Tetrahedron Vol. 34, pp. 3143–3179
0040-4020/78/1101-3143/$05.00/0

TETRAHEDRON REPORT NUMBER 56

THE CHEMICAL SYNTHESIS OF OLIGO- AND POLY-NUCLEOTIDES BY THE PHOSPHOTRIESTER APPROACH

COLIN B. REESE
Department of Chemistry, King's College, Strand, London WC2R 2LS, England

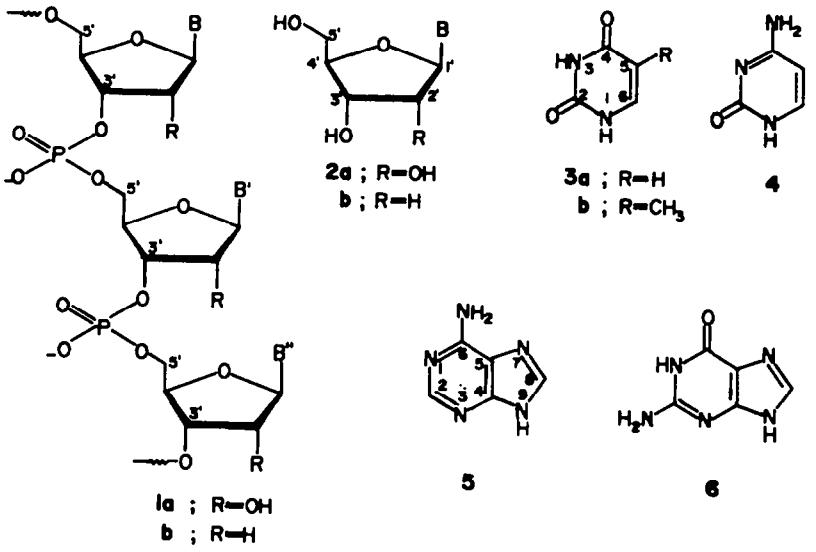
(*Received for publication* 6 *April* 1978)

CONTENTS

INTRODUCTION

One of the most challenging and important areas of research in the chemistry of nucleic acids is that concerned with the development of methods for the chemical synthesis of oligo- and poly-nucleotides. It is a challenging area in that it presents a formidable problem in synthetic organic chemistry and an important area in that chemically-synthesized oligo-ribonucleotides and -deoxyribonucleotides of known base-sequence have proved to be of considerable value in certain biological studies. Thus tri-ribonucleoside diphosphates were used directly in the elucidation of the genetic code[1] and short oligodeoxyribonucleotides have been used as templates in the enzymatic synthesis of high molecular weight poly-deoxyribonucleotides with repeating base-sequences.[2] A more recent and striking example of the biological application of chemically-synthesized oligodeoxy-ribonucleotides is in the synthesis of the genes for alanine[3] and tyrosine suppressor[4] transfer ribonucleic acid (tRNA) by the enzymatic linking together of suitable oligomer segments. There is every reason to believe that such chemically-synthesized oligonucleotides will continue, in the future, to find important applications in biological research.

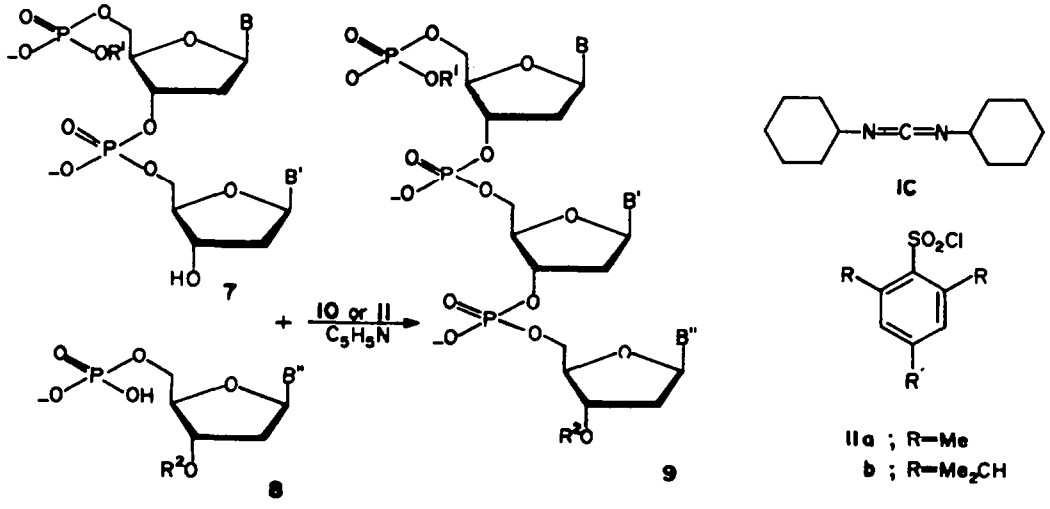
Structurally, oligonucleotides 1 are poly-dialkyl phosphate esters in which the 3′-hydroxy group of one nucleoside unit 2 is joined to the 5′-hydroxy group of another through an internucleotide phosphodiester linkage. In oligoribonucleotides 1a, the nucleoside units are β-D-ribofuranosides of pyrimidine and purine bases, i.e. ribonucleosides 2a. In ribonucleic acids (RNA), there are four principal nucleoside units: uridine, cytidine, adenosine and guanosine (which are, respectively, the 1-β-D-ribofuranosides of uracil 3a and cytosine 4 and the 9-β-D-ribofuranosides of adenine 5 and guanine 6. A number of other ribonucleosides[5] occur naturally in small quantities, especially in tRNA. In oligodeoxyribonucleotides 1b, the nucleoside units are β-D-2-deoxyribofuranosides of pyrimidine and purine bases, i.e. 2′-deoxyribonucleosides 2b. 2′-Deoxycytidine, 2′-deoxyadenosine and 2′-deoxyguanosine, three of the four principal nucleoside units which occur in deoxyribonucleic acids



(DNA), are derived from the same bases (4, 5 and 6, respectively) as three of the above ribonucleosides but the other principal 2′-deoxyribonucleoside, thymidine, is the 1-β-D-2′-deoxyribofuranoside of thymine 3b.

So far, much more success has been achieved in the synthesis of oligodeoxyribo- than of oligoribo-nucleotides. This is due partly to the obvious necessity for the 2′-hydroxy functions to be protected throughout an oligoribonucleotide synthesis and then released under such mild conditions that they do not enter into any reactions with the neighbouring internucleotide phosphodiester linkages (see below). An additional problem which is inherent in oligoribonucleotide synthesis is the steric hindrance to the approach of a phosphorylating agent to the 3′-hydroxy functions which is presented by bulky protected 2′-hydroxy groups. Another important reason why more progress has been made in the deoxyribose series is that the approach which has been most thoroughly investigated so far has proved to be more effective in the synthesis of oligodeoxyribonucleotides. This method, which involves the phosphorylation of intermediates with unprotected internucleotide linkages, is now commonly referred to as the *phosphodiester approach*; it was introduced in 1956 by H. G. Khorana *et al.*[6] and investigated in great detail mainly by the latter group[7] in the subsequent decade or so. An example of the use of the phosphodiester approach in extending a di- to a tri-nucleotide in the deoxyribose series is indicated in Scheme 1.

A 5′-protected dinucleotide 7 is allowed to react with an excess of a 3′-protected mononucleotide 8 in the presence of *N,N′*-dicyclohexylcarbodi-imide[8] 10 or an arenesulphonyl chloride such as mesitylenesulphonyl[9] or 2,4,6-tri-isopropylbenzenesulphonyl[10] chloride 11a or 11b in anhydrous pyridine solution. The protecting groups (R^1 and R^2) for the terminal monophosphate of the dinucleotide 7 and the 3′-hydroxy function of the mononucleotide 8 may, for example, be 2-cyanoethyl[11] ($R^1 = CH_2CH_2CN$) and acetyl ($R^2 = Ac$), respectively. Good yields have often been obtained[7] in such a stepwise synthesis of oligodeoxyribonucleotides by the phosphodiester approach. Indeed, if a large excess of the protected mononucleotide 8 is used, it is possible to extend much larger molecules than dinucleotides 7 in this way and obtain satisfactory yields of products. However, much less satisfactory yields are usually obtained[7] when attempts are made to attach an oligomer (e.g. 7) to another oligomer instead of to a monomer 8. Neverthe-



Scheme 1.

less, despite the often very modest yields obtained, high molecular weight oligodeoxyribonucleotides, including an icosanucleotide sequence of the gene for alanine-tRNA, have been prepared[7] by linking together oligonucleotide blocks by the phosphodiester approach.

It is not proposed to review the phosphodiester approach in this report as it has been dealt with thoroughly elsewhere.[7,12] Undoubtedly a number of biological problems of enormous significance have been solved with the aid of oligodeoxyribonucleotides and indeed of short oligoribonucleotides which were synthesized by the latter approach. However, we first became aware of its limitations during the course of some relatively unsuccessful studies[13] on the synthesis of oligoribonucleotides. Although we believed at the time that the steric properties of the bulky methoxytetrahydropyranyl protecting groups (see below) on the 2'-hydroxy functions contributed to our lack of success, we later formed the opinion[14] that any phosphorylation procedure in which the internucleotide phosphodiester linkages are left unprotected (i.e. any phosphodiester approach) suffers from several inherent disadvantages:

(a) Phosphodiester functions are nucleophilic and are therefore open to attack in subsequent phosphorylation steps. Such side-reactions would upset the stoicheiometry, possibly result in cleavage of the internucleotide linkages and generally lead to lower yields. Thus the phosphorylating species formed by the reaction between 8 and 11b (Scheme 1) could attack the internucleotide phosphodiester linkage of 7 as well as its 3'-hydroxy group.

(b) Salts of phosphodiesters are normally soluble only in water and the more polar organic solvents. Thus they usually cannot be purified by the standard techniques of organic chemistry such as adsorption chromatography on silica gel or alumina. It is therefore necessary to use fractionation techniques such as cellulose or Sephadex anion-exchange chromatography and this tends to limit the scale of the reactions. Clearly, if it were intended to undertake the synthesis of a high molecular weight oligonucleotide, it would be desirable to work with relatively large quantities of material in the early stages of the synthesis.

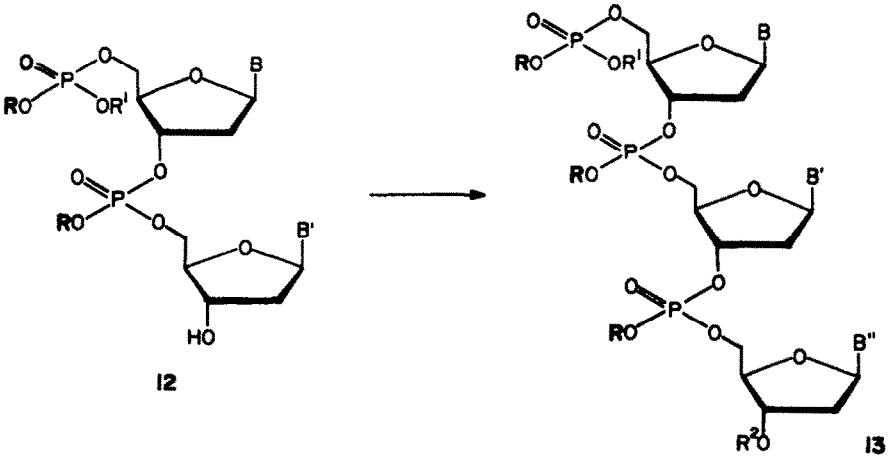
(c) Anion-exchange chromatography generally involves the use of aqueous or aqueous alcoholic buffer solutions. Exceptional care has then to be taken in the purification of partially-protected phosphodiester intermediates as the protecting groups used are usually by necessity very sensitive either to acid- or to base-catalyzed hydrolysis. The purification of phosphodiester intermediates is a particularly formidable task in the ribose series as it is absolutely essential that no unblocking of the 2'-hydroxy functions occurs during chromatography or during the subsequent concentration of fractions.

For these reasons, we thought it likely that ultimate success in the chemical synthesis of even moderately-sized oligoribonucleotides and significant improvement in yields of oligodeoxyribonucleotides would depend on the protection of the internucleotide linkages; i.e. that it would be necessary to develop a synthesis involving *phosphotriester* rather than *phosphodiester* intermediates. In this way, the above disadvantages of the phosphodiester approach would all be removed at one fell swoop.

The simple chain-extension of a di- to a tri-deoxyribonucleotide (7→9) by the *phosphodiester approach* is represented in Scheme 1. The corresponding transformation (12→13) involving the *phosphotriester approach* is illustrated in Scheme 2. Clearly, a new problem has been introduced: what protecting group (R) should be used for the internucleotide linkages. This is a crucial problem which has not yet been solved completely and which will be considered in detail later on in this report.

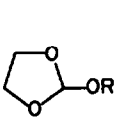
The synthesis of oligonucleotides by either the phosphodiester or the phosphotriester approach involves two main stages: (i) the preparation of suitably-protected monomeric (nucleoside or nucleotide) units derived from each of the common bases, and (ii) the linking together of these monomers in the required order by a suitable phosphorylation procedure. In a final stage, the protecting groups must all be removed. The latter process will be absolutely straightforward if the protecting groups have been correctly chosen or suitably designed in the first place. However, as will become clearer later on, the latter condition is far from easy to fulfil. It is appropriate at this point to include a few general comments on protecting groups before proceeding with a detailed discussion of the main stages of oligonucleotide synthesis.

The expression "suitably-protected" was used above. A protecting group may be regarded[15] as "suitable" for a particular purpose if (i) it is relatively easy to introduce,

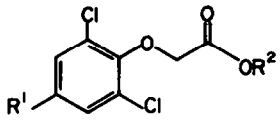


12 13

Scheme 2.



14a ; R=Me
b ; R=Et



15a ; R¹=H
b ; R¹=Me

(ii) it is stable under the reaction conditions and (iii) it is readily removable at the end of the synthesis under conditions under which the required product is stable. It may also be desirable that a protecting group should satisfy several additional criteria including (iv) it should be possible to introduce it by means of a reagent which is both readily accessible and stable, (v) it should be achiral and (vi) it should be designed in such a way that the NMR spectra of resultant protected intermediates will be as simple as possible. The requirement that criteria (i)–(iv) should be met calls for no comment and, as will become clear in the discussion below, the use of chiral groups for the protection of nucleosides leads to the formation of mixtures of diastereoisomers. The rationalization for criterion (vi) is that the protecting group may well be incorporated into a relatively complex molecule and the characterization of the derivative may, especially if it is not crystalline, then depend largely on NMR spectroscopic evidence. Thus the methoxymethylene[16] (as in the ethylene glycol derivative **14a**) is preferred to the ethoxymethylene[17] protecting group (as in **14b**). As a second example, the ^{1}H NMR spectrum of the 2,6-dichlorophenoxyacetyl protecting group (as in **15a**) is much simplified (to the three singlets) by the introduction of a 4-methyl substituent (as in **15b**). Regrettably a number of protecting groups which do not appear to meet the three most essential criteria (i–iii) have been proposed in the literature for use in oligonucleotide synthesis.

STAGE 1—PREPARATION OF NUCLEOSIDE BUILDING BLOCKS

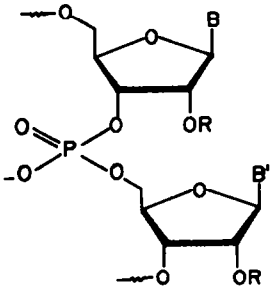
The monomeric building blocks required in the phosphotriester approach are all partially-protected ribo- or 2′-deoxyribo-nucleoside derivatives. One of the great merits of the phosphotriester approach is that once the building blocks have been prepared they can be linked together in the second stage of the synthesis by any phosphorylation method which is available. Furthermore, if, at some time in the future, a better method is developed, it should be possible to use it in conjunction with the same or perhaps slightly modified building blocks. Indeed, a further serious disadvantage of the phosphodiester approach is that it is not versatile in this way.

The choice of protecting groups and the preparation of building blocks presents a much greater problem in the ribose than in the deoxyribose series. In the first place, the orientational problem of distinguishing between one primary (5′-) and two different secondary (2′- and 3′-) hydroxy groups of ribonucleosides is much more difficult than the more conventional problem of distinguishing between the primary (5′-) and the single secondary (3′-) hydroxy groups of 2′-deoxyribonucleosides. Furthermore, the facility with which acyl groups migrate from one secondary hydroxy function to the other in ribonucleoside chemistry[18] adds complexity to synthetic work in this series. For this reason more effort and thought has been put into the synthesis of ribonucleoside building blocks.

(a) RIBONUCLEOSIDE BUILDING BLOCKS

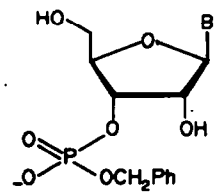
Protection of 2′-hydroxy functions

Arguably the most crucial decision which has to be made in the synthesis of oligoribonucleotides is the choice of the protecting group (R, formula **16**) for the 2′-hydroxy functions. This protecting group has to remain intact until the very last step of the synthesis and must then be removed under conditions which are mild enough to prevent subsequent attack of the released 2′-hydroxy functions on vicinal phosphodiester groups with consequent cleavage or migration of the internucleotide linkages. Thus general criteria (ii) and (iii) (see above) must be applied stringently to this protecting group.

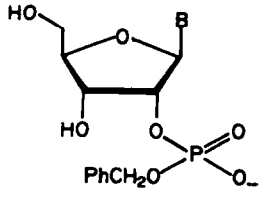


16

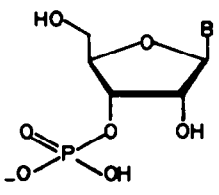
A great deal is known about the action of acids and bases on nucleic acids and their components and it is for this reason that acid-labile (e.g. triarylmethyl, acetal, orthoester) and base-labile (acyl) protecting groups have usually been favoured over those which may be removed in other ways (e.g. reductively or photochemically). Much consideration was, therefore, given to the matter as to whether R (as in **16**) should be an acid-labile or a base-labile group. Brown *et al.*[19] had earlier shown that when cytidine 3′-benzyl phosphate (**17**) was heated in 0.1 M-hydrochloric acid solution at 80° for 2 hr, *ca.* 50% hydrolysis to a mixture of cytidine 2′- and 3′-phosphates (**20** and **19**, respectively) occurred and that the remaining unhydrolyzed material consisted of **17** and the isomeric 2′-benzyl ester (**18**). Thus, under acidic conditions, both hydrolysis and phosphoryl migration (presumably *via* a pentacoordinate phosphorus intermediate) had occurred. Brown *et al.*[19] also reported that alkaline hydrolysis of **17** (to **19** and **20**) was not accompanied by isomerization to **18**.



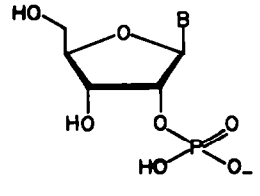
17



18



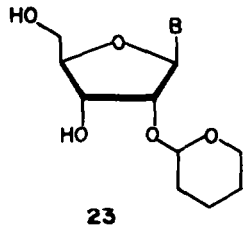
19



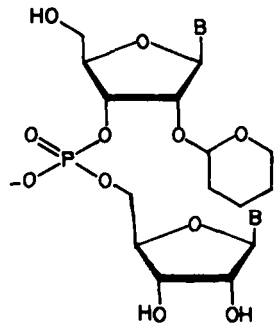
20

B = cytosin-1-yl

It is obviously undesirable that any hydrolysis (i.e. internucelotide cleavage) should accompany the removal of 2'-protecting groups. However, it may well be possible to separate an oligonucleotide from lower molecular weight degradation products by cellulose or Sephadex anion-exchange chromatography. The isomerization process is a much more serious matter as it is virtually impossible to separate oligoribonucleotides containing one or more 2'→5'-internucleotide linkages from isomeric material containing only natural 3'→5'-internucleotide linkages. Thus phosphoryl migration during the final unblocking of the 2'-hydroxy functions must be avoided at all costs. Therefore, if acid-labile protecting groups are to be used for the 2'-hydroxy functions, they must be removable under conditions under which phosphoryl migration does not occur to a significant extent.

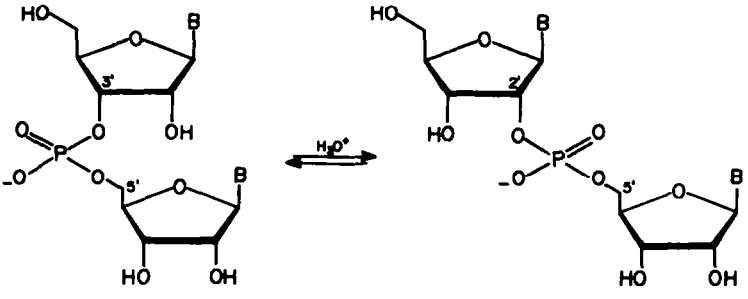


23



24

B = uracil-1-yl



21 B = uracil-1-yl 22

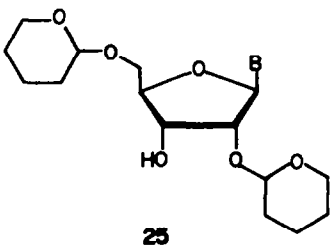
Scheme 3.

In order to ascertain the conditions under which phosphoryl migration occurs in acidic solution, the simple di-ribonucleoside phosphate uridylyl-(3'→5')-uridine (UpU,* 21) was allowed to stand[20] (Scheme 3) in 0.01 M-hydrochloric acid at 25°. After 216 hr, it was possible to detect *ca.* 0.5% degradation (to uridine and uridine 2'(3')-phosphates) and not more than 1% isomerization to 22. Degradation and isomerization were both much faster in 0.1 M-acid but even under these conditions only *ca.* 6% isomerization had occurred[20] in 50 hr at 25°. The acid-labile group which has been used most widely for the protection of isolated alcoholic hydroxy functions is the tetrahydropyranyl[21] acetal system. This group was first used in oligoribonucleotide synthesis by Smrt and Šorm[22] and by Khorana *et al.*[23] and reported[24,25] not to be wholly satisfactory according to the criterion under consideration. However, the half-time ($t_{1/2}$) of hydrolysis of 2'-*O*-tetrahydropyranyluridine 23 in 0.01 M-hydrochloric acid at 22° was found[20] to be 67 min and $t_{1/2}$ for the unblocking of the partially-protected dinucleoside phosphate 24 was found to be 29 min under the same conditions. It should therefore be possible to unblock 24 completely in *ca.* 4–5 hr in 0.01 M-hydrochloric acid at 25° and only *ca.* 0.02% of concomitant migration of the internucleotide linkage would be expected to occur.

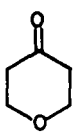
It would therefore appear that the tetrahydropyranyl group is removable under sufficiently mild conditions of acidic hydrolysis to make it suitable for the protection of 2'-hydroxy functions in oligoribonucleotide synthesis. However, this group does not meet general criterion (v) for protecting groups in that it is chiral and its use in connection with optically-active alcohols leads to mixtures of diastereoisomers. Thus the preparation of 2'-*O*-tetrahydropyranyluridine 23 led[20] to a mixture of two diastereoisomers with widely different physical properties (e.g. melting points, solubilities and specific rotations). Although both diastereoisomers of 23 and both diastereoisomers of the 2'-*O*-tetrahydropyranyl derivatives of adenosine[20] and of N^4-benzoylcytidine[26] have been obtained crystalline, attempts to crystallize any one of the four possible diastereoisomers of 2',5'-di-*O*-tetrahydropyranyluridine 25 failed.[27] This is very unsatisfactory as such 2',5'-protected ribonucleoside derivatives are required as building blocks (see below) and it is highly desirable to start with pure crystalline nucleoside building blocks and thereby ensure the absence of position isomers. In this way the presence of 2',5'-internucleotide linkages in the final products may be minimized or possibly avoided altogether.

It was therefore necessary to develop an achiral alternative to the tetrahydropyranyl protecting group. The solution to this problem was not immediately apparent as ketone acetals usually undergo acid-catalyzed hydrolysis[28] at *ca.* 10^3 times the rate of aldehyde acetals (other than those derived from formaldehyde) and are thus too labile to be useful as protecting groups. On the other hand, formaldehyde acetals, although achiral, are much too stable to acidic hydrolysis[28] for this purpose. However, acetals of tetrahydro-4*H*-pyran-4-one 26, prepared by the acid-catalyzed addition of alcohols to 5,6-dihydro-4-

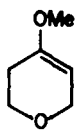
*In general, XpYpZp... denotes an oligonucleotide in which the 3'-hydroxy group of nucleoside X is connected through a phosphodiester linkage to the 5'-hydroxy group of nucleoside Y which, in turn, is similarly connected through its 3'-hydroxy group to the 5'-hydroxy group of nucleoside Z, and so on. The ribonucleosides adenosine, cytidine, guanosine and uridine are denoted by A, C, G and U, respectively and the 2'-deoxyribonucleosides deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine by dA, dC, dG and T, respectively.



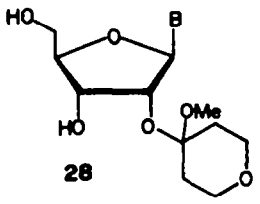
25



26



27

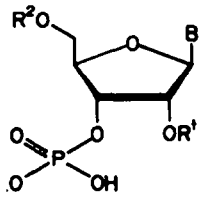


28

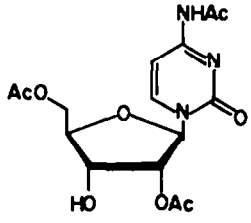
methoxy-2*H*-pyran[27] 27 were found to have the desired hydrolysis properties. Thus $t_{1/2}$ for the unblocking of 2′-*O*-methoxytetrahydropyranyluridine 28, which may readily be prepared as a crystalline compound in high yield (see below), was found[27] to be 24 min in 0.01 M-hydrochloric acid at 20°. The methoxytetrahydropyranyl therefore appears to have even more satisfactory acidic hydrolysis properties than the tetrahydropyranyl group.[20] Furthermore, bis-methoxytetrahydropyranyl derivatives, corresponding to 25, can usually be obtained (see below) as pure crystalline solids in good yields. The enol ether reagent 27[27,29] may readily be prepared from 26, which itself may be prepared[29,30] from 3-chloropropionyl chloride in two steps in *ca.* 50% overall yield.

small quantities of water. Furthermore, 3′-isomers 33 appear invariably to be favoured over 2′-isomers 32 at equilibrium.[18] Thus, if 2′-*O*-acyl ribonucleoside derivatives (such as 30 and 31) are to be used as building blocks in the synthesis of oligoribonucleotides, either by the phosphodiester or the phosphotriester approach, great care must be taken if the formation of a significant proportion of 2′→5′-internucleotide linkages in the products is to be avoided.

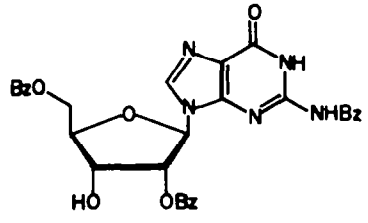
Several groups which may be removed other than by acid- or base-catalyzed hydrolysis have been suggested for the protection of the 2′-hydroxy functions in oligoribonucleotide synthesis. For example, the 2′-*O*-benzyluridine derivative 34 has been used [34] as a building



29 ; R′=Ac or Bz

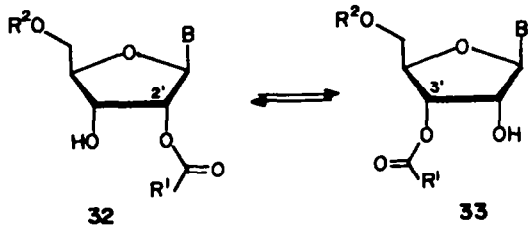


30



31

In their studies on the synthesis of oligoribonucleotides by the phosphodiester approach, Khorana *et al.*[31] favoured the use of base-labile (acetyl and benzoyl) over acid-labile protecting groups for the 2′-hydroxy functions. The building blocks used were 2′-*O*-acyl-3′-ribonucleotide derivatives[32] 29. We showed[33] that the 2′-*O*-acyl ribonucleoside derivatives 30 and 31 could also be used as building blocks in a phosphodiester synthesis. While 30 and 31 would be equally suitable intermediates in a phosphotriester synthesis, 2′-*O*-acyl derivatives of ribonucleosides are, on the whole, likely to be rather unsatisfactory building blocks for oligoribonucleotide synthesis as they can readily undergo base-catalyzed acyl migration[18] to give mixtures of 2′- and 3′-isomers (Scheme 4). Although such acyl migration is, especially for benzoyl groups,[18a] relatively slow in anhydrous pyridine solution, it is accelerated by the presence of

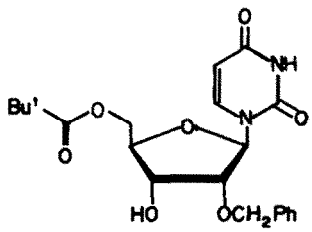


32 33

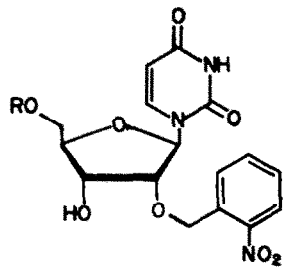
Scheme 4.

block in the synthesis of UpU 21 by the phosphodiester approach. The benzyl protecting group was removed by catalytic hydrogenolysis in the presence of 10% palladized charcoal without any detectable hydrogenation of the 5,6-double bond of the uracil residue. Reitz and Pfleiderer[35] have subsequently used the 2′-*O*-benzyl protecting group in the synthesis of UpU 21 by the phosphotriester approach; however, these workers observed some hydrogenation of the uracil residues during debenzylation. In any case, the use of benzyl groups to protect the 2′-hydroxy functions cannot be recommended as a general procedure in oligoribonucleotide synthesis as it is quite possible that some oligonucleotide benzyl ethers will not be adsorbed on the catalyst surface. Furthermore, in the case of oligomers containing several benzyl protecting groups, it may be difficult to establish by means of a simple analytical procedure whether or not debenzylation is complete.

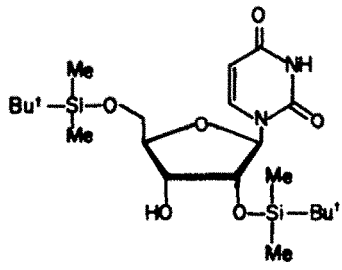
It is not intended to present a complete account of the literature relating to any aspect of oligonucleotide synthesis in this report. However, two other groups which have been used to protect the 2′-hydroxy functions of ribonucleosides in oligoribonucleotide synthesis are of particular interest. Ohtsuka, Tanaka and Ikehara[36] have used the *o*-nitrobenzyl group (as in building block 35), which may be removed photochemically, for this purpose in the synthesis of UpU and UpA by the phosphodiester approach. Ogilvie *et al.*[37] have used 2′,5′-di-t-butyldimethylsilyluridine 36 in the synthesis of UpU



34

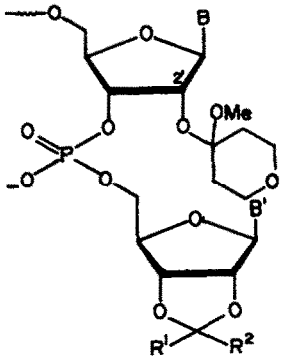


35

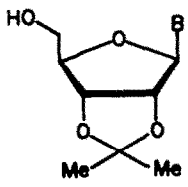


36

21 by the phosphotriester approach and have removed the t-butyldimethylsilyl groups at the end of the synthesis with tetrabutylammonium fluoride in tetrahydrofuran solution. Very recently, these workers have prepared[38] other short oligoribonucleotides from similar building blocks. If the steric properties of the t-butyldimethylsilyl group prove to be favourable enough to permit satisfactory yields of phosphorylation products to be obtained, it seems likely that it could be used as an alternative to the methoxytetrahydropyranyl group in the synthesis of oligoribonucleotides by the phosphotriester approach.



37a ; R^1=R^2=Me
b ; R^1=H, R^2=Ph

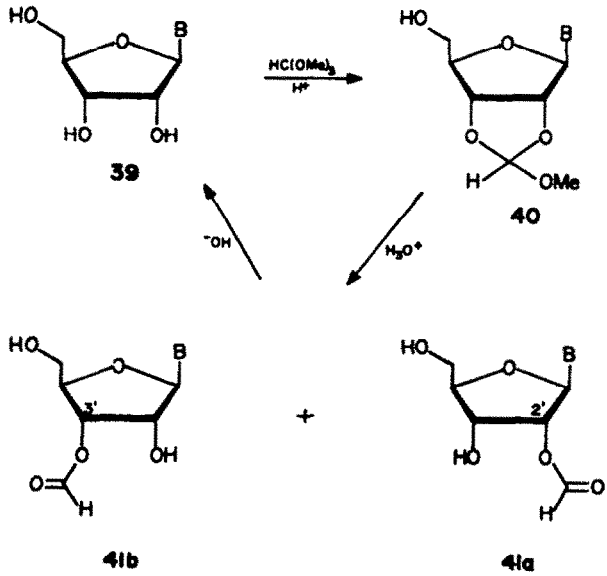


38 ; B=uracil-1-yl

Protection of terminal 2',3'-cis-diol system

In any oligoribonucleotide synthesis, it is essential that the 2',3'-*cis*-diol system should, like the isolated 2'-hydroxy functions, remain protected until the very last step. It is then desirable that the protecting group on the *cis*-diol system should be removable under more or less the same conditions as the protecting groups on the 2'-hydroxy functions. Therefore, if the latter are blocked by methoxytetrahydropyranyl groups, it is desirable that the terminal 2',3'-diol system should be protected (see 37) by a group with a similar acid lability. The commonly used isopropylidene and benzylidene protecting groups[39] (as in 37a and 37b, respectively) are rather unsatisfactory for this purpose in that they undergo acid-catalyzed hydrolysis much too slowly. For example, $t_{1/2}$ for the hydrolysis of 2',3'-*O*-isopropylideneuridine 38 in 0.01 M-hydrochloric acid at 26° is 20 hr.[40] Thus the isopropylidene protecting group undergoes hydrolysis at a rate approximately two orders of magnitude slower than the methoxytetrahydropyranyl group. It would seem that the conditions required for the complete removal of the protecting groups from 37a would lead to *ca.* 1% of phosphoryl migration. Therefore a 1,3-dioxalan system which underwent acid-catalyzed hydrolysis 50–100 times more rapidly than the 2',3'-*O*-isopropylidene group was required.

Cyclic orthoformate esters were very fortunately found to have precisely the required properties. Such 2',3'-*O*-methoxymethylene derivatives 40 are readily

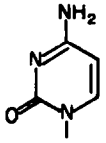


39 40 41b 41a

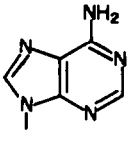
Scheme 5.

prepared[16] by the acid-catalyzed exchange reaction between ribonucleosides **39** or their *N*-acyl derivatives (see below) and trimethyl orthoformate (Scheme 5); these compounds are rapidly converted[16] [$t_{1/2} \sim 10$ min for **40** (B = uracil-1-yl)] into mixtures of the corresponding 2'- and 3'-formate esters **41a** and **41b**, respectively in 0.01 M-hydrochloric acid solution at 20°. Thus the 2',3'-*O*-methoxymethylene appears to be *ca.* twice as labile to aqueous acid as the 2'-*O*-methoxytetrahydropyranyl protecting group. Hydrolysis of the formate esters **41a** and **41b** presents no problem; it proceeds rapidly under mildly alkaline conditions and indeed occurs at a reasonable rate even in pH 7 buffer at room temperature.

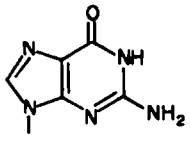
It should be noted that the methoxymethylene group is chiral and that its use can lead to the formation of mixtures of diastereoisomers. In practice,[16] sometimes pure diastereoisomers (as indicated by ^{1}H NMR spectroscopy) and sometimes mixtures of diastereoisomers cyrstallize from the reaction mixture. In either event, the product obtained is quite suitable for use in oligoribonucleotide synthesis as there can be no doubt that it consists solely of a 2',3'-protected ribonucleoside, free from position isomers. The ethoxymethylene protecting group[17] (as in **14b**) has also been used in oligoribonucleotide synthesis; it appears to offer no advantage over the methoxymethylene group. Indeed it would seem that the latter group should be preferred on the basis of its spectroscopic simplicity (see criterion (vi) above).



42



43

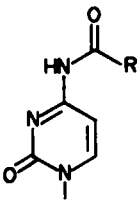


44

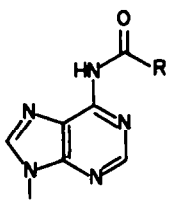
Protection of base residues

Cytosine, adenine and guanine residues **42**, **43** and **44**, respectively, are susceptible to attack by electrophiles such as phosphorylating agents. Thus the matter of the protection of these base residues must be considered both in the synthesis of oligoribo- and oligodeoxyribonucleotides. It seems clear that it is essential to protect cytosine residues **42** to avoid phosphoramidate formation[41] during phosphorylation and it would also seem to be a wise precaution to take in the case of adenine residues **43** as the latter readily undergo acylation on *N*(6). It is not clear that protection of guanine residues **44** prevents attack by phosphorylating agents but it is nevertheless desirable inasmuch as it improves the crystallization and solubility properties of the derivatives.

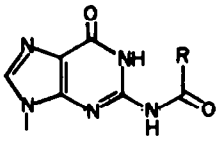
In their work on the synthesis of oligonucleotides by the phosphodiester approach, Khorana *et al.*[7] have used *N*-acyl groups to protect all three base residues **42**, **43** and **44**. This initiative has been followed in most other synthetic work carried out either by the phosphodiester or the phosphotriester approach. In the deoxyribose series,[7b] Khorana *et al.* appear to favour the protection of cytosine residues **42** by N^4-*p*-anisoylation (as in **45b**),



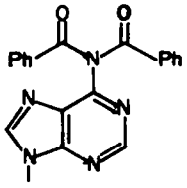
45a ; R=Ph
b ; R=4-$MeOC_6H_4$



46a ; R=Ph
b ; R=4-$MeOC_6H_4$



47a ; R=Me
b ; R=Me_2CH
c ; R=Ph



48

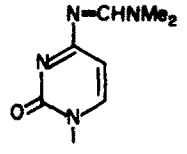
adenine residues **43** by N^6-benzoylation (as in **46a**) and guanine residues **44** as their N^2-isobutyryl derivatives **47b**. In their main studies on the synthesis of oligoribonucleotides,[42] Khorana *et al.* have protected cytosine residues **42** by N^4-benzoylation (as in **45a**), guanine residues **44** by N^2-acetylation (as in **47b**) and adenine residues (as in **43**) as their N^6,N^6-dibenzoyl derivatives (as in **48**).

N-Acyl protecting groups are relatively stable in neutral and acidic media and have the valuable property in the context of oligonucleotide synthesis[43] of being moderately stable at high pH,[b] presumably because of the dissociation of the amide (NHCOR) proton. Thus *N*-acyl groups may be retained while both acid- and base-labile protecting groups are removed from an oligonucleotide. The *N*-acyl groups themselves may generally be removed by ammonolysis.[45]

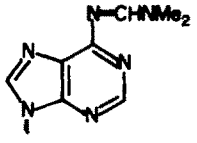
The reasons behind the choices of particular *N*-acyl protecting groups have not always been clear from the literature. Benzoyl and *p*-anisoyl both appear to be sufficiently stable groups for the protection of cytosine[45a] (as in **45a** and **45b**, respectively) and adenine[45b] residues (as in **46a** and **46b**, respectively) and deacylation of all of the latter systems **45a**, **45b**, **46a** and **46b** can readily be effected by ammonolysis.[45] The *p*-anisoyl group, which is somewhat more stable[45a,46] than the benzoyl group, has more satisfactory spectroscopic properties [criterion (vi) for protecting groups; see above]. However, one or other of these groups may ultimately be preferred on the grounds that its use leads to the isolation of crystalline building blocks. N^2-Acylguanine derivatives[47] (as in **47**) are much more resistant to ammonolysis than the corresponding *N*-acyl derivatives of cytosine[45a,46] and adenine.[45b] Indeed N^2-acetylguanine derivatives (as in **47a**) appear to be sufficiently stable[47] for the acetyl group to be used in the protection of guanine residues in oligonucleotide synthesis. N^2-Benzoylguanine residues (as in **47c**) are very stable to ammonolysis but may conveniently be unblocked by treatment with methylamine.[46,48] N^2-Isobutyrylguanine residues (as in **47b**) would be expected[49] to undergo ammonolysis at a rate intermediate between that of N^2-acetyl- and that of N^2-benzoylguanine residues.

[b] This is, of course, not true in the case of di-*N*-acyl derivatives (e.g. **48**) when one of the two acyl groups will be removed rapidly in alkaline solution.[44]

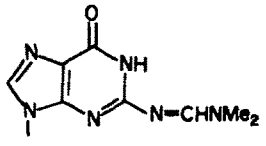
One further consideration relating to *N*-acyl protecting groups may well prove to be important in the phosphotriester approach to oligonucleotide synthesis. Oligonucleotide phosphotriester intermediates tend to become more polar and hence less soluble in organic solvents with increasing molecular weight (see below). This tendency can lead to experimental difficulties in connection with extractions and purification by adsorption chromatography. It may therefore be advisable to attempt to counter this tendency by using lipophilic *N*-acyl (e.g *p*-alkylbenzoyl instead of benzoyl) protecting groups.



49



50



51

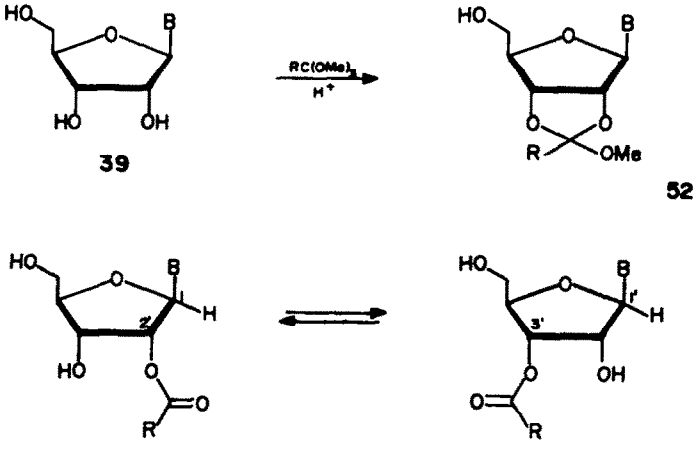
Finally, as an alternative to *N*-acyl groups, the *N*-dimethylaminomethylene group has been suggested[50] for the protection of cytosine (as in **49**), adenine (as in **50**) and guanine (as in **51**) residues in oligonucleotide synthesis. Dimethylaminomethylene groups may be removed by treatment with dilute ammonia[50] but it is not clear that they are more effective than appropriate *N*-acyl protecting groups or that they would remain intact under conditions which would permit the removal of other protecting groups from an oligonucleotide.

Preparation and characterization of 3'-O-acyl derivatives of ribonucleosides

It is appropriate at this point to consider one aspect of ribonucleoside chemistry which has been crucial in the development of methods for the preparation of all but one of the types of building block (see below) required in the synthesis of oligoribonucleotides by the phosphotriester approach. A general procedure for the conversion of a ribonucleoside **39** or its *N*-acyl derivative into its 3'-*O*-acyl **54** and 3',5'-di-*O*-acyl **56** derivatives was required. Fortunately, our study of the chemistry of 2',3'-*O*-methoxymethylene ribonucleosides[16] **40** suggested such a procedure.

The acid-catalyzed reaction (Scheme 6) between a ribonucleoside **39** or its *N*-acyl derivative and a trimethyl orthoester gives[16,51] the corresponding nucleoside orthoester **52** usually in high yield. This appears to be a general reaction; it has, for example, been applied successfully to the preparation of nucleoside orthoformates[16] (**40** or **52**; R = H, see above), orthoacetates[51] (**52**; R = Me), orthomethoxyacetates[52] (**52**; R = $MeOCH_2$) and orthobenzoates[51] (**52**; R = Ph). Such methoxyalkylidene derivatives **52** may be quantitatively converted, by treatment with aqueous acid under mild conditions,[16,51] to give mixtures of the corresponding 2'- and 3'-esters **53** and **54**, respectively. In a number of cases,[51] when the latter mixtures are dissolved in acid-free solvents, crystals of one of the pure isomers **53** or **54** are deposited. Usually,[51] the 3'-esters **54** are obtained crystalline and, as the material remaining in solution can re-equilibrate by acyl migration[16] to give more of the thermodynamically favoured 3'-isomers, isolated yields are often good. For example, both adenosine (**39**; B = **43**) and N^6-benzoyladenosine (**39**; B = **46a**) may be converted,[51,53] *via* their 2',3'-*O*-methoxyethylidene derivatives (**52**; B = **43** and **46a**, respectively, R = Me) into crystalline 3'-acetates (**54**; B = **43** and **46a**, respectively, R = Me) which may be isolated in yields exceeding 75%.

When a crystalline 2'- or 3'-*O*-acyl ribonucleoside derivative is obtained, its orientation is unknown. It was therefore essential to develop an analytical procedure to determine whether such a product was the 2'- or the 3'-isomer **53** or **54**. A general method was developed[54] which depends on the observation that esterification of the 2'-hydroxy function of a ribonucleoside (i.e. transformation **39**→**53**) leads to the deshielding of the glycosidic proton [H(1')] by *ca.* 0.1–0.2 ppm whereas esterification of the 3'-hydroxy function (i.e. transformation **39**→**54**) has virtually no effect on the chemical shift of the H(1') resonance. Thus if the ^1H NMR spectrum of a micture of isomers **53** and **54**, obtained by the

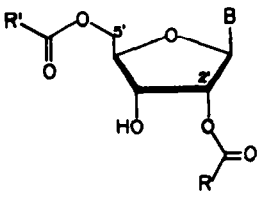


39 52 53 54

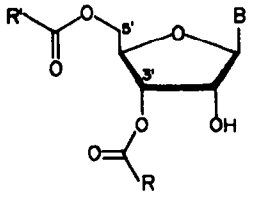
Scheme 6.

hydrolysis of a nucleoside 2',3'-orthoester derivative **52**, is examined, two doublets representing the resonance signals of the glycosidic protons are observed in the region of δ 6. If the spectrum of the crystalline material is then measured, only one doublet is observed in the latter region in the case of a pure isomer. The crystalline material is then identified as the 2'- or 3'-isomer depending on whether the doublet corresponds to the downfield or the upfield signal in the spectrum of the mixture.

Trimethyl orthoesters may be replaced[53] by triethyl orthoesters in the exchange reaction **39** → **52** and hence in the acylation procedure (Scheme 6). Ribonucleoside 2'- and 3'-esters **53** and **54** are readily susceptible to alkaline hydrolysis and, as indicated above, this is particularly so for formate esters **41** and **42** which undergo hydrolysis even under neutral conditions.[18b] Thus care must be exercised if nucleoside 3'-formates **42** are to be used as synthetic intermediates. The isolation of crystalline 2'- or 3'-*O*-acyl ribonucleosides **53** or **54** in high yields depends on the latter isomers being readily able to undergo equilibration by acyl migration[18] in the solvent used for crystallization. For this reason it is generally more satisfactory to work with acetate esters (such as **53** and **54**; R = Me) rather than with the corresponding benzoate esters (**53** and **54**; R = Ph) which undergo equilibration more slowly.[18a] Acetate esters are reasonably stable to alkaline hydrolysis[18b] and are therefore also more convenient intermediates than the corresponding formates **41** and **42**.



55

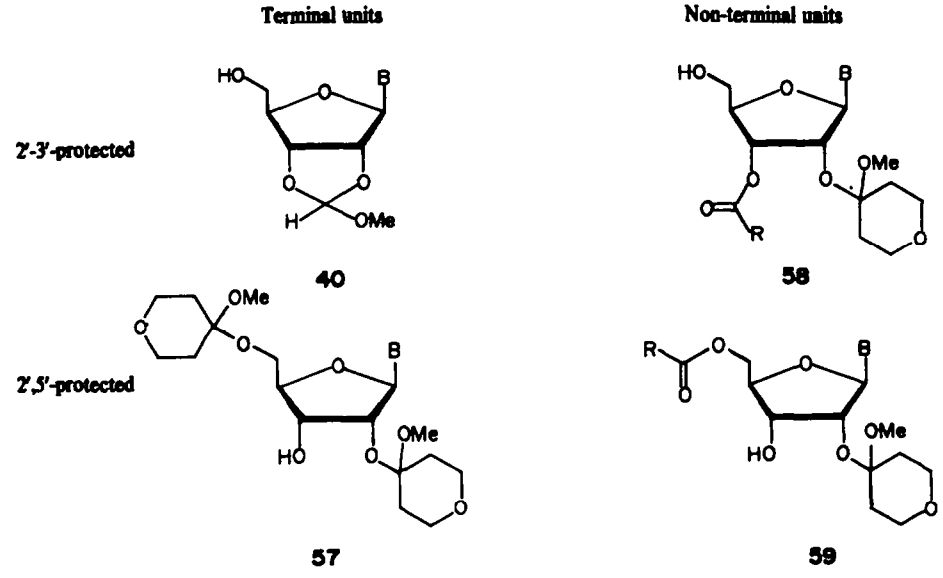


56

The orthoester exchange acylation procedure (Scheme 6) can readily be adapted to provide a general procedure for the synthesis of 2',5'- and 3',5'-di-*O*-acyl ribonucleoside derivatives (**55** and **56**, respectively). If a 2',3'-*O*-methoxyalkylidene derivative **52** is acylated [by treatment with R'COCl or $(R'CO)_2O$ in pyridine solution] before the acidic hydrolysis step, the desired mixture of 2',5'- and 3',5'-di-*O*-acyl derivatives **55** and **56** may usually be obtained[51,52,55] in high yield. In this way, uridine[56] and adenosine[53] (**39**; B = uracil-1-yl and adenin-9-yl, respectively) have been converted into their crystalline 3',5'-diacetates (**56**; B = uracil-1-yl and adenin-9-yl, R = R' = Me) in 90 and over 60% isolated yields, respectively. This extremely versatile procedure may readily be used[52,55,57] in the synthesis of nucleoside 2',5'- and 3',5'-diesters with different acyl groups (i.e. **55** and **56**; R ≠ R'). It is experimentally straightforward and satisfactory yields of diesters are generally obtained provided that care is taken in the manipulation of the acid-sensitive 2',3'-*O*-methoxyalkylidene derivatives **52** prior to their acylation.

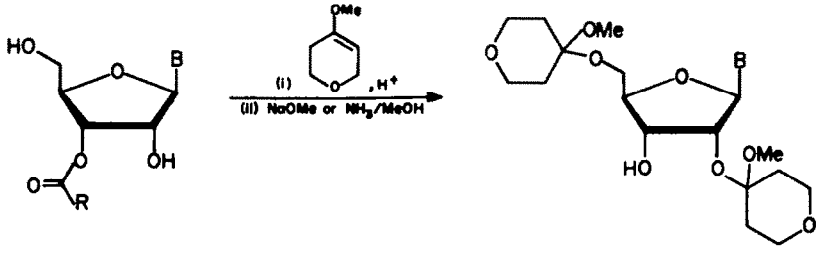
Terminal and non-terminal ribonucleoside building blocks

The actual building blocks required in a general oligoribonucleotide synthesis will clearly depend on the protecting groups which are to be used. If the 2'-hydroxy functions are to remain protected by acid-labile groups until the final step of the synthesis, two *terminal* or chain-end building blocks in which the 3'- or 5'-hydroxy function is also protected by an acid-labile group would appear to be necessary.[14] Such 2',3'- and 2',5'-protected terminal units [**40** and **57**, respectively] are illustrated in Scheme 7. If it is intended that it should be possible to extend the growing oligonucleotide chains in either direction [i.e. either from the terminal 3'- or the terminal 5'-hydroxy function], it is necessary[14] to have 2',3'- and 2',5'-protected *non-terminal* or chain extension building blocks in which the 3'- and 5'-protecting groups, respectively, may be removed under conditions (e.g. alkaline hydrolysis) which do not affect the 2'-acetal groups. It would seem[14] that ribonucleoside 2'-acetal-3'-esters and 2'-acetal-5'-esters [Scheme 7; **58** and **59**, respectively] would make suitable non-terminal building blocks. The procedures which have been developed for the preparation of these four types of building block are outlined below.



Scheme 7.

(i) *Terminal 2',3'-protected units.* The preparation of 2',3'-*O*-methoxymethylene ribonucleosides **40** was described[16] in an earlier section. As indicated above, cytosine, adenine and guanine residues **42**, **43** and **44**, respectively are generally protected in these and in the other three types of ribonucleoside building blocks (see below).

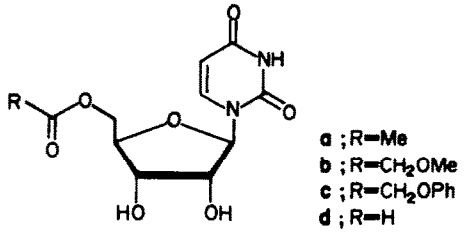


54 **57**

Scheme 8.

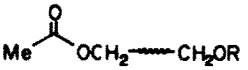
(ii) *Terminal 2',5'-protected units.* The acid-catalyzed reaction between 3'-*O*-acyl (preferably acetyl) ribonucleoside derivatives[51] **54** (see above) and an excess of 5,6-dihydro-4-methoxy-2*H*-pyran[27,29] **27** in dioxan solution leads to fully-protected bis-methoxytetrahydropyranyl derivatives.[58] Treatment of the latter compounds with sodium methoxide (in the case of *N*-acylated intermediates) or ammonia in methanol gives the desired 2',5'-di-*O*-methoxytetrahydropyranyl derivatives **57** in satisfactory to good yields. Crystalline terminal 2',5'-protected building blocks derived from uridine, N^4-*p*-anisoylcytidine, adenosine, N^2-benzoylguanosine, inosine and N^6,N^6-dimethyladenosine have been prepared[58] in this way, thereby confirming the advantage of using an achiral protecting group.

of non-terminal 2',3'-protected units in the following way. A ribonucleoside **39** or its *N*-acyl derivative is first allowed to undergo acid-catalyzed exchange with an orthoester [RC(OMe)$_3$] to give the corresponding 2',3'-*O*-methoxyalkylidene derivative **52**. The latter compound is then acylated with an acid chloride or anhydride derived from a carboxylic acid [R'CO$_2$H], the esters of which undergo saponification more readily than those derived from the acid [RCO$_2$H] corresponding to the orthoester. The product obtained **62** is submitted to mild acidic hydrolysis (Scheme 9) to give a mixture of 2',5'- and 3',5'-di-*O*-acyl derivatives **55** and **56**, respectively, from which it must be possible to obtain the pure 3',5'-isomer **56** crystalline and in good yield. Reaction between **56** and 5,6-dihydro-4-methoxy-2*H*-pyran **27**, followed by selec-



60

a ; R=Me
b ; R=CH_2OMe
c ; R=CH_2OPh
d ; R=H

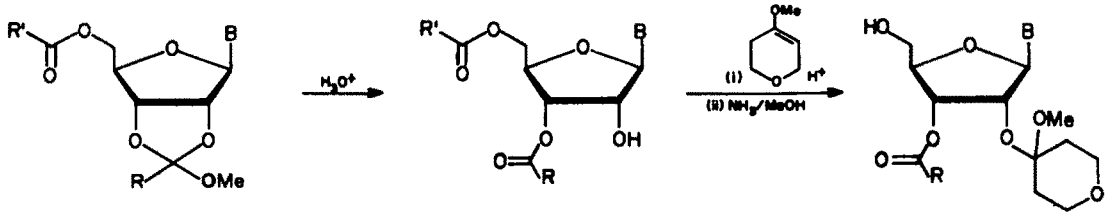


61 a ; R=CHO
b ; R=$COCH_2OPh$
c ; R=H

(iii) *Non-terminal 2',3'-protected units.* The preparation of non-terminal 2',3'-protected units depends on the principle of *selective deacylation.*[55] The relative rates of deacylation[55,57] of 5'-*O*-acetyl-, 5'-*O*-methoxyacetyl-, 5'-*O*-phenoxyacetyl- and 5'-*O*-formyl-uridines **60a–d** in dilute aqueous ammonia solution are approximately

tive removal of the 5'-*O*-acyl group with methanolic ammonia under controlled conditions should give the non-terminal 2',3'-protected building block **58** as a crystalline compound in good yield.[55,57]

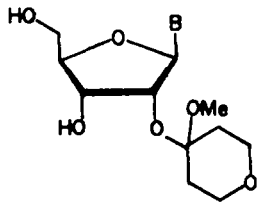
As saponification of an ester derived from a primary alcohol is generally faster than saponification of an ester



62 **56** **58**

Scheme 9.

derived from the same acid and a secondary alcohol, selective deacylation is particularly favourable in the preparation of 2',3'-protected non-terminal units. The combinations (a) R = Me or Ph, R' = CH_2OMe and (b) R = CH_2OMe, R' = H were found[55,57] to be suitable for selective deacylation. Fortunately, good yields of pure crystalline 3',5'-di-*O*-acyl derivatives **56** were obtained[55,57] with both combinations in most cases examined. Combination (b) which involves 5'-*O*-formyl-3'-*O*-methoxyacetyl-ribonucleoside derivatives (**56**; R = CH_2OMe, R' = H) as intermediates led[57] to satisfactory yields of crystalline 2'-*O*-methoxytetrahydropyranyl-3'-*O*-methoxyacetyl-ribonucleoside derivatives (**58**; R = CH_2OMe) which may be further deacylated under comparatively mild conditions of alkaline hydrolysis. The latter point is of considerable importance in connection with the phosphotriester approach to oligonucleotide synthesis as such deacylation must be possible under conditions which do not lead to appreciable concomitant hydrolysis of the phosphotriester groups (see below.).

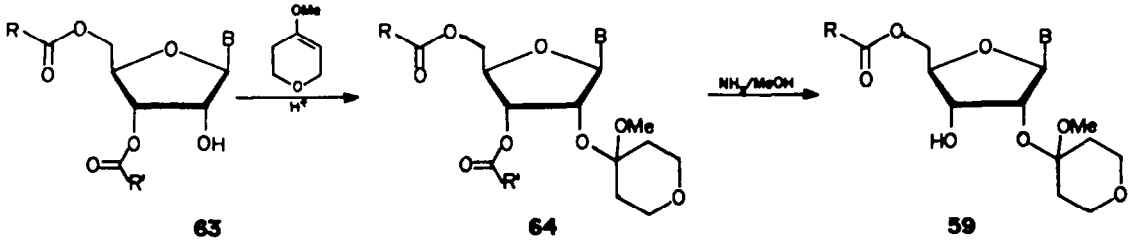


28

2'-*O*-Methoxytetrahydropyranyl derivatives[59] **28** with unprotected 3'-hydroxy functions and the corresponding tetrahydropyranyl derivatives[60] have been used instead of 2',3'-protected non-terminal units in the phosphotriester approach to oligoribonucleotide synthesis. The 5'-hydroxy function is clearly much more accessible to a phosphorylating agent than the comparatively hindered 3'-hydroxy function and undoubtedly natural 3'→5'-internucleotide linkages will be formed predominantly. Although it has been claimed[61] that the formation of 3'→3'-internucleotide linkages cannot be detected when the 3'-hydroxy function is left unprotected, a certain proportion of the latter unnatural linkages must inevitably be formed. If the proportion of 3'→3'-internucleotide linkages is really negligible, and this has yet to be established in precise terms, or if such unnatural internucleotide linkages, once formed, could be cleaved selectively, then simple 2'-*O*-methoxytetrahydropyranyl derivatives **28** could safely be used as alternatives to the above non-terminal 2',3'-protected units **58**.

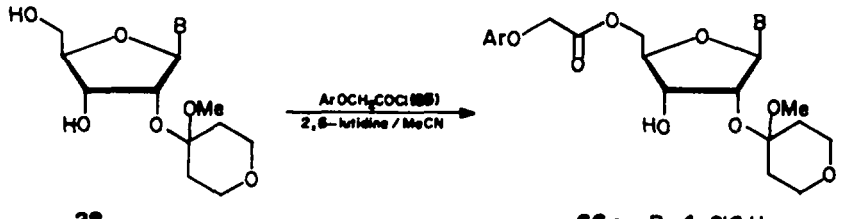
(iv) *Non-terminal 2',5'-protected units.* Selective deacylation may also be used in the preparation of non-terminal 2',5'-protected units **59**.[52] For this approach to be feasible, it must be possible to prepare pure crystalline 3',5'-di-*O*-acyl ribonucleoside derivatives **63** with combinations of acyl groups such that following the methoxytetrahydropyranylation step to give **64** (Scheme 10), the 3'-*O*-acyl groups may be removed selectively to give the desired non-terminal 2',5'-protected units **59**. Selective deacylation is less favourable in this case than in the preparation of the corresponding 2',3'-protected building blocks **58** (see above) as it requires that the saponification of an ester derived from a secondary alcohol should proceed much more rapidly than the saponification of an ester derived from a primary alcohol. Nevertheless combinations (a) R = CH_2OMe, R' = H and (b) R = Ph, R' = CH_2OMe were both found[52] to be suitable and crystalline non-terminal 2',5'-protected units were prepared in satisfactory yields by this method.

Non-terminal 2',5'-protected building blocks are perhaps more conveniently prepared (Scheme 11) by *selective acylation.* Methoxytetrahydropyranylation of *virtually any available crystalline 3',5'-di-O-acyl ribonucleoside derivative* **63**, followed by removal of both of the *O*-acyl groups constitutes a general method[27] of preparing 2'-*O*-methoxytetrahydropyranyl ribonucleosides **28** and their *N*-acyl derivatives. When the latter intermediates are treated with a slight excess of an aryloxyacetyl chloride **65** in the presence of 2,6-lutidine in acetonitrile solution, regioselective acylation occurs[52] to give the non-terminal 2',5'-protected building blocks **66** and usually in good yields. Very little of the isomeric 3'-esters appear to be obtained but it is not possible to avoid the formation of moderate quantities of the 3',5'-diesters. In a number of cases[52,62,63] crystalline *p*-



63 **64** **59**

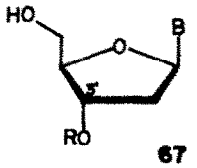
Scheme 10.



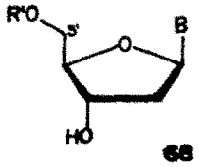
28 **66a**; R=4-ClC_6H_4

Scheme 11.

chlorophenoxyacetates **66a** have been prepared in good yields. These aryloxyacetyl derivatives **66** are particularly suitable as non-terminal 2',5'-protected building blocks in the phosphotriester approach as the saponification of aryloxyacetate esters (see above) can be effected[35] under very mildly basic conditions thereby minimizing the extent of concomitant phosphotriester hydrolysis.



67



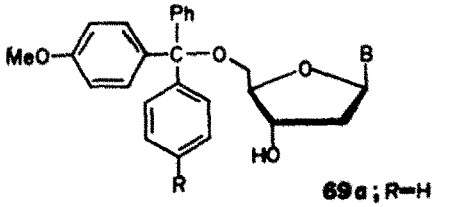
68

(B) 2'-DEOXYRIBONUCLEOSIDE BUILDING BLOCKS

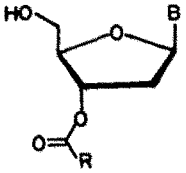
As indicated above, the preparation of building blocks presents much less of a problem in the deoxyribose than in the ribose series. For a completely general stepwise or block synthesis of oligodeoxyribonucleotides by the phosphotriester approach, two types of building blocks are required:[15] a 3'-protected unit **67** and a 5'-protected unit **68**. The synthesis of these building blocks should be relatively straightforward as a number of reagents would be expected to attack 2'-deoxyribonucleosides selectively on their primary 5'-hydroxy functions and it should be possible to convert the 5'-protected units thereby obtained into 3'-protected units.

Protecting groups

The choice of protecting groups for 2'-deoxyribonucleoside building blocks again requires careful consideration. As indicated in the above section on the preparation of ribonucleoside building blocks, Khorana *et al.*[7] used *N*-acyl groups to protect the base residues in their work on the synthesis of oligodeoxyribonucleotides by the phosphodiester approach. Subject to detailed considerations regarding the suitability of particular acyl groups for the individual bases, this would also appear to be the best approach in the synthesis of oligodeoxyribonucleotides by the phosphotriester approach.



69a; R=H
b; R=OMe

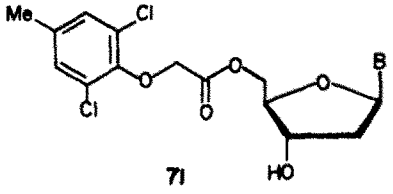


70

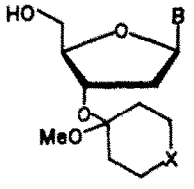
Khorana *et al.*[7] have used acid- and base-labile groups to protect hydroxy functions. These workers[25] first introduced the 4-methoxytrityl (MMTr, as in **69a**) and the 4,4'-dimethoxytrityl (DMTr, as in **69b**) as acid-labile groups for the protection of 5'-hydroxy functions in oligoribonucleotide synthesis. However, the latter protecting groups have subsequently found wide use in oligodeoxyribonucleotide synthesis both in Khorana's laboratory[41] and in a number of other laboratories.[12] As will become apparent later, care must be taken in the removal of acid-labile protecting groups from oligodeoxyribonucleotides containing purine residues. Nevertheless, preliminary experiments (see below) suggest that the above triarylmethyl protecting groups are suitable for use in the synthesis of oligodeoxyribonucleotides by the phosphotriester approach. Clearly, the 5'-*O*-triarylmethyl-2'-deoxyribonucleoside building blocks **69a** and **69b** may be used in conjunction with 3'-*O*-acyl derivatives **70** and other 3'-protected building blocks with acid-stable protecting groups. If 3'-*O*-acyl derivatives **70** are used in the phosphotriester approach and if it is intended that the acyl groups should be removed by alkaline hydrolysis, the latter protecting groups should be chosen in such a way that they can be removed under mild conditions thereby ensuring that concomitant phosphotriester hydrolysis is kept to a minimum. As in the ribose series (see above), aryloxyacetyl groups (e.g. phenoxyacetyl[35] in **70**; R = CH_2OPh) to some extent meet this requirement.

Preparation of building blocks

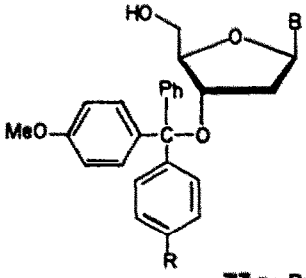
5'-*O*-triarylmethyl derivatives (such as **69a** and **69b**) may be prepared[41] in high yields by the action of the appropriate triarylmethyl chlorides on 2'-deoxyribonucleosides and their *N*-acyl derivatives. The corresponding 3'-*O*-acyl derivatives **70** may clearly be prepared in two steps (acylation followed by acidic hydrolysis) from 5'-*O*-triarylmethyl derivatives **69a** or **69b**. Thus building blocks with 3'-base-labile and 5'-acid-labile protecting groups are readily obtainable.



71



72a; X=O
b; X=S



73a; R=H
b; R=OMe

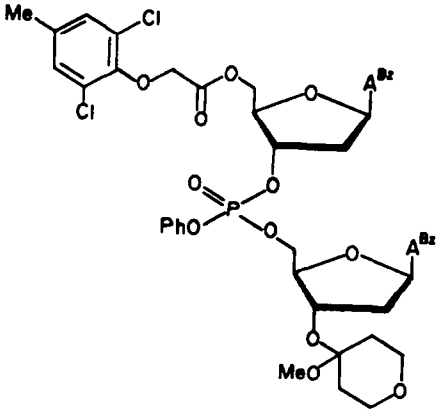
Building blocks with 3'-acid-labile and 5'-base-labile protecting groups have also been prepared. For example, 2'-deoxyribonucleosides and their *N*-acyl derivatives may be acylated regioselectively by reaction with 2,6-dichloro-4-methylphenoxyacetyl chloride[64] to give the 5'-protected building blocks **71** in satisfactory yields.[65,66]

The corresponding 3'-*O*-methoxytetrahydropyranyl derivatives **72a** may then be prepared[67] in two steps (acetalation, followed by deacylation) from the latter **71** or from other 5'-*O*-acyl derivatives. Preliminary experiments (see below) indicate that the removal of methoxytetrahydropyranyl protecting groups by acidic hydrolysis is accompanied[68] by at most a very small amount of depurination. 5' - *O* - Acyl - 2' - deoxyribonucleoside derivatives (such as **71**) may also be converted into the corresponding 3'-*O*-methoxytetrahydrothiopyranyl[69] **72b** and 3'-*O*-triarylmethyl[70] derivatives **73a** and **73b**. The methoxytetrahydrothiopyranyl has been shown[69] to be *ca.* 5 times more labile in aqueous acid solution than the methoxytetrahydropyranyl protecting group and both the 4-methoxy- and 4,4'-dimethoxy-trityl groups appear to be removable under conditions (see below) which lead to no depurination. As 2'-deoxyribonucleosides and their *N*-acyl derivatives can so readily[41] be converted into the corresponding 5'-*O*-triarylmethyl compounds (such as **69**) in high yields and as it appears (see below) that unblocking can be effected without the occurrence of any undesirable side-reactions, it seems that the latter 5'-protected building blocks (e.g. **69**) are particularly suitable for oligodeoxyribonucleotide synthesis by the phosphotriester approach.

Acidic hydrolysis of purine 2-deoxyribosides and derivatives

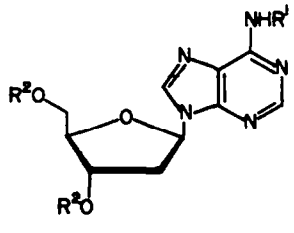
As suggested above, the glycosidic linkages of purine 2-deoxyribosides are very sensitive to acidic hydrolysis. Thus the halftimes for the hydrolysis of 2'-deoxyadenosine **74a** and 2'-deoxyguanosine **75a** in 0.1 M-hydrochloric acid at 23° are reported[71] to be 105 and 100 min, respectively. The corresponding half-times for the hydrolysis of **74a** and **75a** in 0.2 M-hydrochloric acid–dioxan (1:1 v/v) at 20° are *ca.* 8 and 7 hr,[68] respectively and under the latter conditions the time required for the complete removal of the methoxytetrahydroyranyl group from 3'-*O*-methoxytetrahydropyranyl-2'-deoxyadenosine (**72a**; B = **43**) is *ca.* 35 min.[68] It would therefore be expected that the removal of a methoxytetrahydropyranyl group from an oligodeoxyribonucleotide in aqueous dioxan solution would be accompanied by *ca.* 5% cleavage of each of the purine residues. This is clearly unacceptable. Fortunately, however, water–acetic acid (1:4 v/v) is a more suitable unblocking reagent. Although the complete removal of the methoxytetrahydropyranyl group takes *ca.* 6 hr[68] in 80% acetic acid at 20°, very little (possibly *ca.* 1%) depurination of 2'-deoxyadenosine **74a** and 2'-deoxyguanosine **75a** occurs under these conditions. The removal of 4-methoxy- and 4,4'-dimethoxy-trityl protecting groups occurs even more easily in 80% acetic acid: for example, the thymidine derivatives (**69a** and **69b**; B = thymin-1-yl) are reported[41] to undergo complete hydrolysis in 90 and 15 min at 26 and 27°, respectively in this medium. It is further interesting to note[68] that 4-methoxy- and 4,4'-dimethoxy-trityl are both removed more slowly than methoxytetrahydropyranyl groups in 0.2 M-hydrochloric acid–dioxan (1:1 v/v).

A very important factor which must be taken into account if acid-labile protecting groups are to be used in oligodeoxyribonucleotide synthesis is that the glycosidic linkage of N^6-benzoyl-2'-deoxyadenosine **74b** [and presumably other N^6-acyl derivatives of **74a**] is appreciably more labile to acidic hydrolysis than the glycosidic linkage of 2'-deoxyadenosine **74a** itself. For example, the half-time for the hydrolysis of **74b** in 0.2 M-hydrochloric acid–dioxan (1:1 v/v) at 20° is *ca.* 1 hr,[68] that is one-eight of the half-time for the hydrolysis of **74a** under the same conditions (see above). It is further noteworthy[68] that the half-times, in the same medium for the cleavage of the glycosidic linkages of N^2-benzoyl-2'-deoxyguanosine **75b** and 2'-deoxyguanosine **75a** itself are virtually identical. Although the glycosidic linkage of **75b** is more stable than that of **74b** in 80% acetic acid, it is, however, considerably less stable than that of **75a** in the latter medium.[53]

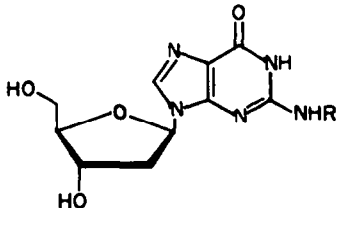


76 ; A^{Bz} = N^6-benzoyladenin-9-yl

Although the exceptional lability of the glycosidic linkage of **74b** (or rather of its 4-methoxytrityl derivative) was noted[41] in the early literature on oligodeoxyribonucleotide synthesis, the consequences of this observation are perhaps not widely appreciated. An obvious consequence in the phosphodiester approach and in the final unblocking steps of the phosphotriester approach is that the *N*-acyl protecting groups should be removed from the base residues *before* any step involving acid



74a ; R^1=R^2=H
b ; R^1=Bz, R^2=H
c ; R^1=Bz, R^2=Ac



75a ; R=H
b ; R=Bz

treatment (preferably with 80% acetic acid) is carried out. The consequences of the lability of the glycosidic linkage of N^6-benzoyl-2'-deoxyadenosine **74b** in the chain extension steps of the phosphotriester approach in which it is necessary to remove acid-labile groups without unblocking the base residues, are not as serious as might have been anticipated. Thus it is possible[68] to remove the 3'-*O*-methoxytetrahydropyranyl group from the fully-protected dinucloside phosphate **76** either by treatment with 80% acetic acid (6 hr, 20°) or with 0.2 M-hydrochloric acid–dioxan (1:1 v/v) (1.5 hr, 20°) with only a small amount of concomitant depurination.[c] It seems likely that the increased stability of the glycosidic linkages of **76** is due to the inductive effects of the 5'-*O*-aryloxyacetyl and the 3',5'-phosphotriester groups. This hypothesis is supported by the observation[68] that the rate of depurination of 3',5'-di-*O*-acetyl-N^6-benzoyl-2'-deoxyadenosine **74c** in 0.2 M-hydrochloric acid–dioxan (1:1 v/v) is only *ca.* one-twentieth that of **74b**.

STAGE 2—OLIGONUCLEOTIDE SYNTHESIS

The development of the phosphotriester approach

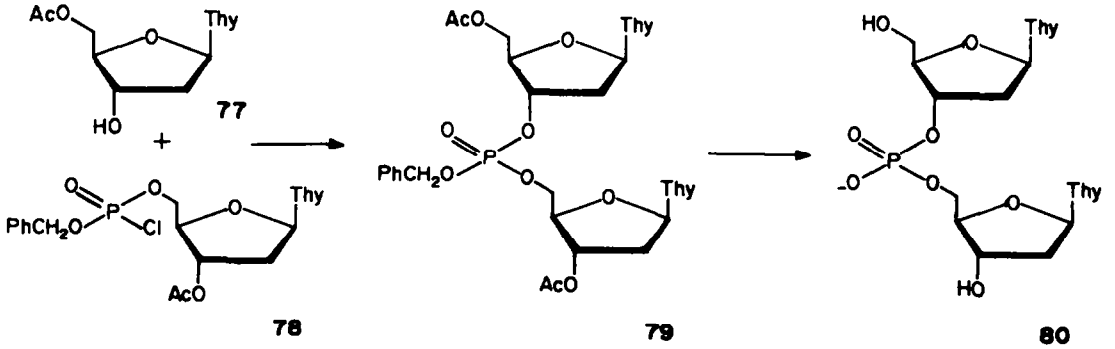
As stated above, the choice of a group R (see Scheme 2) for the protection of the internucleotide linkages is a crucial problem in the synthesis of oligonucleotides by the phosphotriester approach. This group must, of course, fulfil the principal criteria [(i)–(iii)] indicated above for protecting groups. The benzyl protecting group was used[73] in the first phosphotriester synthesis of an oligonucleotide containing a natural 3'→5'-internucleotide linkage. It is interesting to note that the report of this work by Michelson and Todd[73] in 1955 antedates the first paper, by Khorana *et al.*,[6] on the synthesis of an oligonucleotide by the phosphodiester approach.

In their synthesis of TpT **80** by the phosphotriester approach (Scheme 12), Michelson and Todd[73] phosphorylated 5'-*O*-acetylthymidine **77** with the nucleoside 5'-benzyl phosphorochloridate derivative **78** to give the fully-protected dinucleoside phosphate **79**. Removal of the protecting groups gave TpT **80**. In the same paper, the synthesis of pTpT, the 5'-phosphate ester of **80** was also described. Despite these promising initial results, no other work on the synthesis of oligonucleotides by the phosphotriester approach was reported during the following 10 years. In 1965, Letsinger *et al.*[74] re-examined the latter approach using the 2-cyanoethyl group to protect the internucleotide linkages.

In their original studies on oligonucleotide synthesis by the phosphotriester approach, Letsinger *et al.* investigated the possibility of using a polymer support.[74a] Then, in order to carry out preparations on a large scale, these workers[74b] investigated the use of the phosphotriester approach in homogeneous solution according to the procedure outlined in Scheme 13. The 5'-protected thymidine derivative **81** is allowed to react with 2-cyanoethyl phosphate in the presence of an arenesulphonyl chloride in pyridine solution to give the 3'-phosphate ester **82**. In the second phosphorylation step, **82** is allowed to react with an unprotected 2'-deoxyribonucleoside **67a** in the presence of 2,4,6-triisopropylbenzenesulphonyl chloride (TPS, **11b**) in pyridine solution to give **83a**. The latter product **83a**, which is contaminated with a small amount (*ca.* 4%) of material with unnatural 3'→3'-internucleotide linkages, may be completely unblocked by treatment first with 80% acetic acid (to remove the MMTr group) and then with dilute ammonia (to remove the 2-cyanoethyl group); alternatively, **83a** may be converted into its 2-cyanoethyl ester (**83**; R = $PO(O^-)OCH_2CH_2CN$) and the chain extended further. In this way, the oligothymidylic acids $(Tp)_2T$ and $(Tp)_3T$ were prepared from **83a** (B = thymin-1-yl).

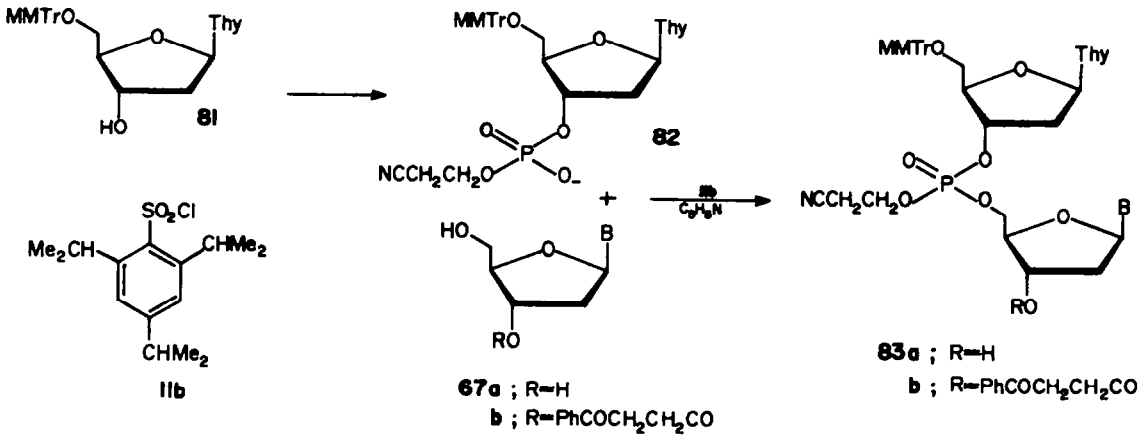
In a later paper, Letsinger *et al.*[75a] abandoned the use of unprotected 2'-deoxyribonucleosides **67a** in the chain-extension steps and instead used the corresponding 3'-*O*-β-benzoylpropionyl derivatives **67b**, thereby preventing the formation of any 3'→3'-internucleotide linkages. The β-benzoylpropionyl group may be removed[75a] by treatment with hydrazine hydrate in pyridine–acetic acid under conditions which do not lead to the unblocking of internucleotide linkages. Using this modification, Letsinger *et al.*[75b] carried out the stepwise synthesis of oligodeoxyribonucleotides containing bases other than thymine and also the block synthesis (i.e. 2+2→4 and

[c] A small amount of depurination of phosphotriester intermediates need not be a particularly serious matter as the resulting "apurinic acid" may possibly be removed by adsorption chromatography. The removal of a 4,4'-dimethoxytrityl protecting group from a similar phosphotriester intermediate, by treatment with 80% acetic acid, would be expected[68] to lead to a negligible amount of cleavage of N^6-benzoyladenine residues. Very recent experiments indicate that 4,4'-dimethoxytrityl[72] and related[53] protecting groups may be removed very rapidly and with virtually no concomitant depurination from phosphotriester intermediates by treatment with an arenesulphonic acid in chloroform containing methanol.



Thy = thymin-1-yl

Scheme 12.



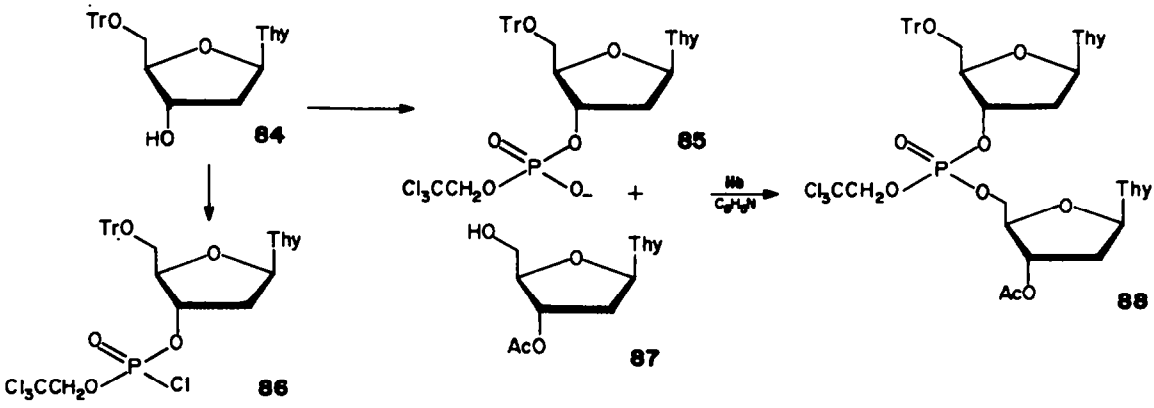
Thy = thymin-1-yl

Scheme 13.

3+3→6) of the tetramer, $(Tp)_3T$ and the hexamer, $(Tp)_5T$.

Both the benzyl and the 2-cyanoethyl protecting groups have the great advantage that they can be removed from the internucleotide linkages at the end of the synthesis under very mild conditions indeed. Debenzylation may readily be effected[76] by thiophenoxide ion at room temperature and 2-cyanoethyl protecting groups are removable[74,75] under very mild basic conditions. Furthermore, both unblocking reactions involve specific *O*-alkyl fission and therefore no cleavage of the internucleotide linkages should occur. Thus criterion (iii) (see above) is fulfilled for both protecting groups. The remaining question is whether criterion (ii) is fulfilled, i.e. whether these protecting groups are completely stable under the reaction conditions. If a comparatively long oligonucleotide is to be synthesized, a number of phosphorylation and purification steps will be necessary. If phosphorylation reactions are to be carried out in pyridine solution or in the presence of another base catalyst, it is conceivable that some concomitant debenzylation might occur. Loss of very sensitive 2-cyanoethyl protecting groups may occur during work-up and chromatography of the phosphotriester intermediates on silica gel. Loss of either protecting group could also occur during the unblocking of terminal hydroxy functions prior to chain-extension. It should nevertheless be emphasized that neither of these protecting groups has been used in the synthesis of high molecular weight oligonucleotides and, therefore, that their possible limitations have not been fully elucidated. However, it would seem unwise to embark on a synthesis of a high molecular weight oligo- or polynucleotide, involving a large number of phosphorylation and purification steps, without first ensuring that the internucleotide linkages are blocked with comparatively stable protecting groups.

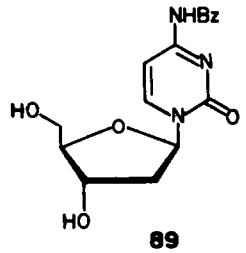
In 1967, Eckstein and Rizk[77] showed that the 2,2,2-trichloroethyl group could be used to protect the internucleotide linkages in oligodeoxyribonucleotide synthesis. In their first study,[77a] these workers converted 5'-*O*-tritylthymidine **84** into its 3'-(2,2,2-trichloroethyl) phosphate **85**. The latter compound (**85**) was then allowed to react (Scheme 14) with 3'-*O*-acetylthymidine (**87**) in the presence of TPS 11b to give **88**. This fully-protected dinucleoside phosphate **88** was completely unblocked by treatment with (i) zinc dust/80% acetic acid at room temperature (to remove the 2,2,2-trichloroethyl group), (ii) hot 80% acetic acid (to remove the trityl group) and (iii) aqueous ammonia (to remove the acetyl group) to give TpT **89**. In a second and closely-related study,[77b] **84** was treated with 2,2,2-trichloroethyl phosphorodichloridate to give the intermediate phosphoroch-



Thy = thymin-1-yl

Scheme 14.

loridate **86** which was then allowed to react with **87** to give **88**. The trimer $(Tp)_2T$ was similarly prepared in a step-wise (1+2→3) synthesis and the tetramer $(Tp)_3T$ in a block (2+2→4) synthesis. These workers also prepared[77c] short oligodeoxyribonucleotides containing adenine and cytosine residues.

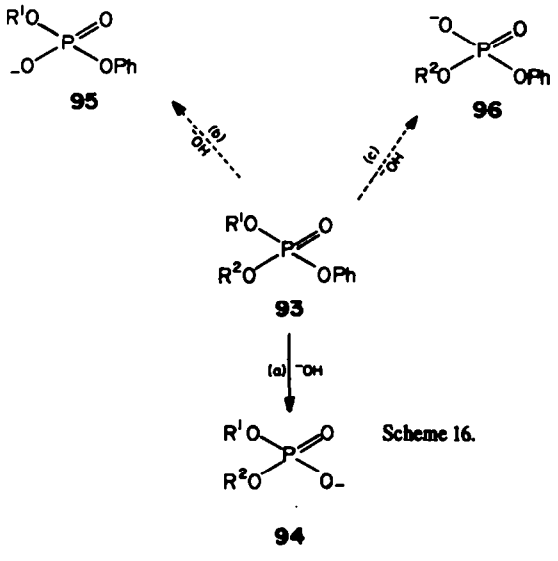


89

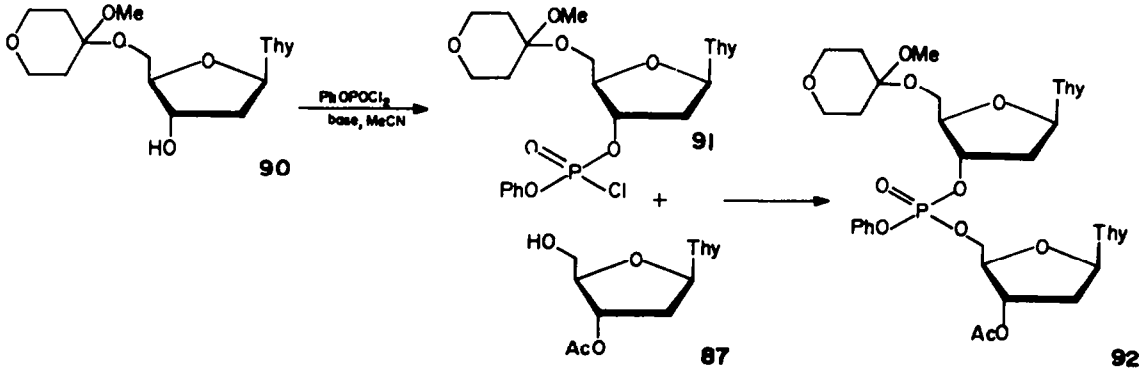
The 2,2,2-trichloroethyl protecting group would be expected to remain intact during the chain-extension and purification steps of oligonucleotide synthesis provided that relatively mild conditions of alkaline hydrolysis were used to remove acyl protecting groups, if any, from the terminal hydroxy functions. A further merit of the 2,2,2-trichloroethyl group is that, like the benzyl and 2-cyanoethyl protecting groups, it may be removed by specific *O*-alkyl cleavage. However, it is by no means clear from the literature that criterion (iii) (see above) for protecting groups is fulfilled, i.e. if 2,2,2-trichloroethyl groups may be removed at the end of the synthesis without the occurrence of undesirable side reactions. The 2,2,2-trichloroethyl group has been removed by treatment with zinc dust or zinc-copper couple in (a) aqueous acetic acid,[77] (b) pyridine containing a protic acid[78,79] and (c) anhydrous dimethylformamide[61,77,80]; it has also been removed[81] by sodium naphthalene in hexamethylphosphoric triamide [procedure (d)]. It would seem that procedure (a) is unsuitable in nucleic acid chemistry in that its use would very likely lead to partial reduction of the pyrimidine bases. Thus Cook[82] reported that a loss in ultraviolet absorption is observed when N^4-benzoyl-2′-deoxycytidine **89** is treated with zinc–copper couple in 90% acetic acid at room temperature. Procedure (c) which involves aprotic reaction conditions would appear, in principle, to be safer than procedure (b). If procedure (d) is followed, strictly anhydrous conditions are necessary in order to ensure that concomitant alkaline hydrolysis of the phosphotriester groups is avoided. As procedures (a)–(c) involve heterogeneous reactions, there is a possibility that the unblocking conditions will not be reproducible. Furthermore, it may be difficult to ensure that, and indeed determine whether all the 2,2,2-trichloroethyl groups are removed from a large protected oligonucleotide.

In 1968, Reese and Saffhill[83] demonstrated that the phenyl protecting group could be used in the synthesis of TpT **80**. 5′-*O*-Methoxytetrahydropyranylthymidine **90** was treated with a very slight excess of phenyl phosphorodichloridate in the presence of 2,6-lutidine in acetonitrile solution to give (Scheme 15) the putative intermediate phosphorochloridate **91**. After a suitable period of time, the 3′-protected building block **87** was added and the second phosphorylation step was allowed to proceed to give the fully-protected dinucleoside phosphate **92** in satisfactory yield. The latter product **92** was unblocked by treatment with (a) alkali for a short time (to remove the acetyl group), (b) acid (to remove the methoxytetrahydropyranyl group) and (c) alkali for a longer time (to unblock the internucleotide linkage). Although it was not recognized at the time, this unblocking procedure[83] led to a mixture of thymidylyl-(3′→5′)-thymidine **80** and its two symmetrical isomers (see below).

The phenyl group is stable enough to remain intact



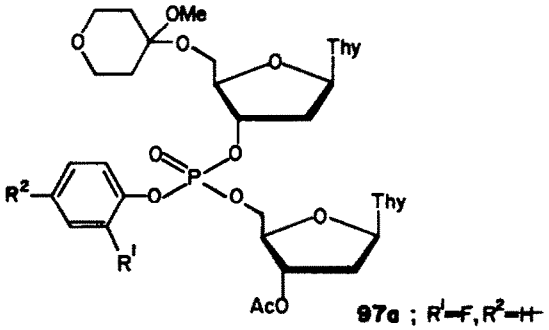
Scheme 16.



Thy = thymin-1-yl

Scheme 15.

during the chain-extension and purification steps of oligonucleotide synthesis again provided[14] that any *O*-acyl protecting groups which are used are removable under, at most, mild conditions of alkaline hydrolysis. The phenyl group has one distinct disadvantage over the benzyl, 2-cyanoethyl and 2,2,2-trichloroethyl protecting groups in that its removal involves attack by hydroxide ion, or by some other nucleophile, on phosphorus with subsequent *O*-phosphoryl cleavage. As phenol is a stronger acid than a simple alcohol by some 5 or 6 orders of magnitude, alkaline hydrolysis of phenyl protected internucleotide linkages (represented by **93**, Scheme 16) might be expected to proceed virtually exclusively by expulsion of phenoxide ion [pathway (a)] to give **94**. Unfortunately, this is not the case: alkaline hydrolysis of phenyl protected oligonucleotides (represented by **93**) also proceeds[84] to some extent with internucleotide cleavage [i.e. by pathways (b) and (c)] to give phenyl alkyl phosphates **95** and **96** in significant quantities. The consequences of such internucleotide cleavage are not serious in the unblocking of oligonucleotides containing only a few residues; however, as will become apparent later, they are very serious indeed in the unblocking of high molecular weight phenyl protected oligo- and polynucleotides.



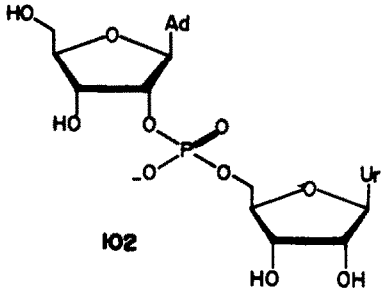
97a ; R^1=F, R^2=H
b ; R^1=Cl, R^2=H
c ; R^1=H, R^2=Cl

Thy = thymin-1-yl

The extent of internucleotide cleavage accompanying alkaline hydrolysis can easily be moderated (see below) by the introduction of electron-withdrawing substituents into the phenyl group as in the fully-protected dinucleoside phosphates **97a–c**. The latter were synthesized[14,85] in satisfactory yields by the procedure indicated in Scheme 15 using *o*-fluoro-, *o*-chloro- and *p*-chloro-phenyl phosphorodichloridates, respectively, instead of phenyl phosphorodichloridate. As well as having the advantage that it can easily be modified by substitution, the phenyl group has two other clear advantages over the 2,2,2-trichloroethyl protecting group. First, the undesirable by-products obtained during unblocking (i.e. the products of internucleotide cleavage rather than of pyrimidine reduction) can usually be separated from the desired products. Secondly, it is relatively easy to ensure that the alkaline hydrolysis of phenyl protected oligonucleotides, which is a homogeneous reaction, goes to completion. Aryl protecting groups also have a general advantage in that they can be incorporated into stable and powerful phosphorylating agents. Taking all these factors into consideration, it seems likely that, if the extent of internucleotide cleavage during unblocking can be decreased substantially, aryl groups will emerge as the protecting groups of choice in the synthesis of oligo- and poly-nucleotides by the phosphotriester approach.

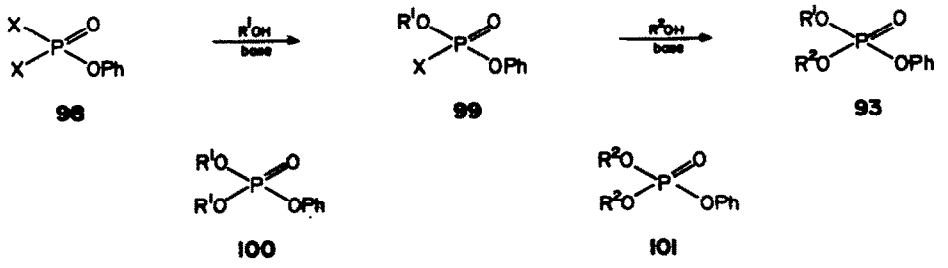
If a bifunctional phosphorylating agent, such as phenyl phosphorodichloridate (**98**; X = Cl) is used in the phosphotriester approach (Scheme 17), it is possible to obtain the symmetrical 3′→3′- and 5′→5′-phosphotriesters **100** and **101**, respectively in addition to the desired unsymmetrical product **93**. The formation of such symmetrical products is indeed observed[67] when bifunctional phosphorylating agents are used in the synthesis of oligodeoxyribonucleotides. Thus, for example, an intermediate phosphorochloridate (**99**; X = Cl) can, as soon as it is formed, compete with the remaining phosphorodichloridate (**98**; X = Cl) for the 5′-protected building block (R^1OH). If stoicheiometric quantities of **98** (X = Cl) and R^1OH are used and some symmetrical 3′→3′-phosphotriester **100** is formed, then the remaining **98** (X = Cl) can react with the 3′-protected building block (R^2OH) when it is added. In this way the symmetrical 5′→5′-phosphotriester **101** may also be formed. It is therefore desirable that, whatever protecting group is used in the phosphotriester approach, the phosphorylating agent should be monofunctional.

All the examples of the use of the phosphotriester approach which have been considered so far relate to the



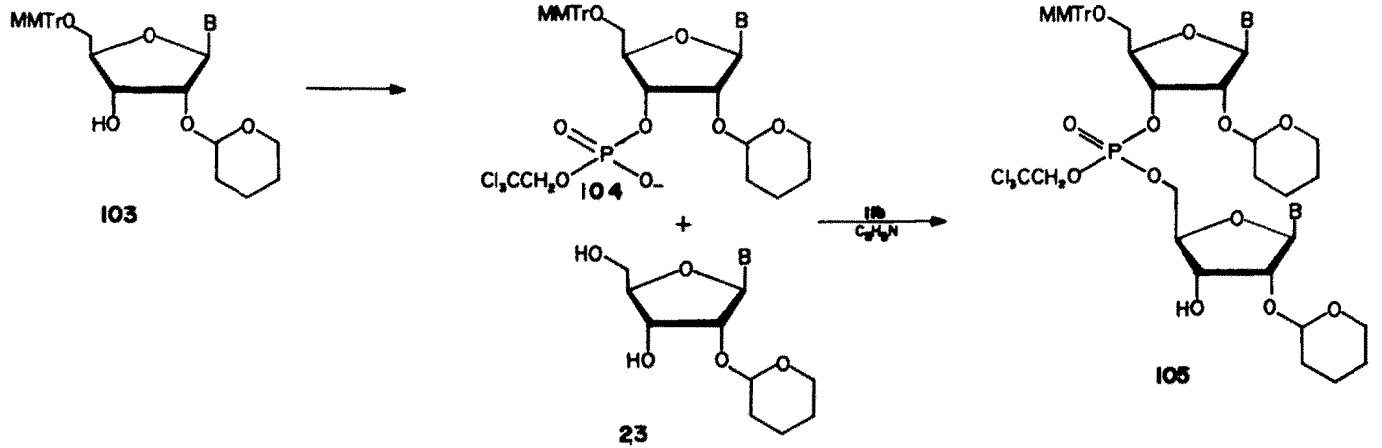
102

Ad = adenin-9-yl; Ur = uracil-1-yl



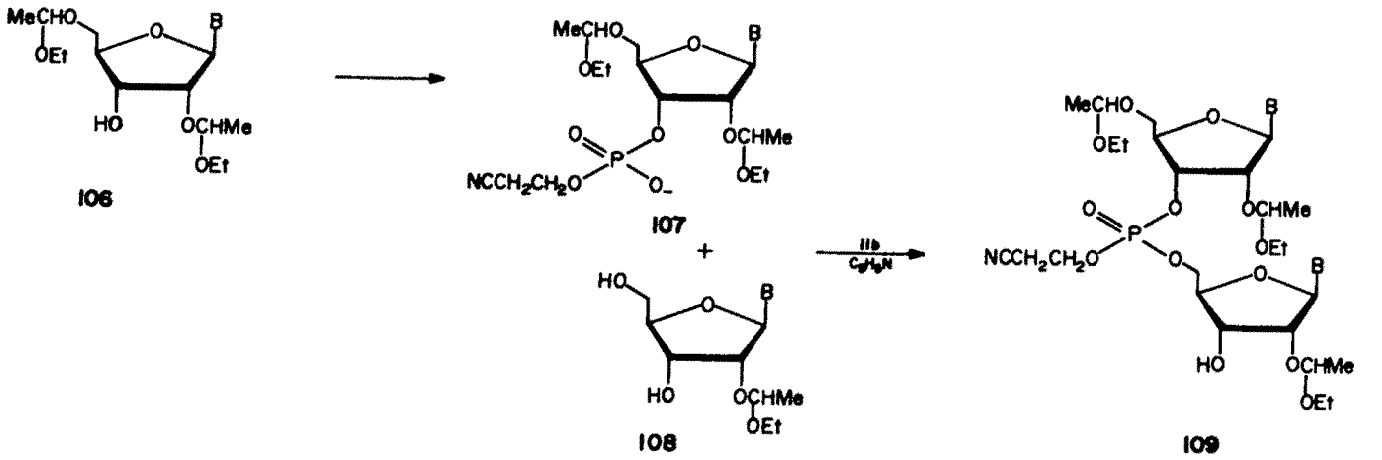
98 **99** **93** **100** **101**

Scheme 17.



B = uracil-1-yl

Scheme 18.



B = uracil-1-yl

Scheme 19.

synthesis of oligodeoxyribonucleotides. The first oligoribonucleotide synthesized by the phosphotriester approach was adenylyl-(2′→5′)-uridine[86] **102** which has an unnatural internucleotide linkage. In 1969, Neilson[60] reported the synthesis of UpU and $(Up)_2U$ by the phosphotriester approach, using the 2,2,2-trichloroethyl protecting group, as indicated in Scheme 18.

The 2′,5′-terminal building block **103**, prepared by the action of 4-methoxytrityl chloride on 2′-*O*-tetrahydropyranyluridine **23**, was phosphorylated by treatment with pyridinium 2,2,2-trichloroethyl phosphate and TPS **11b** in pyridine solution to give **104**. In the second step of the phosphorylation, **104** and **23** were allowed to react together in the presence of TPS **11b** in pyridine solution to give the partially-protected dinucleoside phosphate **105** in satisfactory yield. None of the 3′→3′-isomer of **105**, which may be formed by phosphorylation of the more hindered 3′-hydroxy function of **23**, was detected in the products. Intermediate **105** was then linked together in the same way with **23** to give the partially-protected trinucleoside diphosphate. The latter trimer and **105** were unblocked by treatment with zinc–copper couple in dimethylformamide followed by acidic hydrolysis to give $(Up)_2U$ and UpU.

In a subsequent series of papers, Neilson *et al.*[61,87,88] have extended this procedure and have synthesized a number of oligoribonucleotides both by stepwise and by block synthesis. These workers invariably used 2′-*O*-tetrahydropyranylnucleosides[20,89] (such as **23**) instead of 2′,3′-protected non-terminal building blocks (such as **58**) and in order to avoid possible contamination with material containing 3′→3′-internucleotide linkages, the products were treated with an excess of 4-methoxytrityl chloride before fractionation. Neilson *et al.* have recently noted[88] that the recoveries of unprotected oligoribonucleotides after the unblocking process (including reduction with zinc–copper couple in dimethylformamide) are generally rather poor. Unless a much better unblocking procedure can be devised, these results must cast considerable doubt on the suitability of the 2,2,2-trichloroethyl protecting group in the synthesis of oligonucleotides by the phosphotriester approach. However, these studies clearly indicate that the phosphotriester is more suitable than the phosphodiester approach in the synthesis of oligoribonucleotides.

In 1970, Grams and Letsinger[90] reported a similar synthesis of UpU by the phosphotriester approach using, as in their original work in the deoxyribose series,[74] the 2-cyanoethyl protecting group (Scheme 19). The protected 3′-phosphodiester intermediate **107**, prepared from the corresponding 2′,5′-bisacetal **106**, is allowed to react with the 2′-acetal **108** in the presence of TPS **11b** in pyridine solution to give the partially-protected dinucleoside phosphate **109**. Treatment of the latter intermediate **109** with aqueous ammonia followed by 5% acetic acid gives UpU in satisfactory yield.

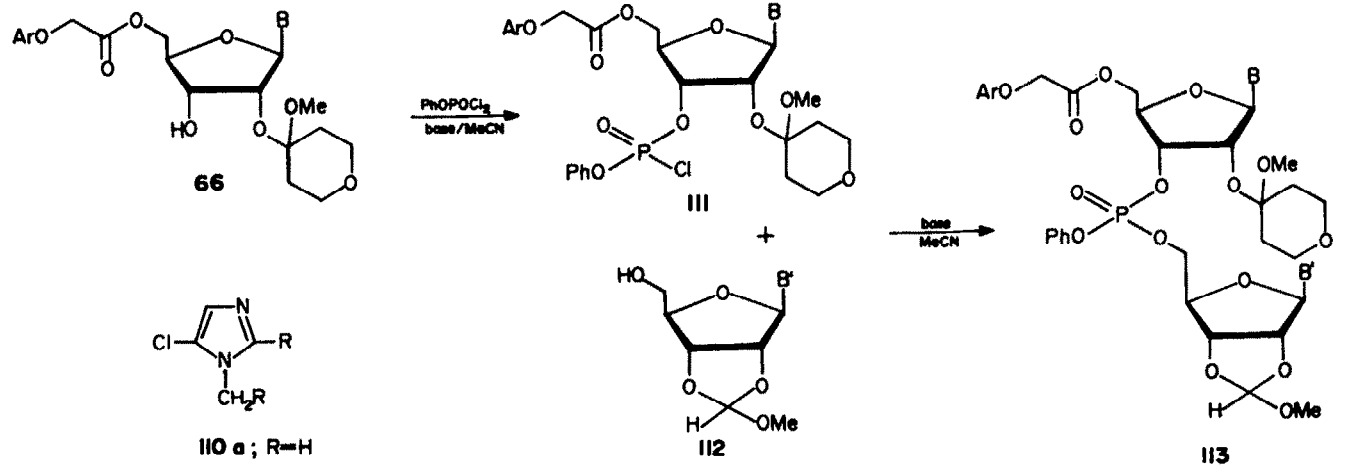
Although the phosphotriester approach, with phenyl protecting groups, had been used successfully[83] in the deoxyribose series, a difficulty was first encountered when it was used in the ribose series. Thus, for example, it was found that the reaction between the 2′,5′-protected non-terminal uridine derivative **66** and phenyl phosphorodichloridate proceeded much more slowly than the reaction between the latter phosphorylating agent and 5′-*O*-methoxytetrahydropyranylthymidine **90**. However, treatment of the non-terminal uridine building block **66** with phenyl phosphorodichloridate (Scheme 20) in the presence of 5-chloro-1-methylimidazole[14] **110a** in acetonitrile solution rapidly gives[56,59] the intermediate phosphorochloridate **111**. Treatment of **111** with the 2′,3′-protected terminal uridine derivative **112** gives the fully-protected dinucleoside phosphate **113** in high yield.[56,59] Unfortunately, the use of **110a** leads[56] to the formation of by-products which are difficult to separate from the desired product **113**. When **110a** is replaced by 5-chloro-1-ethyl-2-methylimidazole **110b**, the formation of such by-products is avoided but both steps of the phosphorylation process (Scheme 20) proceed more slowly and a lower yield of **113** is obtained.[56,59] Although aryl phosphorodichloridates appear to be rather unsatisfactory phosphorylating agents in oligoribonucleotide synthesis, it seems likely that **110b** and other 1-alkyl derivatives of imidazole will prove (see below) to be valuable as catalysts in phosphorylation reactions.

The combination of phenyl dihydrogen phosphate **114** and TPS **11b** constitutes a much more satisfactory phosphorylating agent for the synthesis of oligoribonucleotides than phenyl phosphorodichloridate. Phenyl dihydrogen phosphate[56,91,92] **114** is a non-hygroscopic crystalline solid and TPS **11b** may be used in excess to remove traces of moisture from the reaction medium. Treatment of the 2′,5′-non-terminal uridine building block **66** with **114** and an excess of **11b** in anhydrous pyridine solution gives[56,59] the intermediate **115** (Scheme 21). Reaction between the latter **115** and a small excess of the 2′,3′-terminal uridine building block **112** in the presence of **11b** gives **113** in high yield.[56,59] When the latter fully-protected dinucleoside phosphate **113** is treated with sodium hydroxide in aqueous dioxan for a short time, the partially-protected intermediate **116** is obtained.[56,59]

When **115** is allowed to react (Scheme 22) with the partially protected dinucleoside phosphate **116** in the presence of TPS **11b** in anhydrous pyridine solution and the product subjected to alkaline hydrolysis for a short time, the partially-protected trinucleoside diphosphate **117** is obtained in over 60% yield.[56,59] This stepwise synthesis may be continued and preliminary experiments have indicated that yields of *ca.* 60% are obtained in the overall conversion of **117** into the corresponding tetramer and in the conversion of the tetramer into the corresponding pentamer.[56]

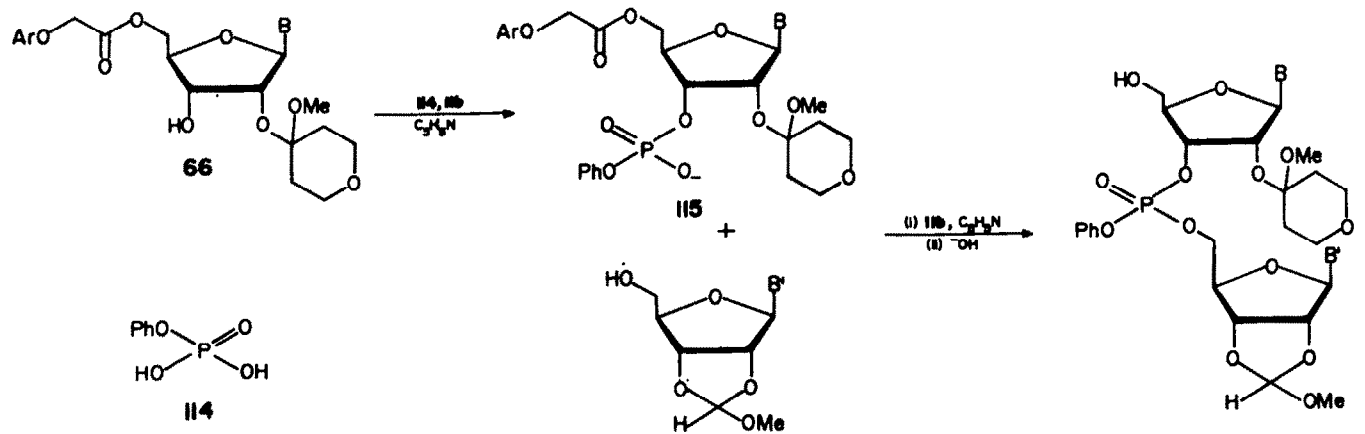
The unblocking of **116**, **117** and other phosphotriester intermediates with free 5′-hydroxy functions to give oligoribonucleotides with solely 3′→5′-internucleotide linkages cannot be effected simply by alkaline, followed by acidic hydrolysis. Thus when **116** is treated with alkali, the 5′→5′-isomer **120** is formed[56,59] in addition to the desired 3′,5′-dinucleoside phosphate **119**. When **118a** is treated with alkali (Scheme 23) under carefully controlled conditions, the cyclic phosphotriester **121** may be isolated[93] from the products in moderate yield and it is clear from further alkaline hydrolysis studies[93] that the conversion of **118a** into **119** and **120** proceeds solely *via* **121**.

Such neighbouring group participation of free 5′- and also free 3′-hydroxy functions leading to partial migration (usually *ca.* 20–30%) of the terminal internucleotide linkage or linkages has been observed both in the ribose[93] and the deoxyribose[93,94] series. Thus, as indicated above, complete unblocking of **92** leads to a mixture of thymidylyl-(3′→5′)-thymidine **80** and the isomeric 3′→3′- and 5′→5′-dinucleoside phosphates. Such phosphoryl migration cannot occur if both of the



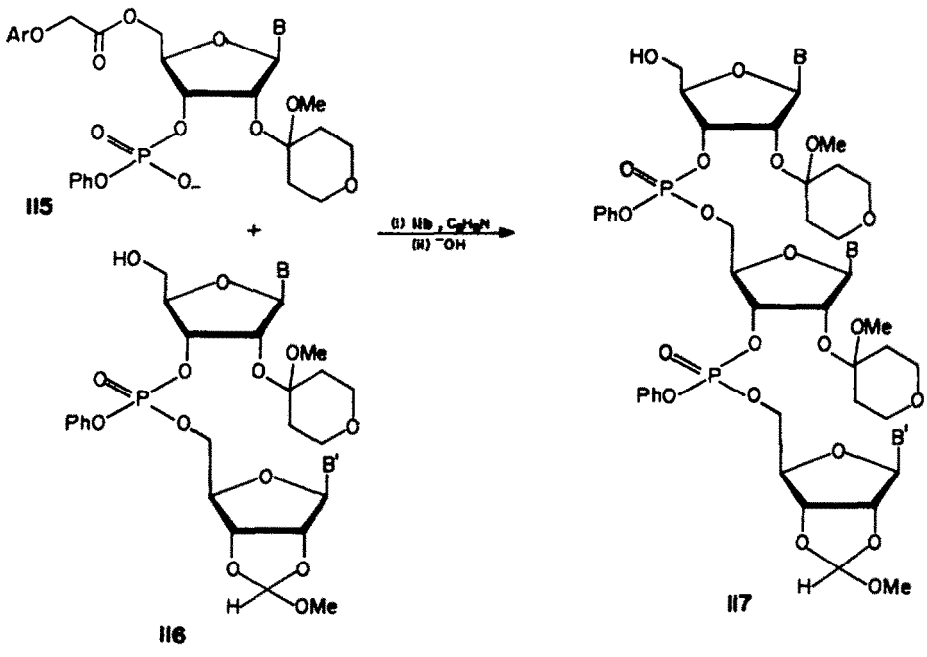
B = B' = uracil-1-yl; Ar = 4-ClC$_6$H$_4$

Scheme 20.



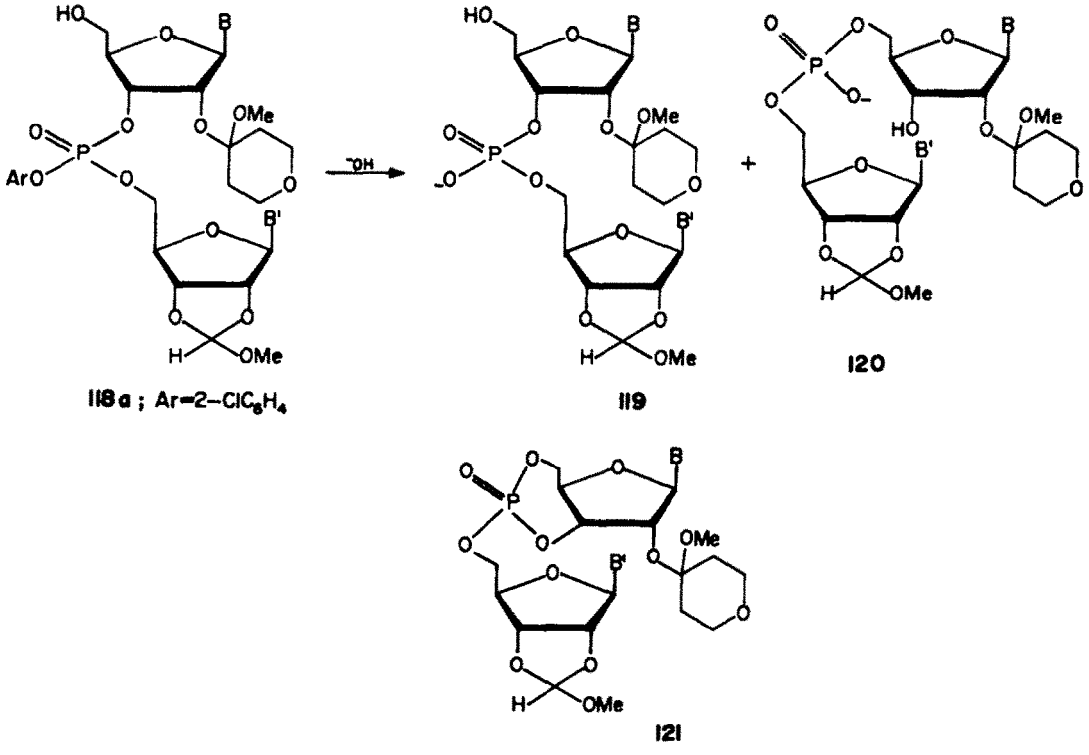
B = B' = uracil-1-yl; Ar = 4-ClC$_6$H$_4$

Scheme 21.



B = B′ = uracil-1-yl; Ar = 4-ClC$_6$H$_4$

Scheme 22.

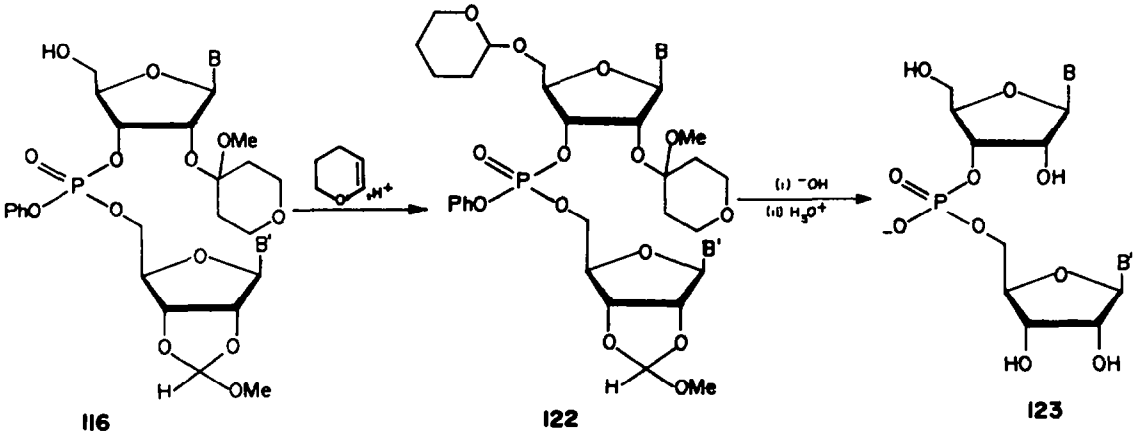


B = B′ = uracil-1-yl;

Scheme 23.

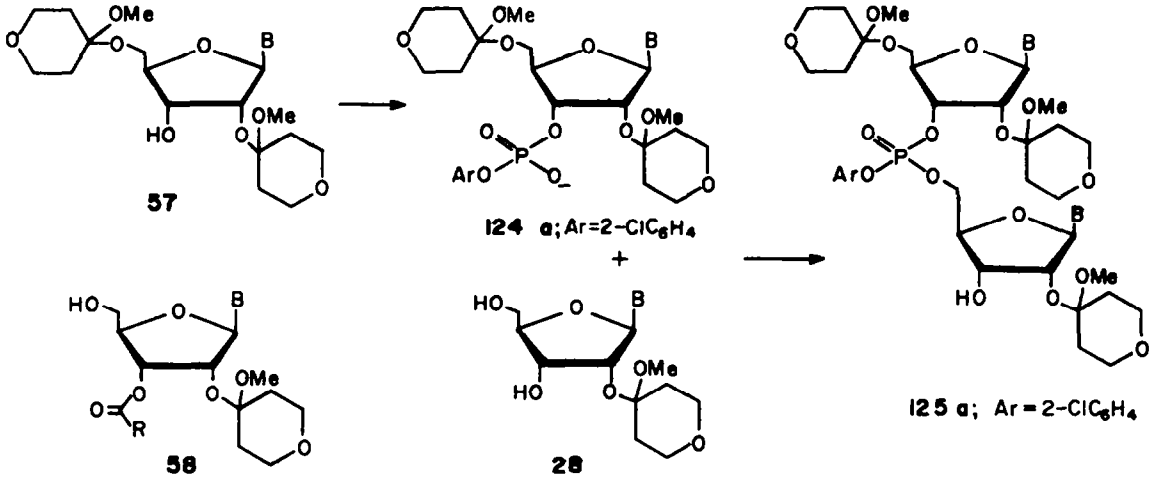
terminal 3′- and 5′-hydroxy functions are protected with base-stable groups before the alkaline hydrolysis step is undertaken. Indeed when **116** is tetrahydropyranylated (Scheme 24) and the fully-protected dinucleoside phosphate **122** subsequently unblocked by treatment first with alkali and then with aqueous acid, uridylyl-(3′→5′)-uridine (**123**; Scheme 24), free from its 5′→5′-isomer, is obtained.[56,59] Similarly, if **117** (Scheme 22) is tetrahydropyranylated before its is unblocked, UpUpU with solely 3′→5′-internucleotide linkages is obtained.[56,59]

The synthesis of oligoribonucleotides with aryl protecting groups, starting from 2′,5′-protected terminal



B = B' = uracil-1-yl

Scheme 24.



B = uracil-1-yl

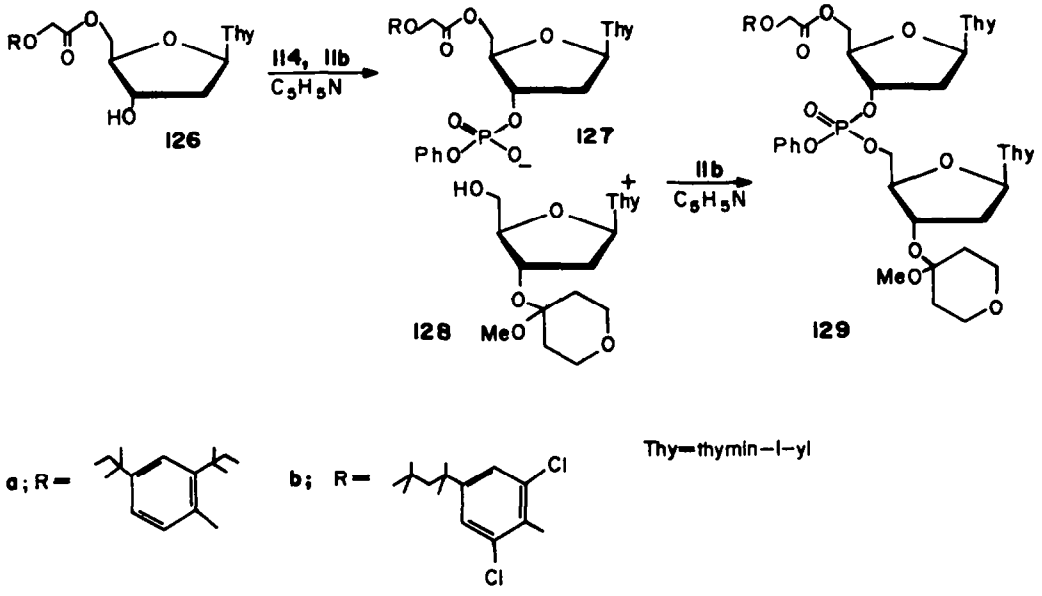
Scheme 25.

ribonucleoside building blocks **57**, has also been undertaken.[39] 2',5'-Di-*O*-methoxytetrahydropyranyluridine (**57**, Scheme 25) reacts with 2-chlorophenyl dihydrogen phosphate and TPS **11b** in pyridine solution to give the 3'-phosphate ester **124a**. Treatment of this intermediate **124a** with **28** in the presence of TPS in pyridine solution gives **125a** in good yield. The latter partially-protected dinucleoside phosphate **125a** may then be linked together with **28** in the same way to give the corresponding partially-protected trinucleoside diphosphate in good yield.[39] Although it was not appreciated at the time,[39] it is now clear[93] that it is also necessary to protect the 3'-hydroxy functions of **125a** and the corresponding partially-protected trinucleoside diphosphate with base-stable groups before the alkaline hydrolysis step is carried out if unblocked products with solely 3'→5'-internucleotide linkages are to be obtained. Although there is no evidence for the formation of the 3'→3'-isomer of **125a** in the reaction between **124a** and **28**, this possibility can be avoided altogether if the corresponding 2',3'-non-terminal ribonucleoside building block **58** is used instead of **28**.

As indicated above, the stepwise synthesis of oligoribonucleotides may be carried out by the phosphotriester approach with aryl protecting groups starting[36,39] from 2',5'-protected non-terminal and 2',3'-protected terminal ribonucleoside building blocks (**66** and **112**, respectively; Scheme 21) or from 2',5'-protected terminal and 2',3'-protected non-terminal ribonucleoside building blocks[39] (**57** and **58**, respectively; Scheme 25). In the first case (Schemes 21 and 22), partially-protected intermediates with free 5'-hydroxy functions (such as **116** and **117**) and in the second case (Scheme 25), partially-protected intermediates with free 3'-hydroxy functions (such as **125**) are obtained. Intermediates of these two types, for example **116** (or **117**) and **125**, may be linked together in a block synthesis. However, block synthesis by the phosphotriester approach with aryl protecting groups was first undertaken in the deoxyribose series.

Block synthesis of oligo- and poly-thymidylic acids by the phosphotriester approach

The block synthesis of oligothymidylic acids was carried out with the 5'-*O*-aryloxyacetylthymidine derivatives **126a**[95] and **126b**[67], phenyl dihydrogen phosphate

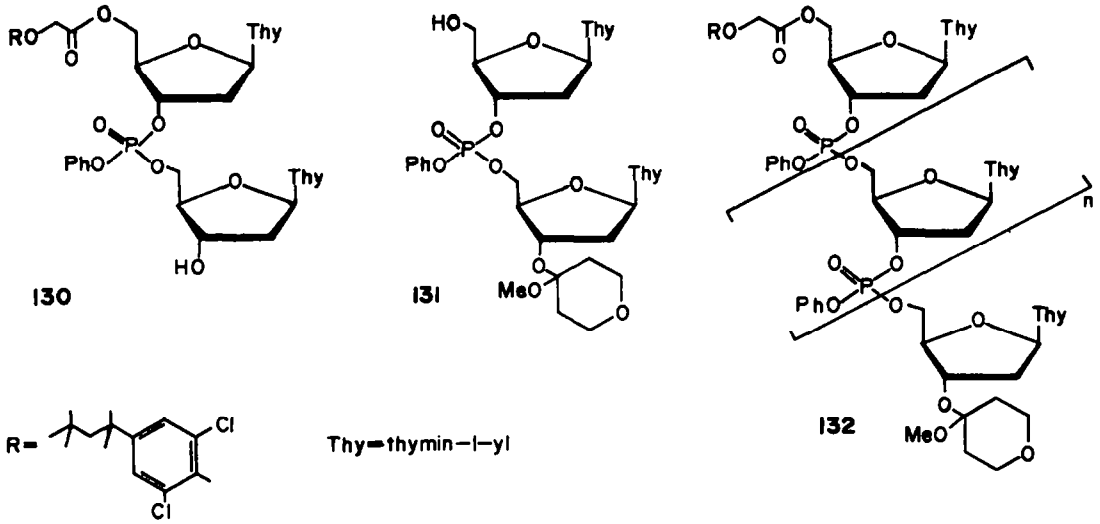


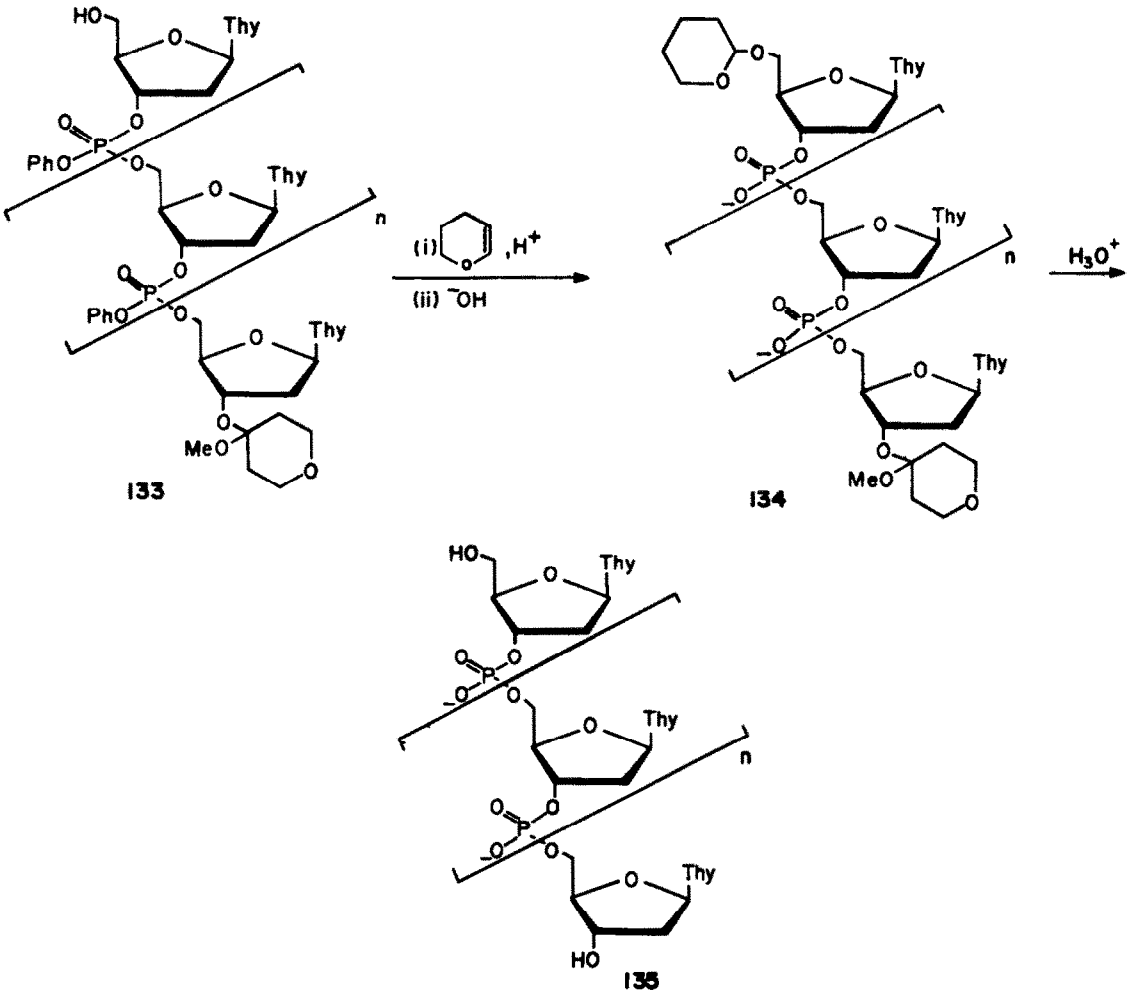
Scheme 26.

114 and 3'-*O*-methoxytetrahydropyranylthymidine 128 as starting materials. The fully-protected dinucleoside phosphates 129a[95] and 129b[67], which may be regarded as the basic units in block synthesis, were prepared according to the procedure outlined in Scheme 26 and isolated pure, following short column chromatography, in 75 and 71% yields, respectively. Preliminary studies[95] were carried out successfully with 129a but even more satisfactory results were obtained[67] (see below) when the block synthesis was based on 129b which differs from 129a only inasmuch as it has a more base-sensitive 5'-*O*-aryloxyacetyl protecting group. Both 129a and 129b were provided with lipophilic aryloxyacetyl protecting groups in order to promote their solubility and the solubility of higher molecular weight oligomers derived from them in organic solvents. Ready solubility in organic solvents and especially in chloroform is advantageous in the work-up of such phosphotriester intermediates and in their fractionation by short column chromatography.[96]

When 129b is treated with dilute acid, 130 is obtained[67] in high yield; when it is treated with dilute ammonia, 131 is obtained also in high yield. These dinucleoside phosphate blocks 130 and 131 may then be linked together in the same way as the nucleoside building blocks (126 and 128, Scheme 26) to give[67] the fully-protected tetramer (132; n = 2) in 62% isolated yield. Partially-protected tetramers with free 3'- and 5'-hydroxy functions, which are obtained in good yields when (132; n = 2) is treated with dilute acid and ammonia, respectively, may be linked together to give the fully protected octamer (132; n = 6) in 54% isolated yield. Two further repetitions of this sequence of steps leads to the fully-protected hexadecamer (132; n = 14) and dotriacontamer (132; n = 30) which may be isolated[67] in 42 and 48% yields, respectively.

Four steps are involved in the unblocking of the fully-protected oligonucleotides 132. The first step involves treatment with ammonia in aqueous dioxan to give the partially-protected oligonucleotides 133. The latter are tetrahydropyranylated (Scheme 27) and the products then subjected to alkaline hydrolysis to give the phosphodiester intermediates 134. The fourth step involves





Thy = thymin-1-yl

Scheme 27.

acidic hydrolysis of the 3'- and 5'-terminal acetal groups to give completely unprotected oligo- and poly-thymidylic acids **135**.

The dimer, tetramer, octamer and hexadecamer (**132**; n = 0, 2, 6 and 14, respectively) may all be unblocked in this way.[67] The dotriacontamer (**132**; n = 30), however, presented a problem in that treatment with ammonia in the first unblocking step led to an appreciable amount of concomitant phosphotriester hydrolysis. For this reason, the second step, i.e. tetrahydropyranylation, was omitted in the unblocking of the dotriacontamer (**132**; n = 30). It is therefore almost certain that migration of the internucleotide linkage at the 5'-end of the molecule occurred to some extent. The unprotected di-, tetra-, octa- and hexadeca-thymidylic acids (**135**; n = 0, 2, 6 and 14, respectively) were further purified by anion-exchange chromatography on DEAE-cellulose or DEAE-Sephadex. The yields of pure products obtained in this way were estimated to be 98, 94, 77.5 and 50%, respectively, of the total nucleoside and nucleotide products eluted from the ion-exchange columns.

As indicated very briefly above (Scheme 16), alkaline hydrolysis of phenyl protected internucleotide linkages proceeds to some extent by internucleotide cleavage. This becomes more and more serious as the number of internucleotide linkages increases, i.e. with increasing molecular weight of the oligonucleotides. Thus, while unblocking of the dimer (**132**; n = 0) which has one internucleotide linkage leads to only 2% loss of material by cleavage, unblocking of the hexadecamer (**132**; n = 14) which has fifteen internucleotide linkages leads to 50% loss of material. It appears that the loss resulting from internucleotide cleavage during the unblocking of phenyl esters by the above procedure amounts to *ca.* 2% per phosphotriester group for low molecular weight and *ca.* 3% per phosphotriester group for high molecular weight oligothymidylic acids.[67] It therefore seems likely that the unblocking of the dotriacontamer (**132**; n = 30) led to virtually no **135** (n = 30).

The elution pattern obtained from the chromatography of the fully unblocked octamer (**135**; n = 6) on DEAE-cellulose is illustrated in Fig. 1. The column was eluted with a linear gradient of triethylammonium hydrogen carbonate buffer (pH 7.5) and it is apparent that the cleavage products with charges of 0, −1, −2, −3, −4, −5 and to some extent −6 are separated from the desired $(Tp)_7T$ (**135**; n = 6) and the other possible products with a charge of −7. It can be seen from Scheme 28 that the unblocking of the fully-protected octamer **136** can lead to one product with zero charge (thymidine), three possible products with a charge of −1 (TpT and thymidine 3'- and 5'-phenyl phosphates), and four possible products with

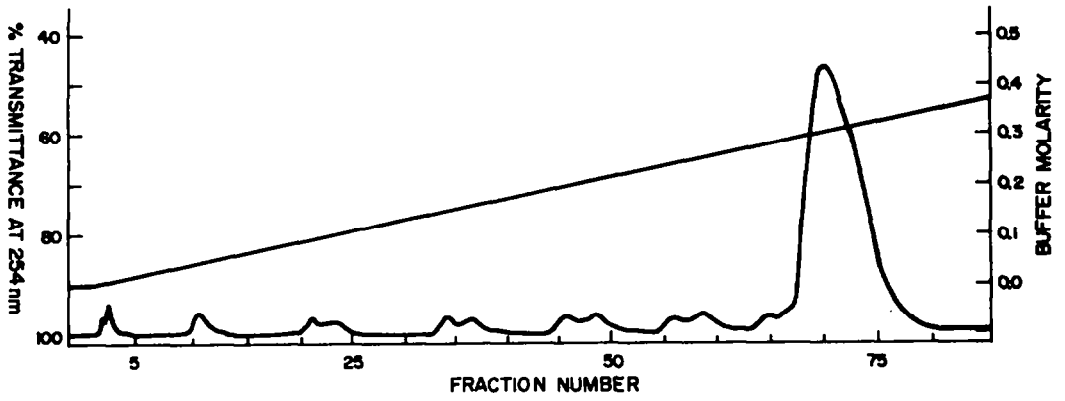
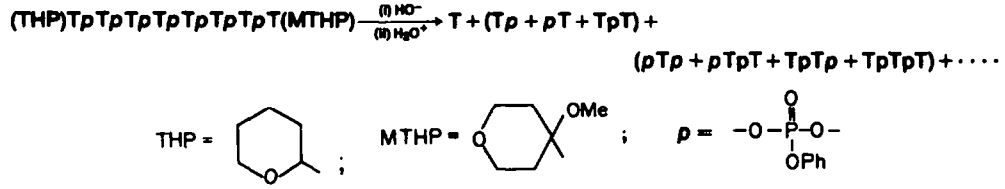


Fig. 1. Elution pattern from chromatography of fully-unblocked $(Tp)_7T$ (135; n = 6) on DEAE-cellulose with triethylammonium hydrogen carbonate buffer (pH 7.5; linear gradient, 0.001–0.6 M; *ca.* 14.5 ml fractions).

charges of −2. There are also four possible corresponding products with charges of −3, −4, −5, −6 and −7. It can be seen from Fig. 1 that either some of the possible products (Scheme 28) are not formed or that they are not fully separated by chromatography on DEAE-cellulose.

phenol itself and again effecting unblocking by hydroxide ion in the most suitable solvent.

(iii) By using phenyl or another aryl protecting group and effecting unblocking with a nucleophile other than hydroxide ion.



Scheme 28.

Although $(Tp)_7T$ (135; n = 6) accounted for over 75% of the total nucleotide products obtained by unblocking the fully-protected octamer (132; n = 6), the material isolated following DEAE-cellulose chromatography (Fig. 1, major peak) was almost certainly contaminated with the isomeric heptanucleotide phenyl esters corresponding to the dinucleotide derivatives (*p*TpT and TpT*p*) indicated in Scheme 28. Therefore the occurrence of significant (i.e. *ca.* 3% per phosphotriester group) internucleotide cleavage during the unblocking of phenyl-protected oligonucleotides is disadvantageous in two ways. Firstly, the yields of unprotected oligonucleotides inevitably become lower with increasing molecular weight. Secondly, the purification of unprotected oligonucleotides by DEAE-cellulose or DEAE-Sephadex chromatography is made more difficult by the presence of internucleotide cleavage products.

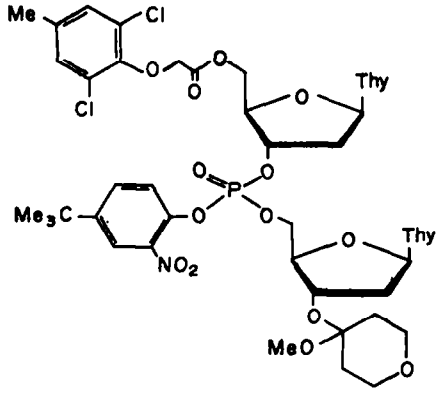
Attempts to suppress internucleotide cleavage during unblocking of the phosphotriester intermediates

If the phosphotriester approach with aryl protecting groups is to become the method of choice for the synthesis of oligo- and poly-nucleotides of high molecular weight, an unblocking procedure must be found which limits the extent of internucleotide cleavage to not more than, say, 0.5% per phosphotriester group. There are, in principle, at least three methods by which this target might be achieved:

(i) By retaining the phenyl protecting group and effecting unblocking with hydroxide ion in a more suitable solvent.

(ii) By replacing phenyl with an aryl protecting group derived from a phenol which is a stronger acid than

It now seems unlikely that the target of not more than 0.5% internucleotide cleavage will be achieved if method (i) is followed. Indeed 2–3% internucleotide cleavage per phosphotriester group occurs even under the most favourable conditions so far found.[67] When sodium hydroxide in water–dioxan (1:1 v/v) is used in the unblocking of the triphenyl ester of $(Tp)_3T$, over 7% internucleotide cleavage per phosphotriester group is observed.[67] Therefore, if hydroxide ion is to be used to effect unblocking, method (ii) must be used, i.e. the phenyl protecting group must be replaced by an aryl group derived from a more acidic phenol. Preliminary studies in the ribose series suggest[24] that an aryl group derived from a phenol with a pK_a of 7.5 or less is required if internucleotide cleavage accompanying attack by hydroxide ion is to be limited to 0.5% per phosphotriester group.



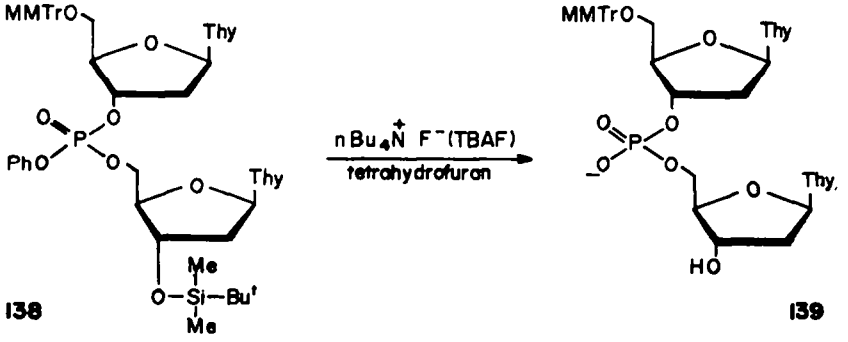
137 Thy = thymin-1-yl

The feasibility of limiting internucleotide cleavage by adopting method (ii) was investigated[65] by carrying out a block synthesis of oligothymidylic acids based on the fully-protected dinucleoside phosphate **137** which was obtained in 66% isolated yield from the appropriate thymidine building blocks, 2-nitro-4-t-butylphenyl dihydrogen phosphate and TPS **11b**. The 2-nitro-substituent was introduced into the aryl protecting group to reduce internucleotide cleavage during unblocking (the pK_a of 2-nitrophenol[97] is 7.23) and the 4-t-butyl substituent was introduced to increase the lipophilicity of the phosphotriester intermediates. It had been found[67] that the solubility of phenyl-protected oligonucleotides in chloroform decreases markedly with increasing molecular weight thus making the work-up and purification of the synthetic reaction products more difficult. The intermediate **137** was treated with acid and with base and thereby converted into the corresponding dinucleoside phosphates with free 3'- and free 5'-hydroxy functions, respectively. The latter were linked together to give the fully-protected tetramer in 58% isolated yield and repetition of this sequence of reactions led to the fully-protected octamer in 37% yield.[65]

The results obtained in the unblocking of the fully-protected dimer **137** and the corresponding tetramer by the general procedure indicated in Scheme 27, with 0.2 M-sodium hydroxide in water–dioxan (1:1 v/v) used to effect hydrolysis of the phosphotriester group, were very satisfactory in that internucleotide cleavage appeared[65] to be less than 0.5% per phosphotriester group. Furthermore, the use of a more lipophilic aryl protecting group constituted an improvement in that the phosphotriester intermediates proved to be readily soluble in chloroform. Unfortunately, however, 2-nitro-4-t-butylphenyl protected intermediates are difficult to work with in that their phosphotriester groups are extremely susceptible to basic hydrolysis. Indeed, it is necessary[65] to use an acidified eluting solvent to limit silica gel promoted hydrolysis during their purification by short column chromatography. It therefore seems unlikely that it will be possible to carry out the synthesis of high molecular weight oligo- and poly-nucleotides by the phosphotriester approach with an aryl protecting group derived from a phenol with a pK_a much below 8.5.

Method (iii) for unblocking aryl-protected internucleotide linkages has so far been little investigated. Indeed fluoride ion is the only alternative to hydroxide ion which has been proposed in the literature. Itakura *et al.*[98] have used fluoride ion [tetraethylammonium fluoride in tetrahydrofuran–pyridine–water (8:1:1 v/v)] to unblock *p*-chlorophenyl-protected internucleotide linkages of comparatively short oligodeoxyribonucleotides but have come to the conclusion that hydroxide ion is more suitable for unblocking higher molecular weight oligomers. However, the latter workers[98] did not discuss the occurrence of internucleotide cleavage during unblocking either by treatment with fluoride ion or by hydrolysis with sodium hydroxide in aqueous dioxan. Ogilvie *et al.*[99] have reported that treatment of the fully protected dinucleoside phosphate **138** with 2 molecular equivalents of tetra-n-butylammonium fluoride (TBAF) in tetrahydrofuran (Scheme 29) gives **139** with no detectable internucleotide cleavage. However, this result needs to be confirmed as treatment of **140a** with 2 molecular equivalents of TBAF in the presence of triethylamine (10 molecular equivalents) and water (3 molecular equivalents) leads[100] to *ca.* 20% internucleotide cleavage. Treatment of **140b** with 2 molecular equivalents of TBAF in tetrahydrofuran–pyridine–water (8:1:1 v/v), under conditions corresponding to those used by Itakura *et al.*,[98] leads[101] to *ca.* 3% internucleotide cleavage. Somewhat less (*ca.* 2%) internucleotide cleavage would be expected in the unblocking of **140b** by hydroxide ion in aqueous dioxan.

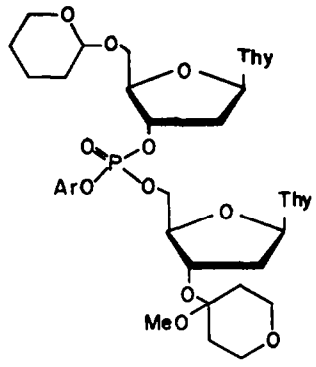
It therefore seems that fluoride ion is unsuitable for the unblocking of relatively high molecular weight aryl-protected oligo- and poly-nucleotides. Nevertheless method (iii) merits further investigation. In the conversion of **141** → **94** (Scheme 30), it is preferable that only one nucleophilic substitution reaction at phosphorus should occur. However, presumably two such substitution reactions occur in the case of fluoride ion. It is possible that formation of an intermediate dialkyl phosphorofluoridate (**142**; X = F) involves some internucleotide cleavage [i.e. R^1O^- or R^2O^- instead of ArO^- as leaving group] but, due to the relatively high strength of the P–F bond, it is even more likely that phosphorofluridate hydrolysis [i.e. conversion of **142** (X = F) to **94**] will proceed with some internucleotide cleavage. Thus X^- should, if possible, be an oxygen nucleophile (RO^-) such that the O–R bond is broken in the conversion of **142** (X = OR) into **94**. Clearly, despite its other limitations, hydroxide ion meets this requirement. It now appears that ultimate success in the synthesis of high molecular weight oligo- and poly-nucleotides by the phosphotriester approach with an aryl



138 **139**

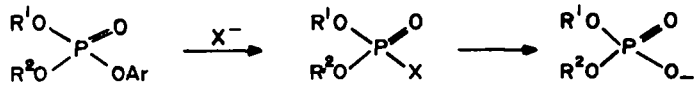
Thy = thymin-1-yl

Scheme 29.



140 a ; Ar=Ph Thy=thymin-1-yl

b ; Ar=2-ClC$_6$H$_4$

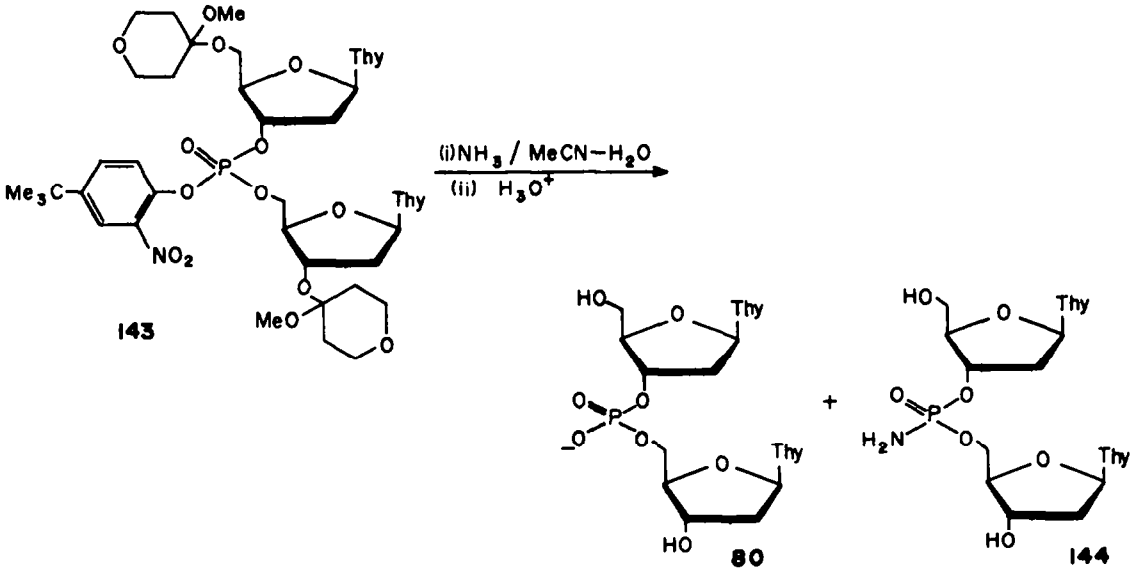


141 142 94

Scheme 30.

protecting group will depend to a large extent on the solution of this problem of internucleotide cleavage during unblocking.†

Finally, it should be noted that, in several laboratories, aqueous ammonia has been used[72,78,102] to effect the unblocking of aryl protected internucleotide linkages. This procedure cannot be recommended as it can lead to both phosphoramidate formation[65,100] and appreciable internucleotide cleavage.[100] Thus when the fully-protected 2-nitro-4-t-butylphenyl ester of TpT 143 is treated with 10 M-ammonia in aqueous acetonitrile (Scheme 31), the phosphoramidate of TpT (144; 10%) is obtained[65] in



143 80 144

Thy = thymin-1-yl

Scheme 31.

†*Footnote added in proof.* We have since found [C. B. Reese, R. C. Titmas and L. Yan, *Tetrahedron Letters* 2727 (1978)] that if unblocking of 2-chlorophenyl protected oligonucleotides is effected with 4-nitrobenzaldoximate or with pyridine-2-carboxaldoximate ion in aqueous dioxan, very little internucleotide cleavage occurs. In the same report, we confirm that fluoride ion is an unsuitable nucleophile for unblocking aryl-protected internucleotide linkages.

addition to TpT (80; 90%). When the corresponding fully-protected tetranucleoside triphosphate (tri-2-nitro-4-t-butylphenyl ester) is unblocked in the same way, the yield of (Tp)$_3$T (135; n = 2) is less than 70%: mono-, di- and possibly tri-phosphoramidates are also obtained.[65] Although it is not clear that treatment[100] of the fully-protected *triphenyl* ester of (Tp)$_3$T (i.e. the 5′-*O*-tetrahydropyranyl derivative of 133) with 10 M-ammonia in

aqueous acetonitrile leads to phosphoramidate formation, the yield of $(Tp)_3T$ (135; n = 2) following acidic hydrolysis is only between 75 and 80%. If no phosphoramidates are formed, then the extent of internucleotide cleavage is *ca.* 7–8% per phosphotriester group.

RECENT DEVELOPMENTS IN PHOSPHORYLATION

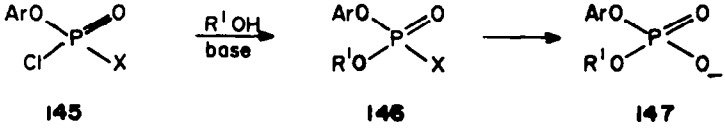
(a) *Monofunctional phosphorylating agents*

As indicated above (Scheme 17 and subsequent discussion), when bifunctional phosphorylating agents, such as phenyl phosphorodichloridate (98; X = Cl) or phenyl dihydrogen phosphate 114 in the presence of TPS 11b, are used, symmetrical products (e.g. 100 and 101, Scheme 17) may be obtained. This is particularly so in the synthesis of oligodeoxyribonucleotides. The formation of such by-products is undesirable not only because it leads to lower yields of the required products but also because it leads to purification problems especially in the case of relatively high molecular weight oligonucleotides.[67] A further disadvantage of using an aryl or alkyl dihydrogen phosphate in the presence of an arenesulphonyl chloride in the first phosphorylation step of a phosphotriester synthesis is that yields appear to be much lower when N^2-acylguanine residues are present.[64,88,103] Recent work (see below) has revealed that, if a suitable monofunctional phosphorylating agent is used, the formation of symmetrical products can be avoided and satisfactory yields are obtained irrespective of the base residues present.

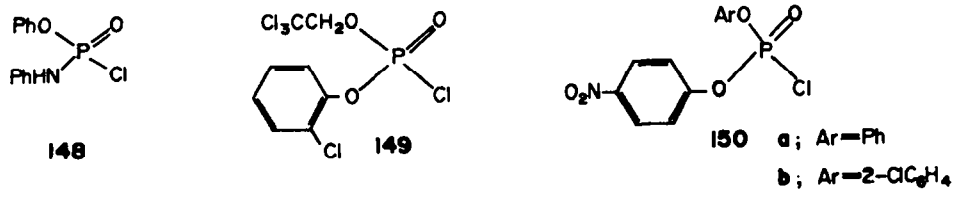
A monofunctional phosphorylating agent (such as 145, Scheme 32) in the first step of the phosphotriester approach must meet at least three requirements. Firstly, it must be reactive enough to phosphorylate the relatively hindered 3'-hydroxy groups of ribonucleoside building blocks (e.g. 59). Secondly, if it is ultimately intended to protect the internucleotide linkages with an aryl group, it must be possible to convert the initial phosphorylation products 146 into the desired intermediate protected nucleoside (or oligonucleotide) 3'-aryl phosphates 147 under conditions which are sufficiently mild for both the acid- and base-sensitive protecting groups to remain intact, and for no other undesired transformations to occur. Thirdly, it must be possible to isolate the latter intermediates 147 as pure salts, free from phosphorylation and other by-products. Clearly, if all three of these requirements are to be met, the choice of X in 145 is likely to be strictly limited.

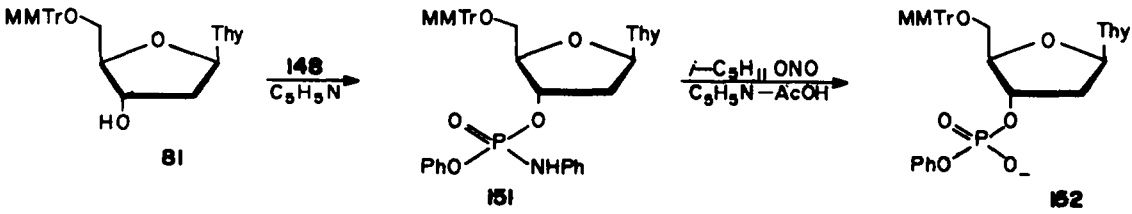
Three monofunctional phosphorylating agents 148, 149 and 150a have recently been suggested for this purpose. Zieliński and Leśnikowski[104] reported that phenyl *N*-phenylphosphoramidochloridate 148 reacts with 81 (Scheme 33) to give 151 in nearly quantitative yield and that treatment of the latter with a large excess of isopentyl nitrite in pyridine–acetic acid at 20° gives the corresponding intermediate phosphodiester 152 which may be isolated by precipitation as its pyridinium salt in 85% yield. However, the Polish workers[104] did not report the use of 152 as an intermediate in oligonucleotide synthesis.

The use of 2-chlorophenyl 2,2,2-trichloroethyl phosphorochloridate 149 as a monofunctional phosphorylating agent has been reported by van Boom *et al.*[105] Thus 5'-*O*-*p*-chlorophenoxyacetylthymidine 126 reacts with 149 in pyridine solution to give 153 in 90% isolated yield. Treatment of 153 with an excess of zinc dust in the presence of acid (0.2 molecular equivalents of 2,4,6-triisopropylbenzenesulphonic acid with respect to 153) in pyridine solution gives the desired intermediate 154 which is then isolated by extraction of its triethylammonium salt into chloroform. The Dutch workers[105] have used this phosphorylation procedure in the synthesis both of oligodeoxyribo- and oligoribo-nucleotides (see below). No difficulties regarding the unfavourable partitioning of charged species (such as 154) between chloroform and aqueous buffer or any evidence of pyrimidine



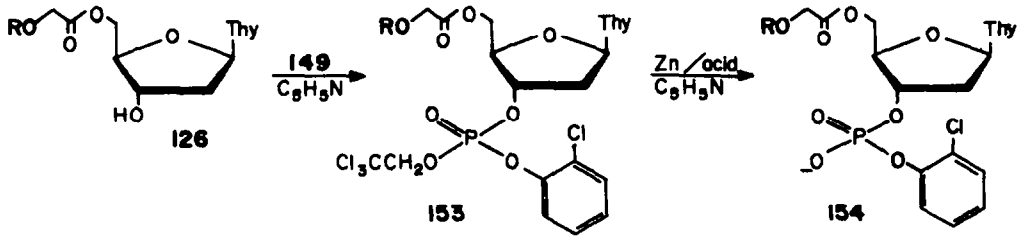
Scheme 32.





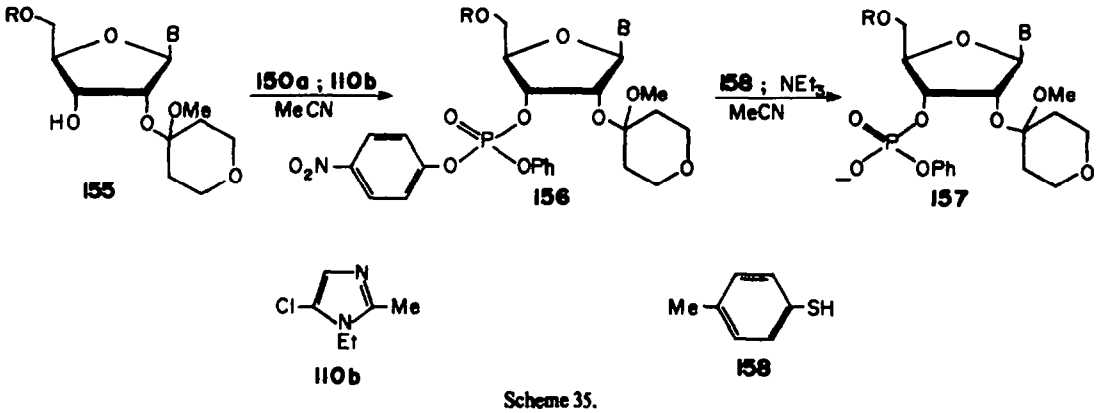
Thy = thymin-1-yl

Scheme 33.



R = 4-ClC$_6$H$_4$; Thy = thymin-1-yl

Scheme 34.



Scheme 35.

(especially cytosine) reduction by zinc dust in the presence of pyridinium ion have been reported.

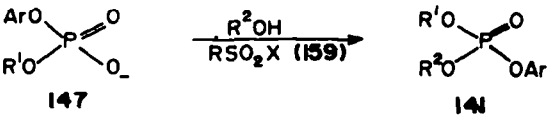
Finally, the use of 4-nitrophenyl phenyl phosphorochloridate[106] **150a** as a monofunctional phosphorylating agent was recently described by Reese and Yan Kui.[107] The latter reagent **150a**, which may easily be obtained as a distillable crystalline solid, reacts readily with 2′,5′-protected ribonucleoside building blocks (**155**; Scheme 35) in the presence of 5-chloro-1-ethyl-2-methylimidazole **110b** in acetonitrile to give the corresponding phosphotriester intermediates **156** in high yields. Thus the isolated yield of an N^2-benzoylguanosine derivative (**156**; B = N^2-benzoylguanin-9-yl) was 92%. This phosphorylating agent **150a** reacts even more readily with 5′-protected 2′-deoxyribonucleoside building blocks **68** to give the corresponding 3′-diaryl phosphates. Treatment of **156** (or one of the latter protected deoxyribonucleotides) with an excess of *p*-thiocresol **158** and triethylamine in acetonitrile at 20° gives a virtually quantitative yield after *ca.* 1 hr of the triethylammonium salt of the desired phosphodiester intermediate (**157** or deoxyribonucleotide analogue).[107] The latter salts may readily be isolated as pure precipitated solids.

Very recently, 2-chlorophenyl 4-nitrophenyl phosphorochloridate[101] **150b**, which is also an easily accessible crystalline solid, has been shown[53,101,108] to be an equally effective monofunctional phosphorylating agent. Thus the phosphorylation procedure is general with respect to substrate and the aryl group intended for the protection of the internucleotide linkages may be varied. As indicated above, no problems are encountered in the isolation of pure phosphodiester intermediates (e.g. **157**) and no side reactions appear to take place under the very mild conditions required to convert the phosphotriesters (e.g. **156**) into triethylammonium salts of the corresponding phosphodiesters (e.g. **157**). Very satisfactory yields are generally obtained when the latter intermediates (**157** and deoxyribonucleotide analogues) are used[53,107,108] in the preparation of oligonucleotides. Thus both **150a** and **150b** appear fully to meet all of the above requirements for monofunctional phosphorylating agents in the synthesis of oligonucleotides by the phosphotriester approach.

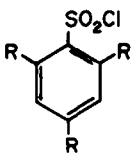
(b) *The second phosphorylation step*

If a monofunctional reagent such as 4-nitrophenyl phenyl phosphorochloridate **150a** is used in the first phosphorylation step (Scheme 35) in a phosphotriester synthesis, an activating agent (**159**) is still required in the second phosphorylation step. As indicated above, TPS **11b**, which was introduced by Khorana *et al.*[10] as an activating agent in the phosphodiester approach, is also suitable for the second step of the phosphotriester approach. On the whole, TPS **11b** has been preferred to mesitylenesulphonyl chloride **11a** in that it appears to react more slowly[10] with the free 5′-hydroxy functions of the second components (R^2OH, Scheme 36) and presumably therefore its use leads to smaller amounts of sulphonate esters (RSO_2OR^2), which are clearly undesirable by-products.

There is no doubt that TPS **11b** is an effective activating agent for the second phosphorylation step and that its use usually leads to satisfactory yields of oligonucleotide products. Indeed some reports in the literature relating to the limitations of TPS in this context may be misleading inasmuch as its suitability for the second step cannot be clearly evaluated unless a pure intermediate

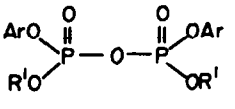


Scheme 36.

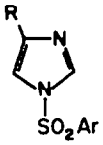


IIa ; R = Me

b ; R = Me_2CH

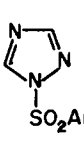


160

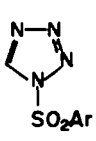


161 a ; R = H

b ; R = NO_2



162



163

phosphodiester **147** is used. The latter condition may well only be met when an effective monofunctional phosphorylating agent (e.g. **158a** or **158b**) is used in the first step. Nevertheless, the use of TPS **11b** does sometimes lead to darkening[78,88,109] of reaction media, some sulphonation[67,78,109] of the second components (R^2OH, Scheme 36) and possibly slower than desirable reaction rates.[78,110]

It has been suggested[77e] on the basis of ^{31}P NMR spectroscopic evidence that the actual phosphorylating species when TPS **11b** is used as the activating agent in the second step of the phosphotriester approach is a tetra-substituted symmetrical pryophosphate (such as **160**). This is plausible inasmuch as such pyrophosphates may be prepared[111] by the action of toluene-*p*-sulphonyl or *p*-nitrobenzenesulphonyl chloride on the corresponding phosphodiesters. If this hypothesis is correct, a reagent which converts **147** into **160** rapidly and without side-reactions is required for the second phosphorylation step. Although further studies should perhaps be directed towards the search for or the development of such a reagent, it has recently been reported that arenesulphonyl derivatives of imidazole[109] and related systems[78,110,112] have advantages as activating agents in oligonucleotide synthesis over the corresponding arenesulphonyl chlorides.

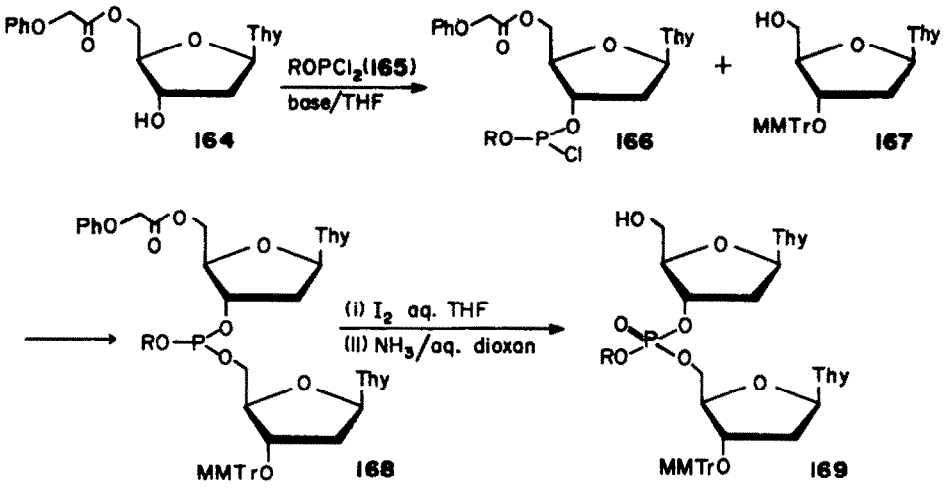
Berlin *et al.*[109] have reported that 1-toluene-*p*-sulphonyl-, 1-mesitylenesulphonyl- and 1-(2,4,6-tri-isopropylbenzenesulphonyl)-imidazoles [**161a**; Ar = 4-MeC_6H_4, 2,4,6-$Me_3C_6H_2$ and 2,4,6-$(Me_2CH)_3C_6H_2$, respectively] are useful activating agents in the synthesis of oligodeoxyribonucleotides by the phosphodiester approach. Although the latter reagents promote phosphorylation without darkening of the reaction media and without concomitant sulphonation of 3'-hydroxy groups, the reactions [especially in the case of **161a** (Ar = 2,4,6-$(Me_2CH)_3C_6H_2$)] proceed very slowly. Narang *et al.*[112] then found that the corresponding arenesulphonyl-1,2,4-triazole derivatives [putative structures **162**] can be used in the second step of oligodeoxyribonucleotide synthesis by the phosphotriester approach. The latter reagents promote phosphorylation reactions more slowly than TPS **11b** but at three times the rates of the corresponding imidazole derivatives **161a**. More recently, workers in the same group have reported[110] that arenesulphonyltetrazoles [putative structures **163**] act as powerful activating agents in the second step of the phosphotriester approach and promote rapid phosphorylation with no concomitant sulphonation of 5'-hydroxy groups. Benzenesulphonyl and mesitylenesulphonyl-tetrazoles [putative structures **163**; Ar = Ph and 2,4,6-$Me_3C_6H_2$, respectively] have been found[110] to be particularly effective activating agents.

Neilson *et al.*[88] have reported that mesitylenesulphonyl -1,2,4-triazole [putative structure **162**; Ar = 2,4,6-$Me_3C_6H_2$], used in conjunction with 2,2,2-trichloroethyl phosphate, is superior to TPS **11b** in both steps of oligoribonucleotide synthesis in that its use leads to no coloration of the reaction medium and to consistently higher yields. van Boom *et al.*[78] have reported that 2,4,6-tri - isopropylbenzenesulphonyl - 4 - nitroimidazole [putative structure **161b**; Ar = 2,4,6-$(Me_2CH)_3C_6H_2$] is a useful activating agent in the second step of oligoribonucleotide synthesis; these workers have found[78] that the latter reagent promotes phosphorylation somewhat more slowly than TPS **11b** in the synthesis of dimers and trimers but more rapidly in the block synthesis of higher molecular weight oligonucleotides. The extent of 5'-sulphonation is reported[78] to be lower and yields of products are reported to be generally higher when **161b** [Ar = 2,4,6-$(Me_2CH)_3C_6H_2$] is used instead of **11b**.

(c) *Other recent developments in phosphorylation*

In the belief that normal phosphorylation reactions proceed too slowly to allow the efficient synthesis of long polynucleotides, Letsinger and Lunsford[81] investigated the possibility of carrying out a phosphotriester synthesis *via* phosphite rather than phosphate intermediates. For example, in the synthesis of fully-protected TpT (Scheme 37), these workers treated 5'-*O*-phenoxyacetylthymidine **164** with a slight deficiency of *o*-chlorophenyl phosphorodichloridite (**165**; R = 2-ClC_6H_4) in the presence of 2,6-lutidine in tetrahydrofuran solution at −78° to give, presumably, **166**. After 6 min, the second building block (**167**, *ca.* 0.5 molecular equivalents with respect to **164**) was added to give **168** (R = 2-ClC_6H_4). Oxidation of the latter intermediate with iodine in aqueous tetrahydrofuran at −10°, followed by treatment with ammonia, gives the corresponding dinucleoside phosphate (**169**; R = 2-ClC_6H_4) in 65% isolated yield (based on **167**). Some symmetrical 3'→3'-dinucleoside phosphate, derived from two molecules of **164**, is also obtained. As aryl phosphorodichloridites **165** and the intermediate phosphorochloridites **166** are remarkably reactive, it is necessary to use an excess of the component with a free 3'-hydroxy group (e.g. **164**) over both the phosphorodichloridite **165** and the second component (e.g. **167**). Symmetrical by-products with 3'→3'-internucleotide linkages are inevitably formed. Letsinger and Lunsford[81] were nevertheless able to carry out a satisfactory stepwise synthesis of $(Tp)_4T$ by this procedure, using 2,2,2-trichloroethyl phosphorodichloridite (**165**; R = Cl_3CCH_2) as the phosphitylating agent.

The use of phosphorodichloridites in oligoribonucleotide synthesis has been reported from two laboratories.[38,76] Despite indications that the 3'-hydroxy groups of 2'-*O*-methoxytetrahydropyranyl ribonucleoside derivatives (such as **57**) are comparatively hindered, substantial quantities of symmetrical products with 3'→3'-internucleotide linkages are obtained when methyl phosphorodichloridite is used[76] as the phosphitylating agent. The use by Daub and van Tamelen[76] of the methyl group for the protection of internucleotide linkages and



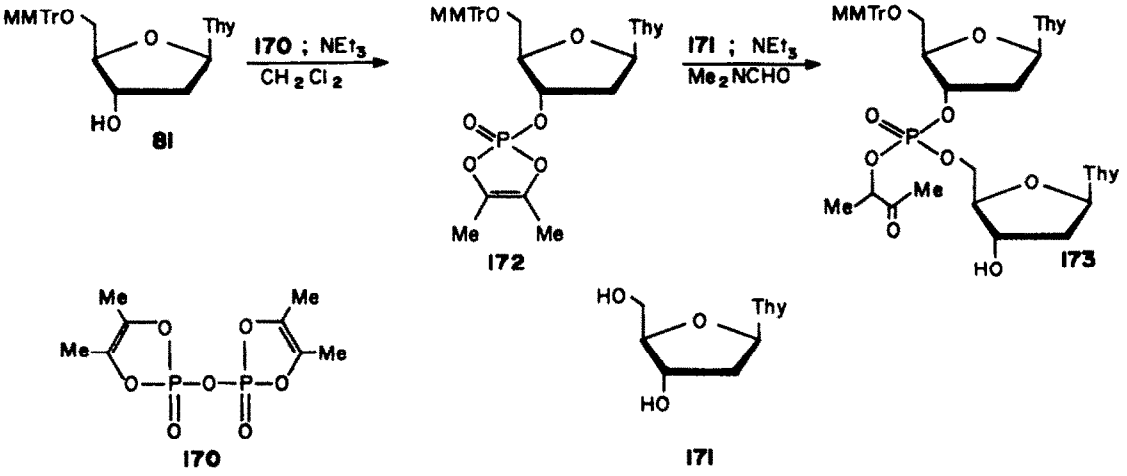
Thy = thymin-1-yl

Scheme 37.

its removal by treatment with thiophenoxide ion is particularly noteworthy. Clearly, a stable monofunctional phosphorochloridite would be of considerable value in oligonucleotide synthesis.

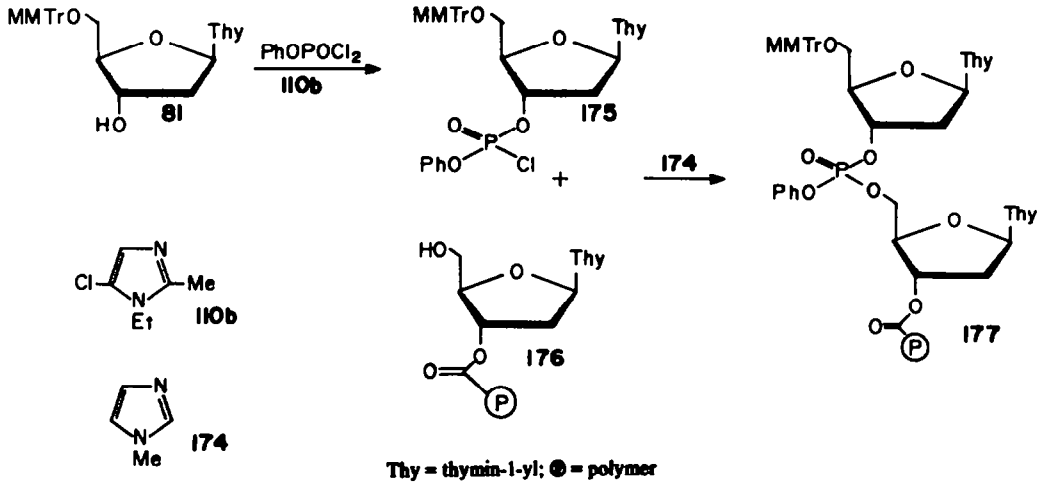
Ramirez *et al.*[113] have investigated the use of di-(1,2-dimethylethenylene) pyrophosphate **170** as a phosphorylating agent in the synthesis of oligothymidylic acids. The synthesis of a partially-protected dinucleoside phosphate **173** by this approach is illustrated in Scheme 38. Treatment of **81** with a stoicheiometric quantity of **170** in the presence of triethylamine in dichloromethane gives the intermediate **172**. When the latter compound **172** is treated with unprotected thymidine **171** in the presence of triethylamine in dimethylformamide, regioselective phosphorylation occurs to give **173** as the main product in 82% yield. This partially-protected dinucleoside phosphate is unblocked to give TpT **80** in 85–90% yield by treatment first with trifluoroacetic acid in dichloromethane and then with triethylamine in aqueous acetonitrile at 0°. In the same way, $(Tp)_2T$ (**135**; n = 1) was prepared from **173**, **170** and **171** and the process was repeated to give $(Tp)_3T$ (**135**; n = 2). It should be noted that the unblocking of 1-methylacetonyl-protected internucleotide linkages (as in **173**) by alkaline hydrolysis is accompanied[113] by a significant amount (21% in the case of the tetramer with three phosphotriester groups) of internucleotide cleavage.

There have been a number of reports in the literature[12] relating to solid support synthesis of oligonucleotides by the phosphodiester approach. Indeed further developments in this area have been reported[114] very recently. On the other hand, the solid support synthesis of oligonucleotides by the phosphotriester approach appears so far to have been little investigated. However, Pless and Letsinger[115] have carried out a synthesis of oligothymidylic acids by the latter approach. An insoluble polymer, obtained by the co-polymerization of styrene, *p*-vinylbenzoic acid and *p*-divinylbenzene, is treated with thionyl chloride and then with **81**. After acid-treatment, **176** (Scheme 39) is obtained. Reaction between **176** and an excess of **175** [obtained by the action of phenyl phosphorodichloridate and **110b** on **81** in dioxan solution] in the presence of 1-methylimidazole **174** gives **177**. Chain extension is effected by treating **177** with 80% acetic acid followed by an excess of **175**. Oligothymidylic acids are finally released from the solid support by



Thy = thymin-1-yl

Scheme 38.



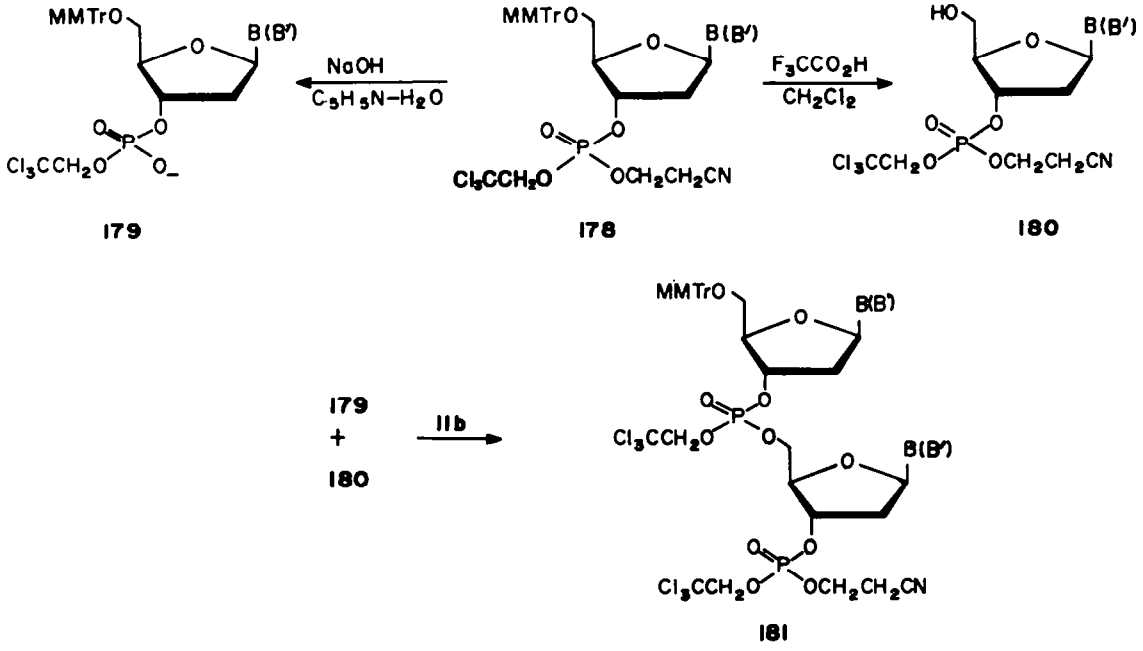
Scheme 39.

alkaline hydrolysis. The unblocked dimer, trimer and tetramer (135; n = 0, 1 and 2) were obtained in this way in 73, 35 and 31% yields, respectively.

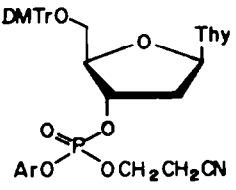
THE STRATEGY OF OLIGO- AND POLY-NUCLEOTIDE SYNTHESIS BY THE PHOSPHOTRIESTER APPROACH

In 1973, Catlin and Cramer[80] proposed a strategy (Scheme 40) for the synthesis of oligodeoxyribonucleotides by the phosphotriester approach. This strategy is based on fully-protected mononucleotide building blocks 178 which may be partially deprotected at the 3'- and 5'-ends by treatment with alkali and acid, respectively, to give 179 and 180. The two types of intermediates 179 and 180 obtained may then be linked together by treatment with, for example, TPS 11b in pyridine solution to give fully-protected dinucleotides 181. Treatment of the latter 181 with alkali and acid similarly leads to partially-protected dinucleotides functionalized at the 3'- and 5'-ends, respectively. Such partially-protected dinucleotides may then be extended in a stepwise manner or condensed with appropriate oligonucleotide blocks. The products obtained may again be functionalized either at their 3'- or 5'-ends and the oligonucleotide chain further extended. Although Catlin and Cramer's original procedure[80] may be modified and possibly improved by changing the phosphorylation procedure and the protecting groups, the overall strategy appears to be sound. Furthermore, with a suitable choice of protecting groups (see below), it is equally applicable to the synthesis of oligoribonucleotides.

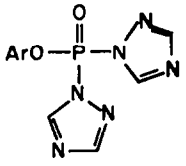
Very recently, Sood and Narang[116] have carried out a synthesis of oligo- and poly-thymidylic acids using essentially Catlin and Cramer's strategy[80] but with aryl protecting groups for the internucleotide linkages. Narang *et al.*[72] had previously shown that fully-protected mononucleotides (such as 182) may be obtained by treating the corresponding 5'-protected 2'-deoxyribonucleosides 69b first with a slight excess of *p*-chlorophenyl phosphorodi-(1,2,4-triazolide) (putative



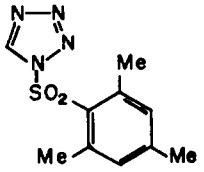
Scheme 40.



182



183

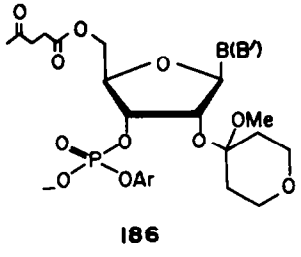


184

Thy = thymin-1-yl; Ar = 4-ClC$_6$H$_4$

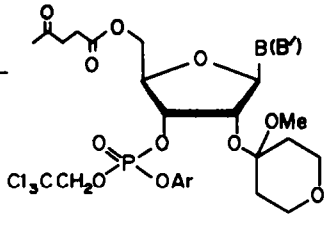
structure 183) and then with 2-cyanoethanol. Apparently very satisfactory yields of mononucleotide building blocks (e.g. 182) may be obtained in this way despite the use of a bifunctional phosphorylating agent 183. The 5'-protecting group is removed from 182 and fully-protected oligonucleotides (corresponding to 181 and higher molecular weight oligomers) by a brief treatment with benzenesulphonic acid in chloroform–methanol.[72] The 2-cyanoethyl group may be removed selectively from 182 and fully-protected oligonucleotides by treatment with a large excess of triethylamine in anhydrous pyridine solution. The latter procedure, which is of great importance to the successful operation of this synthetic strategy, was first reported by Adamiak *et al.*[117] Sood and Narang[116] have used this approach in the block synthesis of oligo- and poly-thymidylic acids containing fourteen, twenty and thirty-eight thymidine residues (135; n = 12, 18 and 36, respectively). In this work the terminal 3'-hydroxy functions were protected as acetate esters and unblocking of the fully-protected products was effected by treatment first with benzenesulphonic acid in chloroform–methanol (to release the terminal 5'-hydroxy functions) and then with concentrated aqueous ammonia at 50° (to remove all of the remaining protecting groups). A noteworthy feature of this work is that mesitylenesulphonyltetrazole (putative structure 184) was used as the activating agent in phosphorylation reactions. The latter reactions proceeded rapidly and the reported yields compare very favourably with yields obtained previously in oligonucleotide synthesis by the phosphotriester approach. Although an appreciable excess of activating agent 184 was used in each condensation, sulphonation of the components with free 5'-hydroxy functions was not observed.

Narang and his coworkers had previously reported[72] the synthesis, by related procedures, of a number of relatively high molecular weight sequences of the *E. coli* lactose operator gene and restriction enzyme recognition sites. These sequences contained all four common bases. However, this work was carried out before the development of the triethylamine–pyridine procedure for the removal of 2-cyanoethyl groups from terminal phosphotriesters and the synthetic strategy adopted was less satisfactory. In their recent work, Narang and his coworkers[72,116] have used concentrated ammonia to unblock the phosphotriester functions of oligo- and poly-deoxyribonucleotide intermediates with unprotected terminal 3'- and 5'-hydroxy functions. Such treatment with ammonia is likely to lead to appreciable internucleotide cleavage[100] (possibly 3–4% per phosphotriester group). Furthermore, there is a risk of phosphoramidate formation[65] and some of the unblocked products are



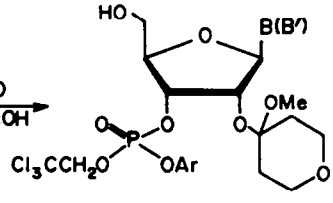
186

Zn

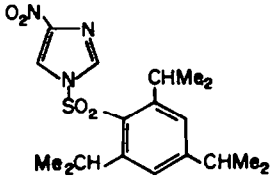


185

$N_2H_4 \cdot H_2O$
C_5H_5N–AcOH



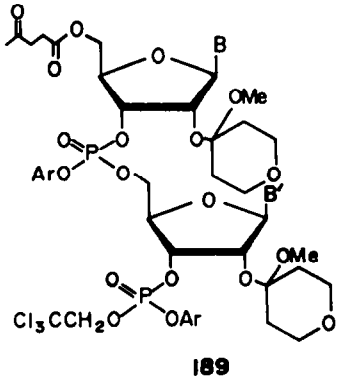
187



188

186
+
187

188



189

Ar = 2-ClC$_6$H$_4$

Scheme 41.

likely to contain significant proportions of terminal 3'→3'- and 5'→5'-internucleotide linkages.[93,94]

van Boom and Burgers[116] have tackled the synthesis of oligoribonucleotides using a strategy which is closely similar to that adopted by Catlin and Cramer[80] and more recently by Sood and Narang[116] in the deoxy-series; these workers[118] have used the acid-labile methoxytetrahydropyranyl group to protect 2'-hydroxy functions and the *o*-chlorophenyl group to protect internucleotide linkages. van Boom and Burgers have avoided the possibility of phosphotriester hydrolysis occurring during the removal of 5'-*O*-acyl groups by using the laevulinyl protecting group (as in **189**, Scheme 41). The use of the latter group was reported previously by Hassner *et al.*[119] and by Ho and Wong[120] who removed it, respectively, by reduction with sodium borohydride in aqueous dioxan and by reaction with hydrazine in boiling methanol. van Boom and Burgers have shown[118] that the laevulinyl group is removed very rapidly by hydrazine hydrate in pyridine–acetic acid, the reagent previously used by Letsinger and Miller[75a] to remove the related β-benzoylpropionyl group. The laevulinyl is more suitable for use in oligonucleotide synthesis than the β-benzoylpropionyl group in that it is cleaved by hydrazine *ca.* 10^2 times more rapidly and apparently under conditions under which the removal of *N*-acyl protecting groups from base-residues and other undesirable side-reactions occur only to a negligible extent.

van Boom and Burgers[118] carried out the synthesis of the decaribonucleotide $(UpAp)_4UpA$ and shorter related sequences based largely on two fully-protected mononucleotide starting materials (**185**; B(B') = uracil-1-yl and N^6-*p*-anisoyladenin-9-yl). Treatment of the latter building blocks (**185**) with zinc in the presence of 2,4,6-tri-isopropylbenzenesulphonic acid in pyridine and with hydrazine hydrate in pyridine–acetic acid, respectively, gives the partially-protected mononucleotides (**186** and **187**). The desired fully-protected dinucleotide (**189**; B = uracil-1-yl, B' = N^6-*p*-anisoyladenin-9-yl) is obtained in 87% yield by allowing the appropriate partially-protected mononucleotides (**186**; B(B') = uracil-1-yl and **187**; B(B') = N^6-*p*-anisoyladenin-9-yl) to react together in the presence of the 2,4,6-tri-isopropylbenzenesulphonyl derivative of 4-nitroimidazole (putative structure **188**) in pyridine solution. As the fully-protected dinucleotide (**189**; B = uracil-1-yl, B' = N^6-*p*-anisoyladenin-9-yl) may, like the fully-protected mononucleotides **185**, be unblocked selectively at its 3'- and 5'-ends by treatment, respectively, with zinc and hydrazine, a block synthesis is possible. The 2',3'-*cis*-diol system of the terminal N^6-*p*-anisoyladenine residue was protected with a methoxymethylene group. Satisfactory yields were reported[118] for all the synthetic steps involved. The fully-protected oligoribonucleotides were unblocked by treatment first with tetra-n-butylammonium fluoride under the conditions suggested by Itakura *et al.*,[98] then with 25% aqueous ammonia and finally with 0.01 M-hydrochloric acid to remove the 2'-*O*-methoxytetrahydropyranyl and 2',3'-*O*-methoxymethylene groups.

CONCLUSIONS

Finally, it is reasonable to conclude that a considerable amount of progress has been made in the phosphotriester approach to the synthesis of oligo- and poly-nucleotides in the past decade or so. At the present time, high molecular weight oligo- and poly-deoxyribonucleotides have already been synthesized in satisfactory yields by this approach and there is no reason to believe that the synthesis of oligo- and poly-ribonucleotides will present any further significant difficulties. Most of the fundamental problems have been solved. Indeed, the final choice of a protecting group for the internucleotide linkages appears to be the only really important remaining problem. It now seems very likely that it will be possible substantially to decrease the extent of internucleotide cleavage accompanying the removal of aryl protecting groups[121] and, if this proves to be the case, there can be very little doubt that the phosphotriester approach will rapidly become established as the method of choice for the chemical synthesis of oligo- and polynucleotides of defined base-sequence.

Acknowledgements—I should like to express my extreme gratitude to a number of excellent coworkers without whose skill and hard work our own studies in this area would not have been possible.

REFERENCES

[1]M. W. Nirenberg and P. Leder, *Science* **145**, 1399 (1964).
[2]C. Byrd, E. Ohtsuka, M. W. Moon and H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **53**, 79 (1965); R. D. Wells, E. Ohtsuka and H. G. Khorana, *J. Mol. Biol.* **14**, 221 (1965); R. D. Wells, T. M. Jacob, S. A. Narang and H. G. Khorana, *Ibid.* **27**, 237 (1967); R. D. Wells, H. Büchi, H. Kössel, E. Ohtsuka and H. G. Khorana, *Ibid.* **27**, 265 (1967).
[3]K. L. Agarwal, H. Büchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. RajBhandary, J. H. van de Sande, V. Sgaramella, H. Weber and T. Yamada, *Nature* **227**, 27 (1970).
[4]H. G. Khorana, K. L. Agarwal, P. Besmer, H. Büchi, M. H. Caruthers, P. J. Cashion, M. Fridkin, E. Jay, K. Kleppe, R. Kleppe, A. Kumar, P. C. Loewen, R. C. Miller, K. Minamoto, A. Panet, U. L. RajBhandary, B. Ramamoorthy, T. Sekiya, T. Takaya and J. H. van de Sande, *J. Biol. Chem.* **251**, p. 565 *et seq.* (1976).
[5]R. H. Hall, *The Modified Nucleosides in Nucleic Acids.* Columbia University Press, New York (1971).
[6]H. G. Khorana, G. M. Tener, J. G. Moffatt and E. H. Pol, *Chem. and Ind.* 1523 (1956).
[7a]H. G. Khorana, *Pure Appl. Chem.* **17**, 349 (1968); [b]K. L. Agarwal, A. Yamazaki, P. J. Cashion and H. G. Khorana, *Angew. Chem. internat. Edit.* **11**, 451 (1972).
[8]H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, *J. Am. Chem. Soc.* **79**, 1002 (1957).
[9]T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.* **86**, 1630 (1964).
[10]R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.* **88**, 829 (1966).
[11]P. T. Gilham and G. M. Tener, *Chem. and Ind.* 542 (1959).
[12]For recent general reviews on oligonucleotide synthesis, see [a]R. I. Zhdanov and S. M. Zhenodarova, *Synthesis* 222 (1975); [b]H. Kössel and H. Seliger, *Fortschr. Chem. Org. Naturstoffe* **32**, 298 (1975); [c]V. Amarnath and A. D. Broom, *Chem. Rev.* **77**, 183 (1977).
[13]B. E. Griffin and C. B. Reese, *Tetrahedron* **25**, 4057 (1969).
[14]C. B. Reese, Colloques Internationaux du C.N.R.S. No. 182, 319 (1970).
[15]C. B. Reese, *Phosphorus and Sulfur* **1**, 245 (1976).
[16]M. Jarman and C. B. Reese, *Chem. and Ind.* 1493 (1964); B. E. Griffin, M. Jarman, C. B. Reese and J. E. Sulston, *Tetrahedron* **23**, 2301 (1967).
[17]J. Žemlička, *Chem. and Ind.* 581 (1964).
[18a]C. B. Reese and D. R. Trentham, *Tetrahedron Letters* 2467 (1965); [b]B. E. Griffin, M. Jarman, C. B. Reese, J. E. Sulston and D. R. Trentham, *Biochemistry* **5**, 3638 (1966).
[19]D. M. Brown, D. I. Magrath, A. H. Neilson and A. R. Todd, *Nature* **177**, 1124 (1956).
[20]B. E. Griffin, M. Jarman and C. B. Reese, *Tetrahedron* **24**, 639 (1968).

[21]W. E. Parham and E. L. Anderson, *J. Am. Chem. Soc.* **70**, 4187 (1948).
[22]J. Smrt and F. Šorm, *Coll. Czech. Chem. Comm.* **27**, 73 (1962).
[23]M. Smith, D. H. Rammler, I. H. Goldberg and H. G. Khorana, *J. Am. Chem. Soc.* **84**, 430 (1962).
[24]D. H. Rammler and H. G. Khorana, *J. Am. Chem. Soc.* **84**, 3112 (1962).
[25]F. Cramer, H.-J. Rhaese, S. Rittner and K.-H. Scheit, *Liebigs Ann.* **683**, 199 (1965).
[26]H. P. M. Fromageot, Ph.D. Thesis, Cambridge University, 1966, p. 70; O. Kennard, W. D. S. Motherwell, J. C. Coppola, B. E. Griffin, C. B. Reese and A. C. Larson, *J. Chem. Soc.* (B), 1940 (1971).
[27]C. B. Reese, R. Saffhill and J. E. Sulston, *J. Am. Chem. Soc.* **89**, 3366 (1967); *Tetrahedron* **26**, 1023 (1970).
[28]M. M. Kreevoy and R. W. Taft, Jr., *J. Am. Chem. Soc.* **77**, 3146, 5590 (1955).
[29]R. Arentzen, C. B. Reese and Y. T. Yan Kui, *Synthesis* 509 (1975).
[30]G. R. Owen and C. B. Reese, *J. Chem. Soc.* (C) 2401 (1970).
[31]D. H. Rammler, Y. Lapidot and H. G. Khorana, *J. Am. Chem. Soc.* **85**, 1989 (1963).
[32]Y. Lapidot and H. G. Khorana, *J. Am. Chem. Soc.* **85**, 3852 (1963).
[33]H. P. M. Fromageot, C. B. Reese and J. E. Sulston, *Tetrahedron* **24**, 3533 (1968).
[34]B. E. Griffin, C. B. Reese, G. F. Stephenson and D. R. Trentham, *Tetrahedron Letters* 4349 (1966).
[35]G. Reitz and W. Pfleiderer, *Chem. Ber.* **108**, 2878 (1975).
[36]E. Ohtsuka, S. Tanaka and M. Ikehara, *Nucleic Acids Res.* **1**, 1351 (1974).
[37]K. K. Ogilvie, K. L. Sadana, E. A. Thompson, M. A. Quilliam and J. B. Westmore, *Tetrahedron Letters* 2861 (1974).
[38]K. K. Ogilvie, N. Theriault and K. L. Sadana, *J. Am. Chem. Soc.* **99**, 7741 (1977).
[39]C. B. Reese, In *Protective Groups in Organic Chemistry* (Edited by J. F. W. McOmie), p. 124 *et seq.* Plenum Press, New York (1973).
[40]A. Hampton, J. C. Frantantoni, P. M. Carroll and S. Wang, *J. Am. Chem. Soc.* **87**, 5481 (1965).
[41]H. Schaller, G. Weimann, B. Lerch and H. G. Khorana, *J. Am. Chem. Soc.* **85**, 3821 (1963).
[42]R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka and H. G. Khorana, *J. Am. Chem. Soc.* **88**, 819 (1966).
[43]See Ref. 7a, p. 352.
[44]P. A. Lyon and C. B. Reese, *J.C.S. Perkin I* 2645 (1974).
[45a]H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961); [b]R. K. Ralph and H. G. Khorana, *Ibid.* p. 2926.
[46]R. Saffhill, Ph.D. Thesis, p. 68. Cambridge University (1968).
[47]R. K. Ralph, W. J. Connors, H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.* **85**, 1983 (1963).
[48]C. B. Reese and R. Saffhill, *J. C. S. Perkin I* 2937 (1972).
[49]R. W. Taft, Jr., In *Steric Effects in Organic Chemistry* (Edited by M. S. Newman), p. 586 *et seq.* Wiley, New York (1956).
[50]J. Žemlička, S. Chládek, A. Holý and J. Smrt, *Coll. Czech. Chem. Commun.* **31**, 3198 (1966).
[51]C. B. Reese and J. E. Sulston, *Proc. Chem. Soc.* 214 (1964); H. P. M. Fromageot, B. E. Griffin, C. B. Reese and J. E. Sulston, *Tetrahedron* **23**, 2315 (1967).
[52]J. H. van Boom, G. R. Owen, J. Preston, T. Ravindranathan and C. B. Reese, *J. Chem. Soc.* (C), 3230 (1971).
[53]J. B. Chattopadhyaya and C. B. Reese, unpublished observations.
[54]H. P. M. Fromageot, B. E. Griffin, C. B. Reese, J. E. Sulston and D. R. Trentham, *Tetrahedron* **22**, 705 (1966).
[55]C. B. Reese and J. C. M. Stewart, *Tetrahedron Letters* 4273 (1968).
[56]G. R. Owen, Ph.D. Thesis, Cambridge University (1971).
[57]C. B. Reese, J. C. M. Stewart, J. H. van Boom, H. P. M. de Leeuw, J. Nagel and J. F. M. deRooy, *J.C.S. Perkin I* 934 (1975).
[58]D. P. L. Green, T. Ravindranathan, C. B. Reese and R. Saffhill, *Tetrahedron* **26**, 1031 (1970).
[59]J. H. van Boom, P. M. J. Burgers, G. R. Owen, C. B. Reese and R. Saffhill, *Chem. Comm.* 869 (1971).
[60]T. Neilson, *Chem. Comm.* 1139 (1969).
[61]T. Neilson and E. S. Werstiuk, *Can. J. Chem.* **49**, 3004 (1971).
[62]J. H. van Boom, P. M. J. Burgers and C. A. G. Haasnoot, *Nucleic Acids Res.* **3**, 2731 (1976).
[63]J. H. van Boom, P. M. J. Burgers, R. Crea, G. van der Marel and G. Wille, *Nucleic Acids Res.* **4**, 747 (1977).
[64]W. T. Markiewicz and C. B. Reese, unpublished observations.
[65]R. W. Adamiak, R. Arentzen and C. B. Reese, *Tetrahedron Letters* 1431 (1977).
[66]R. W. Adamiak and C. B. Reese, unpublished observations.
[67]R. Arentzen and C. B. Reese, *J. C. S. Perkin I* 445 (1977).
[68]W. Krzyzosiak and C. B. Reese, unpublished observations.
[69]J. H. van Boom, P. van Deursen, J. Meeuwse and C. B. Reese, *J.C.S. Chem. Comm.* 766 (1972).
[70]K. K. Ogilvie and R. L. Letsinger, *J. Org. Chem.* **32**, 2365 (1967).
[71]L. Havesi, E. Wolfson-Davidson, J. B. Nagy, O. B. Nagy and A. Bruylants, *J. Am. Chem. Soc.* **94**, 4715 (1972).
[72]J. Stawinski, T. Hozumi, S. A. Narang, C. P. Bahl and R. Wu, *Nucleic Acids Res.* **4**, 353 (1977).
[73]A. M. Michelson and A. R. Todd, *J. Chem. Soc.* 2632 (1955).
[74a]R. L. Letsinger and V. Mahadevan, *J. Am. Chem. Soc.* **87**, 3526 (1965); *Ibid.* **88**, 5319 (1966); [b]R. L. Letsinger and K. K. Ogilvie, *J. Am. Chem. Soc.* **89**, 4801 (1967); *Ibid.* **91**, 3350 (1969).
[75a]R. L. Letsinger and P. S. Miller, *J. Am. Chem. Soc.* **91**, 3356 (1969); [b]R. L. Letsinger, K. K. Ogilvie and P. S. Miller, *Ibid.* **91**, 3360 (1969).
[76]G. W. Daub and E. E. van Tamelen, *J. Am. Chem. Soc.* **99**, 3526 (1977).
[77a]F. Eckstein and I. Rizk, *Angew. Chem. Internat. Edit.* **6**, 695 (1967); [b]*Ibid.* **6**, 949 (1967); [c]*Chem. Ber.* **102**, 2362 (1969).
[78]J. H. van Boom, P. M. J. Burgers, G. van der Marel, C. H. M. Verdegaal and G. Wille, *Nucleic Acids Res.* **4**, 1047 (1977).
[79]R. W. Adamiak, E. Biała, K. Grzeskowiak, R. Kierzek, A. Krazewski, W. T. Markiewicz, J. Stawiński and M. Wiewiórowski, *Nucleic Acids Res.* **4**, 2321 (1977).
[80]J. C. Catlin and F. Cramer, *J. Org. Chem.* **38**, 245 (1973).
[81]R. L. Letsinger and W. B. Lunsford, *J. Am. Chem. Soc.* **98**, 3655 (1976).
[82]A. F. Cook, *J. Org. Chem.* **33**, 3589 (1968).
[83]C. B. Reese and R. Saffhill, *Chem. Comm.* 767 (1968).
[84]J. H. van Boom, P. M. J. Burgers, P. H. van Deursen, R. Arentzen and C. B. Reese, *Tetradedron Letters* 3785 (1974).
[85]J. C. M. Stewart, Ph.D. Thesis, p. 39. Cambridge University (1969).
[86]A. M. Michelson, L. Szabo and A. R. Todd, *J. Chem. Soc.* 1546 (1956).
[87]E. S. Werstiuk and T. Neilson, *Can. J. Chem.* **50**, 1283 (1972); T. Neilson, E. V. Wastrodowski and E. S. Werstiuk, *Ibid.* **51**, 1068 (1973); E. S. Westiuk and T. Neilson, *Ibid.* **51**, 1889 (1973); T. Neilson and E. S. Werstiuk, *J. Am. Chem. Soc.* **96**, 2295 (1974); T. Neilson, K. V. Deugau, T. E. England and E. S. Werstiuk, *Can. J. Chem.* **53**, 1093 (1975).
[88]T. E. England and T. Neilson, *Can. J. Chem.* **54**, 1714 (1976); E. S. Werstiuk and T. Neilson, *Ibid.* **54**, 2689 (1976).
[89]T. Neilson and E. S. Werstiuk, *Can. J. Chem.* **49**, 493 (1971).
[90]G. W. Grams and R. L. Letsinger, *J. Org. Chem.* **35**, 868 (1970).
[91]J. M. A. Hoeflake, *Rec. Trav. chim.* **36**, 24 (1916).
[92]G. R. Owen, C. B. Reese, C. J. Ransom, J. H. van Boom and J. D. H. Herschied, *Synthesis* 704 (1974).
[93]J. H. van Boom, P. M. J. Burgers, P. H. van Deursen, J. F. M. de Rooy and C. B. Reese, *J.C.S. Chem. Comm.* 167 (1976).
[94]A. Myles, W. Hutzenlaub, G. Reitz and W. Pfleiderer, *Chem. Ber.* **108**, 2857 (1975).
[95]N. J. Cusack, C. B. Reese and J. H. van Boom, *Tetrahedron Letters* 2209 (1973).
[96]B. J. Hunt and W. Rigby, *Chem. and Ind.* 1868 (1967).
[97]A. Albert and E. P. Serjeant, *Ionization Constants of Acids and Bases*, p. 130. Methuen, London (1962).
[98]K. Itakura, N. Katagiri, C. P. Bahl, R. H. Wightman and S. A. Narang, *J. Am. Chem. Soc.* **97**, 7327 (1975).

[99]K. K. Ogilvie, S. L. Beaucage and D. W. Entwistle, *Tetrahedron Letters* 1255 (1976).
[100]R. Arentzen, Ph.D. Thesis, p. 99 *et seq.* London University (1977).
[101]C. B. Reese and R. C. Titmas, unpublished observations.
[102]H. Rokos, A. Myles, W. Hutzenlaub and W. Pfleiderer, *Chem. Ber.* **108**, 2872 (1975).
[103]K. Itakura, N. Katagiri and S. A. Narang, *Can. J. Chem.* **52**, 3689 (1974).
[104]W. S. Zieliński and Z. Leśnikowski, *Synthesis* 185 (1976).
[105]J. H. van Boom, P. M. J. Burgers and P. H. van Deursen, *Tetrahedron Letters* 869 (1976).
[106]I. Dilaris and G. Eliopoulos, *J. Org. Chem.* **30**, 686 (1965).
[107]C. B. Reese and Y. T. Yan Kui, *J.C.S. Chem. Comm.* 802 (1977).
[108]C. B. Reese and L. Yau, unpublished observations.
[109]Y. A. Berlin, O. G. Chakhmakhcheva, V. A. Efimov, M. N. Kolosov and V. G. Korobko, *Tetrahedron Letters* 1353 (1973).
[110]J. Stawinski, T. Hozumi and S. A. Narang, *Can. J. Chem.* **54**, 670 (1976).
[111]N. S. Corby, G. W. Kenner and A. R. Todd, *J. Chem. Soc.* 1234 (1952).
[112]N. Katagiri, K. Itakura and S. A. Narang, *J.C.S. Chem. Comm.* 325 (1974).
[113]F. Ramirez, E. Evangelidou-Tsolis, A. Jankowski and J. F. Maracek, *Synthesis* 451 (1977); *J. Org. Chem.* **42**, 3144 (1977).
[114]M. J. Gait and R. C. Sheppard, *Nucleic Acids Res.* **4**, 1135, 4391 (1977).
[115]R. C. Pless and R. L. Letsinger, *Nucleic Acids Res.* **2**, 773 (1975).
[116]A. K. Sood and S. A. Narang, *Nucleic Acids Res.* **4**, 2757 (1977).
[117]R. W. Adamiak, M. Z. Barciszewska, E. Biała, K. Grzeskowiak, R. Kierzek, A. Kraszewski, W. T. Markiewicz and M. Wiewiórowski, *Nucleic Acids Res.* **3**, 3397 (1976).
[118]J. H. van Boom and P. M. J. Burgers, *Tetrahedron Letters* 4875 (1976).
[119]A. Hassner, G. Strand, M. Rubinstein and A. Patchornik, *J. Am. Chem. Soc.* **97**, 1614 (1975).
[120]T. L. Ho and C. M. Wong, *Synth. Comm.* **5**, 91 (1975).
[121]C. B. Reese, R. C. Titmas and L. Yau, *Tetrahedron Letters* 2727 (1978).