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Perspectives

The chemical synthesis of oligo- and poly-nucleotides: a personal commentary

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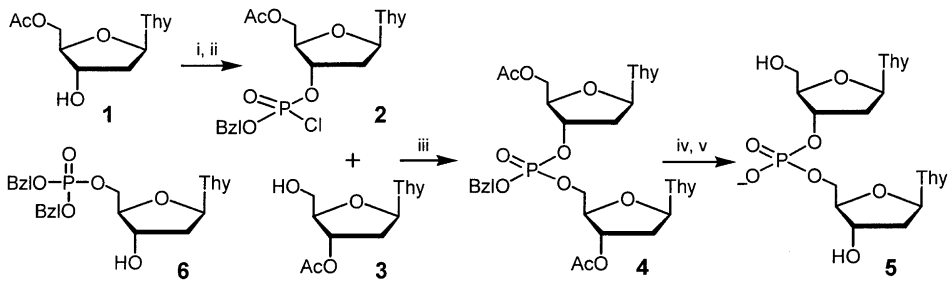
1. Introduction

In October 1953, I began my research career as a Ph.D. student in the laboratory of Alexander Todd (who became Sir Alexander Todd in 1954 and then Lord Todd of Trumpington in 1962), after having graduated from Cambridge University earlier that year. Nineteen fifty-three was of course a momentous year in the history of nucleic acid chemistry. Some six months or so before I started my Ph.D. course, J. D. Watson and F. H. C. Crick had assembled their DNA model[1] in the old Cavendish Physics Laboratory that was almost literally within striking distance of the old University Chemical Laboratory where I was to carry out my Ph.D. studies. I believe that I was very fortunate indeed to start working in what was then most probably the main nucleic acid chemistry laboratory in the world and at a time just before the first ever synthesis of an oligonucleotide with a natural 3′→5′-internucleotide linkage (see below) was to be carried out in that laboratory. As I myself have been actively engaged in oligonucleotide synthesis for the past 40 years, I have been able to follow the development of this field from, so to speak, its birth to its present state. In this Commentary, I shall attempt to highlight what I personally consider to have been significant developments in this field throughout the whole of this period. I shall not attempt to present a comprehensive review of the whole field.

My first research project, which was carried out under the immediate supervision of George Kenner, was concerned mainly with the site of acylation of cytosine residues.[2] This study proved to be relevant to the Watson–Crick structure of DNA inasmuch as it established that the amino form of 1-methylcytosine (a model for 2′-deoxycytidine) was the predominant tautomer. Then, in my second year as a research student, I started working on nucleotide chemistry and more specifically on the synthesis of a nucleotide coenzyme analogue. At that time, the synthesis of nucleotide coenzymes was the main focus of Todd's nucleotide programme and it was partly for his contribution to this area of research that he was awarded the Nobel Prize for Chemistry in 1957. As he makes clear in his autobiography,[3] Todd was not particularly drawn to the chemical synthesis of oligonucleotides. Nevertheless, in 1955 and in collaboration with A. M. Michelson, he published[4] the first chemical synthesis of a natural dinucleoside phosphate [d(TpT)] and a natural dinucleotide [d(pTpT)].

The Michelson and Todd synthesis[4] of d(TpT) **5** is indicated



Thy = thymin-1-yl; Bzl = $PhCH_2$

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.

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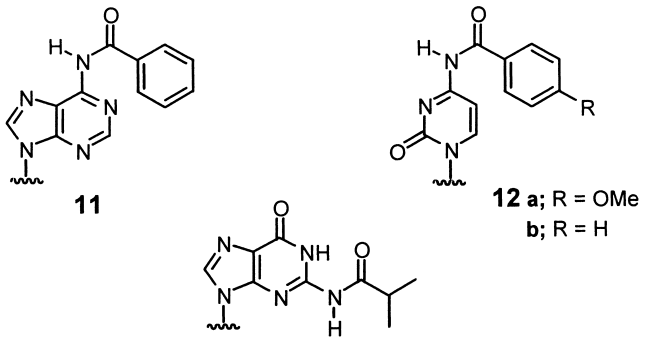
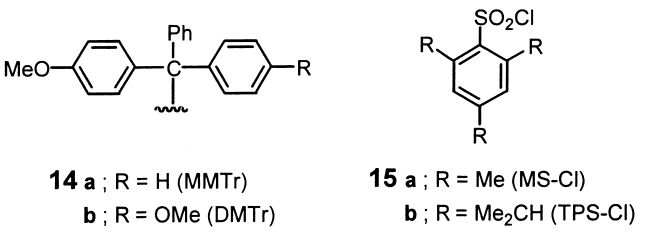
in outline in Scheme 1. The synthesis of the corresponding dinucleotide [d(pTpT)] was very similar except that 5′-*O*-acetylthymidine **1** was replaced by dibenzyl thymidine 5′-phosphate **6** and an additional catalytic hydrogenolysis step was required. The synthetic methodology adopted was based on that used previously in the preparation of mononucleotides and certain nucleotide coenzymes. Thus in the preparation of d(TpT) **5**, 3′-*O*-acetylthymidine **3** was phosphorylated with a dialkyl phosphorochloridate **2** and phosphate esters were protected with benzyl groups. There appeared to be no strategy for extending the oligonucleotide chain and, in any case, benzyl-protected phosphodiesters readily undergo debenzylation, e.g. in pyridine solution. It was probably taken for granted that internucleotide linkages should be protected during synthesis. This approach to oligonucleotide synthesis later became known as the *phosphotriester approach* (see below).

2. The phosphodiester approach

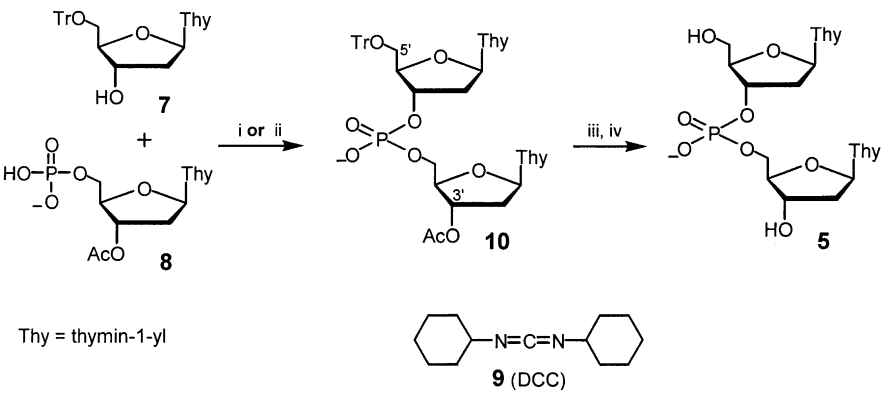
No further work on the phosphotriester approach to oligonucleotide synthesis was carried out in the Cambridge laboratory until the mid-1960s (see below). Indeed, the whole field of oligonucleotide synthesis very soon became dominated, perhaps for a period of 15 years or more, by a completely different approach that was introduced by H. G. Khorana and his co-workers. In this approach,[5–7] which later became known as the *phosphodiester approach*, the internucleotide phosphodiester linkages were left completely unprotected.

An early example of the application of the phosphodiester approach is illustrated in Scheme 2: 5′-*O*-tritylthymidine **7** and 3′-*O*-acetylthymidine 5′-phosphate **8** were allowed to react together[5,6] either in the presence of toluene-4-sulfonyl chloride (TsCl) or N^1,N^3-dicyclohexylcarbodiimide (DCC) **9** in dry pyridine solution. Following the removal of the trityl and acetyl protecting groups, d(TpT) **5** was obtained. The coupling reactions always involved phosphomonoesters and the internucleotide phosphodiester functions were never protected even during block coupling reactions. However, Khorana and his co-workers[8] 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 6-*N*-(*p*-anisoyl) derivatives (as in **11** and **12a**, respectively) and guanine residues were protected as their 2-*N*-isobutyryl derivatives (as in **13**). A very important development was the introduction[9] of the (*p*-anisyl)diphenylmethyl (MMTr, **14a**) and di-(*p*-anisyl)-phenylmethyl (DMTr, **14b**) protecting groups for the 5′-hydroxy functions of nucleosides and oligonucleotide blocks. The acidic conditions required for the removal of trityl protecting groups are drastic enough to promote the cleavage of the glycosidic linkages, especially of purine 2′-deoxyribosides. For this reason, trityl were replaced by the more acid-labile MMTr **14a** protecting groups. Nowadays (see below), the even more labile DMTr **14b** group is used almost exclusively to protect the 5′-hydroxy functions in oligo- and poly-nucleotide synthesis. Initially,[6] DCC **9** was preferred as the coupling reagent but it was soon replaced by mesitylene-[10] and 2,4,6-triisopropylbenzene-[11] sulfonyl chlorides (MS-Cl **15a** and TPS-Cl **15b**, respectively).

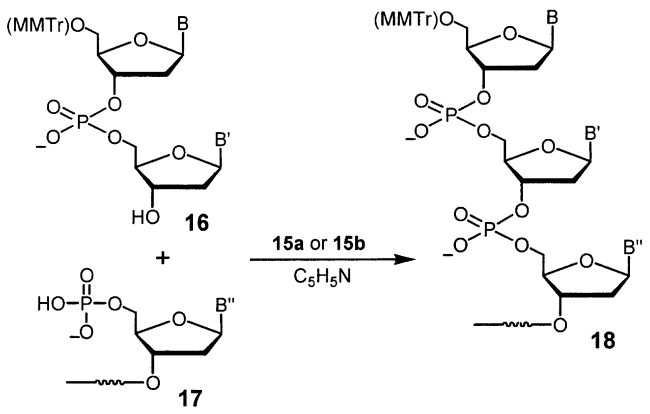




The phosphodiester approach was adapted both to the stepwise[12] and to the block[13] synthesis of moderately high molecular weight oligodeoxyribonucleotides. Alkaline hydrolysis of a 3′-*O*-acyl protected dinucleoside phosphate



Scheme 2. *Reagents*: (i) TsCl, C_5H_5N; (ii) **9**, C_5H_5N; (iii) AcOH–H_2O (4:1 v/v); (iv) NaOH, H_2O.



Scheme 3.

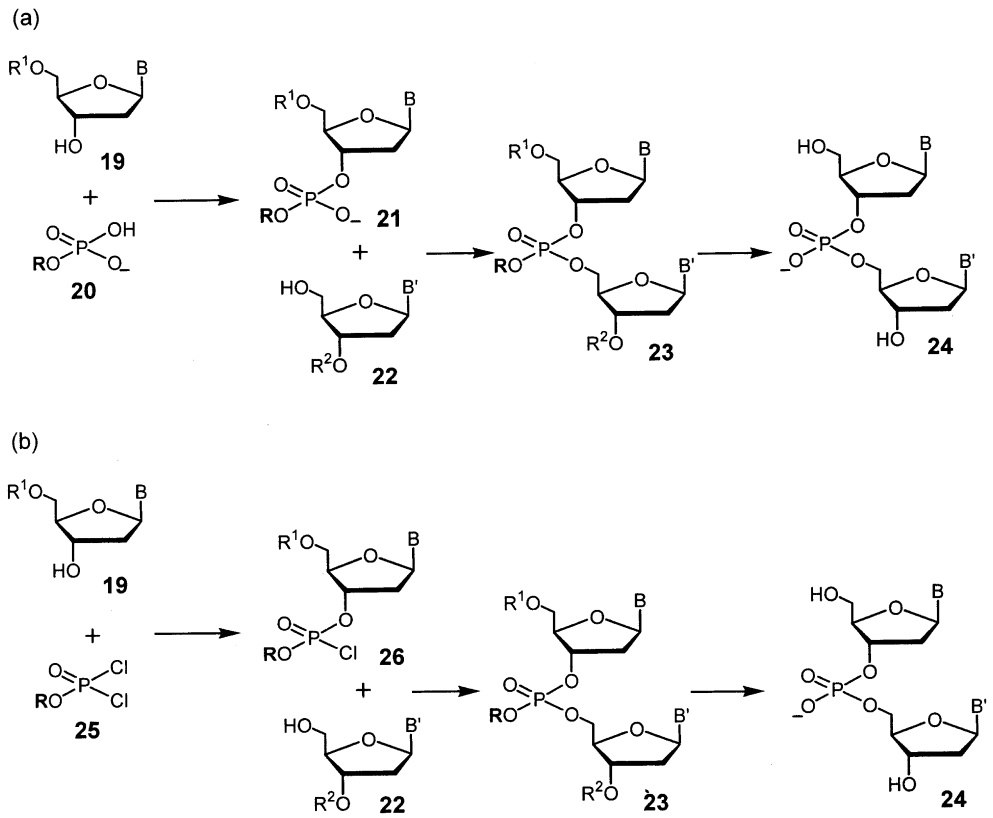
released the 3′-terminal hydroxy function. The product (represented in general by structure **16**) was then coupled (Scheme 3) with a protected 2′-deoxyribonucleoside 5′-phosphate (such as **8**) or with an oligonucleotide **17** terminating in a 5′-phosphate, in the presence of MS-Cl **15a** or TPS-Cl **15b**. Moderately good yields (ca. 50–70%) were obtained[12] in (1+2→3) and other stepwise coupling reactions if a large excess of monomer was used. However, yields were generally lower in block coupling reactions.[13] The presence of guanine residues (protected on N-2 as in **13**) also appeared[14] to lead to diminished yields. Although it became evident that the accumulation of charged phosphodiester internucleotide linkages led to side-reactions and hence to diminished yields, Khorana[15] did not believe that an overall advantage would be gained by protecting the internucleotide linkages. In a 1968 lecture to an IUPAC Natural Products Conference[15] in London, he expressed the opinion that, unless coupling yields were virtually quantitative, adopting the phosphotriester approach would actually mean throwing away what he regarded as 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 conventional chromatographic techniques (e.g. 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.[15] Indeed, it later became clear that nothing is thrown away but much is indeed gained by adopting the phosphotriester approach.

As is apparent from some of the papers on oligonucleotide synthesis by phosphodiester approach published by Khorana and his co-workers,[12,16,17] purification of the intermediate blocks and the final products by chromatography on DEAE-cellulose was both very painstaking and time-consuming. In the course of the purification process, the charged intermediates, which often contained both sensitive protecting groups and nucleoside residues (especially 6-*N*-benzoyl-2′-deoxyadenosine) were necessarily kept in solution in aqueous or aqueous alcoholic buffers for long periods of time and were then concenterated to dryness. Partly for this reason, the success achieved in the implementation of the phosphodiester approach is evidence of the considerable experimental skill of the research workers who were involved. 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 made an enormous contribution[15] to the elucidation of the genetic code by making use of chemically-synthesized oligodeoxynucleotides containing repeating dimer, trimer and tetramer sequences. After the completion of this work, Khorana's group then carried out the total synthesis of DNA duplexes corresponding to yeast alanine transfer RNA[18] and to the precursor of tyrosine suppressor transfer RNA[19] by joining together chemically synthesized oligodeoxyribonucleotide sequences enzymatically. These studies provided a basis for many of the fundamental developments in molecular biology and biotechnology that have since taken place.

3. The phosphotriester approach

Success in oligonucleotide synthesis depends to a large extent on the choice of suitable protecting groups and the development of effective phosphorylation procedures. These two factors are to some extent interconnected. In an earlier article,[20] I listed a number of general criteria that I believe that protecting groups should fulfil. The three absolutely essential criteria are (i) that they should be relatively easy to introduce, (ii) that they should be stable under the reaction conditions and (iii) that they should be removable at the end of the synthesis under conditions under which the desired product is stable. As an extension to the first criterion, it is also desirable that the reagents involved should be relatively easily accessible. Clearly, a matter of crucial importance in the phosphotriester approach is the choice of the protecting group for the internucleotide linkages. It was clear at the outset that the benzyl group, used by Michelson and Todd,[4] would be much too susceptible to nucleophilic attack, particularly by pyridine, to be suitable.

In the second half of the 1960s, when Khorana was in the process of completing his studies on oligonucleotide synthesis by the phosphodiester approach, a reinvestigation of the phosphotriester approach was undertaken in several laboratories. Although almost all of this work was carried out in the solution phase, in 1965 Letsinger and Mahadevan[21] showed considerable foresight by undertaking the first oligonucleotide synthesis on a solid support (see below), using the 2-cyanoethyl group to protect the internucleotide linkages. Letsinger and Ogilvie[22] then switched to solution phase phosphotriester synthesis and continued to use the 2-cyanoethyl protecting group (as in Scheme 4(a), **R**=CH_2CH_2CN). At about the same time, Eckstein and Rizk[23,24] used the 2,2,2-trichloroethyl group (as in Scheme 4(a) and (b); **R**=CH_2CCl_3) to protect the internucleotide linkages in the phosphotriester approach and, soon afterwards, Reese and Saffhill[25] reported the use of the phenyl protecting group (as in Scheme 4(b), **R**=Ph). All three research groups first undertook the preparation of thymidylyl-(3′→5′)-thymidine **5**. Letsinger and his co-workers[26] and Eckstein and Rizk[27] went on to prepare longer sequences (up to hexamers) and carried out

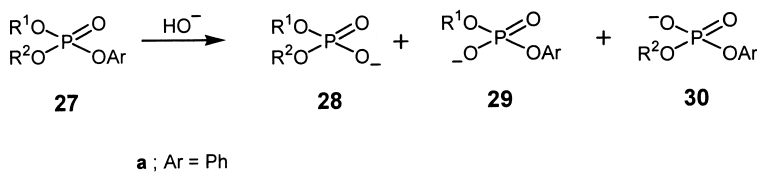


Scheme 4.

sequential (e.g 1+1→2; 1+2→3 etc) and block (e.g. 2+2→4) coupling reactions; both of the latter two research groups also prepared oligonucleotides containing nucleosides other than thymidine.

The basic chemistry used in the early phosphotriester work is indicated in outline in Scheme 4; as it has already been reviewed,[20,28] it will be described only briefly here and will be exemplified only by the synthesis of dinucleoside phosphates. The source of phosphate was either a monoalkyl phosphate **20** (Scheme 4(a)) or a monoalkyl (or monoaryl) phosphorodichloridate **25** (Scheme 4(b)). Letsinger and his co-workers[21,22,26] started with 2-cyanoethyl phosphate **20** (**R**=CH_2CH_2CN), which was allowed to react with a 5′-protected nucleoside building block in the presence of MS-Cl **15a**. The resulting nucleotide derivative **21** (**R**=CH_2CH_2CN) was coupled (Scheme 4(a)) with a 3′-protected nucleoside derivative **22** in the presence of TPS-Cl **15b** to give the fully-protected product **23**. The protecting groups were then removed in an appropriate manner, with aqueous ammonia being used to remove the 2-cyanoethyl group from the internucleotide linkage. The fully unblocked product **24** was thereby obtained. Eckstein and Rizk at first[23] adopted a procedure similar to that of Letsinger et al. but with the 2,2,2-trichloroethyl instead of the 2-cyanoethyl protecting group. Thus, the fully protected dinucleoside phosphate **23** (**R**=CH_2CCl_3) was obtained (Scheme 4(a)) by coupling together the 5′-protected phosphodiester **21** (**R**=CH_2CCl_3) and the 3′-protected nucleoside derivatives **22** in the presence of TPS-Cl **15b**. The 2,2,2-trichloroethyl protecting group was subsequently removed from the internucleotide linkage by treatment with zinc dust in 80% acetic acid at rt. Eckstein and Rizk subsequently used[24,27] the bifunctional reagent 2,2,2-trichloroethyl phosphorodichloridate **25** (**R**=CH_2CCl_3) as the source of phosphate. Thus, the 5′-protected nucleoside derivative **19** was allowed to react (Scheme 4(b)) with slight excess of 2,2,2-trichloroethyl phosphorodichloridate **25** (**R**=CH_2CCl_3) and the putative intermediate phosphorochloridate **26** (**R**=CH_2CCl_3) was then coupled with the 3′-protected nucleoside derivative **22** to give the fully-protected dinucleoside phosphate **23** (**R**=CH_2CCl_3). Reese and Saffhill[25] followed essentially the same strategy (Scheme 4(b)) but used phenyl phosphorodichloridate **25** (**R**=Ph) as the source of phosphate. The phenyl protecting group was removed from the fully protected dinucleoside phosphate **23** (**R**=Ph) by treatment with 0.1 M sodium hydroxide in aqueous dioxane.

The significance of the 1960s phosphotriester studies lies more in the choice of the protecting group for the internucleotide linkages than in the synthetic strategies adopted. Much more work needed to be done before the phosphotriester approach became established as a really effective method for oligonucleotide synthesis. Of the three groups suggested for the protection of the internucleotide linkages, only the 2-cyanoethyl group (as in **23**; **R**=CH_2CH_2CN) proved to be too labile for use in solution phase oligonucleotide synthesis. However, it later became the protecting group of choice in solid phase synthesis (see below). The 2,2,2-trichloroethyl protecting group (as in **23**; **R**=CH_2CCl_3) was subsequently used by Catlin and Cramer[29] in oligodeoxyribonucleotide synthesis and by Neilson and his co-workers[30] in oligoribonucleotide synthesis. However, the latter workers found[31] that recovered yields after removal of the 2,2,2-trichloroethyl groups (with zinc–copper couple in *N*,*N*-dimethylformamide) were



Scheme 5.

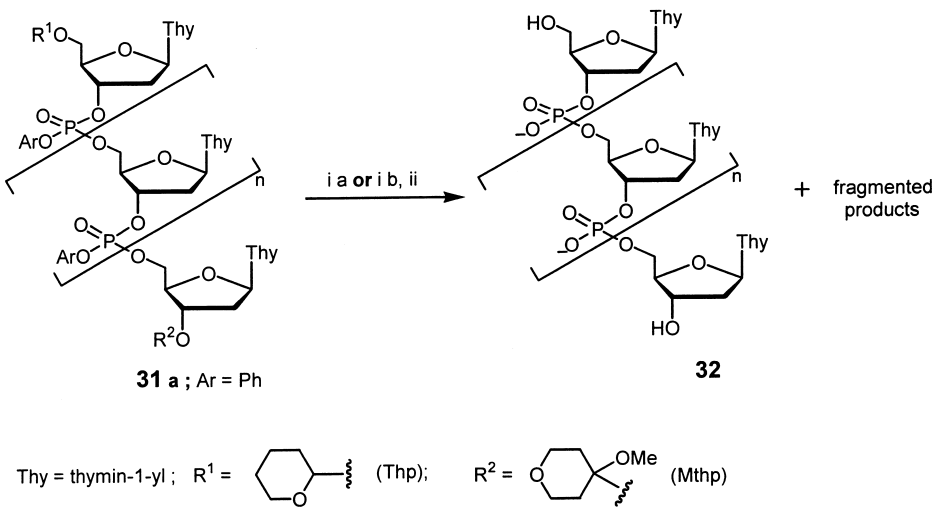
unsatisfactory and this protecting group has subsequently found little favour in oligonucleotide synthesis. Finally, although the phenyl protecting group (as in **23**; **R**=Ph) gave rise to stable phosphotriester intermediates that could readily be manipulated and purified, its use led to a problem that took a number of years to solve in a fully satisfactory way. While the removal of 2-cyanoethyl and 2,2,2-trichloroethyl protecting groups involves *O*-alkyl cleavage, alkaline hydrolysis of phenyl-protected phosphodiesters (such as **27a**) proceeds by the 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 **27a** (e.g. **23**; **R**=Ph) leads (Scheme 5) not only to the corresponding dialkyl phosphate **28** but unfortunately also to significant quantities[32] of the two possible alkyl phenyl phosphates **29a** and **30a**.

The full seriousness of this problem became clear following studies[33] directed towards the block synthesis of a series of oligothymidylic acids **32** (n=0, 2, 6 and 14). The deprotection procedure (Scheme 6) consisted of two steps. First, the internucleotide linkages were unblocked by alkaline hydrolysis, e.g. by treatment either with 1.0 M aqueous potassium hydroxide (step i(a)) or with 0.085 M tetraethylammonium hydroxide in wet dimethyl sulfoxide (DMSO) (step i(b)). The terminal acetal protecting groups were then removed by acidic hydrolysis (step ii).

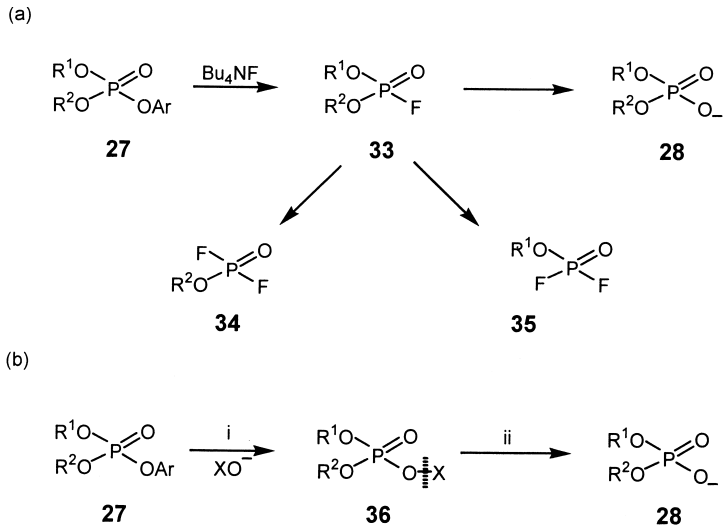
The fully unblocked products were fractionated by chromatography on DEAE-cellulose or DEAE-Sephadex and the percentages of full length oligothymidylic acids **32** were estimated spectrophotometrically. In the case of the tetramer **31a**; n=2, the yield of fully unblocked tetranucleoside triphosphate **32**; *n*=2 was estimated to be 83% (i.e. an average of ca. 6% cleavage per internucleotide linkage) when aqueous potassium hydroxide (as in step i(a)) was used and 94% (i.e. an average of 2% cleavage per internucleotide linkage) when tetraethylammonium hydroxide in wet DMSO (as in step i(b)), which was found[33] to be the reagent of choice, was used. The latter reagent was used in the unblocking of the fully-protected octamer **31a**; n=6 and hexadecamer **31a**; n=14. The recovered yields after chromatography of the fully unblocked octamer **32**; n=6 and the hexadecamer **32**; n=14 were estimated to be 77.5 and 50%, respectively.

A particular advantage of aryl protecting groups is that their properties can easily be modified by substitution. It was very soon found[34] that the unblocking rate could be increased and the extent of internucleotide cleavage decreased by the introduction of electron-withdrawing solution substitutents 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 would be expected to increase the selectivity of the hydrolysis process in Scheme 5 and lead to a greater proportion of the desired dialkyl phosphate **28**. However, it later became apparent that there was a practical limitation to this way of decreasing the extent of oligonucleotide cleavage during unblocking. The results of an appropriate study suggested[35] that if the pK_a of ArOH is less than ca. 8, the phosphotriester intermediates (corresponding to **31**) are likely to become insufficiently stable to allow them to be recoverable in satisfactory yield following their purification and chromatography on silica gel.

An alternative approach to the solution of this problem was to use a nucleophile other than hydroxide ion to unblock the internucleotide linkages. Ogilvie et al.[36] suggested



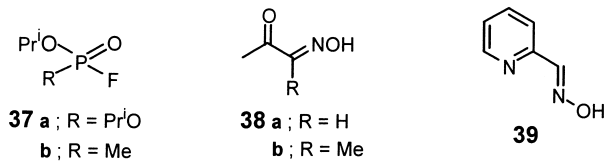
Scheme 6. *Reagents*: (i) (a) 1.0 M aqueous KOH, (b) 0.085 M Et_4NOH in DMSO–H_2O (19:1 v/v); (ii) 0.01 M hydrochloric acid.



Scheme 7.

tetra-n-butylammonium fluoride (TBAF) in tetrahydrofuran (THF) as the unblocking agent (Scheme 7(a)) and Narang and his co-workers[37] recommended the use of TBAF in THF–pyridine–water (8:1:1 v/v) solution. It was later observed[38] that when the fully protected tetranucleoside triphosphate **31** (n=2, Ar=2-ClC_6H_4, R^1=R^2=Mthp), which has three protected internucleotide linkages, was unblocked with 0.05 M TBAF in THF solution, very considerable cleavage of the internucleotide linkages occurred and, following acidic hydrolysis (Scheme 6, step ii), the desired fully unblocked tetranucleoside triphosphate **32**; n=2 was obtained in at most 50% yield. When unblocking was carried out under Narang's conditions,[37] internucleotide cleavage occurred to a lesser extent but the desired product **32**; n=2 was obtained only in at most 89% yield.[38]

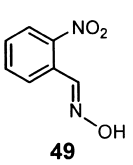
A particular danger of unblocking with fluoride ions under anhydrous conditions is apparent from Scheme 7(a). Thus, the initial product, which is presumably a phosphorofluoridate **33**, is likely to be susceptible to further nucleophilic attack by fluoride on phosphorus to give phosphorodifluoridates **34** and **35**. In the context of oligonucleotide unblocking, the formation of phosphorodifluoridates constitutes internucleotide cleavage. Under Narang's conditions,[37] hydrolysis of the intermediate phosphorofluoridate **33** to give the desired product **28** can compete with phosphorodifluoridate formation. However, hydrolysis of a phosphorofluoridate **33** involves a second nucleophilic substitution at phosphorus and need not necessarily result in displacement of fluoride to give the desired product **28**. It would clearly be advantageous if unblocking by an alternative nucleophile were to involve only one nucleophilic substitution at phosphorus. For this to be possible, it would appear that the nucleophile would need to have the general structure XO^-. A further requirement would be that the initial attack on phosphorus to give intermediate **36** (Scheme 7(b), step i) should be followed by a cleavage of the O–X bond (step ii). Of course, HO^- meets these requirements but the alternative nucleophile would perhaps need to be 'softer' and certainly more selective than hydroxide.



Phosphorofluoridates such as di-isopropyl phosphorofluoridate **37a** and isopropyl methylphosphonofluoridate (Sarin) **37b** are potent inhibitors of cholinesterase. This activity can be reversed by the conjugate bases of certain hydroxamic acids[39] and oximes.[40] Green and Saville examined[40] the reactions of hydroxyiminoacetone **38a** and the monoxime **38b** of butan-2,3-dione with Sarin **37b**, under basic conditions, and concluded that, in both cases, the rate determining step was the formation of an oxime phosphate ester which then underwent rapid fragmentation. These workers also showed[40] that the conjugate base of pyridine-2-carboxaldoxime **39** reacted particularly rapidly with Sarin **37b**. In the course of some studies on the catalytic effect of cetyltrimethylammonium bromide on the reactions between various aldoximate ions and diphenyl 4-nitrophenyl phosphate **40**, Bunton and Ihara found[41] that, at pH 10 and 25°C, the conjugate base of 4-nitrobenzaldoxime **41** reacted readily with the substrate **40** as evidenced by the release of 4-nitrophenoxide **43** (Scheme 8(a)). Very surprisingly, 4-nitrobenzonitrile **44** was not detected in the products. It was concluded that the rate determining step of the reaction was the hydrolysis of the intermediate oxime ester **42**, leading to the formation of diphenyl phosphate **45** and the regeneration of the oxime. The oxime ester **42** was subsequently prepared[42] and isolated as a pure crystalline solid; when it was treated with 2 mol equiv. of N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) in dichloromethane solution at 20°C for 10 min, it was quantitatively converted (Scheme 8(b)) into 4-nitrobenzonitrile **44**, which was isolated as a crystalline solid in 92% yield.

2-Chlorophenyl diethyl phosphate **46** would appear to be a much better model for an aryl-protected internucleotide linkage than 4-nitrophenyl diphenyl phosphate **40**. When

the reaction between this model compound **46** and a large excess each of 4-nitrobenzaldoxime **41** and TMG in dioxane–water (1:1 v/v) (Scheme 8(c)) was monitored[42] by ^{31}P NMR spectroscopy, the proposed intermediate oxime ester **47**, which had been prepared independently, could not be detected. However, 4-nitrobenzonitrile **44** was isolated from the products in good yield. In a separate series of experiments,[42] the reaction between the oxime ester **47**, 4-nitrobenzaldoxime **41** and triethylamine in dichloromethane solution was found to be 2–3 orders of magnitude faster than the corresponding reaction with 2-chlorophenyl diethyl phosphate **46** under the same conditions. The important conclusions to be drawn from these experiments are (i) that, in the reaction between the phosphotriester **46** and the conjugate base of 4-nitrobenzaldoxime **41**, the formation of the oxime ester **47** is rate determining and (ii) that the intermediate oxime ester **47** undergoes rapid base-catalyzed elimination reaction to give 4-nitrobenzonitrile **44** and diethyl phosphate **48**. The conjugate base of 4-nitrobenzaldoxime **41**, like hydroxide ion, is clearly a nucleophile of the type XO^- (Scheme 7(b)) such that the intermediate **36** (i.e. **47**) fragments by cleavage of the X–O bond. It is certainly advantageous that the first step (i.e. the formation of the oxime ester **47**, Scheme 8(c)) is rate determining and that once the oxime ester **47** is formed, it rapidly fragments to give the desired product **48**.



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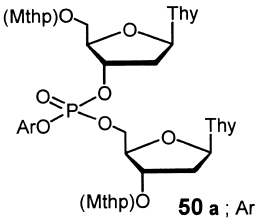
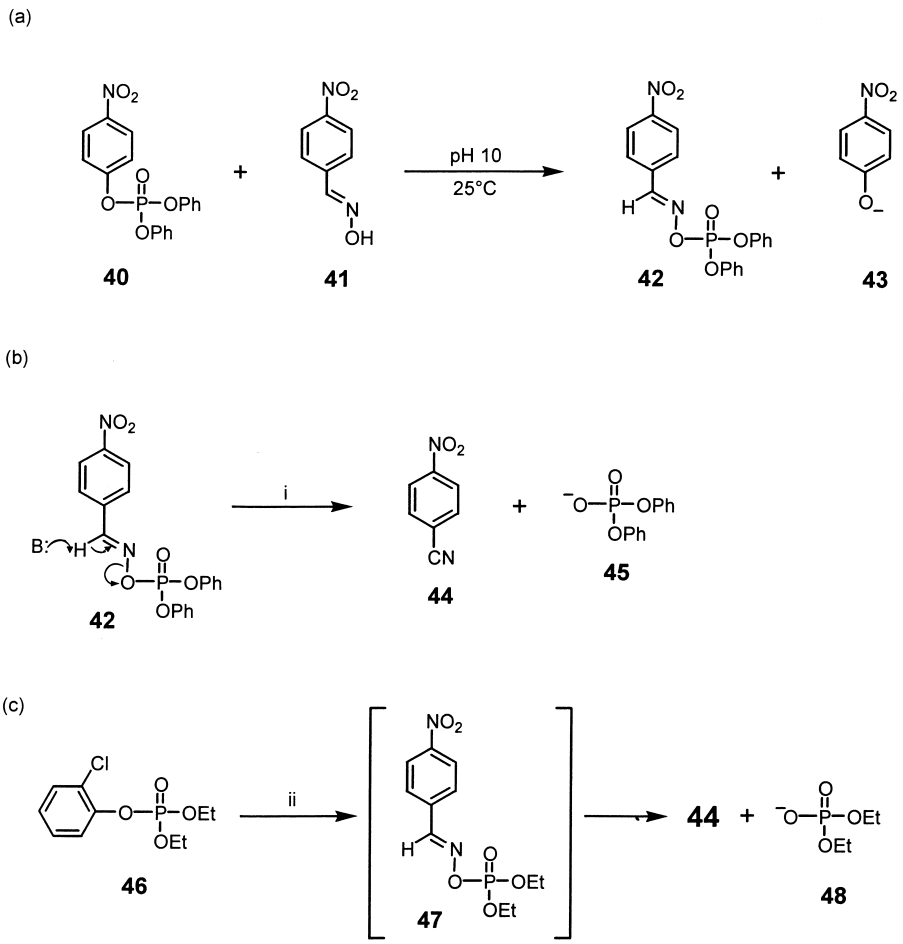


50 **a** ; Ar = 2-ClC_6H_4
b ; Ar = 4-ClC_6H_4
c ; Ar = Ph

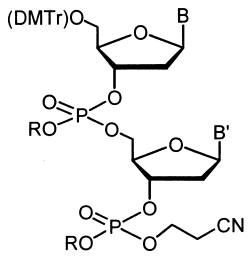
Thy = thymin-1-yl

The conjugate bases both of 4-nitrobenzaldoxime **41** and pyridine-2-carboxaldoxime **39** were found[38] to be very effective in the unblocking of aryl-protected internucleotide linkages. Thus, when the fully protected tetranucleoside triphosphate **31** (n=2, R^1=R^2=Mthp, Ar=2-ClC_6H_4) was allowed to react with a large excess each of 4-nitrobenzaldoxime **41** and TMG in dioxane–water (1:1 v/v) at 20°C for 16 h and the products were then treated with acid to remove the Mthp protecting groups, the d(TpTpTpT) **32**; n=2 obtained accounted for ca. 98% of the total nucleotide products. Virtually the same results were obtained when 4-nitrobenzaldoxime **41** was replaced by pyridine-2-carboxaldoxime **39**. As the starting material **31** (n=2, R^1=R^2=Mthp, Ar=2-ClC_6H_4) was almost certainly not 100% pure, it was not clear that any internucleotide cleavage whatsoever had occurred. Oxymate unblocking

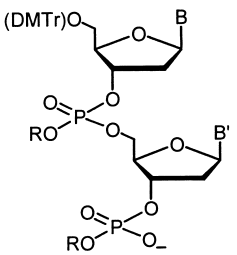


Scheme 8. *Reagents and conditions*: (i) TMG, CH_2Cl_2, 20°C; (ii) **41**, TMG, dioxane–water (1:1 v/v), 20°C.

was carried out in 50% aqueous dioxane in order to ensure that the reaction solution remained homogeneous throughout. It was later found that homogeneity could be maintained with much less or even no water present and dry acetonitrile is now often used as the solvent. In a later study,[43] it was found that the unblocking reaction proceeded more rapidly when 4-nitrobenzaldoxime **41** (pK_a 9.95) was replaced either by 2-nitrobenzaldoxime **49** (pK_a 10.28) or pyridine-2-carboxaldoxime **39** (pK_a 10.05). Studies with three fully-protected dinucleoside phosphates **50a–c** showed that, as expected, the rate of the unblocking reactions could be controlled by substitution of the aryl protecting group. Thus, with the conjugate base of 4-nitrobenzaldoxime **41**, the (2-chlorophenyl)-protected dimer **50a** was unblocked ca. 2.5 times faster than the (4-chlorophenyl)-protected dimer **50b** and ca. 25 times faster than the phenyl-protected dimer **50c**. No detectable (i.e. <0.1%) internucleotide cleavage could be detected when the (2-chlorophenyl)-protected dimer **50a** was unblocked with the conjugate base of 2-nitrobenzaldoxime **49**. This reaction was complete after 30 min at 20°C. Following these studies,[43] 2-chlorophenyl became established as the protecting group of choice in the phosphotriester approach to oligonucleotide synthesis in solution, and 2-nitrobenzaldoxime **49** and pyridine-2-carboxaldoxime **39** became established as the unblocking agents of choice.

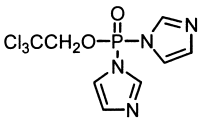


51 a ; R = CH_2CCl_3
b ; R = 4-ClC_6H_4



52 a ; R = CH_2CCl_3
b ; R = 4-ClC_6H_4

In the early and middle 1970s, there were a number of reports of the block synthesis of oligodeoxyribonucleotides in solution. Reese and his co-workers carried out the block synthesis of octathymidine heptaphosphate[33,44] **32**; n=6 and hexadecathymidine pentadecaphosphate[33] **32**; n=14 (Scheme 6 above). Catlin and Cramer[29] developed a synthetic strategy by which a number of di-, tri- and tetranucleotides were prepared from fully-protected dinucleotides of general structure **51a**. 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 **52a**. 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[45,46] followed a similar approach to that of Catlin and Cramer[29] except that the 4-chlorophenyl group was used to protect the internucleotide linkages (as in **51b**); these workers prepared some larger oligodeoxyribonucleotide sequences but most of their studies were carried out before the introduction of the oximate procedure for unblocking the internucleotide linkages. Nevertheless, Itakura and his co-workers[47] followed this approach, using mainly protected tri- rather than di-nucleotide building blocks and aqueous ammonia to unblock the (4-chlorophenyl)-protected internucleotide linkages; these workers thereby prepared a series of 29 oligodeoxyribonucleotides which each contained between 10 and 15 nucleotide residues. These chemically synthesised oligonucleotides were 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[48] completed in the late 1970s, these two insulin genes were successfully expressed.



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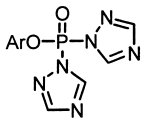
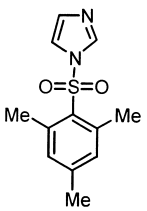


54 a ; Ar = 4-ClC_6H_4
b ; Ar = 2-ClC_6H_4

Considerable progress was made in the 1970s in the development of improved phosphorylation and coupling procedures. Catlin and Cramer[29] used 2,2,2-trichloroethyl phosphorodi-imidazolide **53** as a phosphorylating agent instead of the corresponding phosphorodichloridate **25**; **R**=CH_2CCl_3 and Narang and his co-workers[49] used 4-chlorophenyl phosphorodi-(1,2,4-triazolide) **54a** as a bifunctional phosphorylating agent in the preparation of (2-cyanoethyl) (4-chlorophenyl) phosphate esters of 5′-protected 2′-deoxynucleoside derivatives. Chattopadhyaya and Reese subsequently showed[50,51] that, if 2-chlorophenyl phosphorodi-(1,2,4-triazolide) **54b** 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 (e.g. in the conversion of **19** into **21**; **R**=2-ClC_6H_4; see Scheme 4(a) above). The reactions were relatively fast and following an aqueous triethylamine work-up, the resulting phosphodiesters could readily be isolated as their pure triethylammonium salts in very high yields.



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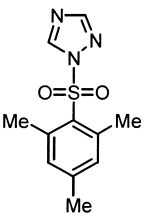

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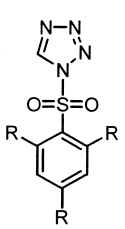
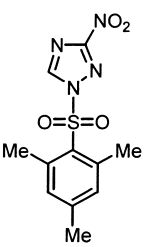
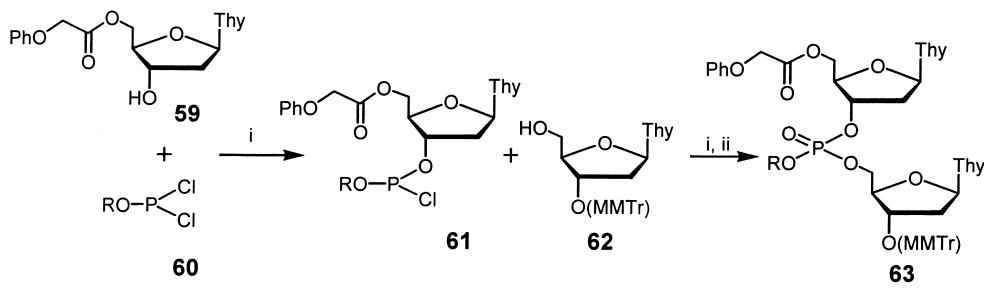


57 a ; R = H
b ; R = Me
c ; R = Me_2CH



58 (MSNT)



Scheme 9. *Reagents and conditions*: (i) 2,6-lutidine, THF, −78°C; (ii) 2,6-lutidine, I_2, THF, H_2O.

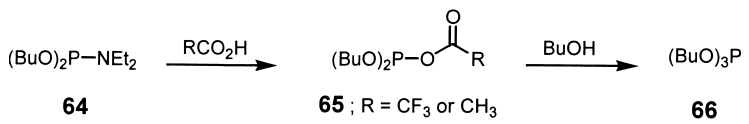
In 1973, Berlin et al. reported[52] that arenesulfonyl derivatives of imidazole (e.g. 1-(mesitylene-2-sulfonyl)-imidazole **55**) could be used as a coupling agent in oligonucleotide synthesis instead of the corresponding arenesulfonyl chlorides (e.g. MS-Cl **15a**). The coupling rates observed with such imidazole derivatives were much slower but no darkening of the reaction medium was observed. Narang and his co-workers[53] subsequently showed that arenesulfonyl derivatives of 1,2,4-1*H*-triazole (e.g. 1-(mesitylene-2-sulfonyl)-1,2,4-1*H*-triazole **56**) effected coupling almost as rapidly as the corresponding arenesulfonyl chloride but higher yields were obtained. These workers then showed[54] that arenesulfonyl derivatives of 1*H*-tetrazole **57a–c** were both faster and more efficient coupling agents than arenesulfonyl chlorides. However, the 1-arenesulfonyl-1*H*-tetrazoles **57a–c** were reported[54] to be relatively unstable. Reese and his co-workers[38,55] then reported that 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT) **58** had similar properties (i.e. its use led to rapid, relatively clean coupling reactions and high yields) to the corresponding tetrazole derivative **57b**, but that it was relatively stable. In the early 1980s, MSNT **58** became established as the coupling reagent of choice both in solution and in solid phase synthesis by the phosphotriester approach.

4. The phosphite triester approach

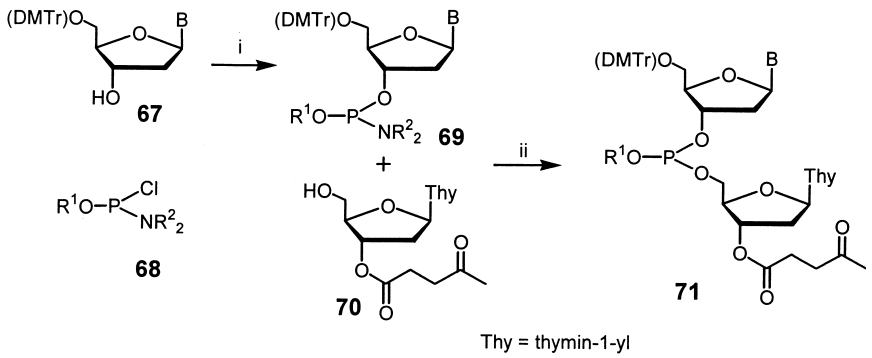
In 1976, Letsinger and Lunsford reported[56] a very important development in phosphorylation methodology. These workers found that P(III) were considerably more reactive than the corresponding P(V) acylating agents. Thus, 2-chlorophenyl phosphorodichloridite **60a** reacted rapidly with the 5′-protected thymidine derivative **59** at −78°C. The putative intermediate phosphorochloridite **61a** obtained was then allowed to react (Scheme 9) with the 3′-protected thymidine derivative **62** to give a fully protected dinucleoside phosphite. The latter product was not isolated but was treated with iodine and water in situ to give the fully protected dinucleoside phosphate **63a**. 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 **60** 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[56] were able to carry out a stepwise synthesis of d(TpTpTpT), based on 2,2,2-trichloroethyl phosphorochloridite **60b** as the phosphitylating agent.

5. The phosphoramidite approach

In 1981, the full importance of the P(III) approach to oligonucleotide synthesis became clear with the introduction by Beaucage and Caruthers[57] of monofunctional nucleoside phosphoramidites **69**. Following an observation by Nifant'ev et al.[58] in 1966 that amine hydrochlorides catalyze the alcoholysis of amides of phosphorous acid (e.g. $(Me_2N)_3P$), Evdakov et al.[59] reported in 1973 that dibutyl *N*,*N*-diethylphosphoramidite **64** reacted instantly with trifluoroacetic (or acetic) acid at 30°C to give (Scheme 10) dibutyl trifluoroacetyl (or acetyl) phosphite **65**. In the presence of an alcohol (e.g. butanol), the intermediate acyl phosphite **65** was converted into a trialkyl phosphite (e.g. tributyl phosphite **66**). In their original study,[57] Beaucage and Caruthers treated 5′-*O*-DMTr-thymidine **67** (B= thymin-1-yl) and 5′-*O*-DMTr-*N*-acyl derivatives of the other main 2′-deoxynucleosides with chloro(dimethylamino)-methoxyphosphine **68**; R^1=R^2=Me to give (Scheme 11) the corresponding protected nucleoside phosphoramidites **69**; R^1=R^2=Me. These products were isolated as colourless powders in high (90% or greater) yield. The reactions (Scheme 11) between these phosphoramidites **69**; R^1=R^2=Me, 1*H*-tetrazole (the acid catalyst) and 3′-*O*-levulinoylthymidine **70** in acetonitrile-d_3 were followed by ^{31}P NMR spectroscopy, which revealed fast reactions and very high yields of the expected fully-protected dinucleoside phosphites. These products were uncontaminated with the symmetrical dimers that were inevitably formed when intermediate protected nucleoside phosphorochloridites **61** (Scheme 9) were used in the coupling reactions. The iodine-promoted oxidation (Scheme 9, step ii) of the phosphite triesters **71** to the corresponding phosphotriesters in the solution phase was not described in Beaucage and Caruthers' original report.[57] However it was reported[57] that the protected nucleoside phosphoramidites **69** could be used successfully in the solid phase synthesis of dinucleoside phosphates. Adams et al.[60] and McBride and



Scheme 10.



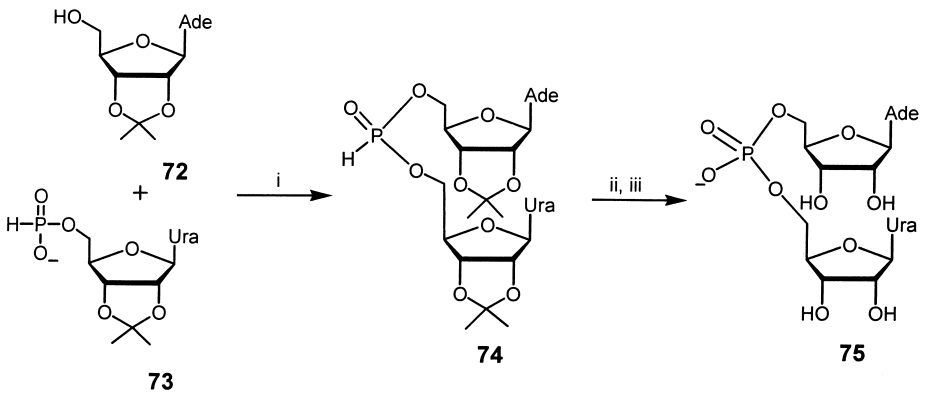
Scheme 11. *Reagents and conditions*: (i) **68**, $Pr^{i}_{2}NEt$, $CHCl_3$, rt; (ii) 1*H*-tetrazole, CH_3CN.

Caruthers[61] later showed that *N*,*N*-di-isopropylphosphoramidites **69**; R^2=Me_2CH were considerably more stable than *N*,*N*-dimethylphosphoramidites **69**; R^2=Me. In another important study, Köster and his co-workers[62] found that 2-cyanoethyl phosphoramidites **69**; R^1=CH_2-CH_2CN were generally more suitable building blocks than the corresponding methyl esters **69**; R^1=Me. 2-Cyanoethyl *N*,*N*-di-isopropylphosphoramidites **69**; 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.

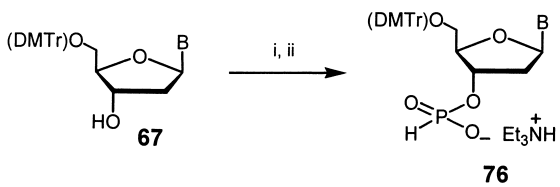
6. The H-phosphonate approach

Like the phosphotriester approach (see above), the H-phosphonate approach to oligonucleotide synthesis was first reported[63] from Todd's Cambridge laboratories in the 1950s. Following an initial study[64] involving a coupling reaction between 2′,3′-*O*-isopropylideneadenosine **72** and monobenzyl phosphite, 2′,3′-*O*-isopropylideneadenosine **72** and 2′,3′-*O*-isopropylideneuridine 5′-H-phosphonate **73** were coupled together (Scheme 12) in the presence of diphenyl phosphorochloridate to give[63] the protected H-phosphonate diester **74**. After chlorination with *N*-chlorosuccinimide, hydrolysis of the putative intermediate phosphorochloridate and removal of the isopropylidene protecting groups, the 5′→5′- dinucleoside phosphate **75** was obtained, albeit in modest yield. However, the true synthetic potential of the H-phosphonate approach was not realized for almost 30 years. In 1979, Hata and his co-workers[65] reported that, when TPS-Cl **15b** was used as the coupling reagent in the reaction between 5′-*O*-tritylthymidine 3′-H-phosphonate and 3′-*O*-acetylthymidine, an oxidative coupling reaction occurred and a poor yield of the desired protected thymidylyl-(3′→5′)-thymidine was obtained. In 1985, Garegg, Stawinski and their co-workers[66] confirmed Todd's original observation that diphenyl phosphorochloridate was an effective coupling reagent. The real significance of the H-phosphonate approach only became apparent in 1986 when Froehler and Matteucci[67,68] and Garegg et al.[69] applied it to solid phase oligonucleotide synthesis. Froehler and Matteucci recommended[67] that pivaloyl chloride should be used as the coupling reagent. Coupling reactions were then very fast and it was found[67,68] that really high molecular weight oligodeoxyribonucleotides could be prepared by solid phase H-phosphonate synthesis (see below). Following Ogilvie and Nemer's initiative,[70] H-phosphonate diesters were oxidized to the desired phosphodiester internucleotide linkages by treatment with iodine in the presence of aqueous base. A later study[71] revealed that adamantane-1-carbonyl chloride was perhaps a marginally more efficient coupling reagent than pivaloyl chloride.

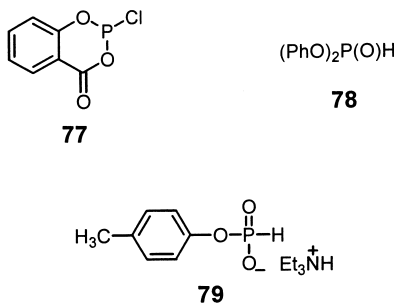
Protected 2′-deoxyribonucleoside 3′-H-phosphonates **76**, the building blocks required for the H-phosphonate approach to oligodeoxyribonucleotide synthesis, may easily be prepared[72] (Scheme 13) by treating the appropriate nucleoside derivatives **67** with the products of the reaction between phosphorus trichloride, 1,2,4-1*H*-triazole (3 mol equiv.) and triethylamine (3 mol equiv.), followed by a hydrolytic work up. This corresponds to the preparation of the protected 2′-deoxyribonucleoside 2-chlorophenyl phosphates[50] **21**; **R**=2-chlorophenyl, the building blocks used in the phosphotriester approach (see above), from the corresponding nucleoside derivative **19** and 2-chlorophenyl phosphorodi-(1,2,4-triazolide) **54b**. Thus, like the 2-chlorophenyl phosphates **21**; **R**=2-chlorophenyl, H-phosphonate building blocks **76** are very easy to prepare and then isolate as pure stable solids. Very high yields of H-phosphonate building blocks are also obtained when 1,2,4-1*H*-triazole is replaced by imidazole.[73] Other convenient and efficient methods for the preparation of H-phosphonate building blocks **76** include the use of (a) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one[74] **77**, (b) diphenyl phosphite[75] **78** and (c) triethylammonium *p*-tolyl phosphite[76] **79** in the presence of pivaloyl chloride.



Scheme 12. *Reagents and conditions*: (i) $(PhO)_2POCl$, 2,6-lutidine, MeCN; (ii) (a) *N*-chlorosuccinimide, 2,6-lutidine, MeCN, (b) $NaHSO_3$, H_2O; (iii) HCl, H_2O.



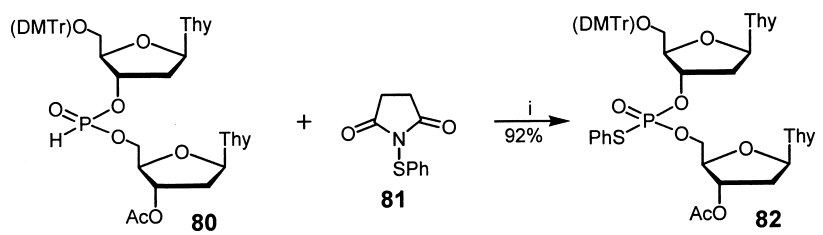
Scheme 13. *Reagents and conditions*: (i) products of the reaction between PCl_3, 1,2,4-1*H*-triazole and Et_3N, CH_2Cl_2; (ii) aqueous triethylammonium bicarbonate.



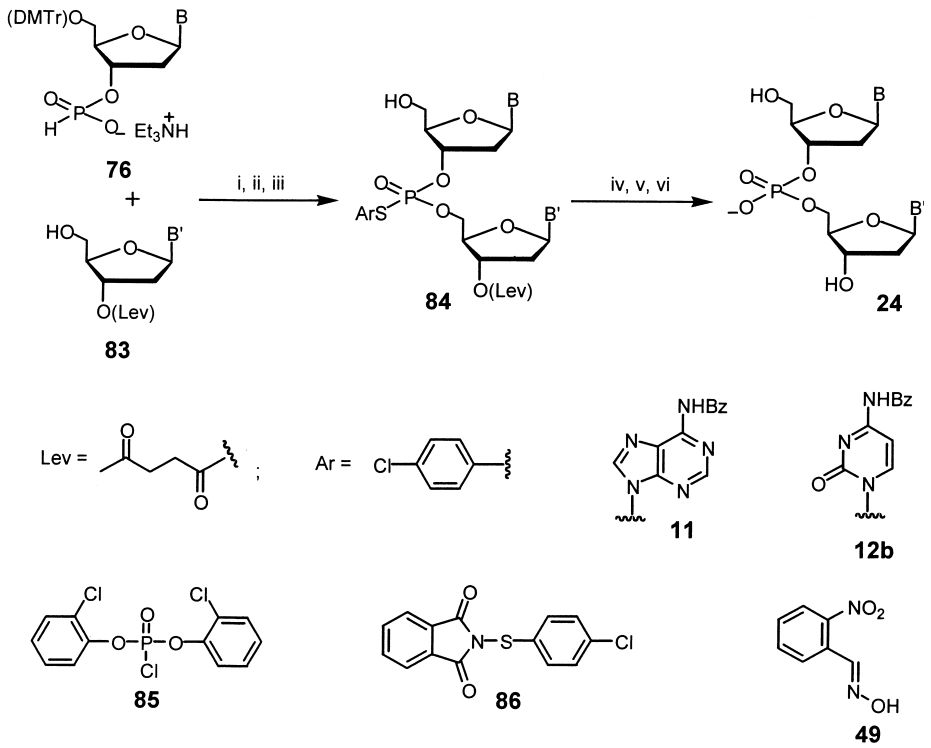
7. The modified H-phosphonate approach

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 and are therefore difficult to manipulate. Westheimer et al.[77] reported that dimethyl phosphite (H-phosphonate) undergoes alkaline hydrolysis at a rate more than four orders of magnitude faster than trimethyl phosphite and more than five orders of magnitude faster than trimethyl phosphate. However, van Boom and his co-workers reported[78] that the protected dithymidine H-phosphonate **80** reacted rapidly with *N*-(phenylsulfanyl)-succinimide **81** in the presence of base (Scheme 14) to give the corresponding much more robust *S*-phenyl phosphorothioate triester **82**. Previously, Hata and his co-workers[79] had developed a modification of the phosphotriester approach in which aryl (e.g. 2-chlorophenyl)-protected phosphotriester linkages (as in **50a**) were replaced by *S*-phenyl phosphothioate triester linkages (as in **82**).

It therefore seemed that if H-phosphonate coupling and the subsequent reaction with an arylsufanylimide (such as **81**) both proceeded in virtually quantitative yield and if the second step could be carried out in situ (i.e. without the isolation of the relatively sensitive intermediate H-phosphonate diester (e.g. **80**)), a new and potentially very powerful approach to the synthesis of oligonucleotides would be feasible. Fortunately, both reactions proved[80,81] to be rapid and virtually quantitative. This modified H-phosphonate approach is exemplified by the preparation of d(ApC). The H-phosphonate building block **76**; B=**11** and the 3′-protected nucleoside derivative **83**; B′=**12b** were first allowed to react together (Scheme 15) in the presence of bis(2-chlorophenyl) phosphorochloridate **85** at −40°C. After 10 min, the arylsufanylimide **86** was added and the products were allowed to warm to rt. After a further period of 15 min, the reaction mixture was quenched with water. Following the removal of the 5′-*O*-DMTr protecting group, the partially-protected dinucleoside phosphorothioate **84**;



Scheme 14. *Reagents and conditions*: (i) Pr^i_2NEt, CH_2Cl_2, rt, 5 min.



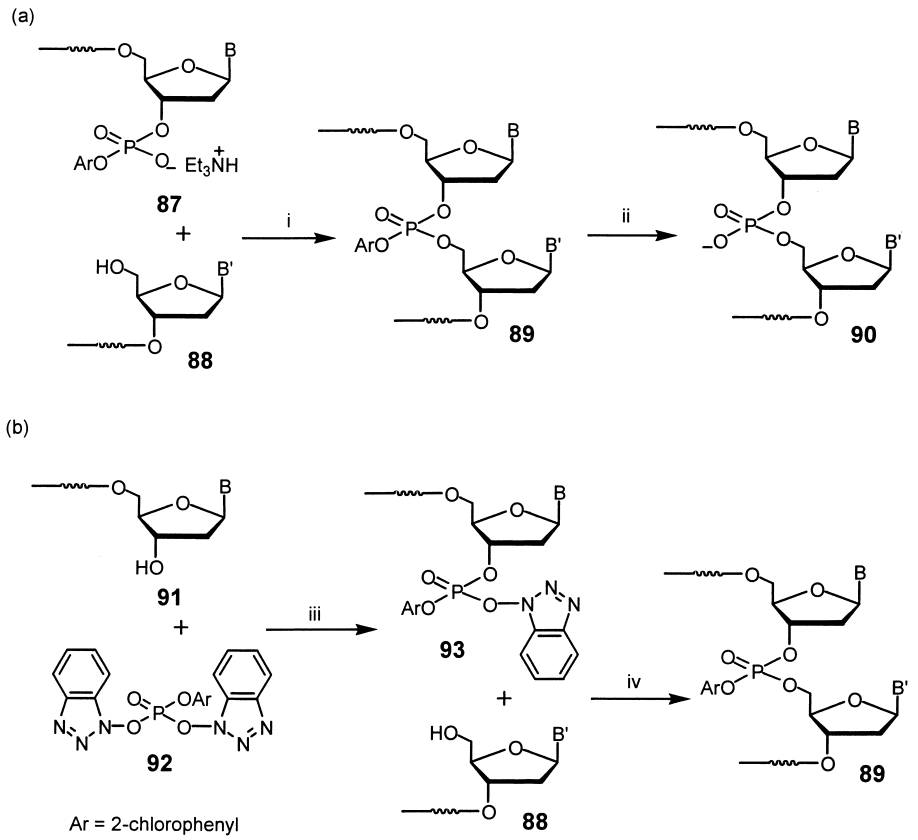
Scheme 15. *Reagents and conditions*: (i) **85**, C_5H_5N, CH_2Cl_2, −40°C, 10 min; (ii) (a) **86**, C_5H_5N, CH_2Cl_2, −40°C, 15 min, (b) C_5H_5N–H_2O (1:1 v/v), −40°C to rt; (iii) HCl, dioxane, CH_2Cl_2, −50°C, 5 min; (iv) Ac_2O, C_5H_5N, rt, 15 h; (v) **49**, TMG, MeCN, rt, 12 h; (vi) conc. aq. NH_3 (*d* 0.88), 50°C, 15 h.

B=**11**, B′=**12b** was isolated[80,81] in 98% yield. This material may then be coupled with another H-phosphonate monomer **76** to give a fully protected trimer.[80,81] This three step process (Scheme 15, steps i–iii) may be repeated until the desired sequence is assembled. Alternatively, block coupling reactions may be carried out. The partially protected dimer **84**; B=**11**, B′=**12b** was unblocked (Scheme 15, steps iv–vi) to give d(ApC) **24**; B=adenin-9-yl, B′=cytosin-1-yl. No further purification of the fully-unblocked dinucleoside phosphate was necessary.[80,81]

Now that the potential use of oligonucleotides and their phosphorothioate analogues as chemotherapeutic agents has become established, the development of a method or methods for their large scale synthesis is a matter of considerable importance and indeed of urgency. So far, the demand for the relatively large quantities of material required for clinical trials has been met by scaling-up solid phase synthetic methodology (see below). However, when really large (i.e. multikilogram to tonne) quantities of a specific sequence or sequences become required for chemotherapeutic purposes, it is likely that solution phase synthesis (quite possibly involving the above modified H-phosphonate approach) will become the method of choice. Although it is not proposed to include a discussion of the synthesis of oligonucleotide analogues in this Commentary, it is worth noting that the modified H-phosphonate approach has been used equally successfully in the synthesis[80,81] of oligonucleotide phosphorothioates which, at present, constitute the main class of oligonucleotide analogues intended for use as chemotherapeutic agents. For example Vitravene, a heneicosamer (21-mer) oligodeoxyribonucleotide phosphorothioate sequence that has been approved by the US Food and Drug Administration for the treatment of cytomegalovirus-induced retinitis[82] has been prepared[83] on a ca. 2 mM scale by means of the modified H-phosphonate approach in solution.

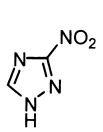
8. Later modifications to the phosphotriester approach

By the beginning of the 1980s, the most generally adopted approach to oligonucleotide synthesis in solution involved the coupling together, in the presence of MSNT[38] **58**, of a protected nucleoside or oligonucleotide 3′-(2-chlorophenyl) phosphate[50,51] **87** and a protected nucleoside or oligonucleotide with a free 5′-hydroxy function **88**. When the coupling process was complete, the internucleotide linkages were unblocked by oximate treatment. This is summarized in Scheme 16(a). van Boom and his co-workers[84] then recommended the use of a bifunctional phosphorylating agent **92**, which was prepared by treating 2-chlorophenyl phosphorodichloridate **25**; **R**=2-chlorophenyl with 1-hydroxybenzotriazole (2 mol equiv.) and pyridine (2 mol equiv.) in THF[84] or dioxane.[85] The protected nucleoside or oligonucleotide **91** with a free 3′-hydroxy function was treated[85] (Scheme 16(b)) with a small (10–15%) excess of reagent **92** and, after an appropriate interval of time, the protected nucleoside or oligonucleotide with a free 5′-hydroxy function **88** and 1-methylimidazole were added. Fully-protected dinucleoside phosphates were obtained in 50–77% yield and good yields were also obtained in block coupling reactions. The products **89** were again unblocked by the oximate procedure, indicated in Scheme 16(a) (step ii). Clearly, one advantage of the hydroxybenzotriazole phosphotriester approach is that a



Scheme 16. *Reagents and conditions*: (i) MSNT, **58**, C_5H_5N; (ii) **49** or **39** or **41**, TMG, dioxane (H_2O) and/or MeCN, followed by other appropriate unblocking steps; (iii) dioxane; (iv) 1-methylimidazole, dioxane, pyridine.

coupling reagent such as MSNT **58** is not required. However, the potential disadvantages of using a bifunctional phosphorylating agent are (a) that it is possible to obtain symmetrical 5′→5′ and 3′→3′-linked products and (b) that traces of moisture can lead to diminished yields. The hydroxybenzotriazole phosphotriester approach has also been used successfully in solid phase oligodeoxyribonucleotide synthesis (see below).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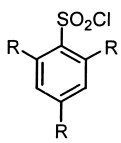
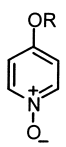
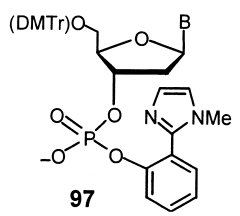

15 **a** ; R = Me
b ; R = Me_2CH


95


96 **a** ; R = Me
b ; R = Et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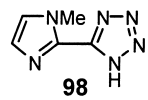
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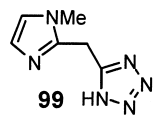
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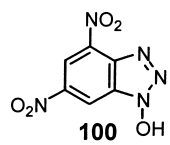
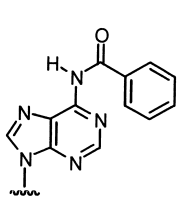

100

In the 1980s and the early 1990s, some other possible modifications to the phosphotriester approach were examined. These studies were mainly concerned with varying the coupling reagent and comparing a number of different nucleophilic catalysts. In this context, it is assumed that 3-nitro-1,2,4-1*H*-triazole (NT) **94** acts as a nucleophilic catalyst. Indeed, it appears[86] that the properties of a stoichiometric mixture of MS-Cl **15a** and NT **94** in pyridine solution are essentially the same as those of MSNT **58**, also in pyridine solution. Efimov and his co-workers recommended the use first of 1-methylimidazole[87] **95** and then a number of pyridine *N*-oxides,[88] including 4-methoxy- and 4-ethoxy-pyridine-1-oxides **96a**,**b**, as nucleophilic catalysts. With both MS-Cl **15a** and TPS-Cl **15b** as coupling

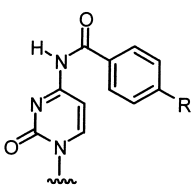
reagents, 1-methylimidazole is virtually as effective[86] a nucleophilic catalyst as NT **94**. 4-Ethoxypyridine-1-oxide **96b** behaves as a powerful nucleophilic catalyst in that it promotes rapid coupling reactions, but with MS-Cl **15a** as the coupling reagent, competitive sulfonation of the 5′-hydroxy function (e.g. the hydroxy function of **88** in Scheme 16(a)) occurs[86] to too great an extent. Froehler and Matteucci[89] investigated the possibility of incorporating the nucleophilic catalyst into the aryl protecting group. Thus, they found that, with MS-Cl **15a** as the coupling reagent, the phosphodiester **97** coupled with the 5′-hydroxy function of a thymidine residue attached to a solid support some 5–10 times more rapidly than the corresponding 4-chlorophenyl derivative. However, it is not clear that oximate-promoted unblocking of a 2-(1-methylimidazol-2-yl)phenyl-protected would proceed as readily as that of a (2-chlorophenyl)-protected internucleotide linkage. Nucleophilic catalysts **98** and **99**, in which a 1-methylimidazole is attached to a 1*H*-tetrazole residue, were also examined.[86] Unfortunately, neither of these catalysts was more effective in terms of coupling rates and yields than NT **94**. However, the dinitro-compound **100** was found[86,90] to be a very powerful and efficient nucleophilic catalyst. When it was used[86] in combination with TPS-Cl **15b** as the coupling agent, a rapid reaction ensured and the isolated yield of coupled product was almost 96%.

9. Base protecting groups and side-reactions in oligonucleotide synthesis

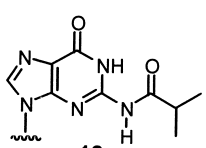
As indicated above, studies relating to the protection of adenine, cytosine and guanine residues as their *N*-acyl derivatives (as in **11**–**13**, respectively) were carried out by Khorana and his co-workers[8] in the course of their work on the synthesis of oligonucleotides by the phosphodiester approach. Thymine residues **101a** (and also uracil residues **101b** in oligoribonucleotide synthesis) were left unprotected in these studies. It seemed clear from 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 oligonucleotide synthesis by the phosphotriester approach had been elucidated.[91,92]



11



12 **a** ; R = OMe
b ; R = H



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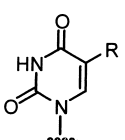
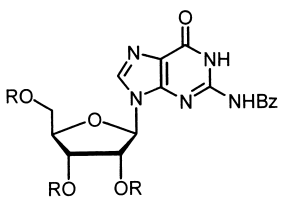
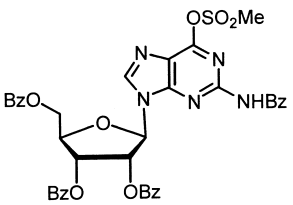


101 **a** ; R = Me
b ; R = H



102 **a** ; R = Bz
b ; R = Ac



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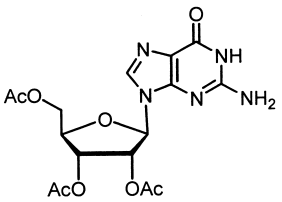

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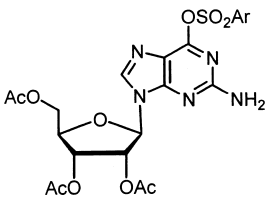
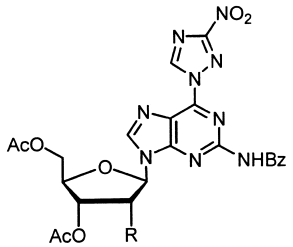
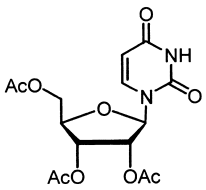


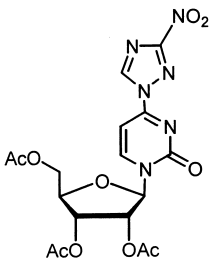
105 **a** ; Ar = 2,4,6-$Me_3C_6H_2$



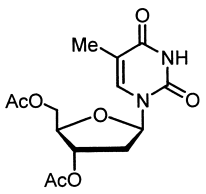
106 **a** ; R = OAc
b ; R = H



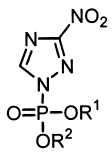
107



108



109



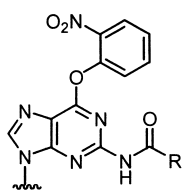
110 **a** ; $R^1 = R^2$ = Ph

The fact that 2-*N*-acylguanine and indeed free guanine residues in guanosine derivatives can undergo acylation on O-6 was first reported[93,94] in 1977. Thus, 2-*N*-benzoyl-2′,3′,5′-tri-*O*-benzoylguanosine **102a** was found[93] to react with methanesulfonyl chloride in the presence of triethylamine to give its 6-*O*-mesyl derivative **103**. Although guanine residues usually react with carboxylic acid chlorides and anhydrides on N-2, it was found that when 2′,3′,5′-tri-*O*-acetylguanosine **104** was treated with MS-Cl **15a** in pyridine solution, its 6-*O*-(mesitylene-2-sulfonyl) derivative **105a** was obtained.[94] Reactions between compound **104** and other arenesulfonyl chlorides (toluene-4-sulfonyl chloride and 4-bromobenzenesulfonyl chloride) also led[94] to the corresponding 6-*O*-(arenesulfonyl) derivatives. In the context of oligonucleotide synthesis, when 2-*N*-benzoyl-2′,3′,5′-tri-*O*-acetylguanosine **102b** was treated with MSNT **58** in pyridine solution, compound **106a** was obtained.[91,92] The reaction was relatively slow but was found to be catalysed by diphenyl phosphate. This was a significant observation as an excess of the phosphodiester component (e.g. **21**; **R**=2-ClC_6H_4, Scheme 4) is generally used[20,51,55] in the phosphotriester approach to oligonucleotide synthesis. When 2′,3′,5′-tri-*O*-acetyluridine **107** was treated with MSNT **58** in pyridine solution, it underwent a similar modification reaction to give[91,92] the corresponding 3-nitro-1,2,4-triazole derivative **108**. It is noteworthy that this reaction was somewhat faster than that involving the guanosine derivative **102b** and that it was also catalysed by diphenyl phosphate. However, the reaction between 3′,5′-di-*O*-acetylthymidine **109** and MSNT **58** in pyridine solution proceeded[91,92] very slowly indeed, both in the absence and presence of diphenyl phosphate. Finally, the reaction between 2-*N*-benzoyl-3′,5′-di-*O*-acetyl-2′-deoxyguanosine and MSNT **58** (to give **106b**) was found to proceed[91,92] at approximately the same rate as the corresponding reaction with the guanosine derivative **102b**. The results suggested that, as far as the phosphorotriester approach is concerned, it would probably be wise 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.

In the phosphotriester approach, activation of a phosphodiester (e.g. **21**; **R**=2-ClC_6H_4) by MSNT **58** probably leads to an intermediate of general structure **110** which, as very little concomitant sulfonation occurs in coupling reactions,[86] must be a much more powerful acylating species than MSNT itself. If this is the case, then 1-(diphenylphosphoryl)-3-nitro-1,2,4-triazole **110a** is likely to be the active intermediate in the above modification reactions involving diphenyl phosphate catalysis. This intermediate would be expected to phosphorylate (i.e. diphenylphosphorylate) guanine residues on O-6 and uracil residues on O-4. The observed modifications would then result from the nucleophilic displacement of diphenyl phosphate anion by the conjugate base of NT **94**. Hata and his co-workers[95] have demonstrated that 2-*N*-benzoyl- and 2-*N*-trityl-guanine residues undergo phosphorylation on O-6.

9.1. Protection of guanine residues

The occurrence of side-reactions was first observed during the synthesis[55] of the 3′-decaribonucleoside nonaphosphate (10-mer) sequence of yeast $tRNA^{Ala}$ (see below) by the phosphotriester approach in solution with MSNT **58** as the coupling reagent. As indicated above, with the help of model compounds, 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 by treatment with oximate ions[91,92] in the course of the unblocking of the internucleotide linkages, an attempt was made to prevent their occurrence by the introduction of appropriate protecting groups. It was decided to investigate the possibility of protecting guanine residues as their 2-*N*-acyl-6-*O*-aryl derivatives. It was clearly essential that the 6-O-aryl protecting groups should be able to withstand the relatively mild acidic and basic conditions that were likely to obtain in the course of the assembly of the desired nucleotide sequences. It was also hoped that it would be possible to regenerate the 1,6-lactam functions of the guanine residues under the oximate conditions used to unblock the 2-chlorophenyl protected internucleotide linkages.



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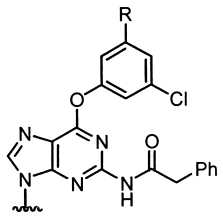
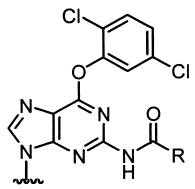
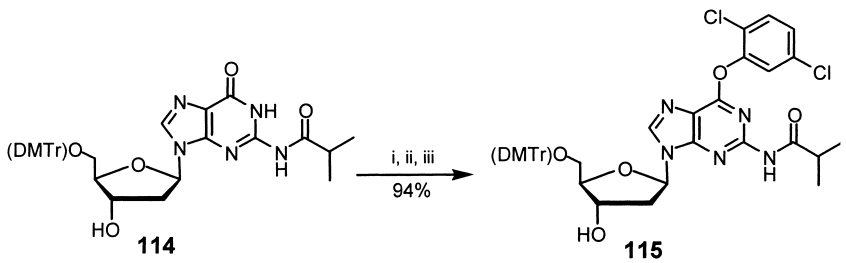


112 a ; R = H
b ; R = Cl

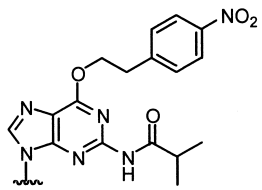


113 a ; R = CH_2Ph

In the initial studies,[96] 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 **111**; R=4-$Me_3CC_6H_4CH_2$. As well as avoiding side-reactions, protection on O-6 made the intermediates more lipophilic and consequently much easier to manipulate. This latter modification together with the protection of uracil residues on O-4 (see below) led[96] to an improved synthesis of the 3′-terminal 10-mer sequence of yeast $tRNA^{Ala}$; it also made it possible to carry out a successful synthesis of the 3′-terminal 19-mer sequence.[97] Later studies[98] in the deoxy-series also indicated that better yields were obtained when guanine residues were doubly protected (as in **111**; R=CH_2Ph), but it was not clear that it was advantageous to protect thymine residues on O-4 (see below). It was subsequently found[99,100] that if guanine residues were protected on O-6 by the somewhat more stable 3-chlorophenyl[99] and 3,5-dichlorophenyl[100] groups (as in **112a** and **112b**, respectively) unblocking (i.e. regeneration of the 1,6-lactam functions) with 2-nitrobenzaldoxime **49** and TMG still occurred quite rapidly in acetonitrile solution. More recently, the 2,5-dichlorophenyl group (as in **113**) has been used successfully both in the



Scheme 17. *Reagents and conditions*: (i) Me_3SiCl, C_5H_5N, rt, 30 min; (ii) MS-Cl **15a**, 1-methylpyrrolidine, C_5H_5N, 0°C, 15 min; (iii) 2,5-dichlorophenol, C_5H_5N, 0°C, 3 h.



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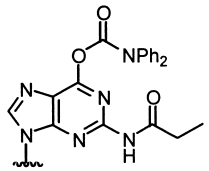


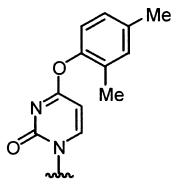
117

deoxyribose[81,101] and ribose[102] series. Although the more easily removable (by ammonolysis) phenylacetyl group[99] (as in **112** and **113a**) is to be preferred for the protection of the 2-amino functions of guanine residues, the isobutyryl group is still used in the deoxyribose series. 5′-*O*-DMTr-2-*N*-isobutyryl-2′-deoxyguanosine **114** is a commercially available building block that can easily be converted[101] in a one pot process (Scheme 17) into its 6-*O*-(2,5-dichlorophenyl) derivative **115** 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[103] and Pfleiderer and his co-workers[104] introduced the use of the 2-(4-nitrophenyl)ethyl group (as in **116**). This protecting group, which may be removed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), has been used[105] effectively in the preparation of 2′-deoxyguanosine-containing oligodeoxyribonucleotides. At almost the same time, Hata and his co-workers[106] introduced the use of the 6-*O*-diphenylcarbamoyl protecting group (as in **117**), which may be removed by ammonolysis in the final unblocking step. There have been several reports in the literature[107–110] dealing with the protection of guanine residues on O-6 in phosphoramidite-based solid phase oligodeoxyribonucleotide synthesis (see below). Apparently, however, modification of the guanine residues can be avoided if 1-methylimidazole is used[108] as the base rather than 4-(dimethylamino)pyridine (DMAP) in the acetic anhydride promoted coupling step, and if capping is carried out before P(III)→P(V) oxidation[107] in each synthetic cycle.

9.2. Protection of uracil and thymine residues

Following the observation that uracil residues were particularly susceptible to modification in the phosphotriester approach to oligoribonucleotide synthesis in solution with MSNT **58** as the coupling reagent,[91,92] uracil residues were effectively protected on O-4 with the 2,4-dimethylphenyl group[96] (as in **118**). Like 6-O-aryl protected guanine residues (e.g. as in **112a**,**b**), the O-4 protected uracil residues (as in **118**) are rapidly unblocked[96] with oximate ions under the conditions used to unblock aryl-protected internucleotide linkages.[43] As indicated above, the combination of O-6 protected guanine residues and O-4 protected uracil residues led[96] 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[97] and 37-mer[111] sequences (see below). Thymine residues may conveniently be protected with the 4-O-phenyl group[100] (as in **119**), which is both easy to introduce and then to remove by oximate treatment. However, as indicated above, it is by no means clear that it is beneficial to protect thymine residues either in phosphotriester or phosphoramidite-based oligonucleotide synthesis. Welch and Chattopadhyaya[112] and Hata and his co-workers[113] have shown that uracil residues may conveniently be protected by acylation on N-3 (e.g. as in **120a**: R^2=Ph or 4-$MeOC_6H_4$). Thymine residues may also be protected by acylation on N-3 (as in **120b**; R^2=4-$MeOC_6H_4$[101]). Unlike 4-O-aryl, 3-N-acyl protecting groups have the advantage that they may be removed from uracil and thymine residues by ammonolysis[101,112] at the same time as the N-acyl protecting groups from other base residues. In this way, an extra unblocking step may be avoided.



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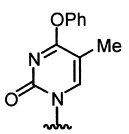

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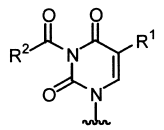


120 a ; R^1 = H
b ; R^1 = Me

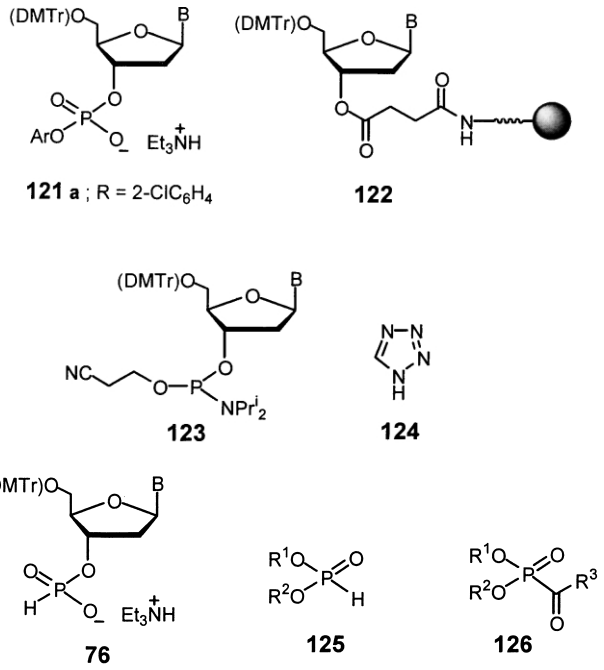
10. Oligonucleotide synthesis on a solid support

It is not proposed to review solid phase oligonucleotide synthesis in any detail in this Commentary as there are several authoritative reviews already in the literature.[114–116] However, its considerable importance is beyond any doubt. Indeed, in the past 20 years, 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. Following Letsinger and Mahadevan's original report[21] 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[20] with aryl (e.g. 2-chlorophenyl) protecting groups for the internucleotide linkages and effective coupling reagents, such as MSNT **58**, had become established. This approach and the modification[84] involving the use of the bifunctional bis-(1-hydroxybenzotriazolide) reagent **92** (see above) were successfully applied to solid phase oligonucleotide synthesis and, at the beginning of the 1980s, were the method of choice. The success of these methods depended to a significant extent on oximate unblocking (see above) of the internucleotide linkages. Several reviews[117–119] on solid phase phosphotriester synthesis were subsequently published.

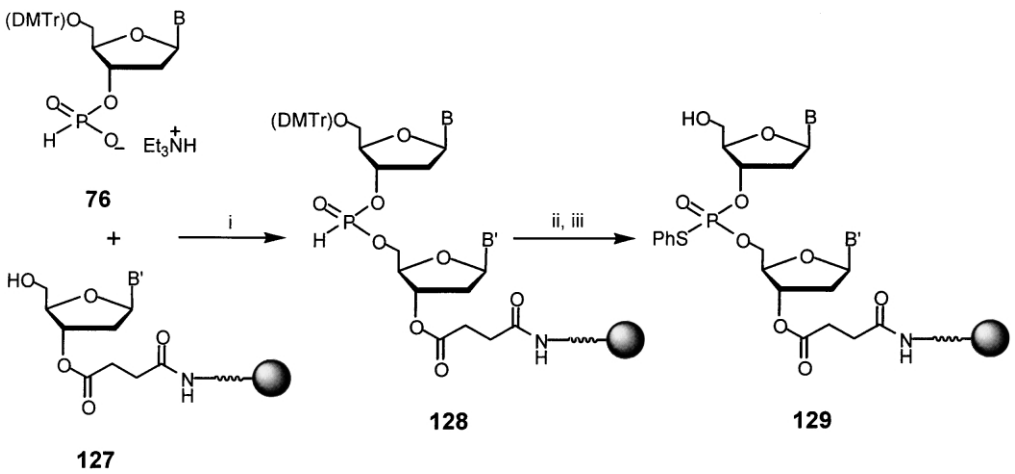
Solid phase synthesis generally involves the addition of one nucleotide residue at a time (e.g. **121a** in the phosphotriester approach) to an immobilized protected nucleoside or oligonucleotide. Controlled-pore glass[120] and highly cross-linked polystyrene,[121] which are both fairly robust materials, have emerged as the solid supports of choice. Controlled-pore glass is usually functionalised 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 **122**). No purification steps are carried out until the fully-assembled sequence is released from the solid support. For at least two reasons, it is crucially important 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 20-mer (involving 19 coupling steps), the calculated overall yields are 83, 56 and 38% 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 of the target sequence 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 is facilitated by 'capping' truncated material after each coupling step and by purifying the crude 'undetritylated' products by reversed phase chromatography, clearly the higher the overall yield of untruncated material the better.

Phosphotriester solid phase synthesis[117–119] was largely superseded by phosphoramidite-based solid phase synthesis[122] by the mid-1980s. It is possible to achieve average coupling yields of 98% or greater with phosphoramidite building blocks of general structure **123** and with 1*H*-tetrazole **124** as the activating agent. Furthermore, the coupling reaction is very rapid indeed and is usually complete within ca. 1 min in the deoxy-series, and irreversible side-reactions can generally be avoided. Although the later modifications to the phosphotriester approach (see above) have led both to increased coupling rates and to 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 20-mers, it is very doubtful if, as it stands, it could be used successfully in the synthesis of say, 50-mers. On the other hand, the synthesis of oligodeoxyribonucleotide 50-mers and, if care is taken, even higher molecular weight DNA sequences is routine.[114] It is perhaps relevant to add that the ready commercial



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 particular 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 probably the best and certainly the most popular of these instruments were designed particularly to accommodate phosphoramidite building blocks and reagents may well be another reason why, in the past 15 years or so, only a limited research effort has been put into the development of alternative methodologies.

Most of the more recent research into alternative synthetic methodologies has been directed towards the development of solid phase H-phosphonate synthesis[67–69] (see above). Like the corresponding building blocks **121a** used in solid phase phosphotriester synthesis, monomeric H-phosphonate building blocks **76** are both easy to prepare[72] and stable. Coupling reactions involving H-phosphonates appear to be at least as fast[72] as those involving phosphoramidites. As indicated above, H-phosphonate building blocks (e.g. **76**) are activated by acylating agents such as pivaloyl chloride.[67] Both overactivation and prolonged coupling times must be avoided in order to prevent the occurrence of undesirable side-reactions.[72,123] Thus particular attention must be paid to the coupling protocol. Nevertheless, the H-phosphonate approach has been used successfully in the solid phase synthesis of high molecular weight oligonucleotides.[67,68] 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 very much more widely. Perhaps an important reason for this is that, in the strategy normally followed, the individual H-phosphonate diester linkages (as in **125**) 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



Scheme 18. *Reagents and conditions*: (i) $(PhO)_2POCl$ or $Me_3C{\cdot}COCl$, C_5H_5N or C_5H_5N–MeCN; (ii) **81**, C_5H_5N or C_5H_5N–MeCN; (iii) Cl_2CHCO_2H, CH_2Cl_2.

problems.[72,123] First, H-phosphonate diesters (as in **125**) are susceptible to acylation on phosphorus (as in **126**). If this happens, the internucleotide H-phosphonate diesters affected will not be converted into 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 diester are converted to phosphodiester linkages in the oxidation step. If oxidation is not complete, the remaining H-phosphonate diester linkages will be cleaved during the ammonolytic step required to remove the N-acyl protecting groups. This would inevitably lead to diminished yields of the target sequences.

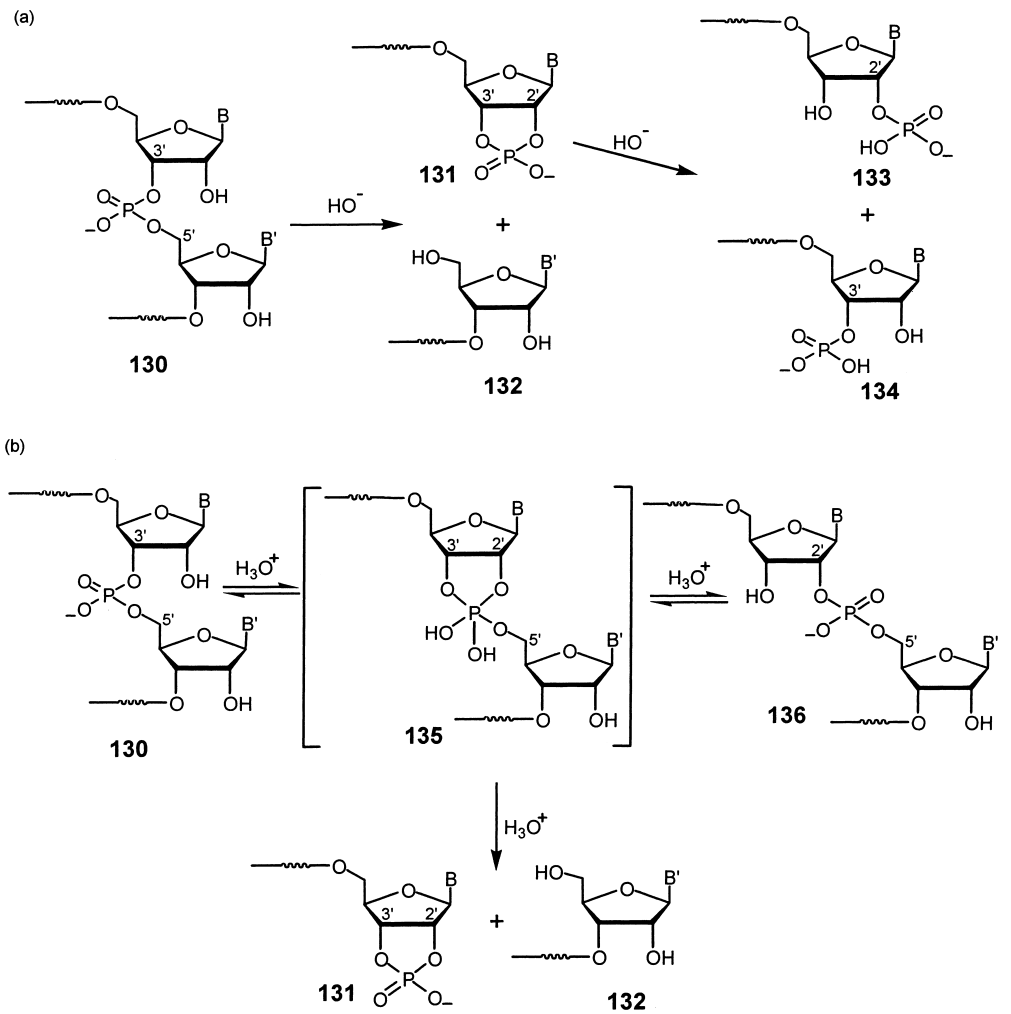
It is possible that solid phase H-phosphonate synthesis could be improved by oxidizing H-phosphonate diester to phosphodiester linkages after each coupling step or by adopting the modified H-phosphonate approach[81] (as in Scheme 15 above). One feasible way of implementing the latter approach to solid phase synthesis is illustrated in Scheme 18. Thus, after coupling the appropriate H-phosphonate building block **76** with the immobilized 3′-terminal nucleoside derivative **127**, the resulting H-phosphonate diester **128** is treated with, for example, *N*-(phenylsulfanyl)succinimide **81**. 'Detritylation' of the product would give the *S*-phenyl phosphorothioate triester **129**, which is now ready for the next coupling step. When the desired oligonucleotide sequence has been fully assembled, it is necessary[81] to carry out an oximate unblocking step (as in Scheme 15, step v) before ammonolysis. If the coupling reagent (e.g. diphenyl phosphorochloridate or pivaloyl chloride) and sulfur transfer reagent (e.g. *N*-(phenylsulfanyl)succinimide **81**) are carefully chosen, it might be possible[83] to add them both at the same time and thereby minimize the lifetime of the potentially sensitive internucleoside H-phosphonate diester linkages. This approach has the advantage that it is, in principle, suitable for solid phase synthesis involving the use either of monomeric or block (e.g. dimer, trimer or tetramer) H-phosphonate building blocks.

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

The synthesis of oligo- and poly-ribonucleotides has been a long-term interest of mine. Early studies[124] in the 1960s involving the phosphodiester approach convinced us that, unless we completely changed our approach, we would be unable to synthesize oligonucleotides larger than dimers or trimers. This led us to investigate the potential of the phosphotriester approach, first of all in the less complex deoxy-series (see above). We then readdressed the problem of oligoribonucleotide synthesis.

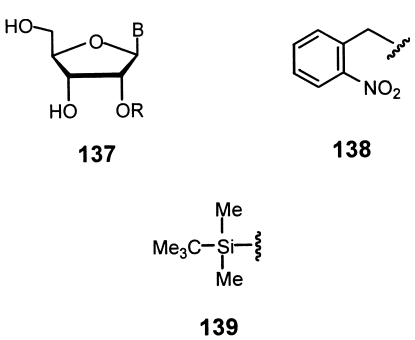
It was clear at the outset that the choice of a protecting group for the 2′-hydroxy functions of ribonucleoside building blocks was the most crucial decision to be made in the development of an overall strategy for polyribonucleotide synthesis. This protecting group has to remain intact throughout the assembly of a target RNA sequence and must then be readily removable in the final unblocking step under conditions under which RNA is completely stable. These are very demanding requirements as, under relatively mild conditions, RNA undergoes base-catalyzed cleavage[125] of the internucleotide linkages and acid-catalyzed cleavage and migration[126] of the internucleotide linkages. The course of base-catalyzed hydrolysis is indicated in Scheme 19(a): two fragments **131** and **132** are formed initially and the 2′,3′-cyclic phosphate **131** then undergoes further hydrolysis to give the corresponding 2′ and 3′-phosphates **133** and **134**. It is believed that acid-catalyzed hydrolysis proceeds via a phosphorane intermediate **135** (Scheme 19(b)). While cleavage of the P–O(2′) bond leads to the regeneration of the starting material **130**, cleavage of the P–O(3′) bond leads to the isomeric product **136** with a 2′→5′-internucleotide linkage. Finally, cleavage of the P–O(5′) bond leads to the same products **131** and **132** that are obtained by base-catalyzed hydrolysis (Scheme 19(a)). The cyclic phosphate fragment **131** is again converted into a mixture of the corresponding 2′- and 3′-phosphates **133** and **134**.

When choosing or designing a protecting group (R) for the 2′-hydroxy functions of a ribonucleoside building block **137**, it is very important indeed to be fully aware of the 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 cleavage of the internucleotide linkages completely to be

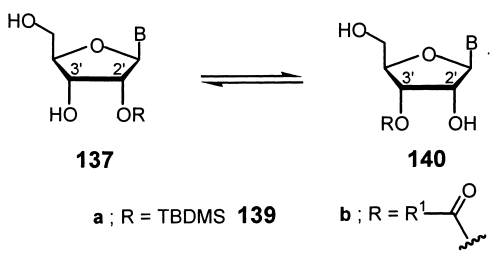


Scheme 19.

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 essential that it should be removable under very mild conditions of acidic hydrolysis indeed as, in practice, it is virtually impossible to free even relatively low molecular weight oligoribonucleotides from contaminating isomeric impurities containing one or more 2′→5′-internucleotide linkages. Finally, it should be born in mind that, after the 2′-protecting group has been removed, the free RNA sequence obtained will be highly susceptible to endonuclease-promoted digestion and must therefore be handled under sterile conditions.



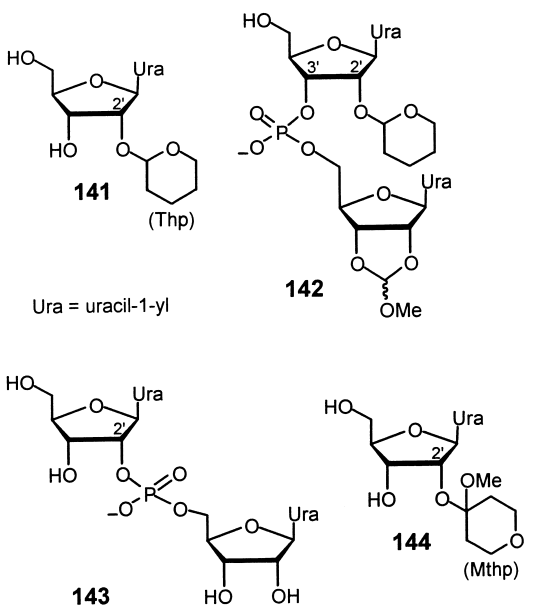
It is not now proposed to review 2′-protection in oligo- and poly-ribonucleotide synthesis in any detail as this subject has recently been reviewed elsewhere.[127] For the reason discussed above, it would in principle appear desirable to protect the 2′-hydroxy functions with a group that is removable at neutral pH, providing that it fully meets the general criteria of stability and ease of removability indicated above. Of the 2′-protecting groups that have been used so far, those removable under more or less neutral conditions include benzyl[128] (as in **137**: R=$PhCH_2$), 2-nitrobenzyl[129] (as in **137**; R=**138**) and *tert*-butyldimethylsilyl[130] (TBDMS, as in **137**; R=**139**). The 2′-*O*-benzyl protecting group, which is removable[128] by catalytic hydrogenolysis, suffers from a serious disadvantage in that



Scheme 20.

it is uncertain whether it can be removed completely even from an RNA sequence of moderate size. Furthermore, it is also possible[131] that concomitant hydrogenation of the 5,6-double bonds of cytosine and uracil residues will occur. The 2-nitrobenzyl group, which is removable photolytically above 280 nm, has been used to a much greater extent. However, it has been reported[132] that photolytic cleavage proceeds more smoothly under acidic conditions (i.e. at pH 3.5) and it is therefore questionable whether it may be considered as a protecting group that is readily removable under neutral conditions. In any case, it is difficult to ensure[132] that such a photolytic process will go to completion. The TBDMS protecting group 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[133] (as in the conversion of **137a** into **140a** and vice versa; Scheme 20). For this reason, 2′-*O*-TBDMS ribonucleoside derivatives with free 3′-hydroxy functions (e.g. **137a**) must be handled with care. The TBDMS group is normally removed by treatment with tetra-*n*-butylammonium fluoride in THF solution[134] or with triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$).[135,136] The former reagent is essentially basic and the latter reagent is more or less neutral. However, there is no evidence that either reagent promotes the cleavage or the migration of internucleotide linkages of unprotected RNA.

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, very little use has in fact been made of them. 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[137] (as in the conversion of **137b** into **140b** and vice versa; Scheme 20). However, unlike mixtures of 2′- and 3′-*O*-TBDMS ribonucleoside derivatives, mixtures of isomeric 2′ and 3′-*O*-acyl ribonucleoside derivatives (e.g. **137b** and **140b**: R^1=Me) are not generally easily separable by chromatography. Despite the potential problems of cleavage and migration of the internucleotide linkages during the unblocking of 2′-protected RNA sequences (see above and Scheme 19(b)), we have always favoured the use of acid-labile groups for the protection of 2′-hydroxy functions. One of the main reasons for this is that the nucleoside aglycone residues and the internucleotide linkages (both in solution phase and solid phase synthesis) are virtually always protected with base-labile groups. Due to the general lability of RNA to acids, bases and, if present, contaminating hydrolytic enzymes, the 2′-protecting groups must always be removed in the final unblocking step, and it is especially important that partial 2′-deprotection should not occur during the initial unblocking step or steps. Clearly, if the 2′-hydroxy functions are to be protected with acid-labile groups, then particular care must be taken to ensure that the final unblocking step is carried out under the mildest possible conditions of acidic hydrolysis.

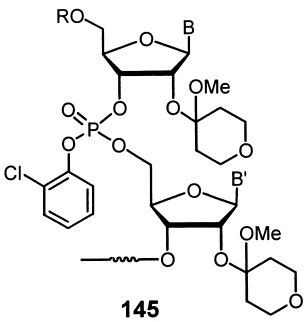


The first acid-labile 2′-protecting group examined[124,138,139] was the tetrahydropyran-2-yl (Thp) group (as in **141**). The Thp group is part of an acetal system. The half-time ($t_{1/2}$) for the deprotection of 2′-*O*-Thp-uridine **141** in 0.01 M hydrochloric acid (pH 2.0) was found[126] to be 54 min at 24°C. However, under the same conditions, $t_{1/2}$ for the removal of the Thp group from the partially-protected uridylyl-(3′→5′)-uridine derivative **142**, which would seem to be a better model for a 2′-protected RNA sequence, was found[126] to be only 29 min. Virtually complete (i.e. 99.9%) 2′-unblocking requires ca. $10\times t_{1/2}$, that is, just under 5 h. Cleavage and migration (to give uridylyl-(2′→5′)-uridine **143**) of the internucleotide linkage were found[126] 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 protected intermediates. For this reason, the Thp group was soon abandoned in favour of the achiral 4-methoxytetrahydropyran-4-yl (Mthp) protecting group[140,141] (as in **144**). The Mthp group has an additional advantage in that it is over twice as labile as the Thp group in 0.01 M hydrochloric acid at rt.[140] It therefore appeared at the outset that the Mthp group met the criteria required for a 2′-protecting group in oligoribonucleotide synthesis.

11.1. Solution phase synthesis of RNA sequences

The 2′-*O*-Mthp protecting group was used successfully in the synthesis of the 3′-terminal decamer (10-mer: r[UCGUCCACCA]),[55,111] nonadecamer (19-mer: r[AUUCCGGACUCGUCCACCA])[97,111] and heptatriacontamer (37-mer: r[GGAGAGGUCUCCGGTΨC-GAUUCCGGACUCGUCCACCA])[111] sequences of yeast $tRNA^{Ala}$ by the phosphotriester approach in solution. The internucleotide linkages were protected with 2-chlorophenyl groups (as in **145**) and MSNT **58** was used as the coupling reagent. It was necessary to block the 5′-hydroxy functions with temporary protecting groups (R in **145**) that were removable under very mild basic conditions so that their removal did not affect the growing numbers of base-sensitive (2-chlorophenyl)-protected internucleotide linkages. The 2-(dibromomethyl)benzoyl (Dbmb)[142] **147** and 2-[(isopropylthio)methoxymethyl]benzoyl[99] **148** protecting groups were developed for this purpose. The adenine, cytosine, guanine and uracil residues were protected as in **149**, **150**, **112a** and **118**, respectively. The 5-methyluridine

(T) and pseudouridine (Ψ) residues in the heptatriacontamer sequence were protected[99] with 4-*O*-phenyl and 1-*N*-(4-bromobenzenesulfonyl) groups, respectively. The synthesis of the 37-mer involved[111] the MSNT-promoted coupling of an 18-mer 3′-phosphodiester block and a 19-mer block with a free 5′-OH function. 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 by treatment with 0.01 M hydrochloric acid at rt to give[111] the fully-unblocked target sequences. This final unblocking step proceeded successfully but later studies (see below) revealed that certain other RNA sequences undergo substantial cleavage and migration of the internucleotide linkages under these conditions (i.e. at pH 2.0 and rt). This has led to the conclusion (see below) that unblocking should generally be carried out at a much higher pH (i.e. at pH 3.25 or above).



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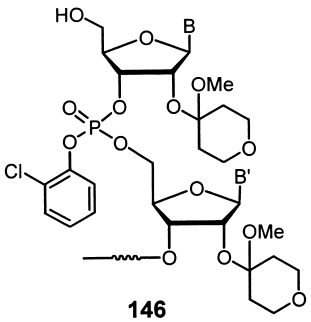

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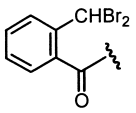
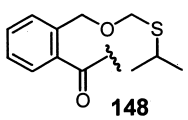


147 (Dbmb)



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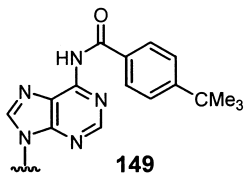

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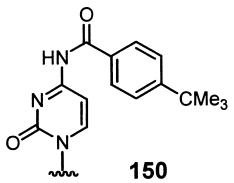

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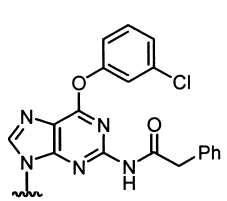
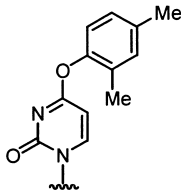


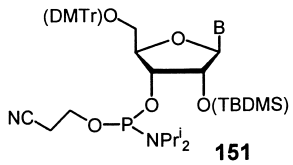
112a



118

11.2. Solid phase synthesis of RNA sequences

The TBDMS protecting group **139** has been used very widely[143] in the solid phase synthesis of RNA sequences. Despite the ease of migration of the TBDMS group, relatively pure monomeric phosphoramidites of general structure **151**, which are contaminated with at most very small quantities of isomeric 3′-*O*-TBDMS-2′-phosphoramidites, are available commercially. Careful quality control is necessary in their manufacture if significant isomeric contamination is to be avoided. The general protocol of RNA synthesis is very similar to that of solid phase DNA synthesis.[116] Again, both controlled-pore glass and polystyrene are used as solid supports. Due no doubt to the bulkiness of the protected 2′-hydroxy functions, coupling times are considerably longer than in DNA synthesis and activators other than 1*H*-tetrazole **124** (e.g. 5-ethylthio-1*H*-tetrazole **152**)[144] 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 relatively labile acyl groups (e.g. as in **153**–**155**, respectively[145]) that are removable by treatment with ammonia or methylamine under very mild conditions. As indicated above, in the final unblocking step, the 2′-*O*-TBDMS protecting groups are best removed by treatment with triethylamine trihydrofluoride.[135,136] The main protocols for solid phase RNA synthesis, based on 2′-*O*-TBDMS-3′-phosphoramidites **151**, have recently been reviewed.[143]



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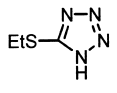

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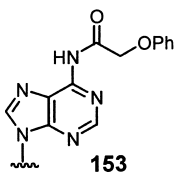

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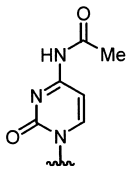

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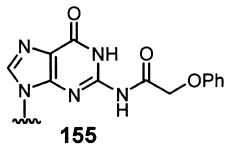
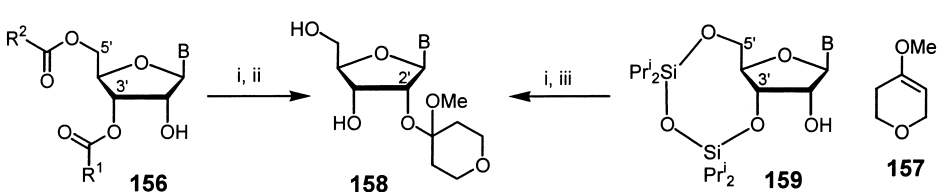


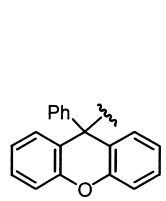
155

Acid-labile (e.g. acetal) protecting groups are completely stable under the basic conditions under which *N*-acyl protecting groups are removed from adenine, cytosine and guanine residues (and, in solid phase synthesis, also under the conditions under which the 2-cyanoethyl protecting groups are removed from the internucleotide linkages and the fully-assembled oligonucleotide sequences are released from the solid supports); they have additional advantages in

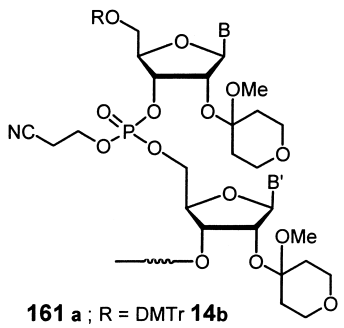


156 **158** **159** **157**

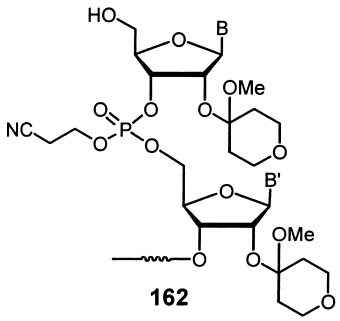
Scheme 21. *Reagents*: (i) **157**, H^+; (ii) NH_3, MeOH; (iii) Et_4NF, MeCN.



160 (Px)



161 a ; R = DMTr **14b**
b ; R = Px **160**

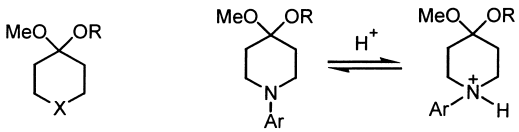


162

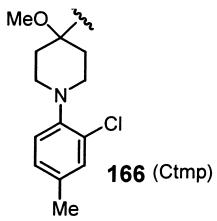
that they may easily be introduced regiospecifically on O-2′ and that, once they are in place, they cannot migrate. 2′-*O*-Mthp-Ribonucleoside derivatives **158** were originally prepared[141] in two steps (Scheme 21) from the corresponding 3′,5′-di-*O*-acyl-ribonucleoside derivatives[146] **156**. However, subsequently[99] they have been more conveniently prepared, also in two steps, from the corresponding 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl) derivatives[147] **159**.

Substituted trityl (e.g. DMTr **14b** and 9-phenylxanthen-9-yl(Px)[148] **160**) groups have been used as temporary protecting groups for the 5′-hydroxy functions in nearly all approaches to the solid phase synthesis of oligonucleotides. 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[114] and the efficiency of the coupling steps thereby monitored. Clearly, a potential problem arises if acid-labile groups are used to protect both the 2′ and 5′-hydroxy functions (as in **161**). Indeed model studies have revealed[149] that the Mthp protecting group cannot withstand the acidic conditions required to remove a 5′-*O*-DMTr or a 5′-*O*-Px group (as in the conversion of **161a** or **161b** into **162**). If the DMTr or Px group is used for the temporary protection of the 5′-hydroxy functions and an acid-labile protecting group is also used for the 2′-hydroxy functions, the 2′-protecting group should be completely stable under the relatively drastic detritylation conditions and then, in the final unblocking step, be susceptible to acidic hydrolysis under mild conditions so that cleavage and migration of the internucleotide linkages in the target RNA sequences are avoided.

It had earlier been found[140,150] that the rate of acid-catalyzed hydrolysis of acetal systems of general structure **163** was 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 had a pK_a of ca. 2, it would be largely protonated (as in **165**) in the presence of an excess of trichloroacetic acid (pK_a 0.66) during the detritylation steps in solid phase synthesis and largely unprotonated (as in **164**) during the final unblocking step, especially if it is carried out at pH 3.25 or above. 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[151] **166** was the first such 1-arylpiperidin-4-one acetal protecting group that was found to have the desired properties: the ratio of its rates of hydrolysis at pH 0.5 and 2.5 was found to be only 1.55. Furthermore it was found to undergo hydrolysis ca. 40 times *more slowly* than the Mthp group at pH 1.0 and ca. 1.5 times *more rapidly* than the Mthp group at pH 3.0.



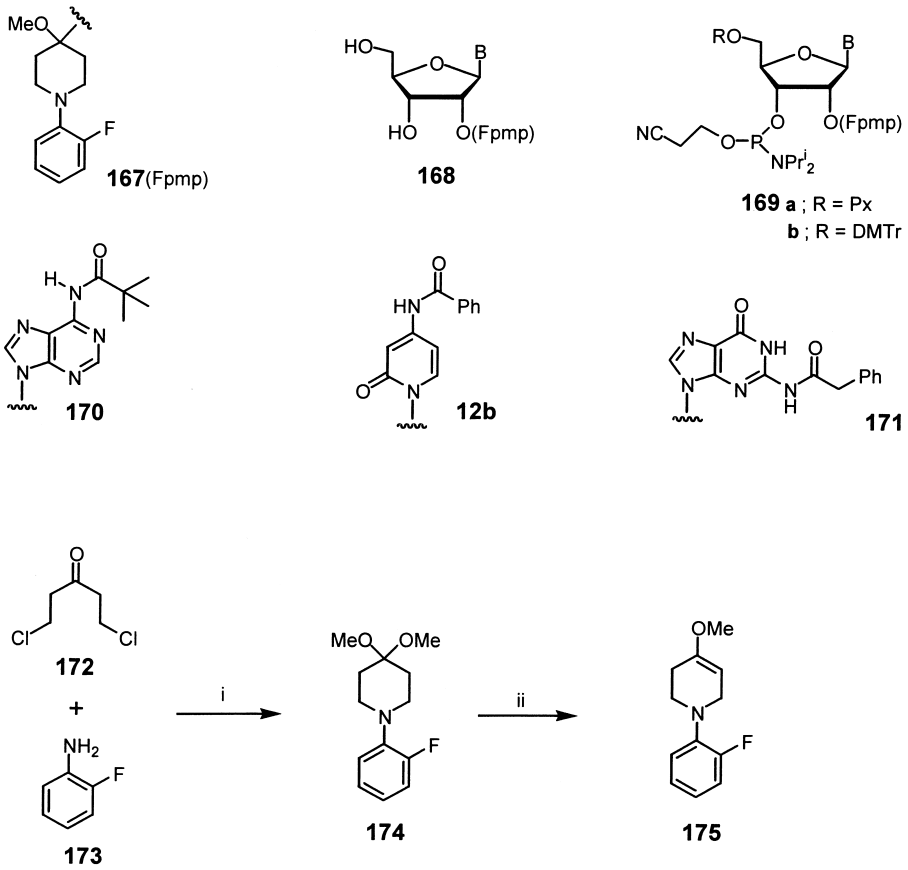
163 **164** **165**



166 (Ctmp)

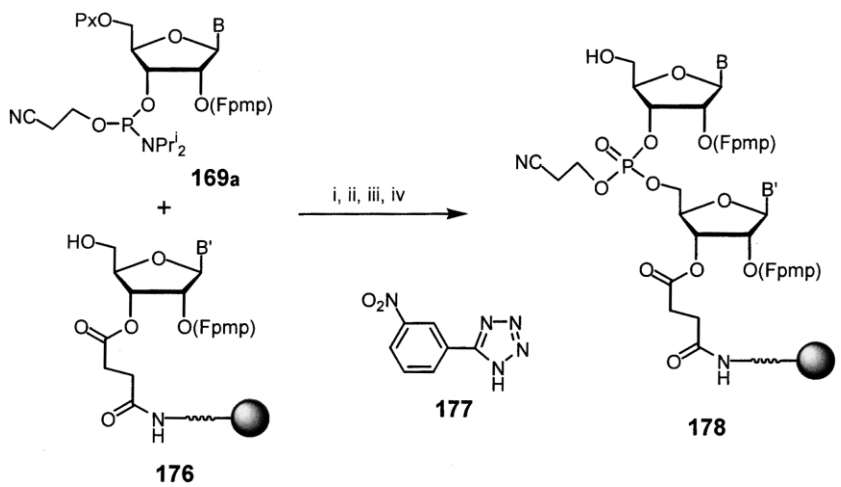
The Ctmp group **166** was found to be compatible with the 5′-*O*-Px protecting group **160** in solid phase oligoribonucleotide synthesis, and has been used successfully to protect the 2′-hydroxy functions both in phosphoramidite[152]- and H-phosphonate[153]-based synthesis.

It was subsequently found[154] that the related Fpmp group **167** had similar hydrolysis properties to the Ctmp protecting group **166**. However, the enol ether reagent[154] **175** required for the preparation of the 2′-*O*-Fpmp nucleoside derivatives **168** and therefore the phosphoramidite building blocks **169** is more readily accessible than the corresponding Ctmp reagent. We later found that the enol ether **175** may be obtained[155] in two steps (Scheme 22) and in good overall yield, starting from 1,5-dichloropentan-3-one[156] **172** and 2-fluoroaniline **173**. The 2′-protected nucleoside derivatives **168** are prepared[157] from the corresponding 3′,5′-*O*-(1,1-3,3-tetraisopropyldisiloxan-1,3-diyl) compounds **159** by the two-step procedure described above (Scheme 21) for the preparation of 2′-*O*-Mthp derivatives **158**, except that the enol ether **175** is used instead of reagent **157**. At first, phosphoramidites with 5′-*O*-Px protecting groups were prepared,[157] but subsequently the corresponding 5′-*O*-DMTr derivatives **169b** became commercially available. In both sets of phosphoramidite building blocks **169a**,**b**, the amino functions of the adenine, cytosine and guanine base residues are protected with pivaloyl, benzoyl and phenylacetyl groups (as in **170**, **12b** and **171**, respectively) and the uracil residues are left unprotected.



Scheme 22. *Reagents and conditions*: (i) (a) TsOH·H_2O reflux, (b) add $(MeO)_3CH$, reflux; (ii) Et_2O–BF_3, Pr^i_2NEt, CH_2Cl_2, 0°C.

The Fpmp group has been used successfully by a number of workers[157–163] for the protection of the 2′-hydroxy functions in the phosphoramidite-based solid phase synthesis of RNA sequences. Initially, the synthesis of the 3′-terminal decamer (r[UCGUCCACCA]), nonadecamer (r[AUUCCGGACUCGUCCACCA]) and heptatriacontamer (r[GGAGAGGUCUCCGGUUCGAUUCCGGACUCGUCCACCA]) sequences of unmodified yeast $tRNA^{Ala}$ was undertaken.[157] Apart from the washing steps, each synthetic cycle (Scheme 23) involved four steps: coupling (step i) with 1-(3-nitrophenyl)-1*H*-tetrazole **177** as the activating reagent, capping (step ii), oxidation (step iii) with an anhydrous solution of *tert*-butyl hydroperoxide and finally detritylation (step iv). The 2′-protected RNA sequences were detached from the controlled-pore glass solid support, and the protecting groups were removed from the base residues and the internucleotide linkages to give the 2′-protected RNA sequences. This 'stabilized RNA', which is resistant to both alkaline hydrolysis and digestion by endonucleases, was purified by liquid chromatography. Finally, the 5′-terminal Px and the 2′-*O*-Fpmp groups were removed by acidic hydrolysis (pH 2.0–2.3, 20°C, 20 h). In virtually

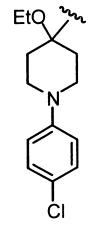


Scheme 23. *Reagents and conditions*: (i) **177**, MeCN; (ii) Ac_2O, AcOH, 2,6-lutidine, 1-methylimidazole, THF; (iii) *t*-BuO_2H, $Me_2CHCH_2CMe_3$, CH_2Cl_2; (iv) $Cl_3C{\cdot}CO_2H$-CH_2Cl_2 (2:98 w/v).

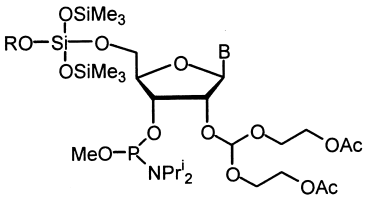
all of the other work[158–163] on the solid phase synthesis of RNA sequences involving the use of 2′-*O*-Fpmp protecting groups, the commercially available 5′-*O*-DMTr phosphoramidite building blocks **169b** rather than the 5′-*O*-Px derivatives **169a** were used.

Although the above tRNA^{Ala} sequences were found to be stable under the conditions of acidic hydrolysis used to remove the 2′-*O*-Fpmp protecting groups, subsequent studies revealed[159] that certain other RNA sequences (e.g. r[(Up)$_9$U] and r[(Up)$_{19}$U]) readily undergo hydrolytic cleavage and phosphoryl migration at pH 2 and rt. However, if unblocking is carried out at rt above pH 3.0 and especially in 0.5 M sodium acetate buffer (pH 3.25),[162] removal of the Fpmp groups from 2′-protected r[(Up)$_{20}$U] may be effected with cleavage or migration of the internucleotide linkages occurring, at most, to a negligible extent. It is important to note that the acid-stability of oligo- and poly-ribonucleotides is sequence dependent.[159] Thus, while the above 3′-terminal yeast tRNA^{Ala} sequences are more acid stable than oligouridylic acids, it is quite possible that certain other oligo- and poly-ribonucleotides may prove to be less stable. Kinetic studies by Kuusela and Lönnberg[125] have revealed that, at 90°C and at pH 6 and below, enzymatically-synthesized polyuridylic acid is more susceptible to internucleotide cleavage than uridylyl-(3′→5′)-uridine. These workers also reported[125] that at 90°C and below pH ca. 4.5, the internucleotide linkages of polyuridylic acid undergo migration more readily than the internucleotide linkage of uridylyl-(3′→5′)-uridine. Morgan et al. subsequently reported[164] that polyuridylic acid is susceptible to internucleotide linkage migration in the pH range of 2.1–2.6 at 25°C.

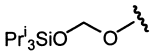
From a consideration of all of the reported studies relating to the acid-catalyzed cleavage and the migration of the internucleotide linkages of RNA sequences, it is clearly desirable to remove Fpmp and related acid-labile protecting groups at as high a pH as possible. The temperature and duration of the unblocking process must also be taken into account. With these points in mind, it was recently shown[165] that it was possible to remove the Fpmp groups from 2′-protected r[(Up)$_{19}$U] in 7 h in 0.5 M sodium acetate buffer (pH 4.0) at 35°C. No cleavage or migration of the internucleotide linkages could be detected in the fully-unblocked r[(Up)$_{19}$U] obtained. Control of the unblocking conditions was considered to be so important that it was decided to examine[165] the hydrolysis properties of twelve other (in addition to the Ctmp and Fpmp groups) 1-aryl-4-alkoxypiperidin-4-yl protecting groups to find the group with the best properties. This proved to be the 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) group **179**. Although the Cpep was found[165] to be 1.3 times *more stable* than the Fpmp group at pH 0.5 and 30°C, it was over 2.2 times *less stable* than the Fpmp group at pH 3.75 and 30°C, thereby permitting milder unblocking conditions. Thus removal of the Cpep groups from 2′-protected r[(Up)$_{19}$U] required only 4 h under the conditions described above (pH 4.0, 35°C) for the unblocking of Fpmp-protected r[(Up)$_{19}$U]. Remarkably, the ratio of the hydrolysis rates of the Cpep protecting group at pH 0.5 and 3.75 was found to be only 3.73 rather than a ratio of 1778 that would be expected for a simple acetal system. The pH 0.5/3.75 rate



179 (Cpep)



180 R = cyclooctyl or cyclododecyl



181 (TOM)

ratios were found[165] to be 10.8 and 9.2, respectively, for the Fpmp and Ctmp protecting groups. Further studies need to be carried out with the Cpep group, which may well prove to be the group of choice for the protection of the 2′-hydroxy functions in both solid and solution phase RNA synthesis.

Recently, Scaringe et al.[166] have described the solid phase synthesis of RNA sequences, based on the phosphoramidite building blocks **180**. The main difference between this and other approaches to solid phase RNA synthesis is that a 'silyl' rather than a 'trityl' group is used to protect the 5′-hydroxy functions. The acid-labile bis-(2-acetoxyethoxy)-methyl group is used to protect the 2′-hydroxy functions. This orthoester group is deacetylated and thereby made more acid-labile in the course of the ammonolytic step required to remove the acyl protecting groups from the base residues in the penultimate unblocking step at the end of the synthesis. The acidic conditions (pH 3.0, 55°C, 10 min) used to deprotect the 2′-hydroxy functions in the final unblocking process are very mild and would not be expected to promote significant cleavage or migration of the internucleotide linkages. The (triisopropylsilyl)oxymethyl (TOM) group **181** has also recently been proposed[167] for the protection of the 2′-hydroxy functions in solid phase RNA synthesis. The TOM is clearly related to the TBDMS **139** protecting group but appears to offer two distinct advantages over it. First, it cannot migrate and secondly its use is reported to lead to faster coupling reactions and higher coupling yields. Like the TBDMS group, it may be removed by treatment with tetra-*n*-butylammonium fluoride in THF. No comparative study has been carried out to determine which group is to be preferred for the protection of the 2′-hydroxy functions in solid phase RNA synthesis. Very recently, Micura[168] has drawn attention to the particular merits of the bis-(2-acetoxyethoxy)methyl and TOM protecting groups. However, the synthesis particularly of the bis-(2-acetoxyethoxy)methyl-protected building blocks is relatively complex and they are therefore likely to be more costly than the corresponding 2′-*O*-TBDMS and 2′-*O*-Fpmp(Cpep) building blocks. This could be an important consideration if large scale synthesis were to be envisaged. Like the Fpmp(Cpep) and TOM groups, the bis-(2-acetoxyethoxy)methyl protecting group has the advantage that it cannot migrate.

Emphasis has been placed on the use of phosphoramidite

building blocks in this discussion of solid phase oligoribonucleotide synthesis. However, H-phosphonate building blocks have also been used successfully in the solid phase synthesis of RNA sequences. The 2′-hydroxy functions have been blocked by a number of protecting groups including the TBDMS,[169] Ctmp,[153] 2-nitrobenzyl[170] and benzoyl[171] groups. The use of monomeric H-phosphonate building blocks in solid phase oligoribonucleotide synthesis has recently been reviewed briefly.[123]

12. Possible future developments in the synthesis of oligo- and poly-nucleotides

Undoubtedly the methodology of oligo- and poly-nucleotide synthesis will undergo numerous further improvements. This is inevitable in an area of such importance. Solid phase synthesis will surely continue to be used in the preparation of relatively small quantities of material. Indeed, solid phase synthesis, based on phosphoramidite monomers, has recently also been used[172] in the preparation of the relatively large (i.e. kilogram) quantities of oligonucleotide analogues required for clinical trials. However, if as indicated above, very large (multikilogram to tonne) quantities of specific sequences become required for chemotherapeutic purposes, and this may happen soon if, for example, a systemic antisense drug becomes licensed, it is not unlikely that either solution phase or a combination of solution and solid phase synthesis will emerge as the method of choice. Thus dimer, trimer and perhaps even higher molecular weight blocks could be prepared on a large scale in solution, quite possible by the modified H-phosphonate approach (see above), and then linked together either in solution or on a solid (or liquid polymer) support. There can be little doubt that considerable developments in synthetic methodology will take place if the manufacture of oligonucleotides for chemotherapeutic purposes develops into an industry with large annual sales.

Despite the considerable progress that has taken place in recent years, the synthesis of RNA sequences still lags behind that of DNA sequences. However, as the demand for synthetic oligo- and poly-ribonucleotides increases, improvements both in solid and liquid phase methodologies will no doubt take place. Recently, the application of the modified H-phosphonate approach to the solution phase synthesis of low molecular weight linear and cyclic oligoribonucleotides[103] has been successful as has the combined application of the phosphotriester and modified H-phosphonate approaches to the synthesis of branched and branched-cyclic oligoribonucleotides.[173] Whether a demand for the very large scale manufacture of RNA sequences for chemotherapeutic purposes will develop is not yet clear. Should this happen, the underlying basis of the necessary synthetic methodology already exists.

Enormous strides have been made in the chemical synthesis of DNA and RNA sequences and their analogues since the mid-1950s when the studies outlined in this Commentary were started. It would, of course, be foolhardy to attempt to predict how far this field will advance in the next four or five decades. It has been really exciting to have been a spectator and to some extent a participant in the past almost 50 years, and it is most gratifying to be able to conclude that the field of polynucleotide synthesis is no less exciting and most certainly no less important now than it was at the outset.

Acknowledgements

I should like to acknowledge the invaluable contributions that my co-workers have made over a period of 40 years to our studies on the chemical synthesis of oligo- and polynucleotides. Some of their names appear in the references below and I owe them all an enormous debt of gratitude.

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