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The Functionalization of Oligonucleotides Via Phosphoramidite Derivatives

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Contents

INTRODUCTION

The rapid growth of biotechnology has triggered an increasing demand for modified oligonucleotides dedicated mostly to diagnostic and therapeutic applications. The plethoric number of such applications has spurred the development of nucleosidic and non-nucleosidic phosphophoramidite derivatives for the functionalization of specific oligonucleotides. While the major contribution of the phosphoramidite approach to the evolution of oligonucleotide synthesis has been addressed earlier,[1] the

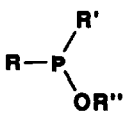
scope of the present Report encompasses the synthethic applications of a wide spectrum of phosphoramidite derivatives allowing the incorporation of fluorescent, lipophilic, intercalating, cross-linking, alkylating and DNA cleaving entities into oligonucleotides. Such derivatizations have been targeted to the 5'-/3'- termini, to the internucleotidic phosphodiester linkages, or to the nucleobases and will be discussed at length.

1. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE 5'-TERMINUS

1.1. Phosphorylation.

The automated phosphorylation of the 5'-OH function of oligonucleotides synthesized by the current solid-phase methods represents an attractive feature, as most biological applications require oligonucleotides carrying a terminal 5'-phosphate group. Engels[2] and others[3-5] have addressed the issue by reporting the preparation of the phosphoramidites **1-3** (Table 1) for this specific application.

Table 1. Phosphoramidite Derivatives for the Functionalization of Oligonucleotides at the 5'-Terminus



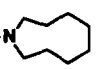
Compound	R	R'	R''	References
1a	$NCCH_2CH_2O$—	—$N(Pr\text{-}i)_2$	—CH_2CH_2CN	2a,3a,4,6
b	CH_3O—			
2	CH_3O—	—$N(Pr\text{-}i)_2$	—CH_2CH_2—  —NO_2	2a-b,5
3	O_2N—  —CH_2CH_2O—	—$N(Pr\text{-}i)_2$	—CH_2CH_2—  —NO_2	2a
4	O_2N—  —CH_2CH_2O—	—N 	—CH_2CH_2—  —NO_2	7
5	O_2N—  —CH_2CH_2O—	—N 	—CH_2CH_2—  —NO_2	7
6	$NCCH_2CH_2O$—	—$N(Pr\text{-}i)_2$	—CH_2—  —Cl	3a,b
7	$H_2C{=}CHCH_2O$—	—$N(Pr\text{-}i)_2$	—$CH_2CH{=}CH_2$	3b
8	$Ph_3SiCH_2CH_2O$—	—$N(Pr\text{-}i)_2$	—CH_2CH_2CN	8
9	$(CH_3)_3CO$—	—NEt_2	—$C(CH_3)_3$	9,10
10	$DMTrO(CH_2)_2SO_2(CH_2)_2O$—	—$N(Pr\text{-}i)_2$	—CH_2CH_2CN	11
11	CH_3O—	—N 	—$(CH_2)_nSTr$ n= 2,3,6	21,62

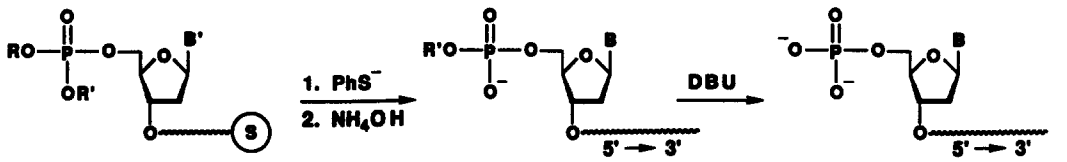
Table 1. CONT'D

Compound	R	R'	R''	References
12	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2)_2NHC(=O)CF_3$	26,27
13a	CH_3O-	$-N(Pr\text{-}i)_2$	$-(CH_2)_6NHC(=O)CF_3$	28-33,49
b	$NCCH_2CH_2O-$			39b
14	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2)_n$NHFmoc n= 2,6	34a-b,35 50
15a	CH_3O-	$-N(Pr\text{-}i)_2$	$-(CH_2)_3$NHMMTr	25,36
b	$NCCH_2CH_2O-$			
16	CH_3O-	$-N(Pr\text{-}i)_2$	$-(CH_2)_5$NHDMTr	37
17a	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2)_6$NHDMTr	39a
b			$-(CH_2)_6$NHMMTr	38,39 40a-b,41a-b
18	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2)_6$NHPx	39a
19	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2CH_2O)_3CH_2CH_2N$ (phthalimide)	42
20	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-CH(CH_2ODMTr)(CH_2NHFmoc)$	48
21	CH_3-	$-N(Pr\text{-}i)_2$	$-(CH_2)_6$NHFmoc	51
22	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2)_n$STr n= 3,6	39,61 63a,64a,65
23	CH_3O-	$-N(Pr\text{-}i)_2$	$-(CH_2)_3$STr	66
24	$NCCH_2CH_2O-$	$-N(Pr\text{-}i)_2$	$-(CH_2)_6O-P(=S)(OCH_2CH_2CN)(OCH_2CH_2CN)$	74a

DMTr = di-(p-anisyl)phenylmethyl
MMTr = (p-anisyl)diphenylmethyl
Tr = triphenylmethyl
Px = (9-phenyl)xanthen-9-yl
Fmoc = 9-fluorenylmethyloxycarbonyl

The reaction of **2** with the 5'-OH group of a protected deoxyribonucleotide, covalently bound to a solid support, and 1*H*-tetrazole afforded the phosphotriester **25** after aqueous iodine oxidation. Following partial deprotection and purification, the oligonucleotide **26** was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to yield **27**. The deprotected oligonucleotide was a substrate for T4-DNA ligase and, hence, demonstrated the biological suitability of chemically 5'-phosphorylated deoxyribonucleotides.[2] Furthermore, the phosphoramidite **1** has been employed in the synthesis of 5'-phospho-2'-deoxyribocytidylyl-(3'→5')-riboadenosine[6] which was aminoacylated and ligated to a truncated amber supressor tRNA. The modified suppressor tRNA could then be used for the site-specific incorporation of unnatural amino acids into proteins *in vitro*.[6]

The phosphoramidites **4-8** were subsequently added to the list of potentially useful reagents for the phosphorylation of oligonucleotides at the 5'-terminus.[3,7,8]



25 R = methyl, R' = p-nitrophenylethyl **26** **27**

DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene
(S) = Silica support
B' = T, C^{bz}, A^{bz} or G^{ib}
B = T, C, A or G

The phosphoramidite **9** has been particularly applied to the 5'-phosphorylation of nucleosides.[9] For example, 3'-azido-3'-deoxythymidine (AZT) was converted into its 5'-di-(*tert*-butyl)phosphate triester upon reaction with **9** and 1*H*-tetrazole followed by oxidation with *m*-chloroperbenzoic acid (MCPBA). The tertiary alkyl phosphate protecting groups were cleaved with trifluoroacetic acid to produce AZT 5'-phosphate in quantitative yield.[9] Interestingly, the *tert*-butyl group could also be quantitatively removed, within 2 h at 75 °C, with trimethylsilyl chloride and triethylamine in acetonitrile.[10] These conditions permitted the synthesis of *N*-protected-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyribonucleoside-3'-phosphates with no loss of the 4,4'-dimethoxytrityl and *N*-acyl groups.[10]

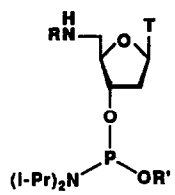
One disadvantage in using the phosphoramidites **1-8** for the 5'-phosphorylation of oligodeoxyribonucleotides was the lack of a convenient way to monitor the coupling reaction. Horn and Urdea circumvented this problem by developing the phosphitylating reagent **10** from sulfonyldiethanol.[11] The phosphoramidite derivative was activated with 1*H*-tetrazole and coupled to the 5'-hydroxyl function of thymidine bound to a controlled-pore glass (CPG) support. Following oxidation, treatment of the solid support with dichloroacetic acid released the bright orange 4,4'-dimethoxytrityl cation which accounted for a coupling efficiency of 96%. Deprotection of the terminal phosphate triester with concentrated ammonium hydroxide at 60 °C afforded thymidine 5'-monophosphate as the sole product. The 5'-phosphorylation of oligodeoxyribonucleotides can alternatively be achieved by the phosphotriester methodology *via* a solid-phase approach[12,13,17] or in solution.[14-16,18-20]

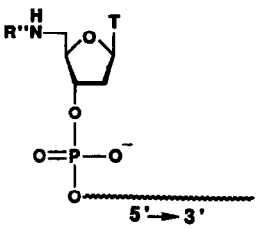
The *S*-triphenylmethyl-2-mercaptoethyl phosphoramidite **11** has also been used for the 5'-phosphorylation of oligonucleotides.[21] Typically, oligomers anchored to a solid support were phosphitylated with **11** in the presence of 1*H*-tetrazole and oxidized under standard conditions. After deprotection and purification, the trityl-sulfur link was cleaved with aqueous silver nitrate affording the corresponding oligonucleotide-5'-mercaptoethylphosphodiester which decomposed quantitatively at pH 8.5 to the desired oligonucleotide-5'-phosphate.[21]

The facile 5'-phosphorylation of oligonucleotides covalently linked to a solid support provided the impetus for the development of specific phosphoramidites that would enable the direct or indirect incorporation of a number of ligands at the 5'-terminus of oligonucleotides.

1.2. Amination, Aminoalkylation, and Ligand Incorporation.

The synthetic challenge involved with the derivatization of oligonucleotides with non-radioactive markers has led to the development of methods for the generation of aliphatic amino groups at the 5'-terminus of these oligonucleotides. One strategy in achieving this goal has been published by Smith *et al.*[22] The approach involved the preparation of 5'-amino-5'-deoxythymine and its conversion into the deoxyribonucleoside phosphoramidites **28-30.** These were incorporated at the 5'-end of oligonucleotides by standard solid-phase synthesis. Once deprotected, the 5'-aminated oligomers were reacted with either fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate, 4-fluoro-7-nitrobenzofurazan or Texas Red sulfonyl chloride yielding the conjugates **31-34.** These oligomers have been used as primers in DNA sequence analysis according to the Sanger approach.[23] The hybridization of these primers to their respective templates was unaltered by the presence of the fluorophores. Furthermore, the facile detection of fluorescent DNA bands on polyacrylamide gels stressed the potential application of oligomers such as **31-34** in automated DNA sequence analysis.[22a,b]

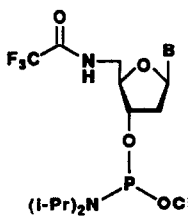




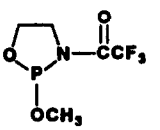
28 R= CCF_3; R'= Me
29 = Fmoc; = Me
30 = MMTr; = CH_2CH_2CN

31 R''= Fluorescein
32 = Tetramethylrhodamine
33 = 7-Nitrobenzofurazan-4-yl
34 = Texas Red

Synthetic routes for the preparation of the protected 5'-amino-2',5'-dideoxyribonucleoside-3'-*O*-phosphoramidites **35a-d** have been developed.[24] Specifically, the phosphoramidite **35b** has been applied to the synthesis of 5'-amino-d(CCGATATCGG) which was, subsequently, reacted with an excess of the pentachlorophenyl ester of a tetrairidium cluster. Co-crystals, of the complex formed between the metallated self-complementary decamer and *Eco*RV, were obtained. These crystals were suitable for electron microscopy studies and X-ray crystallography.[24] Alternatively, the incorporation of the phosphoramidite **30** into oligonucleotides generated 5'-aminated oligomers which were condensed with the *N*-hydroxysuccinimide ester of bathophenanthroline-ruthenium (II) complexes. These metallated oligomers can be detected by time-resolved fluorescence techniques.[25]



35a B= 1-thyminyl
b = 1-(N^4-benzoylcytosinyl)
c = 9-(N^6-pivaloyladeninyl)
d = 9-(N^2-isobutyrylguaninyl)



36

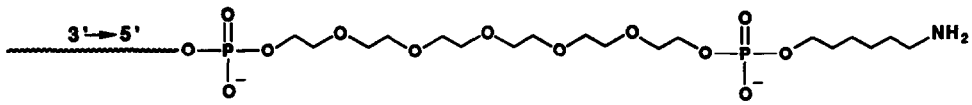
To avoid the preparation of 5'-amino-2,5'-dideoxynucleosides, the phosphoramidites **12**,[26,27] **13a-b**,[28-33] **14**,[34,35] **15a-b**,[25,36] **16**,[37] **17a-b**,[38-41] **18**,[39a] **19**,[42] and **36**[43,44] have been synthesized and applied to the aminoalkylation of synthetic oligodeoxyribonucleotides at the 5'-terminus. The reaction of deprotected 5'-aminoalkylated oligonucleotides with excess biotin *N*-hydroxysuccinimide ester[28,37] or biotin *p*-nitrophenyl ester[36] produced the corresponding 5'-biotinylated oligomers in yields exceeding 85% with no detectable side reaction.[36] The purified 5'-biotinylated oligomers were used as hybridization probes for the detection of specific gene sequences.[26,28] The strong and noncovalent interaction between biotin and avidin or streptavidin leading to the formation of a high affinity complex

(K_d = *ca.* 10^{-15} M) that is easily detectable by enzymatic methods, has offered an alternative to conventional ^{32}P radiolabelling. In addition to hybridization probes, 5'-biotinylated oligonucleotides have found application as primers in experiments involving the Polymerase Chain Reaction (PCR)[28,31,42,45] to facilitate the sequencing of amplified DNA.[46,47]

The versatility of the phosphoramidite **20** has been exemplified by the solid-phase synthesis of an oligonucleotide carrying five primary amine functions attached to the 5'-terminus. The multiple incorporation of the phosphoramidite **20** occurred with a coupling efficiency of 95%.[48] Upon removal of the protecting groups with ammonium hydroxide, the aminoalkylated oligonucleotide was biotinylated and then hybridized to complementary M13mp18 single-stranded DNA immobilized on nitrocellulose filters. Colorimetric detection showed specific hybridization with a sensitivity of 0.5 ng.[48]

In an attempt to facilitate the detection of PCR-amplified DNA sequences through an improved immobilization of sequence-specific oligonucleotide probes to nylon membranes, Zhang *et al.*[49] described the reaction of a hexaethylene glycol-based ß-cyanoethyl-*N,N*-diisopropylphosphoramidite with the 5'-OH of an oligonucleotide linked to a solid support. The resulting "spacer-armed" oligonucleotide was aminolalkylated with the trifluoroacetylated phosphoramidite **13b** yielding, after deprotection, the oligomer **37**. The purified aminoalkylated oligonucleotide was then efficiently coupled to nylon membranes activated with 1-ethyl-3-(*N,N*-dimethylaminopropyl)carbodiimide hydrochloride.[49]

Alternatively, 5'-aminoalkylated oligomers generated from the incorporation of the phosphoramidite **17b** were activated with cyanuric chloride and then coupled to a nylon support covalently coated with poly(ethyleneimine). This support may also find application in the capture of specific RNA and DNA targets.[40]



37

The conjugation of 5'-aminoalkylated oligomers to fluorophores has allowed the classification of *Fibrobacter* strains by fluorescence microscopy on the basis of complementarity to partial 16S rRNA sequences.[44] In addition to enhancing the detection of specific DNA sequences by fluorescence amplification,[32] fluorescently labelled oligodeoxyribonucleotides have served in fluorescence energy transfer experiments[30] and as primers in automated DNA sequence analysis.[43,50]

The phosphoramidite **17b** has been applied to the attachment of a 5'-amino tether to a ribavirin phosphate homopolymer. The amino linker could permit the potential conjugation of the ribavirin oligomer to a monoclonal antibody.[38]

5'-Aminoalkylated oligodeoxyribonucleotides resulting from the incorporation of the phosphoramidite **17b** have also found application in the preparation of oligonucleotide-quinone conjugates.[41] When hybridized to their complementary sequences, these conjugates were activated by cytochrome *c* reductase in the presence of NADH and triggered the formation of cross-linked strands by reductive alkylation.[41b] These oligonucleotide conjugates may find interesting applications in antisense experiments. Similar applications are anticipated for those 5'-aminoalkylated oligomers which have been reacted with *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate or *N*-succinimidyl-4-(*p*-maleimidophenyl)butyrate and conjugated to interleukin-1ß as a means to deliver such conjugates to cells harboring the interleukin-1-receptor.[29]

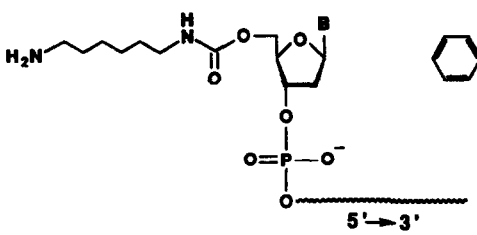
While the synthesis of oligodeoxyribonucleoside methylphosphonates from deoxyribonucleoside methylphosphonamidites has been reported in the mid-eighties, only recently has the 5'-aminoalkylation of a methylphosphonate oligomer been effected by the methylphosphonamidite **21** (Table 1).[51] The deprotected oligomer was biotinylated and, after purification, was immobilized on streptavidin-agarose. Given the stability of the internucleotidic methylphosphonate linkages to the nucleolytic activity of nucleases, the biotinyl-oligonucleoside methylphosphonate was employed in the capture of maxi U2

RNA/ribonucleoprotein complexes from nuclear extracts of cultured human cells.[51]

It is to be noted that solid-phase syntheses of 5'-aminoalkylated oligonucleotides and oligodeoxyribonucleotides containing a 5'-aminoalkylphosphonate function have also been achieved by the phosphotriester approach.[52,53]

Wachter *et al.*[54] further investigated the 5'-aminoalkylation of oligodeoxyribonucleotides. Their approach involved the reaction of the 5'-OH function of solid-phase linked oligomers with carbonyldiimidazole followed by hexamethylenediamine. The deprotected 5'-aminoalkylated deoxyribonucleotide **38** was biotinylated with the *N*-hydroxysuccinimide ester of biotin at pH 7.7. Interestingly, the carbamate linkage in **38** survived the usual basic conditions required for the removal of the base protecting groups.[54,55] This methodology was also employed by Beck *et al.*[56a] toward the development of a chemiluminescent detection of DNA and by others[56b] to evaluate the cellular uptake and trace the intracellular localization of fluorescently-labelled oligonucleotide-neoglycoprotein conjugates.

Along similar lines, De Vos *et al.*[55] described the synthesis of the deoxyribonucleoside phosphoramidite **39a-b** to facilitate the incorporation of an aminoalkyl function at the 5'-end of oligonucleotides. The (*p*-biphenylyl)isopropyloxycarbonyl group was conveniently removed under the acidic conditions required for the cleavage of a DMTr group. The resulting 5'-aminoalkylated oligomer was biotinylated while the oligonucleotide was still attached to the solid support or, alternatively, in solution phase after complete deprotection.[55]



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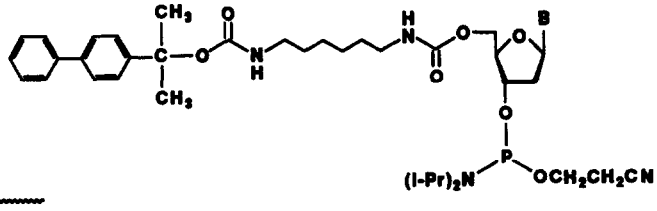
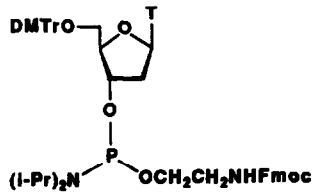


39a B= 1-thyminyl
b = 9-hypoxanthinyl

Seliger *et al.*[57] reported the synthesis of the deoxyribonucleoside phosphoramidite **40** and the dinucleotidic phosphoramidite **41** as a means of inserting aminoalkyl functions in oligonucleotides. The monomeric phosphoramidite **40** led to coupling yields of *ca.* 99% whereas the coupling efficiency of the dimeric phosphoramidite **41** was *ca.* 87%. The purified aminoalkylated oligomers were biotinylated in yields up to 90% with the *N*-hydroxysuccinimide ester of biotin at pH 7.5.[57] A biotinylated hairpin loop was immobilized on an avidin-coated solid support to demonstrate the accessibility to double-stranded DNA manipulations.[57]



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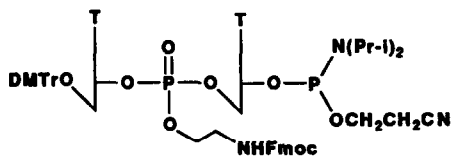
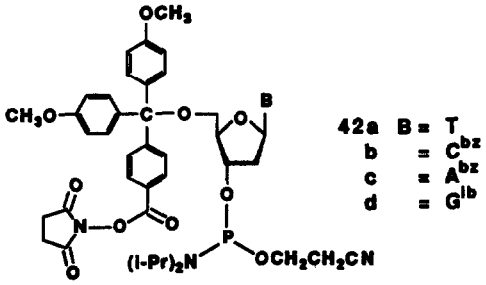


41

A versatile linker for the modification of synthetic biomolecules has been reported by Gildea *et al.*[58] The linker was incorporated into oligonucleotides *via* the phosphoramidites **42a-d.** The solid-phase synthesis of a pentadecamer was performed and the oligomer was reacted with 1,6-diaminohexane followed with the *N*-hydroxysuccinimide ester of biotin. The crude biotinylated oligonucleotide was purified by binding to streptavidin-agarose and washing away non-biotinylated

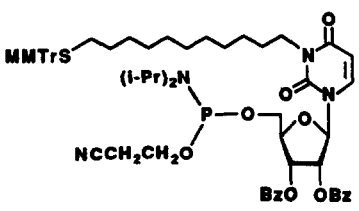
oligonucleotidic sequences. Treatment of the agarose with aqueous acetic acid released the captured oligomer in 85% yield.[58]

In addition to this specific application, the phosphoramidites **42a-d** could potentially lead to the attachment of a variety of ligands at the 5'-end of oligonucleotides with the unique possibility of recovering the unmodified oligomers upon mild acidic conditions.



1.3. Thiolation, Mercaptoalkylation, and Ligand Incorporation.

A strategy for solid-phase purification of oligonucleotides has also been proposed by Bannwarth and Wippler.[59] The approach relied on the incorporation of the ribonucleoside phosphoramidite **43** at the 5'-end of an hexadecaoligonucleotide linked to a solid support. The derivatized oligonucleotide was then deprotected with concentrated ammonium hydroxide and treated with aqueous silver nitrate. The thiol-containing oligonucleotide (**44**) along with the underivatized oligonucleotidic sequences were reacted with the activated disulfide support **45** at pH 7.0. The resulting support **46** was freed from underivatized sequences and the pure 5'-phosphorylated oligonucleotide **47** was released from **46** upon treatment with aqueous sodium metaperiodate and 5% aqueous triethylamine, respectively.[59] The yield of pure **47** has not, however, been reported.



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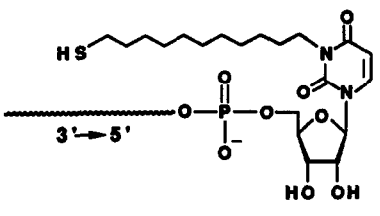
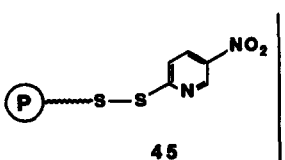


44

Bz = benzoyl

P = Controlled-Pore Glass



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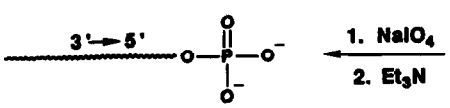

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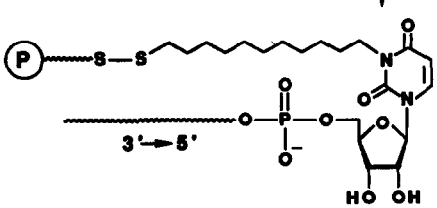

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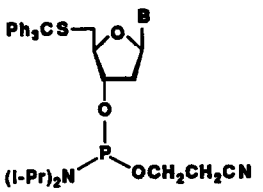
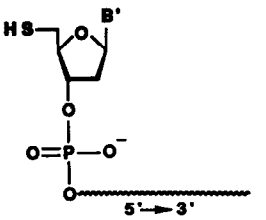
The synthesis of protected 5'-mercapto-2',5'-dideoxyribonucleoside-3'-*O*-phosphoramidites (**48a-d**) and their application toward the preparation of 5'-mercapto-oligodeoxyribonucleotides have been reported.[60] Like 5'-aminodeoxyribonucleotides, 5'-mercaptodeoxyribonucleotides, represented by the structure **49**, can react with a variety of electrophiles, heavy metals and fluorescent markers.[61]





48a B= 1-thyminyl
b = 1-(N^4-benzoylcytosinyl)
c = 9-(N^6-pivaloyladeninyl)
d = 9-(N^2-isobutyrylguaninyl)

49 B'= T, C, A or G

The mercaptoalkylated phosphoramidites **11**[21,62] and **22**[39,61,63a,64a,65] provided an alternate route to the synthesis of 5'-thiolated oligonucleotides. It has been shown that 5'-mercaptopropyloxy-oligodeoxyribonucleotides can be derivatized with tetramethylrhodamine iodoacetamide or fluorescein iodoacetamide to yield fluorescent M13 primers suitable for automated DNA sequencing.[64]

Furthermore, Mori *et al.*[66] described the synthesis of the phosphoramidite **23** and its incorporation at the 5'-end of synthetic oligonucleotides. The 5'-mercaptoalkylated oligonucleotides were then coupled with *N*-9-acridinylmaleimide affording fluorescent oligonucleotides which were used in cellular uptake experiments.[66]

5'-Mercaptoalkylated oligodeoxyribonucleotides have also been applied to the synthesis of sequence-defined polypeptide-oligonucleotide conjugates.[65] Specific polypeptides were prepared by standard solid-phase methodology and were reacted with *N*-hydroxysuccinimidyl-3-maleimido benzoate. Purified maleimido-containing polypeptides were reacted with purified 5'-mercaptoalkylated oligonucleotides at pH 6-7 to yield the desired polypeptide-oligonucleotide conjugates. It is noteworthy that the conjugation of 5'-thiolated oligonucleotides with a maleimido-poly-L-lysine derivative[39a] led to the formation of conjugates that were particularly effective in the selective inhibition of gene expression in intact cells.[67]

Of interest, 5'-thiolated oligomers have been synthesized from the incorporation of a tritylated mercaptotetraehylene glycol phosphoramidite derivative by solid-phase techniques.[68] These thiolated oligomers were condensed with horseradish peroxidase derivatized with maleimide functions to generate valuable conjugates for the sequence-specific detection of PCR-amplified DNA.[68]

The reaction of 5'-mercaptoalkylated oligodeoxyribonucleotides with Tresyl-activated Sepharose 4B or Epoxy-activated Sepharose 6B resulted in the covalent attachment of specific oligomers to Sepharose. The addition of complementary unmodified oligodeoxyribonucleotides yielded DNA duplexes as affinity ligands for the isolation of specific DNA binding proteins.[63]

It is important to mention that 5'-aminoalkylated oligonucleotides can easily be converted into oligonucleotides carrying a 5'-mercapto function upon reaction with dithiobis-(*N*-succinimidyl)propionate at pH 7.7 followed by treatment with dithioerythritol at pH 8.5.[27] 5'-Mercaptoalkylated oligonucleotides were obtained in 70-80% yields. Similarly, Gaur *et al.*[69] reported the reaction of a 5'-aminoalkylated oligonucleotide with *N*-succinimidyl-3-(2-pyridyldithio)propionate[37] instead of dithiobis-(*N*-succinimidyl)propionate. The activated disulfide-containing oligonucleotide produced the 5'-mercaptoalkylated oligonucleotide upon treatment with dithiothreitol (DTT) at pH 9.0.[37,69] Furthermore, the reaction of a 5'-aminoalkylated oligomer with *N*-acetyl-DL-homocysteine thiolactone at pH 8.0[70] or with *N*-succinimidyl-*S*-acetyl thioacetate followed by hydroxylamine

hydrochloride,[71] generated the corresponding oligonucleotide carrying a free thiol group in yields greater than 95%. The thiolated oligomer was then derivatized with *N*-(4-dimethylaminobenzene-4')iodoacetamide or *N*-(3-pyrenyl)maleimide.[70,71]

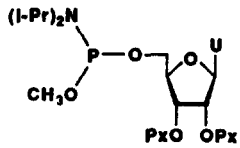
In this context, the reaction of the 5'-hydroxy function of an oligonucleotide linked to a solid support with, successively, carbonyl diimidazole and *S*-DMTr-cysteine yielded, after deprotection and purification, an oligomer derivatized with both mercapto and carboxylic acid functionalities.[72]

Thuong and Chassignol[73a] described the synthesis of oligonucleotides carrying a 5'-terminal thiophosphate monoester function. Their approach was based on the phosphitylation of the 5'-OH function of oligomers anchored to a solid support with the phosphoramidite **1a** followed by sulfurization of the phosphite triester intermediate with elemental sulfur. The purified 5'-thiophosphorylated oligonucleotides were reacted with various electrophiles in aqueous solution, affording the efficient incorporation of photoactivatable cross-linkers,[73] intercalators and chelators[73,74] in addition to fluorescent and radioactive markers.[73,75,76] This strategy has been particularly useful for the introduction of proflavin at the 5'-end of α- or ß-oligodeoxyribonucleotides as a means to direct photosensitized reactions to the complementary DNA sequences.[77,78] In a similar manner, the phosphoramidite **24** (Table 1) enabled the incorporation of a 5'-hexamethylene thiophosphate group into oligonucleotides and the subsequent functionalization of the thioated oligomers with a phenanthroline-copper chelate.[74a]

Recently, the coupling of a specific 5'-thiophosphorylated oligomer with *p*-azidophenacyl bromide was performed to demonstrate that the inhibition of SV40 DNA replication in CV-1 cells resulted from the formation of a triple helix between the viral DNA and the oligonucleotide carrying the cross-linking agent.[79] Psoralen-oligonucleotide conjugates have also been similarly prepared by reacting 5-(6-iodohexyloxy)psoralen with oligodeoxyribonucleotides carrying a 5'-thiophosphate group.[80] The sequence-specific photoinduced cross-linking of the two DNA strands by the triple helix-forming psoralen-oligonucleotide conjugates occurred with efficiencies greater than 80%.[80a] Thus, psoralen-oligonucleotide conjugates can be designed to recognize and irreversibly modify specific DNA sequences.[80] Such modifications are expected to block replication and transcription by preventing the opening of the double helix.

1.4. Oxidation, Esterification, and Ligand Incorporation.

Alternate methods for linking non-radioactive labels to the 5'-end of synthetic oligodeoxyribonucleotides employing phosphoramidite intermediates have been reported. Specifically, the ribonucleoside phosphoramidite **50** was used to generate a (5'→5')-internucleotidic link with an oligomer covalently bound to a solid support.[34b] The deprotected oligomer **51** was oxidized to **52** with sodium metaperiodate. The reaction of **52** with biotin hydrazide followed by reduction with sodium



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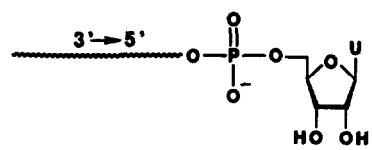

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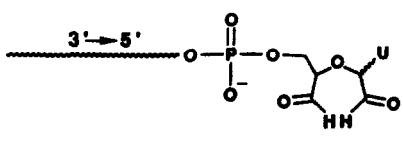

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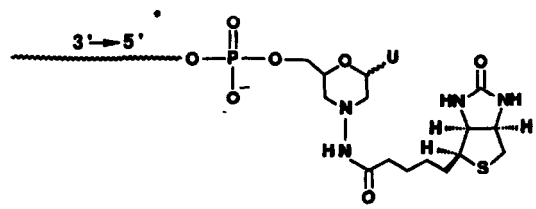
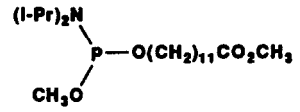


53

borohydride afforded the biotinylated oligonucleotide **53** which has been applied to the detection of M13 DNA immobilized on nitrocellulose filters.[34b]

The phosphoramidite **54** has been recommended by Kremsky *et al.*[81] for the phosphitylation of the 5'-OH function of an oligonucleotide anchored to a solid support. Iodine oxidation followed by complete deprotection yielded the oligomer **55** which upon condensation with biotin hydrazide in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) afforded the biotinylated oligomer **56** in *ca.* 90% yield.



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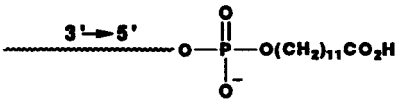

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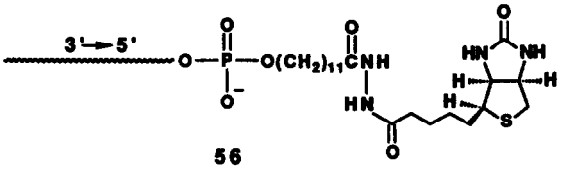
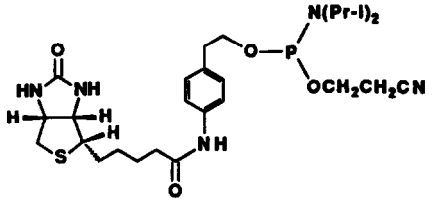


56

Noteworthy procedures for the 5'-biotinylation of synthetic oligonucleotides have also been reported by Kempe *et al.*,[82] Chollet *et al.*,[83] and Chu *et al.*[84]

1.5. Direct Ligand Incorporation.

1.5.1. Biotinyl Phosphoramidites. 5'-Biotinylated oligonucleotides were easily prepared by reacting the phosphoramidite **57** with the 5'-OH group of solid-phase bound oligomers under standard conditions.[85] The synthesis of **57** was accomplished by coupling biotin *N*-hydroxysuccinimide with *p*-(2-hydroxyethyl)aniline. The resulting ethanolamide was then phosphitylated with bis-(*N,N*-diisopropylamino)-ß-cyanoethoxyphosphine and *N,N*-diisopropylammonium tetrazolide.[85] Alternatively, the phosphitylation of a biotinol derivative with chloro-(methoxy)-*N*-morpholinophosphine led to the biotinylated phosphoramidite **58**.[86] The coupling efficiency of **58** with the 5'-OH function of solid-phase bound oligonucleotides can potentially be measured during the removal of the N^1-DMTr group of the biotin moiety.



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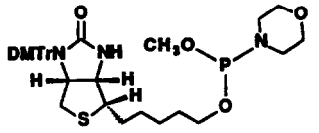
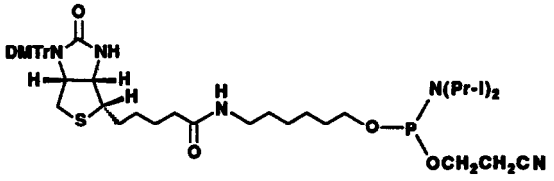


58

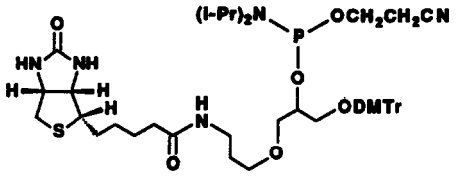
In a recent report, Pon described the preparation of [1-*N*-(4,4'-dimethoxytrityl)-biotinyl-6-aminohexyl]-ß-cyanoethyl-*N,N*-diisopropylphosphoramidite (**59**) for the automated synthesis of 5'-biotinylated oligonucleotides.[87] The N^1-DMTr group provided solubility and purification advantages while the 6-aminohexyl linker facilitated the interaction of biotin with large proteins. The biotinylated phosphoramidite **59** was incorporated into oligonucleotides with an efficiency of *ca.* 85-95%. The biotinylated oligonucleotides could be heated in concentrated ammonium hydroxide for 24 h at 50 °C without significant loss of biotin.[87]



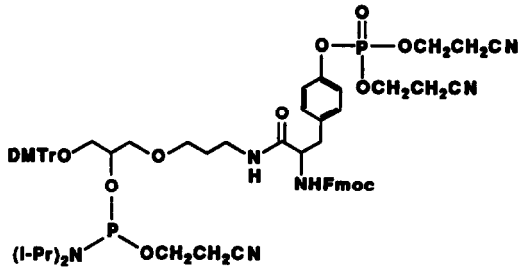
59

Phosphoramidite linkers have been designed for the direct insertion of biotin at multiple sites in synthetic oligonucleotides.[88] For example, the biotinyl phosphoramidite **60** was prepared in six steps from solketal and was repeatedly incorporated at the 5'-end of a universal M13 sequencing primer with a coupling efficiency of *ca.* 99%. Oligonucleotides having up to eight biotin residues were synthesized. Depending on the assay used, it was found that an oligomer having eight biotin residues was detected at a concentration 10 fold lower than that of a singly biotinylated oligomer.[88]

1.5.2. Phosphotyrosinyl Phosphoramidite. The phosphotyrosinyl phosphoramidite **61** was synthesized in nine steps from the *p*-toluenesulfonate salt of L-tyrosine benzyl ester and was also repeatedly inserted in the sequence of the universal M13 sequencing primer (*vide supra*) with a coupling efficiency of *ca.* 96%. Phosphotyrosinyl oligonucleotides enabled the detection of M13 DNA with a signal strength increasing with the number of phosphotyrosinyl residues.[88]

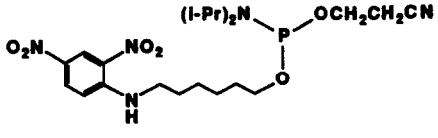


60



61

1.5.3. 2,4-Dinitrophenylyl Phosphoramidites. Will *et al.*[89] reported the synthesis of the phosphoramidites **62** and **63** for the single or multiple incorporation of dinitrophenyls (DNP) at the 5'-terminus of oligonucleotides. The insertion of **63** in oligonucleotides by solid-phase techniques occurred with a coupling efficiency of *ca.* 97% and afforded oligonucleotides having up to ten DNP groups.[89] Due to the sensitivity of the multiple DNP labels to concentrated ammonium hydroxide under the usual deprotection conditions, FOD-amidites[90] carrying base protecting groups that can be removed under milder conditions have been used in the synthesis of DNP-labelled oligonucleotides. The facile detection of such oligonucleotides was effected with anti-DNP monoclonal or polyclonal antibodies harboring suitable markers.[89]



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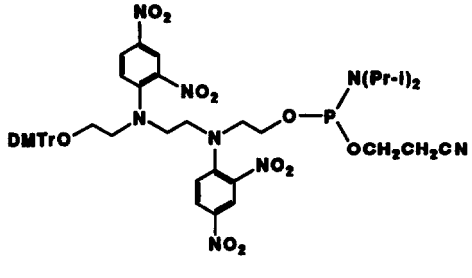
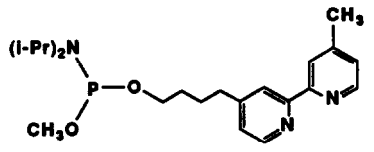


63

1.5.4. Bipyridinyl Phosphoramidite. The phosphoramidite **64** has recently been prepared by Modak *et al.*[91] and employed in the phosphitylation of 3'-*O*-acetyl-2'-deoxythymidine. After oxidation and purification, the bipyridinyl phosphotriester **65** was complexed with Cu(II) and incubated with poly rA_{12-18} at 37 °C, pH 7.1, for 24 or 48 h. The RNA substrate was degraded to the extent of 21% within 48 h. Adenosine (2'-3')-cyclic monophosphate was identified as the major hydrolysis product. No degradation of the RNA homopolymer was detected in the absence of Cu(II) under similar conditions.[91] The phosphoramidite **64** may, therefore, be useful toward the incorporation of a bipyridinyl group at the 5'-end of oligonucleotides and the development of sequence-specific RNA cleaving reagents.



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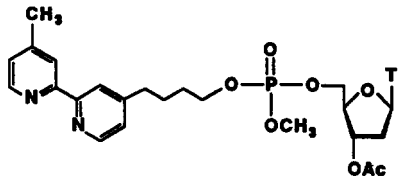
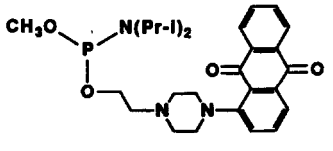


65

1.5.5. Anthraquinonyl Phosphoramidite. Mori *et al.*[92] developed the phosphoramidite **66** for the derivatization of oligonucleotides at the 5'-end with an anthraquinone function. Anthraquinones are potential radical producing entities which do not necessarily require photoactivation or the presence of metal ions. Consequently, anthraquinone-linked oligonucleotides may find application in the delivery of radical-producing anti-cancer drugs to cellular nucleic acid targets.[92]

1.5.6. Psoralenyl Phosphoramidite. The direct incorporation of psoralen derivatives at the 5'-terminus of oligodeoxyribonucleotides was effected by the phosphoramidite **67** *via* solid-phase methods.[93,94] Psoralen derivatives can intercalate between the base pairs of double-stranded nucleic acids and may become covalently attached to thymine residues upon exposure to long-wavelength UV irradiation. Under these conditions, each psoralen-DNA adduct is generated through the formation of a cyclobutane ring. These adducts have been applied to the elucidation of nucleic acid structures and functions.[80,95,96]

1.5.7. Acridinyl and Fagaroninyl Phosphoramidites. The synthesis of the acridinyl phosphoramidites **68**[97a] and **69a-b**[98] and their introduction at the 5'-terminus of modified and unmodified oligodeoxyribonucleotides has been documented.[74c,97b] In addition to providing fluorescence to these oligonucleotides, the acridinyl group stabilized the formation of double- and triple-helical structures with complementary nucleic acid targets by virtue of its intercalating properties.[74c,75,97b,99,100] Such fluorescent oligonucleotides have been particularly useful in kinetic experiments pertaining to the cellular uptake of these biomolecules.[98]



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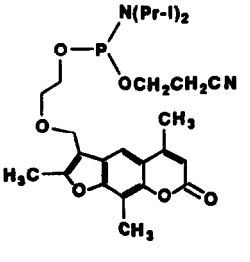
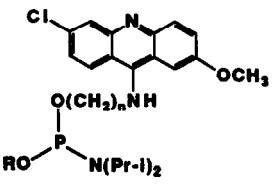
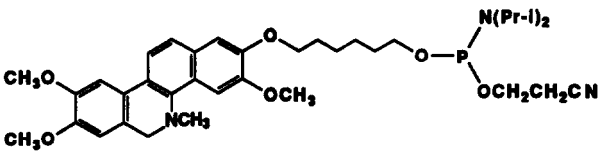


67



68 n= 5; R= 2-cyanoethyl
69a = 3; = methyl
b = 5; = methyl

It has been demonstrated that the benzophenanthridine alkaloid, fagaronine chloride, isolated from *Fagara zanthoxyloides* inhibited the reverse transcriptase activity of several viruses.[101] These findings provided the impetus to determine whether antisense oligonucleotide-fagaronine conjugates would enhance the inhibition of retroviral reverse transcriptases. Fagaronine chloride was converted to the phosphoramidite **70** which was then reacted with the 5'-hydroxy function of oligonucleotides.[101] Relative to unmodified oligomers, oligonucleotide-fagaronine conjugates hybridized better to complementary RNA sequences presumably because of the intercalating abilities of the fagaronine entity. The conjugates were *ca.* 3 times more potent than unmodified oligomers at inhibiting HIV reverse transcriptase but were *ca.* 3 times less inhibitory than fagaronine chloride itself.[101]



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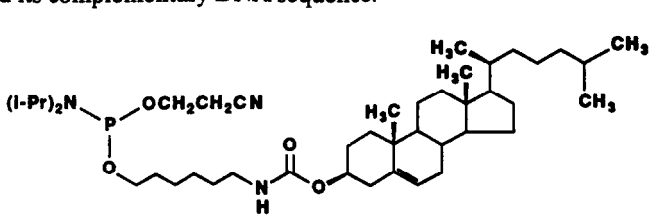
1.5.8. Cholesteryl and Other Lipophilic Phosphoramidites. The application of oligonucleotides as potential therapeutics has been hampered by the low efficiency with which these polyanionic molecules permeated intact cells. It has been rationalized that the attachment of lipophilic groups known to interact with cell membranes, to oligonucleotides, may increase the therapeutic potency of these biopolymers.[102] To test this rationale, the cholesteryl phosphoramidite **71** along with the adamantylethyl phosphoramidite **72** and the polyalkyl phosphoramidites **73-75** were synthesized and incorporated at the 5'-terminus of an oligonucleoside phosphorothioate complementary to a region of the HIV-1 *rev* gene.[102a] It has been shown, so far, that the denaturation temperatures of duplexes composed of the lipophilic oligonucleoside phosphorothioates and complementary DNA strands were identical to that of the duplex resulting from an equivalent underivatized phosphorothioate oligomer and its complementary DNA sequence.[102a]



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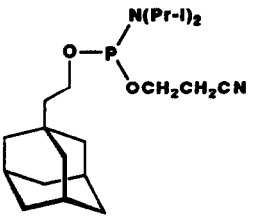

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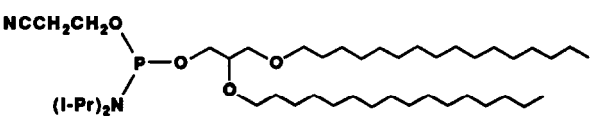

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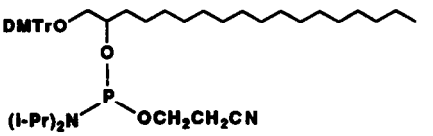
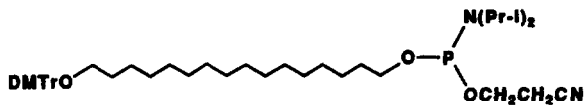


74

Along similar lines, Kabanov *et al.*[102b] reported the synthesis the phosphoramidite **76** as a means to introduce an hydrophobic group at the 5'-end of oligonucleotides. Targeting an undecylated oligonucleotide against a loop forming-site of the influenza A/PR8/34 viral RNA, considerably suppressed the development of the virus in permissive MDCK cells relative to that observed with unmodified oligonucleotides under identical conditions.[102b,c] The attachment of hydrophobic groups to



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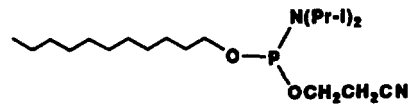
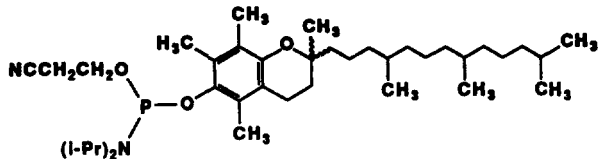


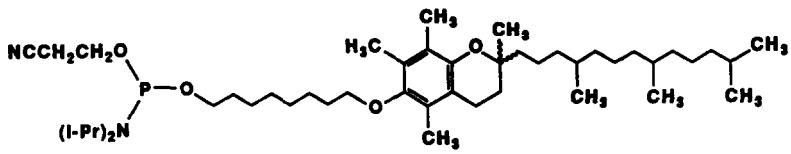
76

oligonucleotides has therefore demonstrated its efficacy at improving the biological activity of theses biomolecules.

In an additional effort to impart lipophilicity to oligonucleotides and improve their cellular uptake and cellular distribution, the synthesis of the DL-α-tocopherol (Vitamin E) phosphoramidites **77** and **78** has been described.[102d] Similar to the phosphoramidite **76**, the incorporation of of **77** and **78** into oligonucleotides occurred in yields greater than 95% according to HPLC analysis of the conjugates.[102d] The anti-HIV activity of these modified oligonucleotides are currently being evaluated in cell cultures.

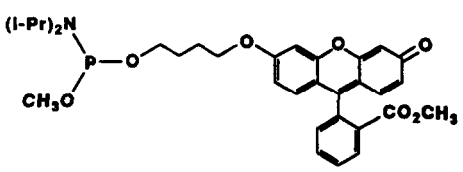


77

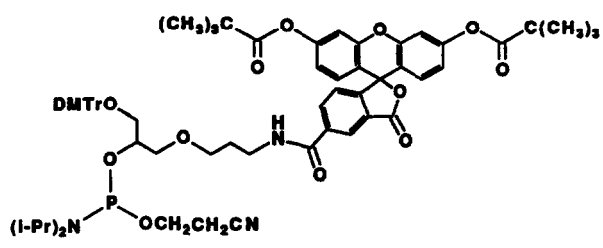


78

1.5.9. Fluoresceinyl Phosphoramidites. The preparation of fluorescently labelled oligonucleotides for automated dideoxy-DNA sequencing has been further simplified by Schubert *et al.*[103a] who reported the facile integration of the fluoresceinyl phosphoramidite **79** into synthetic oligonucleotides. The phosphoramidite was prepared from the alkylation of fluorescein methyl ester with 4-chloro-1-(4,4'-dimethoxytrityloxy)butane followed by detritylation and phosphitylation with bis-(*N,N*-diisopropylamino)methoxyphosphine. The phosphoramidite **79** has been used in the solid-phase synthesis of various M13mp18 sequencing primers. Coupling yields were equivalent as those recorded with the standard deoxyribonucleoside phosphoramidites.[103a]



79



80

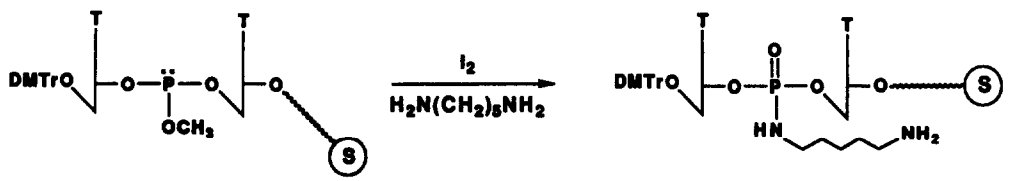
The multistep synthesis of the 5-carboxyfluoresceinyl phosphoramidite **80** from 5-carboxyfluorescein and solketal has recently been reported by Theisen *et al.*[103b] It was argued that because of the steric hindrance imparted by the trityl group, the coupling of **80** at the 5'-OH function of

a solid-phase bound oligonucleotide necessitated a coupling time of 120 sec and proceeded with an efficiency of only 70%. The fluorophore and its linkage to the oligomer were stable during chain assembly and to the conditions required for complete oligonucleotide deprotection.[103b] Like the biotinyl- and phosphotyrosinyl phosphoramidites (**60** and **61**), the fluoresceinyl phosphoramidite **80** can be incorporated at any site within a DNA sequence or can serve in the derivatization of solid supports to yield oligonucleotides labelled at the 3'-terminus.

Aside from the 5'-OH group of oligonucleotides, the internucleotidic phosphodiester function of these biomolecules could also be functionalized. This subject will be dealt with in the forthcoming section.

2. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE INTERNUCLEOTIDIC LINKAGES

Jäger *et al.*[104] demonstrated that aminoalkylated phosphoramidate oligonucleotides can be prepared by routine solid-phase synthesis using deoxyribonucleoside 3'-*O*-(*N*,*N*-diisopropylamino)methoxyphosphine monomers. For example, the oxidation of the dinucleoside phosphite triester **81** with iodine and 1,5-diaminopentane afforded the aminoalkylated phosphoramidate dimer **82** which was subsequently coupled to 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine.[104]



81 **82**

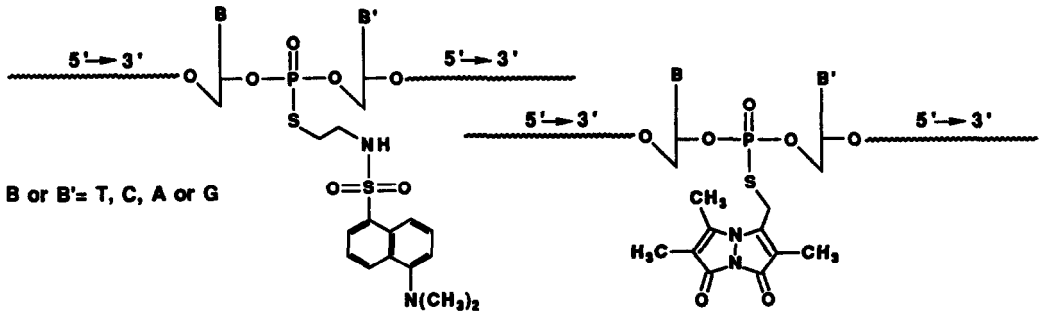
Due to the stability of the phosphoramidate linkage to the conditions used in solid-phase oligonucleotides synthesis by the phosphoramidite method, the combination of deoxyribonucleoside phosphoramidite and *H*-phosphonate monomers has allowed the insertion of cholesteryl,[105] tetramethylpiperidine-*N*-oxyl[106a] or phenazinyl di-*N*-oxide[106b] phosphoramidate links at predetermined sites. The validity of this approach has been previously demonstrated by Letsinger *et al.*[107] in a report delineating the preparation of cationic oligonucleotides.

In a similar manner, Agrawal *et al.*[108] and others[109] introduced either (*N*-1-trifluoroacetyl)hexanediamine arms at specific sites along the oligonucleotide chains[108] or ethylenediamine and hexamethylenediamine tethers at the 5'-end of oligonucleotides.[109] After complete deprotection, the aminoalkylated phosphoramidate oligomers were coupled to ligands such as fluorescein isothiocyanate, rhodamine isothiocyanate or activated esters of biotin. This approach provided multiple labelling capabilities and thereby increased sensitivity for diagnostic purposes.[108]

The specific functionalization of oligonucleotides with two different reporter groups has been described by Agrawal and Zamecnik.[110] Oligonucleotide assembly began with the incorporation of a nucleoside *H*-phosphonate monomer followed by oxidation with carbon tetrachloride and (*N*-1-trifluoroacetyl)hexanediamine to generate a phosphoramidate link. Standard deoxyribonucleoside phosphoramidite monomers were then employed until completion of the synthesis. The last oxidation step was effected with a sulfurizing reagent[111,112] to generate a phosphorothioate link which, after oligonucleotide deprotection, was reacted with monobromobimane at pH 7.2. The aminoalkyl function of the purified bimane-labelled oligomer was then reacted with fluorescein isothiocyanate and the resulting bifunctionalized oligomer was isolated in 60% yield. Such bifunctionalized oligomers may find application in cellular uptake experiments or toward the targeting of specific messenger RNAs.

Numerous reporter groups have been introduced at specific sites in oligonucleotides carrying phosphorothioate diester links.[113] For example, the reaction of an oligonucleoside phosphorothioate with *N*-dansylaziridine afforded the dansylated phosphorothioate oligomer **83** in yields greater than 85%.[114] Substituting monobromobimane for *N*-dansylaziridine at pH 7 led to the complete formation

of the bimane-labelled phosphorothioate oligomer **84**.[115] Interestingly, bimane labels have been incorporated at multiple sites into oligonucleoside phosphorothioates while the oligomers were still embedded in the polyacrylamide gel matrix after electrophoresis. Oligonucleotides containing two hunded to four hundred labels can be detected with the naked eye at the low femtomole level (10-20 fmol).[115,116]



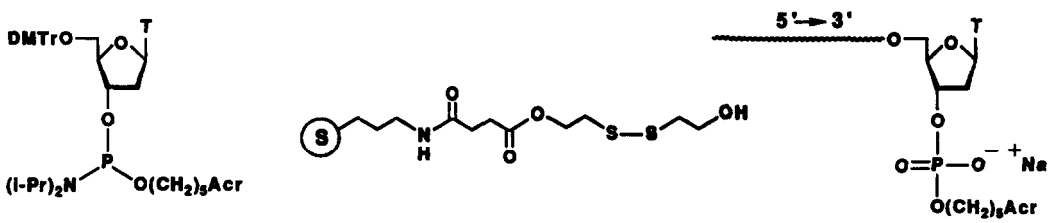
83 **84**

The nucleophilicity of phosphorothioate diesters has been exploited in the sequence determination of DNA and RNA.[117,118] Specifically, the reaction of oligodeoxy- or oligoribonucleotides having randomly distributed phosphorothioate links with iodoethanol or 2,3-epoxy-1-propanol generated hydrolytically labile phosphorothioate triesters which promoted the subsequent cleavage of the oligonucleotidic chains.[117] This approach represents an alternative to the current DNA sequencing procedures.

The following section will assess the recent developments toward the derivatization of oligonucleotides at the 3'-end.

3. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE 3'-TERMINUS

The incorporation of a fluorescent marker at the 3'-terminus of oligonucleotides was reported by Asseline and Thuong[119] Their approach involved the phosphitylation of the solid support **86** with the fluorescent deoxyribonucleoside phosphoramidite **85** in the presence of 1*H*-tetrazole. The labelled oligomers (**87**) were subsequently released from the support by treatment with DTT under alkaline conditions. This methodology also permitted the multiple insertion of fluorescent labels within an oligonucleotidic chain.



85 **86** **87**

Acr(CH$_2$)$_5$OH= 2-methoxy-6-chloro-9-ω-hydroxypentylaminoacridine

(S) = Fractosil 500

In recent years, solid supports have been engineered to facilitate the derivatization of synthetic oligonucleotides with functional groups including masked[56b,75,76,120a] or unmasked 3'-sulfhydryl,[56b,120]

terminal 3'-phosphate,[76,119a,120a,121-123] 3'-thiophosphate,[76,100a,119,124] and 3'-aminoalkyl.[76,119a,125-129] These functional groups can then be further derivatized with various ligands to serve as tools in molecular biology experiments. Solid supports functionalized with acridine,[130a] cholesterol,[130a,b] DL-α-tocopherol[102d] or fluorescein[103] have also been described for the synthesis of oligonucleotides bearing these ligands at the 3'-terminus.

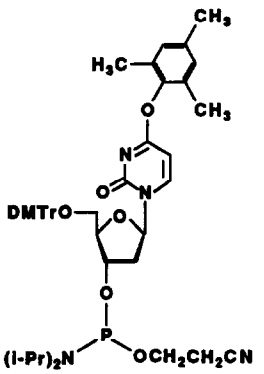
Aside from the internucleotidic linkage and both termini, the functionalization of oligonucleotides at the nucleobases has been thoroughly investigated. The results of these investigations will be summarized in the next section.

4. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE NUCLEOBASES

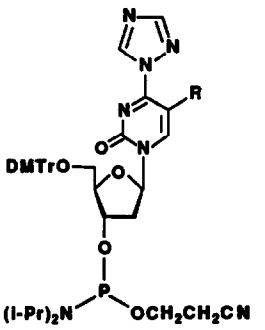
4.1. Derivatization of Activated Purines and Pyrimidines.

It is well-known that the triazolo function of 4-triazolopyrimidinone nucleosides are susceptible to nucleophilic displacement by amines.[131,132] This feature has been exploited by Le Brun *et al.*[133] who inserted 4-triazolopyrimidinones at predetermined sites in oligonucleotides according to the phosphoramidite methodology. The triazolyl groups were then displaced with 1,10-diaminodecane and the corresponding aminoalkylated oligomers were subsequently biotinylated. The specificity of these biotinylated oligomers was tested in hybridization assays with plasmid DNA immobilized on nitrocellulose filters. It was found that the stability of the resulting hybrids was not affected by biotinylated arms located at the 5'-end of the oligomers. Conversely, the stability of the hybrids was strongly impaired by the location of the biotinylated linkers within the oligonucleotidic chains. The denaturation temperature (T*m*) of these hybrids was at least 10 °C lower than that measured with unmodified oligomers.[133] Consequently, the 5'-terminus appears to be the ideal position for the incorporation of these modified nucleobases into oligonucleotides.

MacMillan and Verdine[134a,b] reported the synthesis of functionally tethered oligodeoxyribonucleotides by incorporating the deoxyribonucleoside phosphoramidite **88** at a preselected site during oligonucleotide assembly. The use of deoxyribonucleoside phosphoramidites having base-labile protecting groups (N^4-isobutyryl cytosine, N^6-phenoxyacetyl adenine and N^2-phenoxyacetyl guanine)[135] permitted rapid oligonucleotide deprotection with concentrated ammonium hydroxide without affecting the O^4-arylated uracil residue. Treatment of the modified oligonucleotides with amines such as *N*-methylamine, 1,2-diaminoethane, 1,4-diaminobutane, glycine or bis-(aminoethyl)disulfide at 65 °C resulted in functionally tethered oligonucleotides with a conversion efficiency of 89-100%.[134a,b] A similar postsynthetic generation of oligomers containing O^4-alkylthymine, 5-methylcytosine, N^4-(dimethylamino)-5-methylcytosine and 4-thiothymine residues stemmed from the insertion of the triazolo phosphoramidite derivative **89b** in oligonucleotides.[134c]

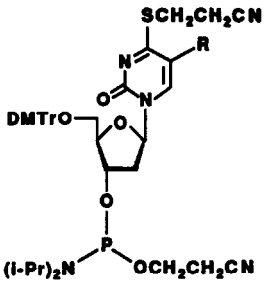


88

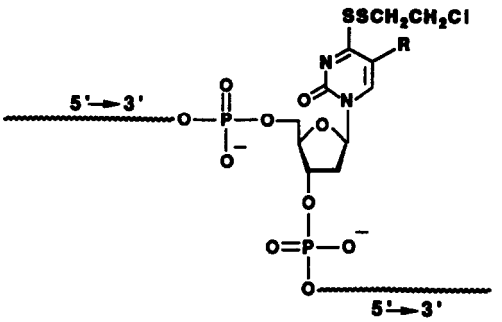


89a R= H
b = Me

It has been recently shown that 4-thio-2'-deoxyuridine-containing oligonucleotides led to tether attachment by postsynthetic *S*-alkylation or mixed disulfide formation.[134d] The 4-thioated-2'-deoxyuridine phosphoramidite **90a** has been synthesized and incorporated into oligothymidylates by solid-phase synthesis.

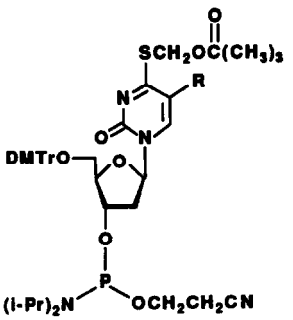


90a R= H
b = Me

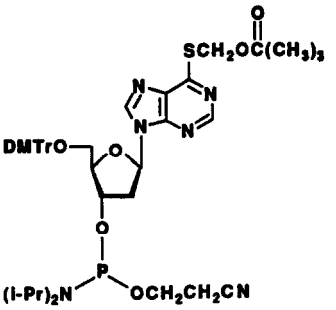


91 R= H

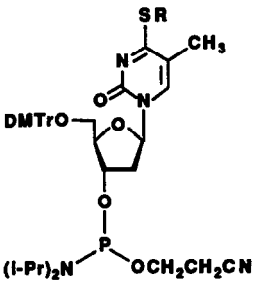
Quantitative *S*-deprotection of each oligonucleotide was effected by treatment of the solid support with 1 M DBU in acetonitrile. The modified oligothymidylate was then released from the stationary phase and reacted with *N*-(2-chloroethylthio)phtalimide in phosphate buffer (pH 8) to yield the oligonucleotide **91** which can be further derivatized with selected reporter groups.[134d] The phosphoramidites **90b** and **92-94** have analogously been applied to the synthesis of oligonucleotides containing thioated nucleobases.[134e-h]



92a R= H
b = Me



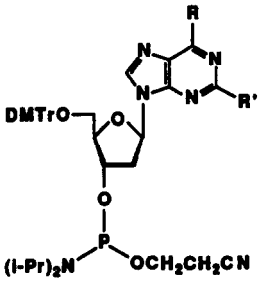
93



94a R= phenyl
b = p-nitrophenyl

To instigate the synthesis of oligonucleotides containing purine modified at *C*-6 with *S*-, *N*- and *O*-derivatives, the 6-(2,4-dinitrophenyl)thiopurine phosphoramidites **95a-b** were prepared in several steps from 3',5'-di-*O*-acetyl-N^2-phenylacetyl-2'-deoxyguanosine and 3',5'-di-*O*-acetyl-2'-deoxyinosine, respectively.[136a,b] The insertion of **95a** into oligonucleotides occurred within 3 min with an efficiency of 98%. A dodecamer was synthesized and fractions of the solid support carrying the fully protected oligonucleotide were each treated with different reagents. For example, the reaction of one of these fractions with 10% mercaptoethanol in concentrated ammonium hydroxide at ambient temperature produced a deprotected dodecamer having a 6-thioguanine residue. Alternatively, the treatment of similar oligonucleotidic fractions with either concentrated ammonium hydroxide containing N^1,N^1,N^3,N^3-tetramethylguanidine (65 mM) and *o*-nitrobenzaldoxime (75 mM), 40% aqueous

methylamine, methanol/DBU (9:1) or 0.5 M aqueous sodium hydroxide at 25 °C, afforded deprotected dodecamers having either 2,6-diaminopurine, 2-amino-6-methylaminopurine, O^6-methylguanine or guanine residues.[136a] This general procedure also allowed the introduction of NMR sensitive groups such as ^{15}N, ^{13}C, and ^{17}O derived from [^{15}N]-ammonia, [^{13}C]-methanol and [^{17}O]-water, at defined locations into synthetic oligonucleotides.[136a] Like **95a-b**, the incorporation of the phosphoramidite **95c** into oligonucleotides enabled the postsynthetic modification of these molecules and yielded oligomers containing O^6-methylguanines, 2,6-diaminopurines and their N^6-aminoalkyl analogues, or fluorescent 6-(*N,N*-dimethylaminopyridinium)-2-aminopurine residues.[136c]



95a R= (2,4-dinitrophenyl)thio; R'= NHCCH$_2$Ph

b = (2,4-dinitrophenyl)thio; = H NHCCF$_3$

c = pentafluorophenoxy; = NHCCF$_3$

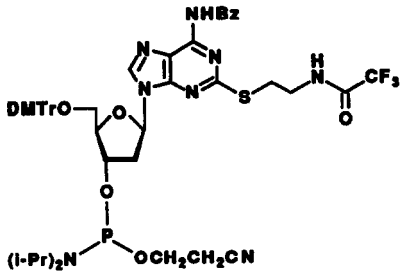
Along similar lines, O^6-phenyl-2'-deoxyinosine was converted to a suitably protected phosphoramidite intermediate and inserted in a self-complementary decanucleotide. The deprotected and purified oligomer was treated with either bis(aminoethyl)disulfide or bis-(aminopropyl)disulfide yielding a DNA duplex having an aminoalkyl disulfide tether at a specific location in consecutive base pairs on opposite strands. Upon reduction with dithiothreitol, the free thiol function formed an interstrand disulfide linkage during aerobic dialysis.[137] Such cross-linked oligonucleotides should facilitate the study of enzyme-mediated unpairing during either transcription, replication or recombination.

The sequence-specific cross-linking of oligodeoxyribonucleotides has also been investigated by Webb *et al.*[138,139] Their approach relied on the incorporation of the modified deoxyribonucleoside phosphoramidites **89a-b** into oligonucleotides by solid-phase methods. The triazolyl nucleobases were converted to their corresponding ethyleneimino derivatives upon treatment with ethyleneimine. The resulting oligomers cross-linked with their complementary oligodeoxyribonucleotides albeit at a slow rate ($t_{1/2}$= 30 h at 22 °C). It was argued that the ethyleneimino function did not provide optimum "interstrand reach" to efficiently cross-link with target sequences.[139]

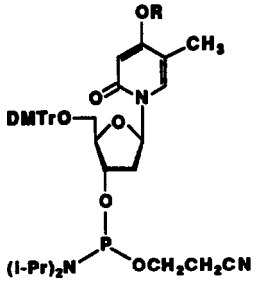
Kido *et al.*[140] described the multistep synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite **96** from 2-mercapto-2'-deoxyadenosine. The single insertion of **96** into oligonucleotides by solid-phase synthesis required a coupling time of 300 sec. Following deprotection and purification, the aminoalkylated oligomers were iodoacetylated and characterized by enzymatic degradation to the nucleosides.[140] Oligonucleotides bearing a 2-(*N*-iodoacatylaminoethyl)thioadenine residue were shown to crosslink and cleave complementary strands at adenines and guanines. It is believed that alkylation occurred at the *N*-3 of adenine from the minor groove or with reactive groups of guanine in the major and minor grooves of the duplex. The efficiency of alkylation was low probably because of the free rotation of the reactive linker around the sulfur atom.[140] More rigid tethers may provide higher alkylation selectivity.

Of interest, the deoxyribonucleoside phosphoramidite **97** has been synthesized to enable the preparation of oligonucleotides containing 3-deazathymine residues.[141] It is anticipated that these modified oligonucleotides will be applied to experiments aimed at estimating the specificity of replication catalyzed by DNA polymerase I. Using nucleosides as models, it has been demonstrated

that 3-deaza-4-*O*-(2,4,6-triisopropylbenzenesulfonyl)thymidine was easily converted to 3-deaza-thymidine upon treatment with concentrated ammonium hydroxide at 55 °C.[141]

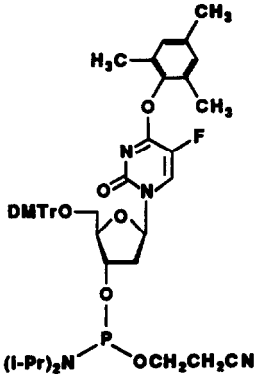


96



97 R= 2,4,6-triisopropylbenzenesulfonyl

In an effort to determine the active-site nucleophile of DNA (cytosine-5)-methyltransferase M.*Hae*III, an enzyme that recognizes dyad-symmetric sites (d[CGCC]) and carries out two sequential methylations of specific cytosine nucleobases, the construction of a suicide DNA duplex was undertaken.[142a,b] One strand of the DNA duplex had a 5-methylcytosine residue at the expected M.*Hae*III methylation site (3'-d[GCGTATC(5-MedC)GGTACTGC]-5'). The complementary strand carried a 5-fluorocytosine nucleobase also at the expected M.*Hae*III methylation site (5'-[CGCATAGG(5-FdC)CATGACG]-3'). These hexadecanucleotides were prepared by the incorporation of the 4-*O*-(2,4,6-trimethylphenyl)-5-fluoro-2'-deoxyuridine phosphoramidite **98** and the 5-methyl-2'-deoxycytidine phosphoramidite **99** by conventional solid-phase synthesis. Upon removal of the protecting groups with concentrated ammonium hydroxide, the 4-*O*-(2,4,6-trimethylphenyl)-5-fluorouracil residue was converted into a 5'-fluorocytosine nucleobase in quantitative yields.[142b] The purified hexadecanucleotides were annealed and then mixed with M.*Hae*III along with the cofactor *S*-adenosyl-L-methionine. The covalent DNA-M.*Hae*III complex was purified and submitted to proteolysis. Sequencing of the proteolytic fragments revealed that Cys_{71} of M.*Hae*III formed a covalent bond with DNA during catalytic methyl transfer.[142b]



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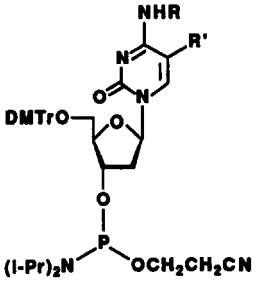


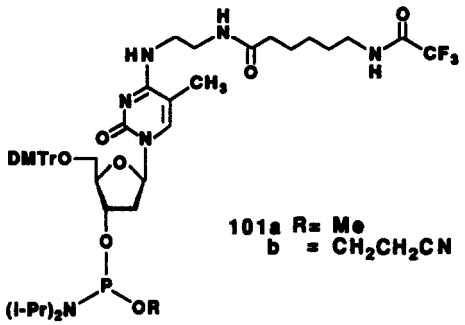
99 R= Bz; R'= Me
100a = Bz; = F
b = H; = F

Schmidt *et al.*[143a] independently described the preparation and the incorporation of the 5-fluoro-2'-deoxycytidine phosphoramidite **100a** into synthetic oligonucleotides to investigate the catalytic mechanism whereby the DNA cytosine methyltransferase of *E. coli* K-12 methylates cytosine residues at

C-5 in a sequence-specific manner. It is important to mention that the 5-fluoro-2'-deoxycytidine phosphoramidite **100b** can be inserted in oligodeoxyribonucleotides by solid-phase techniques without protecting its exocyclic amino function.[143b]

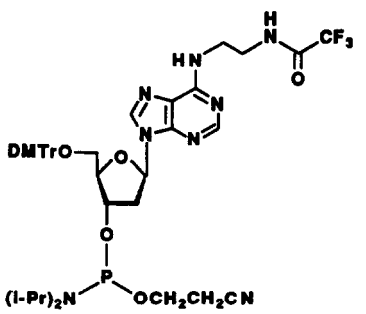
4.2. Ligand Incorporation from Aminoalkylated Purines and Pyrimidines.

Urdea *et al.*[144a] reported the synthesis of the deoxyribonucleoside phosphoramidites **101a-b** from the corresponding 4-triazolopyrimidinone nucleosides. These derivatives led to the solid-phase synthesis of oligonucleotides having aminoalkyl functions at various locations within the oligonucleotidic chains. Reporter groups such as biotin, fluorescein, and isoluminol were coupled to the purified aminoalkylated oligomers and their detection limits were assessed.[144a-b] Furthermore, the reaction of the aminolalkylated oligomers with *p*-phenylene diisothiocyanate followed by incubation with either horseradish peroxidase or alkaline phosphatase afforded the corresponding enzyme-DNA conjugates.[144a]

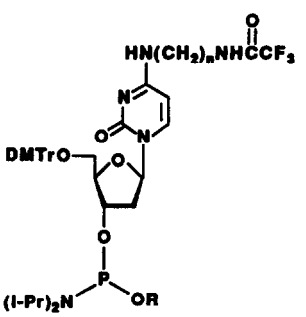


101a R= Me
b = CH_2CH_2CN

Cosstick and Douglas[145] described the synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite **102** and its incorporation into a dimer as a model to evaluate the formation of specific interstrand cross-links. It was argued that the hybridization of an oligonucleotide having a N^6-(2-aminoethyl)adenine nucleobase with a complementary target DNA or RNA sequence would position the nucleophilic aminoethyl function in the viccinity of a pyrimidine residue located on the complementary strand.[145] Activation of the 5,6-double bond of pyrimidines with bisulfites would trigger a nucleophilic attack at *C*-4 by the aminoethyl group and would result in the cross-linking of the two strands. No data supporting this concept have, as yet, been reported.



102

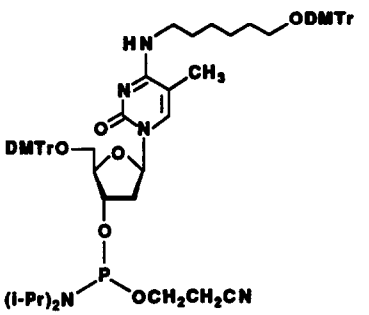


103 R= Me; n= 3
104 = CH_2CH_2CN; = 6
105 = CH_2CH_2CN; = 5

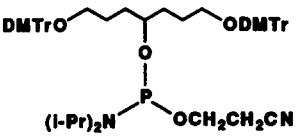
The aminoalkylated phosphoramidites **103** and **104** originated from either the transamination of 2'-deoxycytidine with 1,3-diaminopropane in the presence of sodium metabisulfite[146a,b] or the facile

aminoalkylation of N^4-*p*-toluenesulfonyl 2'-deoxycytidine derivatives with 1,6-diaminohexane.[146d] The deoxyribonucleoside phosphoramidite **104** was incorporated at the 5'-end of an oligonucleotide anchored to a solid support and, after deprotection, the aminoalkylated oligonucleotide was biotinylated.[146d] Along similar lines, the phosphoramidite **105** was prepared in a multistep synthesis from 3',5'-di-*O*-*tert*-butyldimethylsilyl-2'-deoxyuridine and mesitylenesulfonyl chloride.[147] The aminoalkylated phosphoramidite was repeatedly inserted at the 5'-terminus of a 2'-*O*-alkyl oligoribonucleotide and the resulting aminoalkylated oligonucleotide was tetrabiotinylated in an attempt to facilitate the purification of RNA-protein complexes.[147,148]

In an alternate approach, Bazin *et al.*[149] described the synthesis of the deoxyribonucleoside phosphoramidite **106** from the corresponding 4-thiodeoxyribonucleoside and its incorporation into oligonucleotides toward the preparation of polybiotinylated probes. The synthesis of the phosphoramidite **107** was also reported for the same purpose. Incidentally, the coupling of **107** to solid-phase linked oligonucleotides occurred with an efficiency of 94-97%.[149]

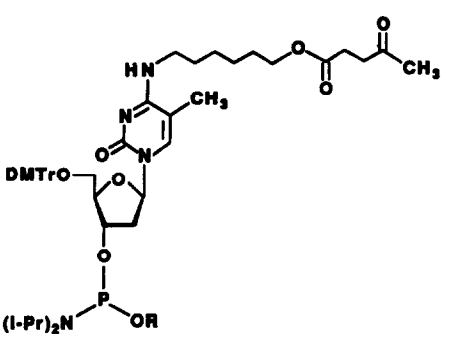


106

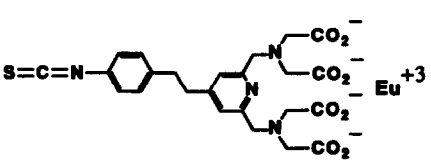


107

The synthesis of "branched" DNA molecules as amplification multimers in bioassays has been reported by Horn *et al.*[150] and by Chang *et al.*[151] This type of branched DNA molecules are composed of two distinctive domains of oligonucleotide sequences. One domain consists of a target specific segment contiguous to an arrangement of branching nucleobases generated by the multiple incorporation of the levulinylated phosphoramidite **108a-b** by standard solid-phase synthesis. The selective removal of the levulinyl groups unmasked hydroxy functions for the solid-phase synthesis of the second domain of oligonucleotides. Labelled probes were hybridized to this second set of DNA sequences thereby providing enhanced detection sensitivity.[150,151]



108a R = Me
b = CH_2CH_2CN

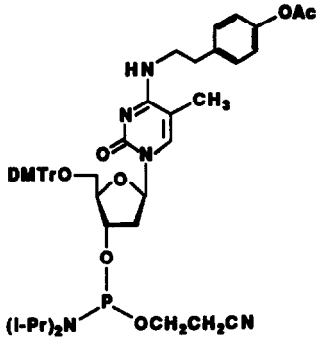


109

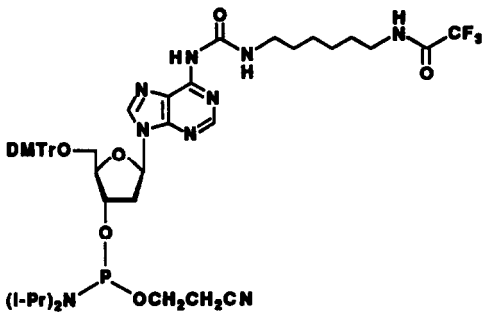
In an effort to prepare Eu^{+3}-labelled DNA probes, Sund *et al.*[152] inserted the deoxyribonucleoside phosphoramidite **105** fifty times at the 5'-end of a synthetic oligomer (50-mer). The purified oligonucleotide (100-mer) was then reacted with the Eu^{+3} chelate **109** at pH 10.5. The Eu^{+3}-labelled probe permitted the detection of 200 picograms of phage lambda DNA by time-resolved fluorometry.[152] It must be noted that a bathophenanthroline-Ru(II) complexe derivatized as a ß-cyanoethyl-*N,N*-diisopropylphosphoramidite derivative, has also been applied to the solid-phase labelling of oligonucleotides.[153] Like Eu^{+3}-labelled oligonucleotides, oligomers labelled with bathophenanthroline-Ru(II) complexes can be detected by time-resolved fluorometry.[25]

The deoxyribonucleoside phosphoramidite **110** was synthesized from 4-thiothymidine and tyramine[154] and inserted at specific locations within oligonucleotides by solid-phase synthesis. The tyramine residues of the deprotected oligonucleotides were radioiodinated by the standard "chloramine T" oxidation. The resulting oligomers have been useful as primers for PCR amplification and for the detection of viral DNA sequences.[154]

The deoxyribonucleoside phosphoramidite **111** has been prepared from N^6-phenoxycarbonyl-2'-deoxyadenosine derivatives[155] and represents an addition to the repertoire of deoxyribonucleoside phosphoramidites dedicated to the preparation of aminoalkylated oligonucleotides.



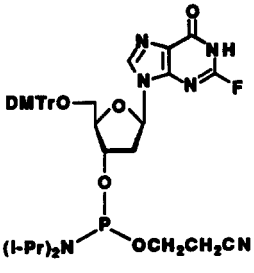
110



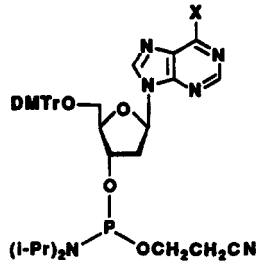
111

4.3. Ligand Incorporation via Halogenated Purines.

Harris *et al.*[156] reported the synthesis and application of the halogenated deoxyribonucleoside phosphoramidites **112** and **113a-b** in the solid-phase synthesis of modified oligonucleotides. Typically, the treatment of these solid-phase bound oligonucleotides with either D-(–)-phenylglycinol or with amines derived from (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-8,9,10,11-tetrahydro-[*a*]-pyrene and (±)-*trans*-8,9-dihydroxy-*anti*-10,11-epoxy-8,9,10,11-tetrahydro-[*a*]-anthracene, under the recommended conditions, afforded the corresponding adducted oligomers.[156] This synthetic method is quite attractive for the preparation of oligonucleotides having structurally defined adducts in the quantities required for structural studies.



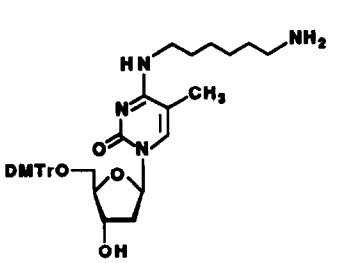
112



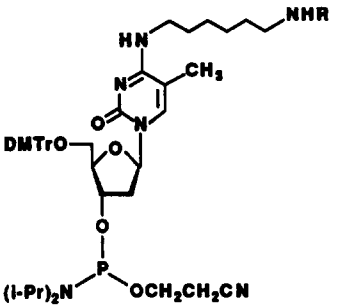
113a X= Cl
b = F

4.4. Modified Nucleoside Phosphoramidites and Direct Ligand Incorporation.

Protected deoxyribonucleoside phosphoramidites carrying various markers such as biotinyl, dinitrophenyl, dansyl and pyrenyl have been synthesized by Roget *et al.*[157] as a means to label synthetic oligonucleotides. Specifically, **114** was prepared in 85% yields from the aminoalkylation of the corresponding 4-thiodeoxyribonucleoside with 1,6-diaminohexane. The reaction of **114** with either biotin *N*-hydroxysuccinimide ester, 1-fluoro-2,4-dinitrobenzene, dansyl chloride or pyrenesulfonyl chloride, followed by phosphitylation, yielded the labelled phosphoramidites **115a-d.** These were activated with 1*H*-tetrazole and incorporated into oligonucleotides with high coupling yields (>97%) by standard solid-phase synthesis. Multiple labelling can be performed on the same oligonucleotide to provide increased detection sensitivity to *in situ* hybridization experiments.[157]

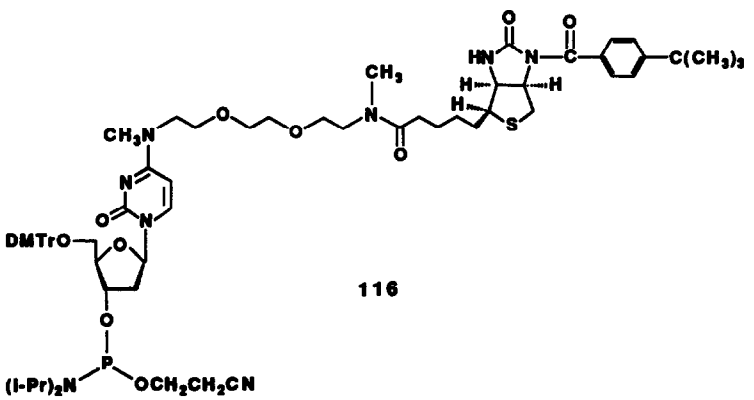


114



115a R= biotinyl
b = 2,4-dinitrophenyl
c = dansyl
d = 3-pyrenesulfonyl

This approach has been further refined by the synthesis of the biotinylated deoxyribonucleoside phosphoramidite **116.**[158] Relative to **115a**, the phosphoramidite **116** carries a larger and more polar spacer arm to facilitate biological applications. Furthermore, **116** is readily soluble in acetonitrile and has been singly or repeatedly inserted at either the 3'-, 5'- or at both termini of 2'-*O*-alkyloligoribonucleotides.[158] The deprotection of DMTr-containing oligonucleotides with concentrated ammonium hydroxide under standard conditions, proceeded without significant loss of biotin. It has been shown that purified tetrabiotinylated oligomers prepared from the incorporation of **116** had reduced non-specific binding to streptavidin-agarose. The tandem incorporation of four biotin residues gave reproducibly superior binding when compared to oligonucleotides containing only one or two biotin residues.[158]

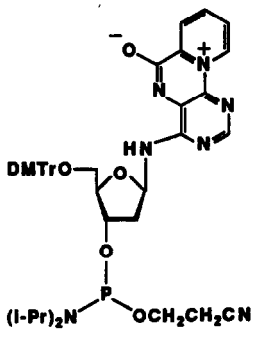


116

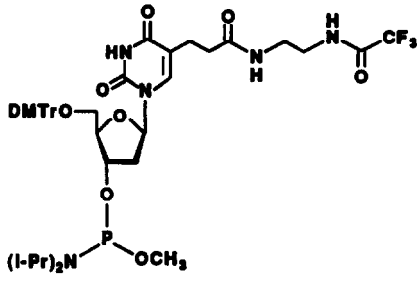
Mielewczyk *et al.*[159] reported the synthesis of the deoxyluminarosine phosphoramidite **117** as a means to generate fluorescent oligonucleotides that could be applied to the non-isotopic detection of specific DNA sequences or the study of nucleic acids stereodynamics. Due to the sensitivity of the luminarosine residue to nucleophilic bases, nucleobase protecting groups different than the benzoyl and isobutyryl groups had to be used to allow complete oligonucleotide deprotection with concentrated ammonium hydroxide at ambient temperature. The solid-phase synthesis of hexadecanucleotides having a single deoxyluminarosine residue has been achieved and deoxyluminarosine-containing oligomers were detected as strong fluorescent bands by polyacrylamide gel electrophoresis analysis.[159]

4.5. Ligand Incorporation at C-5 of Pyrimidines.

The reaction of 5-chloromercury-2'-deoxyuridine with methyl acrylate led to the synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite **118** and its subsequent incorporation into oligonucleotides.[146a-c] Following deprotection and purification, the oligomers were then derivatized with either *N*-succinimidyl-1-pyrenebutyrate, 1-pyrenesulfonyl chloride, fluorescein-5-isothiocyanate, sulfosuccinimidyl-6-(biotinamido)hexanoate, *N*-hydroxysuccinimidyl-anthraquinone-2-carboxylate or the *N*-hydroxysuccinimide ester of 4-carboxy-4'-methyl-2,2'-bipyridine.[146a-c] Incidentally, bipyridine-labelled oligomers reacted with $Ru(bpy)_2(H_2O)_2^{+2}$ to yield oligonucleotides covalently attached to $Ru(bpy)_3^{+2}$ derivatives.[146b] These modified oligonucleotides displayed normal hybridization behavior and ruthenium(II) has been known to effect light-induced DNA strand scission.[146b]

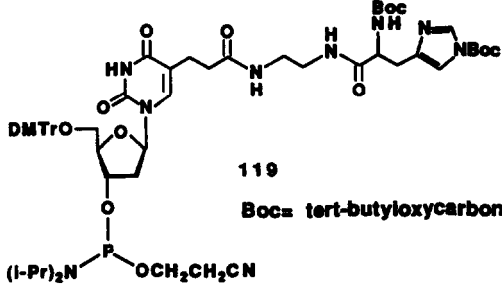


117



118

It is generally accepted that RNA hydrolysis, catalyzed by bovine pancreatic ribonuclease A, requires the participation of two imidazole groups located on histidine residues 12 and 119 in the active site.[160a] In this context, Bashkin *et al.*[160b] proposed the synthesis of ribonuclease mimics by combining the RNA hydrolysis activity of imidazole with the ability of oligonucleotides to bind to RNA with sequence specificity. The deoxyribonucleoside phosphoramidite **119** has been prepared and incorporated at one specific location into oligonucleotides *via* standard solid-phase phosphoramidite methodology. These modified oligonucleotides are now being evaluated as antisense molecules in the control of gene expression and as potential therapeutics.

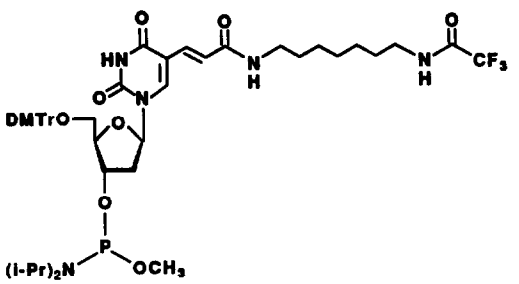


119

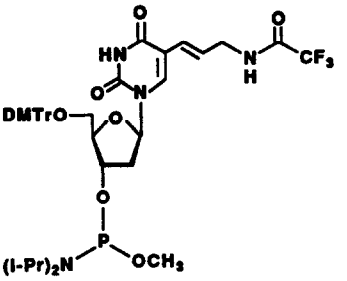
Boc= tert-butyloxycarbonyl

The synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite **120** and its insertion in synthetic oligonucleotides has been reported by Jablonski *et al.*[161a] and others.[161b] These modified oligomers were then reacted with di-(*N*-succinimidyl)suberate and coupled with alkaline phosphatase.[161a,162] The resulting conjugates efficiently hybridized to complementary target sequences and retained the full enzymatic activity associated with the alkaline phosphatase entity. Hybrids were detected with a dye precipitation assay at a the level of 10^6 molecules ($2x10^{-18}$ M) of target DNA. Purified oligonucleotides carrying two aminoalkylated nucleobases could alternatively be reacted with fluorescein isothiocyanate and used as primers in DNA sequencing according to Brumbaugh *et al.*[163]

It is to be noted that the incorporation of the phosphoramidite **121** into oligonucleotides during solid-phase synthesis also produced aminoalkylated oligomers which, upon deprotection, were suitable for reaction with the *N*-hydroxysuccinimide ester of *N*-biotinyl-6-aminocaproic acid.[164] Following purification, the biotinylated oligonucleotides were hybridized to target DNA immobilized on microtiter plates and were detected with a streptavidin-biotinylated horseradish peroxidase complex. Oligonucleotides carrying biotin labels near or at the end of the sequences were more effective probes than those oligomers bearing internal biotin labels.[164]

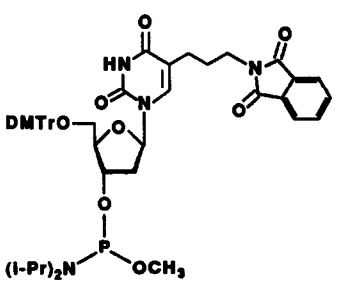


120

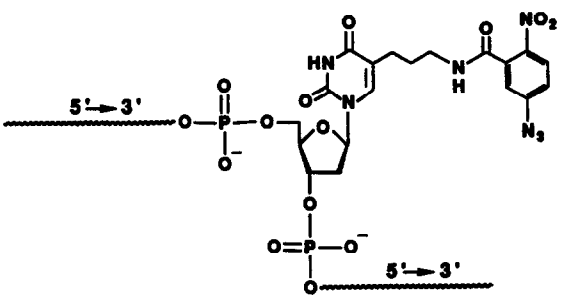


121

In a strategy to investigate protein-DNA interactions Gibson and Benkovic[165a] described the synthesis of the deoxyribonucleoside phosphoramidite **122** from 5-iododeoxyuridine and its integration at the terminus of two undecamers. The deprotected oligomers were treated with the cross-linker (*N*-hydroxysuccinimidyl)-5-azido-2-nitrobenzoate yielding the adduct **123**. Oligomers carrying the photolabile group adjacent to the 3'-end have served as primers for template-directed DNA synthesis with either the Klenow fragment of *E. coli* DNA polymerase I, bacteriophage T4 DNA polymerase, or AMV reverse transcriptase. Irradiation of the primer elongation mixture with 302 nm light produced covalent complexes between DNA and the polymerases. Cross-linking with AMV reverse transcriptase was shown to occur predominantly in the "ß-subunit".[165a]



122



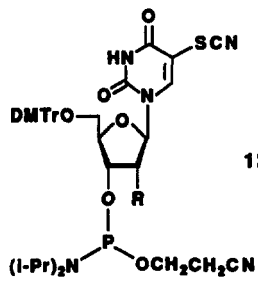
123

Along similar lines, the incorporation of the deoxyribonucleoside phosphoramidite **122** into oligomers has been achieved as a means to generate DNA duplexes biotinylated at specific locations and determine the structural requirements for the exonuclease and polymerase activities of prokaryotic and phage DNA polymerases.[165b] It was demonstrated that, in the presence of avidin, the exonucleolytic and polymerase activities of the Klenow fragment required the primer terminus to be at least fifteen and six base pairs downstream from the biotin-avidin complex, respectively. These results showed that the polymerase and exonuclease sites of the Klenow fragment were physically distinct in solution and displayed different substrate structural requirements for activity.[165b]

The insertion of **122** in oligonucleotides also allowed the derivatization of aminoalkylated oligomers with mansyl or dansyl chloride.[165c,d] Annealing the fluorescent oligomers with complementary oligonucleotides generated duplexes exhibiting fluorescence emissions that increased in intensity upon binding with the Klenow fragment of DNA Polymerase I. By varying the position of the fluorescent label within the DNA duplexes and observing fluorescence emissions, strong enzyme-DNA contact points were identified.[165c]

Bradley and Hanna[166a] described the synthesis of 5-thiocyanato-2'-deoxyuridine and uridine phosphoramidites (**124a-b**) for the functionalization of oligonucleotides without altering Watson-Crick base-pairing. Modified oligonucleotides resulting from the incorporation of **124a** were deprotected under standard conditions without affecting the 5-thiocyanato function. Upon treatment with DTT at 55 °C, the thiocyanato group was reduced to a 5-mercapto function which was then reacted with either *p*-azidophenacyl bromide or 5-iodoacetamidofluorescein to yield functionalized oligonucleotides suitable for the study of protein-nucleic acid interactions.[166a] It must be understood that a variety of functional groups could also be attached to such modified oligonucleotides.

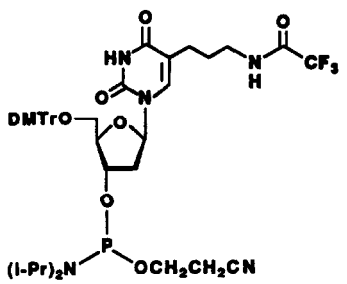
In a different context, dodecamers containing 4-thiocyanatothymine residues and the recognition site d(GATATC) of the endonuclease *Eco*RV or *Eco*RV methyltransferase were found to photochemically cross-link to either enzymes.[166b]



124a R= H
b = OSi(Me)$_2$Bu-t

The protected aminoalkylphosphoramidite **125** was synthesized by Meyer *et al.*[167a] and applied to the solid-phase synthesis of an oligonucleotide carrying an internal 5-(3-aminopropyl)uracil residue. The deprotected oligomer was iodoacetylated with *N*-hydroxysuccinimidyl iodoacetate affording **126**. The incubation of **126** with its complementary DNA target generated observable interstrand cross-linking within 48 h at 37 °C. It was shown that the cross-linking reaction occurred by alkylation of a guanine residue of the complementary strand. Heating the DNA duplex at 51 °C resulted in the depurination of the alkylated guanine residue and scission of the target strand at the depurination site.[167a] In addition, the modified oligonucleotide selectively alkylated the target strand without random alkylation of non-complementary nucleic acids. This feature is quite attractive for the application of these oligonucleotides as chemotherapeutic agents.

Similarly, Povsic *et al.*[167b] reported the synthesis of the deoxyribonucleoside phosphoramidite **127** and its incorporation at the 5'-end of an oligonucleotide by solid-phase synthesis. The deprotected and modified oligonucleotide was treated with *N*-hydroxysuccinimidyl bromoacetate to yield the corresponding *N*-bromoacetylated oligodeoxyribonucleotide. This oligomer hybridized to adjacent



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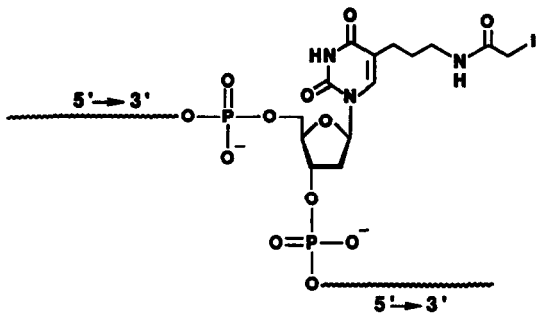
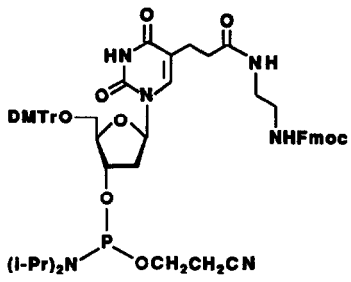


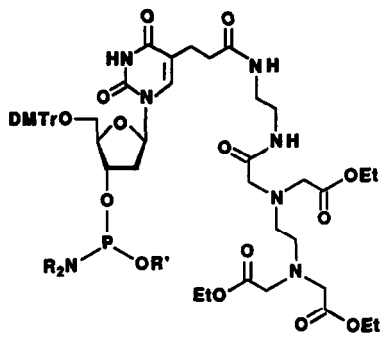
126

inverted purine tracts on double-helical DNA by triple-helix formation and alkylated single guanine residues on opposite strands at 37 °C (pH 7.4). After depurination, double-strand cleavage at a single site within plasmid DNA occurred in yields greater than 85%.[167b] This methodology may become a valuable tool for the sequence-specific cleavage of large DNA molecules.

In their efforts to develop sequence-specific DNA cleaving agents, Dreyer and Dervan[168a] reported the synthesis of a DNA hybridization probe carrying an EDTA-functionalized derivative of thymidine. In the presence of Fe(II), O_2 and DTT the modified probe led to the cleavage of a complementary single-stranded DNA segment.[168a] The EDTA-functionalized deoxyribonucleoside was incorporated into the hybridization probe *via* the phosphoramidite **128a** during routine solid-phase synthesis.[168a,b] The phosphoramidites **128a-b** were similarly inserted in triple-helix forming oligodeoxyribonucleotides which mediated the single site-specific cleavage of double-helical DNA *in vitro*.[168c,d]



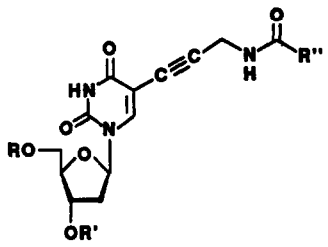
127

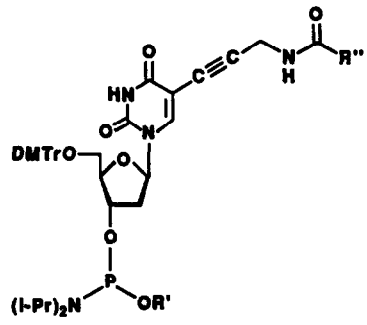


128a R= R'= Me
b R= i-Pr; R'= CH_2CH_2CN

The derivatization of deoxyuridines at *C*-5 with masked primary aliphatic amino groups has also been exploited by Cruickshank *et al.*[169] and Haralambidis *et al.*[170] Their strategies involved the reaction of a suitably protected 5-iododeoxyuridine with a *N*-blocked propargylamine, bis-(triphenylphosphine)palladium chloride, and cuprous iodide to yield the corresponding alkynyl nucleoside **129**[170] or **131**.[169] Additionally, the alkynyl nucleoside **130** was prepared by reacting the deprotected amino function of **129** with the *p*-nitrophenyl ester of 6-*tert*-butyloxycarbonylamidohexanoic acid and DBU.[170] The alkynyl nucleosides **129-131** were converted to their corresponding phosphoramidites (**132-134**) and inserted in oligonucleotides by standard solid-phase synthesis.[169,170] Following the removal of the phosphate protecting groups, the support-linked oligomers were treated with trifluoroacetic acid/ethanedithiol (9:1) for 5 min to cleave the *tert*-butyloxycarbonyl protecting group(s) without extensive depurination.[170] The purified aminoalkylated oligonucleotides were

conjugated to fluorescein[169,170] and the resulting conjugates were hybridized with complementary polyadenylated mRNAs.[170] Probes having short linker arms (derived from **132**) did not hybridize as efficiently as the probes carrying larger linker arms (derived from **133**). Furthermore, 5'-singly labeled probes hybridized better than the multilabelled ones.[170]



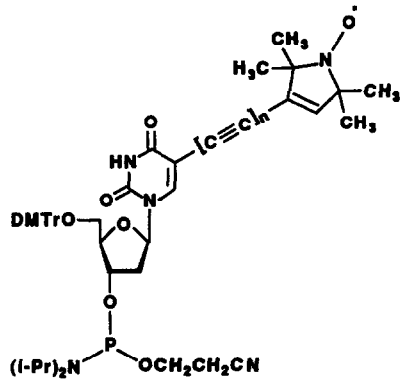


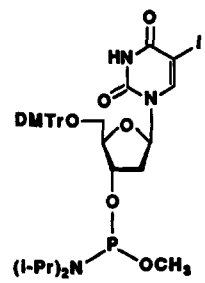
129 R= R'= toluyl; R''= $OCMe_3$
130 = toluyl; = $(CH_2)_5NHBoc$
131 R= DMTr; R'= H; R''= CF_3

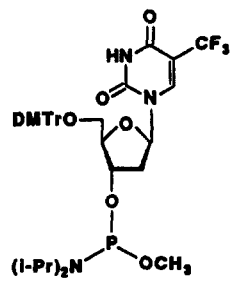
132 R'= Me; R''= $OCMe_3$
133 = Me; = $(CH_2)_5NHBoc$
134 = CH_2CH_2CN; = CF_3

Boc= tert-butyloxycarbonyl

Using a similar synthetic methodology, Spaltenstein *et al.*[171] prepared the deoxyribonucleoside phosphoramidite **135a** for the site-specific incorporation of a paramagnetic probe into a self-complementary oligonucleotide. The bulky and hydrophobic nitroxide moiety did not induce disruption of DNA secondary structures and was sufficiently constrained to permit the correlation of its motion with that of the carrier DNA. However, the length of the alkynyl arm (from the incorporation of **135b**) affected the rotation of the nitroxide moiety about the alkyne axis and should be taken into consideration when monitoring sequence-dependent structural features.[172] These probes may, nonetheless, be useful in investigations pertaining to the dynamics of unusual DNA structures and/or sequences of biological relevance.







135a n= 1
b = 2

136

137

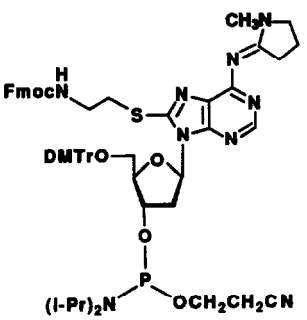
The study of macromolecular structures can be performed by X-ray scattering methods both in solution and in the solid state. The presence of a heavy atom label facilitates the elucidation of the geometric and dynamic structure of the molecule. In this context, Sheardy and Seeman[173] reported the

synthesis of the 5-iododeoxyuridine phosphoramidite **136** and its incorporation into a DNA segment corresponding to an analogue of strand 2 of the immobile nucleic acid junction J1.[174] To minimize the loss of iodine, the oligonucleotide analogue was completely deprotected by treatment with concentrated ammonium hydroxide at ambient temperature.[173] In an effort to unravel the detailed chemistry of genetic recombination, oligonucleotides required for the additional construction and analysis of immobile[175a] and monomobile DNA junctions have also been synthesized from deoxyribonucleoside phosphoramidite derivatives.[175b]

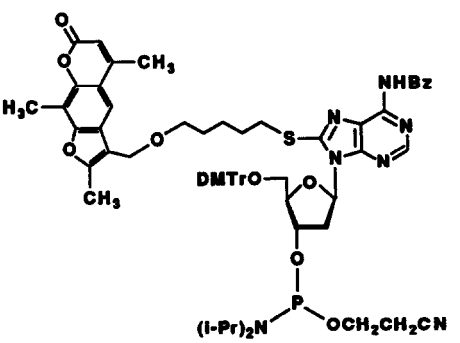
The trifluorothymidine phosphoramidite **137** has been synthesized and incorporated with a coupling efficiency of *ca.* 94% at defined locations into an oligonucleotide designed to inhibit the expression of gene sequences encoding serine proteases in T-lymphocytes.[176] The modified oligomer d(GAGAT*CT*T*CAT*CT*T*CCCCGG), where T* represents trifluorothymidine, was detected by ^{19}F NMR spectroscopy at a concentration of 10 μM with a signal to noise ratio of 10:1.[176] It has been postulated that such limits of detection should be satisfactory for the *in vivo* NMR imaging of the cellular uptake and intracellular distribution of the oligonucleotide analogue.

4.6. Ligand Incorporation at C-8 of Purines.

Roduit *et al.*[177] described a different approach for the introduction of an amino linker into synthetic DNA. The method entailed the reaction of 8-bromodeoxyadenosine with a protected aminoalkylthiolate salt. The deoxyribonucleoside phosphoramidite **138** was prepared in seven steps from 2'-deoxyadenosine and was inserted in oligonucleotides with high coupling yields (96-99%). The purified aminoalkylated oligonucleotides were biotinylated upon reaction with either the *N*-hydroxysuccinimide ester of biotin or caproylamidobiotin in a neutral buffer.[177]



138



139

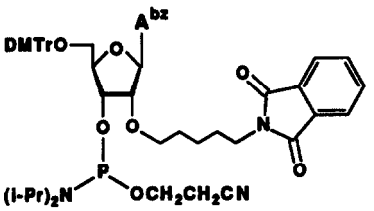
Using a similar concept, Pieles *et al.*[178] described the attachment of 4,5',8-trimethylpsoralen at *C*-8 of deoxyadenosine through a sulfur atom and a five carbon atom linker. The modified nucleoside was converted to the phosphoramidite **139** and incorporated into oligonucleotides. The hybrid formed between a modified oligomer and its unmodified complementary strand was exposed to near UV light (345 nm) for 50 min. It was shown that the extent of cross-linking in this experiment was greater than 90%.[178] The potential biological relevance of this *in vitro* cross-linking experiment should provide the impetus to define the parameters required for such events to occur *in vivo*.

5. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE CARBOHYDRATES

The derivatization of oligonucleotides, particularly, at the internucleotidic phosphodiester linkages or at the nucleobases has inherent limitations. Functional groups attached to these entities may interfere with base pairing and/or stacking interactions. The functionalization of oligonucleotides

by incorporating 2'-*O*-modified ribonucleosides may provide an alternate approach to the multiple labeling of nucleic acids in the minor groove.

Manoharan *et al.*[179] reported the synthesis of the ribonucleoside phosphoramidite **140** in 21% overall yield from adenosine. The synthetic method consisted of the preferential 2'-*O*-alkylation of the nucleoside with an alkyl halide followed by nucleobase/5'-*O*-protection and 3'-*O*-phosphitylation.



140

The insertion of **140** in oligonucleotides (20-mers) by solid-phase synthesis proceeded within 10-15 min with a coupling efficiency better than 95%. Standard deprotection yielded 2'-*O*-aminoalkylated oligonucleotides which, after purification, were reacted with fluorescein isothiocyanate or with the *N*-hydroxysuccinimide ester of either biotin, cholic acid, or digoxigenin-3-*O*-methylcarbonyl-ϵ-aminocaproic acid in aqueous buffers at pH 8-9. This methodology also led to the functionalization of oligodeoxyribonucleoside phosphorothioates and 2'-*O*-methyl oligoribonucleotides.[179] It must be noted that the length of the 2'-aminoalkylated linker can be selected to facilitate minor groove modification for biological applications.

CONCLUDING REMARKS

Inasmuch as nucleoside phosphoramidites have had a tremendous impact on the synthesis of oligonucleotides,[1] this Report further delineates applications of phosphoramidite derivatives to the functionalization of oligonucleotides for, in most cases, biomedical purposes. The incorporation of nucleoside phosphoramidites with modified nucleobases into oligonucleotides has also contributed to a better understanding of the dynamics and thermal stability of DNA duplexes in solution[180,181] and their recognition by proteins. Furthermore, such modified oligonucleotides have recently served as models in mutagenesis experiments and as inhibitors of gene expression by interfering with the transcription of specific genes or with the translation of specific messenger RNAs. These applications will be addressed in detail in a future Report.[182]

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