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Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach

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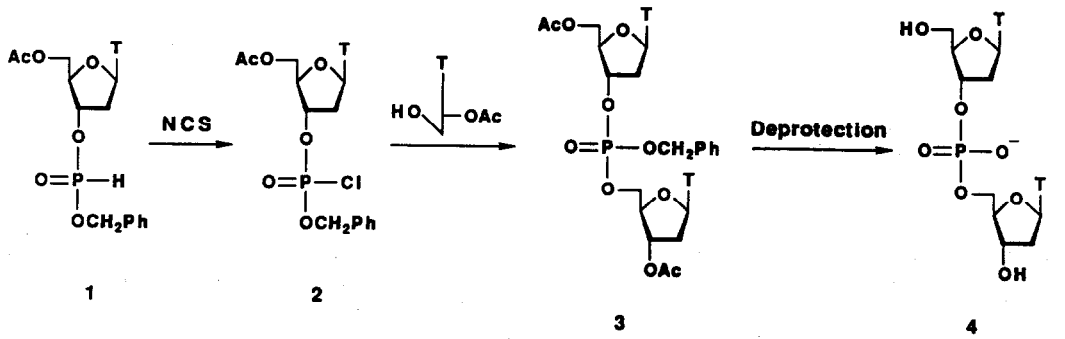
INTRODUCTION

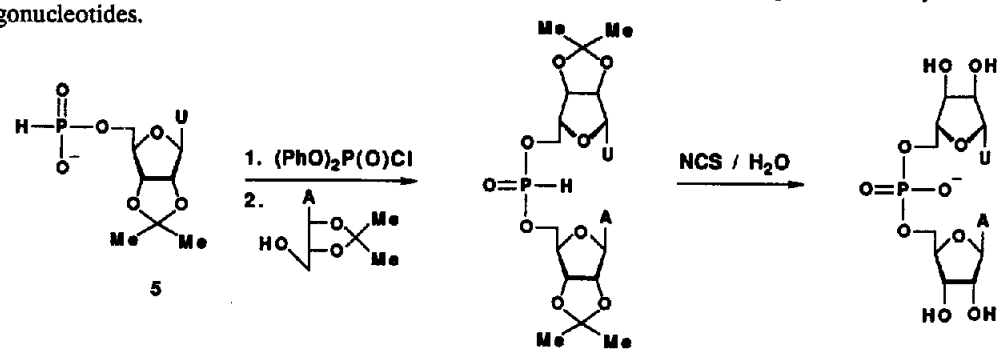
Historically, the discovery of the helical structure of DNA by Watson and Crick[1] provided the impetus to attempt the challenging synthesis of oligonucleotides of defined sequences. Michelson and Todd[2] reported in 1955 the first chemical synthesis of a dinucleotide containing a (3'→5')-internucleotidic linkage identical to natural DNA. Their pioneering approach was based on the activation of 5'-*O*-acetylthymidine-3'-*O*-benzyl hydrogen phosphonate (**1**) by *N*-chlorosuccinimide to the corresponding phosphorochloridate **2** followed by the addition of 3'-*O*-acetylthymidine. The resulting dinucleoside phosphate triester **3** was converted, upon removal of the protecting groups, to the natural thymidylic acid **4**.



1 **2** **3** **4**

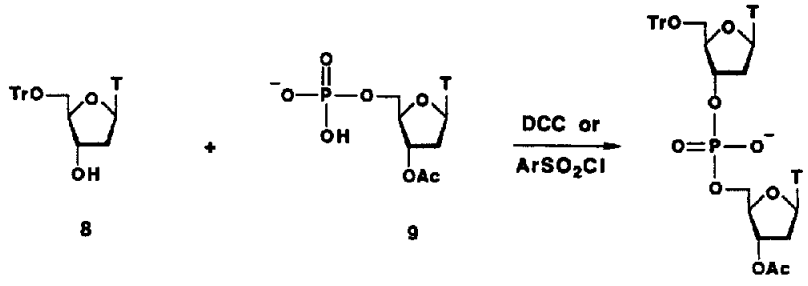
Ac= acetyl
NCS= *N*-chlorosuccinimide

Subsequently, Hall *et al.*[3] demonstrated that 2',3'-*O*-isopropylideneuridine-5'-hydrogen phosphonate (**5**) was activated by diphenylphosphorochloridate to, presumably, a mixed anhydride which reacted with 2',3'-*O*-isopropylideneadenosine affording the corresponding dinucleoside hydrogen phosphonate **6** in high yields. The phosphite link was easily oxidized by *N*-chlorosuccinimide to the natural phosphodiester linkage. These early achievements laid the foundation for the subsequent development of the "phosphotriester" and the "*H*-phosphonate" methodologies for the synthesis of oligonucleotides.



5 **6** **7**

In the late fifties and early sixties, Khorana and his colleagues succeeded in the preparation of short, defined sequences of deoxyribopolynucleotides which were helpful in deciphering the genetic code.[4,5] The chemical methodology used for the synthesis of these oligomers involved the condensation of a nucleoside having a free 3'-hydroxyl group and a 5'-hydroxyl group protected by the acid-sensitive trityl group (**8**) with a nucleoside 5'-phosphate having a 3'-hydroxy function blocked with an acetyl group (**9**), an alkali-labile group. The coupling occurred in the presence of a condensing agent such as 1,3-dicyclohexylcarbodiimide (DCC), mesitylenesulfonyl chloride (MS-Cl) or 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) affording the dinucleoside monophosphate **10**. Extension of the nucleotidic chain was accomplished by the removal of the 3'-*O*-acetyl group from **10** and subsequent condensation with a suitably protected nucleoside 5'-phosphate.[6]



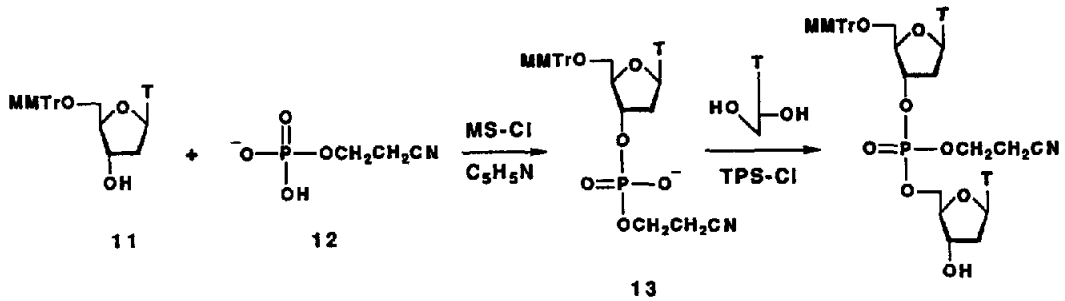
8 **9** **10**

Tr= triphenylmethyl
DCC= 1,3-dicyclohexylcarbodiimide

This "phosphodiester" approach to the synthesis of oligonucleotides became routine for nearly two decades and led to the total synthesis of genes encoding an alanine tRNA from Yeast[7] and a tyrosine suppressor tRNA from *E. coli*,[8] respectively. The biologically active genes were constructed from synthetic DNA segments linked together by T4-DNA ligase.[7-9] During the course of this colossal achievement, the development of trityl group derivatives for the protection of the 5'-hydroxy function of nucleosides along with the benzoyl and isobutyryl groups for the protection of the exocyclic amino

function of adenine and guanine, respectively, became a landmark in the field. These protecting groups are still used in modern oligonucleotide syntheses. One major drawback pertaining to the phosphodiester approach was the ionic nature of the starting material and the various condensation products which had to be separated by tedious and time-consuming ion-exchange chromatography.

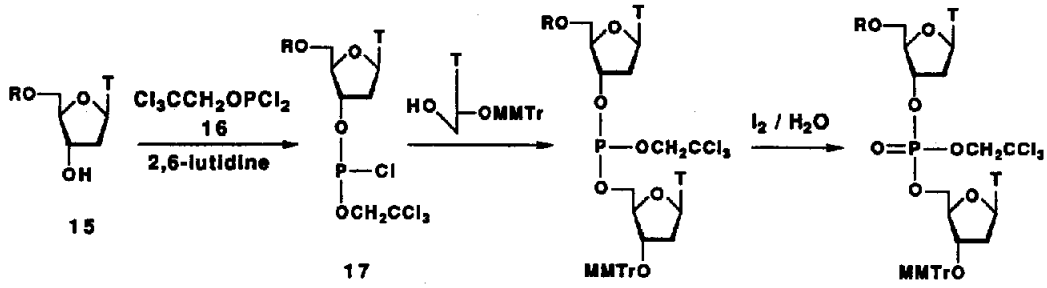
To alleviate this inconvenience, the formation of internucleotidic phosphotriester linkages originally introduced by Michelson and Todd (*vide supra*) was reinvestigated. Letsinger and Ogilvie[10,11] demonstrated that the condensation of a 5'-*O*-protected deoxyribonucleoside (**11**) with a phosphate monoester (**12**) in the presence of MS-Cl generated the corresponding nucleoside phosphodiester intermediate **13**. The addition of a deoxyribonucleoside having a free 5'-hydroxyl group and TPS-Cl afforded the dinucleoside phosphate triester **14** which was easily separated from the ionic starting material by silica gel chromatography. Through the years, this so-called "phosphotriester" approach has been modified and improved by the introduction of new phosphorylating and condensing reagents.[12-16] Nucleophilic catalysts such as *N*-methylimidazole and 4-substituted pyridine-*N*-oxide derivatives with appropriate condensing agents were particularly effective in enhancing the rates of phosphotriester bond formation.[17] Considerable progress has also been achieved in the chemical synthesis of oligoribonucleotides by the phosphotriester approach in spite of the complexity imparted by the presence of the 2'-hydroxy function of ribonucleosides requiring protection. These advances have been reviewed by Ohtsuka *et al.*[18,19] and Reese.[20,21]



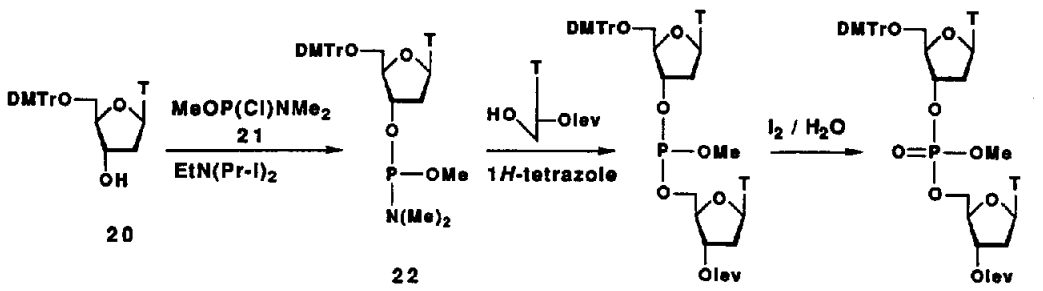
MMTr= *p*-anisyldiphenylmethyl
MS-Cl= 2,4,6-trimethylbenzenesulfonyl chloride
TPS-Cl= 2,4,6-triisopropylbenzenesulfonyl chloride

In the mid-sixties, Letsinger and Mahadevan[22] investigated the use of an organic polymer as a support for the synthesis of oligonucleotides. This concept, originally proposed by Merrifield[23] for the synthesis of peptides, allowed the facile separation of the starting material (the soluble phase) from the product covalently bound to the insoluble phase and, hence, eliminated time-consuming purification steps. Subsequently, Köster introduced inorganic carriers such as silica gel as an alternative to swellable organic supports for the preparation of synthetic oligonucleotides.[24] These approaches met with only moderate success,[25] probably because of the poorly efficient phosphorylation procedures existing at the time.

In the mid-seventies, Letsinger and his coworkers[26,27] revolutionized the chemical synthesis of oligonucleotides through the development of the "phosphite triester" methodology. Essentially, the approach consisted in the reaction of a 5'-*O*-protected deoxythymidine (**15**) with 2,2,2-trichloroethyl phosphorodichloridite (**16**) to generate an intermediate deoxyribonucleoside-3'-*O*-phosphorochloridite (**17**) within 5 min at −78 °C. The addition of a 3'-*O*-protected deoxythymidine resulted in the rapid formation of the dinucleoside phosphite triester **18** which was then oxidized to the corresponding phosphate triester **19** by an aqueous iodine solution.



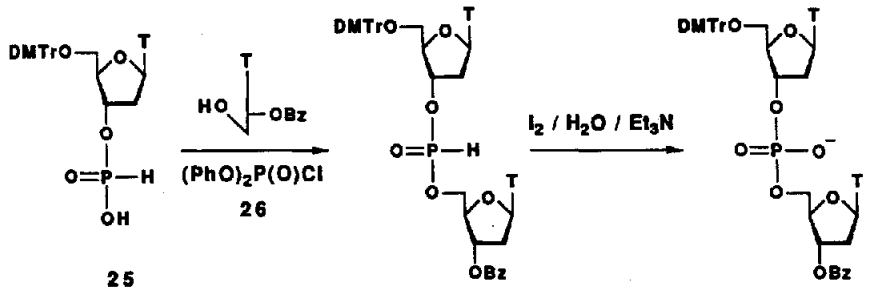
The combination of this efficient approach with the resurgence of silica gel as a solid support in the early eighties intensified research activities toward the automation of oligonucleotide synthesis.[28,29] In spite of its rapidity and efficiency, the phosphite coupling procedure had its limitations. The highly reactive deoxyribonucleoside chlorophosphite intermediates were unstable to moisture and were difficult to handle under normal conditions. Consequently, these intermediates were not suitable for automation. In an attempt to alleviate these problems, Beaucage and Caruthers[30] developed the deoxyribonucleoside phosphoramidites as a new class of intermediates for the synthesis of oligonucleotides. In contrast to the deoxyribonucleoside chlorophosphites, the corresponding phosphoramidite derivatives were isolated as stable powders and could be stored as such for prolonged periods of time. Typically, the monomeric deoxyribonucleoside phosphoramidite **22** was prepared from the reaction of 5'-*O*-dimethoxytrityl deoxythymidine (**20**) with chloro-(*N,N*-dimethylamino) methoxyphosphine (**21**) as a monofunctional phosphitylating agent. The facile activation of **22** by the weak acid 1*H*-tetrazole followed by the addition of 3'-*O*-levulinyl thymidine generated the desired dinucleoside phosphite triester **23** in near quantitative yields.



During the last decade, the only significant structural change to the monomeric deoxyribonucleoside phosphoramidites was the substitution of the *N,N*-dimethylamino function by the *N,N*-diisopropylamino group.[31,32] This substitution facilitated the purification of the monomers and increased their stability in solution. This feature was particularly important for the automation of the procedure.[33] The efficiency of deoxyribonucleoside phosphoramidites allowed coupling reactions mediated by 1*H*-tetrazole with protected nucleosides bound to silica-derived supports to proceed within 1 min in virtually quantitative yields.[34]

Recently, Garegg and his collaborators[35] reported that a 5'-*O*-protected deoxyribonucleoside-3'-*O*-hydrogen phosphonate (**25**) rapidly reacted with 3'-*O*-benzoyl thymidine in the presence of an

activating agent such as TPS-Cl, benzenesulfonyl chloride or diphenylphosphorochloridate (**26**) to yield the corresponding (3'→5')-dinucleoside hydrogen phosphonate **27**. These observations were very similar to those reported by Hall *et al.*[3] more than three decades ago. The activating reagents were subsequently replaced by pivaloyl chloride[36] and, preferably, by adamantoyl chloride[37] to permit the reliable automation of the procedure for the solid-phase synthesis of oligonucleotides. It is generally accepted, however, that the "*H*-phosphonate" approach is not, as yet, as efficient as the phosphoramidite methodology and would, therefore, be limited to the synthesis of shorter oligonucleotide segments to simplify the subsequent purification of the desired nucleic acid sequence from the failure sequences.



25 **27** **28**

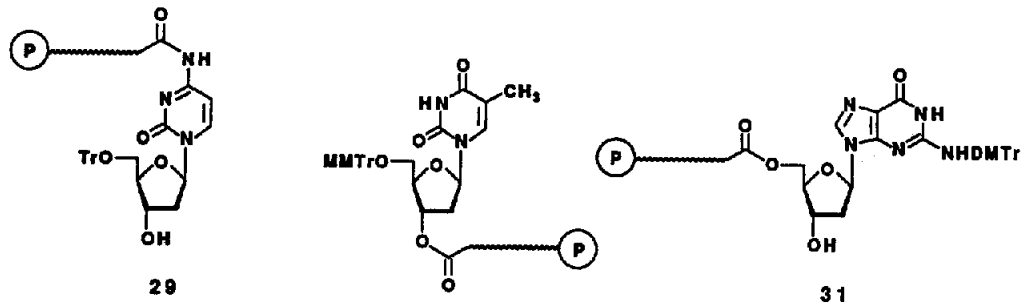
Bz= benzoyl

Due to the overwhelming number of reports that have been published during the last decade about the synthesis of oligonucleotides *via* phosphoramidite intermediates, the scope of this review will be limited to the detailed account of these reports and those pertaining to the protection of nucleosidic and phosphate functions.

1. OLIGONUCLEOTIDE SYNTHESIS ON SOLID SUPPORTS

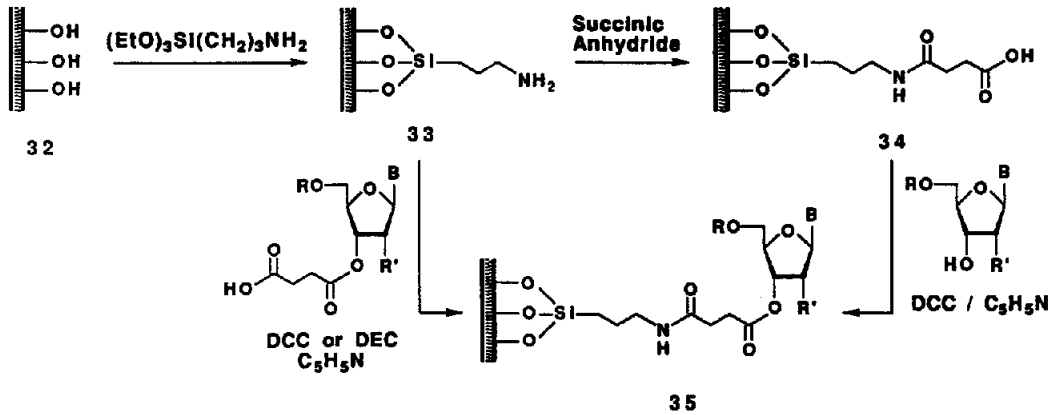
The solid-phase synthesis of peptides was first described by Merrifield[23] in 1962. Since then, considerable efforts have been invested toward the development of a suitable solid-phase strategy for the synthesis of oligonucleotides. The first successful approach was reported by Letsinger and Mahadevan[22] who described the preparation of a "popcorn" copolymer from styrene (88%), *p*-vinylbenzoic acid (12%) and *p*-divinylbenzene (0.2%). This support was insoluble in water, alkaline solutions and organic solvents commonly used in oligonucleotide synthesis. The reaction of the acid chloride form of the polymer with the exocyclic amino function, 3'-OH, or the 5'-OH of properly protected nucleosides afforded the polymer-bound nucleosides **29-31** respectively.[22,38,39] For example, chain extension from **31** was achieved according to the phosphodiester or phosphotriester approach and the resulting oligomer was subsequently cleaved from the support under basic conditions. This technique enabled the separation of the product from the solvents, excess reagents and soluble by-products by simple filtration, thereby, avoiding numerous time-consuming isolation steps.

Through the years, various polystyrene-derived supports[25,40,41] in addition to polyamides[25,41a] and cellulose[40a,42-44] were tested in the solid-phase synthesis of oligonucleotides. The swelling of polystyrene supports with organic solvents inhibited the rapid diffusion of the reagents and solvents through the matrix. Because of this limitation and based on the early findings of Köster,[24] silica supports including controlled-pore glass (CPG) were reinvestigated. During the last decade, the solid-phase synthesis of oligonucleotides according to the phosphoramidite approach[30] has been mainly performed on silica-derived supports. Consequently, these supports will be dealt with in some detail in this section.



P = Poly(styrene-divinylbenzene) copolymer

The first step in the functionalization of silica gel or CPG (**32**) involved a treatment with 3-aminopropyltriethoxysilane.[28,45-50] The resulting aminoalkylated silica **33** was then reacted with succinic anhydride affording the carboxylated matrix **34**.[28,49,50] The coupling of protected nucleosides through either the 2'-OH or the 3'-OH function to the support **34** was mediated by 1,3-dicyclohexylcarbodiimide (DCC)[28,49,50,51] or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC).[52] Alternatively, nucleoside-3'-*O*-succinates[53,54] could also be coupled to the aminated silica **33** in the presence of DCC[54] or DEC[32,55] to yield the derivatized support **35**.

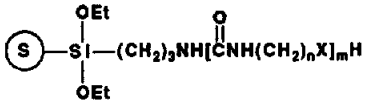


R= Protecting Group
R'= H or OR" where R"= Protecting Group

DEC= 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide

The relative importance of the type of linkage between the 3'-OH of the leader nucleoside and the spacer arm along with the length of the spacer arm and the pore size of the solid support with respect to the efficiency of oligonucleotide synthesis has been investigated by Katzhendler *et al.*[56] and van Aerschot *et al.*[57] It was, indeed, found that the length and the nature of the spacer arm between the matrix and the leader nucleoside were important for optimum synthetic efficiency and product purity. A spacer length of more than 25 atoms was recommended and it was also important to avoid the possibility of folded conformations within the structure of the spacer. These may arise from internal hydrogen bonding, dipole-dipole or π interactions and could decrease the yield and purity of the final product. Spacers having a fully extended conformation such as in **36** or **37** contributed to the

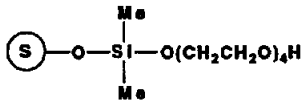
preparation of oligonucleotides in higher yields and purity. These data were in agreement with those reported earlier by Adams *et al.*[32] who described the preparation of 51-mers on a "long chain alkylamine" controlled-pore glass support (LCAA-CPG, **38**)[58] *via* the phosphoramidite approach.



36

where n= 2,3,6,12
m= 1,2,3,4
X= O or NH

(S)= CPG 500, Fractosil 500
Fractosil 1000, Fractosil 2500



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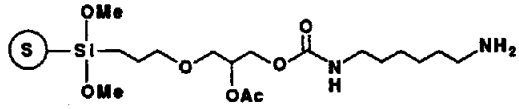
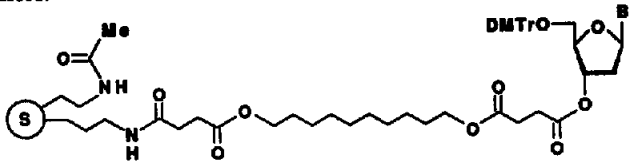


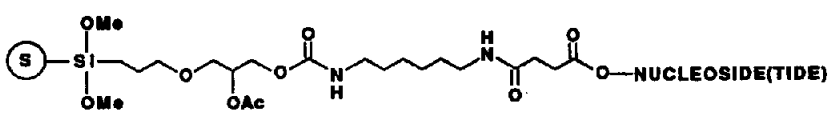
38

The importance of the length of the spacer arm toward efficient solid-phase oligonucleotide synthesis was also stressed by Arnold *et al.*[59] The CPG-derived support **39** was prepared from the activation of 1,10-decanediol bis-succinate with *N*-hydroxysuccinimide followed by successive coupling with a 5'-*O*-DMTr-*N*-protected deoxyribonucleoside and aminopropylated CPG. This support improved the yields of the first chain elongation step to 95-96% by the phosphoramidite approach. It is believed that the spacer arm enhanced the accessibility of the leader nucleoside from a pool of shorter acetylated aminopropyl linkers.



39

In studies aimed at evaluating the 5'-*O*-Fmoc group as a protecting group in oligonucleotide synthesis, it was found that the conventional succinate linkage between the oligonucleotide and the solid support (as in **40**) was unstable to 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) required for the stepwise removal of the 5'-*O*-Fmoc group.[60] Brown *et al.*[61a] recently reported that LCAA-CPG derivatized with a succinyl-sarcosyl linker (**41**) exhibited less than 5% cleavage after an overnight treatment with 10% DBU in dichloromethane. Under these conditions the conventional succinate linkage in **40** was cleaved within 1 h, presumably, as a result of the deprotonation of the amide function followed by intramolecular nucleophilic displacement at the ester carbonyl group.[61a] The solid support **41** was prepared by condensing Fmoc-sarcosine with LCAA-CPG (**38**) in the presence of DCC. After the removal of the Fmoc group, 5'-*O*-DMTr nucleoside-3'-*O*-succinates were activated by DCC and coupled to the *N*-methylamino group of the sarcosine linker. A similar solid support has been developed by others[61b] to avoid similar problems.



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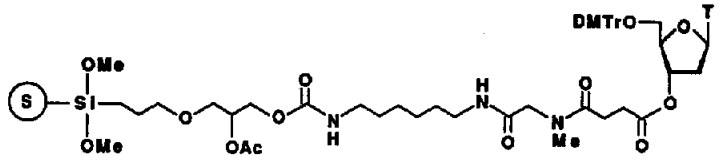
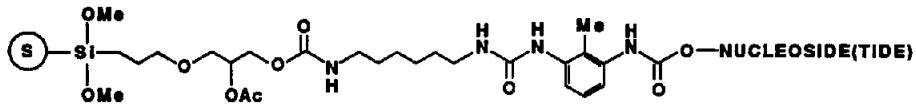


41

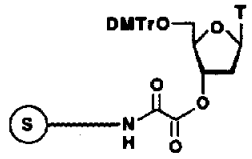
Three other types of linkage between the 3'-OH of the leader nucleoside and the spacer were also evaluated during solid-phase oligonucleotide synthesis by the phosphoramidite methodology.[56] No significant differences in the yields and the purity of the desired oligonucleotides were observed between the syntheses achieved with either an ester, carbonate or carbamate linkage. It was, however, claimed that higher yields of oligodeoxyribonucleotides were obtained *via* the phosphotriester approach when the LCAA-CPG support **42** was used instead of **40**.[62a] The higher stability of the carbamate function linking the nucleoside to the spacer during oligonucleotide assembly was presumably responsible for the modest increase in yields. A prolonged treatment (48 h) with concentrated ammonium hydroxide at 56 °C was required for the release of the oligomers from the support.[62]



42

Recently, Alul *et al.*[40m] reported the derivatization of LCAA-CPG with a suitably *N*-protected-5'-*O*-DMTr nucleoside having a 3'-*O*-oxalyl linker. The support **43** resulted from the reaction of LCAA-CPG with a solution of 5'-*O*-DMTr-thymidine, oxalyl chloride and triazole in acetonitrile-pyridine (5:1). The oxalyl linker was stable to dry pyridine, triethylamine or *N,N*-diisopropylamine for at least 15 h at ambient temperature. Conversely, 5'-*O*-DMTr thymidine was cleaved from **43** within 5 min upon treatment with either wet triethylamine, 40% trimethylamine in methanol, *n*-propylamine in dichloromethane (1:5) or 5% ammonium hydroxide in methanol. Under these conditions, less than 5% of 5'-*O*-DMTr thymidine departed from the standard LCAA-CPG support **40**.[40m]

Oligonucleotide syntheses performed on the solid support **43** according to the phosphoramidite approach proceeded with an average coupling yield of 96%. By comparison, an average coupling yield of 97% was obtained when the standard support **40** was used under identical conditions.[40m] In addition to allowing the automated synthesis of conventional or base-sensitive oligonucleotides, the solid support **43** facilitated the isolation of oligonucleoside methyl phosphotriesters or oligonucleotides with intact *N*-acyl protecting groups.[40m]



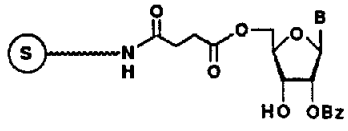
43

The pore size of silica supports is of importance during the synthesis of relatively large oligonucleotides. An abrupt termination of chain propagation occurred when the synthesis of oligomers larger than 100 bases were attempted on CPG with a pore size of 500 Å.[63] This phenomenon was attributed to the increasing steric crowding around the pores and channels of the support caused by

growing oligomer chains which, presumably, reduced the diffusion of the reagents through the matrix. Silica supports with pore size of at least 1000 Å were, nevertheless, satisfactory for the synthesis of large oligonucleotides.[63] For instance, 98-mers were prepared on Fractosil 2500 with an average coupling yield of 99.3% from deoxyribonucleoside phosphoramidite monomers.[56]

Non-porous silica gel microbeads[64] and rigid non-swelling polystyrene beads[40k] have also been applied to the synthesis of larger oligodeoxyribonucleotides by the phosphoramidite approach. The preparation of a 150-mer was achieved with an average stepwise yield of 98-99%.

In an attempt to obviate the tedious preparation of four silica supports derivatized with each of the leader nucleosides, the development of a universal glass support was undertaken by Gough and coworkers.[65,66] Basically, their approach entailed the attachment of N^3-anisoyl-2',3'-*O*-methoxybenzylideneuridine 5'-*O*-succinate to LCAA-CPG. Acidic hydrolysis of the 2',3'-*O*-methoxybenzylidene protecting group generated the 2'(3')-*O*-benzoates **44** and **45**, thereby, exposing 2'(3')-hydroxy functions to chain extension during oligonucleotide synthesis. The release of synthetic oligoribonucleotides from **44** or **45** was effected by concentrated ammonium hydroxide-pyridine (2:1) for 16 h at 50 °C, whereas the release of oligodeoxyribonucleotides required concentrated ammonium hydroxide for 24 h at 60-65 °C. The formation of side-products was not observed under these conditions. This type of support has been useful in the preparation of octamers with alternating phosphorothioate linkages obtained by coupling dimeric phosphoramidites.[67]



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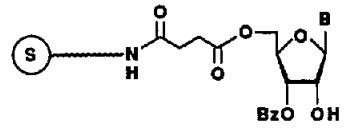
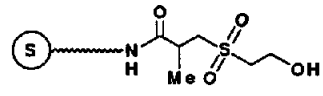


45

B= N^3-anisoyluracil

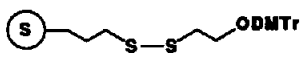
A universal solid support for the synthesis of oligonucleotides with terminal 3'-phosphates has been proposed by Markiewicz and Wyrzykiewicz.[68] The support **46** was prepared by treating LCAA-CPG with methyl methacrylate followed by 2-mercaptoethanol and hydrogen peroxide, respectively. The synthesis of oligonucleotides on **46** was achieved *via* the phosphoramidite approach and the synthetic oligomers were released from the support by concentrated ammonium hydroxide (55 °C, 24 h) in yields comparable to those oligomers attached to LCAA-CPG through a 3'-*O*-succinate linkage. The structure of these oligonucleotide 3'-phosphates was confirmed by chemical ligation with cyanogen bromide which resulted in the formation of polymeric blocks. Analogous sulfonylethyl spacers anchored to polystyrene supports have also been exploited by others toward the synthesis of oligonucleotides bearing a 3'-terminal phosphate group.[69]



46

Alternatively, Kumar *et al.*[70] reacted 3-mercaptopropylated-CPG with 2,2'-dithiobis-(5-nitropyridine) and 4,4'-dimethoxytrityloxyethyl-2-mercaptan to enable the automated synthesis of oligonucleotide 3'-phosphates. The resulting support (**47**) was stable under the conditions required for the synthesis of oligonucleotides by the phosphoramidite approach. Oligonucleotide 3'-phosphates were

obtained by treating the support with 50 mM dithiothreitol (DTT) in concentrated ammonium hydroxide (55 °C, 16 h). Along similar lines, the derivatization of aminopropylated-CPG with 3,3'-dithiobis-(*N*-succinimidyl propionate) and 4,4'-dimethoxytrityloxypentylamine yielded the support **48**.[71] Oligonucleotides with a terminal 3'-sulfhydryl group were readily synthesized and then cleaved from this support under the conditions reported by Kumar *et al.*[70] Thiol-containing oligonucleotides have been useful in the preparation of various fluorescent probes.[72]



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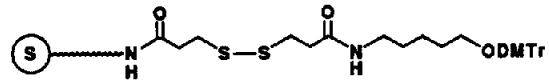
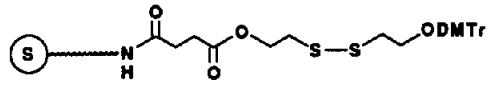


48

Gottikh *et al.*[73] and Asseline and Thuong[74,75] independently engineered the silica-derived support **49** and **50** from aminopropylated-Fractosil 500 and 2,2'-dithiodiethanol for the preparation of oligonucleotides bearing a terminal 3'-phosphate group[73,74] or a 3'-aminoalkyl phosphate diester function.[75] These oligonucleotides were also obtained by treatment of the respective solid support with ammoniacal DTT. The oligomers could then be conjugated to intercalating agents, fluorescent labels, cross-linking agents, affinity binding molecules or DNA cleaving reagents.[75]



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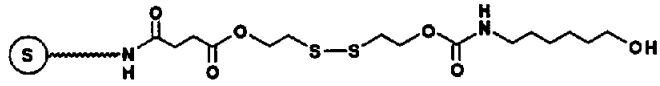
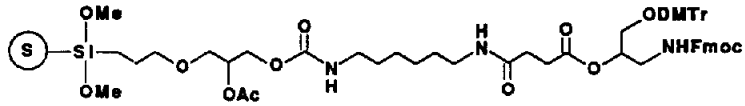


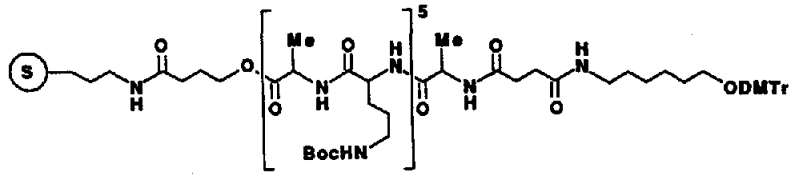
50

An alternate route for the incorporation of 3'-terminal primary aliphatic amines into synthetic oligonucleotides has been reported by Nelson *et al.*[76] Oligonucleotides were synthesized by the phosphoramidite method on the LCAA-CPG support **51** which was prepared by reacting **38** with the *p*-nitrophenyl ester of *N*-Fmoc-*O*-DMTr-3-amino-1,2-propanediol succinate. After standard deprotection with concentrated ammonium hydroxide, the crude 3'-aminoalkylated oligonucleotides were biotinylated. The presence of biotin in the purified oligonucleotide conjugates was confirmed by a *p*-methylaminocinnamaldehyde colorimetric test.[76]



51

In a different approach, Haralambidis *et al.*[77-80] reported the derivatization of aminopropylated-CPG with the peptide (Ala-Lys-)$_5$Ala (as in **52**) toward the preparation of peptide-oligodeoxyribonucleotide conjugates. This strategy would also permit the attachment of non-radioactive labels to the lysine residues of the conjugates. Moreover, a spacing of appropriate dimension between the lysine residues can be accomplished by the insertion of one or several 6-aminohexanoyl units to provide optimum inter-label spacing within the polyamide-oligonucleotide molecules.

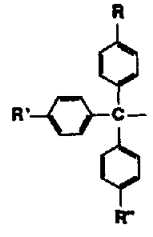


52

In addition to a properly functionalized support, the solid-phase synthesis of oligonucleotides requires the protection of nucleosidic and nucleotidic functionalities. However, the selective deblocking of a specific protecting group is periodically necessary to permit chain extension. Therefore, the selection of compatible hydroxyl, phosphoryl and nucleobase protecting groups represents an important issue. The following section will focus on the protection of the 5'-hydroxyl group of nucleosides.

2. THE PROTECTION OF THE 5'-HYDROXY FUNCTION OF NUCLEOSIDES

The selective incorporation of the trityl derivatives **53-56** at primary hydroxyls is undoubtedly one of the features accounting for the popularity of these groups in the protection of the 5'-hydroxy function of nucleosides. The sensitivity of a specific trityl ether linkage to acidic conditions is dependent on the electron-donating properties of the substituents carried by the trityl group. For instance, each *p*-methoxy substituent enhanced the rate of acid cleavage by approximately a factor of 10.[81] Consequently, the mono- (MMTr, **54**) and to a greater extent the di-*p*-methoxytrityl (DMTr, **55**) groups were commonly used[81-83] in oligonucleotide synthesis, whereas the trityl (Tr, **53**) and the tri-*p*-methoxytrityl (TMTr, **56**) derivatives were too stable[84] or too unstable,[85] respectively, for this purpose.

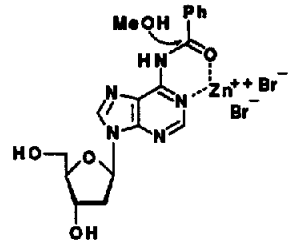


53 R= R'= R"= H
54 R= R'= H; R"= OMe
55 R= H; R'= R"= OMe
56 R= R'= R"= OMe

In the mid-seventies, Letsinger and his coworkers[86] investigated the deblocking of nucleosidic and nucleotidic trityl ethers under non-acidic conditions. They reported the rapid and efficient reductive cleavage of the MMTr group from 5'-*O*-MMTr thymidine and 3'-*O*-MMTr thymidine by naphthalene radical anion in hexamethylphosphoric triamide. Letsinger *et al.*[86b] also demonstrated that anthracene radical anion in tetrahydrofuran converted 5'-*O*-α-naphthyldiphenylmethyl thymidine into thymidine in near quantitative yields whereas 5'-*O*-MMTr thymidine yielded only 2% thymidine under identical conditions. Paradoxically, the acidic hydrolysis (80% aqueous acetic acid) of 5'-*O*-α-naphthyldiphenylmethyl thymidine and 5'-*O*-MMTr thymidine generated thymidine with a half-time of 3.2 h[86b] and 8.5 min,[84] respectively. To our knowledge, the cleavage of 5'-*O*-trityl groups by radical anions has been limited to oligonucleotide syntheses performed in solution phase.

With the advent of automated synthesis of oligodeoxyribonucleotides on polymeric support, the removal of the 5'-*O*-DMTr group under acidic conditions has been particularly useful in evaluating the efficiency of the chain elongation step by simply measuring the release of the di-*p*-methoxytrityl carbocation by spectrophotometry.[87] In an attempt to increase the rates of detritylation and to minimize the potential depurination of N^6-benzoyl deoxyadenosine residues, several deprotecting

reagents have been investigated.[32,40d,69,88-98] Specifically, boron trifluoride etherate in dichloromethane-methanol has been reported to cleave 5'-*O*-trityl groups.[99] A milder Lewis acid, namely, zinc bromide has subsequently been used in nitromethane[100] or in dichloromethane[101] for the cleavage of the 5'-*O*-DMTr group. Unlike protic acids, a saturated solution of zinc bromide in anhydrous nitromethane did not cause significant depurination of N^6-benzoyl deoxyadenosine derivatives within 24 h.[28,97a] This reagent was, nonetheless, slow (ca. 30 min) to cleave a 5'-*O*-DMTr group from an oligomer covalently attached to a solid support.[28] The rates of deprotection were enhanced by increasing the concentration of zinc bromide in solution. This was achieved by using mixed solvents such as nitromethane-methanol (95:5)[102a-c] nitromethane-water (99:1)[54,102d-e,103] and dichloromethane-propan-2-ol (85:15).[104-106] Under these conditions, detritylation without depurination was completed within 5 min. One must, however, mention that the prolonged treatment (ca. 15 h) of N^6-benzoyl-5'-*O*-DMTr deoxyadenosine with a 0.7 M solution of zinc bromide in chloroform-methanol (9:1) led to the generation of deoxyadenosine in 30% yield.[106] It was postulated that methanol effected the nucleophilic displacement of the N^6-benzoyl group *via* the formation of a chelated bidentate intermediate (**57**). Consistent with this postulate, the substitution of methanol by propan-2-ol dramatically decreased the rate of *N*-deacylation.[106]

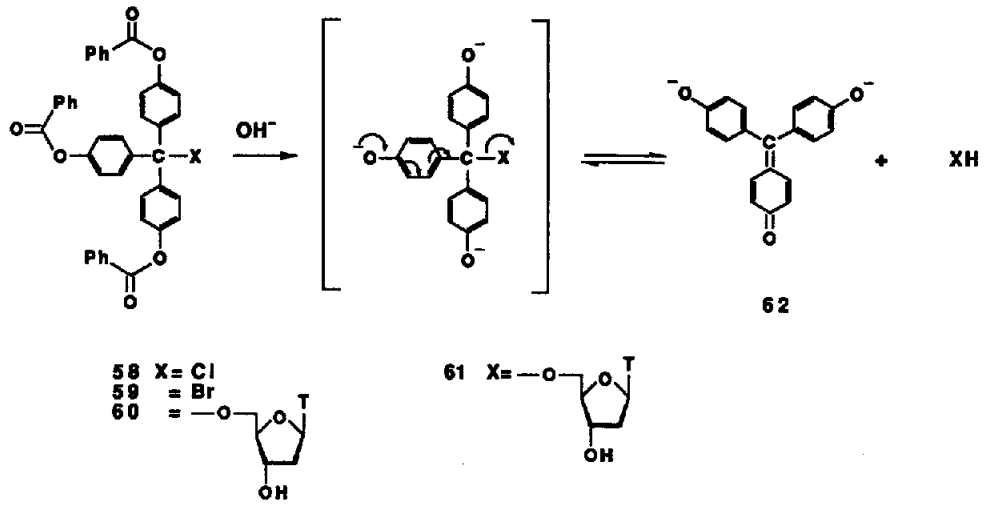


57

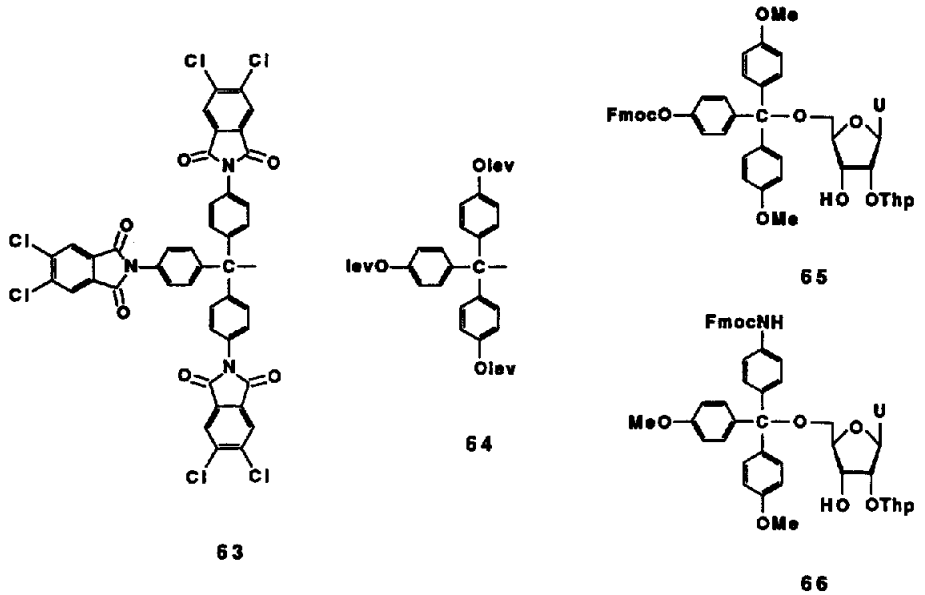
It has been shown that the rate of DMTr group cleavage by zinc bromide rapidly decreased with the length of the DNA chain when polystyrene or a polyamide resin was used as a solid support.[32,105] Consequently, the use of zinc bromide in solid-phase DNA synthesis is limited to the preparation of short oligomers on selected solid supports. Although other Lewis acids such as titanium tetrachloride,[28] aluminum chloride,[28] diethylaluminum chloride[107] and diisobutylaluminum chloride[107] were also reported for the cleavage of nucleosidic 5'-*O*-trityl ethers, these reagents have not been extensively used in the solid-phase synthesis of DNA.

An attractive concept regarding the design of trityl protecting groups was applied by Sekine and Hata.[108] They reported that 4,4',4"-tris-(benzoyloxy) trityl chloride (TBTr-Cl, **58**) and bromide (TBTr-Br, **59**), readily prepared from rosolic acid, selectively reacted with the 5'-hydroxy function of base-protected deoxyribonucleosides and ribonucleosides. As expected from the electron-withdrawing nature of the benzoyloxy substituents, the 5'-*O*-TBTr nucleosides were more resistant to acidic conditions than the corresponding nucleosides protected with the Tr group. However, the treatment of 5'-*O*-TBTr deoxythymidine (**60**) with 0.5 M sodium hydroxide resulted in the complete removal of the TBTr group within 10 min.

It was postulated that the conversion of the electron-withdrawing benzoyloxy groups ($\sigma_p = 0.31$) of the TBTr group into electron-donating oxido functions ($\sigma_p = -0.52$) effected by hydroxide ions, triggered the efficient cleavage of the trityl ether linkage in **61**. The conditions required for the removal of the TBTr group were, nonetheless, incompatible with the base-labile protecting groups of the nucleobases. To circumvent this problem, Sekine and Hata[109,110] introduced the 4,4',4"-tris-(4,5-dichlorophthalimido) trityl group (CPTr, **63**). This trityl group derivative was prepared from pararosaniline and 4,5-dichlorophtha ic anhydride and used as its bromide for the selective 5'-OH tritylation of nucleosides. Similar to the TBTr group, the CPTr group was relatively acid-stable given the strong inductive effect created by the phthalimide moieties. In contrast to the harsh alkaline



conditions required for the cleavage of the TBTr group, the CPTr group was converted into the unstable 4,4',4"-triaminotrityl ether within 20 min by hydrazinolysis.[109-112] The mildness of this treatment permitted the use of 5'-*O*-CPTr nucleosides carrying base-labile protecting groups in the synthesis of oligonucleotides.

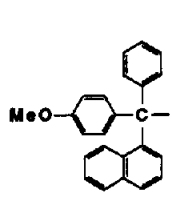


The selective tritylation of the 5'-hydroxy function of deoxythymidine by the 4,4',4"-tris-(levulinyloxy) trityl group (TLTr, **64**) has, alternatively, been described.[113] Like the TBTr group, the TLTr protection was very stable toward acidic conditions and thus allowed its use in the presence of other acid-labile protecting groups. Upon hydrazinolysis, the TLTr group was transformed into the corresponding 4,4',4"-trihydroxytrityl derivative within 3 min. Subsequent heating with pyridine-acetic acid (1:2) at 50 °C for 30 min resulted in the complete cleavage of the trityl ether linkage.

A similar concept was applied by Happ and Scalfi-Happ[114] to the preparation of specific trityl groups which led to the synthesis of the 5'-*O*-protected nucleosides **65** and **66**. When required, the

removal of the trityl protecting group from **65** or **66** can be induced by the ß-elimination of the Fmoc group by DBU followed by a brief treatment (ca. 10 min) with pyridine-formic acid (4:7). The mildness of these conditions were particularly attractive for the synthesis of larger oligonucleotides where depurination could become significant.

The versatility provided by the protection of the 5'-OH function of nucleosides by trityl groups has been further demonstrated by Fisher and Caruthers.[85] In an attempt to monitor the stepwise addition of mononucleotides to deoxyribonucleotides bound to a solid support, several triarylmethyl groups exhibiting similar chemical reactivities but producing different colors under acidic conditions, were prepared. Specifically, the DMTr group **55**(orange), *p*-anisyl-1-napthylphenylmethyl group **67** (red), di-*o*-anisyl-1-napthylmethyl group **68** (blue) and *p*-tolyldiphenylmethyl group **69** (yellow) were assigned to the 5'-OH protection of N^2-isobutyryl deoxyguanosine, deoxythymidine, N^4-benzoyl deoxycytidine and N^6-benzoyl deoxyadenosine respectively.[85,115] These "color-coded" nucleosides were then converted into their respective 3'-*O*-(*N,N*-dimethylamino)methoxyphosphines and used in solid-phase DNA synthesis. The monitoring of the relative extent of condensation when more than one mononucleotide was added during the preparation of mixed-sequence probes, could be achieved because of the large difference in the visible absorption spectra of the various triarylmethyl cations generated during the stepwise deblocking step.[85]



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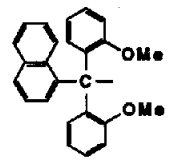

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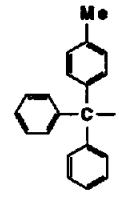
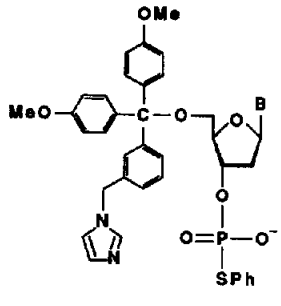


69

Added versatility was also provided by the 3-(imidazolylmethyl)-4,4'-dimethoxytrityl group (IDTr).[116] In addition to selectively react with the 5'-OH function of properly protected nucleosides it has been shown that, presumably because of the presence of the imidazole moiety acting as a catalyst, the condensation of **70** with the nucleoside **71** in the presence of isodurene disulfonyl chloride resulted in the formation of the dinucleoside phosphotriester **72** in near quantitative yields within 30 s.[116,117] The considerable rate enhancement observed in the phosphorylation raction was, however, not tested during solid-phase DNA synthesis.



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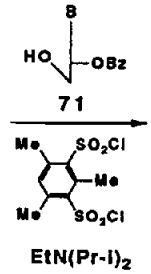
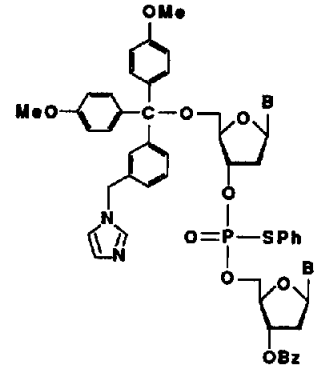
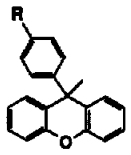


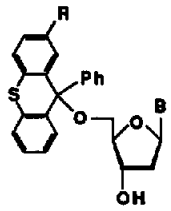


72

The selective protection of the 5'-OH of *N*-acylated nucleosides was also achieved with the 9-phenylxanthen-9-yl group **73** (pixyl, Px)[118-120a,b;121-125] and the 9-(*p*-methoxyphenyl)xanthen-9-yl group **74** (Mox).[121-125] The stability of the Px and DMTr groups towards acidic conditions was comparable. The pixyl group provided crystalline properties to nucleoside derivatives and improved their detection on thin layer chromatography to the nanomolar range.[120a] Additionally, the 9-phenylthioxanthen-9-yl (*S*-pixyl) and 7-chloro-9-phenylthioxanthen-9-yl (chloro-*S*-pixyl) groups were reported by Balgobin *et al.*[126] as potential 5'-*O*-protecting groups for deoxyribonucleosides. These groups were completely cleaved from the deoxynucleoside derivatives **75a-d** and **76a-d** within 90 s at 20 °C with 1.1 eq of *p*-toluenesulfonic acid monohydrate in chloroform-ethanol (98:2). Like the pixyl group, the *S*-pixyl group and the chloro-*S*-pixyl groups exhibited fluorescence at 366 nm and were detected at a concentration as low as 2 nM. When applied to the preparation of an octathymidylic acid according to the phosphotriester approach in solution phase, the *S*-pixyl group was preferred to the chloro-*S*-pixyl group because the former could still be removed within 90 s under the above conditions, whereas the latter required at least 180 s for complete cleavage.[126]

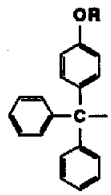


73 R= H
74 = OMe

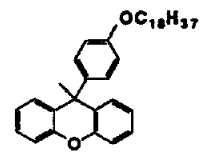


75a B= thymin-1-yl; R= H
b B= N^4-benzoylcytosin-1-yl; R= H
c B= N^6-*m*-chlorobenzoyladenin-9-yl; R= H
d B= N^2-*t*-butylbenzoylguanin-9-yl; R= H
76a-d R= Cl

It is well-known that terminal 5'-*O*-trityl or pixyl group derivatives were resistant to the basic conditions required for the deprotection of synthetic oligomers. This feature permitted the considerable retention of the desired nucleotidic sequence on reverse-phase high performance liquid chromatography (HPLC) relative to the failure sequences generated during the synthesis. An enrichment in the correct sequence was thus realized by this simple procedure.[121a,127-131] After purification, the terminal 5'-*O*-trityl or pixyl group could then be removed by treatment with aqueous 80% acetic acid. In addition to the DMTr and Px groups[88] the 4-decyloxytrityl (C_{10}Tr, **77a**),[130,131] the 4-hexadecyloxytrityl (C_{16}Tr, **77b**),[127,128] the 9-(4-octadecyloxyphenyl)xanthen-9-yl (C_{18}Px, **78**)[121a,129] and the 1,1-bis-(4-methoxyphenyl)-1'-pyrenyl methyl (BMPM, **79**) group[132] were also used as lipophilic groups for the purification of synthetic oligomers. Particularly, 5'-*O*-BMPM oligonucleotides were detected at the 100 pM level at 360 nm by virtue of the fluorescent nature of the BMPM group.[132]



77a R= $C_{10}H_{21}$
b = $C_{16}H_{33}$



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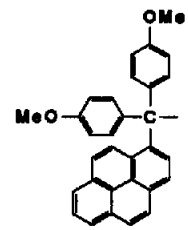
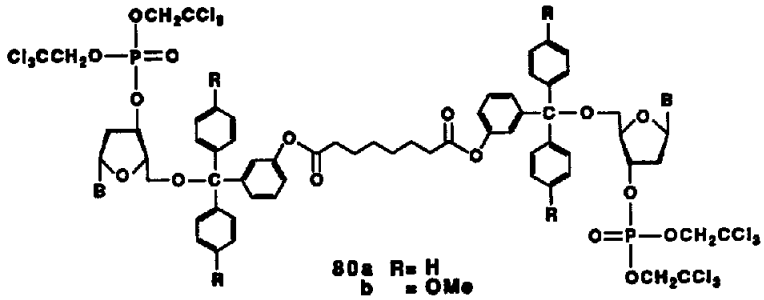


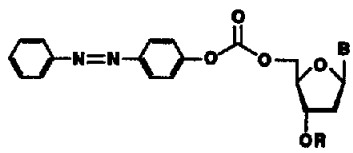
79

An additional application of trityl derivatives as a purification tool has been reported by Biernat *et al.*[133] A "bridged" bis-trityl chloride was used for the protection of the 5'-OH function of nucleoside-3'-*O*-phosphotriesters. Chain elongation from **80a** or **80b** led to the concomitant preparation of two

oligonucleotide strands. These were purified, in a stepwise manner, from the condensing reagent and unreacted monomers by gel filtration. The major drawback of this elegant approach was the time consumed for each elongation step (ca. 4 h).



In spite of the popularity of substituted trityl groups for the protection of the 5'-OH of nucleosides and nucleotides, the search for 5'-hydroxyl protecting groups that can be selectively introduced at this position and subsequently removed under near neutral conditions led Seliger *et al.*[134,135] to investigate the *p*-phenylazophenyloxycarbonyl group (PAPoc) for this purpose. The PAPoc group was introduced by treating deoxyribonucleosides with *p*-phenylazophenyloxycarbonyl chloride. The 5'-*O*-PAPoc nucleosides **81** were isolated in ca. 70% yields and were converted into 3'-*O*-phosphoramidites (**82** and **83**) toward the solid-phase preparation of oligodeoxyribonucleotides.



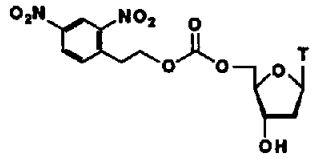
81 R= H
82 = $P(OMe)NMe_2$
83 = $P(OMe)N(Pr\text{-}i)_2$

The removal of the PAPoc group was achieved at ambient temperature by a 4 min treatment with ß-cyanoethanol-triethylamine-water (1:1:1) followed by 0.01 M DBU in pyridine (1 min).[135] Under these conditions, the colored *p*-phenylazophenolate anion thus generated allowed the visual monitoring of the coupling effiency. The deprotection mechanism, apparently, involved consecutive transesterification and ß-elimination reactions. This hypothesis was supported by the characterization of the ß-cyanoethyl carbonate intermediate.[134] The stepwise yields recorded during the preparation of dT_9 and dA_9 were only 66% and 85% respectively. A striking enhancement in stepwise yields was realized when the removal of the PAPoc group was effected by 8% *N,N*-dimethylaminopyridine (DMAP) in *N,N*-dimethylformamide for 2 min. For instance, dT_8 and d(CACGGGGCGC) were synthesized with an average stepwise yield of 98% and 93% respectively.[135]

The base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group was introduced for the selective protection of the 5'-OH group of deoxyribonucleosides to minimize the depurination of N^6-acylated deoxyadenosine moieties[60,61a,136-139] observed during the stepwise removal of the 5'-*O*-DMTr group. This group was also used to selectively protect the 5'-hydroxy function of 2'-*O*-protected ribonucleosides[140-143] to ensure the stability of specific acid-labile 2'-*O*-protecting groups throughout oligonucleotide assembly. These 5'-*O*-Fmoc nucleosides were converted into their corresponding 3'-*O*-(*N,N*-diisopropylamino)methoxy or 2-cyanoethoxyphosphines[60,137,138,142] and successfully used in solid-

phase oligonucleotide synthesis. The stepwise removal of the Fmoc group was effected by base-induced ß-elimination. Specifically, 10% piperidine in acetonitrile[138] was effective in deblocking the Fmoc (ca. 3 min) and in neutralizing the reactive benzofulvene generated during the deprotection. However, this reagent caused the partial cleavage (ca. 5%) of the 3'-*O*-succinate linkage within 3 h along with the removal of the N^4-benzoyl group of deoxycytidine and the demethylation of the phosphate ester function with a half-life of ca. 30 h and 3 h respectively. Despite these limitations, the stepwise coupling yield was 95-96%. Alternatively, Lehmann *et al.*[142] reported the use of 0.1 M DBU in acetonitrile for the deprotection of the 5'-*O*-Fmoc group during the solid-phase synthesis of ribonucleotides using the cyanoethyl group as phosphate protecting group. These researchers indicated that some or all of the cyanoethyl phosphate protecting groups of the support-bound oligonucleotide were likely to be removed under these conditions. Nevertheless, no adverse effect on the stepwise coupling yield (ca. 96%) was observed.

In an analogous context, Pfleiderer *et al.*[144,145] described the selective incorporation of the 2,4-dinitrophenylethoxycarbonyl group (DNPEoc) at the 5'-OH of deoxyribonucleosides (as in **84**) in yields ranging from 70-80%. The dinucleoside phosphotriester **86** was prepared from the 5'-*O*-DNPEoc deoxyribonucleoside phosphoramidite **85** and 3'-*O*-benzoyl deoxythymidine in the presence of 1*H*-tetrazole. The removal of the DNPEoc group from **86** was mediated by triethylamine *via* a ß-elimination reaction. These conditions allowed the selective removal of the DNPEoc group in the presence of *p*-nitrophenylethyl (NPE) (**86** →**87**) and *p*-nitrophenylethoxycarbonyl (NPEoc) protecting groups.[145] Therefore, the DNPEoc group may be considered as a potential substitute for the DMTr group in the synthesis of oligodeoxyribonucleotides.



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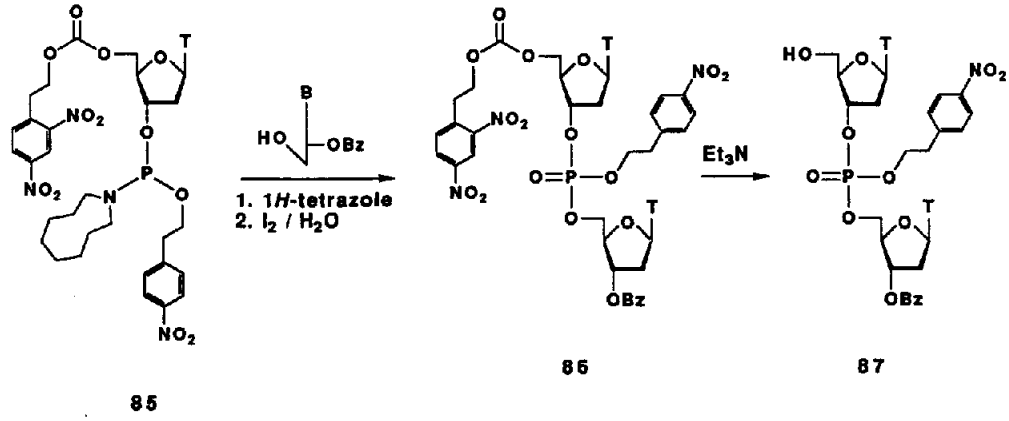
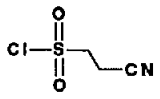


85 **86** **87**

Reiner and Pfleiderer[146] pointed out the potential usefulness of ß-substituted ethylsulfonyl chlorides (**88-90**) as "capping" reagents during solid-phase oligonucleotide synthesis. Basically, these reagents would react with the 5'-OH function of failure sequences generated during the synthesis and terminate the further growth of these sequences which otherwise would complicate the subsequent

purification of the desired sequence. Like the NPE and the NPEoc protecting groups, the ß-substituted ethylsulfonyl groups derived from **88-90** were also labile to DBU. It is, therefore, possible to synthesize oligonucleotides which upon treatment with DBU would leave on the solid support the desired 5'-*O*-DMTr-containing sequences and the failure sequences carrying free 5'-hydroxyls. The truncated sequences could then be digested by specific phosphodiesterases[147] and further simplify the isolation of 5'-*O*-DMTr oligonucleotides. This strategy has been limited by the slow reaction (ca. 12 min) of **88-90** with the 5'-OH of nucleosides even in the presence of *N*-methylimidazole. Consequently, the practical utilization of these capping reagents in solid-phase oligonucleotide synthesis would require a significant enhancement of their reaction kinetics.



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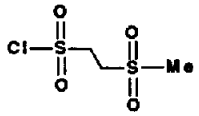

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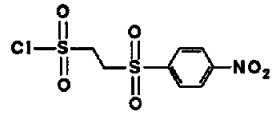
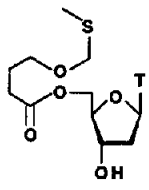


90

Other types of protecting groups that can regioselectively react with the 5'-hydroxy function of nucleosides are the 4-(methylthiomethoxy)butyryl (MTMB, as in **91**), 2-(methylthiomethoxymethyl) benzoyl (MTMT, as in **92**) and the 2-(isopropylthiomethoxymethyl)benzoyl (PTMT, as in **93**) groups.[148-151] The introduction of the MTMB and MTMT groups was achieved by the condensation of the corresponding acid with either *N,N*-bis-(2-oxo-oxazolidin-3-yl)phosphorodiamidic chloride or 2,6-dichlorobenzoyl chloride and thymidine in pyridine. The 5'-*O*-protected thymidine derivatives **91** and **92** were both isolated in 70% yields. The incorporation of the PTMT group was, alternatively, realized by coupling the corresponding acid with 2'-*O*-methoxytetrahydropyranyl-4-*O*-(2,4-dimethylphenyl) uridine in the presence of 1,3-diisopropylcarbodiimide.



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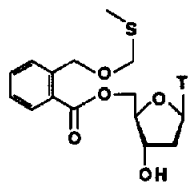

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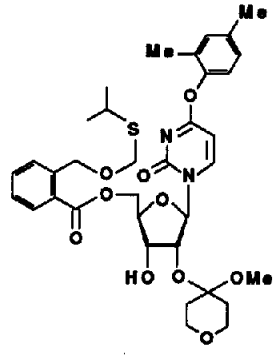
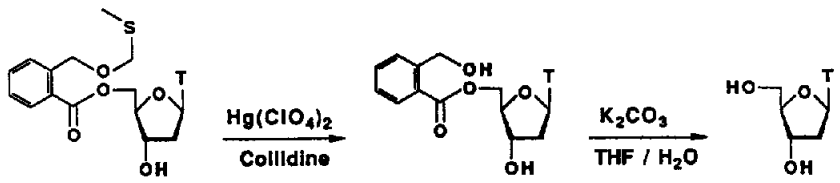


93

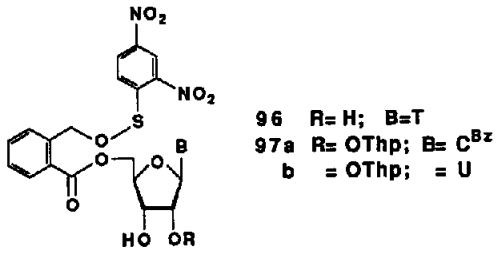
The stability of **92** toward ammonolysis was remarkable. In concentrated ammonium hydroxide, **92** was converted into thymidine with a half-life of ca. 6 h at 20 °C. By comparison, 5'-*O*-acetylthymidine was transformed into thymidine under the same conditions with a half-life of 5 min. However, the reaction of **92** with mercury (II) perchlorate and 2,4,6-collidine afforded the intermediate **94** within ca. 3 h at 20 °C. The subsequent treatment of **94** with potassium carbonate produced thymidine within 30 s. Under similar conditions, **93** reacted with mercury (II) perchlorate to yield the corresponding 2-hydroxymethyl benzoyl intermediate within 5 min. The complete removal of the protecting group was effected by triethylamine within 1 min at ambient temperature.[149,150] It has been reported that the PTMT group was selectively removed from a fully protected nonadecaribonucleotide in 88% isolated yield.[152] Thus, the PTMT group in addition to the DNPEoc group may also represent a

substitute for the DMTr group, especially in the synthesis of oligoribonucleotides.



92 94 95

Christodoulou *et al.*[153,154] described the 2-(2,4-dinitrobenzenesulphenyloxymethyl)benzoyl group (DNBSB) as a potentially useful protecting group for the 5'-hydroxy function of nucleosides. The desired 5'-*O*-DNBSB nucleosides **96** and **97a-b** were isolated in only 25-33% yields from the condensation of suitably protected nucleosides with DNBSB-acid and pivaloyl chloride. The conversion of 5'-*O*-DNBSB nucleosides into their respective 3'-*O*-(*N,N*-diisopropylamino)-2-cyanoethoxyphosphines has not been encouraging probably because the polarized S-O linkage was sensitive to intra and/or intermolecular nucleophilic attack by the phosphoramidite function. Despite the incompatibility of the DNBSB group with the phosphoramidite approach, the protecting group was completely removed from nucleosides within 2 min by treatment with *p*-toluenethiol.



96 R= H; B=T
97a R= OThp; B= C^{Bz}
b = OThp; = U

Several other 5'-OH protecting groups have been reported during the last decade, most of which were introduced *via* acyl chlorides or chloroformates. The conditions required for the deprotection of these groups were often incompatible with either the aglycone or phosphate protecting groups. The application of such 5'-OH protecting groups has been reviewed elsewhere[15,20] and will not be reported herein.

It is common knowledge that the most efficient chemical approaches to the synthesis of oligonucleotides required the protection of the aglycone moiety of the nucleosides to prevent potential side reactions during the formation of internucleotidic linkages. For instance, Khorana *et al.*[82] have used acyl groups for the protection of the nucleobases. Specifically, the exocyclic amino function of deoxycytidine was protected with either a *p*-anisoyl or a benzoyl group.[84,155] The half-lives of these protecting groups in 9 N ammonium hydroxide were 64 and 16 min respectively.[155] The 6-amino function of deoxyadenosine was also protected with a benzoyl group[84] whereas the exocyclic amino function of deoxyguanosine was protected with an isobutyryl group.[156] No protecting group for thymine and uracil aglycones was apparently needed.

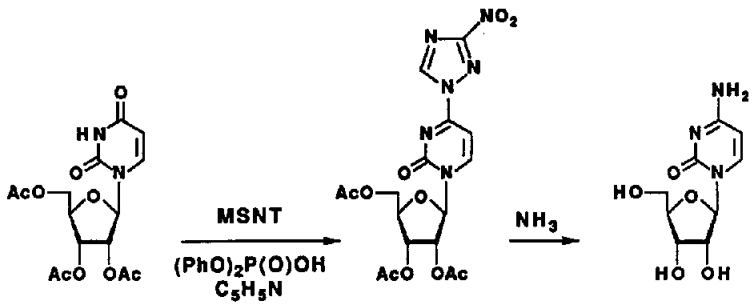
The above nucleobase protecting groups are still popular in modern solid-phase oligonucleotide synthesis not only *via* the deoxyribonucleoside phosphoramidite approach but also *via* the phosphotriester and the *H*-phosphonate approaches as well. However, various studies have demonstrated that modification of the thymine, uracil and guanine rings can occur during oligonucleotide synthesis.[157-159] Additionally, N^6-benzoyl deoxyadenosine and N^2-isobutyryl deoxyguanosine are notoriously susceptible to depurination under the acidic conditions required for the

deblocking of the strategic 5'-*O*-DMTr group. A survey of the attempts aimed at eliminating these potential problems through the development of nucleobase protecting groups is presented in the forthcoming section.

3. PROTECTION OF THE AGLYCONES

3.1. Imide and lactam function of thymine and/or uracil and guanine nucleosides.

The intrinsic acidity of the N-H function of thymine/uracil (*pKa* = 9.8)[160] and guanine (*pKa* = 9.2)[160] nucleobases is responsible for their sensitivity to chemical modifications under basic conditions. Reese *et al.*[158,159] reported that 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), a condensing reagent used in the synthesis of oligonucleotides, reacted with 2',3',5-tri-*O*-acetyluridine (**98**) in the presence of diphenyl hydrogen phosphate in pyridine to generate the 4-(3-nitro-1,2,4-triazolo)pyrimidinone **99**.

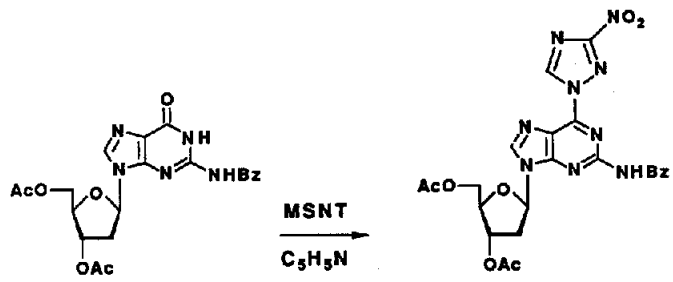


98 **99** **100**

MSNT= 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole

Treatment of **99** with ammonium hydroxide afforded cytidine (**100**) as the sole nucleoside product. Alternatively, the reaction of **99** with either N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-4-nitrobenzaldoximate or tetra-*n*-butylammonium fluoride led to the quantitative recovery of the uridine derivative **98**.[161] Li *et al.*[162] subsequently demonstrated that the reaction of 3',5'-bis-*O*-(methoxyacetyl)thymidine with diphenyl phosphorochloridate, 3-nitro-1,2,4-triazole and triethylamine produced the corresponding 4-(3-nitro-1,2,4-triazolo)pyrimidinone which reacted with methanol in DBU to yield the 4-*O*-methylthymidine derivative.

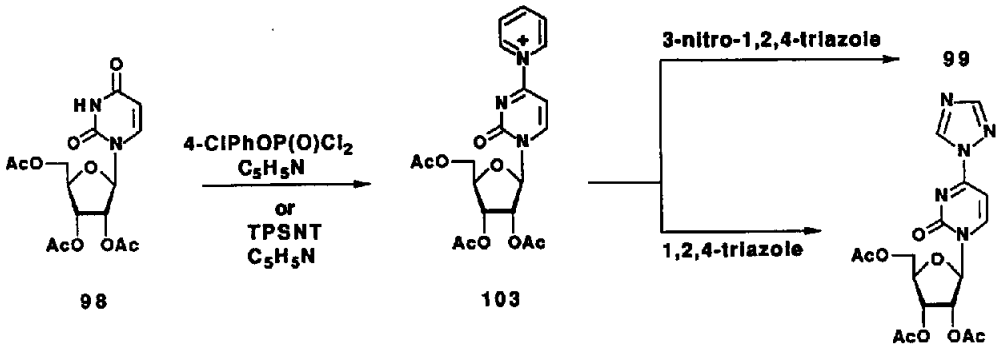
Furthermore, Reese and Ubasawa[159] pointed out that the reaction of the deoxyguanosine derivative **101** with MSNT in the presence or absence of diphenylphosphate in pyridine afforded the triazolonucleoside **102** in 70% isolated yield.



101 **102**

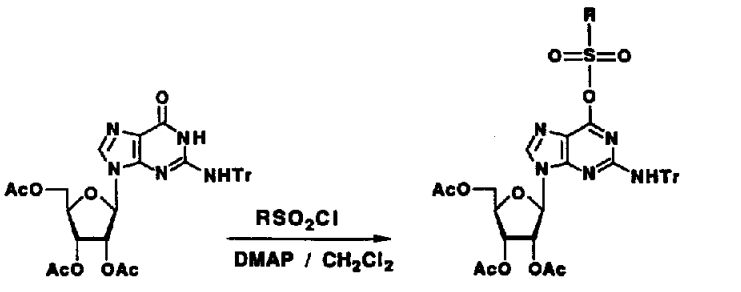
Such a triazolo nucleoside derivative has been reported to react with concentrated ammonium hydroxide to generate the corresponding 2-amino adenosine derivative.[163a]

Sung *et al.*[164] and others[163] described a similar modification of pyrimidine nucleoside derivatives with *p*-chlorophenyl phosphorodichloridate and 1,2,4-triazole or 1*H*-tetrazole in pyridine. Moreover, Adamiak *et al.*[165a,b] discovered that the reaction of 2',3',5'-tri-*O*-acetyluridine (**98**) with *p*-chlorophenyl phosphorodichloridate and 1,2,4-triazole or 2,4,6-triisopropylbenzenesulfonyl-3-nitro-1,2,4-triazole (TPSNT) in pyridine generated the fluorescent pyridinium salt **103** which in the presence of 1,2,4-triazole or 3-nitro-1,2,4-triazole afforded the 4-triazolopyrimidinone **104** or **99** reported earlier by Reese *et al.*[163b] and Sung.[164d] In addition to these findings, Reese and Richards[165c] reported that 2-chlorophenyl phosphorodichloridate activated with 1-hydroxybenzotriazole also attacked thymidine and uridine in the presence of *N*-methylimidazole.



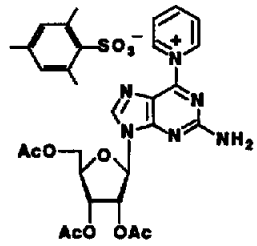
TPSNT= 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole

In contrast to thymine or uracil nucleosides, sulfonated guanine nucleosides can be isolated. For instance, the reaction of 2',3',5'-*O*-N^2-tetrabenzoyl guanosine with an excess of methanesulfonyl chloride and triethylamine afforded the crystalline O^6-mesyl derivative in 75% yield.[166a] It has also been documented that the reaction of N^2-trityl-2',3',5'-tri-*O*-acetyl guanosine (**105**) with MS-Cl or TPS-Cl and DMAP generated the respective O^6-arylsulfonyl guanosine derivatives **106-108**.[166b,c]



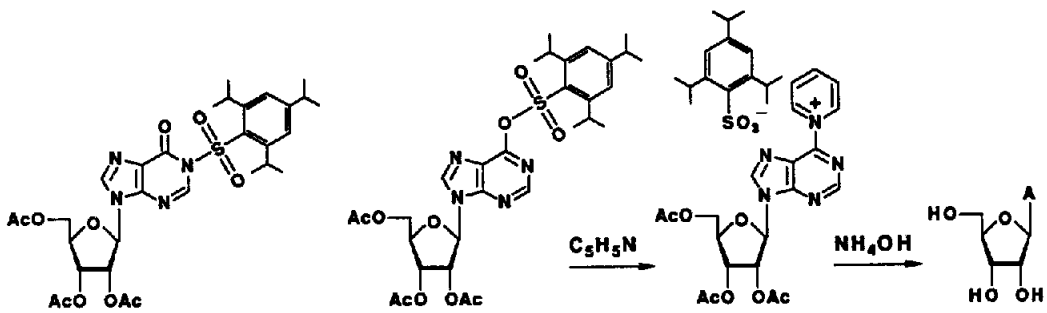
DMAP= 4-dimethylaminopyridine

O^6-arylsulfonyl guanosine derivatives were, nevertheless, sensitive to nucleophiles.[166c] Indeed Francois *et al.*[167] isolated and characterized the pyridinium salt **109** from the reaction of 2',3',5'-tri-*O*-acetyl guanosine with MS-Cl in pyridine.



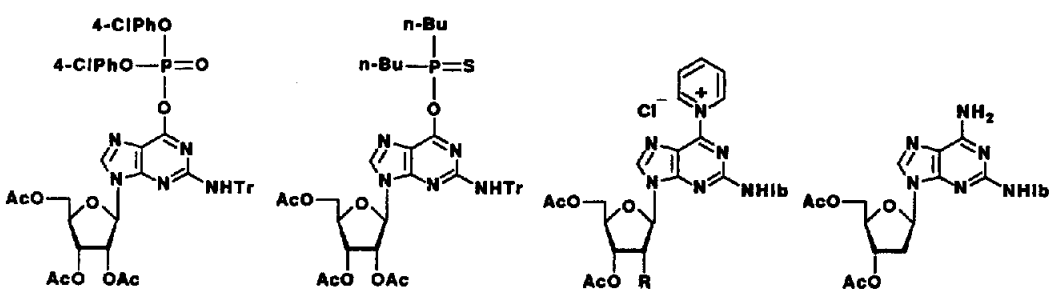
109

These findings were supported by Adamiak *et al.*[165a-b,168-170] who reported the reaction of 2',3',5'-tri-*O*-acetylinosine with TPS-Cl and triethylamine.[168] The crystalline N^1-sulfonated inosine **110** was isolated in 65% yield along with the O^6-sulfonated isomer **111** (ca. 15%). Only **111** reacted with pyridine to generate the pyridinium salt **112**. The latter readily reacted with concentrated ammonium hydroxide affording adenosine (**113**) quantitatively within 1 h at 20 °C.[168] These observations were applied to the derivatization of guanine and hypoxanthine nucleosides at *C*-6.[166d,169,171,172]



110 111 112 113

Phosphorylating agents such as dialkyl phosphoryl, diaryl phosphoryl and phosphinothioyl halides are also known to produce O^6-phosphorylated guanosine derivatives (**114** and **115**) in the presence of DMAP and triethylamine.[166b,c] These compounds have been isolated and fully characterized by elemental analysis, ^{1}H NMR and UV spectroscopy.[166c]

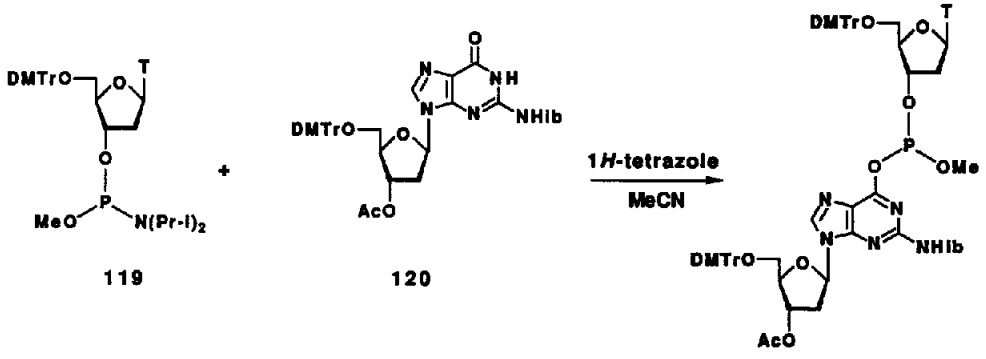


114 115 116 R= OAc 117 = H 118

Most of the O^6-phosphorylated guanosine derivatives are quite labile in basic and acidic

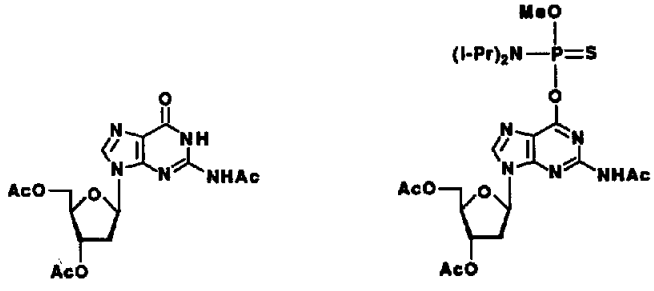
media.[166c,173,174] When either N^2-isobutyryl-2',3',5'-tri-*O*-acetyl guanosine or 3',5'-di-*O*-acetyl deoxyguanosine was reacted with *p*-chlorophenyl phosphorodichloridate and 1,2,4-triazole in pyridine, neither the O^6-phosphorylated guanosine nor the 6-triazolopurine nucleoside was formed. Instead, the pyridinium salt **116** or **117** was isolated.[165a,175] The conversion of **117** into the 6-amino derivative **118** was effected by concentrated ammonium hydroxide.

Pon *et al.*[176,177] and others[178] reported the potential phosphitylation of the guanine ring at *O*-6 during the synthesis of oligonucleotides by the phosphoramidite approach.[30] This possibility was investigated by reacting the guanine nucleoside **120** with the deoxyribonucleoside phosphoramidite **119** activated by 1*H*-tetrazole. ^{31}P NMR analysis of the reaction indicated the presence of new phosphorus resonances at 133.95 and 133.79 ppm presumably resulting from the diastereomeric compound **121**. This compound was, however, not formed when **120** was protected at *O*-6 with a *p*-nitrophenylethyl group.



119 **120** **121**

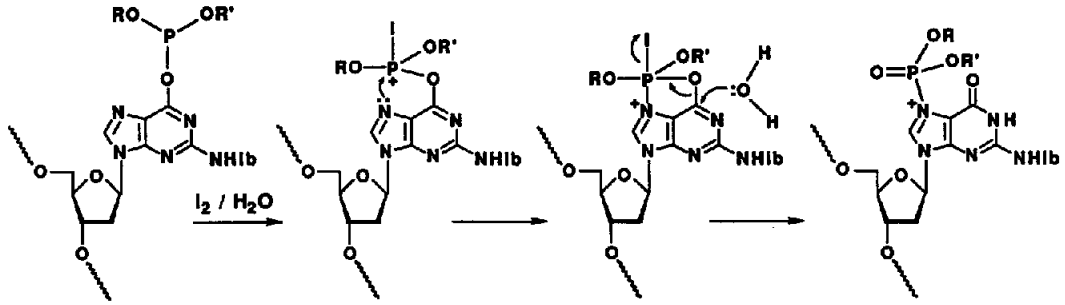
Additional evidence supporting the phosphitylation of guanine nucleosides at *O*-6 during the preparation of oligonucleotides by the phosphoramidite approach was provided by Nielsen *et al.*[179] They reported that the reaction of the guanosine derivative **122** with methoxy-(*N,N,N',N'*-tetraisopropylamino)phosphine and *N,N*-diisopropylammonium tetrazolide led to the formation of the adduct **123** after oxidation with elemental sulfur. The compound was isolated and characterized.



122 **123**

The practical implication stemming from the observations reported by Pon *et al.*[176,177] is that d[$(Gp)_{23}$G] cannot apparently be isolated without protecting the guanine residues at *O*-6. Pon *et al.*[177] speculated that an adduct such as **125** could induce depurination upon oxidation with aqueous iodine. The generation of apurinic sites would be accompanied by chain cleavage during the deprotection conditions and thereby prohibit the facile isolation of d[$(Gp)_{23}$G].

Pon *et al.*[180] pointed out, however, that the adduct **121** was readily cleaved by water or acetate ion to regenerate the deoxyguanosine derivative **120**. A satisfactory source of acetate ions was provided by the solution of acetic anhydride-2,6-lutidine-DMAP normally used as the "capping" reagent during the solid-phase synthesis of oligonucleotides. Thus, performing the capping step before the oxidation reaction allowed the solid-phase preparation of d[$(Gp)_{23}$G] in yields similar to those obtained when the guanine residues were protected at *O*-6 with a ß-cyanoethyl group.[180]

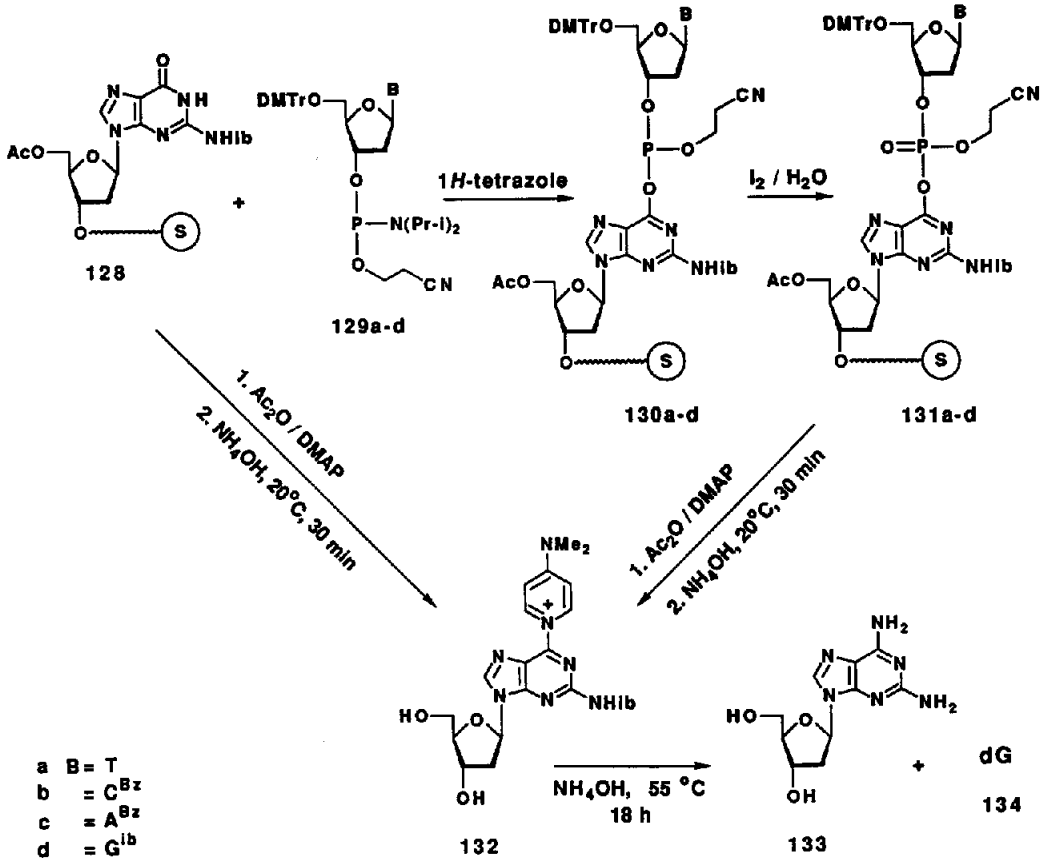


124 **125** **126** **127**

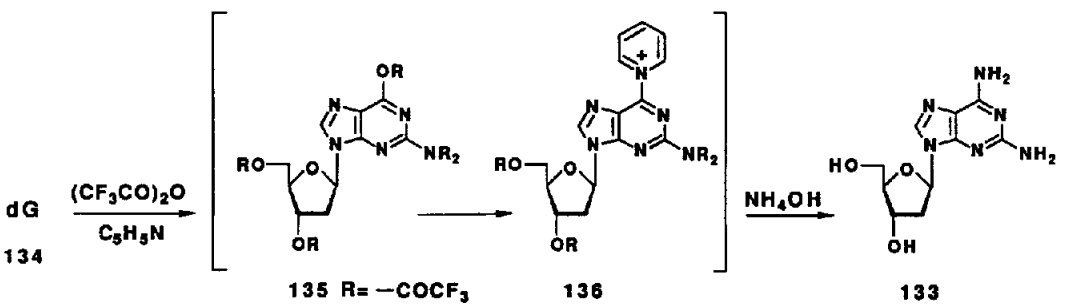
Yeung *et al.*[181] described the modification of guanine nucleobases during the synthesis of oligonucleotides when using "methyl deoxyribonucleoside phosphoramidite" synthons. It was found that modified guanine residues were more susceptible to potassium permanganate oxidation than thymine residues, thereby, allowing their detection by chemical DNA sequencing techniques. Additionally, double-stranded DNA segments prepared from methyl deoxyribonucleoside phosphoramidite monomers had "bulky" base modifications and became substrates for the *Uvr*ABC endonuclease of *E. coli*, an excision repair enzyme. When analyzed on a DNA sequencing gel, the incision bands resulting from the endonucleolytic action of *Uvr*ABC were mapped to guanine residues. The extent of guanine modifications in a specific sequence (54-mer) was about 0.5-3% per guanine residue. One might question the validity of this estimate since random DNA sequences did not show any "bulky" modifications detectable by the *Uvr*ABC endonuclease.[181] Oligonucleotides prepared with "cyanoethyl deoxyribonucleoside phosphoramidite" synthons did not generate guanine residues sensitive to potassium permanganate and were unaffected by the *Uvr*ABC endonuclease.

In a systematic study aimed at evaluating nucleobase modifications during the solid-phase synthesis of oligodeoxyribonucleotides by the phosphoramidite approach, Eadie and Davidson[182] reported that guanine residues produced a fluorescent side-product with a relative abundance of less than 0.1% of the parent nucleoside. Consistent with the data reported by Pon *et al.*,[180] a capping step consisting of acetic anhydride and DMAP performed before the aqueous iodine oxidation, reduced the amount of the fluorescent side-product by at least 30% relative to the converse experiment in which the capping step was performed after the oxidation reaction. The omission of DMAP in the latter capping reaction inhibited the formation of the fluorescent side-product, whereas the omission of acetic anhydride increased (4-fold) the formation of the fluorescent side-products. These data indicated that DMAP caused the formation of a fluorescent guanine adduct at *C*-6 presumably by displacement of the phosphate triester **131a-d** resulting from the oxidation of the O^6-phosphite triester **130a-d**. It is well documented that most phosphitylation of nucleoside bases occurred either at the *O*-6 of the guanine ring[176,177,179,180,182,183] or at *O*-4 of the thymine residues.[184] Moreover, the formation of **132** is similar to the generation of the fluorescent pyridinium adduct at *C*-6 of guanine and hypoxanthine residues reported by others.[165a-b,167,168,171,175]

The reaction of the deoxyguanosine derivative **128** with acetic anhydride and DMAP also generated after mild treatment with ammonium hydroxide the dimethylaminopyridinium salt **132** and,



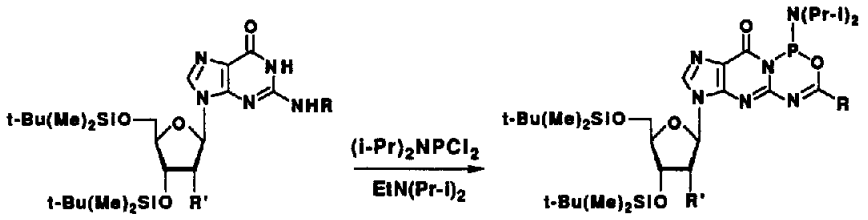
therefore, suggested that an acetyl adduct at the *O*-6 position of guanine residues could also be displaced by DMAP.[182] This observation is consistent with the data reported by Fathi *et al.*[185] describing the reaction of deoxyguanosine (**134**) with trifluoroacetic anhydride in pyridine. It has been postulated that the trifluoroacetylation of deoxyguanosine at *O*-6 occurred and led to the formation of the fluorescent *C*-6 pyridyl derivative **136** which upon reaction with concentrated ammonium hydroxide afforded **133**.



The substitution of DMAP for *N*-methylimidazole in the capping step of the solid-phase synthetic cycle prevented the generation of fluorescent oligonucleotides. Indeed, the enzymatic digestion of these

oligomers revealed the absence of a fluorescent deoxyguanosine derivative and a 15-fold reduction in the formation of the 2,6-diaminopurine nucleoside **133**.[182] It has been concluded that this improved capping step enhanced the chemical authenticity and biological fidelity of the synthetic oligomers.[186]

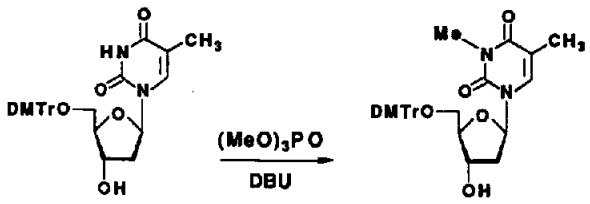
Certain guanine nucleosides acylated at *N*-2 (**137-140**) were reported by Damha and Ogilvie to rapidly react with dichloro-(*N,N*-diisopropylamino)phosphine and *N,N*-diisopropylethylamine to produce the tricyclic guanosine derivatives **141-144** in 60-96% yields.[187] Specifically, **141** was isolated by silica gel chromatography and fully characterized. The quantitative conversion of **141** into **137** was observed under mild acidic conditions.



137 R= Bz; R'= OSi(Me)$_2$Bu-t
138 = Bz; = H
139 = Ib; = H
140 = Ac; = H

141 R= Ph; R'= OSi(Me)$_2$Bu-t
142 = Ph; = H
143 = i-Pr; = H
144 = Me; = H

In addition to being susceptible to sulfonylation, phosphorylation and phosphitylation, thymine and uracil nucleosides are also sensitive to alkylation under basic conditions as a consequence of the acidity of their N-H function.[188a] For instance, Gao *et al.*[188b] described the efficient alkylation of 5'-*O*-di-*p*-methoxytrityl thymidine (**20**) at *N*-3 by a 10% solution of trimethylphosphate and DBU in pyridine. This experiment would argue against the use of the methyl group as a phosphate protecting group[189] during solid-phase oligonucleotide synthesis to prevent the potential alkylation of thymine nucleobases by internucleotidic methyl phosphotriesters. Urdea *et al.*[190] reported that large oligodeoxyribonucleotides (50-120-mers) prepared with methyl deoxyribonucleoside phosphoramidites had as much as 30% of the thymine bases converted into N^3-methylthymine. The latter findings were contested by McBride *et al.*[191] who reported the synthesis of four oligomers (18, 34, 72 and 150-mers) using methyl deoxyribonucleoside phosphoramidites. These oligomers were then treated with a thiophenoxide solution and fully deprotected according to a standard protocol. Following purification, the synthetic DNA segments were digested with snake venom phosphodiesterase and alkaline phosphatase to nucleosidic components. Only ca. 0.2% of N^3-methylthymidine relative to thymidine were observed from HPLC analysis of the hydrolysates pertaining to the 72 and 150-mers. Interestingly, the omission of the thiophenoxide treatment during the deprotection protocol yielded 10% of N^3-methylthymidine from the synthesis of d(GCGCGCTT).[191]



20 **145**

DBU= 1,8-diazabicyclo[5.4.0]undec-7-ene

Andrus and Beaucage[192] have also investigated the potential methylation of the thymine residues during the solid-phase synthesis of a polythymidylic acid methyl ester (65-mer) using methyl deoxyribonucleoside phosphoramidite monomers. The crude oligomer was treated with an equimolar solution of 2-mercaptobenzothiazole and *N,N*-diisopropylethylamine in 1-methyl-2-pyrrolidinone for 2 h at 55 °C. After being released from the solid support, the crude 65-mer was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase. HPLC analysis of the digest revealed the presence of negligible amounts of N^3-methylthymidine (0.2% relative to thymidine). These experiments indicated that methylation of the thymine residues does occur when the methyl group is used as a phosphate protecting group. The extent of thymine methylation was, nevertheless, negligible when the deprotection of the internucleotidic methyl phosphotriesters was effected by selected thiolates.

Depending upon the precautionary measures taken to minimize side-reactions during the synthesis and the subsequent deprotection of oligonucleotides, thymine or uracil and guanine residues may become irreversibly modified. To ensure the genetic integrity of synthetic oligonucleotides, several research groups have developed during the last decade protecting groups for the *N*-3/*O*-4 and *O*-6 function of thymine or uracil and guanine nucleobases respectively. These protecting groups are tabulated below:

Table 1. Protecting Groups for the Imide and Lactam Function of Thymine/Uracil and Guanine Nucleosides.

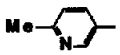
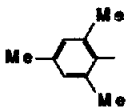
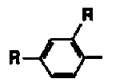
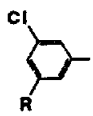
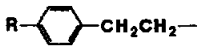
Protecting Group	Nucleobase	Deblocking Conditions	References
	Guanine (O-6) Uracil (O-4)	*syn*-4-nitrobenzaldoxime tetramethylguanidine	232-234,238
	Guanine (O-6) Uracil (O-4)	*syn*-4-nitrobenzaldoxime tetramethylguanidine	150,235-238, 298a
	Uracil (O-4)	*syn*-4-nitrobenzaldoxime tetramethylguanidine	234,238,326, 327
 R= H or Me	Thymine (O-4) Uracil (O-4)	*syn*-2-nitrobenzaldoxime tetramethylguanidine	112,150,151, 236,237,239
 R= H or Cl	Guanine (O-6)	*syn*-2-nitrobenzaldoxime tetramethylguanidine	111,150,151, 237,239,255b
 R= NO_2 or CN	Guanine (O-6) Thymine/Uracil (O-4) Xanthine (O-2, O-6 or N-1, O-2) Hypoxanthine (O-6, N-1)	pyridine-2-aldoxime tetramethylguanidine or 0.5 M DBU/pyridine	144,145,172b, 178,240-249, 250a,250d,253

Table 1. CONT'D

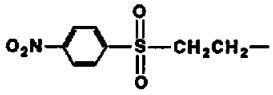
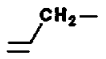
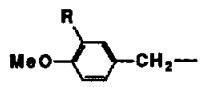
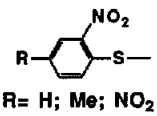
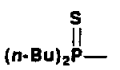
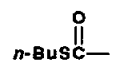
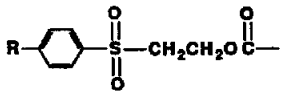
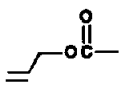
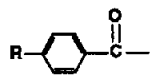
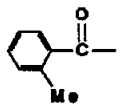
Protecting Group	Nucleobase	Deblocking Conditions	References
	Guanine (N-1 or O-6) Thymine/Uracil (N-3)	concentrated NH_4OH, Δ	251-254
RCH_2CH_2— R= Me_3Si; PhS; CN 4-NO_2PhS	Guanine (O-6)	R= Me_3Si: *n*-Bu_4NF/THF or $ZnBr_2/MeNO_2$ R= PhS; 4-NO_2PhS: $NaIO_4$/conc. NH_4OH/Δ R= CN: DBU or NH_4OH	172b,253,255b
	Guanine (O-6)	$Pd[P(C_6H_5)_3]_4/P(C_6H_5)_3$ *n*-$BuNH_3^+HCO_2^-$	330
$PhCH_2$—	Guanine (O-6)	10% Pd/C/H_2	256,257,286
	Guanine (O-6) Uracil (N-3) 5-Fluorouracil (N-3)	R= OMe: DDQ/MeCN/H_2O R= H: $AlCl_3$/Anisole or $(NH_4)_2Ce(NO_3)_6$/MeCN/H_2O	258-260,328
$PhCH_2OCH_2$—	Thymine/Uracil (N-3)	10% Pd/C/H_2	329
$MeOCH_2CH_2OCH_2$—	Uracil (N-3)	concentrated NH_4OH, Δ or $(Ph)_3C^+BF_4^-$/MeCN/H_2O	259,261-263
$(Ph)_3CS$—	Uracil (N-3)	0.1 M I_2/THF/Collidine/H_2O	264,270
	Uracil (N-3)	Pyridine/H_2O or 0.2 M *n*-Bu_4NF/THF	265,270
	Guanine (O-6)	60% Formic acid	166b,266
	Guanine (N-2, O-6) Uracil (N-3)	NH_4OH/MeOH	267-269

Table 1. CONT'D

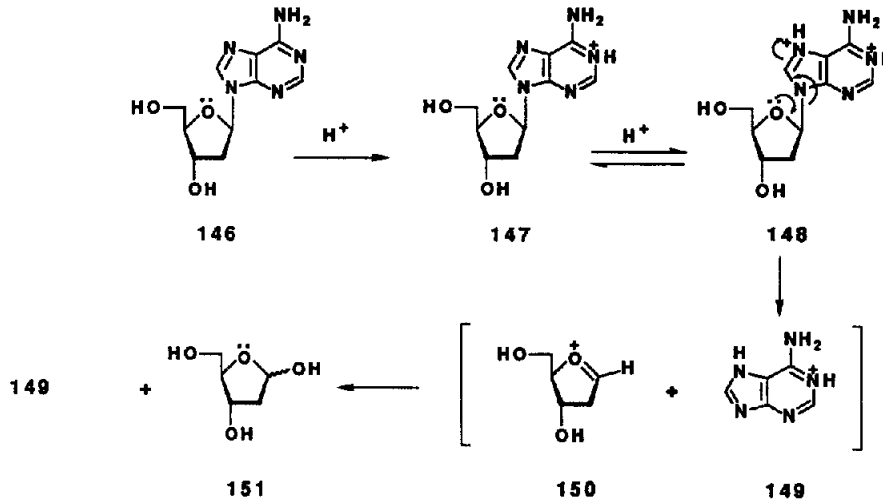
Protecting Group	Nucleobase	Deblocking Conditions	References
 R= H; Me	Uracil (N-3)	DBU/Morpholine/C_5H_5N	301
	Uracil (N-3, O-4)	$Pd[P(C_6H_5)_3]_4/P(C_6H_5)_3$ *n*-$BuNH_2$/THF	270-272
	Uracil (N-3, O-4)	Zn/acetyl acetone	270,273-276
	Guanine (O-6)	concentrated NH_4OH	122,123,215, 277-281a,282,283, 286,287,325b,325e
 R= H; Me; OMe; Cl	Uracil (N-3, O-4)	concentrated NH_4OH	122,123,139,215, 238,270,273,275, 278-281,283-289
	Uracil (N-3)	concentrated NH_4OH	139,270,289a

3.2. Exocyclic amino function of cytosine, adenine and guanine nucleosides.

The 5'-*O*-DMTr group is currently the preferred protecting group for the 5'-OH function of nucleosides during solid-phase oligonucleotide synthesis. The introduction of this protecting group, however, required the prior protection of the exocyclic amino function of cytosine, adenine and guanine nucleosides to prevent these from being tritylated as well.[193]

Selecting a protecting group for the exocyclic amino function of deoxyadenosine is problematic because the chemical stability of the nucleoside is considerably affected by the protection. Purine deoxyribonucleosides, nucleotides and oligonucleotides are sensitive to moderately acidic conditions which promote the cleavage of the glycosidic linkages.[194] Typically, guanosine is 520 times more resistant to depurination than deoxyguanosine at 100 °C and deoxyadenosine depurinates 2.2 times faster than deoxyguanosine at 30 °C.[194] These data are in agreement with the findings of Tanaka and Letsinger[97a] indicating that N^6-benzoyldeoxyadenosine has a greater tendency to depurinate than that of N^2-isobutyryldeoxyguanosine. The mechanism of this depurination, at least in the case of deoxyadenosine (**146**), involved the protonation of *N*-1 (**147**) and *N*-7 (**148**).[195] The departure of the protonated purine **149** and the concomitant formation of the oxonium-carbonium ion **150** were rate-limiting.[194,196-198] The acidic depurination of deoxyadenosine occurred only at very low pH because of the low *pKa* of protonated *N*-7 (*pKa* = −1.48).[199]

Should the adenine residue be acylated at *N*-6, a suppression of the amidine resonance in the pyrimidine ring would result and decrease the electronic density at *N*-1 while increasing it at *N*-7.[200] According to ^{15}N NMR measurements the site of first protonation predominantly become *N*-7,[201,202] thereby, significantly enhancing depurination rates. The extent to which depurination occurs may become significant during the preparation of larger oligodeoxyribonucleotides. Efcavitch and Heiner[203] reported that the cleavage of apurinic sites by concentrated ammonium hydroxide at 55 °C was detected by high resolution polyacrylamide gel electrophoresis. The direct implications of this study were that internal depurination did not interfere with chain elongation, at least *via* the phosphoramidite approach, but reduced the isolated yield of the desired oligomer.



The search for an acid capable of cleaving the 5'-*O*-DMTr group within a short period of time under the mildest possible conditions and the design of specific protecting groups for the amino function of deoxyadenosine that can further reduce depurination under acidic conditions, have attracted considerable attention. The various amidine protecting groups[204,205] listed in Table 2 were developed for this purpose. Moreover, these protecting groups were chemoselectively targeted to the exocyclic amino function of purine and pyrimidine nucleosides. For example, the reaction of deoxyadenosine (**146**) with *N,N*-dimethylacetamide dimethylacetal (**152**) afforded the amidine **153** in 70% yield.[206] This amidine-protected deoxyadenosine was ca. 20 times more stable than the corresponding N^6-benzoyl deoxyadenosine toward depurination (2% dichloroacetic acid in dichloromethane).[183,207] ^{15}N NMR spectroscopy showed that protonation predominantly affected the *N*-1 and *N*-6 resonances of the amidine-protected deoxyadenosine instead of the *N*-1 and *N*-7 resonances observed with N^6-benzoyl deoxyadenosine.[201] Amidine-protected deoxyribonucleoside phosphoramidites have demonstrated their usefulness in the solid-phase synthesis of deoxyribonucleotides.[40k,59,183,206,208,209]

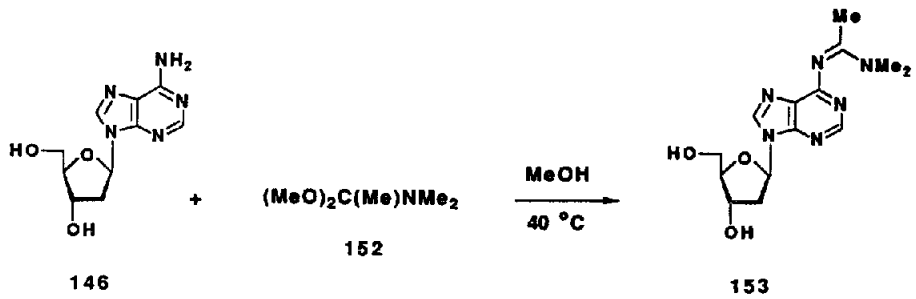
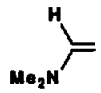
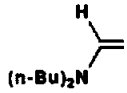
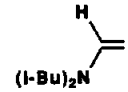
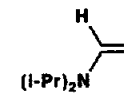
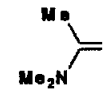
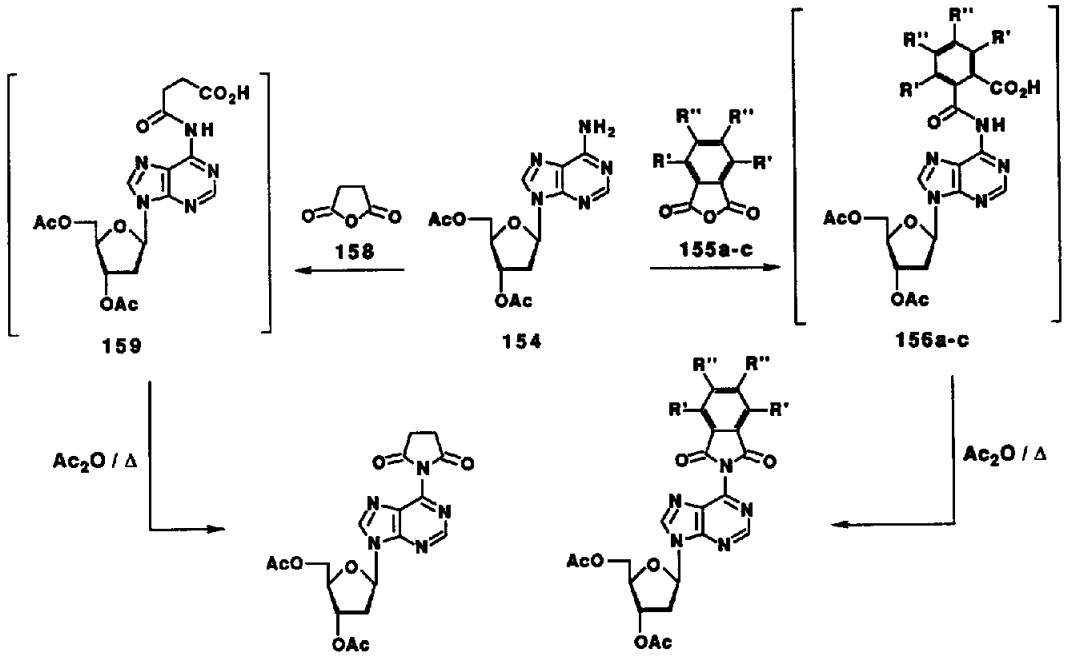


Table 2. Amidine Protecting Groups for the Exocyclic Amino Function of the Aglycones

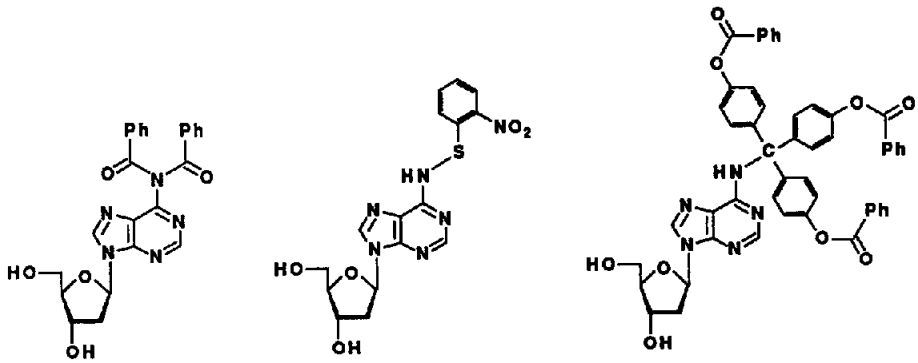
Protecting Group	Nucleobase	Deblocking Conditions	References
	Guanine (N-2) Adenine (N-6)	concentrated NH_4OH/Δ	40k,59,183, 206-209,248, 255b,290-293
	Guanine (N-2) Adenine (N-6)	concentrated NH_4OH/Δ or 0.5 M $NH_2NH_2.H_2O/C_5H_5N/AcOH$	62b,183, 205-207,294
	Adenine (N-6)	conc. NH_4OH/10% NH_4OAc/Δ or 0.5 M $NH_2NH_2.H_2O/C_5H_5N/AcOH$	205
	Adenine (N-6)	conc. NH_4OH/10% NH_4OAc/Δ or 0.5 M $NH_2NH_2.H_2O/C_5H_5N/AcOH$	205
	Guanine (N-2) Adenine (N-6)	concentrated NH_4OH/Δ	59,183, 206-208,294
	Cytosine (N-4)	concentrated NH_4OH/Δ	183,206,207

Other protecting groups have also been designed for the *N*-6 amino function of deoxyadenosine to inhibit its depurination under acidic conditions. Kume *et al.*[40d,93,210] introduced cyclic diacyl groups to fulfill this objective. The reaction of 3',5'-di-*O*-acetyl deoxyadenosine (**154**) with the cyclic anhydride **155a-c** or **158** afforded the phthaloyl derivatives **157a-c** or the succinoyl derivative **160**.[40d] The deoxyadenosine derivatives **157a-c** and **160** were ca. 4 to 6 times and ca. 8 times, respectively, more resistant toward depurination than the corresponding N^6-benzoyl deoxyadenosine derivative in 80% acetic acid at 70 °C. These protecting groups were, however, not very stable under weakly basic conditions such as pyridine-water (1:1) and were readily cleaved by concentrated ammonium hydroxide-pyridine (3:2) at ambient temperature.[40d] Unlike amidine protecting groups, the introduction of the phthaloyl and succinoyl groups at the *N*-6 amino function of deoxyadenosine required the transient protection of the 3'- and 5'-OH functions with trimethylsilyl chloride.[211] Along similar lines, Takaku *et al.*[96] protected the exocyclic amino group of deoxyadenosine as the N^6,N^6-dibenzoyl derivative **161** which, apparently, was more resistant toward depurination than N^6-benzoyl deoxyadenosine. In contrast to the N^6-phthaloyl deoxyadenosine derivatives, **161** was more stable in pyridine-water (1:1).[96]

Enhanced resistance to depurination was also achieved when the 6-amino group of deoxyadenosine was protected with the *o*-nitrophenylsulfenyl group (Nps) as in **162**. In 80% aqueous acetic acid at 30 °C, **162** remained intact for 24 h. Under identical conditions, the depurination of N^6-benzoyl deoxyadenosine and N^6-phthaloyl deoxyadenosine occurred with a half-life of 30 and 120 min respectively.[212] The Nps group also protected the exocyclic amino function of cytosine and guanine nucleosides. Although this protecting group appeared satisfactory during the synthesis of DNA and RNA *via* the phosphotriester approach,[212] no phosphoramidite monomer bearing the Nps group, to our knowledge, has been reported.



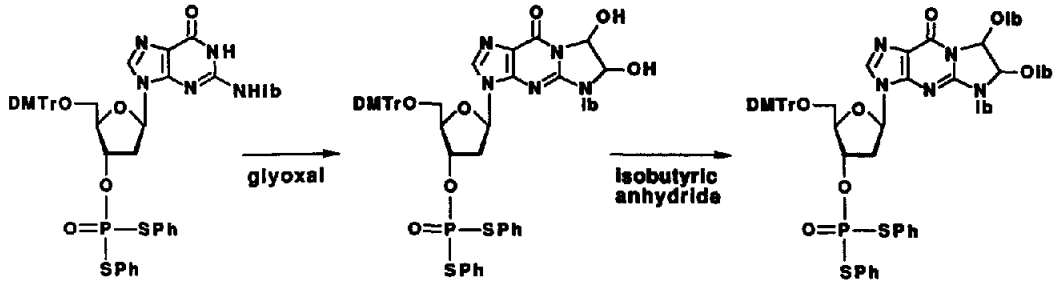
Of interest, the N^1-oxide of deoxyadenosine derivatives[213,214] were claimed to improve the stability of the glycosidic linkage under acidic conditions.[214] When needed, the deoxygenation of deoxyadenosine N^1-oxide derivatives was effected by trimethyl phosphite and cerium chloride in methanol.[214] However, this type of protection has not been thoroughly tested in the synthesis of oligonucleotides.



Although trityl derivatives such as Tr, MMTr and DMTr served as protecting groups for the 5'-OH of nucleosides, these trityl groups have also been applied toward the protection of the exocyclic amino function of nucleosides.[39,84,215-219] For instance, it has been shown that the protection of deoxyadenosine at *N*-6 with the 4,4',4''-tris-(benzoyloxy)trityl group (TBTr) (**163**) provided resistance to depurination by ca. 4 times (2% dichloroacetic acid in dichloromethane) with respect to N^6-benzoyl

deoxyadenosine.[220] Additionally, N^6-TBTr deoxyadenosine derivatives were easily converted to methyl-(*N*-morpholino)phosphoramidite monomers and used in solid-phase DNA synthesis.[220a] The TBTr group was also used for the protection of the exocyclic amino function of deoxycytidine and deoxyguanosine. It has been, nonetheless, observed that monomeric N^2-TBTr-5'-*O*-DMTr deoxyguanosine phosphoramidites did not produce satisfactory coupling yields during solid-phase DNA synthesis presumably because of the steric hindrance created by the N^2-TBTr group. The deblocking of the TBTr group was achieved by 2 M sodium hydroxide within 10 min or, alternatively, by treatment with N^1,N^1,N^3,N^3-tetramethylguanidinium 2-pyridinecarboxaldoximate during 3 h.[220b] The application of the TBTr and 4,4',4"-tris-(anisoyl)trityl groups to the protection of the amino function of adenosine in oligoribonucleotide synthesis was also described.[221]

An original way of simultaneously protecting the lactam and N^2-amino functions of deoxyguanosine was reported by Sekine *et al.*[222] and Matsuzaki *et al.*[223] The phosphorodithioate **164** was readily converted into the 1:1 adduct **165** upon reaction with glyoxal in pyridine which without isolation was treated with isobutyric anhydride affording **166** in 97% overall yield.[222] This protection method prevented the modification of the guanine ring at *O*-6 during oligonucleotide synthesis. The natural guanine aglycone was easily recovered upon treatment with concentrated ammonium hydroxide.



164 **165** **166**

Several acyl protecting groups were developed during the last decade for the protection of the exocyclic amino function of cytosine, adenine and guanine nucleosides. These are listed in Table 3. In most cases, these protecting groups were introduced by peracylation of the nucleoside followed by selective saponification of the 2'- and/or 3'- and 5'- ester functions as described by Schaller *et al.*[84] An alternative approach invoked the transient protection of the hydroxy groups of the sugar moiety by trimethylsilylation[211] followed by acylation of the aglycone. A few acyl groups such as the α-phenyl cinnamoyl group[224] and the napthaloyl group[225] were chemoselective for the exocyclic amino function of deoxycytidine, deoxyadenosine and deoxyguanosine. Importantly, deoxyadenosine protected at *N*-6 with either of these aromatic protecting groups in addition to the 3-methoxy-4-phenoxybenzoyl group[226] and the fluorenylmethoxycarbonyl group,[227] exhibited better resistance to depurination under acidic conditions, relative to N^6-benzoyl deoxyadenosine.

The phenoxyacetyl group represents a convenient protecting group for guanine, adenine and cytosine residues[228,229] as its half-time of deprotection with concentrated ammonium hydroxide at 20 °C is 8 min, 7 min and 2 min respectively.[229a] Under identical conditions, the half-time of deprotection of the corresponding *N*-benzoylated nucleosides were, in the same order, 10 h, 11 h and 3 h.[229a] The rapid deprotection of the nucleobases under basic conditions is particularly important in oligoribonucleotide synthesis to minimize chain scission should the 2'-*O*-protecting group be partially cleaved in the process. The use of phenoxyacetylated nucleobases should also facilitate the preparation of oligonucleotide analogues carrying modified nucleosides sensitive to alkaline conditions. Moreover, the rate of depurination of N^6-phenoxyacetyl deoxyadenosine in 80% acetic acid was 20% lower than that observed with N^6-benzoyl deoxyadenosine under the same conditions.[230] However, the partial replacement of the N^2-phenoxyacetyl group of the guanine residues by an acetyl group was reported to occur during the

Table 3. Protecting Groups for the Exocyclic Amino Function of the Nucleobases.

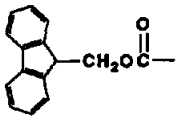
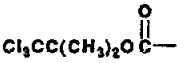
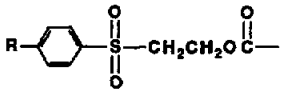
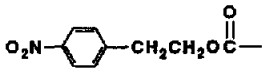
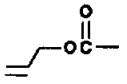
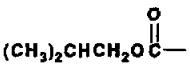
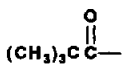
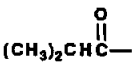
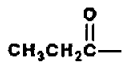
Protecting Group	Nucleobase	Deblocking Conditions	References
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	conc. NH_4OH-C_5H_5N (1:1) or Et_3N-C_5H_5N (1:5) or 1 M DBU/MeCN	112,139,227, 255,295-297
	Guanine (N-1, N-2) Adenine (N-6) Cytosine (N-4)	Lithium Co(I) Phthalocyanine	276,298-300
 R= H, Cl, NO_2	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	Tetramethylguanidine:morpholine: C_5H_5N or DBU:morpholine:C_5H_5N or NH_4OH:morpholine:C_5H_5N	301
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	DBN or DBU/C_5H_5N	144,145,242,247, 250a-e,302,303
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	10% Pd/C/H_2	256,257
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	$Pd[P(C_6H_5)_3]_4/HCO_2H/Et_2NH$	270-272, 304-307,330
	Cytosine (N-4)	conc. NH_4OH-C_5H_5N (3:1)	308
	Cytosine (N-4)	0.2 M NaOH-MeOH (1:1)	314
	Guanine (N-2) Cytosine (N-4)	concentrated NH_4OH, Δ	40k,82,156,209, 228b,230,309-311, 314
	Guanine (N-2)	conc. NH_4OH-C_5H_5N (2:1)	122,123,215,277, 280,281a,282,283, 287,325b,325e
	Cytosine (N-4)	NH_3-EtOH (1:1)	111,228, 312-314
	7-deaza-6-methyl guanine (N-2)	concentrated NH_4OH	318

Table 3. CONT'D

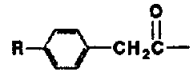
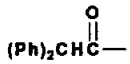
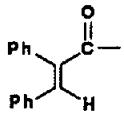
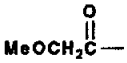
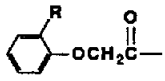
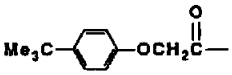
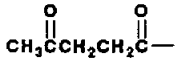
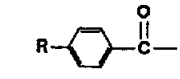
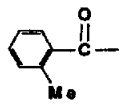
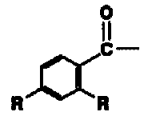
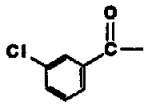
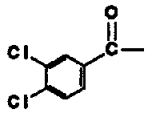
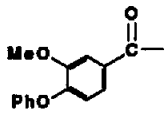
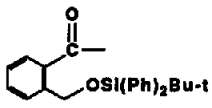
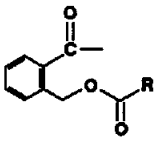
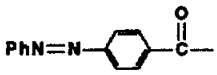
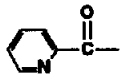
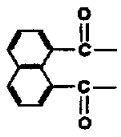
Protecting Group	Nucleobase	Deblocking Conditions	References
 R= H, CMe_3	Guanine (N-2)	5 M NH_3-MeOH	118,120a,150, 151,236,237,239, 314,323,324
	Guanine (N-2)	concentrated NH_4OH, Δ	325a-d
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	concentrated NH_4OH, Δ	224
	Guanine (N-2)	concentrated NH_4OH	230,309,310
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	concentrated NH_4OH	316
 R= H, Cl	Guanine (N-2) Adenine (N-6)	concentrated NH_4OH	228,230,293, 309-313,317
	Guanine (N-2)	0.2 M NaOH-MeOH (1:1)	314
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	0.5 M $NH_2NH_2.H_2O$ C_5H_5N:AcOH (4:1)	315
 R= H, OMe, Cl, NO_2 NMe_2, CMe_3	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	concentrated NH_4OH	82,84,118,119, 120-123,126,150, 151,155,156,223, 239,281-283,287, 289b-c,314,319,325d
	Cytosine (N-4)	0.2 M NaOH-MeOH (1:1) or conc. NH_4OH-Dioxan (4:1), Δ	314,325b,325e

Table 3. CONT'D

Protecting Group	Nucleobase	Deblocking Conditions	References
 R= Me, OMe	Cytosine (N-4)	0.2 M NaOH-MeOH (1:1)	314
	Adenine (N-6)	concentrated NH_4OH	120,126
	Guanine (N-2)	0.2 M NaOH-MeOH (1:1)	314
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	concentrated NH_4OH, Δ	226,320
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	n-$Bu_4NF/C_5H_5N/H_2O$	321,322
 R= Ac; Ph	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	0.05 M K_2CO_3/MeOH or conc. NH_4OH, R.T	231
	Cytosine (N-4)	0.2 M NaOH-MeOH (1:1)	314
	Cytosine (N-4)	0.2 M NaOH-MeOH (1:1)	314
	Guanine (N-2) Adenine (N-6) Cytosine (N-4)	concentrated NH_4OH, Δ	225

capping step of the solid-phase synthetic cycle.[228a] This problem was circumvented by substituting acetic anhydride for phenoxyacetic anhydride during the capping reaction.[228a]

The 2-(acetoxymethyl)benzoyl group[231] has also been proposed as an alternative protecting group for the amino function of nucleosides to facilitate the synthesis of base-labile modified oligonucleotides. Although the protecting group was readily cleaved (ca. 1-2 h) from nucleosides by concentrated ammonium hydroxide or 0.05 M potassium carbonate in methanol at ambient temperature, it took ca. 2-6 h to deprotect small oligonucleotides (dimers and trimers) under the same conditions.[231]

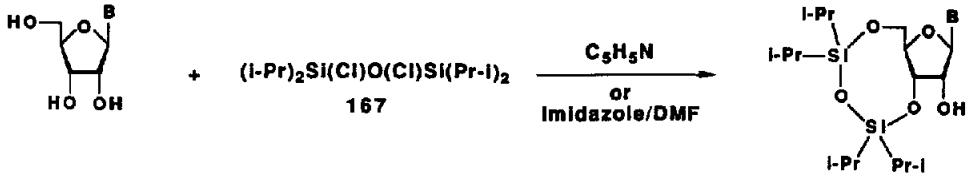
From the plethoric number of nucleobase protecting groups reviewed in this section, the *N,N*-dimethylformamidine group represents the most promising protecting group for purine nucleosides. In addition to being chemoselectively introduced at the exocyclic amino function of deoxyadenosine and deoxyguanosine, the protecting group conferred resistance to depurination and was readily cleaved by concentrated ammonium hydroxide (1 h at 50 °C) during the final deprotection of synthetic oligonucleotides.[40k,209] Because of these attributes, the *N,N*-dimethylformamidine group may become the protecting group of choice for purine nucleobases in the synthesis of oligonucleotides.

The next section is dedicated to the recent developments pertaining to 2'-OH protecting groups, an important issue in the synthesis of oligoribonucleotides.

4. THE PROTECTION OF THE 2'-HYDROXY FUNCTION OF RIBONUCLEOSIDES

The complexity of the chemical synthesis of oligoribonucleotides relative to oligodeoxyribonucleotides emerged from the additional protecting group required for the 2'-hydroxy function of ribonucleosides. This protecting group must remain intact throughout the synthesis of the oligoribonucleotide and, yet, it must be removed during the final deblocking step under conditions that will not promote the cleavage and/or migration of the internucleotide linkages. Consequently, the choice of the 2'-OH protecting group will dictate the nature of the aglycone and 5'-OH protecting groups. Moreover, the steric bulk of the 2'-*O*-protecting group may impact on processes occurring at the 3'-position.

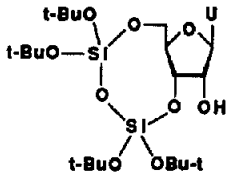
To facilitate the regioselective incorporation of a 2'-hydroxyl protecting group in the *cis*-2',3'-diol system of ribonucleosides, Markiewicz and Wiewiórowski[331] reported, more than a decade ago, the use of the 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane **167** as a means to simultaneously protect the 5'-OH and 3'-OH functions of ribonucleosides. The silylation of the primary hydroxy function of ribonucleosides occurred at least 1000 times faster than the secondary hydroxy functions[332] which, if sterically possible, was achieved by an intramolecular cyclization.[331,333] The formation of 7-membered disiloxane diether rings was preferred. However, 8-membered rings were formed when the formation of the 7-membered rings was either impossible or unfavored.[334,335]



B= purine or pyrimidine **168**

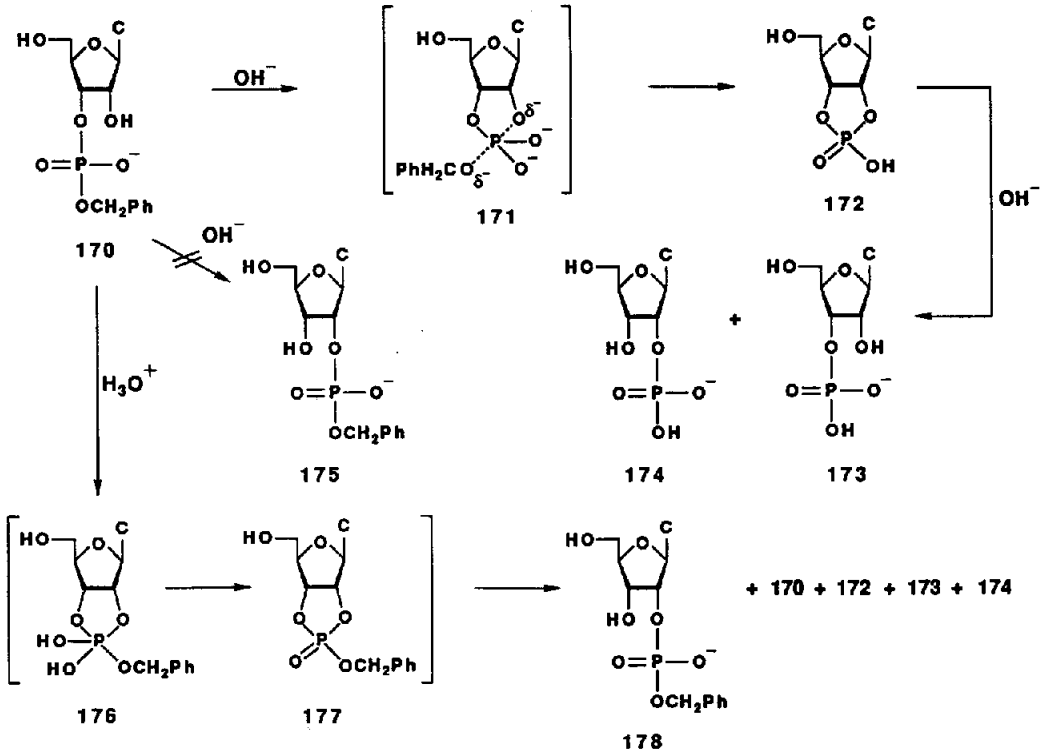
The 1,1,3,3-tetraisopropyldisiloxane-1,3-yl (TIPDS) group (as in **168**) was stable to water, 0.3 M *p*-toluenesulfonic acid in dioxan, 10% trifluoroacetic acid in chloroform, 5 M ammonium hydroxide in dioxan-water (4:1), isobutylamine-methanol (9:1) and tertiary amines in pyridine.[331] Conversely, the TIPDS group was quantitatively cleaved by 1 M tetra-*n*-butylammonium fluoride in tetrahydrofuran within 10 min. The TIPDS group was also removed by 0.2 M hydrochloric acid in dioxan-water (4:1) within 24 h.[331] Due to its facile introduction, stability and compatibility with many other protecting

groups, the TIPDS group has been generally used for the incorporation of most of the ribonucleoside 2'-hydroxyl protecting groups that will be mentioned in this section. In addition to the functionalization of ribonucleosides[336,337] and their analogues, the TIPDS group has also found wide applicability in carbohydrates and open chain polyhydroxy compounds.[332] Recently, Markiewicz *et al.*[338] described the tetra-*tert*-butoxydisiloxane-1,3-diyl (TBDS) group as a new type of bifunctional silyl protective group. This group (as in **169**) was more stable to hydrolytic conditions than the TIPDS group. Indeed, the TBDS group hydrolyzed ca. 25 times slower than the TIPDS group under alkaline conditions (0.2 M sodium hydroxide in dioxan) and while the TBDS group was stable to 0.2 M hydrochloric acid in dioxan the TIPDS group was completely cleaved from the 5'-end of **168** within 2.5 h.[332,333b,337,338] The enhanced stability of the TBDS group relative to the TIPDS group toward hydrolysis under acidic or basic conditions should provide greater specificity in the functionalization of nucleosides.



169

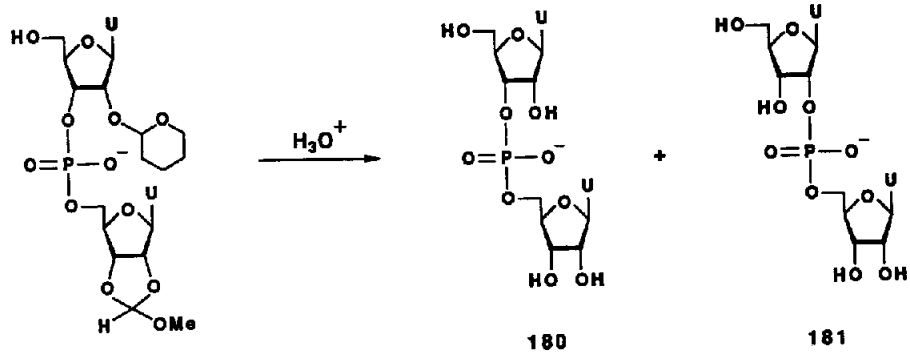
The question of whether the 2'-OH protecting group should be an acid-labile or a base-labile group has received considerable attention. Several decades ago, Brown *et al.*[339] reported that the partial hydrolysis of cytidine 3'-*O*-benzyl phosphate (**170**) by 0.5 N sodium hydroxide did not yield the ester **175**. Instead, the mononucleotides **173** and **174** were generated from the hydrolysis of the unstable cyclic phosphate **172**. Alternatively, the partial acid hydrolysis of **170** afforded the aralkylphosphate ester **178** along with **173** and **174**.



This model experiment indicated that under acidic conditions the conversion of a (3'→5')-internucleotidic linkage, regardless of it being a phosphodiester or a phosphotriester, into a (2'→5')-internucleotidic linkage can occur and represents a serious problem.[140] It would be difficult to separate oligoribonucleotides containing one or more (2'→5')-internucleotidic linkages from the natural oligoribonucleotides. Therefore, it is of crucial importance to avoid phosphoryl migration and/or chain cleavage during the deblocking of the 2'-hydroxy function.

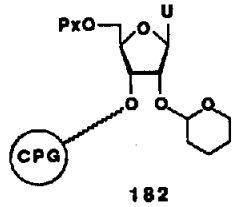
With the above considerations in mind, the acid-labile tetrahydropyranyl group (Thp) has been tested as a 2'-OH protecting group in oligoribonucleotide synthesis.[340,341] The conditions necessary for its complete removal were relatively mild (0.01 N hydrochloric acid, ca. 3-4 h at 20 °C) and did not promote significant phosphoryl migration. It would take 216 h under these conditions to generate 1% of uridilyl-(2'→5')-uridine (**181**) from the uridilyl-(3'→5')-uridine derivative **179**.

During the last decade, the application of the Thp group in the synthesis of oligoribonucleotides *via* the phosphotriester approach has been thoroughly investigated.[40e,111,215,218,219,274,287,325d,342-346]



179 **180** **181**

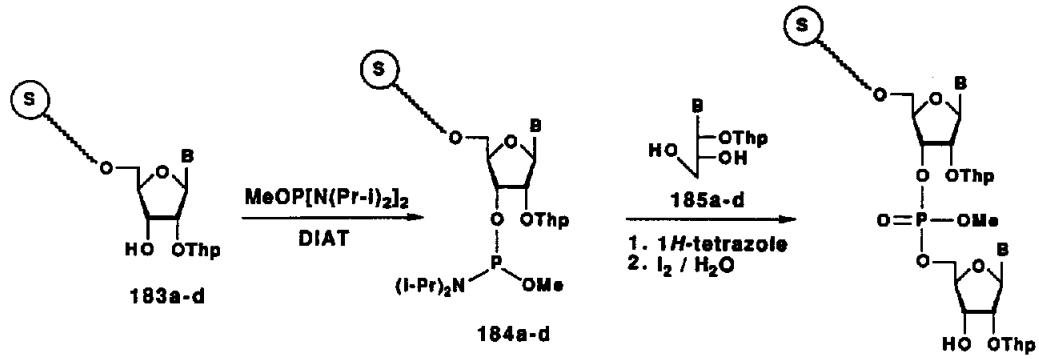
Recently, monomeric 2'-*O*-Thp ribonucleoside phosphoramidites have been applied to the solid-phase synthesis of oligoribonucleotides.[122-125,346-350] The 5'-OH function of the purine phosphoramidite derivatives was protected with a DMTr or Px group whereas the 5'-OH of the pyrimidine phosphoramidite derivatives was protected with a 9-(*p*-anisyl)xanthen-9-yl group in an attempt to reduce the cleavage of the 2'-*O*-Thp group during the stepwise removal of the 5'-*O*-protecting group.[122-125] It has been shown, however, that the treatment of the uridine derivative **182** with 3% dichloroacetic acid in 1,2-dichloroethane not only cleaved the 5'-*O*-Px group but also the 2'-*O*-Thp group with a half-life of ca. 560 s.[351] The application of both 5'-*O*-Px (or 5'-*O*-DMTr) and 2'-*O*-Thp protecting groups in oligoribonucleotide synthesis has, therefore, been restricted to the preparation of relatively short oligomers (less than 20 bases long) to minimize the cleavage and/or isomerization of internucleotidic linkages that may result from the partial loss of the 2'-*O*-Thp group during the deblocking of the 5'-*O*-Px group (or 5'-*O*-DMTr).[288,352] In a brief report, Seliger *et al.*[135] described the solid-phase synthesis of a pentadecauridylate using the 5'-*O*-tri-*p*-methoxytrityl group (TMTr) and the 2'-*O*-Thp group for the protection of the ribonucleoside phosphoramidite monomers. The detritylation



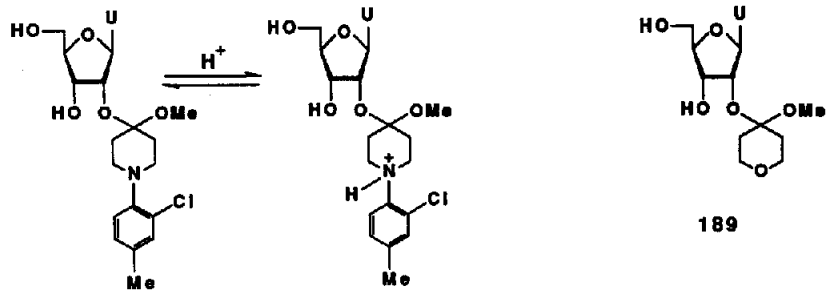
182

step was effected by 10% monochloroacetic acid in methylene chloride at room temperature for 5 min. No loss of the 2'-*O*-Thp group was detected under these conditions. Chain extension occurred with an average stepwise yield of 95%. This approach has not been extensively used in the solid-phase synthesis of oligoribonucleotides presumably because of the notorious instability of the 5'-*O*-TMTr group.[85]

To avoid acidic treatment during the solid-phase synthesis of oligoribonucleotides on polystyrene support Iwai *et al.*[40g,h] reported the preparation of an octaribonucleotide *via* the phosphotriester approach by extending the ribonucleotidic chain in a (3'→5')-direction instead of the usual (5'→3')-direction. Similarly, Caruthers *et al.*[348,353,354] described the attachment of 2'-*O*-Thp-*N*-protected ribonucleosides through their 5'-OH to either silica or CPG affording **183a-d**. The reaction of the 3'-OH function of these support-bound nucleosides with bis-(*N,N*-diisopropylamino)methoxyphosphine and *N,N*-diisopropylammonium tetrazolide yielded the corresponding phosphoramidites **184a-d**. The coupling of the 2'-*O*-Thp-*N*-protected ribonucleosides **185a-d** with **184a-d** in the presence of 1*H*-tetrazole led, after aqueous iodine oxidation, to the regioselective formation of the (3'→5')-dinucleoside phosphotriesters **186a-d**. A pentadecaribonucleotide was synthesized according to this approach and was shown to have exclusively (3'→5')-internucleotidic linkages by enzymic hydrolysis.[354]

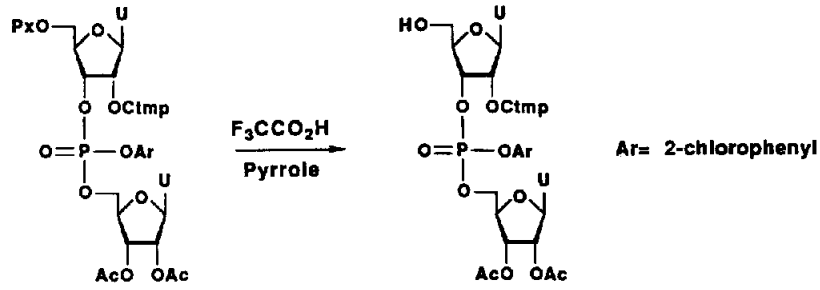


Several years ago, Reese *et al.*[355,356] introduced the 4-methoxytetrahydropyran-4-yl group (Mthp) as an alternative to the Thp group for the 2'-OH protection of ribonucleosides. The Mthp group was cleaved from oligoribonucleotides by 0.01 M hydrochloric acid (pH 2) at 20 °C without significant cleavage and migration of the internucleotide linkages.[149,357] Through the years the Mthp group has been extensively used in the synthesis of oligoribonucleotides.[40b,112,121b,150,151,255,333b,358-362] However, like the Thp group, the 2'-*O*-Mthp group was incompatible with the 5'-*O*-Px or 5'-*O*-DMTr group during solid-phase oligoribonucleotide synthesis.[152,351,363] The search for a 2'-*O*-protecting group that was totally stable under the acidic conditions required for the complete removal of the 5'-*O*-Px (or 5'-*O*-DMTr) group motivated Reese *et al.*[152,364] to develop the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp) group as a new protecting group for the 2'-hydroxy function of ribonucleosides. This protecting group was designed in such a way that the tertiary anilino function of the acetal **187** would be largely unprotonated at pH 2-2.5, thereby, allowing the hydrolysis of the acetal function to occur at a rate similar to that observed with the Mthp group. The protonation of the anilino function as in **188** would occur at lower pH and would retard the hydrolysis of the acetal function by virtue of the strong inductive effect generated by the protonated amine. Typically, the rate of hydrolysis of the 2'-*O*-Mthp uridine **189** was 140 times faster at pH 1.0 than at pH 3.0. In contrast, the rate of hydrolysis of 2'-*O*-Ctmp uridine (**187**) was only ca. 2.25 times faster at pH 1.0 than at pH 3.0 and yet the Ctmp and Mthp groups hydrolysed at a similar rate at pH 2.0 ($t_{1/2}$ = 41 and 21 min respectively).[364]

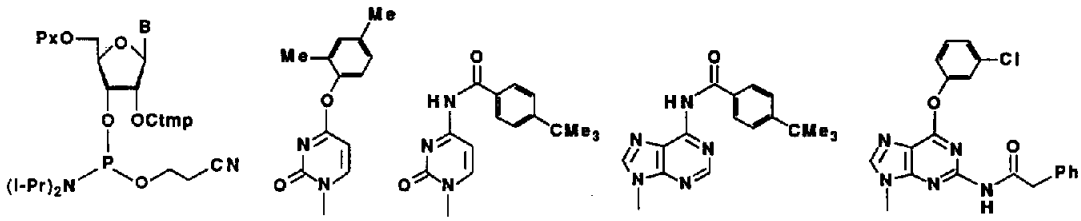


187 188 189

In an experiment aimed at evaluating the stability of the 2'-*O*-Ctmp group under the conditions required to completely remove a 5'-*O*-Px (or a 5'-*O*-DMTr) group, the dinucleoside phosphate triester **190** was treated with trifluoroacetic acid and pyrrole for 30 s at 20 °C. The partially protected dinucleoside phosphotriester **191** was isolated in 96% yield. It was estimated that less than 0.2% of the 2'-*O*-Ctmp group was cleaved during the complete acidolysis of the 5'-*O*-Px group.[364] The compatibility of the 5'-*O*-Px group and the 2'-*O*-Ctmp group was further tested during the solid-phase synthesis of a nonadecaribonucleotide corresponding to a sequence of yeast alanine tRNA. The oligonucleotide was prepared by activating the ribonucleoside phosphoramidites **192a-d**[239] with 5-(*p*-nitrophenyl)-1*H*-tetrazole.[365] A coupling time of 15 min led to an average stepwise yield of 93%. Recently, the *H*-phosphonate approach has also been used to evaluate the combination of the 5'-*O*-DMTr and 2'-*O*-Ctmp protecting groups during the solid-phase synthesis of an octadecaribonucleotide.[366,367]



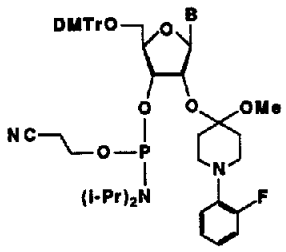
190 191

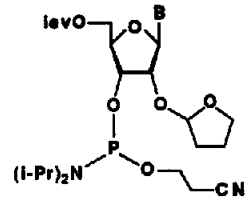


192 B= a-d a b c d

Although the Ctmp group has been satisfactory for the protection of the 2'-hydroxy function of ribonucleosides, Reese and Thompson[368] described the more accessible 1-(2-fluorophenyl)-4-

methoxypiperidin-4-yl (Fpmp) group as an alternative to the Ctmp group in the synthesis of oligoribonucleotides. It is to be noted that the acidic hydrolysis properties of the Fpmp group are virtually identical to those observed for the Ctmp group.[368] Recently, Beijer *et al.*[313] reported the preparation of the ribonucleoside phosphoramidites **193a-e** and the application of these monomers in the solid-phase synthesis of oligoribonucleotides (18-20 mers).



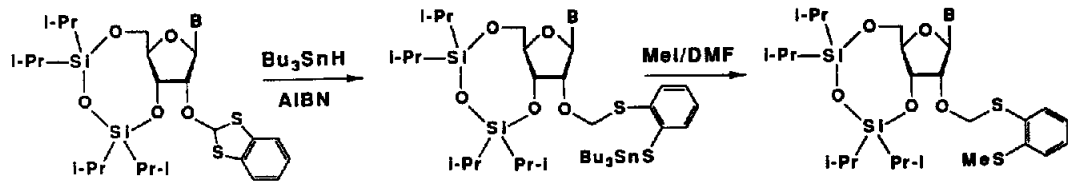


193a B= U
b = N^4-acetylcytosin-1-yl
c = N^6-phenoxyacetyladenin-9-yl
d = N^2-phenoxyacetylguanin-9-yl
e = I

194a B= U
b = C^{bz}
c = A^{Bz}
d = G^{Ib}

An alternate strategy toward the solid-phase synthesis of RNA oligomers was proposed by Iwai *et al.*[369] In this approach, *N*-acylated ribonucleosides were protected at the 5'-OH with a levulinyl group and at the 2'-OH with a tetrahydrofuranyl (Thf) group. These were converted into the ribonucleoside phosphoramidites **194a-d**[369b,370] which, upon activation by 1*H*-tetrazole, were coupled to 2'-*O*-Thf uridine covalently attached to a CPG support. The stepwise removal of the 5'-*O*-levulinyl group was performed by 0.5 M hydrazine hydrate for a period of 10 min. Under these conditions, the partial cleavage of the N^6-benzoyl group of adenine residues was observed. Despite this limitation, an oligoribonucleotide (21 bases long) was obtained in ca. 8% yield after purification.[369b]

Prior to these findings, the combination of 5'-*O*-levulinyl and 2'-*O*-Mthp groups had been exploited for the solid-phase synthesis of oligoribonucleotides *via* the phosphotriester approach.[40b] Furthermore, 5'-*O*-levulinyl-2'-*O*-DMTr ribonucleosides led Decout and Ogilvie[371] to the successful preparation of an hexaribonucleotide according to the phosphite coupling procedure in solution phase. The combination of the 5'-*O*-levulinyl or 5'-*O*-Fmoc group[136] with the acid-labile 2'-*O*-(1,3-benzodithiol-2-yl) group (BDT)[372] was also proposed for the synthesis of oligoribonucleotides but has not been thoroughly tested. Interestingly, the 2'-*O*-BDT group of the ribonucleoside **195** can be converted into a 2'-*O*-[2-(methylthio)phenyl]thiomethyl (MPTM) group as in **197** upon a reductive ring opening reaction followed by S-methylation.[281] No loss of the 2'-*O*-MPTM group was observed upon prolonged treatment (30 min) with 1% trifluoroacetic acid in dichloromethane. Consequently, the 5'-*O*-DMTr (or Px) and the 2'-*O*-MPTM group would be compatible toward the solid-phase synthesis of oligoribonucleotides.



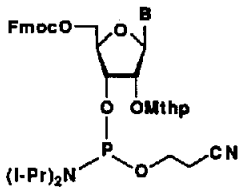
195 **196** **197**

AIBN= 2,2'-azobis(2-methylproprionitrile)

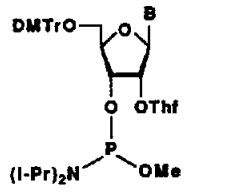
The release of the MPTM group from nucleosides can be achieved by treatment with mercury (II) chloride in acetonitrile-water (4:1) or by silver nitrate in *N,N*-dimethylformamide-water (9:1).[281a,373] The latter reagent was more efficient in cleaving the 2'-*O*-MPTM group from oligoribonucleotides. It is to be noted, however, that in the presence of *N,N*-diisopropylethylamine these metallic salts promoted the formation of (2'→5')-internucleotidic linkages *via* isomerization to the extent of 0.12% within 24 h.[281a] The application of the 2'-*O*-MPTM group in the solid-phase synthesis of oligoribonucleotides has not, as yet, been investigated.

Chattopadhyaya *et al.*[374,375] proposed a strategy for the synthesis of oligoribonucleotides involving the protection of the 5'-OH function of ribonucleosides with the base-labile Fmoc or 2-(4-chlorophenyl) sulfonylethoxycarbonyl (CPSEC) group while the 2'-OH function was protected with the Px group or pixyl derivatives such as 7-chloro-9-(*p*-anisyl)thioxanthen-9-yl, 7-chloro-9-phenylthioxanthen-9-yl or 9-phenylthioxanthen-9-yl (S-Px) groups. Particularly, the Px and the S-Px groups were easily cleaved by 80% acetic acid within 15 min at 20 °C.[139] Conversely, the complete deprotection of the 5'-*O*-CPSEC group by triethylamine was slow (18 h at 20 °C). This limitation casted doubts about the suitability of the 5'-*O*-CPSEC group toward solid-phase oligoribonucleotide synthesis.

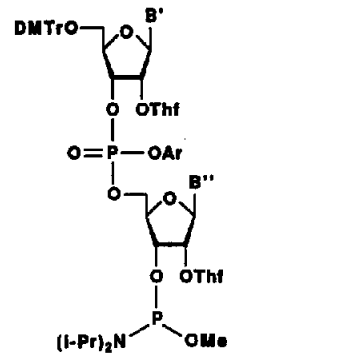
In a similar context, the combination of the 5'-*O*-Fmoc and 2'-*O*-Mthp protecting groups for ribonucleosides has been tested in the solid-phase synthesis of oligoribonucleotides (up to 20 bases long).[142,143] The activation of the ribonucleoside phosphoramidites **198a-d** with 5-(*p*-nitrophenyl)-1*H*-tetrazole promoted a stepwise coupling efficiency of ca. 96% within a coupling time of 2-10 min. The 5'-*O*-Fmoc group was removed in a stepwise manner by DBU and the release of the Fmoc was spectrophotometrically monitored at 305 nm.[142,143]



198a B= U
b = C^{bz}
c = A^{Bz}
d = G^{ib}



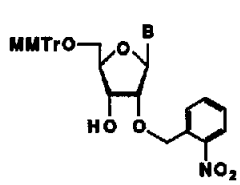
199a B= U
b = C^{bz}
c = A^{Bz}
d = G^{ib}



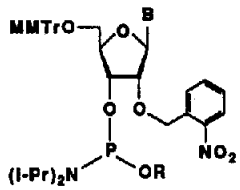
200a B'= C^{Bz}; B''= U
b = G^{ib}; = C^{Bz}

The compatibility of the 2'-*O*-Thf group with the 5'-*O*-DMTr group was investigated during the solid-phase synthesis of oligoribonucleotides *via* the phosphoramidite approach. The monomers **199a-d** and the ribonucleotide phosphoramidites **200a-b** were activated by 5-(*p*-nitrophenyl)-1*H*- tetrazole and were reacted with *N*-acetylated 2'-*O*-Thf nucleosides bound to LCAA-CPG.[376] The stepwise removal of the DMTr group with 1 M zinc bromide in dichloromethane-propan-2-ol (85:15) indicated that **199b** or **200a** produced coupling yields of 98%.[376] Tetra-, hepta- and octaribonucleotides were synthesized according to this approach. The practicability of this method was, however, limited by the slow and often incomplete removal of the 5'-*O*-DMTr group effected by zinc bromide.[40f,40h,377]

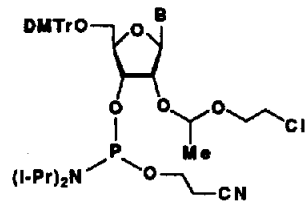
Like the 2'-*O*-Thf protecting group[40f-h,173,377-379] the 2'-*O*-(*o*-nitrobenzyl) group has been extensively used by Ohtsuka *et al.*[380,381] and others[40j,382] in the synthesis of RNA oligomers. Recently, Tanaka *et al.*,[383] Sakata *et al.*,[384] and Odai *et al.*[385] reported the preparation of the ribonucleoside phosphoramidites **202a-d** and **203a-d** from the 5'-*O*-MMTr-*N*-acylated-2'-*O*-(*o*-nitrobenzyl) nucleosides **201a-d** according to the procedure of Barone *et al.*[184] Oligoribonucleotides (11-16 mers) were synthesized by coupling either **202a-d** or **203a-d** to *N*-acylated 2'(3')-*O*-acetyl ribonucleosides covalently



201a B= U
b = C^{bz}
c = A^{Bz}
d = G^{ib}



202a-d R= Me
203a-d R= CH_2CH_2CN



204a B= U
b = C^{An}
c = A^{Bz}
d = G^{ib}

attached to LCAA-CPG in the presence of 5-(*p*-nitrophenyl)-1*H*-tetrazole.[383,384] The 2'-*O*-(*o*-nitrobenzyl) group was removed from the partially deprotected and purified oligonucleotides by long wave UV irradiation in 0.1 M ammonium formate (pH 3.5).[384-386] Ribonucleoside-*H*-phosphonate derivatives prepared from **201a-d** were also applied to the solid-phase synthesis of oligoribonucleotides.[387]

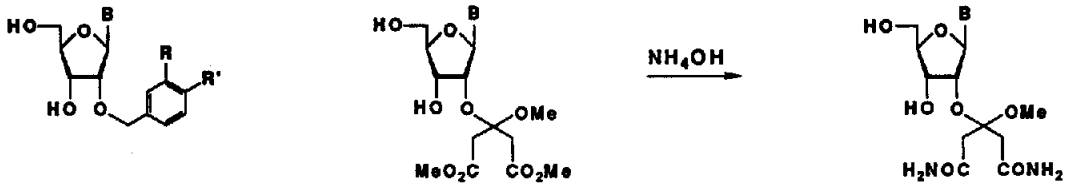
Very recently, Yamakage *et al.*[388] introduced the 1-(2-chloroethoxy)ethyl group as an acid-labile protecting group for the 2'-hydroxy function of ribonucleosides. To evaluate the potential of this protecting group in the solid-phase synthesis of oligoribonucleotides, 2'-*O*-1-(2-chloroethoxy)ethyl uridine was treated with 1.5% dichloroacetic acid in dichloromethane. Under these conditions the acetal had a half-life of 420 min. By comparison, the tetrahydropyranyl group had a half-life of 90 min under the same conditions. It was concluded that the 1-(2-chloroethoxy)ethyl group was completely stable under the conditions required for the complete removal of the 5'-*O*-DMTr group. The ribonucleoside phosphoramidites **204a-d** were prepared according to standard procedures and activated by 1*H*-tetrazole during the solid-phase synthesis of a dodecaribonucleotide. At the end of the synthesis, the 2'-*O*-protecting group was removed last by treatment with 0.01 N hydrochloric acid (pH 2.0) for 20 h at ambient temperature without detectable (3'→2')-isomerization of the internucleotidic linkages.

Takaku *et al.*[268b] reinvestigated the (1-methyl-1-methoxy)ethyl group (MME) which was originally introduced by Reese *et al.*[355] as a 2'-OH protecting group. The 2'-*O*-MME group had a half-life of only 20 min when treated with 5% acetic acid. Consequently, the 2'-*O*-MME and the 5'-*O*-DMTr protecting groups were incompatible for the solid-phase synthesis of oligoribonucleotides. Alternatively, Takaku *et al.*[259,263,389-392] evaluated the 4-methoxybenzyl and the 3,4-dimethoxybenzyl groups as potential 2'-OH protecting groups. These groups were introduced mainly at the 2'-OH of ribonucleosides by alkylation with either 4-methoxybenzyl bromide or 3,4-dimethoxy benzyl bromide in the presence of sodium hydride. Both the 2'-*O*-(4-methoxy)benzyl and 2'-*O*-(3,4-dimethoxy)benzyl protecting groups were stable to basic and acidic conditions. The 2'-*O*-(4-methoxy)benzyl group could be removed from **205a-c** by triphenylmethyl fluroborate,[389,390] whereas the 2'-*O*-(3,4-dimethoxy)benzyl group could be cleaved from **206a-c** under milder conditions by 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ).[259] Small oligoribonucleotides (3-9 mers) carrying either the 2'-*O*-(4-methoxylbenzyl) or the 2'-*O*-(3,4-dimethoxybenzyl) group were prepared according to the phosphotriester approach.[389-391]

The (methoxyethoxy)methyl group (MEM) has also been proposed as a 2'-OH protecting group for uridine and has been applied to the synthesis of an uridylate trimer by the phosphotriester method.[261] The 2'-*O*-MEM group was cleaved from the trinucleotide by triphenylmethyl fluoroborate in acetonitrile-water (4:1) within 2 h at room temperature. However, no detailed studies concerning the stability of the 2'-*O*-MEM group under the conditions required for the complete removal of the 5'-*O*-DMTr group were reported.

It has been claimed that the 2'-*O*-Thp and the 2'-*O*-Mthp groups of ribonucleosides were stabilized against acidic hydrolysis by an adjacent 3'-phosphotriester function.[121b] Conversely, an adjacent 3'-phosphodiester destabilized the 2'-*O*-Thp protecting group under the same conditions.[341] These observations led Sandström *et al.*[289b,c] to design and evaluate the 3-methoxy-1,5-

dicarbomethoxypentan-3-yl (MDMP) group as an achiral and acid-labile 2'-hydroxyl protecting group for ribonucleosides. The stability of the 2'-*O*-MDMP group in **207a-d** under acidic conditions (80% acetic acid) was comparable to that of the 2'-*O*-Mthp group.[289b,c] Moreover, the 2'-*O*-MDMP group was completely stable under the conditions required for the removal of the 5'-*O*-Px group from ribonucleoside phosphotriesters. An inherent feature of the 2'-*O*-MDMP group entailed the conversion of the ribonucleosides **207a-d** into the 2'-*O*-diamide ribonucleoside derivatives **208a-d** upon treatment with concentrated ammonium hydroxide. This transformation enabled the acidic cleavage (80% acetic acid) of the 2'-*O*-protecting group in **208a-d** to proceed seventeen times faster than that of the 2'-*O*-MDMP group in **207a-d**.[289b,c] This feature may reduce the potential (3'→2')-migration of the internucleotidic linkages of ribonucleotides during the final acidic deprotection step. The synthesis of a pentaribonucleotide using the MDMP as a 2'-hydroxy function protecting group was reported.[289c]



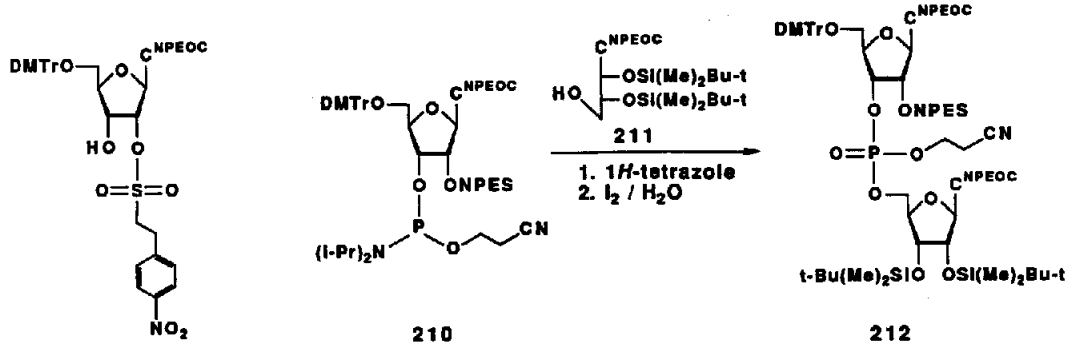
205a B= C; R=OMe; R'= H
b B= A
c B= G^{Bz} or G^{ib}
206a-c R= R'= OMe

207a B= N^3-benzoyl-1-uracil
b = N^4-benzoyl-1-cytosinyl
c = N^6-benzoyl-9-adeninyl
d = N^2-(*t*-butylbenzoyl)-9-guaninyl

208a B= U
b = C
c = A
d = G

In their studies pertaining to the reaction of arenesulfenyl chlorides with the hydroxyls of ribonucleosides, Bazin *et al.*[393] suggested the *o*-nitrobenzenesulfenyl (Nbs) group for the protection of the 2'-hydroxy function. The 2'-*O*-Nbs group was stable under normal laboratory conditions but was readily cleaved under near neutral conditions by triethylammonium thiocresolate. The synthesis of oligoribonucleotides from protected 2'-*O*-Nbs ribonucleosides has not, as yet, been reported.

Recently, Pfister *et al.*[249,250b] and others[144,243,250c,d] tested the *p*-nitrophenylethylsulfonyl (NPES) group for the 2'-OH protection of ribonucleosides. The NPES group was incorporated at the 2'-OH of suitably protected ribonucleosides by treatment with *p*-nitrophenylethylsulfonyl chloride in pyridine. The 2'-*O*-NPES ribonucleoside **209** was isolated in 57% yield.[250b] It is important to mention that the 2'-*O*-NPES group in **209** did not undergo (2'→3')-migration. It was postulated that the relatively high stability of this group resulted from an unfavorable alignment of dipoles in the transition state of the nucleophilic displacement at *C*-2. The NPES group could be removed by cleaving the carbon-sulfur linkage *via* a ß-elimination process.[144,249]



209 **210** **212**

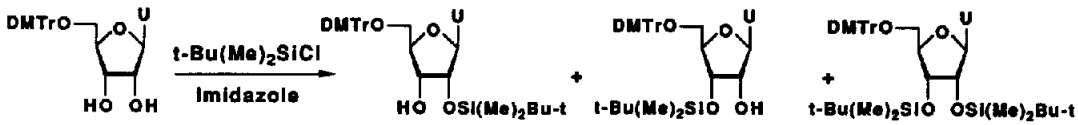
NPEOC= *p*-nitrophenylethyloxycarbonyl

The ribonucleoside phosphoramidite **210** was prepared from **209** according to the procedure reported by Sinha *et al.*[394] The coupling of the silylated ribonucleoside **211** with **210** in the presence of 1*H*-tetrazole afforded after aqueous iodine oxidation the dinucleoside phosphotriester **212**. The dinucleotide was deprotected by first removing the 5'-*O*-DMTr goup under acidic conditions (80% acetic acid) while the 2'-*O*-NPES, phosphate and aglycone protecting groups were removed simultaneously by DBU. Finally, the removal of the terminal 2'- and 3'-*O*-*tert*-butyldimethylsilyl (TBDMS) groups by fluoride ion followed by DEAE sephadex purification afforded CpC in 79% yields. Similarly, ApA[249,250c] and GpG[250d] were also prepared. Nevertheless, the preparation of UpU[243] led to the formation of anhydronucleotides in the presence of DBU. This unwanted reaction was prevented by protecting the uracil residue at *O*-4 with a *p*-cyanophenylethyl goup which could be removed by DBU only at elevated temperature (50 °C). This protection allowed the isolation of UpU in 80% yield.[249] It has also been shown by Pfister and Pfleiderer that the NPES group can serve as a 3'-OH protecting group in the synthesis of (2'→5')-oligoribonucleotides.[250b]

Subsequently, the *p*-cyanophenylethyl sulfonyl (CPES) and the carbomethoxyethylsulfonyl (CMES) groups were introduced for the protection of the 2'-hydroxy function of ribonucleosides.[250e] It was found that the 2'-*O*-CPES group of the dinucleoside phosphotriester analogous to **212** was more stable to fluoride ion than the ß-cyanoethyl phosphate protecting group. Moreover, the N^6-*p*-nitrophenylethoxycarbonyl residues were virtually undisturbed under these conditions. These findings implied that the use of the 2'-*O*-CPES group could reduce the cleavage of internucleotide linkages and the formation of anhydronucleotides during deprotection.[250e] Alternatively, the application of the CMES group in nucleoside and nucleotide chemistry was limited because the protecting group was too base-labile.

Acyl groups for the protection of the 2'-hydroxyl groups of ribonucleosides have not been popular because it has been shown several years ago that these groups underwent facile (2'=3')-isomerization.[395,396] Despite these convincing data, Kempe *et al.*[397] reported that the 2'-*O*-benzoylation of ribonucleosides can be achieved in high isomeric purity at low temperature (−40 °C). The properly protected 2'-*O*-benzoyl ribonucleosides were converted into ribonucleoside phosphoramidite monomers and used in the solid-phase synthesis of mixed sequences of DNA and RNA. Great care had to be exercised during the removal of the protecting groups under basic conditions to minimize the cleavage of the phosphodiester linkages.

In the early seventies, Ogilvie and coworkers applied the TBDMS group and a variety of alkylsilyl groups to the protection of the hydroxy functions of deoxyribonucleosides[315b,398-401] and ribonucleosides.[315b,402] It was shown that, under controlled conditions, the reaction of *tert*-butyldimethylsilyl chloride with 5'-*O*-DMTr uridine (**213**) and imidazole in *N,N*-dimethylformamide afforded the desired 2'-*O*-TDBMS derivative **214** in 51% yield along with the ribonucleosides **215** and **216** in 36% and 11% yield respectively.[403]



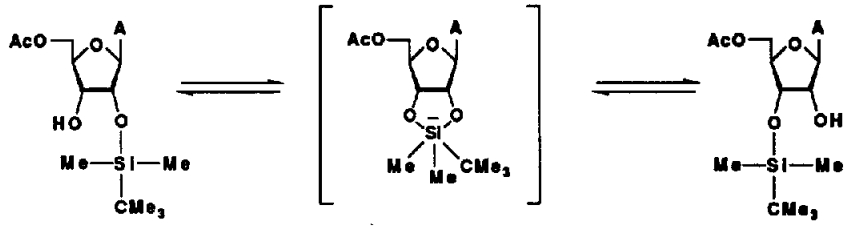
213 **214** **215** **216**

The silylation of *N*-acylated-5'-*O*-DMTr ribonucleosides under similar conditions did not occur with greater regioselectivity.[404] However, when the silylation reaction was performed in the presence of silver nitrate or silver perchlorate and pyridine in tetrahydrofuran, the ribonucleoside **214** was obtained in 70% yield, whereas **215** was isolated in only 15% yield.[405,406] It is noteworthy that under similar conditions the silylation of 5'-*O*-DMTr-α-uridine resulted in the selective incorporation of the TBDMS group at the 3'-OH[407] instead of the 2'-OH observed with ß-ribonucleosides.[406]

An improved regioselectivity was obtained when the silylation reaction was performed in the

presence of silver perchlorate and 1,4-diazabicyclo[2.2.2]octane (DABCO) in tetrahydrofuran. Under these conditions, the 3'-*O*-TBDMS ribonucleoside **215** was isolated in 91% yield along with small amount (5%) of **214**.[406,408] Similarly, the hydroxyls of arabinonucleosides were also regioselectively protected by the TBDMS group.[409,410]

The usefulness of the TBDMS group as a 2'-OH protecting group for ribonucleosides in the synthesis of oligoribonucleotides was questioned by Jones and Reese[411] who reported the migration of the 2'- and 3'-*O*-TBDMS groups of the ribonucleosides **217** and **219** in methanolic solutions at 36 °C. Under these conditions **217** and **219** were interconverted with a half-life of ca. 1 h. In anhydrous pyridine the conversion of **217** into **219** was much slower ($t_{1/2}$ = 1140 min at 36 °C). This slow rate of isomerization was only apparent as **219** interconverted to **217** with a half-life of 380 min under identical conditions.[411]



217 **218** **219**

In earlier reports Ogilvie *et al.*[403] and others[412] indicated that the (2'⇌3')-isomerization of the TBDMS group did occur in aqueous pyridine. No isomerization was, however, observed in aqueous pyridine containing 2,4,6,-triisopropylbenzenesulfonic acid.[403] No detectable isomerization occurred within 25 h under acidic conditions (80% acetic acid or 0.1 M methanolic hydrochloric acid at 20 °C)[402,403] or in pure solvents such as chloroform and anhydrous *N,N*-dimethylformamide.[315b,413,414] Less than 1% of (2'⇌3')-isomerization of the TBDMS group was observed in either tetrahydrofuran or acetonitrile within 24 h.[315b,413,414]

The 2'-*O*-TBDMS group of ribonucleosides was reasonably stable to acidic conditions. For instance, 5'-*O*-MMTr-2'-*O*-TBDMS uridine was converted into 2'-*O*-TBDMS uridine in 98% yield upon treatment with 80% acetic acid for 4 h at 22 °C. Only ca. 2% of the 2'-*O*-TBDMS group was cleaved under these conditions.[403] However, 2'-*O*-TBDMS uridine was converted into uridine in ca. 90% yield upon treatment with 9 M ammonium hydroxide at 70 °C.[403] More importantly, the 2'-*O*-TBDMS protecting group was completely cleaved by fluoride ion within 30 min at 20 °C.[403] These desirable features promoted the extensive utilization of 2'-*O*-TBDMS ribonucleosides in oligoribonucleotide synthesis *via* the phosphotriester and the phosphite coupling procedures whether in solution or on solid-phase.[41b,49,51,403,404,412,414-424]

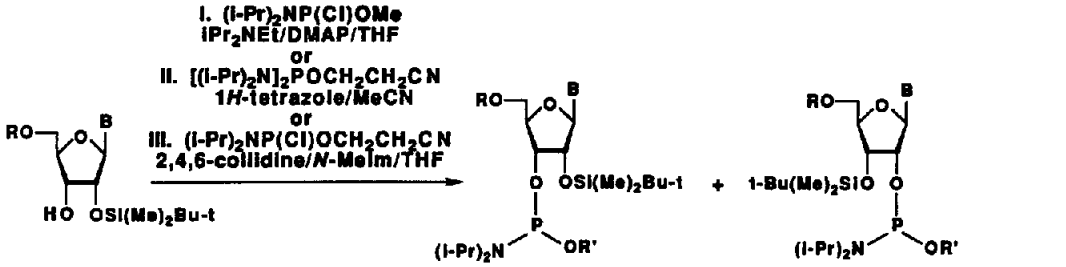
Usman *et al.*[425] reported the conversion of the 5'-*O*-MMTr-2'-*O*-TBDMS ribonucleosides **220a-c** into the corresponding ribonucleoside phosphoramidites **221a-c**. These monomers were then activated with 1*H*-tetrazole and reacted with 3'-*O*-TBDMS ribonucleosides covalently attached to LCAA-CPG through a 2'-*O*-succinate linkage. Using a 15 min coupling step, this approach enabled the preparation of oligoribonucleotides (43-mer and a 77-mer) with an average stepwise yield of 98%.[426,427] Of particular importance, the sequence corresponding to an *E. coli* $tRNA_f^{met}$ analogue (77-mer), demonstrated modest methionine acceptance activity.[426b] Other synthetic oligoribonucleotides prepared according to this methodology were useful for probing RNA-protein interactions in the MS2(R17) translational repression complex.[428]

In spite of these successful syntheses, the coupling efficiency of ribonucleoside phosphoramidites was less than that observed with deoxyribonucleoside phosphoramidite synthons. In an attempt to overcome this limitation, Pon[429] investigated the parameters involved in the coupling reaction. It was found that the best coupling results were obtained when 5-(*p*-nitrophenyl)-1*H*-tetrazole and catalytic amounts of DMAP were used in the activation of the ribonucleoside phosphoramidite **223a**. Under

these conditions and with a stepwise coupling time of 3 min, a nonauridylic acid was synthesized on a CPG support with coupling yields averaging 96%.[429]

Ribonucleoside phosphoramidites having different nucleobase protecting groups with respect to **224a-d** were synthesized by Chaix *et al.*[312] and applied toward the solid-phase synthesis of oligoribonucleotides corresponding to the 5'-half of the initiator tRNA from *B. subtilis*. The preparation of a 35-mer was achieved with an average coupling yield exceeding 95%.

Milecki *et al.*[430] and Wu and Ogilvie[431] reinvestigated the potential (2'⇌3')-isomerization of the TBDMS group during the preparation of the ribonucleoside phosphoramidites **221a-c**, **223a-d**, and **224a-d** by ^1H and ^{31}P NMR spectroscopy. Specifically, the reaction of **222a-d** under conditions "I" afforded exclusively **223a-d**. Similarly, the reaction of **222a-d** under conditions "II"[430] or "III"[432] resulted in the exclusive formation of **224a-d**. However, the substitution of 1*H*-tetrazole for *N,N*-diisopropylammonium tetrazolide under conditions "II", generated **226a-d** (4-5%)[430] and thus precluded the use of this activator during the preparation of **224a-d**.



220a B= U; R= MMTr
b = C^{Bz}
c = A^{Bz}

222a-c, R= DMTr
222d B= G^{ib} or G^{Bz}; R= DMTr

221a-c; R= MMTr; R'= Me

223a-d; R= DMTr; R'= Me

224a-d; R= DMTr; R'=CH_2CH_2CN

225a-d; R= DMTr; R'= Me

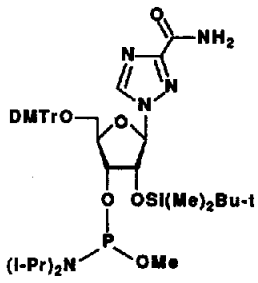
226a-d; R= DMTr; R'=CH_2CH_2CN

The regioisomeric integrity of the internucleotidic phosphate linkage in synthetic RNA prepared from the 2'-*O*-TBDMS phosphoramidites **224a-d** was further examined by Wang *et al.*[433] Two purified mixed DNA-RNA nonamers, T_3XT_5 and T_5XT_3 (X= U, rC, rA and rG) were submitted to digestion by ribonuclease T_2 which cleaves (3'→5')-phosphodiester linkages but not (2'→5')-linkages. It was found that T_5XT_3 and T_3XT_5 were greater than 99% hydrolysed at the XpT linkage while the control nonamer $T_5A(2'\rightarrow5')TT_2$ was 100% resistant under the same conditions. This experiment demonstrated that RNA with the correct (3'→5')-internucleotidic phosphodiester linkages could be prepared on solid supports from **224a-d** without significant isomerization.[433]

The 2'-*O*-TBDMS group was also applied toward the solid-phase synthesis of a ribavirin pentadecamer.[434] With a coupling time of 8 min the ribonucleoside phosphoramidite **227** generated an average coupling yield of ca. 94%. The purified oligomer was completely hydrolyzed by ribonuclease T_2 thereby indicating the exclusive presence of (3'→5')-phosphodiester linkages.[434]

Moreover, the α-ribonucleoside phosphoramidite **228** has been successfully used in the solid-phase synthesis of α-U_6 and α-U_{12}.[407] A 15 min coupling reaction mediated by 1*H*-tetrazole resulted in an average coupling efficiency of 97%. The possible occurrence of phosphoryl migration during the deprotection conditions was evaluated by HPLC analysis of α-(UpU). Only 0.3% of α-[uridilyl-(2'→5')-uridine] contaminated the desired α-[uridilyl-(3'→5')-uridine]. These results were consistent with the extent of isomerization observed with the ß-anomeric oligomers.

The above results suggest that the use of the 2'-*O*-TBDMS group in conjunction with the phosphoramidite approach may be the method of choice for the synthesis of oligoribonucleotides. One must also mention that ribonucleoside-*H*-phosphonates carrying 5'-*O*-DMTr and 2'-*O*-TBDMS groups



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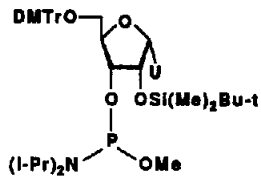

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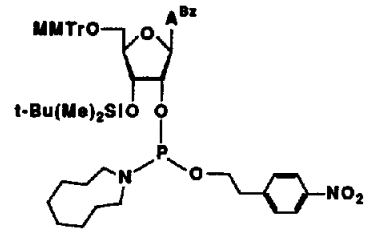
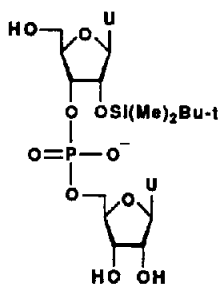


229

were prepared without significant (2'⇌3')-isomerization (less than 0.5%) of the TBDMS group.[430,435,436] These monomers led to the solid-phase synthesis of a 21-mer with an average stepwise yield of 97-99%.[436]

The solid-phase synthesis of (2'→5')-linked oligoadenylates has also been realized according to the phosphoramidite methodology using 5'-*O*-DMTr and 3'-*O*-TBDMS protecting groups.[437] An average coupling efficiency of 99% was obtained during the preparation of (2'→5')-$(pApA)_5$. Charubala and Pfleiderer reported the synthesis of a tetrameric (2'→5')-adenylyl phosphorothioate in solution phase from the 5'-*O*-MMTr-3'-*O*-TBDMS ribonucleoside phosphoramidite **229**.[438] The conversion of the phosphite triester linkages into phosphorothioates was effected by sulfur oxidation and the various diastereoisomers were separated in an attempt to mechanistically probe the mode of action of (2'→5')-oligoadenylates.

In spite of the efficiency with which oligoribonucleotides could be synthesized from ribonucleoside phosphoramidites having 5'-*O*-MMTr and 2'-*O*-TBDMS groups, the final product was contaminated with a number of shorter sequences accounting for 5-10% of the desired oligomer.[229] It is reasonable to argue that under the alkaline conditions required for the removal of the aglycone protecting groups, the 2'-*O*-TBDMS group was cleaved to some extent and resulted in partial chain breakage. Stawinski *et al.*[435,439] have indeed shown that the 2'-*O*-TBDMS group in **230** was cleaved by concentrated ammonium hydroxide at 55 °C at a rate of 27% after 8 h and 65% after 24 h. The internucleotidic phosphodiester cleavage resulting from this loss occurred at a rate of 2.7% after 8 h and 23% after 24 h.[435,439]



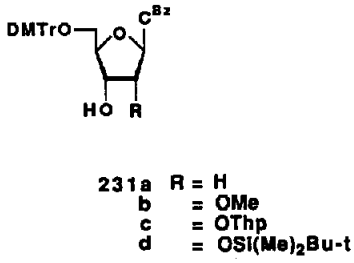
230

The addition of ethanol to concentrated ammonium hydroxide dramatically decreased the rate of 2'-*O*-TBDMS cleavage.[426,427] For instance, the treatment of **230** with concentrated ammonium hydroxide-ethanol (3:1) at 55 °C produced ca. 4% of TBDMS group cleavage after 8 h. The phosphodiester bond was completely stable under these conditions. However, ca. 10% of the TBDMS group and 0.9% of the internucleotidic phosphodiester linkage were cleaved after 24 h. These

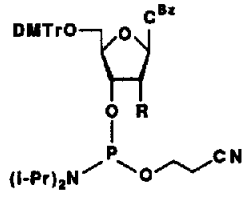
conditions were, therefore, satisfactory for the deacylation of medium-sized oligoribonucleotides. The final removal of the 2'-*O*-TBDMS group from **230** with tetra-*n*-butylammonium fluoride occurred smoothly within 4 h without cleavage or isomerization of the phosphodiester linkage.[435,439]

In an effort to reduce the damage caused to the 2'-*O*-TBDMS group by ammonium hydroxide during the removal of the aglycone protecting groups, Wu *et al.*[229] and Chaix *et al.*[228,312] reported the solid-phase synthesis of oligoribonucleotides *via* *N*-phenoxyacetylated 2'-*O*-TBDMS ribonucleoside phosphoramidites. The removal of the nucleobase protecting groups was achieved under milder conditions by a short 4 h treatment with concentrated ammonium hydroxide-ethanol (1:1) at ambient temperature.[312] It is worth noting that ethanolic ammonia has been effective in the deprotection of oligoribonucleotides carrying *N*-benzoyl and *N*-isobutyryl groups as nucleobase protecting groups. Complete deprotection without detectable cleavage of the 2'-*O*-TBDMS was achieved within 16 h at 55 °C.[432]

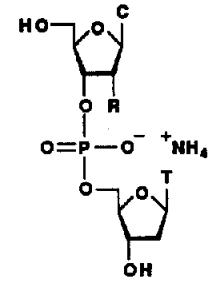
One important issue in the synthesis of oligoribonucleotides is the effect of the steric hindrance generated by the 2'-*O*-protecting group on the 3'-phosphitylation of the ribonucleosides and the coupling rates of the ribonucleoside phosphoramidite monomers during chain elongation. In a competitive phosphitylation experiment involving bis-(*N,N*-diisopropylamino)-2-cyanoethoxyphosphine and ribonucleosides carrying different 2'-*O*-protecting groups (**231a-d**), Kierzek *et al.*[440] noticed that the rates of the phosphitylation reaction when performed under the conditions described by Barone *et al.*,[184] decreased in the order: 2'-H; 2'-*O*-Me; 2'-*O*-Thp; 2'-*O*-TBDMS. These observations were also valid for *N*-benzoylated adenosine derivatives. Also in a competitive experiment, the coupling of **232a-d** with deoxythymidine bound to a solid support led, after treatment with ammonium hydroxide, to the analysis and quantitation of the dimers **233a-d.** It was shown that the coupling efficiency of **232a-d** decreased as follows: 2'-H; 2'-*O*-Me; 2'-*O*-Thp; 2'-*O*-TBDMS. Consequently, the steric bulk of the 2'-*O*-protecting group represents an additional factor deserving consideration in the design of such groups.



231a R = H
b = OMe
c = OThp
d = $OSi(Me)_2Bu\text{-}t$



232a-d



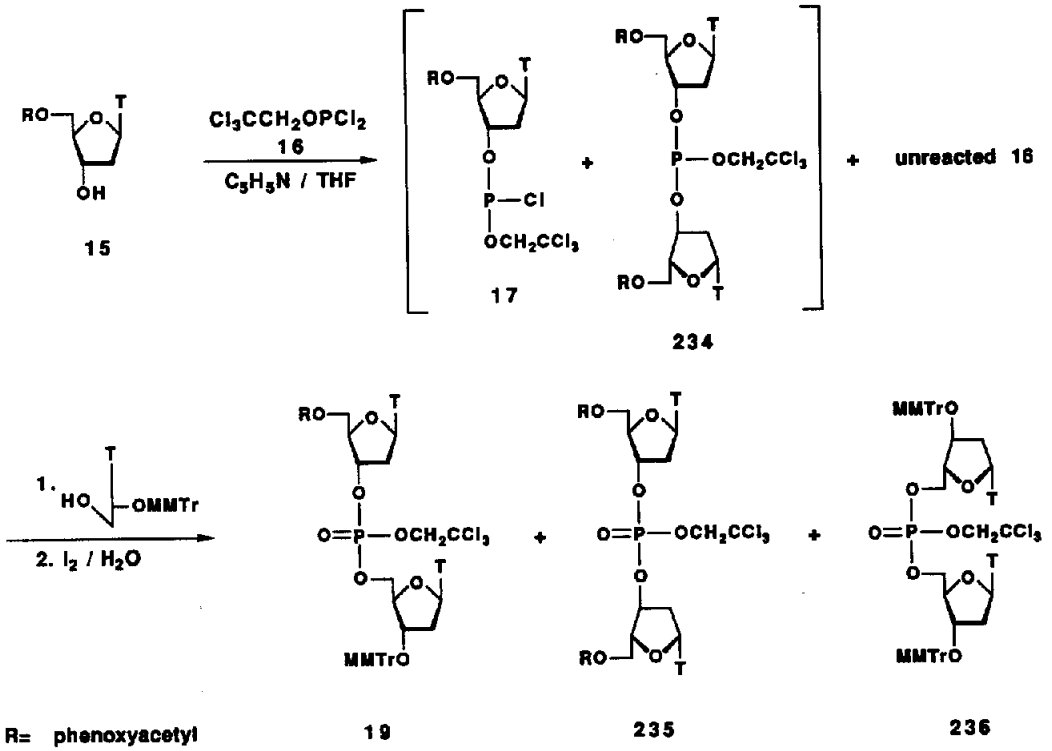
233a-d

The next topic will emphasize the formation of internucleotidic phosphite triester linkages *via* nucleoside chlorophosphite and activated nucleoside phosphoramidite intermediates. The oxidation of oligonucleoside phosphite triesters and the protection of the phosphate function with respect to solid-phase oligonucleotide synthesis will also be discussed.

5. THE FORMATION OF INTERNUCLEOTIDE LINKAGE IN THE SYNTHESIS OF OLIGONUCLEOTIDES AND THE PROTECTION OF THE PHOSPHATE FUNCTION

In the mid-seventies, Letsinger *et al.*[26,27] pioneered the phosphite coupling procedure for the generation of internucleotidic links. Basically, the approach involved the reaction of 5'-*O*-phenoxyacetylthymidine (**15**) with 2,2,2-trichloroethyl phosphorodichloridite (**16**) at −78 °C for 5-10 min. The subsequent addition of 3'-*O*-MMTr thymidine yielded after treatment with aqueous iodine

the dinucleoside phosphate triester **19** along with the symmetrical (3'→3')- and (5'→5')-dinucleoside phosphate triesters **235** and **236** as side-products. Under optimal conditions, **19** was prepared within 1 h in 82% isolated yield.[27] In those days, the formation of internucleotidic linkages with such kinetics was unprecedented.

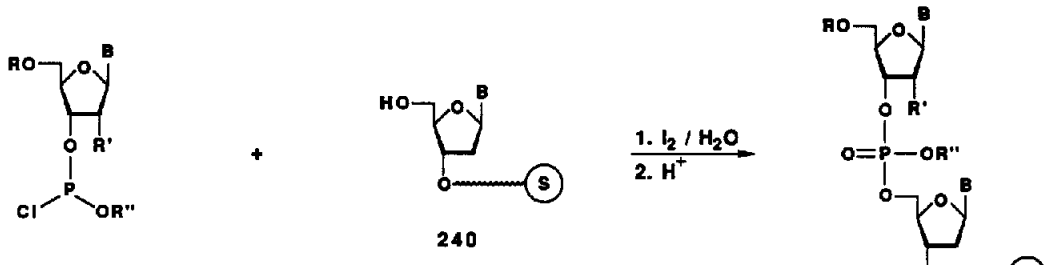


The phosphite coupling procedure was then applied to the preparation of oligodeoxyribonucleotides[27,441] and their analogues[442] in addition to the synthesis of oligoribonucleotides either by a stepwise[403,404,415,417,418,443,444] or a block condensation approach.[416,420] The compatibility of various phosphate protecting groups with respect to the hydroxyl and aglycone protecting groups along with the conditions for their sequential removal were also evaluated. The phosphate protecting groups studied were: 2,2,2-trichloroethyl,[422,445,446] 2,2,2-tribromoethyl,[422,447] 2-cyanoethyl,[10,422,448] benzyl,[2,189,422] methyl,[189,192,422,449] *p*-chlorophenyl[422,450,451] and *p*-nitrophenylethyl.[422,452]

The formation of significant amounts of (3'→3')-dinucleoside phosphite triesters during the synthesis of oligodeoxyribonucleotides with phosphorodichloridites was reduced by increasing the steric bulk of the phosphitylating agent. For instance, the reaction of 2,2,2-trichloro-1,1-dimethylethyl phosphorodichloridite[97b,453-455] or 2-cyano-1,1-dimethylethyl phosphorodichloridite[456] with properly protected nucleosides generated only traces of (3'→3')-dinucleoside phosphite triesters. Moreover, the 2,2,2-trichloro-1,1-dimethylethyl (TCDME) group can be removed by tributylphosphine within 1 h at 80 °C.[97b] This feature allowed the use of the TCDME protecting group in the synthesis of oligonucleotides on silica supports, as neither zinc[445,446] nor radical ions[27,441,457] were effective in cleaving 2,2,2-trichloroethyl phosphotriesters bound to these supports.[453] The removal of the 2-cyano-1,1-dimethylethyl phosphate protecting group was effected by 0.2 M sodium hydroxide in dioxan-methanol (15:5) within 1 min at 20 °C.[456a]

More than a decade ago, Caruthers *et al.*[50] described the reaction of the deoxyribonucleoside chlorophosphite **237** with a deoxyribonucleoside covalently attached to a silica support *via* a 3'-*O*-

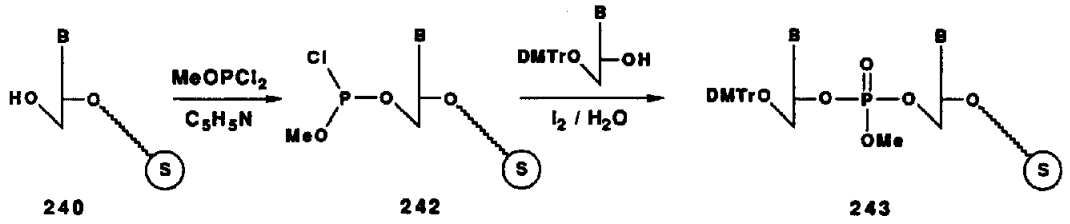
succinate linkage (as in **240**). The dinucleoside phosphate triester **241** was generated in yields greater than 90%. Two nonanucleotides were subsequently prepared and isolated in ca. 25% yields. In addition to **237**, the deoxyribonucleoside chlorophosphite **238** and the ribonucleoside chlorophosphite **239** have been employed in the solid-phase synthesis of oligodeoxy-[29,54,97,453,458] and oligoribonucleotides.[41b,49,51,419]



237 R= DMTr; R'= H; R"= Me
238 = DMTr; = H; = $C(Me)_2CCl_3$
239 = MMTr; = $OSi(Me)_2Bu$-t; R"= Me

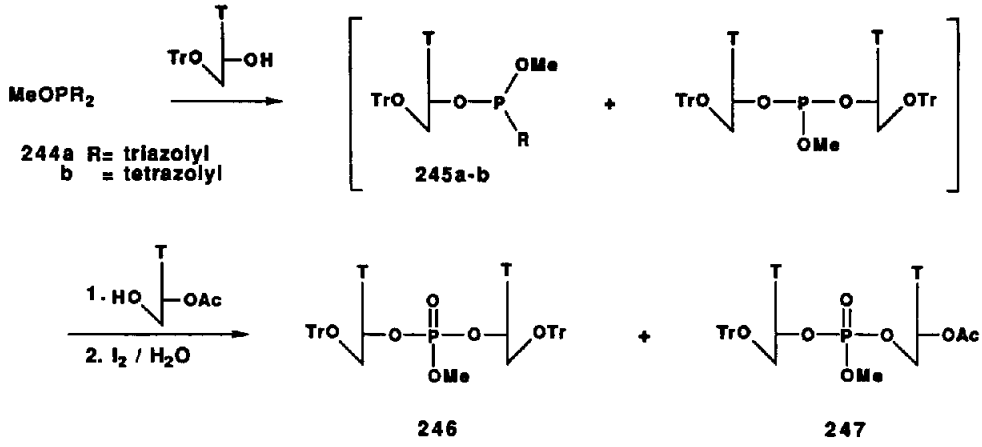
B= Thymine or protected purine/pyrimidine nucleobases

A different strategy in the solid-phase synthesis of oligodeoxyribonucleotides *via* the phosphorodichloridite approach was described by Jayaraman and McClaugherty.[459] To circumvent the formation of side-products during the preparation of deoxyribonucleoside chlorophosphites, a deoxyribonucleoside bound to a silica support (**240**) was phosphitylated with methoxydichlorophosphine at ambient temperature affording the corresponding chlorophosphite **242**. The subsequent addition of a 5'-*O*-DMTr deoxyribonucleoside followed by iodine oxidation resulted in the formation of the dinucleoside phosphate triester **243** in ca. 95% yield. Although an octanucleotide was prepared by this approach, an excess of phosphitylating reagent was necessary to avoid the potential hydrolysis of **242** by trace amounts of moisture present in the solvents and environment. A large excess of valuable 5'-*O*-DMTr deoxyribonucleoside was, therefore, required to react with both **242** and the excess methoxydichlorophosphine.



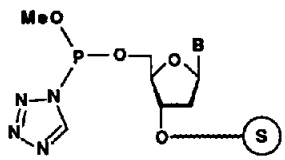
In their studies regarding the application of deoxyribonucleoside chlorophosphites to solid-phase DNA synthesis, Caruthers *et al.*[28,102c] discovered that adding 1*H*-tetrazole to these intermediates improved the coupling yields to greater than 95% and enhanced the condensation rates relative to the parent chlorophosphites. These improvements led to the solid-phase preparation of a dodecanucleotide in a 55% overall yield. Independently, Fourrey and Shire[460] found that the amount of (3'→3')-dinucleoside phosphite triesters formed during the preparation of oligodeoxyribonucleoside methylphosphotriesters *via* the phosphorodichloridite approach could be dramatically reduced by converting methoxydichlorophosphine into the corresponding bis-triazolyl and bis-tetrazolyl derivatives. Thus, the reaction of 5'-*O*-trityl thymidine with either bis-triazolyl (**244a**) or bis-tetrazolyl methoxyphosphine (**244b**) generated *in situ* at −70 °C, followed by the addition of 3'-*O*-acetylthymidine,

afforded after aqueous iodine oxidation the desired (3'→5')-dinucleoside phosphotriester **247** in ca. 75% yield. Only trace amounts of the (3'→3')-isomer **246** was generated when **244a** was used as the phosphitylating agent.



246 **247**

In a strategy identical to that reported by Jayaraman and McClaugherty (*vide supra*), Cao *et al.*[461] employed the bifunctional phosphitylating agent **244b** to generate the deoxyribonucleoside phosphorotetrazolide (**248**) covalently attached to a solid support through a standard 3'-*O*-succinate linker. The reaction of 5'-*O*-DMTr deoxyribonucleosides with **248** proceeded with very high efficiency (ca. 99% yield). A tetradecanucleotide was synthesized according to this procedure in an overall yield of 94%. It was postulated that the efficiency of the procedure was due to the high reactivity of **248**. In spite of these attractive features, the reproducibility of the methodology has not, as yet, been clearly demonstrated.



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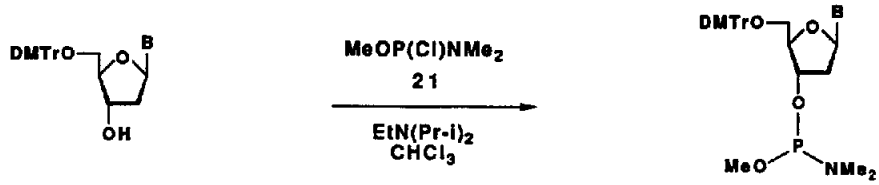
The application of nucleoside chlorophosphites or the corresponding tetrazolides to the solid-phase synthesis of oligonucleotides was generally problematic. The preparation of these reagents from reactive bifunctional phosphitylating agents had to be performed at low temperature in the absence of moisture and under an inert atmosphere. In addition to being contaminated by variable amounts of undesired (3'→3')-dinucleoside phosphite triesters, the nucleoside chlorophosphites and/or corresponding tetrazolides were sensitive to hydrolysis and, hence, difficult to handle. Although these synthons enabled the rapid formation of internucleotidic linkages and efficient preparation of a large number of DNA segments on silica supports, their relative instability precluded their reliable use in automated systems.

To satisfy the requirements for automation, the phosphitylated nucleosidic monomers must be stable to hydrolysis and air oxidation under normal laboratory conditions. In addition to being easily isolated and stable upon storage, these synthons must be readily activated to reactive species to permit rapid and efficient formation of internucleotidic links. The pioneering work of Beaucage and Caruthers[30] in the development of deoxyribonucleoside phosphoramidites as a new class of key

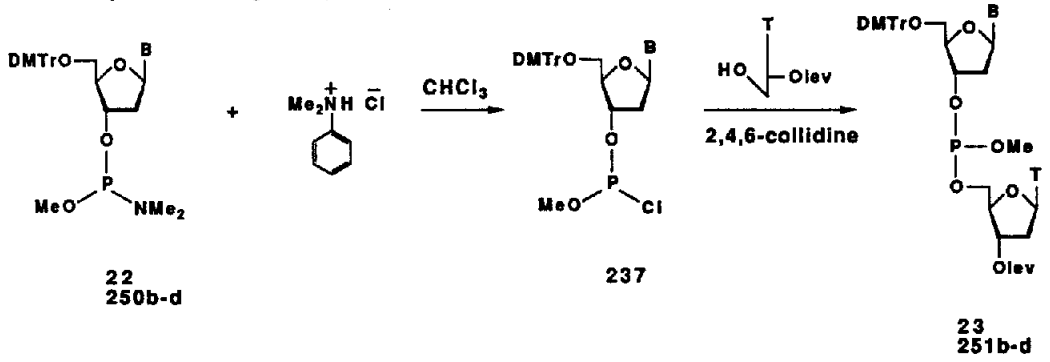
intermediates for deoxyribonucleotide synthesis led to the convenient and reliable automation of DNA synthesis.[462] Essentially, the approach consisted in the reaction of 5'-*O*-DMTr deoxyribonucleosides (**20** and **249b-d**) with chloro-(*N,N*-dimethylamino)methoxyphosphine (**21**) in the presence of *N,N*-diisopropylethylamine in dry chloroform at 20 °C.[30,102a,d,e] After work-up, the resulting deoxyribonucleoside phosphoramidites **22** and **250b-d** were isolated by precipitation in cold hexane in 90-94% overall yields. These were stored as dry powders under an inert atmosphere at 20 °C for at least a month without significant decomposition.



20 B= 1-thyminyl
249b = 1-(N^4-benzoylcytosinyl)
c = 9-(N^6-benzoyladeninyl)
d = 9-(N^2-isobutyrylguaninyl)

22
250b-d

The monofunctional phosphitylating agent **21** prevented the formation of (3'→3')-dinucleoside phosphite triesters often observed with the phosphorodichloridite methodology. Interestingly, the activation of the phosphoramidite **22** with a weak acid such as *N,N*-dimethylaniline hydrochloride in dry chloroform generated the nucleoside chlorophosphite **237** in quantitative yields as judged by ^{31}P NMR spectroscopy.[30] As expected the condensation of **237** with 3'-*O*-levulinyl thymidine afforded the (3'→5')-dinucleoside phosphite triester **23** in essentially quantitative yields. These experiments demonstrated that a stable deoxyribonucleoside phosphoramidite could be activated to a very reactive intermediate toward the synthesis of oligodeoxyribonucleotides.



22
250b-d

237

23
251b-d

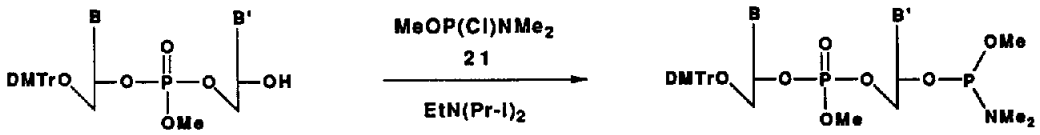
The hygroscopic nature of tertiary amine hydrochlorides was, nonetheless, incompatible with the anhydrous conditions required for optimum coupling reactions. The search for non-hygroscopic weak acids capable of activating phosphoramidites led to 1*H*-tetrazole, a commercially available compound which could be purified and dried in one step by sublimation. The activation of **22** and **250b-d** by 1*H*-tetrazole in the presence of 3'-*O*-levulinyl thymidine in dry acetonitrile afforded **23** and **251b-d** in quantitative yields within the time required to record a ^{31}P NMR spectrum (ca. 5 min).[30]

The usefulness of **22** and **250b-d** as synthons and 1*H*-tetrazole as an activator was first demonstrated by the solid-phase synthesis of various dimers in yields varying from 93-100%.[30] This approach was then applied to the synthesis of much larger oligodeoxyribonucleotides (up to 45 bases long).[103] Coupling yields ranged from 85-100%. Despite the fact that the phosphoramidites **22** and **250b-d** were only ca. 85% pure, the stability of these reagents in solution was quite impressive. After

four weeks in acetonitrile at 20 °C, **22** and **250b-d** produced coupling yields only slightly lower than those obtained from fresh reagents.[103]

Soon after the introduction of deoxyribonucleoside phosphoramidites as a new class of intermediates in the synthesis of oligodeoxyribonucleotides, considerable efforts were devoted to the elaboration of an automated system that would permit the utilization of these monomers and enable the chain assembly of oligonucleotides on solid supports. The design of such an instrument has been reported by Horvath *et al.*[463] Several types of DNA/RNA synthesizers are now commercially available.

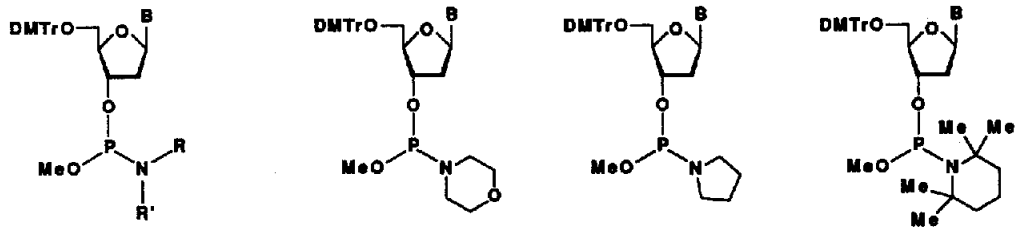
The dinucleotide phosphoramidites **253** were also applied to the solid-phase synthesis of oligodeoxyribonucleotides.[464] These were prepared by the reaction of **22** and **250b-d** with suitably protected 3'-*O*-levulinyl deoxyribonucleosides and 1*H*-tetrazole in acetonitrile. After aqueous iodine oxidation and hydrazinolysis, the resulting dinucleoside phosphotriesters **252** were reacted with chloro-(*N,N*-dimethylamino)methoxyphosphine (**21**) affording the phosphoramidite derivatives **253**.



252 **253**

The coupling of **253** to a deoxyribonucleoside bound to CPG occurred as efficiently as with the monomers **22** and **250b-d**. With 1*H*-tetrazole as activator, coupling yields of 99% were observed and the synthesis of a 29-mer was achieved by this approach.[464] One major disadvantage of this methodology entailed the time-consuming preparation of the 16 dimers that may be required for the synthesis of one oligomer. Consequently, the monomers **22** and **250b-d** have been preferred for the solid-phase synthesis of oligonucleotides.[102b,115,465-468]

In spite of the usefulness of the *N,N*-dimethylaminophosphoramidites **22** and **250b-d** in the solid-phase synthesis of oligodeoxyribonucleotides, their application in automated systems has been unreliable because their stability in acetonitrile varied from hours to weeks depending on their purity. In an attempt to alleviate this impediment, McBride and Caruthers[31] investigated the deoxyribonucleoside phosphoramidites **119**, **254b-d** and **255-257a-d** as potential intermediates for the automated solid-phase synthesis of oligodeoxyribonucleotides.



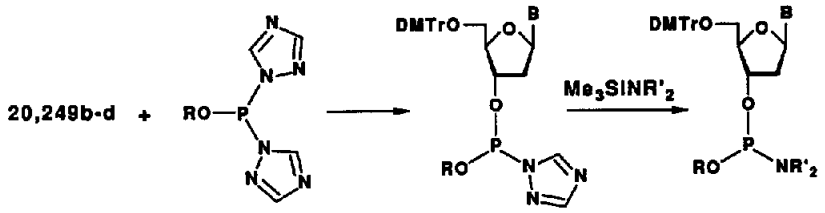
119 B= T; R= R'= i-Pr **255a-d** **256a-d** **257a-d**
254b-d R= R'= i-Pr
258a-d R= R'= Et
259a-d R= Me; R'= i-Pr

a, B= T; b, B= C^{Bz}; c, B= A^{Bz}; d, B= G^{ib}

Particularly, **255a-d** could be purified by silica gel chromatography. Moreover, **255a** was stable in

acetonitrile for a least 42 days without significant decomposition (less than 4%). Similarly, **119** was still 85% pure after being in acetonitrile for 40 days.[31] These findings were supported by Dörper and Winnacker[469] and others[470-473] who also reported the preparation and purification of **255a-d**. These intermediates were successfully used in the synthesis of deoxyribonucleotides (up to 52 bases long)[473] on either silica,[469,474] CPG[470,471] or cellulose filter discs.[44]

A different approach in the preparation of various deoxyribonucleoside phosphoramidites was proposed by Fourrey and Varenne.[475,476] Basically, the approach involved the reaction of suitably protected 5'-*O*-DMTr deoxyribonucleosides (**20** and **249b-d**) with the phosphoroditriazolides **244a** and **260-262** affording the corresponding deoxyribonucleoside phophorotriazolides **263-266a-d**.



244a R= Me
260 = $(CH_2)_2CN$
261 = $(CH_2)_2SO_2Ph$
262 = o-ClC_6H_4

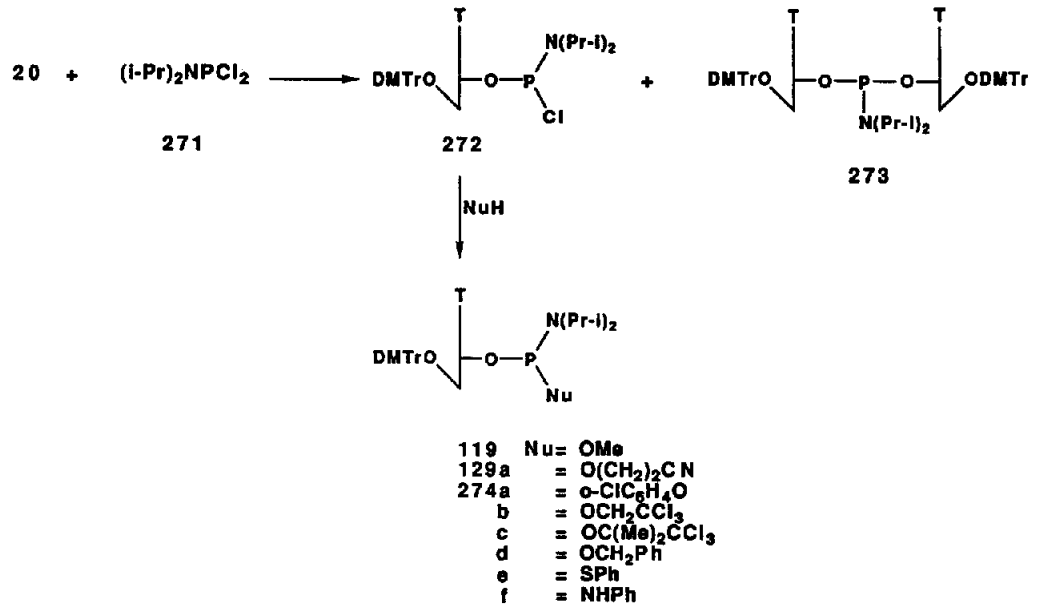
263a-d
264a-d
265a-d
266a-d

22, 250b-d R= R'= Me
254c R= Me; R'= i-Pr
255a-d R= Me; NR'$_2$= morpholino
267a-d R= o-ClC_6H_4; R'= Me
268a-d R= o-ClC_6H_4; NR'$_2$= morpholino
269d R= $(CH_2)_2CN$; NR'$_2$= morpholino
270a R= $(CH_2)_2SO_2Ph$; NR'$_2$= morpholino

The reaction of selected trimethylsilylamines with either **263a-d**, **264a-d**, **265a-d** or **266a-d** resulted in the formation of the desired phosphoramidites in good overall yields. This methodology allowed the preparation of phosphoramidites that would otherwise have been difficult to synthesize. For instance, the purification of chloro-(*o*-chlorophenoxy)*N*-morpholinophosphine which would normally be used for the preparation of **268a-d**, would have been uneasy. An obvious disadvantage of the approach was the unavoidable presence of (3'→3')-dinucleoside phosphite triesters (< 15%) resulting from the use of **244a** and **260-262**. These side-products could be eliminated by silica gel chromatography. Fourrey and Varenne[476] demonstrated that the activation of **268a** with *N*-methylanilinium trifluoroacetate promoted the coupling reaction with deoxythymidine anchored to a silica support to occur with an efficiency of ca. 95%. Thus, *N*-methylanilinium trifluoroacetate represents an alternative to 1*H*-tetrazole as an activator.

Although conceptually similar to the approach reported by Fourrey and Varenne,[475,476] an alternate pathway to the synthesis of deoxyribonucleoside phosphoramidites was advanced by Tanaka *et al.*[477] 5'-*O*-DMTr thymidine (**20**) was reacted with the bifunctional phosphitylating agent **271** generating **272** with the concomitant formation of some (3'→3')-dinucleoside phosphoramidite **273**. The addition of a nucleophile (NuH) like an alcohol, a thiol or an amine resulted in the formation of the deoxyribonucleoside phosphoramidites **119**, **129a** and **274a-f** in yields varying from 38-81%.[477] The methodology permitted the preparation of a large variety of monomeric phosphoramidites potentially useful in the solid-phase synthesis of DNA and its analogues despite the loss of expensive nucleosides through the formation of (3'→3')-dimers.

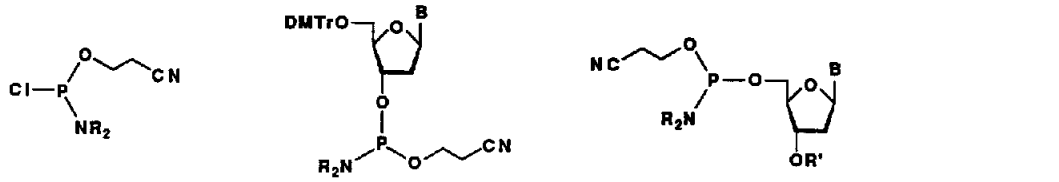
Adams *et al.*[32] also observed that increasing the steric hindrance about the P-N bond increased the stability of the phosphoramidites in acetonitrile. Specifically, they showed that **254b** was more stable than **258b** which in turn was more stable than the corresponding *N,N*-dimethylamino phosphoramidite



119 Nu= OMe
129a = $O(CH_2)_2CN$
274a = o-ClC_6H_4O
b = OCH_2CCl_3
c = $OC(Me)_2CCl_3$
d = OCH_2Ph
e = SPh
f = NHPh

derivatives. Virtually no decomposition of **119** and **254b-d** was observed after 4 days. The effectiveness of these phosphoramidites was demonstrated by the synthesis of 51-mer deoxyribonucleotides on CPG in an overall yield of ca. 10%.

Despite the popularity of the deoxyribonucleoside phosphoramidites **119** and **254b-d**[478a] in the automated synthesis of polydeoxyribonucleotides,[478b] the removal of the methyl phosphate protecting groups from synthetic oligomers required thiolates.[189,192] In particular, the use of thiophenol for this purpose[189] was an unpleasant step in the deprotection of these macromolecules. To simplify and accelerate this post-synthesis step, Sinha *et al.*[394,479] reported the preparation of deoxyribonucleoside (ß-cyanoethoxy)-*N,N*-dialkylamino-/*N*-morpholinophosphines (**129a,c,d, 277b** and **278a-d**) as monomers in the solid-phase synthesis of oligodeoxyribonucleotides. These were prepared from the reaction of properly protected 5'-*O*-DMTr deoxyribonucleosides with the monofunctional phosphitylating agents **275** or **276** essentially as reported by Beaucage and Caruthers.[30] **275** and **276** were in turn synthesized from ß-cyanoethoxy dichlorophosphine[422] and *N*-trimethylsilylated secondary amines according to the procedure of McBride and Caruthers.[31] The ß-cyanoethyl phosphate protecting group[11] was removed from oligonucleoside phosphotriesters under the basic conditions required for the cleavage of the aglycone protecting groups.



275 R= i-Pr
276 NR_2= morpholino

129a,c,d R= i-Pr
277b R= i-Pr
278a-d NR_2= morpholino

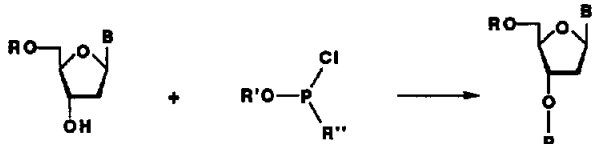
279a-d R= i-Pr; R'= levulinyl
280a-d = i-Pr; = $Si(Ph)_2Bu$-t
281a-d = i-Pr; = DMTr

a, B=T; b, B= N^4-(2-methylbenzoyl)cytosinyl; c, B=A^{Bz}; d, B= G^{ib}

Köster *et al.*[480] described the solid-phase preparation of a decanucleotide by reacting either **279a-d** or **280a-d** with a deoxyribonucleoside anchored to CPG *via* a 5'-ester linkage in the presence of 1*H*-tetrazole or 5-(*p*-nitrophenyl)-1*H*-tetrazole. However, deoxyribonucleoside phosphoramidites structurally related to **129a-d** have been by far the most popular monomers used in the automated solid-phase synthesis of oligonucleotides. Additionally, the stability of the phosphoramidites **129a-d** in wet acetonitrile was superior to that observed with the phosphoramidites **119** and **254b-d** as evidenced by ^{31}P NMR spectroscopy.[481]

A variety of other monomeric deoxyribonucleoside phosphoramidites prepared from the corresponding monofunctional phosphitylating agents have been used as a means to evaluate the properties of the P-N linkage and the various phosphate protecting groups incorporated into oligonucleotides. These are reported in Table 4.

Table 4. Various Deoxyribonucleoside Phosphoramidites as a Valuable Class of Intermediates in the Synthesis of Oligodeoxyribonucleotides



B= suitably protected nucleobase
R= DMTr or MMTr

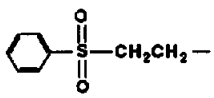
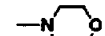
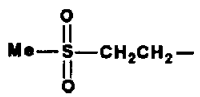
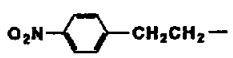
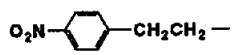
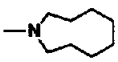
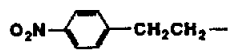
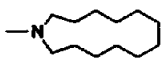
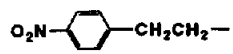
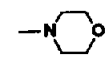
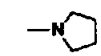
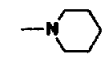
Compound	R'	R''	References
282			120c
283			537
284			482,484
285			145,482,484
286			482,484
287			145,247,483
288			482
289			482

Table 4. CONT'D

Compound	R'	R''	References
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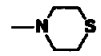
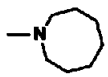
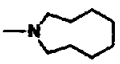
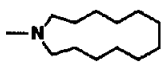
291	Me—		482
292	Me—		482
293	Me—		482
294	Me—		482
295	Me—		482
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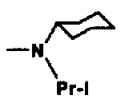
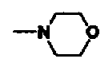
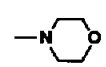
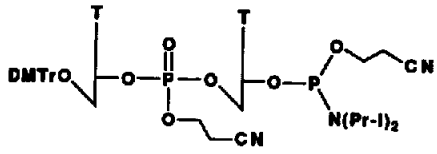
297	Me—		482
298	$NCCH_2C(Me)_2$ —		456a
299	$Cl_3CC(Me)_2$ —	—NMe_2	298a,485
300	$Cl_3CC(Me)_2$ —	—NEt_2	538
301	$Cl_3CC(Me)_2$ —	—$N(Pr\text{-}i)_2$	485
302	$Cl_3CC(Me)_2$ —		485
303	Cl_3CCH_2 —	—NMe_2	485

Particularly interesting is the reported purification of the deoxyribonucleoside phosphoramidites **282** by silica gel chromatography.[120c] It has been postulated that the phenylsulfonylethyl group stabilized the phosphoramidites and enhanced their solubilities in organic solvents. The phenylsulfonylethyl phosphate protecting group could be removed along with other *N*-protecting groups under alkaline conditions.

Alternatively, Pfleiderer *et al.*[145] demonstrated that the *p*-nitrophenylethyl phosphate protecting group along with the *p*-nitrophenylethyloxycarbonyl and *p*-nitrophenylethyl groups used for aglycone protection could simultaneously be cleaved by treatment with DBU in aprotic solvents. Consequently, the monomeric synthons **284-287** became quite attractive for the solid-phase synthesis of oligodeoxyribonucleotides.[482-484]

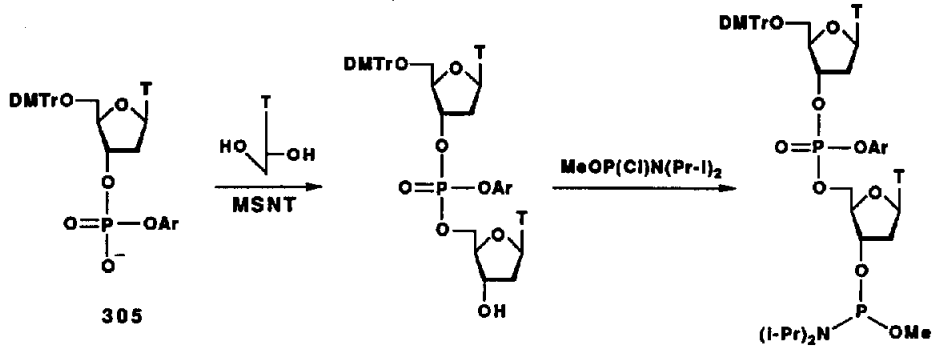
The usefulness of the deoxyribonucleoside phosphoramidites **299** and **303** in the synthesis of oligonucleotides was tested during the solid-phase preparation of a dodecathymidylic acid. Typically, the activation of **303** with 5-trifluoromethyl-1*H*-tetrazole and *N*-methylimidazole hydrochloride produced an average coupling yield of 90% whereas only a 70% stepwise yield was recorded when **301** was used under identical conditions.[485] The difference in coupling yields was attributed to the steric bulk of the 2,2,2-trichloro-1,1-dimethylethyl group.

Using a strategy similar to that reported by Kumar and Poonian,[464] Wolter *et al.*[486] delineated the preparation of the dimeric phosphoramidite **304** and reported the synthesis of a large oligomer (101-mer) on CPG using 5-(*p*-nitrophenyl)-1*H*-tetrazole as activator.



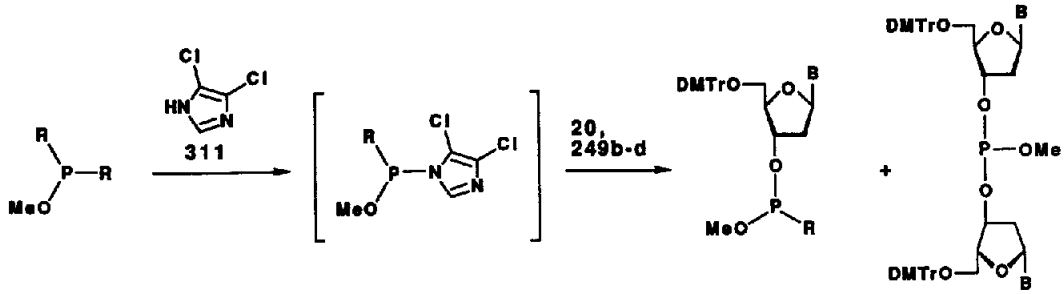
304

A different approach in the preparation of dimeric phosphoramidites for the solid-phase synthesis of oligonucleotides was described by Miura *et al.*[487] The procedure involved the preparation of the dinucleoside phosphotriester **306** from the condensation of the diester **305** with deoxythymidine in the presence of 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT). The phosphitylation of **306** with chloro-(*N,N*-diisopropylamino)methoxyphosphine afforded **307** which was successfully used in the automated preparation of a pentadecathymidylic acid. The cleavage of the oligonucleotide from the support and the removal of both methyl and *o*-chlorophenyl phosphate protecting groups were achieved by treatment with concentrated ammonium hydroxide.[487]



The preparation, isolation and purification of monomeric deoxyribonucleoside phosphoramidites was time consuming. In an attempt to shorten this process, Beaucage reported the highly selective activation of bis-(pyrrolidino)methoxyphosphine (**309**) by 4,5-dichloroimidazole (**311**) as a rapid route to the preparation of deoxyribonucleoside phosphoramidites *in situ*.[488] The objective of this approach was to automate the preparation of either **22**, **119**, **250b-d**, **254b-d** or **256a-d** and use, within a short

period of time, the monomeric phosphoramidites in standard solid-phase DNA synthesis. In addition to alleviating the problems associated with the isolation and purification of deoxyribonucleoside phosphoramidites this strategy would eliminate the problems pertaining to the stability of the phosphoramidites in solution for extended periods of time.



308 R= N,N-dimethylamino **309** = N-pyrrolidino **310** = N,N-diisopropylamino

312 R= N,N-dimethylamino **313** = N-pyrrolidino **314** = N,N-diisopropylamino

22,250b-d **119,254b-d** **256a-d** **315a-d**

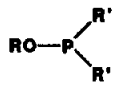
It was shown that **256a-d** could be prepared in yields exceeding 86% from the reaction of **20** and **249b-d** with **309** and **311** in 1-methyl-2-pyrrolidinone. The reaction was complete within 10 min according to ^{31}P NMR spectroscopy and less than 10% of the (3'→3')-dimers **315a-d** were detected. Although the generation of **22, 250b-d** and **256a-d** occurred in high yields within minutes, no formation of **119** and **254b-d** was observed during the same period of time. According to the data reported by Moore and Beaucage[489] the selective activation of **308-310** by **311** led to the formation of the reactive intermediates **312-314** which could be detected by ^{31}P NMR spectroscopy. The rates at which the phosphitylation of **20** and **249b-d** by **312-314** would occur, were presumably dependent on the steric bulk of the phosphitylating agent. The phosphoramidites **256a-d** generated *in situ* were successfully activated by 1*H*-tetrazole and used in the solid-phase synthesis of a deoxyribonucleotide (22 bases long) with an average coupling yield of 95%. Despite its attractive features this approach has not been evaluated in the automated synthesis of oligodeoxyribonucleotides.

Subsequently, Barone *et al.*[184] along with Lee and Moon[490] also described the selective activation of bis-(*N,N*-dialkylamino)alkoxyphosphines. Specifically, the interaction of 5'-*O*-DMTr deoxyribonucleosides (**20** and **249b-d**) with the phosphorodiamidite **310** and *N,N*-diisopropylammonium tetrazolide[87,184] or 1*H*-tetrazole[184,490] in dichloromethane or acetonitrile afforded the corresponding deoxyribonucleoside phosphoramidites **119** and **254b-d** in isolated yields varying between 82-92%. Less than 1% of the (3'→3')-dimer **315a** was detected when **20** was used as starting material. The average time required to generate deoxyribonucleoside phosphoramidites *in situ* under these conditions was about 1 h.[184]

Because of the facile preparation of deoxyribonucleoside phosphoramidites from the selective activation of phosphorodiamidites by 1*H*-tetrazole or its *N,N*-diisopropylammonium salt, a variety of these bifunctional phosphitylating agents have been prepared. (see Table 5)

Nielsen *et al.*[491] pointed out that those phosphorodiamidites with alkoxy groups capable of ß-elimination (**316** and **321**) were thermally unstable. However, their conversion into the corresponding alkylphosphonic diamides was inhibited by bulky *N*-substituents. For example, **317** and **322** were thermally stable and could be distilled without significant decomposition. Unexpectedly, the (2-cyano-1,1-dimethylethoxy)-bis-(diethylamino)phosphine **319** was thermally stable and reacted with suitably protected deoxyribonucleosides and 1*H*-tetrazole to yield the corresponding deoxyribonucleoside phosphoramidites.[325b] These were utilized in the solid-phase preparation of an heptadecanucleotide. The stepwise oxidation reaction was effected by *tert*-butyl hydroperoxide during the synthesis because the standard aqueous iodine oxidation was apparently unsatisfactory.

Table 5. Various Phosphorodiamidites Useful in the Synthesis of Nucleoside Phosphoramidites.



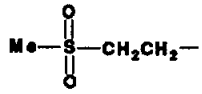
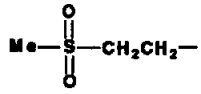
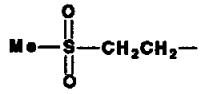
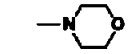
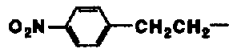
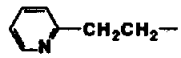
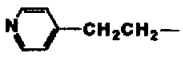
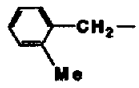
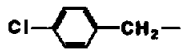
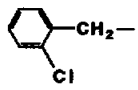
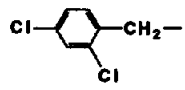
Compound	R	R'	References
316	$NCCH_2CH_2$—	—NEt_2	491
317	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$	87,325e,491 500b,539,540
318	$NCCH_2CH(Me)$—	—NEt_2	491
319	$NCCH_2C(Me)_2$—	—NEt_2	491
320	$NCCH_2C(Me)_2$—	—$N(Pr\text{-}i)_2$	491
321		—NEt_2	491
322		—$N(Pr\text{-}i)_2$	491
323			347b
324	Cl_3CCH_2—	—$N(Pr\text{-}i)_2$	500b
325	$Cl_3CC(Me)_2$—	—$N(Pr\text{-}i)_2$	500b
326		—$N(Pr\text{-}i)_2$	500b
327		—$N(Pr\text{-}i)_2$	500a-c
328		—$N(Pr\text{-}i)_2$	501
329		—$N(Pr\text{-}i)_2$	347b
330		—$N(Pr\text{-}i)_2$	347b
331		—$N(Pr\text{-}i)_2$	347b
332		—$N(Pr\text{-}i)_2$	347b

Table 5. CONT'D

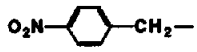
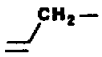
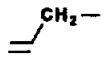
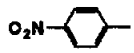
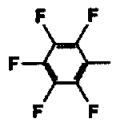
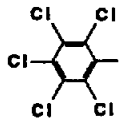
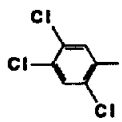
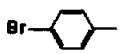
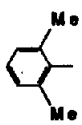
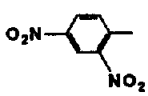
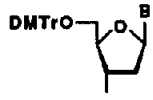
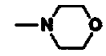
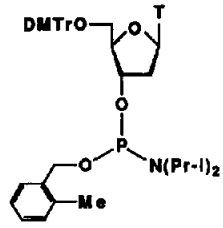
Compound	R	R'	References
333		—N(Pr-i)$_2$	347b
334		—N(Pr-i)$_2$	305,307
335		—NMe$_2$	503
336	Ph—	—NEt$_2$	492
337		—NEt$_2$	492
338		—NEt$_2$	492
339		—NEt$_2$	492
340		—NEt$_2$	492
341		—NEt$_2$	492
342		—NEt$_2$	492
343		—NEt$_2$	492
344		—NEt$_2$	492
345		—NEt$_2$	492

Table 5. CONT'D

Compound	R	R'	References
346	$(CF_3)_2CH$—	—$N(Pr\text{-}i)_2$	504
347		—$N(Pr\text{-}i)_2$	325c,494
348		—NEt_2	496
349			495

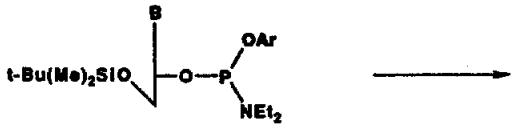
An analogous observation concerning the behavior of the *o*-methylbenzyl group as a phosphate protecting group was reported by Caruthers *et al.*[347b] The deoxyribonucleoside phosphoramidite **350** prepared from **20** and **329** in the presence of *N,N*-diisopropylammonium tetrazolide was used in the solid-phase synthesis of an oligothymidylic acid. It was observed that during the aqueous iodine oxidation step, the *o*-methylbenzyl group was quantitatively removed. Instead of searching for a compatible oxidizing reagent, Caruthers *et al.*[347b] realized that the absence of phosphate protecting groups did not inhibit the subsequent elongation of the DNA segment. It was postulated that mixed anhydrides resulting from the interaction of phosphate diesters and activated deoxyribonucleoside phosphoramidites could be cleaved by excess 1*H*-tetrazole to regenerate deoxyribonucleoside phosphorotetrazolide intermediates. Under these conditions, the synthesis of the oligothymidylic acid (20-mer) was achieved with an average stepwise yield of 96%. These observations demonstrated that deoxyribonucleotides could be prepared in the absence of phosphate protecting groups from specific monomeric deoxyribonucleoside phosphoramidites.



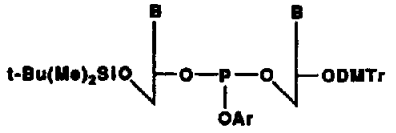
350

The application of *O*-arylphosphoramidites toward the preparation of natural and modified internucleotide linkages was investigated by Eritja *et al.*[492] The monomeric deoxyribonucleoside phosphoramidites **351a-j** were prepared *in situ* from **336-345** in the presence of one equivalent of 1*H*-tetrazole. After purification by chromatography on silica, **351a-j** were activated by *N*-methylanilinium trifluoroacetate[476] and coupled with 3'-*O*-DMTr deoxyribonucleosides affording the corresponding dinucleoside phosphite triesters **352a-j**. The rate of coupling depended on the phenyl ring substituents

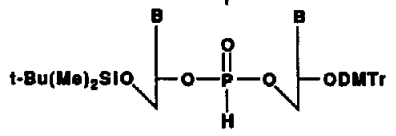
and the electron-withdrawing character of the substituents. For example, **351g** reacted faster than **351f** and both were slower than **351h**.



351a Ar= 2,4-dinitrophenyl
b = pentafluorophenyl
c = pentachlorophenyl
d = 2,4,5-trichlorophenyl
e = 4-nitrophenyl
f = 2-bromophenyl
g = 4-bromophenyl
h = phenyl
i = 2-methylphenyl
j = 2,6-dimethylphenyl



352a-j

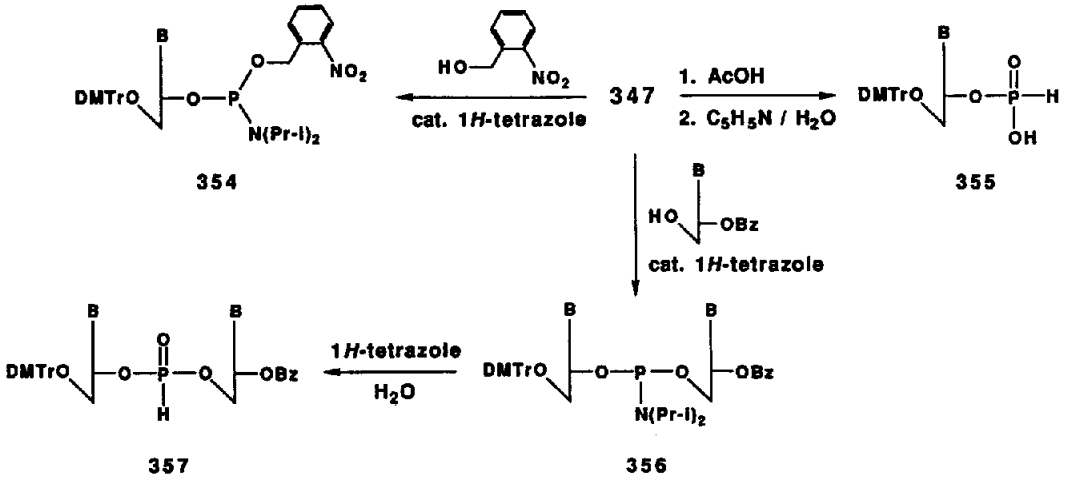


353

Interestingly, **352h** was converted under mild acidic hydrolysis to the dinucleoside *H*-phosphonate **353** within 10 min.[492] The latter could be transformed into dinucleotides having either a natural or a modified internucleotidic link.[493]

Alternatively, the interaction of 5'-*O*-DMTr-2'-deoxythymidine-3'-*O*-(*N,N*-diethylamino) phenoxyphosphine with *N*-methylanilinium trifluoroacetate and 2'-deoxythymidine bound to a silica support generated coupling yields of only 40-60%. The low yields were apparently caused by hydrolysis of the deoxyribonucleoside phosphoramidites during the coupling step.[492] Further investigation is needed to improve the preparation of oligonucleoside *H*-phosphonates on solid support *via* the *O*-aryl phosphoramidite approach.

Deoxyribonucleoside phosphorodiamidites (**347**) prepared from suitably protected deoxyribonucleosides and bis-(*N,N*-diisopropylamino)chlorophosphine represent valuable intermediates in the preparation of various deoxyribonucleoside phosphoramidites (*e.g.* **354**) potentially useful in the solid-phase synthesis of oligonucleotides.[325c] Alternatively, the reaction of **347** with 3'-*O*-benzoyl deoxyribonucleosides under identical conditions afforded the dinucleoside phosphoramidites **356**. Treatment of the latter with 1*H*-tetrazole and water generated the dinucleoside *H*-phosphonates **357** in quantitative yields.[325c]



354 **355** **357** **356**

As reported above, the internucleotide linkage in **357** could be converted into a natural linkage or an analogue. For example, Nielsen *et al.*[494] have synthesized and characterized dinucleoside phosphorodithioates. Thus, **347** was reacted with a 3'-*O*-acylated deoxyribonucleoside leading to a dinucleoside phosphoramidite analogous to **356** which upon treatment with hydrogen sulfide and 1*H*-tetrazole generated a dinucleoside *H*-thiophosphonate also analogous to **357**. Oxidation of the latter with elemental sulfur afforded the corresponding dinucleoside phosphorodithioate as a new class of potential therapeutic agents.[494]

Uznanski *et al.*[495] described the preparation of **349** and its reaction with excess 1*H*-tetrazole and a 3'-*O*-protected deoxyribonucleoside to yield the corresponding dinucleoside phosphoromorpholidite. The hydrolysis of the phosphoromorpholidite with a 0.5 M solution of 1*H*-tetrazole containing 20% water led to a dinucleoside *H*-phosphonate analogous to **357**. Similarly, the alcoholysis of the dinucleoside phosphoromorpholidite with anhydrous ethanol or isopropanol afforded the corresponding dinucleoside phosphite triesters. This strategy was further exploited by Yamana *et al.*[496] as they reported the preparation of **348** by reacting 5'-*O*-DMTr deoxyribonucleosides with tris-(diethylamino)phosphine and *N,N*-diisopropylammonium tetrazolide. Without purification, **348** was activated by 5-(*p*-nitrophenyl)-1*H*-tetrazole and used in the solid-phase synthesis of a decanucleotide. Each coupling step was followed by a hydrolytic step effected by a solution of 1*H*-tetrazole and water to generate an *H*-phosphonate internucleotide link. An average coupling yield of 97% was obtained according to this methodology.

Monomeric deoxyribonucleoside *H*-phosphonates (**355**) based on the original findings of Hall *et al.*,[3] have been used by Garegg *et al.*[35,497] and later by others[36,493,498] in the synthesis of oligonucleotides and their analogues. **355** was conveniently prepared and isolated in yields exceeding 85% from the reaction of the phosphorodiamidite **348** with acetic acid followed by hydrolysis with pyridine-water. The phosphorodiamidite **347** could also be used for the preparation of **355** despite a much slower acidolysis rate.[499]

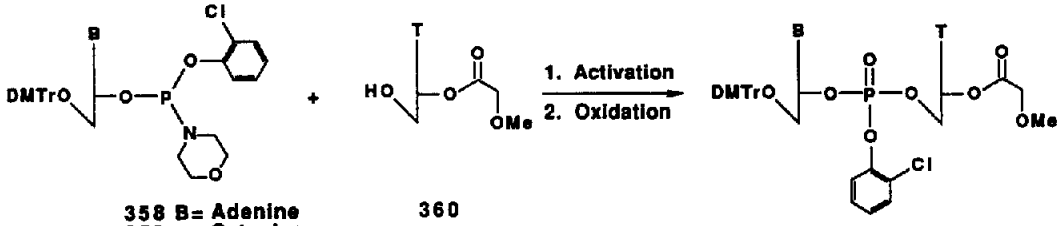
Deoxyribonucleoside phosphoramidites carrying the 2-(2-pyridyl)ethyl[500] and the 2-(4-pyridyl)ethyl group[501] have also been generated *in situ* and used in solid-phase DNA synthesis upon activation with 1*H*-tetrazole or 5-(*p*-nitrophenyl)-1*H*-tetrazole. The removal of the 2-(2-pyridyl)ethyl or the 2-(4-pyridyl)ethyl group was accomplished by treatment with phenyl chloroformate followed by concentrated ammonium hydroxide[501] or by treatment with methyl iodide without any apparent alkylation of the nucleobases.[502]

Allyl groups were shown by Hayakawa *et al.*[503] to be useful protecting groups for internucleotidic phosphodiester linkages. Typically, 5'-*O*-MMTr deoxythymidine was reacted with the allyloxy phosphorodiamidite **335** and 1*H*-tetrazole followed by 3'-*O*-TBDMS deoxythymidine affording, after oxidation with dinitrogen tetroxide at −78 °C, the dinucleoside allylphosphate triester in 86% isolated yields. A variety of mild conditions for the removal of the allyl group involving $Pd[P(C_6H_5)_3]_4$, amines or ammonium formates were also reported.[503] This approach has been applied to the synthesis of branched oligoribonucleotides.[306] Recently, Hayakawa *et al.*[305,307] demonstrated the usefulness of the allylic protection method in solid-phase oligonucleotide synthesis. Deoxyribonucleoside phosphoramidites prepared *in situ* from the reaction of properly protected deoxyribonucleosides with **334** in the presence of *N,N*-diisopropylammonium tetrazolide were efficiently used to generate DNA segments in sizes varying between 32-60 bases long. The allyl phosphate protecting group and the allyloxycarbonyl group protecting the exocyclic amino function of cytosine, adenine and guanine were deblocked rapidly and efficiently in one step by treatment with a mixture of tris-(dibenzylideneacetone)dipalladium-(0)-chloroform complex, triphenylphosphine and *n*-butylamine-formic acid.[305,307]

The 1,1,1,3,3,3-hexafluoro-2-propyl (HFP) group for the protection of internucleotidic linkages has been evaluated by Takaku *et al.*[504] The reaction of bis-(diisopropylamino)chlorophosphine with 1,1,1,3,3,3-hexafluoro-2-propanol afforded **346** in 78% yield. Properly protected deoxyribonucleoside phosphoramidites carrying the HFP group were prepared *in situ* from **346** and purified by silica gel chromatography. Activation of the pure deoxyribonucleoside phosphoramidites by 1*H*-tetrazole

permitted the synthesis of a decanucleotide on a CPG support with stepwise coupling yields of ca. 95%. The removal of the HFP phosphate protecting group was achieved by N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-4-nitrobenzaldoximate in dry acetonitrile.[504] The HFP group thus adds to the repertoire of suitable phosphate protecting groups in the synthesis of oligonucleotides *via* the phosphoramidite approach.

Early reports by Letsinger *et al.*[505a] and Ogilvie *et al.*[404,418] mentioned that the protection of the exocyclic amino function of the nucleobases was not required with the phosphorodichloridite procedure. Hayakawa *et al.*[506] have shown that the chemoselective phosphorylation of *N*-unprotected nucleosides and the formation of internucleotide linkages could also be achieved by activation of hydroxy functions with organometallic reagents. Additional lines of evidence provided by Shimidzu *et al.*[507] indicated that the polymerization of unprotected ribonucleosides with phosphorus tris-azole led to a rapid synthesis of short homooligoribonucleotides composed of either uracil, adenine or cytosine bases with mixed (3'→5')- and (2'→5')-phosphodiester linkages. Moreover, Fourrey and Varenne[508a] demonstrated that monomeric *N*-unprotected deoxyribonucleoside phosphoramidites (**358** and **359**) could be used in the preparation of dinucleoside phosphate triesters. For example, the phosphoramidite **358** was prepared from the reaction of 5'-*O*-DMTr-2'-deoxyadenosine with 2-chlorophenoxy-bis(triazolyl)phosphine at −78 °C followed by an excess of morpholine. After purification, **358** was activated with *N*-methylanilinium trifluoroacetate and reacted with 3'-*O*-methoxyacetyl thymidine (**360**) affording, upon oxidation, the dimer **361** in high yields. Subsequently, Gryaznof and Letsinger[505b] described the solid-phase synthesis of oligonucleotides from 5'-*O*-DMTr-2'-deoxyribonucleoside-3'-*O*-(*N*,*N*-diisopropylamino)methoxy phosphines having unprotected nucleobases. The synthetic protocol included a treatment with an equimolar solution (0.1 M) of pyridine hydrochloride and aniline in acetonitrile after each coupling to cleave the nucleobase adducts stemming from the oligonucleotidic chain prior to the oxidation step. This strategy allowed the preparation of a 20-mer and should facilitate the synthesis of oligonucleotides containing base-sensitive functional groups *via* an appropriate solid support (such as **43**).

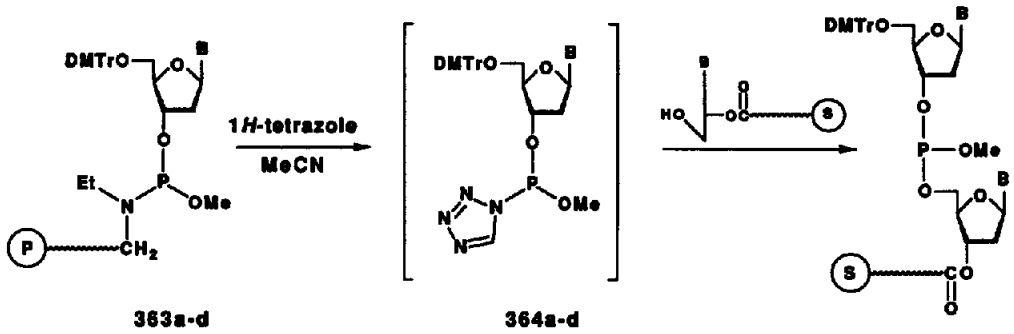


358 B= Adenine
359 = Cytosine

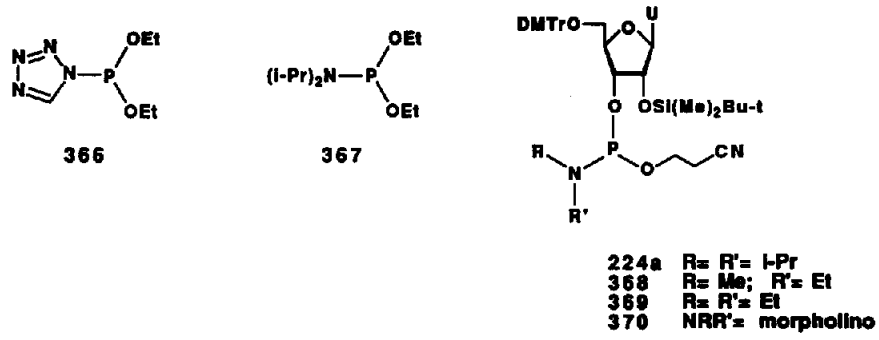
360

361 B= Adenine
362 = Cytosine

The key step in the automated synthesis of an oligonucleotide by the phosphoramidite approach is the 1*H*-tetrazole-mediated coupling reaction of a 5'-*O*-DMTr-nucleoside-3'-*O*-phosphoramidite with the 5'-OH group of the oligonucleotide anchored to the polymeric support. The mechanism of this coupling reaction has been investigated and it is generally accepted that the process is acid-catalyzed.[30,509] The proposed mechanisms include *N*-protonation,[488,510] *P*-protonation[511,512] and/or nucleophilic catalysis by the conjugate base of the acid.[510] However, only few studies have, so far, been carried out to delineate the mechanistic details of the coupling step. A major problem, has been the extremely rapid kinetics (less than 30 s) of the coupling reaction which precluded the isolation and characterization of the proposed intermediates. In an elegant approach, Seliger and coworkers[135,513] demonstrated that the activation of the solid-phase bound deoxyribonucleoside phosphoramidites **363a-d** by 1*H*-tetrazole generated the putative intermediates **364a-d** which efficiently phosphitylated deoxyribonucleosides covalently attached to another solid support. This approach enabled the solid-phase preparation of the oligothymidylate dT_8 with an average coupling yield greater than 95%. This experiment showed that protonation of the phosphoramidites was necessary but, in this specific case, not sufficient for the phosphitylation of deoxyribonucleosides bound to a solid support.



Recently, Berner *et al.*[514] described the preparation of the diethoxy-(*N*-tetrazolyl)phosphine **366** as a model compound to further assess the intermediacy of **364a-d** during the coupling step mediated by 1*H*-tetrazole. The ^{31}P NMR spectrum of **366** exhibited a resonance at 126 ppm. This resonance was also observed when the diethoxy-(*N,N*-diisopropylamino)phosphine **367** or the deoxyribonucleoside phosphoramidite **119** was treated with 1*H*-tetrazole in acetonitrile. Moreover, Stec and Zon[515] reported that the *Rp* or the *Sp* diastereoisomers of **119** and **254b-d** underwent rapid epimerization at phosphorus under similar conditions, thereby generating racemic deoxyribonucleoside phosphite triesters upon reaction with ethanol. These experiments were consistent with the notion that the activation of deoxyribonucleoside phosphoramidites by 1*H*-tetrazole occurred *via* a rapid protonation followed by the reversible and slower formation of the phosphorotetrazolides **364a-d**.[514]



Interestingly, the activation of deoxyribonucleoside phosphoramidites with octanoic acid which has a *pKa* similar to 1*H*-tetrazole (*pKa* = 4.9 and 4.8 respectively) did not promote the formation of internucleotide links at an appreciable rate.[515] It has, however, been shown that the activation of **367** with acetic acid (*pKa* = 4.76) to yield the corresponding acyl phosphite derivative, occurred as rapidly as the activation of **367** by 1*H*-tetrazole according to ^{31}P NMR spectroscopy.[514] Collectively, these results indicated that the protonation of the deoxyribonucleoside phosphoramidites by 1*H*-tetrazole during the coupling step was not rate-limiting as incorrectly inferred by others.[365] In addition to the activation mechanism, other factors affecting the coupling rates of activated deoxyribonucleoside phosphoramidites were investigated by Dahl *et al.*[516] It was found that the coupling rates varied with the nature of the *N,N*-dialkylamino and *O*-alkyl/aryl functions of the phosphoramidites in the following order: *N,N*-dimethylamino > *N,N*-diisopropylamino > *N*-morpholino > *N*-methylanilino and *O*-methyl > *O*-(2-cyanoethyl) > *O*-(1-methyl-2-cyanoethyl) > *O*-(1,1-dimethyl-2-cyanoethyl) >> *O*-(*o*-chlorophenyl). It is to be noted that these findings resulted from the addition of 1*H*-tetrazole to

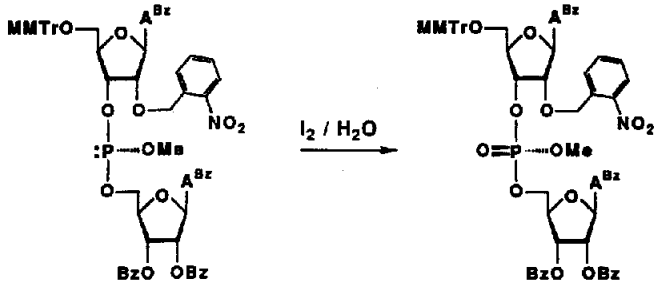
premixed phosphoramidites and 5'-OH deoxyribonucleosides. These conditions were different than those normally used during oligonucleotide synthesis which involved the addition of premixed phosphoramidites and 1*H*-tetrazole to 5'-OH deoxyribonucleosides. Consequently, caution must be exercised in the interpretation of these data.

In agreement with the observations of Dahl *et al.*,[516] Gasparutto *et al.*[517] demonstrated that the ribonucleoside phosphoramidites **368** and **369** upon activation with 1*H*-tetrazole led to faster coupling reactions than that observed with **224a** or **370** under the same conditions. Coupling yields of 96-97% (average of 8-14 synthesis cycles) were obtained within 4 min. The preparation of **368** was performed according to a published procedure[426a] without detectable (2'⇌3') migration of the TBDMS group.

Although 1*H*-tetrazole has been commonly used in the activation of nucleoside phosphoramidites, 5-(*p*-nitrophenyl)-1*H*-tetrazole,[365,429,480,486,501,512] *N*-methylanilinium trifluoroacetate,[476,508] *N*-methylanilinium trichloroacetate,[518] 5-trifluoromethyl-1*H*-tetrazole,[485] 1-hydroxybenzotriazole,[456a,537] *N*-methylimidazole hydrochloride[485] and *N*-methylimidazolium trifluoromethanesulfonate[59,519] have also been proposed as activators.

Despite the high efficiency of the phosphoramidite approach, the chain elongation step was not accomplished in quantitative yields. Consequently, at the end of the synthesis the desired oligomer was mixed with a sub-population of shorter oligomers (by at least 1 base). With increasing chain lengths the purification of the desired *n*-mer oligonucleotide from the (*n*-1)-mer may be difficult. To simplify this problem the stepwise acetylation of the unphosphitylated chains (capping step) was performed to terminate the extension of these unwanted oligomers. The preferred capping reagent consists of acetic anhydride, 2,6-lutidine and *N*-methylimidazole in tetrahydrofuran.[182,186] In addition to preventing further elongation of unphosphitylated oligomers, the capping reagent has been effective in reducing the concentration of the O^6-phosphitylated guanine residues generated during the coupling step.[180,182] It is therefore recommended that the capping reaction be performed before the oxidation step which, otherwise, would convert O^6-phosphitylated guanine residues into the considerably more stable O^6-phosphorylated guanine derivatives. Unwanted chain growth and ramification would stem from these modified guanine bases.

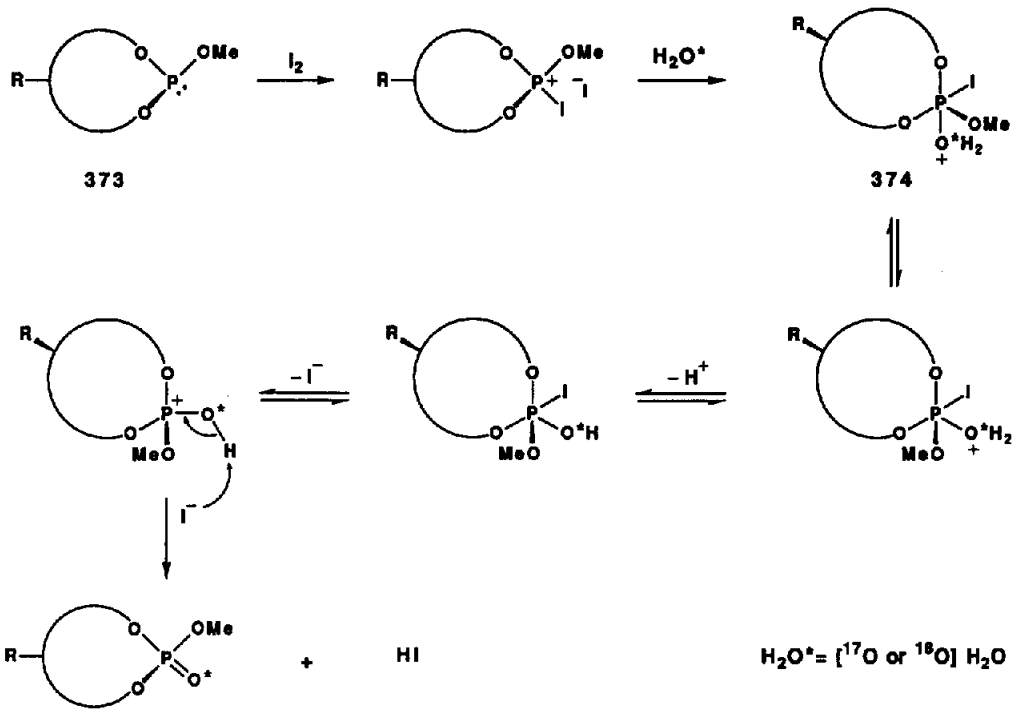
The stepwise oxidation of the internucleotide phosphite triester to the phosphotriester also represents a crucial step in the automated synthesis of oligonucleotides by the phosphoramidite approach. First introduced by Letsinger and coworkers, an aqueous solution of iodine containing 2,6-lutidine[26] or pyridine[186,425,520] has been the most commonly used reagent for this task. The mechanism and stereochemistry of this reaction have been investigated by Cullis.[521] It was shown that the oxidation of the diastereoisomeric mixture of **371a** and **371b** (2:1) by aqueous iodine under the conditions described by Letsinger *et al.*[26] yielded the phosphate triesters **372a** and **372b** in a ratio of 2:1 respectively. Model reactions unambiguously confirmed that this oxidation occurred with overall retention of configuration.[521] Interestingly, the oxidation of diastereoisomeric dinucleoside *H*-phosphonates with iodine-[^{18}O]H_2O was not stereospecific and preferentially led to the formation of the *Sp* [^{18}O] phosphodiester.[522]



371a major
b minor, epimeric at P

372a major
b minor, epimeric at P

Bentrude *et al.*[523] rationalized the retentive stereochemistry observed in the aqueous iodine oxidation of cyclic phosphites (373) by invoking the apical introduction of water and the equatorial positioning of iodine during the formation of the initial pentacovalent intermediates 374.



The development of non-aqueous oxidizing agents as a means to eliminate the potential interference of water with the anhydrous steps involved in the solid-phase synthesis of oligonucleotides, has attracted some attention. Oxidizing reagents such as dinitrogen tetroxide,[523,524] *tert*-butyl hydroperoxide,[525-527] di-*tert*-butyl hydroperoxide,[527] cumene hydroperoxide,[527] hydrogen peroxide,[527] bis-(trimethylsilyl) peroxide in the presence of catalytic amounts of trimethylsilyl triflate,[527,528] *m*-chloroperbenzoic acid,[97a,529] dimethyl sulfoxide,[527] trimethylamine-*N*-oxide,[527] pyridine-*N*-oxide,[527] *N*-methylmorpholine-*N*-oxide,[527] iodobenzene diacetate,[508b] tetra-*n*-butylammonium periodate[508b] and O_2/AIBN under thermal or photochemical conditions[523] were applied to the oxidation of nucleoside phosphite triesters. Of these, only bis-(trimethylsilyl) peroxide-trimethylsilyl triflate[528] and *m*-chloroperbenzoic acid in the presence of pyridine[97,453] provided oxidation rates comparable to those observed with aqueous iodine during the solid-phase synthesis of oligonucleotides. Thus, aqueous iodine continues to be the reagent of choice for the oxidation of nucleotidic phosphite triesters, given its mildness, stability, efficiency and rapid reaction kinetics.

Dinucleotides and oligonucleotides having chiral phosphodiester linkages were readily obtained from the stepwise oxidation of phosphite triesters with iodine and isotopically enriched [^{17}O or ^{18}O] water.[530-534] Using a similar protocol, Seela *et al.*[535,536] have also prepared *N,N*-diisopropyl phosphoramidites of appropriately protected *Rp* and *Sp* d{T-[P(^{18}O)]-A} toward the synthesis of d{GAGT-[(*Rp*)-P(^{18}O)]-ACTC} and d{GAGT-[(*Sp*)-P(^{18}O)]-ACTC}. These octamers were prepared to probe the stereochemical course of hydrolyses catalysed by phosphodiesterases.

The most popular techniques involved in the purification and characterization of synthetic oligonucleotides have been extensively reviewed[15,34,541,542] and, consequently, will not be discussed

herein. Precautionary measures must, however, be applied while working with oligoribonucleotides. The ubiquitous nature of RNase required the use of sterile equipment and reagents during the purification of RNA oligomers. These measures have been delineated by Usman *et al.*[426a]

CONCLUDING REMARKS

Due to the scope of this Report the applications of phosphoramidite intermediates toward: (*i*) the 5'- and 3'- functionalization of oligonucleotides; (*ii*) the incorporation of modified nucleosides into oligonucleotides; (*iii*) the preparation of "branched" oligoribonucleotides; (*iv*) the synthesis of sterol-nucleoside phosphates, glycosyl phosphates, *myo*-inositol phosphates, phosphopeptides and nucleopeptides, have not been discussed. We will review these applications in a forthcoming Report.

It is nonetheless clear that the discovery and utilization of nucleoside phosphoramidites along with the recent developments in solid support derivatization and improved strategies for the protection of nucleosidic functions have had an impact on biological sciences and have resulted in an increasing demand for oligonucleotides. These molecules provide powerful tools to scientists in their efforts to unravel the mechanism(s) by which complex biological processes occur both *in vitro* and *in vivo*.

However, much remains to be done to further improve the coupling efficiency of the phosphoramidite monomers to enable the synthesis of larger DNA segments and provide a simpler and faster preparation of specific genes. With the emerging field of gene therapy, the availability of engineered genes shall open the door to exciting and challenging biomedical applications for the benefit of mankind.

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