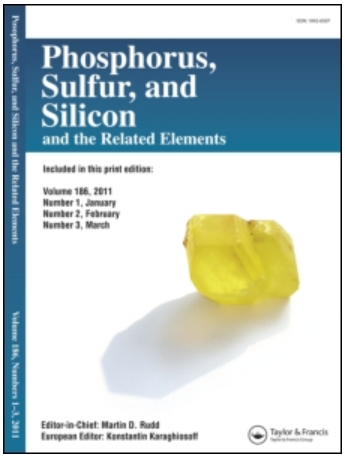

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AN APPROACH TO OLIGONUCLEOTIDE SYNTHESIS BY THE PHOSPHOTRIESTER METHOD

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AN APPROACH TO OLIGONUCLEOTIDE SYNTHESIS BY THE PHOSPHOTRIESTER METHOD

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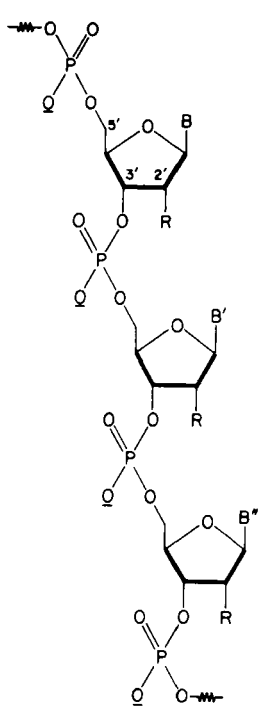
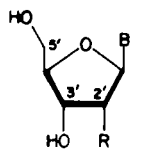
A systematic approach to the chemical synthesis of oligoribo- and oligodeoxyribo-nucleotides is described. Synthesis in both series of oligonucleotides has been tackled as a two-stage problem. The first stage, which is concerned with the preparation of correctly-orientated, suitably-protected nucleoside building blocks, has been completed. The second stage, which is concerned with the development of an effective phosphorylation procedure for linking the building blocks together, is presently being investigated. From the results obtained so far, it is apparent that the phosphotriester approach to the synthesis of oligonucleotides, especially with phenyl (and other aryl) protecting groups, is superior to approaches which involve unprotected internucleotide linkages during synthesis.

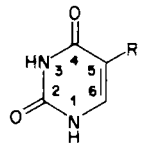
We have been concerned for a number of years with the development of methods for the chemical synthesis of oligonucleotides of known base-sequence. Although we originally tackled this problem because it presented a formidable challenge in synthetic organic chemistry, our continuing enthusiasm has been sustained by the value of synthetic oligonucleotides in the investigation of a number of important problems in biochemistry and molecular biology. A notable example of a biological application of chemically-synthesized oligoribo- and oligodeoxyribo-nucleotides was in the elucidation of the genetic code.[1,2] The ultimate objective of work in this field is the synthesis of a large sequence of a naturally-occurring deoxyribonucleic acid (DNA) molecule and perhaps the total synthesis of a transfer ribonucleic acid (tRNA) molecule.



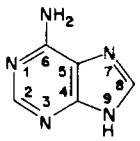
1 **a**; R=H
b; R=OH



2 **a**; R=H
b; R=OH



3 **a**; R=CH_3
b; R=H



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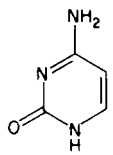

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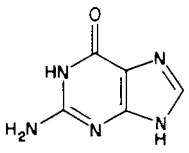

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† Plenary Lecture. Vth International Conference of Organic Phosphorus Chemistry, Gdansk, Poland, September 1974.

Structurally, oligonucleotides (**1**) are poly-dialkyl phosphate esters in which the 3′-hydroxyl function of one nucleoside building block (**2**) is joined to the 5′-hydroxyl function of another, through an internucleotide phosphodiester linkage. There are two series of oligonucleotides: the first series, the oligodeoxyribonucleotides (**1a**) are comparatively low molecular weight DNA-like molecules in which the building blocks are 2′-deoxyribonucleosides (**2a**). The principal 2′-deoxyribonucleosides (**2a**) are β-D-2-deoxyribofuranosides of two pyrimidine bases [thymine (**3a**) and cytosine (**4**)] linked through their 1-positions, and two purine bases [adenine (**5**) and guanine (**6**)] linked through their 9-positions. The second series of oligonucleotides, the oligoribonucleotides (**1b**), are comparatively low molecular weight RNA-like molecules. The only significant difference between the two series is the presence of the 2′-hydroxyl functions vicinal to the internucleotide linkages in the latter. Thus the principal nucleoside building blocks (**2b**) of oligoribonucleotides are β-D-ribofuranosides of two pyrimidine bases [uracil (**3b**), instead of thymine (**3a**) and cytosine (**4**)] and the same two purine bases [(**5**) and (**6**)]. A large number of other nucleosides have been found to occur[3] to a lesser extent, especially in tRNA.

The presence of the 2′-hydroxyl functions make oligonucleotide synthesis a more complex problem in the ribose than in the 2-deoxyribose series. The 2′-hydroxyl functions must be protected throughout an oligoribonucleotide synthesis to ensure that only natural 3′→5′-internucleotide linkages are formed. This leads to an orientational problem: a chemical method (or methods) of distinguishing between the 2′- and 3′-secondary hydroxyl functions is obviously required. The 2′-hydroxyl functions must then be blocked in such a way that they remain protected throughout the synthesis but may easily be released in the last step. It is not therefore surprising that progress in the chemical synthesis of oligonucleotides has been faster in the deoxyribose than in the ribose series.

We have tackled the synthesis of oligonucleotides in both series as a two-stage problem.[4] The first stage is concerned with the development of methods for the synthesis of protected nucleoside building blocks and the second stage involves joining the building blocks together by means of a suitable phosphorylation procedure. The second stage also involves the removal of all the protecting groups but this should present no difficulty if the latter have been correctly chosen or suitably designed in the first place. However, as will become clearer later on, this condition is not always easy to fulfil.

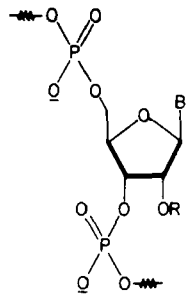
Nevertheless, we feel convinced that satisfactory methods have now been developed for the synthesis of correctly-orientated, suitably-protected nucleoside building blocks both in the ribose and deoxyribose series. Thus we believe that the first stage of the problem of oligonucleotide synthesis has essentially been completed. Some progress has been made with the second stage but, in our opinion, the outstanding remaining problem in this field is the development of a phosphorylation procedure which would permit really large oligomers to be synthesized in good yields.

THE FIRST STAGE – NUCLEOSIDE BUILDING BLOCKS

(a) Protecting Groups

It is relevant to include a short discussion on protecting groups before the actual matter of nucleoside building blocks is considered. The expression "suitably-protected" was used above. We consider a protecting group to be "suitable" for a particular purpose if (i) it is relatively easy to introduce, (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 desired product is stable. Regrettably, a number of protecting groups which do not meet all three of these criteria have been proposed in the literature for use in this field.

We have always favored the use of acid-labile (acetal, ketal, orthoester) and base-labile (acyl) protecting groups over those which may be removed in other ways (e.g. reductively or photochemically). Our reason for this attitude is that a great deal more is known about the action of acids and bases than of other reagents on nucleic acids and their components. The choice of protecting groups and the preparation of building blocks presents a more difficult problem in the ribose than in the deoxyribose series. Indeed perhaps the most crucial decision which has to be made in the planning of an oligoribonucleotide synthesis is the choice of protecting group (R, formula **7**) for the 2′-hydroxyl functions. This is largely because it must be possible to release the 2′-



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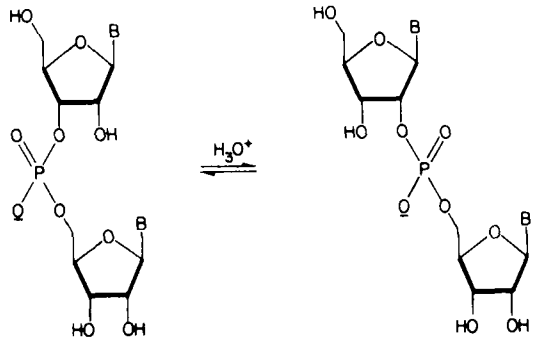
hydroxyl functions in the final step of the synthesis under conditions which are mild enough to prevent their attack on vicinal phosphodiester groups with consequent cleavage or migration of the internucleotide linkages.



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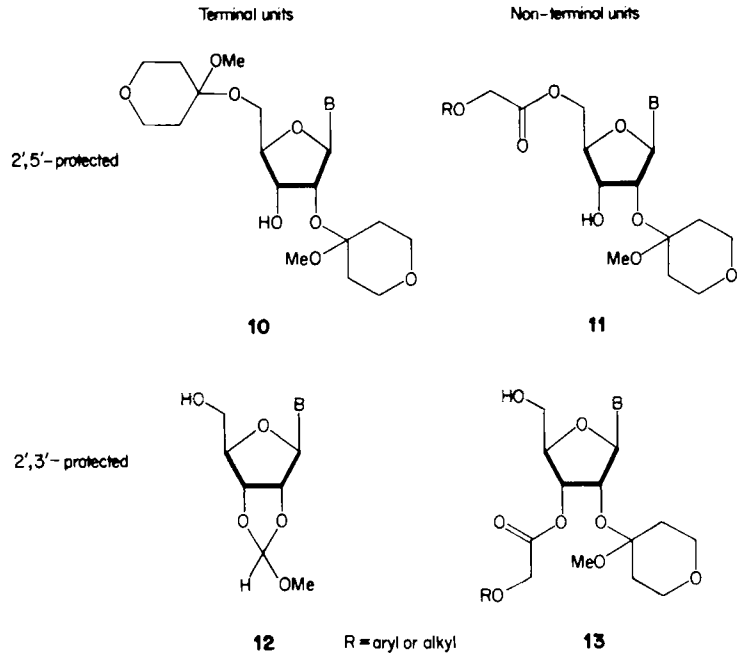
B = uracil-1-yl

Scheme 1

A few years ago there was some difference of opinion as to whether acid- or base-labile protecting groups should be used to block the 2′-hydroxyl functions in oligoribonucleotide synthesis. Khorana[5] favored the use of base-labile protecting groups on the grounds that migration of the internucleotide linkages can occur[6] in acidic media. Some internucleotide cleavage (involving intermediate 2′, 3′-cyclic phosphates) occurs[6] both in basic and acidic media but phosphoryl migration is a much more serious problem in that it is virtually impossible to separate oligoribonucleotides with one or more unnatural 2′→5′-internucleotide linkages. However, on the basis of a study[7] of the behaviour of uridylyl-(3′→5′)-uridine (Scheme 1, 8) in acidic media, we concluded that phosphoryl migration, to give (9), occurs to a negligible extent under the conditions required to remove an otherwise suitable acid-labile protecting group. We have subsequently always used acid-labile groups (see below) to protect the 2′-hydroxyl functions of ribonucleoside building blocks.

(b) Ribonucleoside Building Blocks

It is perhaps unnecessary to discuss here the methods which have been developed for the preparation of nucleoside building blocks as all of the main procedures have been in the literature for several years. Furthermore, as the design and preparation of deoxyribonucleoside building blocks are relatively simple matters, it is desirable to consider the ribonucleoside derivatives first and in more detail. Pure crystalline building blocks



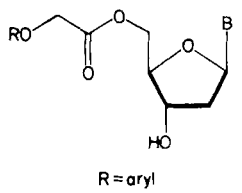
10 11 12 13

Scheme 2

of four types (**10**,[8] **11**,[9] **12**[10] and **13**;[11] Scheme 2) have been prepared from each of the four principal ribonucleosides and also from some of the minor nucleosides which occur in tRNA. The procedures are all completely general and depend only on the availability of the parent ribonucleosides. It can be seen that the 2′-hydroxyl functions of each of the building blocks is protected by an acid-labile protecting group: it is intended that these groups should remain intact until the final stage of the synthesis. We developed the methoxytetrahydropyranyl[12] (building blocks **10**, **11** and **13**) and the methoxymethylene[10] (building block **12**) protecting groups specifically for this purpose. Unlike the more commonly-used tetrahydropyranyl group,[13] the methoxytetrahydropyranyl protecting group is achiral and therefore its use in the protection of chiral alcohols does not lead to the formation of mixtures of diastereoisomers. Thus even *bis*-methoxytetrahydropyranyl derivatives, such as (**10**), may be isolated as pure crystalline solids in good yields.[8] We attach much importance to starting with pure crystalline building blocks as only in this way can the absence of impurities which would lead to incorrectly-orientated internucleotide linkages be ensured.[14] The conditions of acidic hydrolysis required[12] for the removal of methoxytetrahydropyranyl protecting groups are such that migration and cleavage of the internucleotide linkages (see above) occur to a negligible extent. The methoxymethylene (cyclic orthoformate) group protects both the 2′- and 3′-hydroxyl functions of building block (**12**); this group undergoes acidic hydrolysis at a rate[10] close to that of the methoxytetrahydropyranyl group.

Of the four types of building block prepared (Scheme 2), each has either a 3′- (the 2′, 5′-protected unit) or a 5′- (the 2′, 3′-protected unit) hydroxyl function free. The terminal units (**10** and **12** respectively) have the 5′- or 3′-hydroxyl functions also protected with acid-labile protecting groups while the non-terminal (or chain-extension) units (**11** and **13** respectively) have the 5′- or 3′-hydroxyl functions protected with base-labile protecting groups. The latter, which are generally aryloxy- or alkoxy-acetyl groups, have been selected to be particularly sensitive to base for the reason considered below. As will soon become apparent, all four types of building block are required for each ribonucleoside if stepwise synthesis in both directions (i.e. from the 3′- and 5′-ends of the growing oligomers) and block synthesis of oligoribonucleotides are to be possible.

(c) Deoxyribonucleoside Building Blocks



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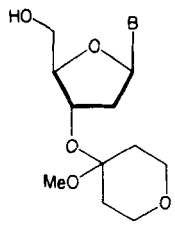

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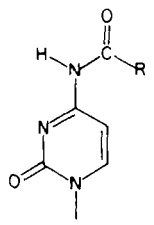
Only two types of building block are required for each 2′-deoxyribonucleoside: one with a protected 5′-hydroxyl function (**14**) and one with a protected 3′-hydroxyl function (**15**). If one building block has a base-labile and the other an acid-labile protecting group, stepwise synthesis in both directions and block synthesis are possible. For technical reasons, we decided[15] to work with 5′-esters (**14**) and 3′-ketals (**15**). Both types of building block are easy to prepare:[15,16] the 5′-esters may be made directly from the corresponding free (or *N*-protected) deoxynucleosides and the 3′-ketals may be prepared from the 5′-esters in two steps. As will become apparent, in oligodeoxyribonucleotide synthesis we also need acyl protecting groups which are particularly sensitive to base. Although the methoxytetrahydropyranyl group was designed specifically for the protection of 2′-hydroxyl functions in oligoribonucleotide synthesis, it is very fortunately also suitable for the protection of 3′-hydroxyl functions in oligodeoxyribonucleotide synthesis. Thus it is removable under very mild conditions of acidic hydrolysis which lead[16] to a negligible amount of cleavage of the glycosidic linkages of 2′-deoxyadenosine and 2′-deoxyguanosine residues.

Like Khorana[2b] and other workers in this field, we have protected cytosine, adenine and guanine residues by *N*-acylation (as in **16**, **17** and **18** respectively). It is necessary to do this to avoid *N*-phosphorylation in the case of the cytosine derivatives[17] and it is a wise precaution to take in the case of adenine derivatives. As far as guanine derivatives are concerned it is hard to purify let alone to crystallize the nucleoside building blocks unless they are first protected by *N*-acylation. However, simple acylation (e.g. as in **18**; R = Ph) does not appear to provide sufficient protection for the guanine residues during phosphorylation. Thus the yields of oligonucleotides obtained in the phosphotriester approach (see below) when *N*-benzoylguanine (**18**; R = Ph) derivatives are involved are usually[16] substantially lower than the yields obtained otherwise. Possible solutions to this problem are currently under investigation.



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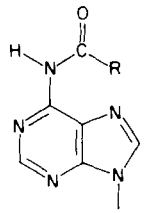

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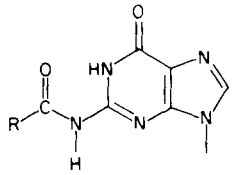

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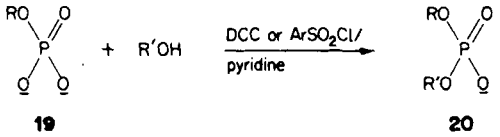
THE SECOND STAGE. PHOSPHORYLATION PROCEDURES. OLIGONUCLEOTIDE SYNTHESIS

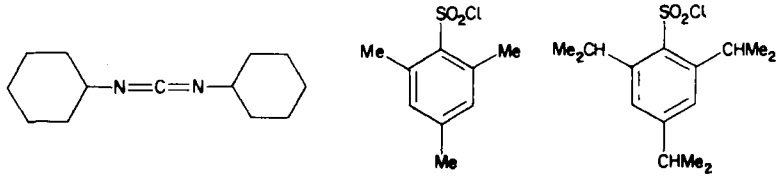
(a) The Phosphodiester Approach

The phosphorylation procedures which have been used most widely in oligonucleotide synthesis are summarized in Scheme 3. These procedures, which were introduced by Khorana and his coworkers,[18] involve the reaction between a monoalkyl phosphate (**19**), an alcoholic hydroxyl function and a condensing agent in

anhydrous pyridine solution to give the desired dialkyl phosphate (**20**). The main condensing agents which have been used[19] are *N,N'*-dicyclohexylcarbodiimide (**21**) (DCC) and arenesulfonyl chlorides such as mesitylene- and 2,4,6-triisopropylbenzenesulfonyl chlorides (**22** and **23** (TPS), respectively). One of the essential features of this approach is that the internucleotide phosphodiester linkages are left unprotected during subsequent phosphorylation steps.



19 **20**



21 **22** **23**

Although this phosphodiester approach has been used successfully in the deoxyribose series,[2b] it did not prove[20] to be suitable for the synthesis of oligonucleotides from our ribonucleoside building blocks. We found[20] that whether DCC (**21**) or TPS (**23**) was used as condensing agent, yields of oligomers larger than dinucleoside phosphates were unsatisfactory. Possibly this was due to the presence of bulky 2'-O-methoxytetrahydropyranyl groups but, in any case, a phosphorylation procedure in which the internucleotide phosphodiester linkages are left unprotected (i.e. any phosphodiester approach) has several inherent disadvantages:

i) Phosphodiester functions are nucleophilic and are thus 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.

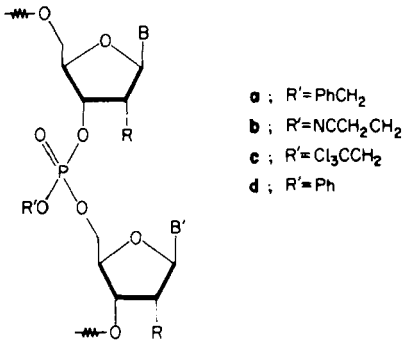
ii) Salts of phosphodiesters are normally only soluble in water and the more polar organic solvents. Thus they cannot usually be purified by the standard techniques of organic chemistry such as adsorption chromatography on silica gel or alumina: it is therefore necessary to use fractionation techniques such as cellulose or Sephadex anion-exchange chromatography and this tends to limit the scale of the reactions.

iii) Anion-exchange chromatography usually involves the use of aqueous or aqueous alcoholic buffer solutions. Exceptional care has then to be taken in the purification of the partially-protected phosphodiester intermediates as the protecting groups used are necessarily very sensitive to acid- or base-catalyzed hydrolysis.

For these reasons, we formed the opinion[4] that ultimate success in the chemical synthesis of oligoribonucleotides, and probably also 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 disadvantages of the phosphodiester approach would all be removed at one fell swoop. However, a new problem is then introduced: what protecting group should be used for the internucleotide linkages?

(b) The Phosphotriester Approach

The phosphotriester approach to the synthesis of oligonucleotides with natural 3'→5'-internucleotide linkages was first demonstrated in 1955 by Michelson and Todd,[21] who used the benzyl protecting group (as in **24a**) in the synthesis of thymidylyl-(3'→5')-thymidine. This approach then remained out of favor for ten years until



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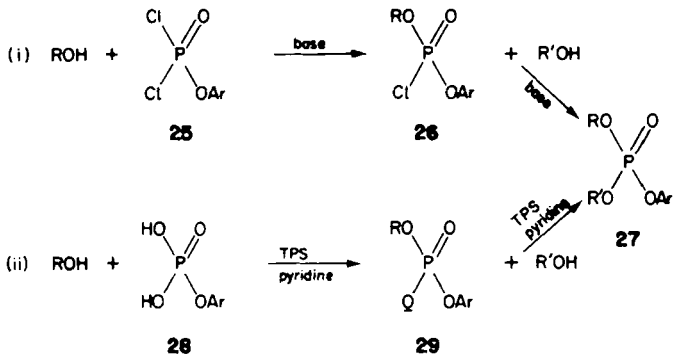
Letsinger and Mahadevan[22] used the 2-cyanoethyl group for the protection of internucleotide linkages (as in **24b**). Shortly afterwards, Eckstein and Rizk[23] proposed the use of the 2,2,2-trichloroethyl protecting group (as in **24c**) and we proposed[24] the use of the phenyl protecting group (as in **24d**) for this purpose. It now seems probable that both benzyl and 2-cyanoethyl groups are too labile to be used in the synthesis of moderately-sized or large oligonucleotides. The 2,2,2-trichloroethyl group does not suffer from this disadvantage and has been used by Neilson[25] in the synthesis of oligoribonucleotides. However we feel that phenyl and other aryl groups are particularly suitable for the protection of internucleotide linkages and all of our recent work on the synthesis of oligonucleotides has involved their use.



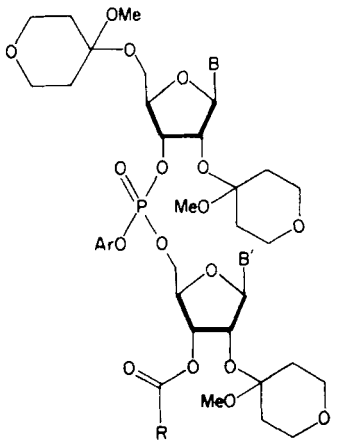
Scheme 4

If the phosphotriester approach with an aryl protecting group is adopted, the desired initial products are unsymmetrical dialkyl aryl phosphates (**27**). In Scheme 4, ROH and R'OH represent the protected nucleoside (or oligonucleotide) building blocks with free 3'- and 5'-hydroxyl functions, respectively. Triester synthesis is clearly a two-step process which requires a bifunctional phosphorylating agent. Perhaps the most obvious procedure to follow is (i) which involves the use of an aryl phosphorodichloridate (**25**). This procedure may

be used but the second step is very slow unless a nucleophilic catalyst (such as 5-chloro-1-methylimidazole, **30**) is added.[26] Even then the yields of oligomers larger than dinucleoside phosphates are unsatisfactory.

Procedure (ii) (Scheme 4), in which phosphorylation is effected by an aryl dihydrogen phosphate (**28**) in the presence of TPS (**23**) in pyridine solution, is much more satisfactory: good yields even of quite large oligonucleotides may then be obtained. Procedure (ii) has two other important advantages over procedure (i):

1) Aryl dihydrogen phosphates (**28**) may be usually obtained[27] as crystalline, non-hygroscopic solids and thus precisely stoicheiometric amounts of phosphorylating agent may be used.
2) TPS (**23**) acts as a drying agent as well as a condensing agent; thus if a sufficient excess of it is used, high yields of products may be obtained even if the reactants are not rigorously anhydrous.



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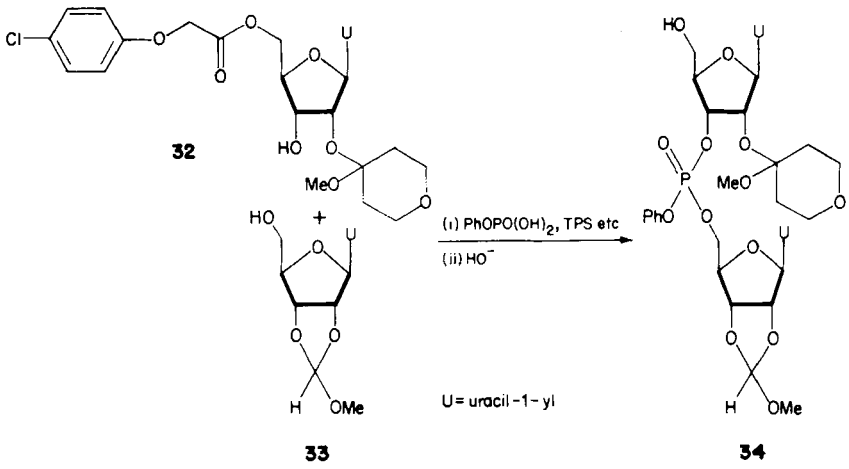
Before the phosphotriester approach to oligonucleotide synthesis is considered in detail, another factor relating to the choice of protecting groups should be mentioned. If, for example, the fully-protected diribonucleoside phosphate (**31**) is to be extended to a trimer or larger oligomer, it is essential that the phosphotriester system should be stable under the basic conditions required to remove the 3′-*O*-acyl protecting group (RCO). For this reason, exceptionally base-labile protecting groups, such as aryloxy- and alkoxy-acetyl,[11] have been incorporated into the appropriate types of nucleoside building blocks (**11**, **13** and **14**) (see sections I (b) and I (c) above).

(c) Stepwise Synthesis of Oligoribonucleotides

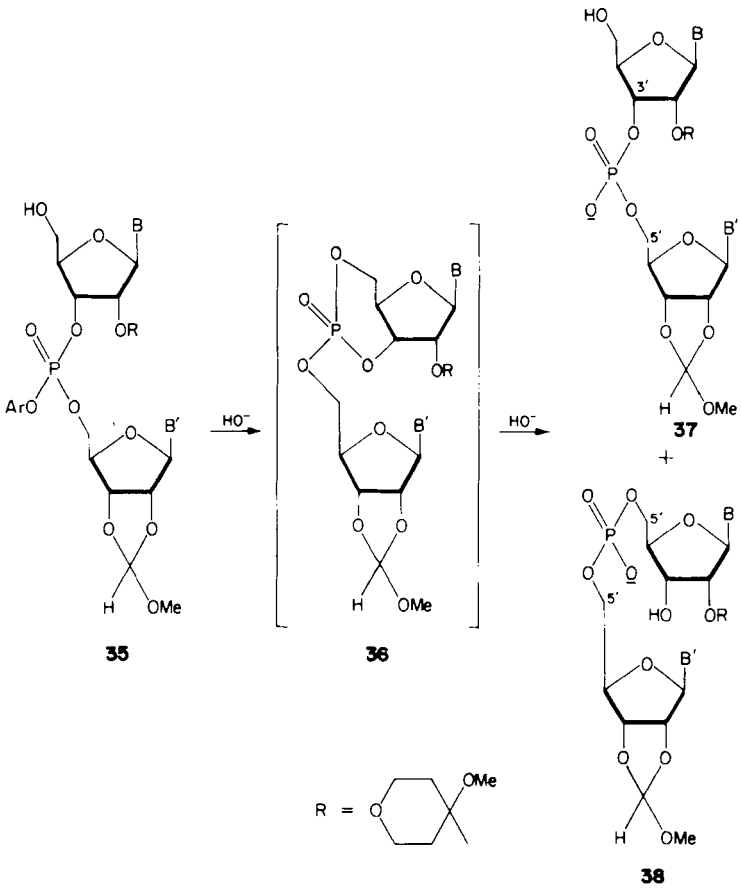
A stepwise synthesis of oligoribonucleotides, involving extension from the terminal 5′-hydroxyl function of the growing oligomer, is suggested in Scheme 5. A 2′, 5′-non-terminal uridine building block (**32**) is allowed to react[26] with a stoicheiometric amount of phenyl dihydrogen phosphate (**28**; Ar = Ph) in the presence of an excess of TPS (**23**) in pyridine solution. After several hours, a 2′, 3′-terminal uridine building block (**33**) and more TPS is added. The second stage of the phosphorylation (Scheme 4 (ii)) is allowed to proceed for ca. 48 hours before the products are worked-up. Treatment with base, under very mild conditions, then leads to selective 5′-deacylation to give the partially protected uridylyl-(3′→5′)-uridine derivative[26] (**34**). The latter material may be purified by adsorption chromatography and isolated as a colourless solid in over 75% yield. If the 2′, 5′-non-terminal building block (**32**) is treated again with a stoicheiometric amount of phenyl dihydrogen phosphate and an excess of TPS, and then allowed to react with (**34**) instead of the 2′, 3′-terminal building block (**33**) in the second phosphorylation step, a trimer may be obtained[26] in ca. 60% yield. Thus a stepwise synthesis is possible. Preliminary experiments have indicated[28] that the yields of partially-protected

oligouridylic acids (with free 5′-hydroxyl functions, corresponding to **34**) up to and including the pentamer all appear to be ca. 60%.



32 **33** **34**

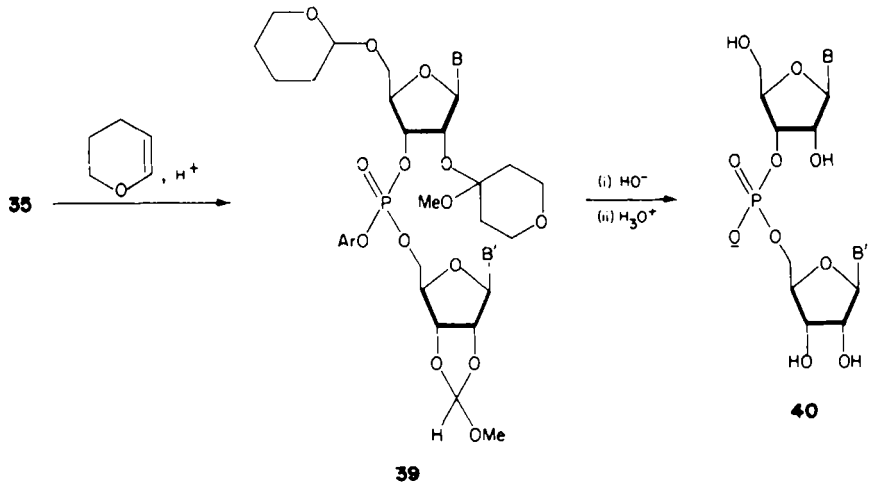
Scheme 5



35 **36** **37** **38**

Scheme 6

In order to remove the protecting groups from partially-protected dinucleoside phosphates (**35**) or corresponding larger oligomers, treatment with alkali (to unblock the internucleotide linkages), followed by treatment with acid (to unblock the hydroxyl functions) is necessary. However, unless the terminal 5′-hydroxyl functions are first protected, treatment with alkali leads[26] to some material with terminal 5′→5′-internucleotide linkages in addition to the desired products. Thus, in the case of (**35**), treatment with alkali (see Scheme 6) leads[26] to (**37**), contaminated with (**38**). The formation of the latter compound presumably results from the intermediacy of (**36**) but attempts to isolate such an intermediate 3′, 5′-cyclic dinucleoside phosphate have, so far, been unsuccessful.† Recent experiments[26b] have shown that both unprotected 5′- and 3′-hydroxyl functions participate to an appreciable extent in the hydrolysis of thymidylyl-(3′→5′)-thymidine aryl esters. If the 3′-hydroxyl function is left unprotected, thymidylyl-(3′→3′)-thymidine derivatives are obtained. These results are not unexpected in that thymidine 3′, 5′-cyclic phosphate may be obtained[29] in high yield by the action of base on either thymidine 3′- or 5′-*p*-nitrophenyl phosphate.



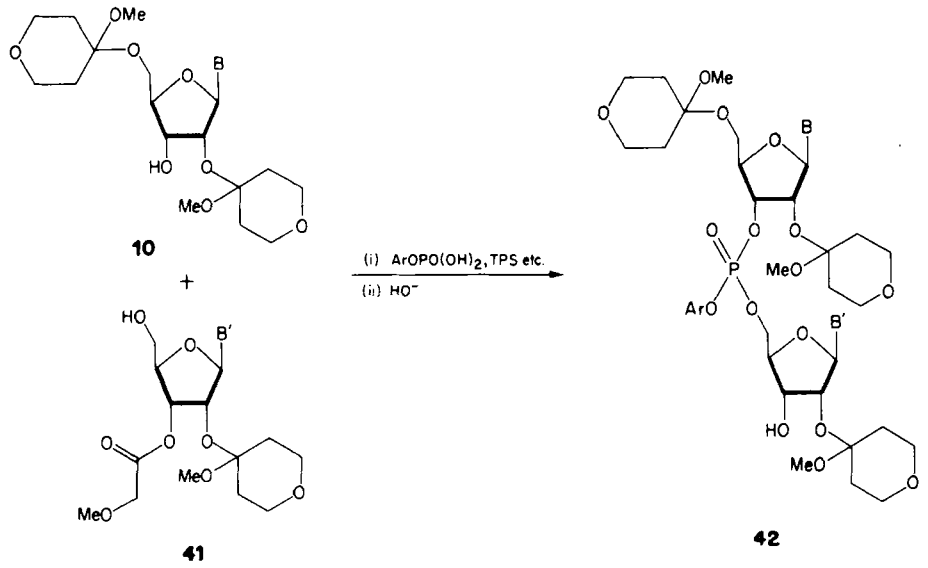
Scheme 7

The isomerization problem in the unblocking of partially-protected oligomers with free 5′-hydroxyl functions (such as **35**) can be overcome[26] by introducing a tetrahydropyranylation step before treatment with alkali (see Scheme 7). Tetrahydropyranylation[13] is usually a rapid and virtually quantitative reaction. The unblocking steps then proceed in high yield to give a dinucleoside phosphate or larger oligoribonucleotide. This procedure may also be used[16] in the unblocking of a partially-protected oligonucleotide with a free 3′-hydroxyl function (see below).

An alternative stepwise approach to the synthesis of oligoribonucleotides *via* phosphotriester intermediates is suggested in Scheme 8; this approach involves extension from the terminal 3′-hydroxyl function of the growing oligomer. A 2′, 5′-terminal building block (**10**) is phosphorylated with an aryl dihydrogen phosphate (**28**) and TPS (**23**) in pyridine solution and then a 2′, 3′-non-terminal building block (**41**) is added. Work-up and treatment with base under mild conditions gives the partially-protected dinucleoside phosphate (**42**) with a free 3′-hydroxyl function. If this process is repeated, except that the terminal ribonucleoside building block (**10**) is replaced by (**42**), a trinucleoside diphosphate is obtained.

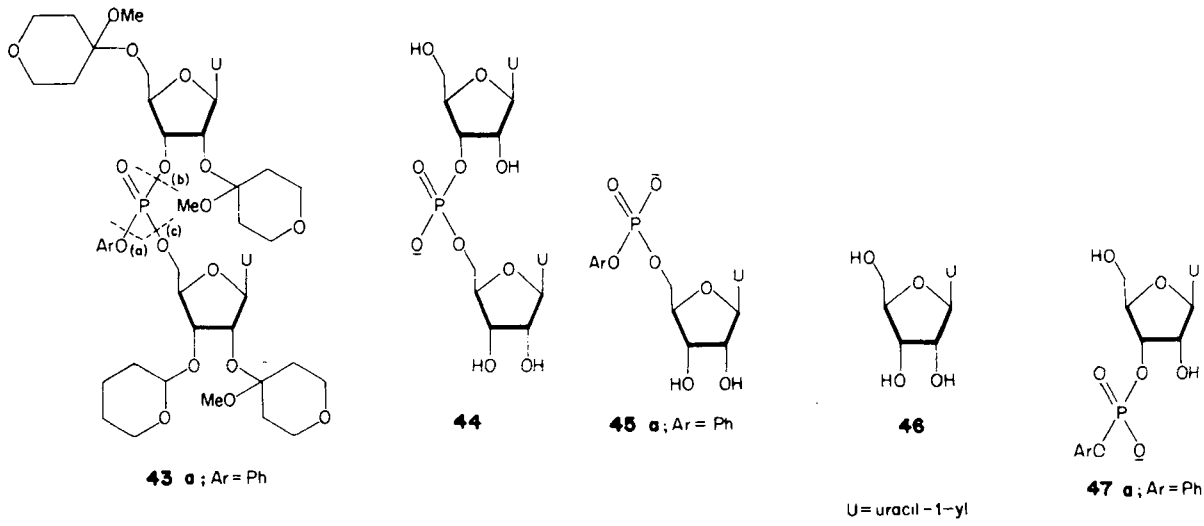
Both phenyl and *o*-chlorophenyl dihydrogen phosphates have been used as the phosphate source in this approach. Reactions were carried out[16] with ribonucleoside building blocks derived from uridine, N^4-*p*-anisoylcytidine, N^6-*p*-anisoyladenosine and N^2-benzoylguanosine to give satisfactory yields (60-70%) of partially-protected dinucleoside phosphates (**42**) except when guanosine derivatives were involved[16] (see

† *Footnote added in proof.* This was the case in September 1974 when this lecture was delivered. However, it has recently been possible (Ref. 26 b) to isolate (**36**, B = B′ = uracil-1-yl).



Scheme 8

section I (c) above). Several trinucleoside diphosphates (derived from uridine, N^4-*p*-anisoylcytidine and N^6-*p*-anisoyladenosine) were prepared from partially-protected dimers (**42**) and 2′, 3′-nonterminal building blocks (**41**): satisfactory yields (60-70%) were again obtained.[16] As in the case of partially-protected oligomers with free 5′-hydroxyl functions, (**42**) and the corresponding trinucleoside diphosphates were tetrahydropyranylated (Scheme 7) before the alkaline hydrolysis step of the unblocking procedure was effected.



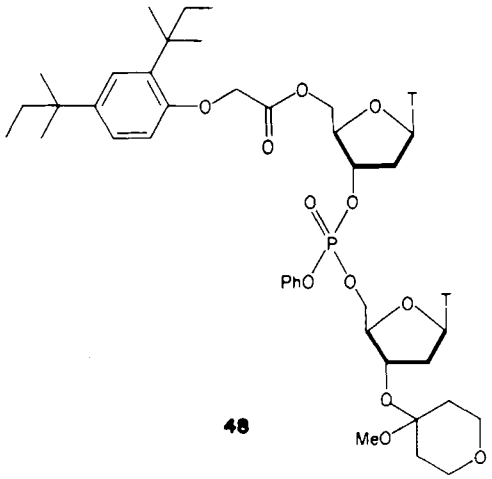
Scheme 9

The choice of the phenyl group for the protection of internucleotide linkages was made on the basis of phenol being a stronger acid by ca. 5 orders of magnitude than a simple alcohol (such as the 3′- or 5′-hydroxyl function of a nucleoside). It was originally hoped that, for example, alkaline hydrolysis of the fully-protected dinucleoside phosphate phenyl ester (**43a**) would proceed virtually exclusively by mode (a) (with phenoxide ion as leaving group; see Scheme 9) to give, after the removal of the ketal and acetal protecting groups, solely the desired uridylyl-(3′→5′)-uridine (**44**). Unfortunately,[30] only ca. 96% phosphotriester hydrolysis occurred by mode (a); hydrolysis also proceeded by modes (b) and (c) to give, following acidic hydrolysis, uridine 5′-phenyl phosphate (**45a**), uridine (**46**) and products derived from uridine 3′-phenyl phosphate (**47a**). As might be expected, internucleotide cleavage due to hydrolysis by modes (b) and (c) could become a serious problem in the unblocking of oligomers with several phenyl-protected internucleotide linkages. Fortunately, however, as will become clear later, there are at least two ways of lowering the amount of internucleotide cleavage accompanying phosphotriester hydrolysis so that it does not rise above an acceptable level.

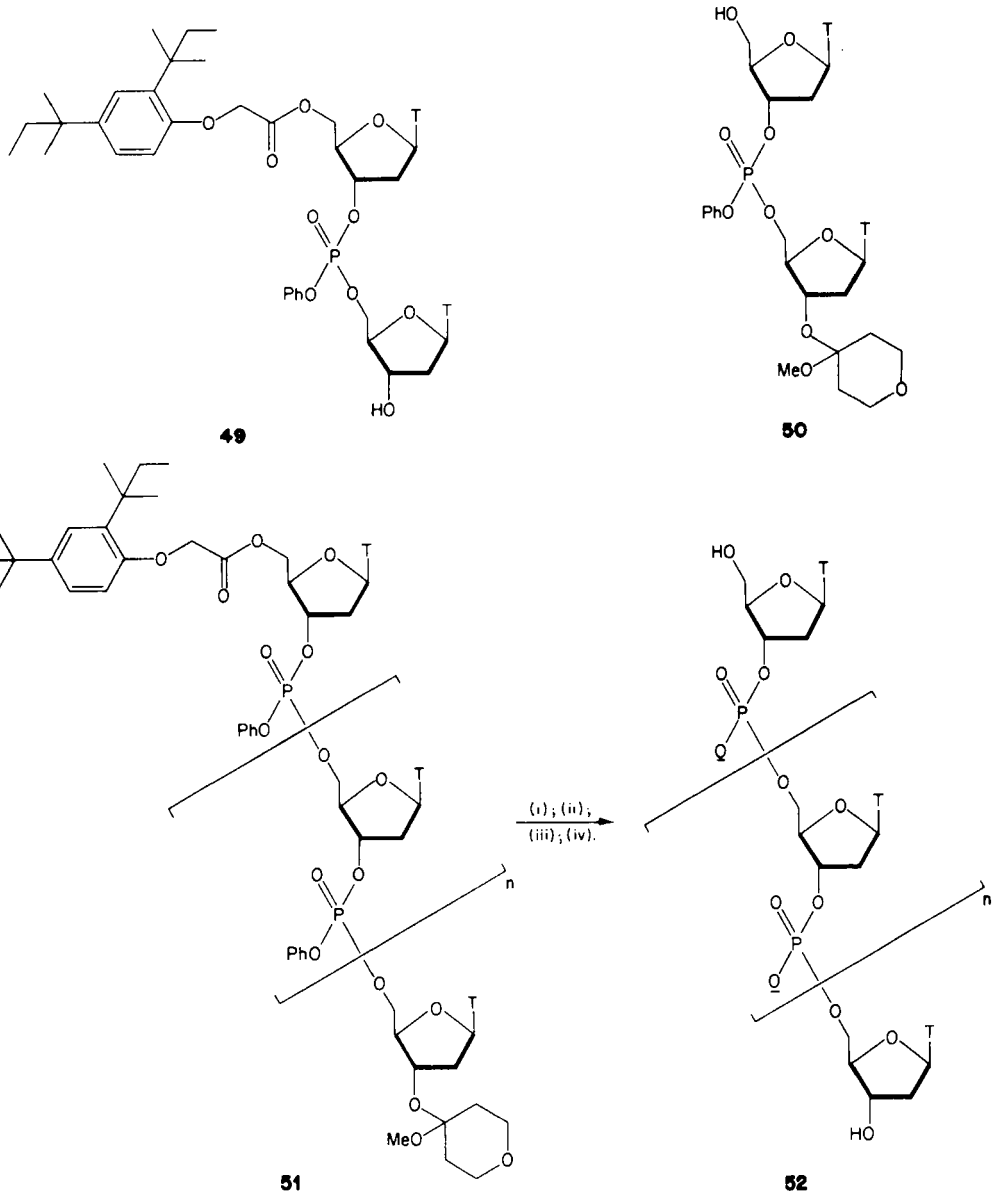
(d) Block Synthesis of Oligodeoxyribonucleotides

As indicated above, the availability of four types of ribonucleoside building block makes it possible for the stepwise synthesis of oligoribonucleotides to be carried out in both directions. The first stepwise synthesis (Scheme 5) leads to partially-protected oligomers (such as **34**) with free 5′-hydroxyl functions whereas the second synthesis (Scheme 8) leads to partially-protected oligomers (such as **42**) with free 3′-hydroxyl functions. The two types of partially-protected oligomers are designed in such a way that it should be possible to link them together in a block synthesis. We have not yet carried out such a block synthesis in the ribose series but have little doubt that it would be possible to do so. We thought that it might, in the first instance, be more instructive to investigate the potential of block synthesis in the deoxyribose series. We therefore undertook[15] the block synthesis of oligothymidylic acids of different chain lengths.

The fully-protected thymidylyl-(3′→5′)-thymidine derivative (**48**) (Scheme 10) was prepared[15] from the appropriate thymidine building blocks (see formulae **14** and **15**), phenyl dihydrogen phosphate (**28**; Ar = Ph) and TPS (**23**); it was isolated as a pure colourless solid, in 75% yield, following short column chromatography[31] of the products. The latter technique has been found to be particularly useful for the purification of nucleoside building blocks and phosphotriester intermediates. The lipophilic aryloxyacetyl protecting group was used to facilitate chromatographic separations.



48



Scheme 10

The partially-protected dinucleoside phosphates (**49** and **50**) were obtained, in high yields, by submitting (**48**) to mild conditions of acidic and basic hydrolysis, respectively. Treatment of (**49**) with a stoicheiometric amount of phenyl dihydrogen phosphate and an excess of TPS, followed by the addition of a stoicheiometric amount of (**50**), gave the fully-protected tetranucleoside triphosphate (**51**; n = 2). This block synthesis was carried out on a 2.8 millimolar scale and the latter compound was isolated as a colourless solid in 63% yield. This process was repeated. Thus the partially-protected tetramers (corresponding to **49** and **50**) with free 3′- and 5′-hydroxyl functions, respectively, were prepared by treating (**51**; n = 2) with acid and base; these tetramers (0.49 millimoles of each) were then joined together by the same procedure to give the fully-protected octamer (**51**; n = 6) in 57% isolated yield. A preliminary experiment, carried out on a small scale, indicated that the fully-protected hexadecamer (**51**; n = 14) could be prepared from the two corresponding partially-protected octamers without any appreciable fall-off in yield.

The success of the block synthesis depends ultimately not on the yields of fully-protected oligomers (**51**) but on the yields of unprotected oligothymidylic acids (**52**) obtained. The procedure adopted (Scheme 10) for the unblocking of the fully-protected oligomers consists of four steps: (i) treatment with base for a short time to unblock the terminal 5′-hydroxyl function; (ii) protection of the latter hydroxyl function by tetrahydropyranylation to prevent its participation during phosphotriester hydrolysis; (iii) alkaline hydrolysis of the phosphotriester groups to liberate the internucleotide phosphodiester linkages; and (iv) removal of the terminal methoxytetrahydropyranyl and tetrahydropyranyl protecting groups by acidic hydrolysis. This procedure was used in the unblocking of the fully-protected dimer (**51**; n = 0), tetramer (**51**; n = 2) and octamer (**51**; n = 6).

In each case, the unprotected oligothymidylic acid (**52**) was purified by chromatography on DEAE-cellulose. The yields of pure oligothymidylic acids obtained in the unblocking processes, expressed as percentages of the total amount of nucleoside and nucelotide material eluted from the DEAE-cellulose columns, were: dinucleoside phosphate (**52**; n = 0), 92%; tetranucleoside triphosphate (**52**; n = 2), 79%; octanucleoside heptaphosphate (**52**; n = 6), 52%. Thus, as anticipated above, internucleotide cleavage becomes a serious problem in the unblocking of relatively large fully-protected oligonucleotide phenyl esters by the above procedure.

The most obvious way to decrease the amount of internucleotide cleavage accompanying phosphotriester hydrolysis is to use an aryl protecting group derived from a more acidic phenol. It can be seen from the table[30] that alkaline hydrolysis of the fully-protected uridylyl-(3′→5′)-uridine 2-chlorophenyl and 3,5-dichlorophenyl esters (**43**; Ar = 2-ClC_6H_4 and 3,5-$Cl_2C_6H_3$, respectively) leads to ca. 40% and 30% of the amount of internucleotide cleavage observed in the hydrolysis of the corresponding phenyl ester (**43a**). It can further be seen that it is possible to keep the amount of internucleotide cleavage below 1% by, for example, the use of the 2,4- or the 2,5-dichlorophenyl protecting group.

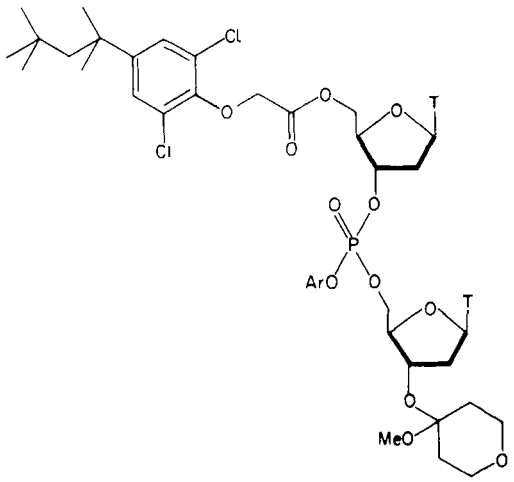
TABLE I

Alkaline hydrolysis of fully-protected uridylyl-(3′→5′)-uridine aryl esters[a] (**43**) with 0.1 *M*-NaOH/dioxan-water (1:4, v/v) at 20°.

Ar	$t_{1/2}$ (min) [b]	Yield of uridine (%) [c]
C_6H_5–	40	4.2
2-FC_6H_4–	7.5	2.4
2-ClC_6H_4–	6.5	1.8
3,5-$Cl_2C_6H_3$–	3.0	1.2
2,4-$Cl_2C_6H_3$–	2.5	0.8
2,5-$Cl_2C_6H_3$–	1.8	0.7

[a] The initial substrate concentrations were 0.001 *M*.
[b] First-order kinetics were observed.
[c] Uridine is formed following acidic hydrolysis (0.01 *M* hydrochloric acid) of the products; its yield is based (see Scheme 9) on the possible formation of one molecule of uridine per molecule of (**43**). The percentage of uridine released is a direct measure of the percentage of internucleotide cleavage (i.e. hydrolysis by modes (b) and (c)).

However an additional problem may be introduced if a halogenated aryl protecting group is used. Thus although the use, for example, of the 3,5-dichlorophenyl instead of the phenyl protecting group lowers internucleotide cleavage by a factor of 3.5, the rate of triester hydrolysis is increased by a factor of more than 13. This is disadvantageous in that all of our synthetic procedures require the selective unblocking of hydroxyl functions by base-catalyzed de-acylation with, if possible, no concomitant phosphotriester hydrolysis. This problem has already been considered in section II (b) above.



53 **a** ; Ar = C_6H_5
b ; Ar = 3,5−$Cl_2C_6H_3$

Thus it is possible to remove the particularly base-sensitive aryloxyacetyl protecting group from the fully-protected thymidylyl-(3′→5′)-thymidine phenyl ester[32] (**53a**) without any detectable phosphotriester hydrolysis. Indeed, preliminary experiments suggest[32] that it should be possible to unblock the 5′-end of the corresponding octamer (heptaphenyl ester) without any of the seven phosphotriester groups undergoing hydrolysis to a significant extent. This is clearly essential if the synthesis of really large oligomers is to be undertaken by this approach. However we anticipate that, while it should be possible to unblock the 5′-end of the fully-protected thymidylyl-(3′→5′)-thymidine 3,5-dichlorophenyl ester (**53b**) with good recovery, an attempt to unblock the 5′-end of the corresponding octamer (hepta-3,5-dichlorophenyl ester) would almost certainly lead to an unsatisfactory yield of the desired product.

A consideration of the possible solutions to this problem leads to two obvious approaches. First, an acyl group, much more sensitive to base, could be used to protect the 5′-hydroxyl functions. Secondly, a group, removable by means other than basic or acidic hydrolysis, could be used to protect the 5′-hydroxyl functions. We are currently investigating the first approach and are considering the possibility of the second. However, rather than discuss these two approaches here, we should like to indicate a third, perhaps less obvious approach.

This approach is particularly simple. The two media (0.1 *M*-sodium hydroxide/water-dioxan, 4:1, v/v, and 0.2 *M* sodium hydroxide/water-dioxan, 1:1, v/v), which we have previously used for alkaline hydrolysis of phosphotriester groups, were apparently unfortunate choices in that their use led to appreciable internucleotide cleavage. Following observations made in connection with recent studies[33] on the synthesis of nucleoside 3′, 5′-cyclic phosphates, we have carried out some phosphotriester hydrolysis experiments with 0.1 *M*-potassium hydroxide/dimethyl sulfoxide-water (9:1, v/v) and have observed[30] an approximately threefold decrease in internucleotide cleavage compared with the above media. We are now investigating the possibility of using other alkaline media and our immediate aim is to find a medium in which hydrolysis of oligonucleotide phenyl esters will lead to not more than 0.5% cleavage of each internucleotide linkage. If this aim is

achieved, we shall feel justified in concluding that the phosphotriester approach with phenyl protecting groups for the internucleotide linkages is, at present, the method of choice for the chemical synthesis of oligonucleotides both in the ribose and in the deoxyribose series.

ACKNOWLEDGEMENTS

I should like to acknowledge the contributions which a number of excellent coworkers have made over the past twelve years to our studies on the chemical synthesis of oligonucleotides. In making this acknowledgement, I should like to emphasize that our work over the past four years has been the result of close collaboration between myself and my coworkers in Cambridge (and more recently in London) and Dr J. H. van Boom and his coworkers in Leiden. I should like to add that during this latter period the contributions of Dr van Boom's laboratory to the overall project have been outstanding.

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