PERSPECTIVE

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Oligo- and poly-nucleotides: 50 years of chemical synthesis

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It is fifty years since the first chemical synthesis of a dinucleoside phosphate and a dinucleotide with natural 3′→5′-internucleotide linkages was reported. The main developments in the methodology of oligo- and poly-nucleotide synthesis that have taken place since are described.

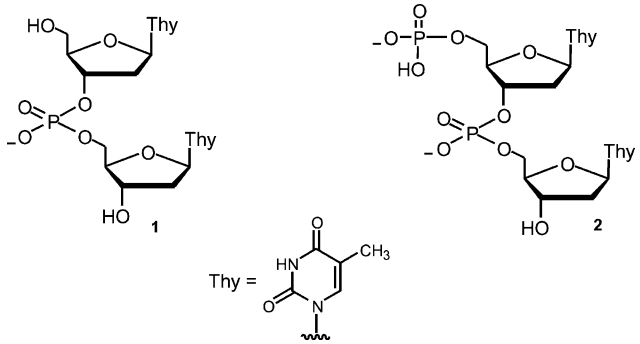
Introduction

A one-day symposium was held in Cambridge on 25th April 2003 to celebrate, precisely to the day, the fiftieth anniversary of the publication[1] in *Nature* of J. D. Watson and F. H. C. Crick's historic article on the structure of DNA. This, of course, was one of the most important scientific publications of the twentieth century. In the old Pembroke Street University Chemical Laboratory, which was virtually within striking distance of the old Cavendish Physics Laboratory, where Watson and Crick assembled their model of DNA, A. R. Todd and his co-workers had, for almost a decade, been carrying out fundamental research on the chemistry of nucleosides, nucleotides and nucleic acids. One of Todd's main aims, which may have developed from his interest in the chemistry of the B group of vitamins, was the synthesis of the nucleotide coenzymes, and this work was highlighted in the citation when he received the Nobel Prize for Chemistry in 1957. However, the scope of the nucleotide research in Todd's laboratory was very broad indeed and much fundamental research on nucleoside chemistry and chemical phosphorylation was carried out in it.

The publication[2] from Todd's laboratory in the early 1950s that was most relevant to the structure of DNA, and therefore to the Watson–Crick model, related mainly to the conclusion that the alkaline hydrolysis products of RNA (*i.e.* a pair of isomeric mononucleotides derived from each of the four nucleoside components) could be rationalised in terms of RNA having solely 3′→5′- or 2′→5′-internucleotide linkages. It had been shown[3] that DNA underwent digestion in the presence of a phosphodiesterase to give a mixture of all four 2′-deoxyribonucleoside 5′-phosphates and, of course, in the case of DNA only 3′→5′-internucleotide linkages are then possible. It thus became of particular interest to undertake the synthesis of short DNA sequences with natural 3′→5′-internucleotide linkages. This goal was first achieved[4] in 1955 when Todd, in collaboration with A. M. Michelson, reported the synthesis of the dinucleoside phosphate, thymidylyl-(3′→5′)-thymidine [d(TpT)] **1** and the corresponding dinucleotide, 5′-*O*-phosphorylthymidylyl-(3′→5′)-thymidine[d(pTpT)] **2**. A. M. Michelson was, at that time, a senior research worker who had contributed to a number of Todd's important nucleotide and nucleoside projects including the chemical synthesis of adenosine 5′-triphosphate (ATP).[5] Michelson left Cambridge in the late 1950s; he first took a post in the research laboratories of Arthur Guinness Ltd. in Dublin and then moved to l'Institut de Biologie Physico-Chimique in Paris. In 1963, he published a substantial monograph[6] on nucleoside and nucleotide chemistry. It is particularly important to emphasize Michelson's contribution to this field as this Perspective is being written in connection with a one-day symposium that is being held in Cambridge on 9th December 2005 to celebrate the fiftieth anniversary of Michelson and Todd's report[4] in the *Journal of the Chemical Society* of the first synthesis of an oligonucleotide with a natural 3′→5′-internucleotide linkage.

Colin Reese is a graduate of Cambridge University. He also received his PhD training (under the supervision of George Kenner) at Cambridge in Alexander Todd's laboratory during the period when the first synthesis of a natural dinucleoside phosphate and a dinucleotide was being carried out. Following a postdoctoral year in R. B. Woodward's laboratory at Harvard University, he returned to Cambridge and held faculty positions (as a University Demonstrator, an Assistant Director of Research and a University Lecturer) for a period of over 14 years. Throughout this time, he was an Official Fellow and Director of Studies in Chemistry at Clare College. In 1973, he was appointed to the Daniell Professorship of Chemistry at King's College London, a post that he held for 25 years. In 1981, he was elected a Fellow of the Royal Society. Through his career, his principal field of interest has been the chemistry of nucleosides, nucleotides and nucleic acids with an emphasis on the development of synthetic methodology. Other research interests have included inositol phosphate chemistry and aspects of the chemistry of heterocyclic and small- and medium-ring alicyclic compounds.

Colin Reese



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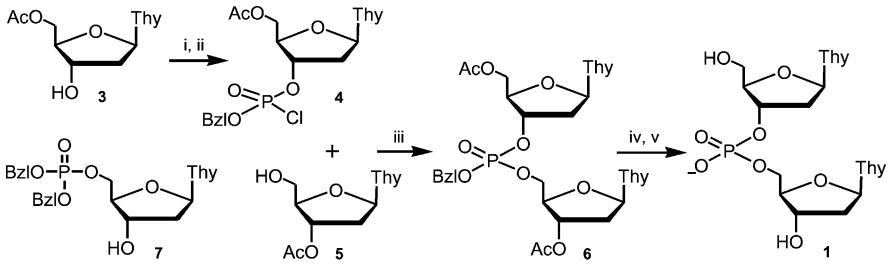
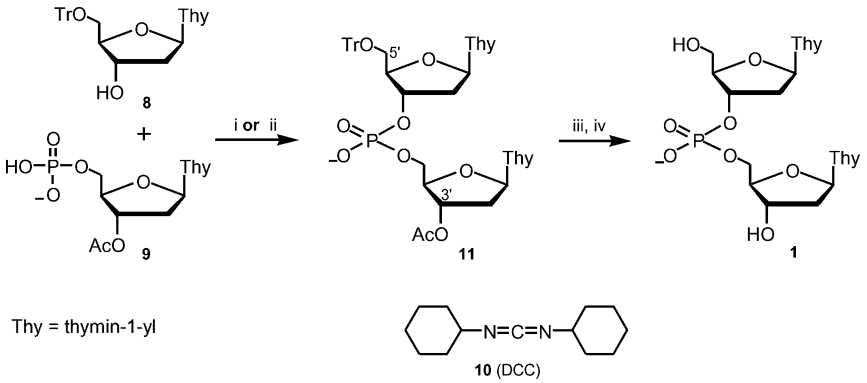


Scheme 1 *Reagents*: i, product obtained from the reaction between ammonium monobenzyl phosphite and $(PhO)_2P(O)Cl$, 2,6-lutidine, benzene; ii, *N*-chlorosuccinimide, MeCN, benzene; iii, 2,6-lutidine, MeCN; iv, H_2SO_4, EtOH, H_2O; v, $Ba(OH)_2$, H_2O.



Scheme 2 *Reagents*: (i) TsCl, C_5H_5N; (ii) **10**, C_5H_5N; (iii) AcOH–H_2O (4 : 1 v/v); (iv) NaOH, H_2O.

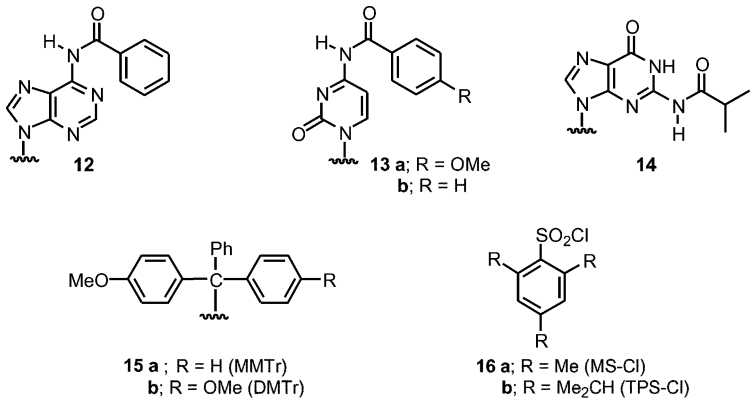
The Michelson and Todd synthesis[4] of d(TpT) **1** is indicated in outline in Scheme 1. The synthesis of the dinucleotide [d(pTpT)] **2** was very similar except that 5′-*O*-acetylthymidine **3** was replaced by the dibenzyl ester **7** of thymidine 5′-phosphate, and an additional catalytic hydrogenolysis step was required. The synthetic methodology adopted was based on that used previously in Todd's laboratory in the preparation of mononucleotides and certain nucleotide coenzymes. Thus, in the preparation of d(TpT) **1**, 3′-*O*-acetylthymidine **5** was phosphorylated with the phosphorochloridate **4**, and the phosphate function was protected with a benzyl group. The structures of the synthetic d(TpT) **1** and d(pTpT) **2** were confirmed[4] by enzymatic digestion. There appeared to be no strategy for extending the oligonucleotide chain and, in any case, benzyl-protected internucleotide linkages (as in **6**) readily undergo debenzylation, for example, in pyridine solution. It was probably taken for granted that internucleotide linkages should be protected during synthesis and it is now clear that this is indeed desirable. This approach to oligonucleotide synthesis later became known as the *phosphotriester approach*.

The phosphodiester approach

Rather surprisingly, no further work on the phosphotriester approach was carried out in the Cambridge laboratory for more than a decade. There are perhaps several reasons for this. Michelson soon left Cambridge and, as Todd makes clear in his autobiography,[7] he was not particularly attracted by the apparently repetitive nature of oligonucleotide synthesis. However, probably the most important reason was that the whole field of oligonucleotide synthesis became dominated, perhaps for a period of 15 years or more, by a completely different approach that was introduced[8] by H. G. Khorana and his co-workers. In this approach, which later became known as the *phosphodiester approach*, the internucleotide linkages were left completely unprotected throughout the assembly of the oligonucleotide sequence.

The first published examples of the phosphodiester approach[8a,b] are illustrated in Scheme 2: 5′-*O*-tritylthymidine **8** and 3′-*O*-acetylthymidine 5′-phosphate **9** were allowed to react together in the presence of toluene-4-sulfonyl chloride (TsCl) or N^1,N^3-dicyclohexylcarbodiimide (DCC) **10** in pyridine solution. Following the removal of the trityl and acetyl protecting groups, d(TpT) **1** was obtained. The coupling reactions always involved phosphomonoesters and internucleotide phosphodiester functions were left unprotected even during block coupling reactions. However, Khorana and his co-workers[9] gave much consideration to the protection of the base residues and the hydroxy functions that were not involved in the coupling reactions. Adenine and cytosine residues were generally protected as their 6-*N*-benzoyl and 4-*N*-(*p*-anisoyl) derivatives (as in **12** and **13a**, respectively), guanine residues were protected as their 2-*N*-isobutyryl derivatives (as in **14**) and, as in the Michelson and Todd synthesis[4] of d(TpT), thymine residues were left unprotected. A very important innovation was the use[10] of the (*p*-anisyl)diphenylmethyl (MMTr, **15a**) and the di-(*p*-anisyl)phenylmethyl (DMTr, **15b**) protecting groups for the 5′-hydroxy functions of nucleosides and oligonucleotide blocks. The MMTr and DMTr protecting groups are *ca.* 10 and 100 times, respectively, more labile to acid-promoted cleavage than the parent trityl group, and their removal leads to much less cleavage of the glycosidic linkages, especially of purine 2′-deoxyribonucleosides. Nowadays the more labile DMTr group **15b** is used almost exclusively to protect the 5′-hydroxy functions in oligo- and poly-nucleotide synthesis. Initially, DCC **10** was the preferred coupling agent, but it was soon replaced[11] by mesitylene-2- and 2,4,6-triisopropylbenzene-sulfonyl chlorides (MS-Cl **16a** and TPS-Cl **16b**, respectively).

The phosphodiester approach was adapted both to the stepwise and block synthesis[12] of moderately high molecular weight oligodeoxyribonucleotides. Reasonably good (*ca.* 50–70%) yields were obtained[12a] in (1 + 2 → 3) and other stepwise coupling reactions if a large excess of monomer was used. Yields were generally lower in block coupling reactions.[12b] The presence



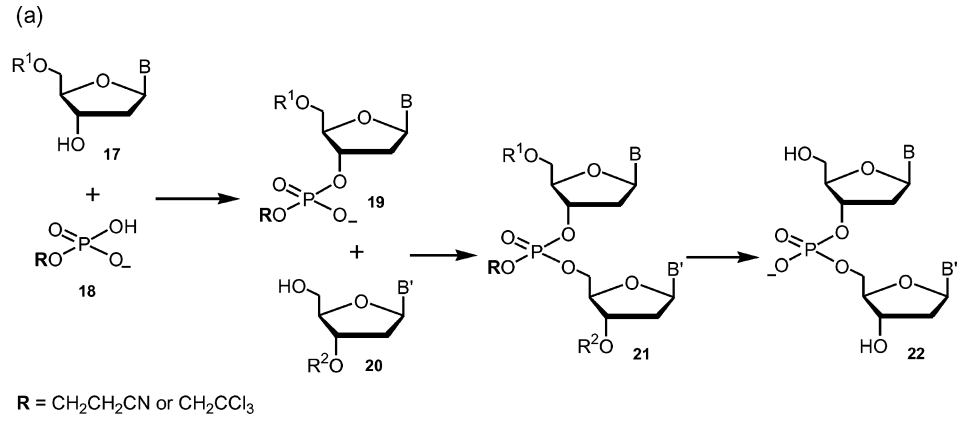
of guanine residues (protected on *N*-2 as in **14**), also appeared[12c] to lead to lower yields. Although it became evident that the accumulation of charged phosphodiester internucleotide linkages led to side-reactions and thereby to diminished yields, Khorana did not believe that an overall advantage would be gained by protecting the internucleotide linkages. Indeed, in a lecture, presented[13] in London in 1968, he expressed the view that, unless coupling yields were virtually quantitative, adopting the phosphotriester approach would actually mean throwing away what he considered to be the most important property that anyone had hitherto exploited in the separation of polynucleotides, namely their polyelectrolyte character. Fortunately, this misgiving has proved to be unwarranted. In the phosphotriester approach (see below), purification may be effected at the triester level by standard chromatographic techniques, such as chromatography on silica gel and then, following the unblocking of the internucleotide linkages, again if necessary, at the diester level thereby taking advantage of the polyelectrolyte properties alluded to by Khorana. Indeed, it later became clear that nothing is thrown away but much is gained by adopting the phosphotriester approach.

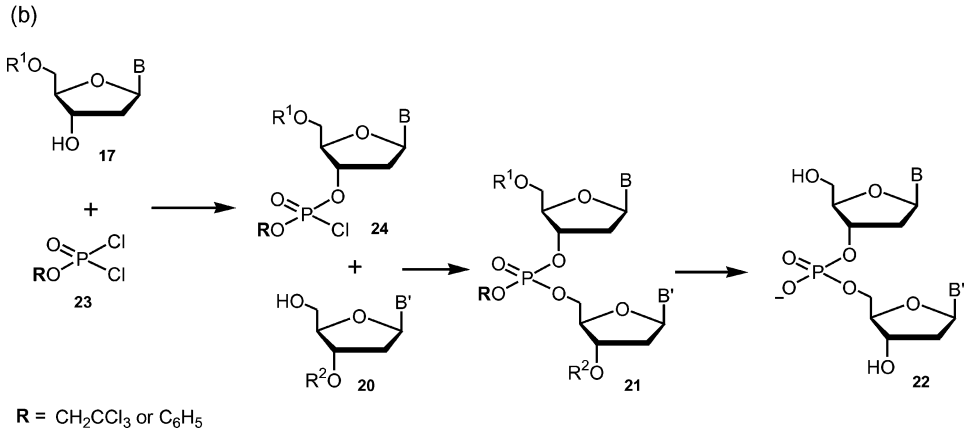
Due to side-reactions, the relatively modest yields obtained and the purification difficulties encountered, the phosphodiester approach was more or less abandoned in the 1970s. However, what is indisputable is the importance of the fundamental problems in biology that were solved by means of oligodeoxyribonucleotides that had been synthesized by the phosphodiester approach. Khorana and his co-workers[13] made an enormous contribution to the elucidation of the genetic code by making use of chemically-synthesized oligodeoxyribonucleotides containing repeating dimer, trimer and tetramer sequences. After the completion of this work, Khorana's group carried out the total synthesis[14] of DNA duplexes corresponding to yeast alanine transfer RNA and the precursor of tyrosine suppressor transfer RNA by joining together chemically-synthesized oligodeoxyribonucleotide sequences enzymatically. These studies provided the basis for many of the fundamental developments in molecular biology and biotechnology that have since taken place.

The phosphotriester approach

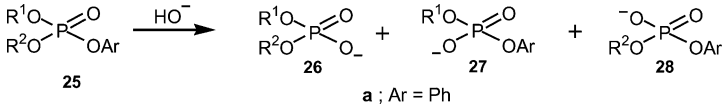
In the second half of the 1960s, the phosphotriester approach to oligodeoxyribonucleotide synthesis was reinvestigated in several laboratories. At first, Letsinger and Mahadevan[15a] carried out a phosphotriester synthesis on a solid support (see below), using the 2-cyanoethyl group to protect the internucleotide linkages. Letsinger then switched[15b,c] to solution phase synthesis and continued to use the 2-cyanoethyl group. At about the same time, Eckstein and Rizk[16] used the 2,2,2-trichloroethyl group to protect the internucleotide linkages and, soon afterwards, Reese and Saffhill[17] reported the use of the phenyl protecting group. The dinucleoside phosphate d(TpT) **1** was synthesized in all three laboratories. However, Letsinger and his co-workers[15c] and Eckstein and Rizk[16c] went on to prepare longer sequences, derived from thymidine and other 2′-deoxyribonucleoside building blocks, both by stepwise and block coupling reactions. In the first phosphorylation step, Letsinger and his co-workers[15] allowed the component **17** (Scheme 3a) with a free 3′-hydroxy function to react with 2-cyanoethyl phosphate (**18**; **R** = CH_2CH_2CN) in the presence of MS-Cl **16a** and then coupled the product **19** (**R** = CH_2CH_2CN) with the component **20** with a free 5′-hydroxy function in the presence of TPS-Cl **16b**. This was followed by an ammonolysis step to remove the 2-cyanoethyl protecting group or groups. Eckstein and Rizk[16a] first adopted virtually the same procedure (Scheme 3a) except that 2,2,2-trichloroethyl phosphate (**18**; **R** = CH_2CCl_3) was used as the phosphate source. The 2,2,2-trichloroethyl protecting group or groups were then removed from the fully-protected product (**21**; **R** = CH_2CCl_3) by treatment with zinc dust in acetic acid. Eckstein and Rizk subsequently used,[16b,c] 2,2,2-trichloroethyl phosphorodichloridate (**23**; **R** = CH_2CCl_3) as the phosphate source. The 5′-protected component **17** (Scheme 3b) was allowed to react with a slight excess of the phosphorodichloridate (**23**; **R** = CH_2CCl_3) and the intermediate phosphorochloridate (**24**; **R** = CH_2CCl_3) was then coupled with the 5′-hydroxy component **20** to give the same fully-protected product (**21**; **R** = CH_2CCl_3). Reese and Saffhill[17] used essentially the same strategy (Scheme 3b) except that phenyl phosphorodichloridate (**23**; **R** = Ph) was used as the phosphate source. The phenyl protecting group was removed from the fully-protected product (**21**; **R** = Ph) by treatment with 0.1 M sodium hydroxide in aqueous dioxane.

The significance of the 1960s phosphotriester studies lay more in the choice of the protecting group for the internucleotide linkages than in the synthetic strategies adopted. Of the three groups suggested for the protection of the internucleotide linkages, only the 2-cyanoethyl group (as in **21**; **R** = CH_2CH_2CN) proved to be too labile for use in solution phase oligonucleotide synthesis, but it later became the protecting group of choice in solid phase synthesis (see below). The 2,2,2-trichloroethyl protecting group was subsequently used[18,19] by other workers. However, yields of material with deprotected internucleotide linkages were later found[19b] to be unsatisfactory. Finally, although the phenyl protecting group[17] (as in **21**; **R** = Ph) gave rise to stable phosphotriester intermediates that could readily be manipulated and purified, its use and that of substituted aryl protecting groups led to a problem that took a number of years to solve in a fully satisfactory way (see below). While removal of 2-cyanoethyl and 2,2,2-trichloroethyl protecting groups involve *O*-alkyl cleavage, alkaline hydrolysis of phenyl- and other aryl-protected phosphodiesters **25** proceeds by direct attack of hydroxide ions on phosphorus with resulting *O*-phosphoryl cleavage. Although phenol is a much stronger acid (by *ca.* 5 pK_a units) than the 3′- or 5′-hydroxy function of a 2′-deoxynucleoside derivative, the action of hydroxide ions on a phenyl dialkyl phosphate **25a** (*e.g.* **21**; **R** = Ph) leads (Scheme 4)





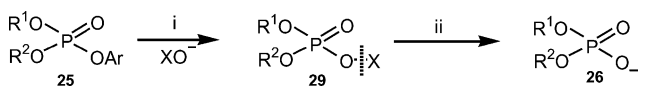
Scheme 3



Scheme 4

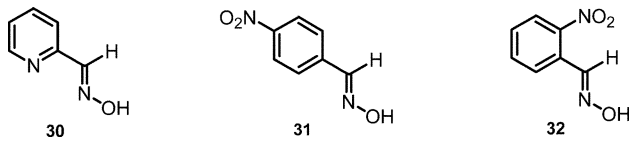
not only to the desired corresponding dialkyl phosphate **26** (*e.g.* **22**), but unfortunately also to significant quantities[20] of the two possible alkyl phenyl phosphates **27a** and **28a**.

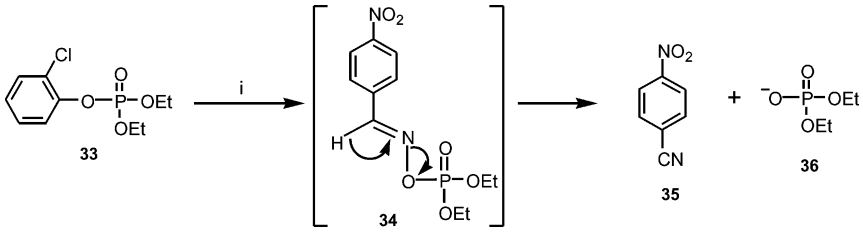
A particular advantage of aryl protecting groups is that their properties can easily be modified by substitution. It was soon found that the deblocking rate could be increased and the extent of internucleotide cleavage decreased by the introduction[21] of electron-withdrawing substituents such as *ortho*-fluoro, *ortho*- or *para*-chloro. A decrease in the pK_a of the phenol (ArOH) from which the aryl protecting group (Ar) is derived was found to increase the selectivity of the hydrolysis process indicated in Scheme 4 and lead to a greater proportion of the desired dialkyl phosphate **26**. An alternative approach to the solution of this problem was to use a nucleophile other than hydroxide ion to deblock the internucleotide linkages. It seemed that it would be particularly advantageous if the use of such a nucleophile were to involve only one substitution reaction at phosphorus. For this to be possible, the nucleophile would need to have the general structure XO^-. A further requirement would be that the initial attack on phosphorus to give intermediate **29** (Scheme 5, step i) should be followed by cleavage of the O–X bond (step ii). Of course, HO^- meets both of these requirements, but it seemed likely that the alternative nucleophile would need to be 'softer' and certainly more selective than HO^-.



Scheme 5

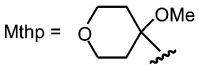
In a preliminary study,[22a] the conjugate bases of *E*-pyridine-2-carboxaldoxime **30** (pK_a 10.05) and *E*-4-nitrobenzaldoxime **31** (pK_a 9.95) were both found to be very selective nucleophiles for deblocking (2-chlorophenyl)-protected internucleotide linkages. In a subsequent mechanistic study,[22b] 2-chlorophenyl diethyl phosphate **33** was chosen as the substrate as it seemed to be a good model for a (2-chlorophenyl)-protected internucleotide linkage. When this model compound **33** was allowed to react with large excesses each of *E*-4-nitrobenzaldoxime **31** and N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) in dioxane-water (1 : 1 v/v), the presumed intermediate oxime ester **34**, which had been prepared independently, could not be detected in the products (Scheme 6). However, diethyl phosphate **36** was obtained and 4-nitrobenzonitrile **35** was isolated in good yield. In a separate experiment, the reaction between the oxime ester **34**, 4-nitrobenzaldoxime **31** and triethylamine in dichloromethane solution was found[22b] to be between 2 and 3 orders of magnitude faster than the corresponding reaction with 2-chlorophenyl diethyl phosphate **33** under the same conditions. In a later more comprehensive study,[22c] it was found that deblocking of the aryl-protected internucleotide linkage proceeded more rapidly when the conjugate base of either pyridine-2-carboxaldoxime **30** or 2-nitrobenzaldoxime **32** (pK_a 10.28) was used rather than that of 4-nitrobenzaldoxime **31**. Experiments with three fully-protected dinucleoside phosphates **37a–c** showed[22c] that, as expected,



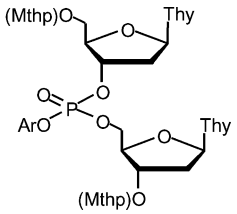


Scheme 6 *Reagents and conditions*: i, **31**, TMG, dioxane–water (1 : 1 v/v), 20 °C.

the rate of the deblocking reactions could be controlled by substitution of the aryl protecting group. Thus, with the conjugate base of 4-nitrobenzaldoxime **31**, the (2-chlorophenyl)-protected dimer **37a** was deblocked *ca.* 2.5 times more rapidly than the (4-chlorophenyl)-protected dimer **37b** and *ca.* 25 times more rapidly than the phenyl-protected dimer **37c**. No detectable (*i.e.* <0.1%) internucleotide cleavage could be detected when the (2-chlorophenyl)-protected dimer **37a** was deblocked with the conjugate base of 2-nitrobenzaldoxime **32**. This reaction was complete after 30 min at 20 °C. Following this study, 2-chlorophenyl became established as the protecting group of choice in the phosphotriester approach to oligonucleotide synthesis, and 2-nitrobenzaldoxime **32** and pyridine-2-carboxaldoxime **30** became the deblocking reagents of choice.

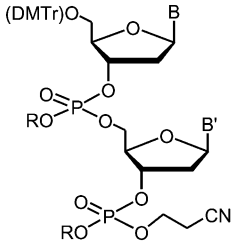


Thy = thymin-1-yl

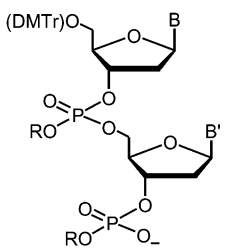


37 a; Ar = 2-ClC$_6$H$_4$
b; Ar = 4-ClC$_6$H$_4$
c; Ar = Ph

In the early and middle 1970s, there were a number of reports of the block synthesis of oligodeoxyribonucleotides by the phosphotriester approach in solution. Arentzen and Reese[23] carried out the block synthesis of octathymidine heptaphosphate and hexadecathymidine pentadecaphosphate. Catlin and Cramer[18] developed a synthetic strategy by which a number of di-, tri- and tetra-nucleotides were prepared from fully-protected dinucleotides of general structure **38a**. When these building blocks were treated with acid, the 5′-hydroxy functions were released and, under mild basic conditions, they were converted into the corresponding 3′-phosphodiesters **39a**. Possibly due to difficulties encountered in the removal of 2,2,2-trichloroethyl protecting groups, these studies were not developed further. Narang and his co-workers[24] followed an approach similar to that of Catlin and Cramer except that the 4-chlorophenyl (as in **38b**) was used instead of the 2,2,2-trichloroethyl group; these workers prepared some larger oligodeoxyribonucleotide sequences but most of their studies were carried out before the introduction of the oximate procedure for deblocking the internucleotide linkages. Nevertheless, Itakura and his co-workers[25] followed essentially this approach, using mainly protected tri- rather than di-nucleotide building blocks and aqueous ammonia to deblock the (4-chlorophenyl)-protected internucleotide linkages; these workers thereby prepared a series of 29 oligodeoxyribonucleotides, each of which contained between 10 and 15 nucleotide residues. These chemically-synthesized oligonucleotides were then successfully joined together by enzymatic ligation to form two double helices containing 77 and 104 base pairs, respectively, which corresponded to the genes for the A and B chains of human insulin. In a landmark study[26] completed in the late 1970s, these two insulin genes were successfully expressed.

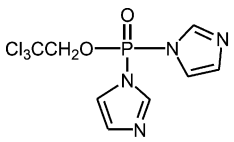


38 a ; R = CH_2CCl_3
b ; R = 4-ClC$_6$H$_4$



39a ; R = CH_2CCl_3
b ; R = 4-ClC$_6$H$_4$

Considerable progress was made in the 1970s in the development of improved phosphorylation and coupling procedures. Catlin and Cramer[18] used 2,2,2-trichloroethyl phosphorodiimidazolide **40** as a bifunctional phosphorylating agent instead of the corresponding phosphorodichloridate **23**; **R** = CH_2CCl_3, and Narang and his co-workers[24] used 4-chlorophenyl phosphorodi-(1,2,4-triazolide) **41a** as a bifunctional phosphorylating agent in the preparation of building blocks such as **38b**. Chattopadhyaya and Reese subsequently showed[27] that, if 2-chlorophenyl phosphorodi-(1,2,4-triazolide) **41b** was used in a two- to three-fold excess, it behaved effectively as a monofunctional phosphorylating agent in the conversion of protected nucleosides or oligonucleotides into the corresponding 3′-(2-chlorophenyl) phosphates (**19**; R = 2-ClC$_6$H$_4$) (see below). The reactions were relatively fast and, following an aqueous triethylamine work-up, the products (**19**; R = 2-ClC$_6$H$_4$) could be isolated as their pure triethylammonium salts in very high yields.



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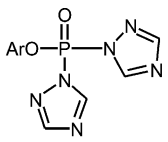
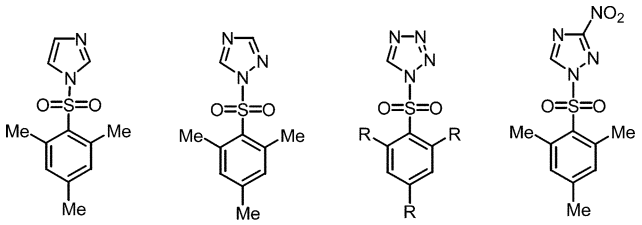


41 a; Ar = 4-ClC$_6$H$_4$
b; Ar = 2-ClC$_6$H$_4$

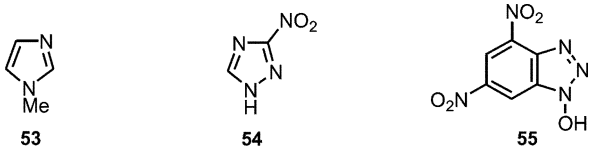
In 1973, Berlin *et al.* reported[28] that arenesulfonyl derivatives of imidazole (*e.g.* 1-(mesitylene-2-sulfonyl)imidazole **42** could be used as a coupling agent in oligonucleotide synthesis instead of the corresponding arenesulfonyl chlorides (*e.g.* MS-Cl **16a**). The coupling rates observed with such imidazole derivatives were much slower but no darkening of the reaction medium was observed. Narang and his co-workers subsequently showed[29a] that arenesulfonyl derivatives of 1,2,4-1*H*-triazole (*e.g.* 1-(mesitylene-2-sulfonyl)-1,2,4-1*H*-triazole **43**) effected coupling almost as rapidly as the corresponding arenesulfonyl chloride **16a** but that higher yields were obtained. These workers then showed[29b] that arenesulfonyl derivatives of 1*H*-tetrazole **44a–c**, were both faster and more efficient coupling agents than the corresponding arenesulfonyl chlorides. However, the 1-arenesulfonyl-1*H*-tetrazoles **44a–c** were reported[29b] to be relatively unstable. Reese and his co-workers then reported[22a,30] that 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-triazole (MSNT) **45** had similar properties (*i.e.* its use led to rapid, relatively clean coupling reactions and high yields) to the corresponding tetrazole derivative **44b**, but that it was relatively stable. In the



early 1980s, MSNT **45** became established as the coupling agent of choice both in solution and in solid phase (see below) synthesis by the phosphotriester approach.

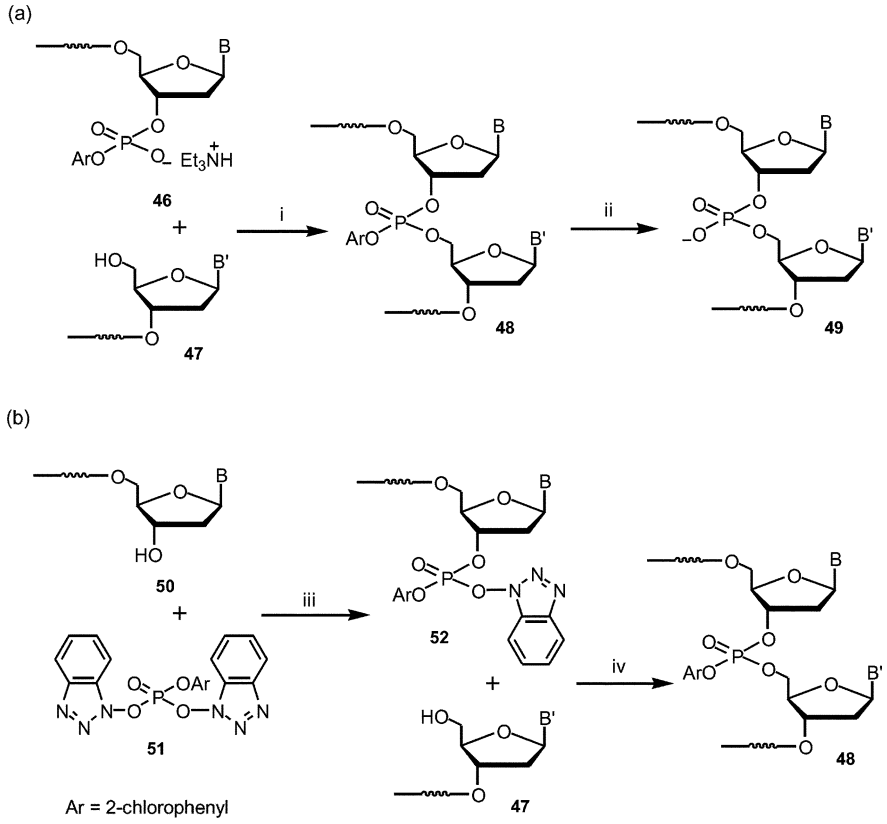
Indeed, at the beginning of the 1980s, the most generally adopted approach to oligonucleotide synthesis both in solution and on a solid support (see below) involved the coupling together, in the presence of MSNT **45**, of a protected nucleoside or oligonucleotide 3′-(2-chlorophenyl) phosphate **46** and a protected nucleoside or oligonucleotide with a free 5′-hydroxy function **47**. When the coupling process was complete, the internucleotide linkages were deblocked by oximate treatment. This protocol is summarized in Scheme 7(a). van Boom and his co-workers[31] then recommended the use of a bifunctional phosphorylating agent **51**, which was prepared by treating 2-chlorophenyl phosphorodichloridate **23**; **R** = 2-ClC_6H_4 (1 mol equiv.) with 1-hydroxybenzotriazole (2 mol equiv.) and pyridine in THF or dioxane. The protected nucleoside or oligonucleotide **50** with a free 3′-hydroxy function was treated (Scheme 7(b)) with a small (10–15%) excess of reagent **51** and, after an appropriate interval of time, the protected nucleoside or oligonucleotide **47** with a free 5′-hydroxy function and 1-methylimidazole **53** were added. Fully-protected dinucleoside phosphates were obtained in 50–77% yields and satisfactory yields were also obtained in block coupling reactions. The fully-protected products **48** were again deblocked by the oximate procedure, as indicated in Scheme 7(a) (step ii). Clearly, one advantage of the hydroxybenzotriazole phosphotriester approach is that a coupling agent, such as MSNT **45**, is not required. However, the potential disadvantages of using a bifunctional phosphorylating agent are (a) that it is possible to obtain both symmetrical 5′→5′- and 3′→3′-linked products and (b) that traces of moisture can lead to diminished yields. Nevertheless, the hydroxybenzotriazole phosphotriester approach has been used successfully, especially in solid phase oligodeoxyribonucleotide synthesis (see below).

A number of other modifications to the phosphotriester approach were reported in the 1980s and 1990s. These modifications mainly involved varying the coupling agent and the nucleophilic catalyst. Thus, in the coupling step (Scheme 7(a), step i), it was found[32b] that a mixture of MS-Cl **16a** and 3-nitro-1,2,4-1*H*-triazole (NT) **54** was as effective as MSNT **45** and that 1-methylimidazole **53** appeared[32b] to be as efficient a nucleophilic catalyst as NT **54**. Furthermore, a mixture of TPS-Cl **16b** and 1-hydroxy-4,6-dinitrobenzotriazole[32a,b] **55** was found to constitute a fast and high-yielding coupling agent.

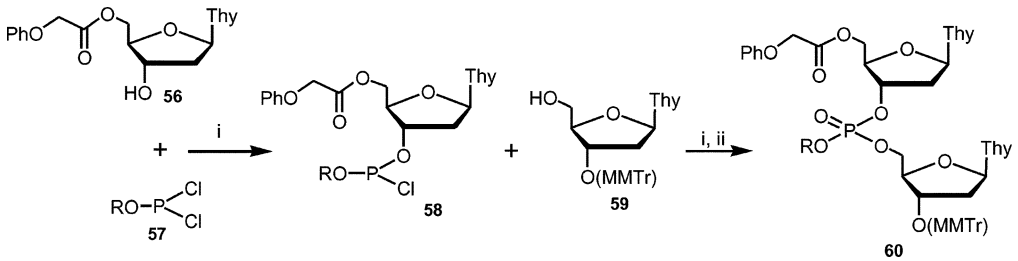


The phosphite triester and phosphoramidite approaches

In 1976, Letsinger and Lunsford reported[33] a very important development in phosphorylation methodology. These workers found that P(III) were considerably more reactive than the corresponding P(V) acylating agents. Thus, 2-chlorophenyl phosphorodichloridite **57a** reacted very rapidly with the 5′-protected thymidine derivative **56** at −78 °C. The putative



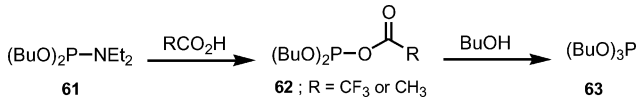
Scheme 7 *Reagents and conditions*: i, MSNT **45**, C_5H_5N; ii, **30** or **32**, TMG, dioxane (H_2O) and/or MeCN, followed by other appropriate unblocking steps; iii, dioxane; iv, 1-methylimidazole, dioxane, pyridine.



Scheme 8 *Reagents and conditions*: i, 2,6-lutidine, THF, −78 °C; ii, 2,6-lutidine, I_2, THF, H_2O.

intermediate phosphorochloridite **58a** obtained (Scheme 8) was then allowed to react with the 3′-protected thymidine derivative **59** to give a fully-protected dinucleoside phosphite. The latter product was not isolated but was treated *in situ* with iodine and water to give the corresponding fully-protected dinucleoside phosphate **60a**. This may be regarded as a modification of the phosphotriester approach in which a phosphite triester is obtained first and is then immediately oxidized to the corresponding phosphotriester. The use of a very reactive bifunctional phosphorodichloridite **57** inevitably leads to a mixture of the desired product with a 3′→5′-internucleotide linkage and the two possible symmetrical products with 3′→3′- and 5′→5′-internucleotide linkages. Nevertheless, Letsinger and Lunsford[33] were able to carry out a stepwise synthesis of the tetranucleoside triphosphate d(TpTpTpT), based on 2,2,2-trichloroethyl phosphorodichloridite **57b** as the phosphitylating agent.

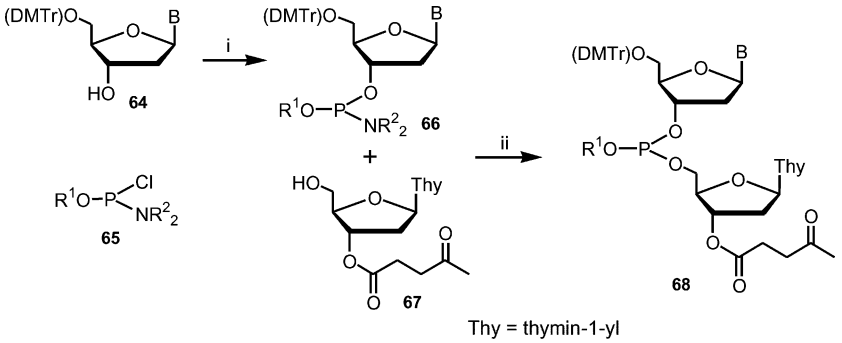
In 1981, the full importance of the P(III) approach to oligonucleotide synthesis became clear with the introduction of monofunctional nucleoside phosphoramidites **66** by Beaucage and Caruthers.[34] Following an observation[35] by Nifant'ev *et al.* in 1966 that amine hydrochlorides catalyze the alcoholysis of amides of phosphorous acid (*e.g.* $(Me_2N)_3P$), Evdakov *et al.* reported[36] in 1973 that dibutyl *N*,*N*-diethylphosphoramidite **61** reacted instantly with trifluoroacetic (or acetic) acid at 30 °C to give (Scheme 9) dibutyl trifluoroacetyl (or acetyl) phosphite **62**. In the presence of an alcohol (*e.g.* butanol), the intermediate acyl phosphite **62** was converted[36] into a trialkyl phosphite (*e.g.* tributyl phosphite **63**). In their original study, Beaucage and Caruthers[34] treated 5′-*O*-DMTr-thymidine **64** (B = thymin-1-yl) and the 5′-*O*-DMTr-*N*-acyl derivatives of the other main 2′-deoxyribonucleosides with chloro(dimethylamino)-methoxyphosphine **65**; $R^1 = R^2 = Me$ to give (Scheme 10) the corresponding nucleoside phosphoramidites **66**; $R^1 = R^2 = Me$. The products were isolated as colourless powders in high (90% or greater) yield. The acid-catalyzed reactions (Scheme 10) between these phosphoramidites **66**; $R^1 = R^2 = Me$, 1*H*-tetrazole (the acid catalyst) and 3′-*O*-levulinoylthymidine **67** in acetonitrile-d_3 were followed by ^{31}P NMR spectroscopy, which revealed fast reactions and very high yields of fully-protected dinucleoside phosphites **68**. These products were uncontaminated with the symmetrical dimers that were formed[33] when alkyl or aryl phosphorodichloridites **57** (Scheme 8) were used as the phosphitylating agents. It was also reported[34] that the protected nucleoside phosphoramidites **66**; $R^1 = R^2 = Me$ could be used successfully in the solid phase synthesis of dinucleoside phosphates. These nucleoside *N*,*N*-dimethylphosphoramidites **66**; $R^2 = Me$ proved to be unstable in solution. Adams *et al.*[37] and McBride and Caruthers[38] later showed that *N*,*N*-diisopropylphosphoramidites **66**; $R^2 = Me_2CH$ were considerably more stable than *N*,*N*-dimethylphosphoramidites **66**; $R^2 = Me$. In another important study, Köster and his co-workers[39] indicated that 2-cyanoethyl phosphoramidites **66**; $R^1 = CH_2CH_2CN$ were to be preferred to the corresponding methyl esters **66**; $R^1 = Me$. 2-Cyanoethyl *N*,*N*-di-isopropylphosphoramidites **66**; $R^1 = CH_2CH_2CN$, $R^2 = Me_2CH$ have since been used virtually exclusively in phosphoramidite-based solid phase oligonucleotide synthesis (see below), which has proved to be a process of enormous practical importance.



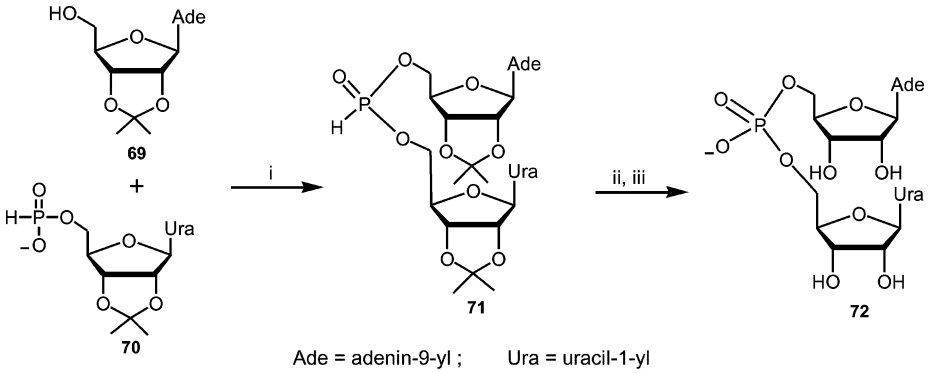
Scheme 9

The H-phosphonate approach

Like the phosphotriester approach, the H-phosphonate approach to oligonucleotide synthesis was first reported[40] from Todd's Cambridge laboratory in the 1950s. When 2′,3′-*O*-isopropylideneadenosine **69** and 2′,3′-*O*-isopropylideneuridine 5′-H-phosphonate **70** were allowed to react together in the presence of diphenyl phosphorochloridate (Scheme 11), the protected dinucleoside H-phosphonate **71** was obtained. Following chlorination with *N*-chlorosuccinimide, hydrolysis of the putative intermediate phosphorochloridate and removal of the isopropylidene protecting groups, the 5′→5′-dinucleoside phosphate **72** was obtained,[40] albeit in modest yield. However, the true potential of the H-phosphonate approach was not realized for almost 30 years. In 1985, Garegg, Stawinski and their coworkers[41a] confirmed the Cambridge laboratory's observation that diphenyl phosphorochloridate was an effective coupling



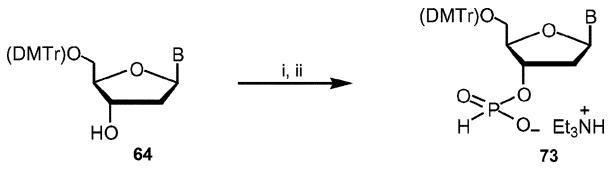
Scheme 10 *Reagents and conditions*: i, **65**, Pr^i_2NEt, $CHCl_3$, room temp.; ii, 1*H*-tetrazole, CH_3CN.



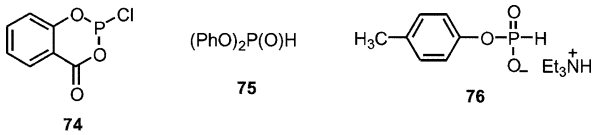
Scheme 11 *Reagents and conditions*: i, $(PhO)_2P(O)Cl$, 2,6-lutidine, MeCN; ii, a, *N*-chlorosuccinimide, 2,6-lutidine, MeCN, b, $NaHSO_3$, H_2O; iii, HCl, H_2O.

agent. The real significance of the H-phosphonate approach only became apparent in 1986 when Froehler and Matteucci[42] and Garegg *et al.*[41b] applied it to solid phase oligonucleotide synthesis. Froehler and Matteucci recommended that pivaloyl chloride should be used as the coupling agent. Coupling reactions were then very fast and it was found[42a,b] that really high molecular weight oligodeoxyribonucleotides could be prepared by solid phase H-phosphonate synthesis (see below). The H-phosphonate diesters were oxidized to the corresponding phosphodiesters by treatment with iodine in the presence of aqueous base.

Protected 2′-deoxyribonucleoside 3′-H-phosphonates **73**, the building blocks required for the H-phosphonate approach to oligodeoxyribonucleotide synthesis, may readily be prepared[42c] (Scheme 12) by treating the appropriate nucleoside derivatives **64** with the products of the reaction between phosphorus trichloride, 1,2,4-1*H*-triazole (3 mol equiv.) and triethylamine (3 mol equiv.), followed by a hydrolytic work up. These monomeric H-phosphonate building blocks **73** are very easy to prepare and then isolate as pure stable solids. Other convenient and efficient methods for the preparation of H-phosphonate building blocks **73** include the use of (a) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one[43] **74**, (b) diphenyl phosphite[44] **75** and (c) triethylammonium *p*-tolyl phosphite **76** in the presence of pivaloyl chloride.[45]



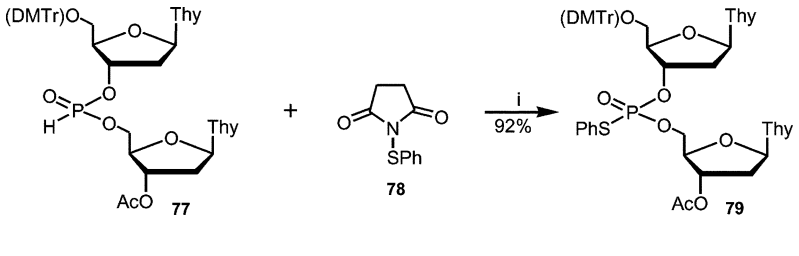
Scheme 12 *Reagents and conditions*: i, products obtained from the reaction between PCl_3, 1,2,4-1*H*-triazole and Et_3N, CH_2Cl_2; ii, aqueous triethylammonium bicarbonate.



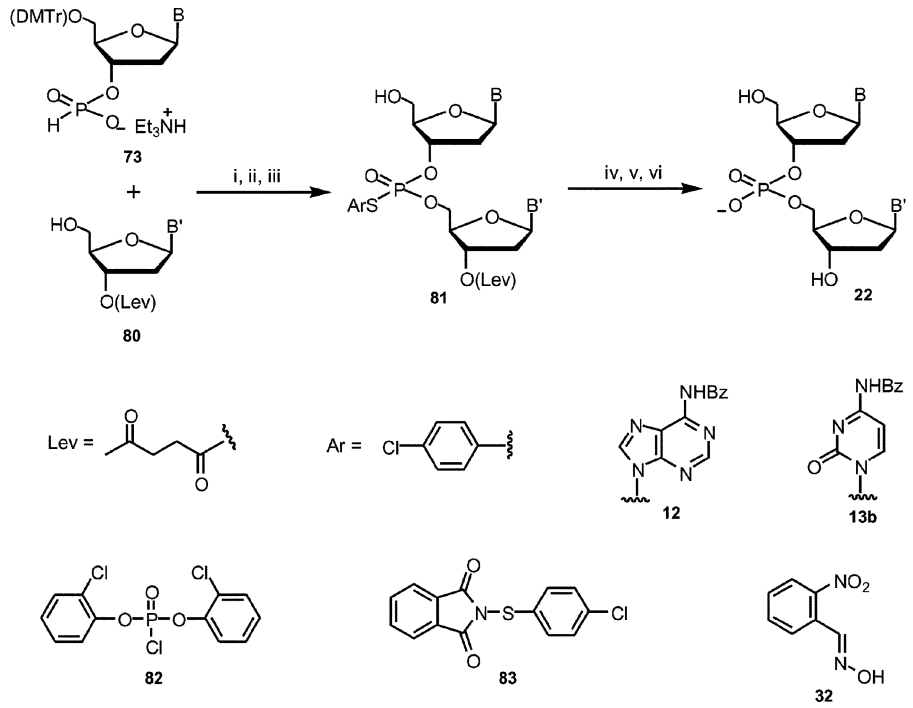
The modified H-phosphonate approach

Unlike the phosphotriester approach, which has been used successfully both in solution and in solid phase synthesis, the H-phosphonate approach in its original form is suitable only for solid phase synthesis. The reason for this is that H-phosphonate diesters are very susceptible to base-catalyzed hydrolysis[46] and are therefore difficult to manipulate. However, van Boom and his co-workers[47] reported that the protected dithymidine H-phosphonate **77** reacted rapidly with *N*-(phenylsulfanyl)succinimide **78** in the presence of base (Scheme 13) to give the corresponding much more robust *S*-phenyl phosphorothioate triester **79**. Previously, Hata and his co-workers[48] had developed a modification of the phosphotriester approach in which aryl (*e.g.* 2-chlorophenyl)-protected phosphotriester linkages (as in **37a**) were replaced by *S*-phenyl phosphorothioate triester linkages (as in **79**).

It therefore seemed that if H-phosphonate coupling and the subsequent reaction with an arylsulfanylimide (such as **78**) both proceeded in virtually quantitative yield and if the sulfur-transfer step could be carried out *in situ* (*i.e.* without the isolation of the relatively sensitive H-phosphonate diester (*e.g.* **77**)), a new and potentially very efficient approach to the synthesis of oligonucleotides would be feasible. Fortunately, both reactions proved[49a,b] to be rapid and virtually quantitative. This modified H-phosphonate approach is exemplified (Scheme 14) by the preparation of d(ApC). The H-phosphonate building block **73**; B = **12** and the 3′-*O*-levulinoylnucleoside derivative **80**; B′ = **13b** were first allowed to react together in the presence of bis(2-chlorophenyl) phosphorochloridate **82** at −40 °C. After 10 min, the products were allowed to warm up to room temperature. After a further period of 15 min, the sulfur-transfer reagent **83** was added and the reaction mixture was quenched with water. The 5′-*O*-DMTr protecting group was then removed and the partially-protected dinucleoside phosphorothioate **81**; B = **12**, B′ = **13b** was isolated[49a,b] in 98% yield. This material may then be coupled with another H-phosphonate monomer **73** to give a fully-protected trimer. This three step process (Scheme 14, steps i–iii) may be repeated until the desired sequence is assembled. Alternatively, block coupling reactions may be carried out. The partially-protected dimer **81**; B = **12**, B′ = **13b** was deblocked



Scheme 13 *Reagents and conditions*: i, Pr^i_2NEt, CH_2Cl_2, room temp., 5 min.

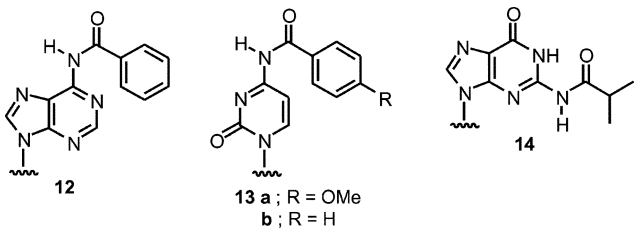


Scheme 14 *Reagents and conditions*: i, **82**, C_5H_5N, CH_2Cl_2, −40 °C, 10 min; ii, a, **83**, C_5H_5N, CH_2Cl_2, −40 °C, 15 min, b, C_5H_5N–H_2O (1 : 1 v/v), −40 °C to room temp.; iii, HCl, dioxane, CH_2Cl_2, −50 °C, 5 min; iv, Ac_2O, C_5H_5N, room temp., 15 h; v, **32**, TMG, MeCN, room temp., 12 h; vi, conc. aq. NH_3 (d 0.88), 50 °C, 15 h.

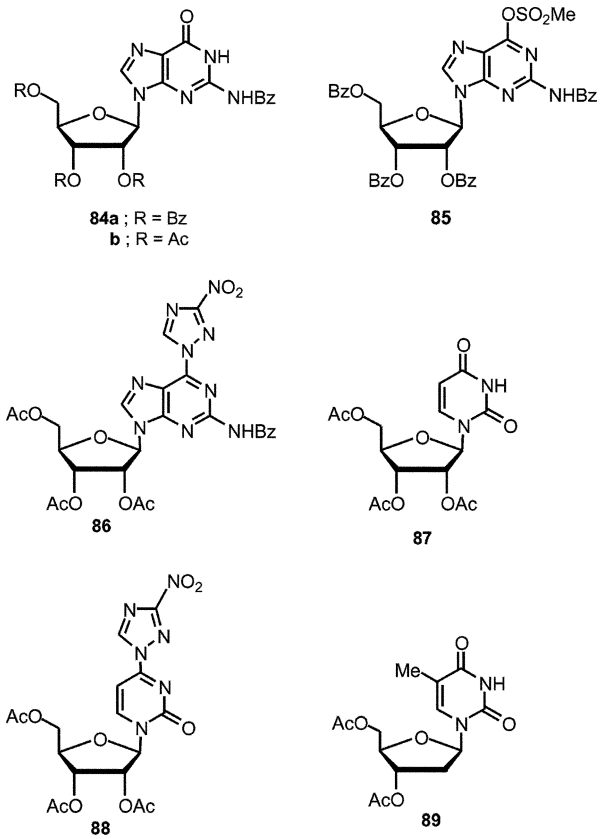
(Scheme 14, steps iv–vi) to give d(ApC) **22**; B = adenine-9-yl, B′ = cytosine-1-yl. It was unnecessary to carry out any further purification of the fully-deblocked dinucleoside phosphate. The synthetic protocol indicated in Scheme 14 has recently been simplified in that coupling reactions have been carried out at room temperature and the coupling and sulfur-transfer reagents have been added simultaneously.[49c]

Base protecting groups and side-reactions in oligonucleotide synthesis

As indicated above, in the course of their work on the synthesis of oligonucleotides by the phosphodiester approach, Khorana and his co-workers[9] protected adenine, cytosine and guanine residues as their *N*-acyl derivatives (as in **12–14**, respectively). Thymine residues (and also uracil residues in oligoribonucleotide synthesis) were left unprotected. It seemed clear from this early work that yields were poorer when guanine residues were present but the reason for this was unknown at the time. This base protection strategy remained virtually unchanged for about 20 years and was revised only after the side-reactions that accompany oligoribonucleotide synthesis by the phosphotriester approach had been elucidated.[50]



The fact that 2-*N*-acylguanine and indeed free guanine residues in guanosine derivatives can undergo acylation on O-6 was first reported[51] in 1977. Thus, for example, 2-*N*-benzoyl-2′,3′,5′-tri-*O*-benzoylguanosine **84a** was found to react with methanesulfonyl chloride in the presence of triethylamine to give[51] its 6-*O*-mesyl derivative **85**. When 2-*N*-benzoyl-2′,3′,5′-tri-*O*-acetylguanosine **84b** was treated with MSNT **45** in pyridine solution, compound **86a** was obtained.[50] The reaction was relatively slow but was found to be catalyzed by diphenyl phosphate. This was a significant observation because an excess of the phosphodiester component (*e.g.* **19**; **R** = 2-ClC_6H_4, Scheme 3) is generally used in the phosphotriester approach.[27,30] When 2′,3′,5′-tri-*O*-acetyluridine **87** was treated with MSNT **45** in pyridine solution, it underwent[50] a similar modification reaction to give the 3-nitro-1,2,4-triazole derivative **88**. This reaction was



somewhat faster than that involving the guanosine derivative **84b** and it was also catalyzed by diphenyl phosphate. However, the reaction between 3′,5′-di-*O*-acetylthymidine **89** and MSNT **45** in pyridine solution was very slow, both in the absence and presence of diphenyl phosphate. These results suggested that, as far as the phosphotriester approach is concerned, it would probably be advisable to protect both the guanine and uracil residues (on O-6 and O-4, respectively) in oligoribonucleotide synthesis and the guanine residues on O-6 in oligodeoxyribonucleotide synthesis.

The occurrence of side-reactions was first observed during the synthesis[30] of the 3′-terminal decaribonucleoside nonaphosphate (10-mer) sequence of yeast $tRNA^{Ala}$ (see below) by the phosphotriester approach in solution with MSNT **45** as the coupling reagent. With the help of model compounds, as indicated above,[50] it was possible to show that the side-reactions led to the 6- and 4-*O*-(3-nitrotriazolation) of the guanine and uracil residues, respectively. Although these base modifications could be reversed[50] by treatment with oximate ions in the course of the deblocking of the internucleotide linkages, it seemed to be preferable to attempt to prevent their occurrence by the introduction of appropriate protecting groups.

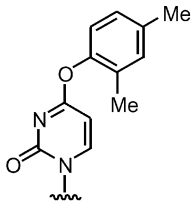
a Protection of guanine residues

In the initial studies,[52a,b] which were carried out in the ribose series, the guanine residues were protected on N-2 and O-6 by (*tert*-butyl)phenylacetyl and 2-nitrophenyl groups, respectively, as in **90**; R = 4-$Me_3CC_6H_4CH_2$. In addition to the avoidance of side-reactions, protection on O-6 made the intermediates more lipophilic and consequently much easier to manipulate. Later studies in the deoxy-series[52c] indicated that better yields were obtained when guanine residues were also doubly protected (as in **90**; R = CH_2Ph), but it was not clear that it was advantageous to protect thymine residues on O-4. It was subsequently found that, if guanine residues were protected on O-6 by the somewhat more stable 3-chlorophenyl[52d] and 3,5-dichlorophenyl[52e] groups (as in **91a** and **91b**, respectively), deblocking (*i.e.* regeneration of the 1,6-lactam functions) with 2-nitrobenzaldoxime **32** and TMG still occurred quite rapidly in acetonitrile solution. More recently, the 2,5-dichlorophenyl group (as in **93**) has been used successfully both in the deoxyribose,[49b,53a] and ribose[53b] series. Although the more easily removable (by ammonolysis) phenylacetyl group[52d] (as in **91**) is to be preferred for the protection of the 2-amino functions of guanine residues, the isobutyryl group (as in **92**) is still being used in the deoxyribose series. 5′-*O*-DMTr-2-*N*-isobutyryl-2′-deoxyguanosine **92** is a commercially available starting material that can easily be converted[53a] in a one-pot process into its 6-*O*-(2,5-dichlorophenyl) derivative **93** in very high yield.

Two other groups have also been used successfully for the O-6 protection of guanine residues in solution phase phosphotriester synthesis. Gaffney and Jones[54a] and Pfleiderer and his co-workers[54b] suggested the use of the 2-(4-nitrophenyl)ethyl group (as in **94**). This protecting group, which may be removed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), has been used effectively[54c] in the preparation of 2′-deoxyguanosine-containing oligodeoxyribonucleotides. At almost the same time, Hata and his co-workers[54d] introduced the use of the 6-*O*-diphenylcarbamoyl protecting group (as in **95**), which may be removed by ammonolysis in the final deblocking step. There have been several reports[55a–d] in the literature dealing with the protection of guanine residues on O-6 in phosphoramidite-based solid phase oligodeoxyribonucleotide synthesis (see below). Apparently, modification of the guanine residues can be avoided if 1-methylimidazole **53** is used[55b] as the base in the acetic anhydride promoted capping step, and if capping is carried out before the P(III) → P(V) oxidation step[55a] in each synthetic cycle.

b Protection of uracil and thymine residues

Following the observation[50] that uracil residues were particularly susceptible to modification in the phosphotriester approach to oligoribonucleotide synthesis in solution with MSNT **45** as the coupling agent, it was found that they could be effectively protected on O-4 with the 2,4-dimethylphenyl group[52a] (as in **96**). Like 6-*O*-aryl protected guanine residues (*e.g.* as in **91a,b**), O-4 protected uracil residues (as in **96**) may be rapidly deblocked[52a] with oximate ions under the conditions used to deblock (2-chlorophenyl)-protected internucleotide linkages. As indicated above, the combination of O-6 protected guanine residues and O-4 protected uracil residues led[52a] to an improvement in the synthesis of the 3′-terminal 10-mer sequence of yeast $tRNA^{Ala}$ and also made possible the synthesis of the 3′-terminal 19-mer[52b] and 37-mer[56] sequences (see below). Thymine residues may conveniently be protected with the 4-*O*-phenyl group[52e] (as in **97**), which is both easy to introduce and then remove by oximate treatment. However, it is by no means clear that it is beneficial to protect thymine residues either in phosphotriester- or



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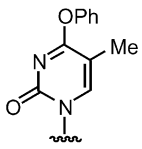

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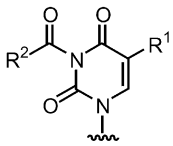
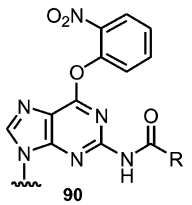


98 a ; R^1 = H
b ; R^1 = Me



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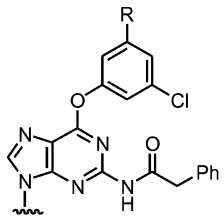
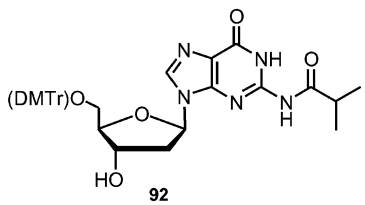


91 a ; R = H
b ; R = Cl



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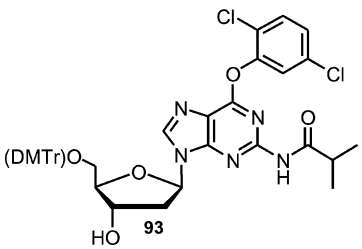

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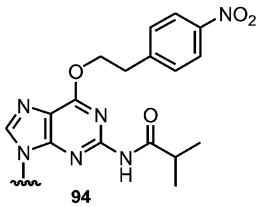

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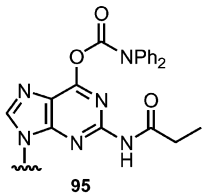


95

phosphoramidite-based oligonucleotide synthesis. Welch and Chattopadhyaya[57a] and Hata and his co-workers[57b] have shown that uracil residues may conveniently be protected by acylation on N-3 (*e.g.* as in **98a**; R^2 = Ph or 4-$MeOC_6H_4$). Thymine residues may also be protected by acylation on N-3 (as in **98b**; R^2 = 4-$MeOC_6H_4$).[53a]

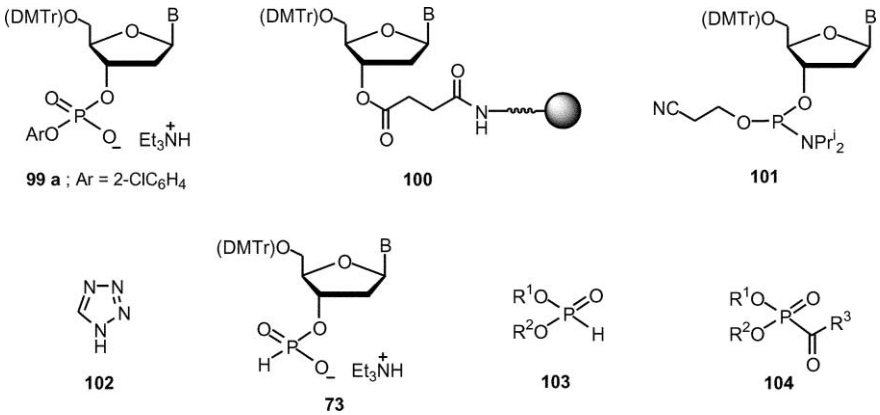
Oligonucleotide synthesis on a solid support

It is not proposed to discuss solid phase oligonucleotide synthesis in any detail in this Perspective as there are a number of authoritative reviews already in the literature.[58a–c] However, its great practical importance is beyond any doubt. Indeed, in the past 20 years or so, nearly all oligonucleotide synthesis has been carried out on a solid support and this is clearly the most rapid and efficient method of synthesizing the generally small quantities of material required for biological research. Solid phase synthesis has also been used successfully in the synthesis of the multigram quantities of material required for clinical trials of potential oligonucleotide drugs. Following Letsinger and Mahadevan's original report[15a] in 1965, it took almost 15 years before the real potential of this approach became clear. Probably the main reason for this was that no really satisfactory synthetic procedure was available. By the late 1970s, the phosphotriester approach (Scheme 7a) with aryl (particularly 2-chlorophenyl) protecting groups for the internucleotide linkages and effective coupling agents, such as MSNT **45**, had become established. This approach and the modification involving the use of the bifunctional bis-(1-hydroxybenzotriazolide) reagent[31a] **51**; Ar = 2-ClC_6H_4 (Scheme 7b) were successfully applied to solid phase oligonucleotide synthesis and, at the beginning of the 1980s, were the methods of choice. The success of these methods depended to a significant extent on oximate deblocking[22] of the internucleotide linkages. Several reviews[59a–c] on solid phase synthesis by the phosphotriester approach were subsequently published.

Solid phase synthesis generally involves the addition of one nucleotide residue (*e.g.* **99a** in the phosphotriester approach) at a time to an immobilized protected nucleoside or oligonucleotide. Controlled-pore glass[60] and highly crosslinked polystyrene,[61] which are both fairly robust materials, have emerged as the solid supports of choice. Controlled-pore glass is usually functionalized with a long-chain alkylamine and polystyrene with an aminomethyl group. The 3′-terminal nucleoside residue is commonly attached to these solid supports *via* a succinoyl group (as in **100**). No purification steps are carried out until the fully-assembled sequence is released from the solid support. For at least two reasons, it is of the utmost importance that the coupling efficiency is very high indeed. First, the yield of the target sequence falls off very rapidly with decreasing coupling efficiency. For example, in the synthesis of a 25-mer (involving 24 coupling steps), the calculated overall yields are *ca.* 79, 48 and 29% when the average coupling yields are 99, 97 and 95%, respectively. Even more dramatically, in the synthesis of a 50-mer (involving 49 coupling steps), the calculated overall yield falls from 61 to 37% when the average coupling yield falls from 99 to 98%. Secondly, unless the coupling efficiency is very high, the separation of the target sequence from truncated material becomes more difficult. Although the final purification process is facilitated by 'capping' truncated material after each coupling step and by purifying the crude 'undetritylated' products by reversed phase chromatography, it is clearly desirable that the overall yield of untruncated material should be as great as possible.

Phosphotriester solid phase synthesis[59a–c] had largely been superseded by phosphoramidite-based synthesis[62] by the mid-1980s. It is possible to achieve average coupling yields of 98% or greater with phosphoramidite building blocks of general structure **101** and with 1*H*-tetrazole **102** as the activating agent. Furthermore, the coupling reaction is very rapid indeed and, in the deoxy-series, it is usually complete within *ca.* 1 min. Irreversible side-reactions can generally be avoided. Although modifications to the phosphotriester approach have led both to increased coupling rates and increased coupling yields, the phosphoramidite approach has remained the method of choice. While phosphotriester-based solid phase synthesis is likely to be effective in the synthesis of 25-mers, it is very doubtful if, as it stands, it could be used as successfully in the synthesis of say, 50-mers. On the other hand, the phosphoramidite-based synthesis of oligodeoxyribonucleotide 50-mers and, if care is taken, even higher molecular weight DNA sequences is almost routine.[58a] It should perhaps be added that the ready availability of phosphoramidite building blocks and their decreasing cost in recent years may have made the search for alternative synthetic methodologies appear not to be a matter of urgency. A crucially important factor in the success of solid phase oligonucleotide synthesis has been the commercial availability of easy-to-operate automatic synthesizers. The fact that the best and probably the most popular of these instruments were designed especially for the use of phosphoramidite building blocks and reagents may well be another reason why, in the past 20 years, only a very limited research effort has been put into the development of alternative methodologies.

Indeed, most of the more recent research into alternative methodologies for solid phase oligonucleotide synthesis has involved the H-phosphonate approach[42] (see above). Like the corresponding building blocks **99a** used in solid phase phosphotriester synthesis, monomeric H-phosphonate building blocks **73** are both stable and easy to prepare.[42c] Coupling reactions involving H-phosphonates appear to be at least as fast[42c] as those involving phosphoramidites. As indicated above, H-phosphonates (*e.g.* **73**), are activated by acylating agents such as pivaloyl chloride. Both overactivation and prolonged coupling times should be avoided in order to prevent the occurrence of undesirable side-reactions.[42c,63] Therefore particular attention must be paid to the coupling protocol. Nevertheless, the H-phosphonate approach has been used successfully in the solid phase synthesis of relatively high molecular weight



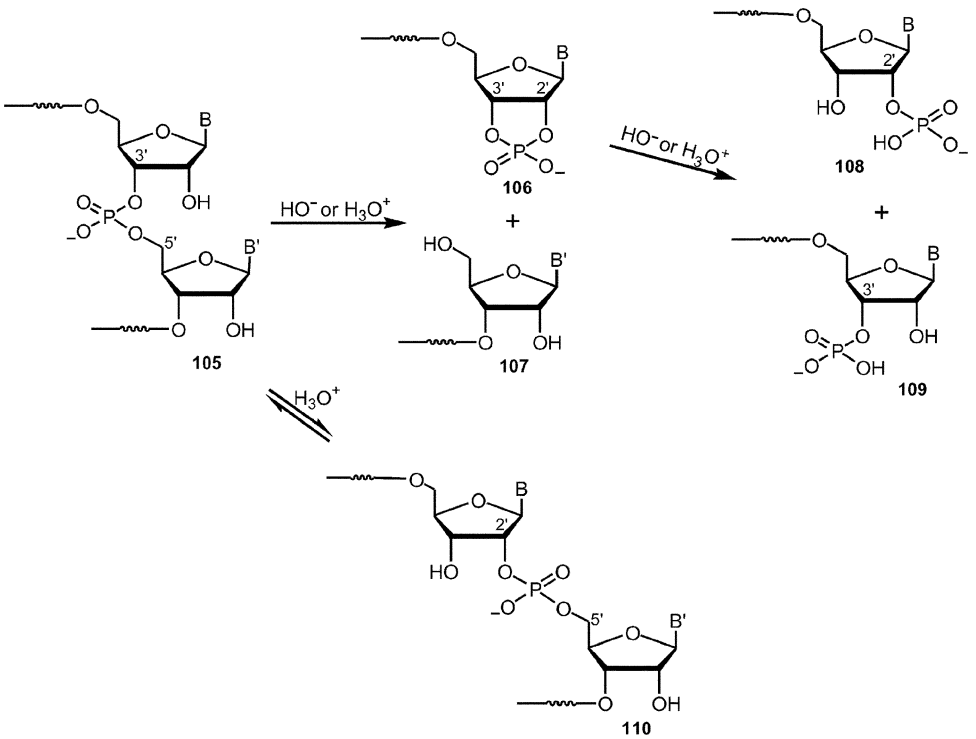
oligonucleotides.[42a,b] However, the general perception is that the phosphoramidite approach to solid phase synthesis is superior to the H-phosphonate approach in its present form and, for this reason, it is used much more widely. A possible explanation for this is that, in the strategy normally followed, the individual H-phosphonate diester linkages (as in **103**) are not oxidized after each coupling step but are all oxidized together after the entire oligonucleotide sequence has been assembled. This could give rise to a number of problems.[42c,63] First, H-phosphonate diesters (as in **103**) are susceptible to acylation on phosphorus (as in **104**). If this occurs, the internucleotide linkages affected will not be converted into the desired phosphodiester internucleotide linkages in the final oxidation step. Secondly, even if this side-reaction does not occur, it is difficult to ensure that all of the H-phosphonate diesters are converted into phosphodiester linkages in the oxidation step. If oxidation is incomplete, the remaining H-phosphonate diester linkages will be cleaved during the ammonolytic step required to removed the *N*-acyl protecting groups from the base residues. This would inevitably lead to diminished yields of target sequences. It is possible that solid phase H-phosphonate synthesis could be improved by oxidizing H-phosphonate diester to phosphodiester internucleotide linkages after each coupling step or perhaps better still by adopting the modified H-phosphonate approach[49a,b] (Scheme 14), which would involve a sulfur-transfer step (resulting in P(III) → P(V)) in each synthetic cycle.

Synthesis of oligo- and poly-ribonucleotides (RNA sequences)

It was clear at the outset that the choice of a protecting group for the 2′-hydroxy functions of ribonucleoside building blocks was the most crucial decision to be made in the overall strategy for polyribonucleotide synthesis. This protecting group has to remain intact throughout the assembly of the target RNA sequences and must then be easily removable in the final deblocking step under conditions under which RNA is completely stable. These are very demanding requirements. Under relatively mild basic conditions, the inter-ribonucleotidic linkage (as in **105**, Scheme 15) undergoes hydrolytic cleavage[64] to give at first a fragment terminating in a 2′,3′-cyclic phosphate (as in **106**) and a fragment **107** with a free 5′-hydroxy function. The cyclic phosphate fragment **108** can then undergo further base-catalyzed hydrolysis to give an isomeric mixture of the corresponding 2′- and 3′-monophosphates (**108** and **109**, respectively). The same fragments **106** (leading to **108** and **109**) and **107** are also obtained under conditions of acid-catalyzed hydrolysis.[65] However, under acidic conditions, migration of internucleotide linkages,[65] as indicated (Scheme 15) by the conversion of **105** into **110**, which has one or more 2′→5′-internucleotide linkages, can also occur. This isomerization process is reversible.

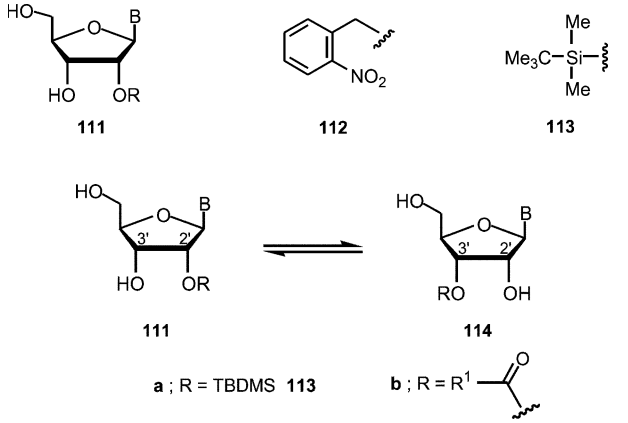
In choosing or designing a protecting group R for the 2′-hydroxy function of a ribonucleoside (as in **111**), it is very important indeed fully to be aware of the above hydrolysis properties of RNA. If a base-labile protecting group is selected, it is obviously desirable that it should be removable under basic conditions that are mild enough for the cleavage of the internucleotide linkages (Scheme 15) completely to be avoided. However, if a small amount of cleavage does occur, it is generally possible to remove the contaminating truncated sequences by chromatographic or other purification methods. If an acid-labile protecting group is selected, it is absolutely essential that it should be removable under very mild conditions indeed of acidic hydrolysis as, in practice, it is virtually impossible to free even relatively low molecular weight oligoribonucleotides from contaminating impurities containing one or more 2′→5′-internucleotide linkages (as in **110**). Finally, it should be borne in mind that, once the 2′-protecting groups have been removed, the free RNA sequences obtained will be highly susceptible to endonuclease-promoted digestion and must therefore be handled under sterile conditions.

It is not proposed to consider 2′-protection in oligo- and poly-ribonucleotide synthesis in any detail here as this subject has recently been discussed fully elsewhere.[66] For the reasons indicated above, it would in principle appear desirable to protect the 2′-hydroxy functions with a group that is removable at neutral pH, providing that it fully met the general criteria of stability and ease of removability required for all protecting groups. The 2′-protecting groups used so far that are removable under more or less neutral conditions include benzyl[67a] (as



Scheme 15

in **111**; R = CH_2Ph), 2-nitrobenzyl[67b] (as in **111**; R = **112**) and *tert*-butyldimethylsilyl[67c] (TBDMS, as in **111**; R = **113**). The 2′-*O*-benzyl protecting group, which is removable[67a] by catalytic hydrogenolysis, suffers from a serious disadvantage in that it is difficult to ensure that complete 2′-deblocking of an RNA sequence even of moderate size will occur. Furthermore, concomitant hydrogenation of the 5,6-double bonds of cytosine and uracil residues may also occur.[68] The 2-nitrobenzyl group **112**, which is removable photolytically[67b] above 280 nm, has been used to a much greater extent. However, it has been reported[69] that photolytic cleavage proceeds more smoothly under acidic conditions (*i.e.* at pH 3.5) and it is therefore questionable whether 2-nitrobenzyl may be considered as a protecting group that is readily removable under neutral conditions. The TBDMS protecting group **113** has been used very widely in solid phase oligoribonucleotide synthesis (see below); it nevertheless suffers from a notable disadvantage in that it readily undergoes base-catalyzed migration[70] (as in the conversion of **111a** into **114a** and *vice versa*; Scheme 16). For this reason, 2′-*O*-TBDMS-ribonucleoside derivatives (*e.g.* **111a**) with free vicinal 3′-hydroxy functions must be handed with care. The TBDMS group is normally removed by treatment with tetra-n-butylammonium fluoride in THF solution[71a] or with triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$).[71b,c] The former reagent is essentially basic and the latter reagent is more or less neutral, and there is no evidence that either reagent promotes the cleavage or migration of the internucleotide phosphodiester linkages of unprotected RNA.



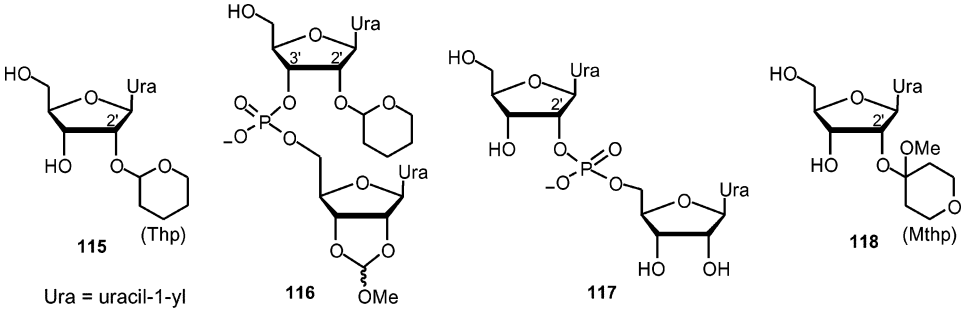
Scheme 16

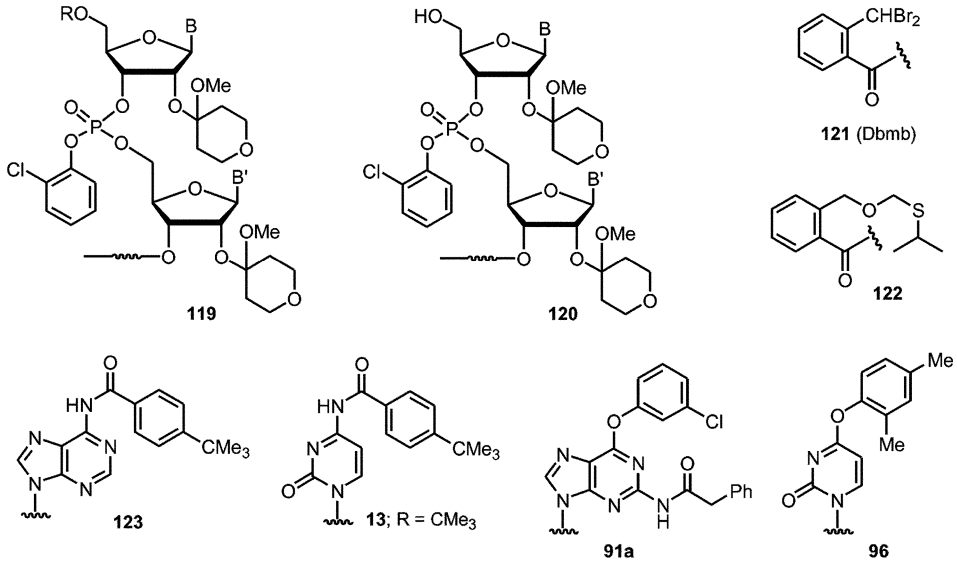
Although from the above discussion, base-labile would in principle appear to be more suitable than acid-labile groups for the protection of the 2′-hydroxy functions in oligoribonucleotide synthesis, in fact very little use has been made of them. Perhaps the main reason for this is that acyl groups, which are the most common base-labile protecting groups for hydroxy functions, very readily undergo base-catalyzed migration[72] (as in the conversion of **111b** into **114b** and vice versa; Scheme 16). However, unlike mixtures of 2′- and 3′-*O*-TBDMS derivatives (**111a** and **114a**), isomeric 2′- and 3′-*O*-acyl ribonucleoside derivatives (*e.g.* **111b**; R^1 = Me and **114b**; R^1 = Me) are usually not readily separable by chromatography. Despite the potential problems of cleavage and migration of the internucleotide linkages during the deblocking of 2′-protected RNA sequences (see above and Scheme 15), the use of acid-labile groups for the protection of the 2′-hydroxy functions merits serious consideration as nucleoside aglycone residues and internucleotide linkages (both in solution phase and solid phase synthesis) are virtually always protected with base-labile groups. Due to the lability of RNA to acids, bases and, if present, contaminating hydrolytic enzymes, it is highly desirable that the 2′-protecting groups should remain fully intact until the final deblocking step. However, if the 2′-hydroxy functions are to be protected with acid-labile groups, then particular care must be taken to ensure that the final deblocking step is carried out under the mildest possible conditions of acidic hydrolysis.

The first acid-labile 2′-protecting group examined was the tetrahydropyran-2-yl (Thp) group[73a–c] (as in **115**), which is part of an acetal system. The half-time ($t_{\frac{1}{2}}$) for the deprotection of 2′-*O*-Thp-uridine **115** in 0.01 M hydrochloric acid (pH 2.0) was found[65] to be 54 min at 24 °C. However, under the same conditions, $t_{\frac{1}{2}}$ for the removal of the Thp group from the partially-protected dinucleoside phosphate **116**, which would seem to be a better model for a 2′-protected RNA sequence, was found[65] to be only 29 min. Virtually complete (*i.e. ca.* 99.9%) 2′-deblocking should require *ca.* $10t_{\frac{1}{2}}$, that is, just under 5 h. Cleavage and migration (to give uridylyl-(2′→5′)-uridine **117**) of the internucleotide linkage of the dinucleoside phosphate **116** was found to occur to a negligible extent in 5 h under these conditions. As the Thp group is chiral, its use leads to diastereoisomeric mixtures of products. For this reason, the Thp group was abandoned in favour of the achiral 4-methoxytetrahydropyran-4-yl (Mthp) protecting group[74a,b] (as in **118**). The Mthp group has an additional advantage in that it is over twice as labile as the Thp group at pH 2.0 and room temperature.[74a]

a Solution phase synthesis of RNA sequences

The 2′-*O*-Mthp protecting group was used successfully in the synthesis of the 3′-terminal decamer[30,56] (10-mer: r[UCGUCCACCA]), nonadecamer[52b,56] (19-mer: r[AUUCCG-GACUCGUCCACCA], and heptatriacontamer[56] (37-mer: r[GGAGAGGUCUCCGGTψCGAUUCCGGACUCGUCC-ACCA] sequences of yeast $tRNA^{Ala}$ by the phosphotriester approach in solution. The internucleotide linkages were protected with 2-chlorophenyl groups (as in **119**) and MSNT **45** was used as the coupling agent. It was necessary to block the 5′-hydroxy functions with temporary protecting groups (R in **119**) that were removable under very mild basic conditions indeed in such a way that their removal did not affect the growing number of base-sensitive (2-chlorophenyl)-protected internucleotide linkages. The 2-(dibromomethyl)benzoyl (Dbmb)[75] **121** and 2-[(isopropylthio)methoxymethyl]-benzoyl[52d] **122** protecting groups were developed for this purpose. The adenine, cytosine, guanine and uracil residues were protected as in **123**, **13**; R = CMe_3, **91a** and **96**, respectively. The 5-methyluridine (T) and pseudouridine (ψ) residues





in the 37-mer sequence were protected[52d] with 4-*O*-phenyl and 1-*N*-(4-bromobenzenesulfonyl) groups, respectively. Following the removal of the protecting groups from the internucleotide linkages and the base-residues by treatment first with 2-nitrobenzaldoximate ions and then with concentrated aqueous ammonia, the Mthp groups were removed from the 2′-hydroxy functions with 0.01 M hydrochloric acid at room temperature to give the fully-unprotected target sequences.[56] Other phosphotriester approaches to the solution phase synthesis of RNA sequences, involving different 2′-protecting groups, have also been reported.[76]

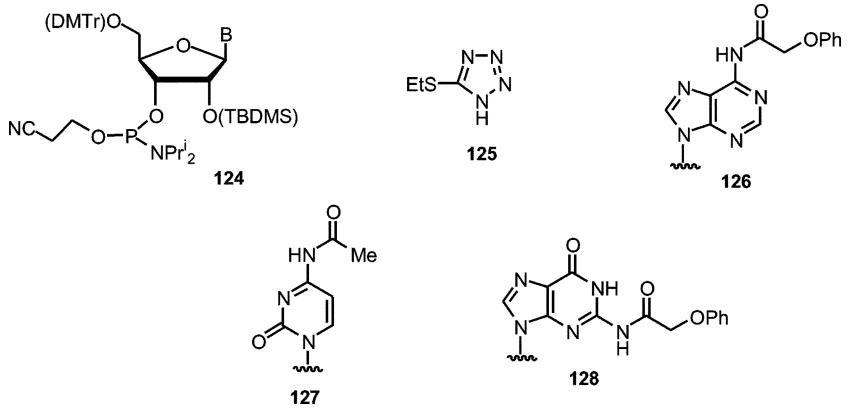
b Solid phase synthesis of RNA sequences

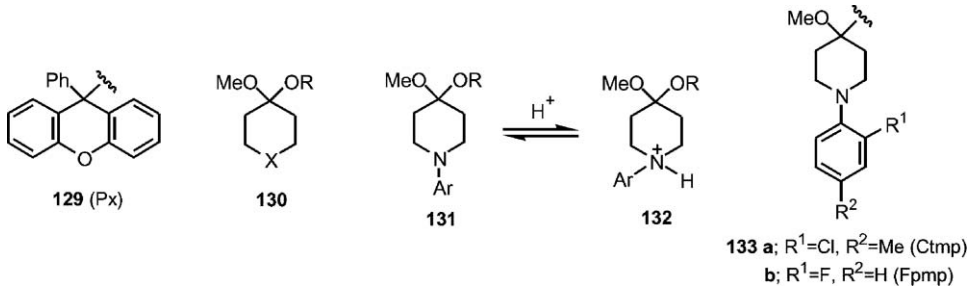
The TBDMS protecting group **113** has been used very widely[77] in the solid phase synthesis of RNA sequences. Despite the ease of migration of the TBDMS group, relatively pure monomeric phosphoramidites of general structure **124**, which are contaminated with at most very small quantities of isomeric 3′-*O*-TBDMS-2′-phosphoramidites, are commercially available. The general protocol of solid phase RNA synthesis is very similar to that of solid phase DNA synthesis.[58c] Again, both controlled-pore glass and polystyrene are generally used as solid supports. Due presumably to the bulkiness of the protected 2′-hydroxy functions, coupling times are generally much longer than in DNA synthesis and activators other than 1*H*-tetrazole **102** (*e.g.* 5-ethylsulfanyl-1*H*-tetrazole[78] **125**) have sometimes been used. In order to ensure that the 2′-*O*-TBDMS protecting groups remain largely intact until the final unblocking step, it is advisable that the adenine, cytosine and guanine residues should be protected with particularly labile acyl groups (*e.g.* as in **126–128**, respectively[79]) that are removable by treatment with ammonia or methylamine under very mild conditions. In the final deblocking step, the 2′-*O*-TBDMS protecting groups are best removed by treatment with triethylamine trihydrofluoride.[71b,c]

As suggested above, acid-labile (*e.g.* acetal) 2′-protecting groups have the considerable advantage that they remain intact under the basic conditions required to remove the protecting groups from the nucleoside aglycone residues and the internucleotide linkages. Fully-assembled RNA sequences are generally also released from the solid support under mild basic conditions. Acetal protecting groups have two additional very considerable advantages in that they can be introduced regiospecifically onto the 2′-hydroxy function of a ribonucleoside derivative and, once in place, they do not migrate.

Substituted trityl (*e.g.* DMTr **15b** and 9-phenylxanthen-9-yl (Px)[80] **129**) groups have been used for the temporary protection of 5′-hydroxy functions in nearly all approaches to the solid phase synthesis of oligoribonucleotides. Such ‘trityl’ protecting groups have the advantage that they are readily cleaved under acidic conditions. ‘Trityl’ protecting groups have a further advantage in that the liberated ‘trityl’ cations may be assayed spectrophotometrically[58a] and the efficiency of the coupling steps thereby monitored. Clearly, a potential problem of selectivity arises if acid-labile groups are used to protect both the 2′- and 5′-hydroxy functions.

It had earlier been found[74a,81] that the rate of acid-catalyzed hydrolysis of acetal systems of general structure **130** is very sensitive to the inductive effect of the atom or group X. It then seemed reasonable to assume that if the tertiary amine function in a 1-arylpiperidin-4-one acetal system **131** had a pK_a of *ca.* 2, it would be largely protonated (as in **132**) in the presence of an excess of trichloroacetic acid (pK_a 0.66) during the ‘detritylation’ step in solid phase synthesis and largely unprotonated (as in





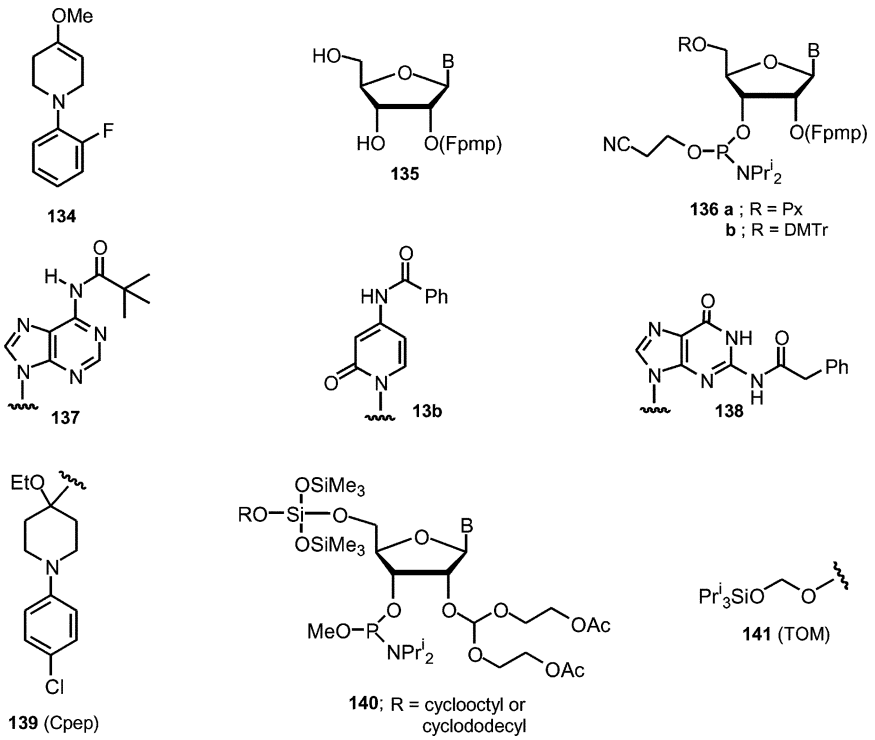
129 (Px) **130** **131** **132**

133 a; R^1=Cl, R^2=Me (Ctmp)
b; R^1=F, R^2=H (Fpmp)

131) during the final deblocking step, especially if it is carried out above, say, pH 3. It was then concluded that, as a first approximation, the rate of hydrolysis should be pH independent in a pH range from somewhere below to somewhere above its pK_a. The Ctmp group[82a] **133a** was the first such 1-arylpiperidin-4-one acetal system that was found to have the desired properties: the ratio of its hydrolysis rates at pH 0.5 and 2.5 was found to be only 1.55. The Ctmp group **133a** was found to be compatible with the 5′-*O*-Px protecting group **129** in solid phase oligoribonucleotide synthesis, and has been used successfully to protect the 2′-hydroxy functions both in phosphoramidite-based[82b] and in H-phosphonate-based[82c] synthesis.

It was subsequently found[83] that the related Fpmp group **133b** had similar hydrolysis properties to the Ctmp protecting group **133a**. However, the enol ether reagent[83,84] **134** required for the preparation of 2′-*O*-Fpmp nucleoside derivatives **135** and hence for the preparation of the phosphoramidite building blocks **136a,b** is more readily accessible than the corresponding Ctmp reagent. At first, phosphoramidites **136a** with the more acid-labile 5′-*O*-Px protecting groups were used,[85] but subsequently the corresponding 5′-*O*-DMTr derivatives **136b** became commercially available. In both sets of phosphoramidite building blocks[85,86] **136a,b** the amino functions of the adenine, cytosine and guanine base residues were protected with pivaloyl, benzoyl and phenylacetyl groups (as in **137**, **13b** and **138**, respectively), and the uracil residues were left unprotected. The Fpmp protecting group has been used successfully in solid phase RNA synthesis by a number of workers.[86a–f] After the completion of the coupling steps, the products are generally treated with concentrated aqueous ammonia (to release the protected RNA from the solid support and remove the protecting groups from the internucleotide linkages and the base residues). The stabilized (*i.e.* stable to base- and endonuclease-catalyzed hydrolysis) 2′-*O*-Fpmp RNA may safely be purified before it is subjected to acidic hydrolysis to give fully-deblocked RNA. Although acid treatment at pH 2.0–2.3 and room temperature proved to be satisfactory for the deblocking even of some relatively high molecular weight RNA sequences[85] (*e.g.* the 3′-terminal 37-mer sequence of unmodified yeast $tRNA^{Ala}$), it was subsequently found[86b] that the internucleotide linkages of other sequences (*e.g.* r[$(Up)_9$U] and r[$(Up)_{19}$U]) underwent appreciable cleavage and migration under the latter acidic conditions. Indeed it is now believed[86e] that it is generally advisable to remove 2′-*O*-Fpmp and related protecting groups from RNA sequences at or above pH 3.25 and room temperature in order to avoid these undesirable side-reactions. An examination of twelve other related piperidinyl protecting groups revealed[87] that the 1-(4-chlorophenyl)-1-ethoxypiperidin-4-yl (Cpep) **139** group had an even better acid hydrolysis profile than the Fpmp group **133b** in that it is both more stable to acidic hydrolysis in the pH range 0.5–2.5 and more labile in the pH range 3.25–3.75.

Recently two other approaches to solid phase RNA synthesis have been developed and commercialized successfully. Scaringe *et al.*[88] have reported the use of the phosphoramidite building blocks **140** which have since been widely used in a custom synthesis of RNA sequences. The particular features of this approach are (i) that instead of an acid-labile 5′-protecting group, a 5′-*O*-silyl group, which is removable by treatment with fluoride ions, is used, (ii) methyl rather than 2-cyanoethyl phosphoramidite building blocks are used, and (iii) orthoester 2′-protecting groups



134

135

136 a ; R = Px
b ; R = DMTr

137

13b

138

139 (Cpep)

140; R = cyclooctyl or cyclododecyl

141 (TOM)

are used. The orthoester group is deacetylated and thus made more acid-labile in the penultimate deblocking step. In the second approach, the (triisopropylsilyl)oxymethyl (TOM) **141** group[89] is used to protect the 2′-hydroxy functions. The TOM group **141** may be regarded as an alternative to the TBDMS protecting group **113** in that it is also removed by treatment with fluoride ions; however, it has two advantages over the TBDMS group in that (i) it cannot migrate and (ii) it appears to be effectively less bulky and therefore its use leads to shorter coupling times.

So far emphasis has been placed on the use of phosphoramidite building blocks in solid phase RNA synthesis. However, H-phosphonate building blocks have also been used successfully. The 2′-hydroxy functions have been blocked by a number of protecting groups including TBDMS[90] **113** and Ctmp[82c] **133a**. The use of monomeric H-phosphonate building blocks in solid phase oligoribonucleotide synthesis has recently been reviewed.[63]

Present conclusions and possible future directions of oligo- and poly-nucleotide synthesis

It is clear that enormous strides have been made in the methodology of the chemical synthesis of DNA and RNA sequences since the publication of Michelson and Todd's seminal paper[4] in August 1955. It would, of course, be foolhardy to attempt to predict how this field will progress in the next five decades. As it is an area of such intrinsic and practical importance, it is inevitable that, in the future, synthetic methodology will undergo numerous significant developments. It is also very likely that the course of these developments will be strongly influenced by the requirements of molecular biology and biotechnology. Solid phase synthesis, probably based on phosphoramidite building blocks, will surely continue to be used in the preparation of the relatively small quantities of material required by molecular biologists. A more uncertain matter is what influence the licensing of a systemic oligonucleotide drug, which may be required in multikilogram or even tonne quantities would have on the development of synthetic methodology. While solid phase synthesis has so far provided the multigram quantities of material[91] required for clinical trials, it seems not unlikely that either solution phase synthesis or a combination of solution and solid phase synthesis will emerge as the method of choice if really large quantities of material become required. Thus, dimer, trimer or even larger oligonucleotide blocks could be prepared on a large scale in solution, quite possibly by the modified H-phosphonate approach[49a,b] (see Scheme 14 above) and then linked together either in solution or on a solid (or liquid polymer) support.

Despite the considerable progress that has taken place in recent years, the synthesis of RNA still lags somewhat behind that of DNA sequences. This is due largely to the demanding requirements of 2′-protection. As there has so far been no success in the licensing of a systemic antisense drug, it is quite likely that research in oligonucleotide drug discovery will become directed more to the development of RNAi-based[92] chemotherapy. This would inevitably lead to research effort in the field of oligo- and poly-nucleotide synthesis becoming more and more directed towards the synthesis of RNA and modified RNA sequences.

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