a sulfited spruce bark extract/copper system with ammoniacal copper naphthenate found the former system was more effective in soil block tests (Table 4).

For commercial applications, a treatment method where the tannin-containing extract and copper salt could be applied in one step would be very desirable. Simply combining the two components in water results in formation of an insoluble complex. An aqueous ammonia solvent, however, will dissolve the complex. Unfortunately, exposing the extract to the alkaline conditions of the ammonia solution reduces its preservative efficacy. Wood blocks treated with the ammoniacal complex have a greater weight loss than samples exposed to the dual treatment (Table 2). This is probably due to alkaline rearrangements occurring in the tannin during the treating process that reduce its preservative efficacy.[26] We have subsequently found that the polyflavonoid/copper complex is soluble in water-ethanol combinations and are currently evaluating the efficacy of this type of treating solution, as well as oil-borne formulations.

Table 4. Results from Soil Block (Birch) Tests (ASTM D1413-81) Comparing Copper-Complexed, Black Spruce Bark Sulfited Extract (Dual Treatment) to Ammoniacal Copper Naphthenate, After Exposure to *Coriolus versicolor*.

Treatment	Retention (Kg/m^3)	Copper Retention (Kg/m^3)	Average Weight Loss (%)[a]
Extract + $CuCl_2$	2.96 + 1.46	0.36	7.3
	5.93 + 2.91	0.70	2.7
	12.13 + 5.91	1.41	0.0
	22.25 + 11.32	2.84	0.0
Extract	2.05		51.08
	4.42		55.5
	8.56		50.3
	17.45		48.4
Ammoniacal Copper Naphthenate	3.06	0.75	39.4
	5.99	1.48	11.9
	12.07	2.92	4.0
	24.24	5.74	0.0
Untreated Controls	–		>50.0

[a] Average of five replicates

CONCLUSIONS

Over many millions of years of evolution, plants have developed very sophisticated mechanisms to protect themselves from the predations of fungi and insects. These mechanisms can be very effective, giving individuals of some tree species lifetimes measured in the thousands of years. Flavonoids are important components of

plant protection systems through their role as phytoalexins – natural biocides produced in response to injury or infection. We have synthesized two sets of flavonoid phytoalexin analogues and correlated their lipophilicity with their antifungal and antibacterial activity. The structure/activity relationships observed suggest that these analogues, and perhaps flavonoid phytoalexins in general, function primarily as uncouplers of oxidative phosphorylation. The main factors affecting the antifungal activity of the flavonoids examined were the relative acidity and number of hydroxyl groups per molecule.

In laboratory evaluations, wood preservative formulations based on polyflavonoid/copper complexes have been found to have good efficacy. The advantages of a commercial formulation based on tannins are many. Since tannins are a significant component of the human diet, it would be expected that a wood preservative based on them would have a reduced environmental impact during manufacture and treatment and also be safer for the users of the treated wood than conventional treatments. The system would also be based on a renewable resource, avoiding all the economic and social problems associated with petrochemicals. Work is continuing toward development of naturally-sourced wood preservatives.

ACKNOWLEDGMENTS

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REFERENCES

1. Gottleib, O.R. Evolution of natural products. *In*: Rowe, J.W., (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants. Springer-Verlag, New York (in press).
2. Harun, J.; Labosky, P. Antitermitic and antifungal properties of selected bark extractives. *Wood Fiber Sci.* 17(3):327 (1985).
3. Rao, S.S.R.; Rao, K.V.N. Fungitoxic activity of proanthocyanidins. *Indian J. Plant Physiol.* 19(3):278 (1986).
4. Samejima, M.; Yoshimoto, T. Effect of hot-water extracts from coniferous barks on mycelial growth of edible and pathogenic fungi. *Mokuzai Gakkaishi* 30(5):413 (1984).
5. Ingham, J.L. Phytoalexins from the leguminosae. *In*: Bailey, J.A.; Mansfield, J.W. (eds.) Phytoalexins. John Wiley and Sons, New York p. 21 (1982).
6. Pan, S.; Mukherjee, B.; Ganguly, A. Mitra, R.R.; Bhattacharyya, A. Antifungal activity of some naturally occurring flavonoids. *J. Plant Dis. Protect.* 92(4):392 (1985).
7. Mori, A.; Nishino, C.; Enoki, N.; Tawata, S. Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. *Phytochemistry* 26(8):2231 (1987).
8. Selway, J.W.T. Antiviral activity of flavones and flavans. *In*: Cody, E.; Middleton, E.; Harborne, J.B. (eds.) Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships. Alan R. Liss, Inc., New York p. 521 (1986).
9. Isobe, T.; Fukushige, T.; Noda, Y. A new flavonoid glycoside from *Polygonum nodosum*. *Chem. Lett.* :27 (1979).
10. Laks, P.E. Flavonoid biocides: Phytoalexin analogues from condensed tannins. *Phytochemistry* 26(6):1617 (1987).

11. Laks, P.E.; Pruner, M.S. Flavonoid biocides: Structure/activity relations of flavonoid phytoalexin analogues. *Phytochemistry*I 28:87 (1989).

12. Dekker, J. *In*: Green, M.C.; Spilker, D.A. (eds.) Fungicide Chemistry: Advances and Practical Applications. ACS Symp. Ser. 304:107 (1986).

13. Hait, G.N.; Sinha, A.K. Protection of wheat seedlings from *Helminthosporium* infection by seed treatment with chemicals. *J. Phytopathol.* 115:97 (1986).

14. VanEtten, H.D. Importance of phytoalexin detoxification for plant pathogenesis. Presentation at ACS National Meeting, New Orleans, Aug. 30 - Sept. 4 (1987).

15. Hansch, C.; Lien, E.J. Structure-activity relationships in antifungal agents. A survey. *J. Med. Chem.* 14(8):653 (1971).

16. Bush, I.E. Applications of the RM treatment in chromatographic analysis. *Methods Biochem. Anal.* 13:357 (1965).

17. Ingham, J.L. Phytoalexins from the Leguminosae. *In*: Bailey, J.A.; Mansfield, J.W. (eds.) Phytoalexins. John Wiley and Sons, New York. (1982).

18. Stossel, P. Structure-activity relationship of some bean phytoalexins and related isoflavonoids. *Physiol. Plant Pathol.* 26:269 (1985).

19. Weinstein, L.I.; Albersheim, P. Host-pathogen interactions XXIII. The mechanism of the antibacterial action of glycinol, a pterocarpan phytoalexin synthesized by soybeans. *Plant Physiol.* 72:557 (1983).

20. Boydston, R.; Paxton, J.D.; Koeppe, D.E. Glyceollin: A site-specific inhibitor of electron transport in isolated soybean mitochondria. *Plant Physiol.* 72:151 (1983).

21. Adesanya, S.A.; O'Neill, M.J.; Roberts, M.F. Structure-related fungitoxicity of isoflavonoids. *Physiol. Molec. Plant Pathol.* 29:95 (1986).

22. Anderson, A.B. The influence of extractives on tree properties. 1. California redwood (*Sequoia sempervirens*). *J. Inst. Wood Sci.* 8:14 (1961).

23. Zavarin, E.; Snajberk, K.; Smith, R.M. The chemistry of the natural phlobaphenes II. Further pyrolysis studies of the phlobaphenes from redwood (*Sequoia sempervirens* Endl.). *TAPPI* 48:574 (1965).

24. Laks, P.E. Wood preservation as trees do it. *Proceed. of the Amer. Wood Preservers' Assoc.*, Minneapolis, p. 147 (1988).

25. Laks, P.E.; McKaig, P.A.; Hemingway, R.W. Flavonoid biocides: wood preservatives based on condensed tannins. *Holzforschung* 42(5):299 (1988).

26. Laks, P.E.; Hemingway, R.W. Condensed tannins. Structure of the 'phenolic acids'. *Holzforschung* 41(5):287 (1987).

TANNINS AS SPECIALTY CHEMICALS: AN OVERVIEW

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ABSTRACT

Apart from their traditional use for leather manufacture and more recent applications as wood adhesives, tannins have seen limited success as specialty chemicals. Previous attempts to develop tannin-based chemicals have suffered because of our failure to appreciate fully economic requirements, purity and stability factors, and application needs of industrial processes. This chapter presents an overview of some of the chemical, physical, and structural features of tannins that have a bearing on future specialty chemical development. Information also is provided on areas where tannins have recently been utilized.

INTRODUCTION

The worldwide abundance of plant tannins has created continual interest in their chemical utilization. While biogenesis and function of polyflavanoids in our ecosystem have often been debated (refer to previous sections on biogenesis and biological signifiance), many beneficial applications of tannins have been discovered by mimicking nature's uses. The chemical variety of polyflavanoids present in plants, their strong association with other organic plant materials, and the difficulties of isolating and concentrating specific tannins all are impediments to utilization. Future acceptance of tannins as specialty chemicals will depend upon how well these limitations are overcome.

Primary sources of tannin are bark, wood, and nutshells. Most industrial tannins are derived from bark (wattle) and wood (quebracho), imported to North America from South Africa and South America. However, a large potential domestic source is tree bark readily available in large quantities at many mill locations.

Historically, bark products have had significant use over many centuries in the fabrication of clothing, rope, paper, furniture, roofs, and for agricultural and horticultural purposes.[1] Often, the presence of tannins has played a role in extending the life of these materials.

One of the more important commercial applications of tannins has been for tanning hide leather, a complex process that seems more art than science. The historical and chemical basis of this process has been aptly described by others[2] (also Chapter 1 and Chapter 31).

Another major use of bark tannins is for bonding wood. An extensive literature exists that describes a variety of developed tannin adhesive formulations.[2-3] These tannins are frequently used as a co-adduct with other synthetic polymers to form effective adhesives. Tannins have also been noted for both their medicinal and antiseptic properties.

Based on their polyphenolic structure, tannins have a number of other potential uses,[2-4] some of which are listed in Table 1.

Table 1. Potential and Past Uses of Tannin

Treatment or Modification	Product Process
Condensation with formaldehyde	- wood adhesives - paper and cardboard binder - phenolic molding compounds
Reaction with diisocyanate	- wood adhesives - urethane foam - coatings
Sulfonation	- drilling mud additive - mineral flotation - slow-release encapsulation
Mannich type reaction	- cationic flocculant - water treatment
Hydrogenolysis/oxidation	- monomeric phenolics and their derivatives
Complexation/heat/pressure	- dispersants - slow-release biocide - bark boards - heavy metal and oil scavengers
Isolation and purification	- leather tanning - antioxidants

FACTORS AFFECTING TANNIN'S USE FOR SPECIALTY CHEMICALS

Chemical Features

The condensed tannins are made up of a unique chemical family, the flavonoids, consisting of two aromatic rings joined through a pyran ring. The chemical properties of condensed tannins are largely a function of hydroxyl pattern within the flavanol unit, the stereochemistry at the three chiral centers of the heterocyclic ring, and the location of the interflavanoid bond. Reactivity and substitution orientation at the tannin A-ring are controlled primarily by hydroxyl pattern and steric influences (Chapter 14). The chemistry of the B-ring is also related to hydroxyl pattern, exhibiting properties that are characteristic of the phenol parent (Chapter 15). Ring C (the pyran ring) reactivity is a function of how rings A and B affect its electronic structure and also reflects the steric influence of the total condensed tannin molecule (Chapter 16). Together, these characteristics help to determine stability of tannin molecules, their reactivity with formaldehyde or other co-reactants, and tannin's potential as a specialty chemical. With many tannins, it is often not a question of enhancing reactivity, but rather of controlling reactions, for example, when dealing with formaldehyde-based co-reactants.

Studies on wattle and pine tannin have clearly demonstrated that reactivity is a function of species and primarily reflects the differences of A- and B-ring substitution pattern.[3] Other factors influencing overall tannin reactivity include: tree age and location, climate, bark harvesting season, extraction procedure, and age of extract.[1-3] In particular, recognition that extraction conditions can significantly change structure and reactivity of the flavanoid (i.e., formation of catechinic acid from conifer tannin under alkaline conditions) has been a major step in understanding how to utilize tannin chemicals.[5,6]

Physical Features

Many condensed tannin extracts are composed of polymers with a number average molecular weight corresponding to 5 to 9 flavanoid units. Intramolecular association between units and complex formation with carbohydrate and protein molecules (Chapters 19 - 21) yield structures that behave like much higher molecular weight molecules. Conformational behavior of these structures adds further complexity to tannin's physical properties. The interflavanoid bond imposes significant restrictions on allowable conformations in these systems (Chapter 7). Consequently, tannin physical properties are highly sensitive to temperature, solvent effects, pH conditions, solution concentrations, and presence of salts.[7] The polymeric nature of many tannin extracts limits their solubility, often resulting in colloidal solutions. Physically, some of these molecules may have difficulty penetrating the cell wall of wood, thus limiting their use as wood adhesives.

Application Factors

Successful tannin utilization requires extracts that can be applied under a variety of conditions. Of importance are stable solutions that are soluble and of reason-

ably low viscosity. Early attempts to formulate tannin adhesives were confronted with high-viscosity solutions having limited stability.[7] Cleavage of interflavanoid bonds often is needed to yield lower molecular weight components with proper application characteristics. Many research programs tend to minimize application requirements, resulting in situations where laboratory performance may prove excellent but working properties and application needs of the industrial user cannot be satisfied. Both wettability and diffusion characteristics also are important in determining the suitability of an adhesive for wood applications.

Economic Factors

To be considered as synthetic chemical substitutes, tannins must compete in price or offer manufacturing process advantages that give overall lower production costs. The more steps required to isolate, purify, and transform tannins into a specialty chemical, the more valuable the worth of this specialty chemical must be. At present, the value of tannin as a substitute for phenol, for example (current U.S. list price $0.43/lb), is such that only simple, one-step extraction processes provide the economic incentive for tannin use.

TECHNOLOGICAL REQUIREMENTS FOR INDUSTRIAL UTILIZATION

For tannin chemicals to gain a share of the marketplace, a competitively priced, non-disruptable supply of material must be available having both consistent quality and known reactivity.

Insufficient information is available on the influence tannin aging has on chemical performance both prior to and following extraction. For instance, tannin molecular weight is higher in outer bark and heartwood material than in inner bark and sapwood products. Consequently, tannin extracts from the top portion of a tree can differ from those at the base, depending upon tree age. During harvesting and log storage, bacterial, enzymatic, and oxidative effects may alter tannin structure and mobility. Leaching of soluble, lower molecular weight flavanoids can occur when logs are transported by water or stored in wet climates. During extraction, the multitude of factors that can lead to extract variations is well-known.[7,8] Product quality problems often arise because of the economic need to obtain the highest possible tannin yield.

Harvesting and extraction strategies can be undertaken to enhance tannin quality. A good example is provided by South Africa where plantation growing conditions, harvesting at specific tree age, and use of highly controlled extraction procedures all contribute to yield successful wattle tannin products.[3,8] This is feasible because of low labor costs and the presence of a strong tannery industry, which has traditionally required a higher quality extract.

In North America, where large quantities of conifer bark are available, successful tannin utilization requires a more diligent approach in materials management, especially in bark sorting, storage, and extract quality control procedures. There is a need to develop analytical techniques for rapid characterization of extract quality and to establish purification criteria. In addition, methodology to preserve tannin

quality after extraction needs further development. In this regard, some consideration should be given to storage conditions, addition of chemical stabilizers, or conversion to solid form by spray-drying. Improved procedures to convert tannins to monomeric units or reduce their structure into simple, but reactive, phenols also would be beneficial.

To be effective, all these approaches require a thorough understanding of tannin chemistry and structure. Consequently, fundamental research activities in this area must be maintained. Recent utilization advances made with tannin adhesives are a direct result of basic knowledge developed over the last two decades.

As a commodity product, tannins must be low cost to be attractive to the industrial user. As a specialty product, tannins must be of higher quality to command higher costs. This is the reality of most commercial enterprises and one that should be recognized by all applied researchers in this field.

SPECIALTY CHEMICAL DEVELOPMENTS

Leather tanning remains the major use for tannin extracts throughout the world. Wattle and quebracho are renowned for hide tanning quality because they penetrate hides rapidly and impart light color to the final product. Although leather tanning technology has a long history, there is still only a limited understanding of the mechanism governing the interaction of tannins and animal protein. Flavanoid structure, tannin penetration rate, and the extent of fixation in the animal skin all appear to be important factors governing finished hide quality (Chapter 31).

An increasing use of tannins is for adhesives, specifically wood bonding adhesives. Tannin adhesives are extensively used in South Africa, with Australia and New Zealand also having limited commercial applications. While others have experimented with tannin adhesives, only those countries having access to quebracho or wattle type tannins have made significant commercial use of these systems.

Although resin formulations have been developed using conifer bark tannins as plywood, particleboard, and laminating adhesives,[2,3] the most promising application has been for cold-setting laminates, where tannins have been used to partially replace resorcinol[9] (and Chapter 29). These two-component systems were based initially on wattle tannins, but more recently, successful formulations have been developed using pine tannin extracts. Resorcinol substitution may also be feasible in rubber-cord bonding applications where tannins could partially replace the resorcinol-formaldehyde latex system (Chapter 30).[10]

In the future, other tannin adhesive systems of importance may include tannin-isocyanate formulations having little or no formaldehyde emission and thus satisfying increasingly stringent formaldehyde emission standards for wood composite materials.[11] Another potential adhesive combination is tannin-protein formulations that form strongly bound systems under acidic conditions.

Flakeboards and oriented strandboards are products for which tannins could be very effective as adhesives. The higher pressing temperature (200 °C) used to form these composites would increase the cure speed of crude tannin extracts. Recent trends in bonding wood at higher moisture contents also favor consideration of the tannin adhesive option for these applications. Tannin modified resins are known to be more moisture tolerant than standard PF resins.[3]

Additional tannin uses as specialty chemicals have been limited, but several categories of application include:[2,4] 1) *Drilling fluids* – mixtures of clay, minerals, and tannin extracts used to form a uniform slurry having viscosity characteristics suitable to lubricate and cool the drill bit while sealing the well hole walls to prevent fluid loss. 2) *Grouting systems* – combinations of tannin extracts and metal salts injected as a water solution into sand, gravel, or porous rock. These mixtures quickly set into an immobile gel-like system that reinforces and stabilizes the formation to allow for improved load-bearing capacity. 3) *Complexing agents* – sulfonated tannins that provide excellent treatment systems for removing scale-forming minerals from boilers and cooling water pipes. Similar complex-forming capabilities have been utilized to form slow-release agricultural nutrients. 4) *Biocides and wood preservatives* – natural or semi-synthetic tannin-based compounds that offer potential as biologically active agents to inhibit fungal and bacterial growth (Chapter 32). So far, active flavanoids have been shown to consist of a variety of structures, suggesting different mechanisms of action are operating. Tannins require complexation with copper or other metals to have efficacy as wood preservatives. 5) *Medicines* – tannins that are the basis for traditional Chinese medications. Natural tannins are active as antiviral, antibacterial, and antiflammatory agents, suggesting potential in the development of future pharmaceuticals.

IMPLICATIONS FOR FUTURE DEVELOPMENTS

While there have been many attempts to utilize tannins commercially, most of these ventures have failed, not necessarily due to either lack of research funds or limited research activity, but, more often to factors beyond a scientist's control. By considering the history of tannin chemicals, one can gain important insight that may assist future developments.

The lack of commercial success in tannin utilization can be attributed to 1) failure to be cost competitive with synthetic chemicals that the tannins were targeted to replace (a prime example is phenol for adhesive applications), 2) inherent variability and limited stability of many tannin extracts, 3) failure to recognize industrial application and performance requirements.

In terms of economics, tannin producers need to consider whether their products are to be marketed as commodity items or specialty items. The commodity approach of low price and high volume has suffered from extraction quality problems. Alternatively, for the specialty chemical directive to succeed, unique (preferably high-value) markets must be identified and their chemical needs fully understood. Tannin quality is of prime importance here.

Future success in tannin utilization requires uniform and stable extracts. Improved chemical understanding of extraction procedures, better characterization techniques, and simplified purification methods would help achieve this goal. It is likely that criteria for extract quality will need to be established for each type of tannin application.

Process application requirements is a subject frequently ignored by tannin researchers although it is likely the most important factor determining tannin utilization. The tannin extract must satisfy the user's need or it will not be utilized.

Ideally, tannin products should be developed that both accommodate process needs and compensate for extract variability.

Before significant progress can be achieved in future tannin utilization, these issues need to be addressed seriously. The challenge for researchers is to increase basic knowledge of tannin chemistry and structure while adapting this information to benefit the development of cost-effective and useful tannin products.

REFERENCES

1. Chow, S. Some physical properties of bark related to utilization. Western Forest Products Lab Report (Forintek Canada), Canadian Forestry Service, Vancouver, B.C. 22 p. (1976).
2. Porter, L.J.; Hemingway, R.W. Significance of condensed tannins. *In*: Rowe, J.W. (ed.) Natural Products Extraneous to the Lignocellulosic Cell Wall of Woody Plants, Springer-Verlag (in press).
3. Pizzi, A. Tannin-based wood adhesives. *In*: Pizzi, A. (ed.) Wood Adhesives; Chemistry and Technology, Marcel Dekker Inc., New York, pp. 177-246 (1983).
4. Herrick, F.W. Chemistry and utilization of hemlock bark extractives. *Agric. Food Chem.* 28:228 (1980).
5. Sears, K.D.; Casebier, R.L.; Hergert, H.L.; Stodt, G.M.; McCandish, L.E. The structure of catechinic acid, a base rearrangement product of catechin. *J. Org. Chem.* 39:3244 (1975).
6. Hemingway, R.W.; Kreibich, R.E.; McGraw, G.W.; Ohara, S. Modifying phenolic adhesives with condensed tannins. *In*: Structural Wood Composites: New Technologies for Expanded Markets. FPRS Proceedings 47359, Memphis, Tennessee. pp. 88-92, (1988).
7. Hillis, W.E. Natural polyphenols (tannins) as a basis for adhesives. *In*: Phenolic Resins - Chemistry and Applications. Weyerhaeuser Science Symposium Proceedings 2, Tacoma, Washington, pp. 171-188 June (1979).
8. Pizzi, A. Research vs. industrial practice with tannin-based adhesives. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds) Adhesives from Renewable Resources. ACS Symposium Series No. 385., American Chemical Society, Washington, DC, pp. 254-270 (1989).
9. Kreibich, R.E.; Hemingway, R.W., Tannin-based adhesives for finger-jointing wood. *In*: Hemingway, R.W., Conner, A.H. Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385., American Chemical Society, Washington DC, pp. 203-216 (1989).
10. Hamed, G.R.; Chung, K.H.; Hemingway, R.W. Condensed tannins as substitutes for resorcinol in bonding polyester and nylon cord to rubber. *In*: Hemingway, R.W.; Conner, A.H.; Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385., American Chemical Society, Washington DC, pp. 242-253 (1989).
11. Dix, B., Marutzky, R., Modification of diisocyanate-based particleboard and plywood glues with natural polymers; polyphenols, carbohydrates, and proteins. *In*: Hemingway, R.W.; Conner, A.H., Branham, S.J. (eds.) Adhesives from Renewable Resources. ACS Symposium Series No. 385, American Chemical Society, Washington, DC., pp. 229-241, (1989).

Conclusions

FUTURE CONFERENCES

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ABSTRACT

Given the success of the first North American Tannin Conference, another is being planned for 1991 and it is envisaged that additional conferences will be held at approximately 3-year intervals. This meeting of about 70 scientists with widely different backgrounds demonstrates that the objective of the conference – to bring together people with a common interest in condensed tannins and to promote interdisciplinary interactions that will lead to a better understanding of these important substances – was achieved. Because of the strong interest, our planning for the next conference is for a full week allowing for more time for discussion between formal presentations and an afternoon of poster sessions. The excellent representation from overseas suggests that future tannin conferences should take on a more international perspective. A second objective of the conference was the publication of this book. The work presented in this book demonstrates that research on condensed tannins has progressed well beyond an exercise in structure elucidation and is addressing important questions about the biological and commercial significance of these unique renewable phenolic polymers. Early plans for future conferences are summarized.

INTRODUCTION

The first North American Tannin Conference evolved from the informal discussions of a group of tannin chemists at Kincaid Lake near Pineville, Louisiana, in the summer of 1983. Because we felt that this area of natural products chemistry was quite specialized, we only continued to talk about the prospect of a meeting for a number of years. In the years between, we came to appreciate that research on condensed tannins was not only of interest to a few groups of chemists scattered about the world but, in fact, that these compounds were receiving attention from a broad spectrum of the scientific community. This gave us the needed encouragement to plan the first conference. To release this first trial balloon was not without concern that the investment in time and scarce funds might fall flat. However, the conference and the preparation of this book developed from the proceedings proved to be a rewarding endeavor.

The objective of our first North American Tannin Conference was to bring together people with a common interest in condensed tannins and to promote interdisciplinary interactions that would lead to a better understanding of these important substances. Judged from the widely varying backgrounds of the participants (see a List of Participants in the Index) our objective was met. When questioned about what participants liked best about the conference, the most common answer was the breadth and comprehensiveness of the topics covered. Many were anxious to have another conference in the next 2 to 3 years. What follows are some thoughts we have about the organization of future conferences.

PLANNING FOR FUTURE CONFERENCES

Now that interest has been kindled, it is tempting to hold another North American Tannin Conference in the near future. There are, to be sure, plenty of interested scientists and a wide array of possible themes. However, as one respondent wrote, "it would be useful to have meetings regularly but it should be also borne in mind that something fresh be available to talk about each time. A balance should be struck." It is our opinion that we should hold meetings at 3-year intervals, hence the planning for a second conference in 1991. The feeling that there are too many conferences to prepare for already is a commonly held belief, and fresh approaches might become hard to come by if the meetings were held more frequently.

Our experience in developing the conference and this book has been that it is important that a monograph be produced from the proceedings. Prior knowledge that the papers will be published in a book such as this adds significant incentives for authors to produce outstanding contributions. Reflection on the chapters presented in this book shows that considerable time and effort went into their preparation. Editing of a proceedings like this is certainly much easier when working with manuscripts such as those that were presented to us.

It is also important that each conference be centered on a theme different from previous meetings and that the theme chosen be such that it continues to build on the interdisciplinary relationships that evolved from the first meeting. The

concentration of the first North American Tannin Conference on the condensed tannins was in some respects unfortunate. Some discussions of the hydrolyzable tannins occurred, but these compounds deserve more attention in the future. At the present stage of our planning, the theme – "Structure-Property Relationships in Plant Polyphenols" – would seem to permit presentations of work on fundamental chemistry as well as on the biological and commercial significance of these compounds. While it is expected that most contributions will be centered on hydrolyzable and condensed tannin polymers, this theme also leaves the door open for studies of monomeric phenolics that can be related to these polymers.

Professor Haslam's concerns about the use of the term "tannins" have also caused us to reflect on the name of the conference. However, for the next meeting we decided that we should keep the name "North American Tannin Conference" because of the recognition this first meeting received.

At the first North American Tannin Conference, we found that far too little time was allowed for discussions between papers and informally during the meeting. That is partly a reflection of the interest that resulted from excellent presentations and the strong interest in condensed tannins by all the participants. For the next meeting, it is planned to lengthen the time to a full week with more time between presentations and an afternoon of poster sessions to allow more people to present current research. Blocks of time at midday will be provided to allow participants to have more informal discussion time.

Because this was the first effort to organize this meeting, we invited all authors to present papers, and the subjects considered had to be somewhat restricted. In preparation for the next conference, a call for papers will be made from the whole scientific community through advertisements in widely read Journals. A planning committee will select papers for formal presentation and poster sessions on the basis of their scientific merit and relevance to the theme. This should permit much broader participation by all those interested in these compounds. Because of the time required to select a publisher of the proceedings, the call for papers will be approximately 1 year ahead of the meeting. Authors of papers selected will be expected to submit their manuscripts on computer disks in common IBM compatible files. These files will then be "typeset" using LaTex software as in this book.

We plan to continue to present the North American Tannin Conference Award. We will ask for nominations from all those who attended the first tannin conference, and the planning committee will then vote on those nominations. The recipient will present a plenary lecture.

Several of the overseas participants expressed interest in hosting the conference. If the second North American Tannin Conference is as successful as the first one, then we should begin to seriously consider making it an international conference and simply call it The Tannin Conference or something similar. Our opinion is that it is important that it is clearly separate but coordinated with "Groupe Polyphenols" and that the North American Tannin Conference remain a comparatively small but intensely focused family of scientists interested in phenolic polymers from plants other than lignin.

SUMMARY

Considering that the first North American Tannin Conference was an inaugural meeting, held independently of other organizations, it must be considered quite successful. Preliminary plans for a second meeting in 1991 are presented here in hopes of nurturing the sense of optimism about the near-term breakthroughs that seem possible for increased understanding of the chemistry and significance of tannins. We have a nice start in developing a family of scientists interested in these compounds, and future conferences will form more productive bonds.

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