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Phosphite Synthesis of Oligodeoxyribonucleotides

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Data in the literature published between 1983 and 1986 on the synthesis of oligodeoxyribonucleotides from nucleoside derivatives of phosphorous acid are reviewed. Attention is mainly focused on the techniques employed for preparing initial monomers for condensation. The individual stages in the solid-phase phosphite method are presented, and the potential of various approaches for achieving this type of synthesis is discussed. In addition, studies on the synthesis of oligonucleotides with the aid of monoesters of phosphorous acid (phosphonate synthesis) are assessed.
The bibliography includes 101 references.

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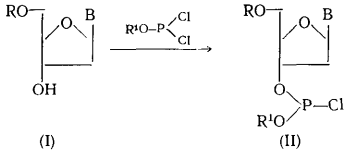
I. INTRODUCTION

Chemical synthesis of DNA fragments is of key importance for the advancement of molecular biology and genetic engineering. Many commercial organisations in a number of Advanced Nations offer a range of reagents and efficient automatic synthesisers and also provide a service for synthesising oligodeoxyribonucleotides with the required sequence. Moreover many scientific centres are involved in the elaboration of synthetic methods and therefore one would anticipate further progress in this field.

Until recently advances in solid-phase synthesis of DNA had been mainly determined by developments in the chemistry of derivatives of tri-coordinated phosphorus, the nucleoside phosphites. The reviews present in the literature at the time when the present communication was submitted for publication did not consider aspects of phosphite synthesis in any detail.[1-5] The present review focuses on reports published between 1983 and 1986 in which nucleoside phosphites were used for oligonucleotide synthesis and which deal with various practical aspects of this type of synthesis.

II. SYNTHESIS OF NUCLEOSIDE PHOSPHORAMIDITES

It is generally accepted that the original monomeric units in solid-phase phosphite synthesis were the nucleoside chlorophosphites (II) obtained by the reaction between nucleosides‡ (I) and alkyl dichlorophosphites:[6-8]



(I) (II)

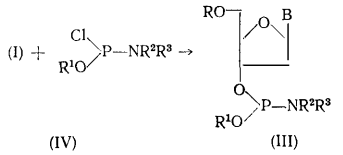
†The prefix *d* (deoxy) is omitted throughout for brevity.

‡The following arbitrary terms are used to describe the formulae: $(CH_3O)_2Tr$ – 4,4'-4,4'-dimethoxytrityl; throughout where not specially indicated R, triarylmethyl: R^1, alkyl or aryl; R^2 and R^3, alkyl; B, the protected heterocyclic base of the nucleoside.

Although this appears to be a straightforward process, the monomers (II) are relatively unstable during storage, and it is necessary to employ low temperatures (–78 °C), in order to reduce secondary reactions. Although until recently certain groups of research workers have adhered to the chlorophosphite approach[9] for this synthesis, the use of phosphoramidites (III) has gained universal acceptance, since the latter compounds may be converted into active intermediates by reaction with acidic reagents *in situ*.[10] A feature of this approach is the relative ease of use of these compounds, and they can be stored for months or even years under conditions which are easy to provide in practice. There are currently several techniques and modifications for preparing various phosphoramidites (III). Naturally not all of these modifications are used in practice but they all represent "progress steps" along the route towards a more efficient and cost effective method for the chemical synthesis of DNA.

1. Main Methods for Monomer Synthesis

The most extensively used method for preparing phosphoramidites (III) involves the reaction between the corresponding nucleoside (I) and the following type of phosphorylating agent (IV):[10-17]



(IV) (III)

This method is quite straightforward and well-defined provided that the phosphorylating agent (IV) is pure. In addition to synthesis of normal phosphoramidites (III) this method is also used for resolving certain non-routine problems, for example preparation of phosphoramidites of unusual nucleosides,[18] and also in a number of lipids.[19]

A considerable number of type (IV) agents have already been synthesised and characterised. Key information on certain of these compounds is presented in Table 1. Nevertheless, the preparation of compounds (IV) is labour-intensive and is not always a safe process (on account of their very high reactivity). Moreover, reagent (IV)

cannot be purified by distillation, when relatively high molecular weight substituents are present in positions R^1, R^2, or R^3. Hence, several alternative routes for the synthesis of nucleoside phosphoramidites (III) have been proposed which circumvent the phosphorylating agent (IV) preparation stage. In one of these methods,[20-24] a substituted dichlorophosphite is treated with excess of triazole or its trimethylsilyl derivative. The resultant bis-triazolide subsequently reacts sequentially with nucleoside (I) and the corresponding amine:

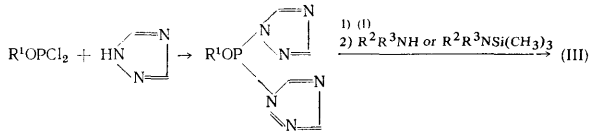


Table 1. Physicochemical properties of certain amidochlorophosphites (IV).

R^1—	—NR^2R^3	Boiling point, °C (mm Hg)	^{31}P NMR, p.p.m.	Refs.
CH_3—	—$N(CH_3)_2$	40—42 (13)	—179.5 (a)	[10]
CH_3—	—N⟨morpholino⟩	52—54 (0.1)	—172.1 (a)	[11,17]
CH_3—	—$N[CH(CH_3)_2]_2$	35—36 (0.02)	—183.9 (a)	[11]
CH_3—	—N⟨pyrrolidino⟩	28—30 (0.01)	—182.7 (a)	[11]
CH_3—	—N⟨2,6-dimethylpiperidino⟩	61—64 (0.02)	—194.4 (a)	[11]
$N{\equiv}CCH_2CH_2$—	—$N(CH_3)_2$	90—92 (0.6)	—176.0 (6)	[12,16]
$N{\equiv}CCH_2CH_2$—	—$N[CH(CH_3)_2]_2$	103—104 (0.08)	—179.8 (6)	[12,16]
$N{\equiv}CCH_2CH_2$—	—N⟨morpholino⟩	—	—168.2 (6)	[12,16]
$CH_3S(=O)CH_2CH_2$—	—N⟨morpholino⟩	—	—167.5 (6)	[13]

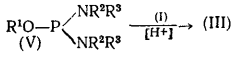
*Relative to H_3PO_4, solvent is indicated in the brackets: a) deuterochloroform; b) deuteroacetonitrile.

Conversion of the dichlorophosphite into the bis-triazolide leads to enhanced selectivity with respect to phosphorylation of nucleoside (I), i.e. a reduction in the proportion of by-products, 3',3'-dinucleoside phosphite. A similar result may also be obtained, when nucleoside (I) is treated with a large excess of dichlorophosphite R^1OPCl_2 with subsequent introduction of an adequate amount of amine:[25]

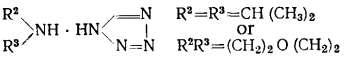
$$R^1OPCl_2 \xrightarrow{(I)} [(II)] \xrightarrow{R^2R^3NH} (III)$$

The latter method is of course only practicable, when a sufficiently effective technique (e.g. chromatography) is available for isolating the required phosphoramidite (III) from the multi-component reaction mixture.

The research workers who introduced the phosphoramidite method accept that this technique, used for activation of amides (III) with weak acids thereby creating an internucleotide linkage, is based on fundamental studies (mainly Soviet) on the protonation of amides of phosphorous acids (see communication[10] and the references in this article). Although specific questions in regard to the mechanism of protonation are still under debate,[26] this in no way imposes restrictions on the practical exploitation of the reaction in nucleic acid chemistry. For example, since bis(dialkylamino)alkoxyphosphines (V) are protonated by weaker acids than the phosphoramidites (III), the latter may be obtained by the reaction between nucleosides (I) and reagents (V) in the presence of specially selected proton donors:

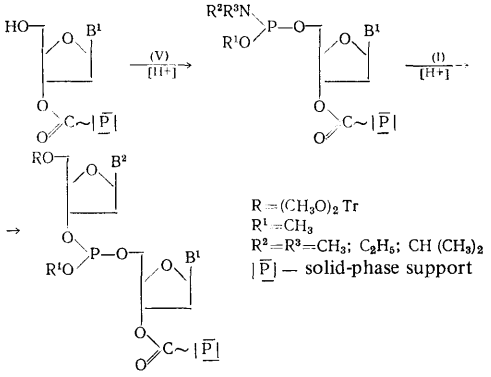


A number of azoles, of which 4,5-dichloro-imidazole proved to be the most effective,[27,28] were selected as weak acids for activation of bis(pyrrolidino)methoxyphosphine (V), $R^1 = CH_3$, $R^2R^3 = (CH_2)_4$. Another group of workers[29] have proposed that tetrazolium salts of the corresponding dialkylamines should be used for the selective activation of phosphines (V), $R^1 = CH_3$.



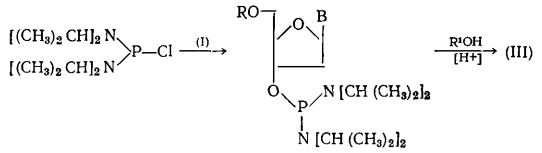
The possiblity of obtaining phosphoramidites (III) of acceptable purity immediately prior to condensation is the main advantage of the selective activation method, since difficulties associated with the isolation and storage of the reactive monomers may be avoided (III). In contrast to the corresponding amidochlorophosphites (IV), it is also of importance that type (V) compounds are extremely stable and safe to use. From recent studies in this direction the use of bis(dialkylamino)phosphite containing a β-cyanoethyl substituent (V) [$R^1 = CNCH_2CH_2$; $R^2 = R^3 = CH(CH_3)_2$] is noteworthy.[30]

A somewhat different approach has been suggested by Korean workers[33] for the use of diamidophosphites (V). The method recommended resembles previously-described techniques for two-stage phosphite condensation.[31,32]



In this case (V) is initially activated with an equimolar quantity of tetrazole, and the nucleoside component attached to the solid phase reacts with the protected compound (I) in solution even in the presence of excess tetrazole. However the active component in the phosphite synthesis, the phosphoramidite, is irreversibly inactivated by traces of water. Therefore, like the early methods, the main defect of this method[33] is that this active component is associated with the polymer and it is impossible to maintain an excess of this component.

Recently yet another modification has been proposed for the synthesis of phosphoramidites (III) which is based on selective activation:[34]



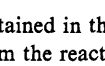
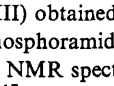
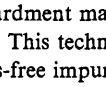
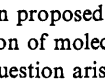
The phosphorylating agent, bis(diisopropylamino)chlorophosphine, is quite stable and may be purified by vacuum distillation. The intermediate nucleoside bis(diisopropylamido)phosphite subsequently reacts with an alcohol R'OH to yield the required phosphoramidite (III). This variant has none of the limitations associated with the problems in obtaining dichlorophosphites or dialkylamidochlorophosphites.

A stage in which the preparation is obtained in the form of a dry powder by re-precipitation or directly from the reaction mixture or after chromatography in a silica gel column is quite typical for most above-mentioned modifications used for synthesis of nucleoside phosphoramidites (III). This is an extremely important stage in regard to the purity of the compounds (III) obtained and consequently their stability. The purity of phosphoramidites can be most conveniently monitored according to ^{31}P NMR spectra and in certain cases by thin-layer chromatography.[17] The use of fast atom bombardment mass spectrometry has been proposed for this purpose. This technique permits the detection of molecules of phosphorus-free impurities.[35] However the question arises of whether it is absolutely essential to obtain dry and extremely pure phosphoramidites (III) for satisfactory long-term storage and further use in the synthesis of oligonucleotides. In an earlier study[36] phosphoramidites (III) were prepared by a very conventional method, which involved the interaction between an equimolar ratio of nucleosides (I) and amidochlorophosphite (IV) [$R^1=R^2=R^3=CH_3$] in the presence of the base, ethyldiisopropylamine. After removal of the base as the hydrochloride and replacement of the solvent (tetrahydrofuran by actonitrile) the phosphoramidite (III) solutions were used for solid-phase synthesis of oligonucleotides. Preparations of morpholidophosphites (III) [$R^1=CH_3$, $R^2R^3=(CH_2CH_2)_2O$] obtained by a similar method were even more stable, and after storage for many months they were used for the synthesis of oligonucleotides of differing length up to 52 bases.[37]

2. The Effect of the Protecting Group at the Phosphorus Atom and the Nature of the Original Amine on the Properties of the Nucleoside Phosphoramidites (III)

Historically the first acid used in phosphoramidite synthesis was dimethylaniline hydrochloride, which on interaction with phosphite (III) [$R^1=R^2=R^3=CH_3$] quantitatively (according to ^{31}P NMR data) generated the corresponding nucleoside chlorophosphite (II).[10] Both the nature of the substituents R^1, R^2 and R^3 and also the acidity of the selected activator should naturally have an effect on the rate at which the reaction occurs. From the practical viewpoint phosphoramidites (III) with more electron-accepting substituents at the P atom are more stable during the isolation procedure and storage and they require stronger proton donors for activation. Compounds (III) with more lipophylic and bulky substituents at the N atom are also found to be more stable. When the R^1, R^2, and R^3 substituents are selected, any potential difficulties in the preparation of the respective phosphorylating agents (IV) should be taken into account or alternative routes for synthesis of the phosphoramidites (III) should be considered. The main criterion for selection of the R^1 substituent should be the ease with which it can be removed and the selectivity of the removal process following oligonucleotide synthesis.

Table 2. Certain phosphoramidites (III) used in the synthesis of oligonucelotides.

R^1	$-NR^2R^3$	Method of activation (solvent, activation period, min)	Refs.
CH_3-		5-*p*-nitrophenyltetrazole (acetonitrile, 1-3)	[38]
		tetrazole (acetonitrile, 10)	[39]
		oxybenzotriazol(tetrahydrofuran, 3)	[25]
CH_3-	$-N(CH_3)_2$	tetrazole (acetonitrile, 5)	[36]
CH_3-	$-N[CH(CH_3)_2]_2$	tetrazole (acetonitrile, 5)	[40]
$C_6H_5SO_2CH_2CH_2$	$-N(CH_3)_2$	tetrazole (acetonitrile, 10)	[23]
$CH_3SO_2CH_2CH_2$		oxybenzotriazole (tetrahydrofuran, 3)	[13]
$NCCH_2CH_2-$	$-N[CH(CH_3)_2]_2$	5-*p*-nitrophenyltetrazole (acetonitrile, 5)	[16]
		N-methylaniline trifluoroacetate (acetonitrile, 5)	[21]

Certain known and currently used monomers (III) are presented in Table 2. It should be noted that one can only speculate on the possible advantages of certain specific phosphoramidites (III) over others. These assessments, which are based on yield comparisons at the condensation stages, final oligonucleotide yields, and certain other criteria, may not necessarily take into account the diversity of the condensation conditions, the properties of the solid-phase supports used, the apparatus, and the authors' individual interpretations. Nevertheless, as a result of the findings of many research groups the diisopropyl phosphoramidites (III) [$R^1=CH_3$, $R^2=R^3=CH(CH_3)_2$] [11,40] and morpholidophosphites (III) [$R^1=CH_3$, $R^2R^3=(CH_2CH_2)_2O$] [11,15,17] can be currently regarded as the most important agents practically. The former compounds are extremely stable and may be obtained in an appropriately pure form by direct precipitation in hexane without the use of chromatography, and they are therefore commercially most readily available. The latter compounds have even higher stability and may be carefully purified by means of silica gel chromatograpy. The methyl group in oligonucleotides, which have been obtained by using these phosphoramidites (III) [$R^1=CH_3$], may be easily removed by triethylammonium thiophenolate. However, a number of research groups use alternative protecting groups, which may be removed by the β-elimination mechanism under conditions in which the oligonucleotides are completely deblocked (concentrated aqueous ammonia, 50 °C): cyanoethyl [$R^1=NCCH_2CH_2$],[16] methylsulphonylethyl [$R^1=CH_2.CH_2SO_2CH_3$],[13] and phenylsulphonylethyl [$R^1=CH_2CH_2SO_2C_6.H_5$].[23] Monomers containing the cyanoethyl group are also commercially available. Apart from the absence of a separate stage for

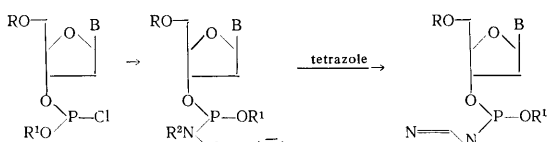
removal of the protecting group R after synthesis of the oligonucleotides, when these agents are used the obnoxious compound, thiophenol, may be omitted. Certain authors question the use of the cyanoethyl group and note that it is unstable in the presence of bases used when unreacted hydroxyl groups on the support are blocked.[5]

o-Chlorophenyl protection could be a possible alternative.[20,21] These phosphoramidites (III) [$R^1 = C_6H_4Cl$] are even more stable than their methyl analogues (III) [$R^1 = CH_3$, $R^2R^3 = (CH_2CH_2)_2O$] and may be readily purified in short columns of silica gel. In this case a five-fold excess of phosphoramidite over the nucleoside component in the polymer has been found adequate for oligonucleotide chain elongation in solid-phase synthesis, whereas in all other studies a 10–20-fold excess of phosphoramidites (III) with $R^1 = CH_3$ has been used.[21] The *o*-chlorophenyl protection can be quantitatively removed by tetramethylguanidinium *p*-nitrobenzaldoximate, but this procedure is much more time-consuming than treatment with thiophenolate and requires subsequent gel-filtration of the deblocked sample.

Phosphoramidites (III) containing a 1,1-dimethyl-2,2,2-trichlorethyl protecting group [$R^1 = C(CH_3)_2CCl_3$] have also been proposed, and the latter may be removed under reducing conditions, for example with tributyl phosphine or zinc dust.[41] This group has previously been used in the chlorophosphite approach.[9]

3. Amidophosphites (III) of Especial Interest

Immobilisation of the protected nucleoside chlorophosphite (II) on polystyrene containing NH-groups has effectively resolved the problem of isolating the required phosphoramidite (III).[42] After this type of polymer had been washed with tetrazole in acetonitrile, a solution of the nucleoside phosphite tetrazolide was obtained, and the latter may be effectively used in solid-phase synthesis.

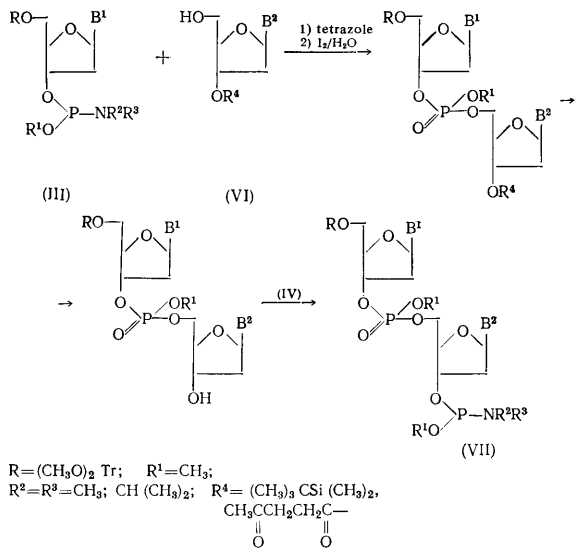


This original scheme would probably be most useful in a study of the mechanisms of phosphite (III) activation rather than from a practical standpoint.

Even in the initial stages in the elaboration of the phosphite synthesis it was suggested that the synthesis should be performed by protecting the exocyclic amino groups in the heterocyclic rings.[43] The development of this concept as applied to the phosphoramidite variant led to the formation of phosphites (III) in which the bases had unprotected amino groups and also to the synthesis of certain dimers based on these phosphites.[22] Speed and cost efficiency are the attractions of this strategy. However it is inconsistent with the trend in solid-phase synthesis in accordance with which protection of amino and hydroxyl groups in the heterocyclic ring is becoming more reliable and perfected, and even in the distant future it is unlikely that this method could be used for preparing oligonucleotides of significant length. Some authors[22] consider that the principle of minimal protection could be useful in the synthesis of many short oligomers on cellulose discs.

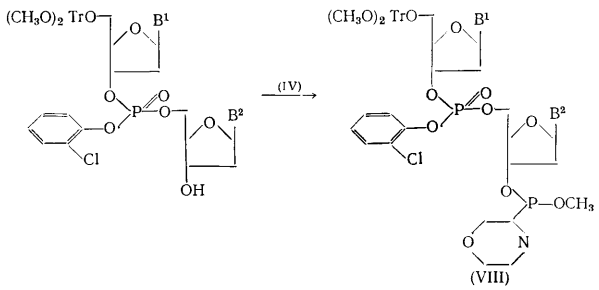
4. Phosphoramidite Dimer Blocks

For some considerable time the use of pre-formed blocks in oligonucleotide synthesis monopolised conventional triester strategy. It appeared that during use of the monomers (III), on account of the speed and high yield of the phosphite condensation, any desired result could be achieved. However, a number of papers have now been published which describe the use of dimer blocks in phosphite synthesis.[9,44–49] There is normally a phosphate internucleotide linkage in such dimers which differs only in regard to the method employed for creating this bond (the latter may be both phosphotriester and phosphite and may be subsequently oxidised to phosphate). For example in a previous study[44] phosphoramidite blocks may be obtained as follows:



R=$(CH_3O)_2$ Tr; $R^1=CH_3$;
$R^2=R^3=CH_3$; CH $(CH_3)_2$; R^4= $(CH_3)_3$ CSi $(CH_3)_2$, $CH_3CCH_2CH_2C$—

The levulinic protecting group R^4 was used which may be removed by mild hydrazinolysis. In the case of cytosine derivatives the use of (VI) containing the levulinic group is complicated by the poor solubility of this type of component in virtually any convenient solvent. This problem is resolved by substitution of levulinic by butyldimethylsilyl protecting groups. The resultant dimers are precipitated with cold hexane and may be stored until required. By using such dimers a number of oligonucleotides 29 bases in length have been synthesised. Other workers[45] have used pre-formed nucleoside blocks containing *o*-chlorophenyl protection of the internucleotide phosphate obtained by the normal triester method. By phosphorylation of these preparations it has been possible to construct 16 dimer blocks (VIII):



The blocks were purified by column chromatography and were characterised with the aid of ^{31}P NMR spectra.

The present authors consider that the above-mentioned methods for preparing phosphoramidite dimers require approximately the same number of man hours, and the choice of tactics has evidently depended on which method of synthesis in solution (phosphate or phosphite) the research worker has more effectively elaborated. However, since there were two different protective groups on the phosphorus in oligonucleotides synthesised with dimers (VIII), two different deblocking procedures should be carried out.

The use of phosphite blocks is undoubtedly accompanied by an increase in the yield of synthesised oligomers, since in this case there is a decrease in the number of essential condensations and secondary reactions are also reduced. The most outstanding example of the use of this method has been the synthesis of a 101-base polynucleotide.[48] However, construction of the blocks is a laborious process which demands very highly qualified personnel, and it is unlikely that phosphite dimers will be used extensively in practice for solid-phase synthesis. Increases in yield of oligonucleotides may apparently be achieved by less expensive methods, for example by scale-down of the process, by increasing the concentration of the monomeric phosphoramidites (III), and by optimising the solid-phase cycle with the aim of minimising secondary reactions.

III. REACTIONS AND REAGENTS FOR SOLID-PHASE PHOSPHITE TRIESTER SYNTHESIS

The main aspects of the solid-phase phosphite synthetic cycle are well conserved and comprise a sequence of stages including detritylation, condensation of nucleoside and nucleotide components, oxidation of phosphite to phosphate, and blocking of unreacted hydroxyl groups. However, the duration of the complete cycle has been reduced from 30–60 minutes to 10 minutes or less over a five-year period by the modern automatic synthesisers. This more rapid cycle time has resulted from the shortening of non-productive time in the automated devices, improvements in the mass-transfer processes as a consequence of solid-phase supports, and also the selection of the most appropriate reactions and reagents for rapid synthesis.

1. Detritylation and 5′-Protecting Groups in Phosphite Synthesis

The dimethoxytrityl group is often used for protection of the 5′-position of nucleotides in the phosphite synthesis. Similar types of groups may be removed in acid solution and in principle they may be accompanied by cleavage of purine bases. There is a large amount of data on the actual effect of depurination on the composition of the final reaction mixtures but these data are inconsistent.[6,50–55] It has been reported that removal of the dimethoxytrityl group under normal conditions (3% trichloracetic acid in dichlorethane) is not accompanied by any noticeable depurination throughout the treatment period (1.5 minutes per cycle).[54,55] Nevertheless certain authors as mentioned above prefer to use zinc bromide in nitromethane as a slowly acting but extremely selective detritylating agent.[45,46]

The monomethoxytrityl group is even more inconvenient for solid-phase synthesis if one considers the suggestion that secondary reactions may occur during detritylation. However there is an experiment cited in the literature in which this group has been effectively employed in phosphoramidite synthesis.[56] Various alternative detritylating solutions have been proposed by means of which the monomethoxytrityl group can be removed in the solid phase as rapidly as the dimethoxytrityl group: 0.1 M trifluoroacetic acid in dichloromethane, 1 minute;[56] 5% trichloroacetic acid in dichlorethane, 3.5 minutes,[57] 1 M $ZnBr_2$, 0.02 M 1,2,4-triazole in a dichloromethane-isopropanol mixture (85:15), 1.5–3 minutes.[58]

In a previous study[59] a number of triphenylcarbinol derivatives has been examined and different protecting groups have been proposed for each of the four nucleosides. Under normal detritylation conditions these groups give solutions of triarylmethyl cations in different colours (orange, blue, red, and black) and it is thus usually possible to monitor the sequence of bases in the oligomer under synthesis.

In principle weak acids used for promoting phosphoramidites may also have a detritylation action. Thus apparently the 9*H*-9-phenylxanthenyl (pixyl) protecting group, being more acid-labile than the dimethoxytrityl group[60] has not gained popularity among those who favour phosphite synthesis. The proposed protecting groups for the 5′-terminus in phosphite synthesis do not only consist of derivatives of triarylmethanes. The alkali-labile *p*-phenylazophenyloxycarbonyl group[61] has recently been reported, although at present difficulties would be encountered in the extensive use of this grouping.

2. Blocking of Unreacted Hydroxyl Groups on the Support (Capping)

As with detritylation, at this stage there are no major differences in any of the modifications of the solid-phase synthesis. In the early studies this stage was carried out by using phenyl isocyanate[7] or diethyl chlorophosphite.[8] The use of mixtures based on acetic anhydride in pyridine, lutidine, or collidine[46] is currently preferred, since they are more convenient in practice and quite effective, especially in the presence of the nucleophilic catalysts, dimethylaminopyridine[13,25,55] and N-methylimidazole.[56] This stage is essential for the synthesis of extended sequences, since it facilitates markedly subsequent isolation of the required oligonucleotides. It has already been mentioned in Section II that under certain conditions capping may lead to undesirable secondary reactions.[5]

3. Phosphoramidite Condensation and Factors Affecting the Efficiency of the Condensation

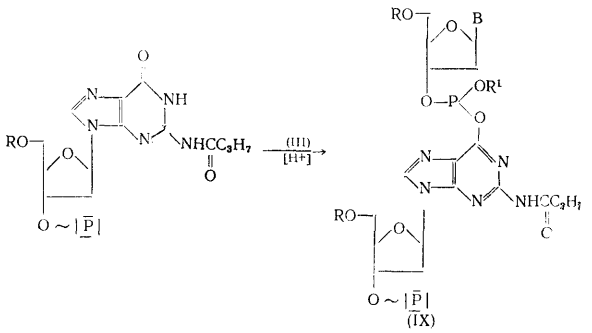
This stage is most important in regard to the efficiency of solid-phase synthesis. Acid activation of phosphoramidites has been briefly discussed by the present authors in Section II. In this Section weak acids normally used for this purpose are presented (Table 2). Tetrazole is most frequently used and may be obtained in a suitably pure and dry form by vacuum sublimation of commercially available preparations. However, tetrazole is not suitable when using the more stable type (III) compounds, for example the morpholidophosphites (III) [$R^2R^3=(CH_2CH_2)_2O$], and more effective proton donors are used in such cases. Selection of an acid should be made on the basis of the following properties: a) high stability in the medium; b) ability to protonate the selected phosphoramidite (III) at a rate compatible with the practical conditions; c) the absence of any detectable deblocking of the nucleotide component under the condensation conditions; d) chemical stability of the working acid solution for the entire duration of oligomer synthesis.

A distinctive feature of phosphite synthesis is the large (10–20-fold) excess of reagent (III) over the nucleoside component in the polymer. This excess is essential not only for ensuring an adequate rate of reaction for chain elongation but also for compensating the

consumption of phosphite (III) in secondary reactions, for example with the residual moisture in the solvents (in the phosphotriester condensation in the traditional variants an excess of condensing agent fulfils this function). However, in work involving micro-scale solid-phase synthesis even these excess quantities are found to be acceptable, and in certain instances authors have changed the method by repeating each condensation twice, and this modification guarantees that the process will proceed quantitatively.[28,48,62]

A significant excess of phosphite is undoubtedly an essential prerequisite for a highly efficient condensation. However, is this condition sufficient? A completely negative answer has been given to this question, viz. steric factors play a key role on solid-phase supports with a high degree of nucleoside modification.[62] It has been established empirically that an extremely high yield on silica supports is ensured, when the nucleoside loading does not exceed 0.4 μmol/m^2. The currently extensively used solid-phase supports based on glass modified with long-chain aminoalkyl "spacers" in particular satisfy this condition. It is relevant that the authors are aware of only one study in which phosphoramidites (III) have been used for the synthesis of cellulose discs.[65] The difficulties associated with mass-exchange on the cellulose matrix outweigh the main advantage of phosphite synthesis, viz. speed, and compound the problems associated with secondary reactions.

Secondary reactions can play an important role in reducing the yield at the condensation stage. For example, it has been shown that guanidine may be phosphorylated at position O(6) with the formation of "pseudo" chains, which are subsequently destroyed on treatment with aqueous ammonia:[54,55,66]



This process may to a marked extent complicate the synthesis and subsequent isolation of oligonucleotides with a high guanosine content. Difficulties may be avoided by the use of phosphoramidites (III) comprising O(6)-*p*-nitrophenylethyl-protected guanosine derivatives[54,55] or β-cyanoethyl group.[68] However, data are available which indicate that on introduction of a bulky diphenylcarbamoyl goup at this position there is a decrease in the rate of condensation due to steric hindrance.[67]

The product formed by phosphorylation at the heterocyclic ring (IX) is sufficiently reactive to be attacked rapidly by certain nucleophiles. This approach is the basis of a number of techniques for the decomposition of compound (IX) immediately after the condensation before oxidation to the phosphate, which markedly increases the possibility of synthesising oligonucleotides with a high guanosine content.

1) Regeneration of guanosine with water or the acetate ion before oxidation with iodine; the regeneration process also takes place, when a mixture of acetic anhydride and pyridine is used for capping prior to oxidation;[68]

2) the use of N-methylalanine as a phosphoramidite (III) activator;[68]

3) oxidation with an iodine solution in a pyridine-acetic acid mixture; in this case the product (IX) is attacked by the acetate ion more rapidly than oxidation of phosphite to phosphate.[56]

The instability of phosphoramidite derivatives of guanosine in solution during storage was the object of a thorough investigation in an earlier report.[69] This instability presents certain difficulties in the synthesis of mixed oligonucleotide probes, since the mixture becomes depleted of oligonucleotides with a high guanosine content. With the aid of ^{31}P NMR spectroscopy it was shown that a substance with a chemical shift corresponding to a secondary phosphate is predominant among the phosphoramidite (III) degradation products. The mechanism of this process has not been precisely determined, but there are grounds for suggesting that products from the type (IX) phosphorylation are formed as intermediates. These authors[69] also note that in the case of phosphoramidites containing a cyanoethyl group (III) [$R^1 = CH_2.CH_2CN$] the guanosine derivatives are not as unstable as the methyl analogues (III) [$R^1 = CH_3$].

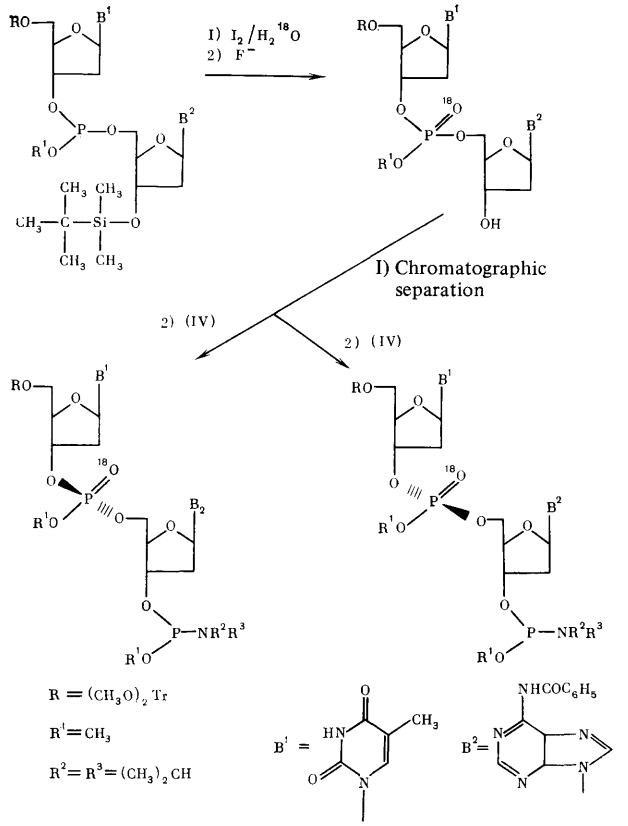
With phosphoramidites (III) [$R^1 = CH_3$] methylation of the thymine heterocycle may also occur,[70] although the practical importance of this type of secondary reaction under the actual conditions of solid-phase synthesis is questionable.[5] On the whole the effect of secondary reactions on oligonucleotide synthesis is clearly illustrated by the following example. When extended products from automatic phosphoramidite synthesis (60–90 bases) are cloned, on average there are base substitutions or deletions in 40% of the clones.[71]

4. Oxidation

This process is traditionally achieved with the aid of iodine in an aqueous-organic solution. The reaction is rapid and selective, although alternation of treatment of the support with water when the latter possesses a certain sorption capacity with subsequent condensation in "absolute" solution cannot be regarded as a satisfactory procedure. In an earlier study[6] *m*-chloroperbenzoic acid in pyridine was used as the oxidising agent, although this agent rapidly lost activity in the course of the synthesis. Currently bis-trimethylsilyl peroxide[72] is recommended for the conversion of phosphite to phosphate in the solid phase including the trimethylsilyl ester of pertrifluoromethane sulphonic acid as catalyst,[73] tetrabutylammonium periodate,[74] and iodosobenzene diacetate.[74] The latter compound is of interest, since it may be used directly in the capping solution. In regard to their application to solid-phase synthesis the new reagents are still under investigation although extensive testing of these compounds will be restricted by their lower availability in comparison with iodine. Nevertheless taking into account the modern-day arsenal of oxidising agents one would anticipate that new studies in this direction will be performed. The use of iodine in a pyridine-acetic acid mixture is also a promising method.[56]

The oxidation stage or generally the transition from tricoordinated phosphorus derivatives to tetracoordinated phosphorus derivatives is of great significance for the synthesis of oligomers modified at the internucleotide linkage. It has been shown that during condensation in the presence of phosphoramidites (III) phosphite triester

enantiomers are obtained, and that the ratio of the latter is dependent on the reaction conditions.[45,75,76] If $H_2{}^{18}O$ is used for the oxidation with iodine, the corresponding isotopically chiral phosphates are obtained and they may be used for the synthesis of chiral blocks.[47]



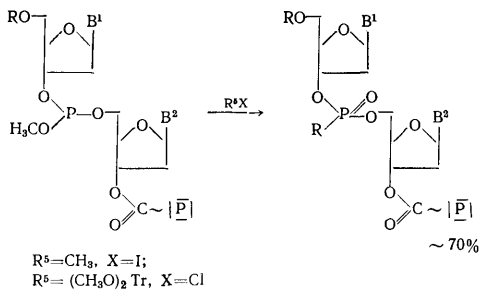
Both of these blocks are further used for the synthesis of oligonucleotides, which differ only in regard to the configuration of one of the internucleotide phosphates.

The phosphite synthesis is extremely convenient for the preparation of thiophosphate analogues of oligonucleotides, which are often used as model compounds in enzyme studies (see communication[77] and the references cited in this article for recent studies on the use of these analogues). In the automated solid-phase method of Stec et al.[78] the thiophosphate bond was introduced at the "oxidation" stage, aqueous iodine having been replaced by sulphur (0.4 M S_8 in lutidine; 60 °C; 15 minutes). The resultant chiral oligomers with respect to thiophosphate in most cases could be separated chromatographically into the individual enantiomers.

In a similar way to the thiophosphates, selenium phosphate analogues could in principle be obtained, the latter compounds being of interest in regard to X-ray structural studies on DNA and for studies on $^{31}P-{}^{77}Se$ spin–spin interactions. However, the authors[78,79] were only able to obtain a few di- and tri-nucleotide analogues (0.1 M KSeCN in acetonitrile; 60 °C; one hour), since selenium is lost by some mechanism unknown to the investigators during the repeating cycles at the stage involving oxidation with iodine.

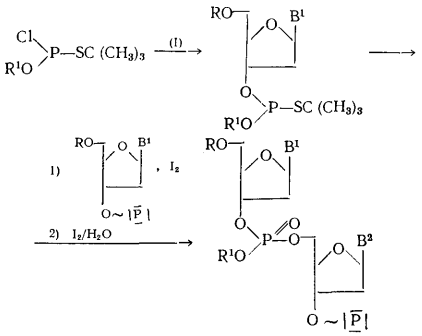
In the interaction between trialkylphosphite intermediates in solid phase and an iodine/alanine/lutidine mixture several phosphoramidite analogues of di- and trinucleotides were obtained, although the yield of the required products was low (less than 20%) and the methodology is in need of development.[79]

The internucleotide linkage may also be modifed by using the Arbuzov rearrangement.[80]



Oligomers obtained by means of this technique, in which one or several internucleotide groupings are phosphonate, may also be separated into the individual enantiomers with the aid of reversed-phase high performance liquid chromatography.

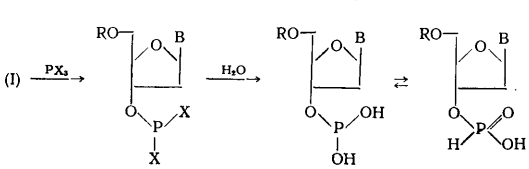
A new modification of the phosphite synthesis has been proposed in which the oxidation and condensation stages may essentially be integrated. Thiolphosphites[81] are used as the original monomers in this method:



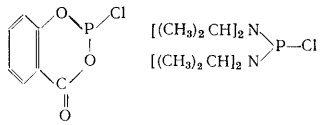
The condensation is achieved in the presence of iodine in an anhydrous solvent, and oxidation may be subsequently carried out by simply adding water. However, it is unlikely that in the near future this method will provide a realistic alternative to the well developed phosphoramidite condensation.

IV. NUCLEOSIDE PHOSPHITES IN THE SYNTHESIS OF OLIGONUCLEOTIDES (PHOSPHONATE SYNTHESIS)

It has recently been proposed that nucleoside phosphites (X) mainly in the H-phosphonate form should be used as monomers in solid-phase synthesis. The procedure used for synthesis of oligonucleotides is known as the "phosphonate" method.[82–85] The methods for preparing nucleoside phosphites (X) proposed by the pioneers of solid-phase phosphonate synthesis are based on the interaction between nucleosides (I) and tris(azolyl)phosphines:



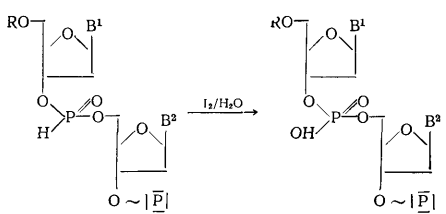
Naturally, in order to prevent the formation of di- and tri-esters of phosphorus acid, a great excess of these phosphorylating agents was used. Later a report was published on the use of other phosphorylating agents:[86]



The use of these agents reduces the possibility that by-products are formed instead of tris(azolyl)phosphines. In all cases[82–86] monomers (X) obtained as a result of phosphorylation and subsequent hydrolysis were purified with the aid of adsorption or reversed-phase column chromatography. The nucleoside phosphites (X) are sufficiently stable in the form of tertiary amine salts and may be stored for considerable periods.

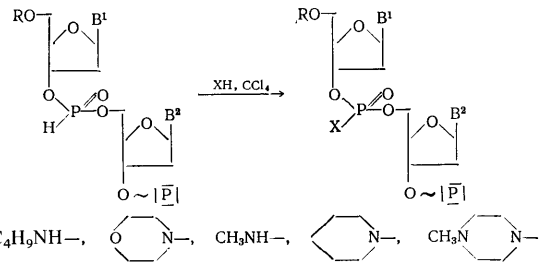
It has been shown that various anhydrides and acid chlorides of phosphorous acids and carboxylic and sulphonic acids may serve as monomer (X) activators during oligonucleotide synthesis, although the acid chloride of trimethyl acetic acid was found to be the best of the agents tested.[84,85] It has been suggested that the active phosphorylating compound in this case is a mixed anhydride of phosphorous and carboxylic acids. It is considered that pre-mixing of the nucleoside phosphite (X) and pivaloyl chloride leads to the formation of a bifunctional phosphorylating agent, diacyl nucleoside phosphite. It is therefore recommended that the reagents should be mixed directly during the condensation process in the reactor.[85]

A separate capping stage is evidently not required in the phosphonate synthesis, since non-phosphorylated hydroxyl groups on the support should be acylated in the condensation process. As there is a five-fold excess of condensing agent (pivaloyl chloride) over monomer (X), in the phosphonate method a lower amount of nucleotide component is consumed than in the phosphoramidite method. The internucleotide phosphite diester groups formed during the synthesis are chemically quite inert and do not require protection. They are oxidised with iodine in an aqueous-organic solution at the end of oligomer synthesis.



However the oxidation process is slower than with the trialkyl phosphites and in order to promote the oxidation, catalysis with stronger bases than pyridine is necessary.[84] Thus in the phosphonate synthesis the chain elongation cycle consists essentially of a condensation stage (1 – 2 minutes), detritylation (1 – 2 minutes), and a washing procedure, and therefore greatly extended oligonucleotides containing up to 100 bases may be synthesised rapidly.[84] At present there are certain unknown factors and reservations in regard to this synthesis. For example, partial detritylation in the condensation process[84] or incomplete capping is thought to occur. However, the phosphonate method is extremely straightforward and cost effective, and very shortly many research workers will undoubtedly favour this method.

This method also shows promise in regard to the synthesis of oligonucleotide analogues. Unfortunately the phosphite diester group itself is unstable in the presence of bases, and an unoxidised oligomer containing phosphite diester internucleotide groups cannot be removed from the support by conventional methods. However, this group may be susceptible to attack by a nucleophile, for example the Atherton – Todd reaction. This property has been exploited by Gryaznov et al.[87] for the synthesis of dinucleotides containing an internucleotide phosphamide linkage. By the application of this reaction to the oligomeric product from the solid-phase phosphonate synthesis it has been possible to obtain a number of amidophosphate analogues of oligonucleotides:[88]



This communication[88] describes the synthesis of nine undecathymidylate analogues which contained different numbers of morpholidophosphate internucleotide groups, and also mentions the possibility of preparing thiophosphate [0.1 M S_8 in a triethylamine – carbon disulphide (1:9) mixture 5 min] and triester [10% alcohol in an N-methylimidazole – triethylamine – carbon tetrachloride (5:5:90) mixture] analogues of oligonucleotides via the phosphite diesters on a support.

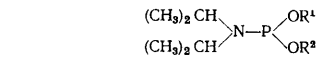
V. PHOSPHITE REAGENTS FOR THE INTRODUCTION OF DIFFERENT FUNCTIONAL GROUPS INTO OLIGONUCLEOTIDES

Oligonucleotides are nowadays increasingly used as carriers for various groupings, for example chemically active (complementarily addressed modification) and reporter (nonradioactive probes) groups. Research workers are focusing attention on the replacement of a number of enzyme processes in genetic engineering (for example kination and ligation) by purely chemical processes. There would be strong grounds to use for such purposes the same chemical arsenal as for the synthesis of the oligomers themselves.

1. Introduction of the Phosphate Group at the 5′-Terminus of the Oligonucleotide

This operation represents a chemical alternative to the kinase reaction and has been thoroughly worked out in the phosphotriester synthesis. The main problem associated with the introduction of the 5′-terminal phosphate is the selection of a phosphorylating agent with easily removable protecting groups at the phosphorus atom, confirmation that the phosphorylation has taken place, and also the separation of the 5′-phosphorylated oligonucleotide from the normal oligonucleotide (when the chain is long enough, there is an insignificant difference between the chromatographic and electrophoretic mobility of both oligomers).

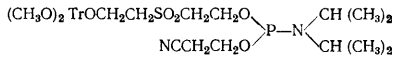
Several phosphorylating agents prepared by conventional methods of phosphite synthesis have been proposed.[89,90]



$R^1=R^2=CH_2CH_2C_6H_4NO_2$; $R^1=R^2=CH_2CH_2CN$;
$R^1=CH_3$, $R^2=CH_2CH_2C_6H_4NO_2$; $R^1=CH_3$, $R^2=CH_2CH_2CN$

Solid-phase phosphorylation of the oligomers by such agents with the aid of acid catalysis, oxidation, and subsequent deblocking yields the required oligonucleotide-5′-phosphates. Methyl and β-cyanoethyl groups are accordingly removed before the support is treated with ammonia and during this treatment. The *p*-nitrophenylethyl protection may be removed only by strong bases such as diazabicycloundecene. It may therefore be used as an hydrophobic probe during the removal of the 5′-phosphate of the oligomer by reversed-phase chromatography.

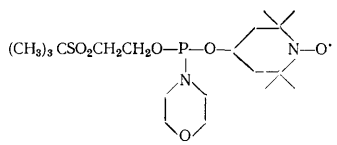
A phosphorylating agent containing the dimethoxytrityl group has also been described:[91]



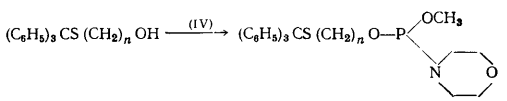
After oxidation of the phosphorylated oligomer and treatment with acid it is possible to determine whether phosphorylation is complete according to the concentration of the dimethoxytrityl cation. The remaining ethoxysulphonylethyl fragment is removed together with the β-cyanoethyl protection when the support is treated with ammonia.[91]

2. Introduction of the Reporter and Functional Groups

The synthesis of a phosphoramidite containing a nitroxyl fragment is an example of the direct introduction of a reporter group with the aid of a phosphorylating agent:[92]



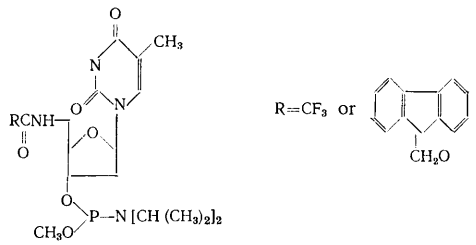
However, reporter groups such as coloured or fluorescent chromophores are often complex in structure. It is easier to introduce these groups under milder conditions into oligonucleotides, which have been preliminarily modified with certain functional groups. The authors of a study on 5′-phosphorylation with the aid of phosphoramidites[89–91] note that by using phosphite reagents it is possible to introduce a 5′-terminal thiophosphate, a potential acceptor group for different modifiers. A morpholidophosphite with trityl protection has been used to introduce the thiol group:[93]



$n=2, 3, 6$

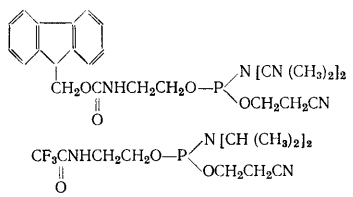
After phosphorylation of the oligonucleotide and oxidation with iodine the trityl group was removed with silver nitrate.

The amino group was introduced by means of phosphoramidite derivatives of 5′-deoxy-5′-aminothymidine:[94]



Alkali-labile groups (fluorenylmethoxycarbonyl or trifluoracetyl) were removed while the support was being treated with ammonia. Oligomers containing amino groups obtained in this way have been used in the synthesis of primers for sequencing according to the method of Sanger which carried a fluorescent marker at the 5′-terminus.[94]

The fluorenylmethoxycarbonyl[95] and trifluoracetyl[96] groups have also been used in the construction of other phosphorylating agents, in order to introduce the amino group.



In the latter case in order to increase the yield of the required oligonucleotides, the protecting groups were removed not with ammonia but with diazabicycloundecane.[96]

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As a result of the development of methods for nucleophilic catalysis it is now possible to achieve a phosphotriester condensation quite rapidly and efficiently.[67,97–101]. However, commercial manufacture of nucleoside phosphoramidites and automatic synthesisers, which operate according to the phosphoramidite cycle, has indeed become a mass-production venture. Phosphonate synthesis is also being rapidly commercialised. Operations in the synthetic cycle are constantly being improved and simplified with the result that the chemical synthesis of DNA is a rapidly-developing and refined procedure. The demand for modified oligonucleotides is also attracting increasing attention in regard to nucleoside phosphites and fundamental questions on the chemistry of organophosphorus compound, which should ensure further advances in phosphite synthesis.

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