Tetrahedron Vol. 49, No. 28, pp. 6123-6194, 1993
Printed in Great Britain

0040-4020/93 $6.00 + .00
Pergamon Press Ltd

TETRAHEDRON REPORT NUMBER 335

The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and Their Applications

Serge L. Beaucage* and Radhakrishnan P. Iyer

Division of Biochemistry and Biophysics, Center for Biologics Evaluation and Research
Food and Drug Administration, Bethesda, Maryland 20892.

(*Received* 15 *March* 1993)

Contents

INTRODUCTION

Although the application of nucleosidic and non nucleosidic phosphoramidite derivatives to the preparation and functionalization of oligonucleotides has recently been reviewed,[1,2] the synthesis of

oligonucleotide analogues *via* nucleosidic phosphoramidites and their applications will be addressed in this Report. Specifically, the relevance of modified oligonucleotides to the study of the physicochemical properties of DNA duplexes in solution and toward a better understanding of proteins-DNA interactions will be discussed along with experimental models pertaining to DNA damage and mutagenicity. Furthermore, the application of modified nucleoside phosphoramidites to the synthesis of oligonucleotide analogues as inhibitors of gene expression and as potential therapeutics will be emphasized. Given the efficacy of oligonucleotides as chemotherapeutics is largely dependent on their cellular permeation, their stability to extracellular and intracellular nuclease degradation, and their affinity to selected targets, the synthesis of oligonucleotides with modified internucleotidic phosphodiester linkages and/or carbohydrates will be reviewed in detail.

1. THERMODYNAMIC PROPERTIES OF DNA/RNA, PROTEIN-DNA INTERACTIONS, AND DNA/RNA RECOGNITION

1.1. DNA/RNA Thermodynamics and Protein-DNA Interactions.

The insertion of 7-deaza-2'-deoxyguanosine d(c^7G) *via* the phosphoramidite **1a** (Table 1) in the self-complementary hexamer d(c^7GCc^7GCc^7GC) generated a duplex having a lower T*m* than that obtained with an unmodified duplex (ΔT*m* = 10 °C).[3a] A possible explanation for this phenomenon could be the higher *pKa* of the N-H function of d(c^7G) (*pKa* = 10.3) relative to that of 2'-deoxyguanosine (*pKa* = 9.3) which resulted in a destabilization of the Watson-Crick d(c^7G)-dC base pair. Similarly, the phosphoramidites **2a-b** and **3a-b** were utilized in the preparation of modified alternating d(G-C)$_3$ sequences to probe the structure of these DNA duplexes.[3b]

Seela and coworkers demonstrated that the integration of 7-deaza-2'-deoxyadenosine [d(c^7A)][4a,b] or 8-aza-7-deazaadenosine [d(c^7z^8A)][4c] *via* **4a**, **5a-b** or **6** into oligonucleotides provided stabilization of the d(c^7A-T)$_3$, d(c^7A-T)$_6$, d(c^7z^8A-T)$_9$, and d(c^7z^8A-T)$_{12}$ duplexes relative to the corresponding d(A-T) sequences. The nature of the adjacent nucleobases was nonetheless important to the stabilization or destabilization of duplexes containing d(c^7A).[4a]

The incorporation of d(c^7G) at the cleavage site of the palindromic octamer corresponding to the recognition sequence of the endonuclease *Eco*RI [d(pGc^7GAATTCC)][5a] resulted in an enzymatic hydrolysis rate of less than 2% than that observed with the unmodified palindromic duplex.[5b] It was concluded that the *N*-7 of guanine was a binding site between the DNA fragment and the enzyme but was not a prerequisite for the recognition of the DNA segment. The substitution of d(c^7G) for dG lowered the T*m* of the duplex by 4 °C implying that the substitution had minor influence on its secondary and tertiary structure.[5b]

Numerous modified nucleosides represented by the phosphoramidites **3-30** (Table 1) have been incorporated into synthetic oligomers to evaluate duplex stabilities, and to provide a better understanding of the restriction site determinants required by *Bam*HI,[6a-b] *Dpn*I,[6d] *Eco*RI,[5,6a-c,7-10] *Eco*RV,[11-13] *Hin*dII,[14] *Hin*dIII,[15] *Sal*I,[14] *Sau*3A,[4c,7a] and *Taq*I[14] for sequence recognition and phosphodiester cleavage. In addition, the deoxyribonucleoside phosphoramidites **17** and **18** have been inserted in dodecamers to study ligand-DNA and protein-DNA interactions. The 5-methyl-4-pyrimidinone residues were resistant to the conditions used during the synthesis and deprotection of DNA sequences. However, the 2-thiothymine nucleobases decomposed to various products upon treatment with an iodine solution.[16b]

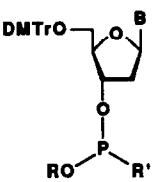
Diekmann and McLaughlin[17a] reported that the ligation of a decadeoxyribonucleotide containing the *Eco*RI recognition site formed a series of "curved" multimers on the basis of anomalous migration in polyacrylamide gels. It was shown that the insertion of modified base pairs in the *Eco*RI recognition sequence using the deoxyribonucleoside phosphoramidites **10b**, **19**, **28** or **29**, led to increased migration anomaly or increased DNA curvature when the purine substituent at *C*-6 was an amino group rather than a hydrogen or an oxo group. The deletion of the 2-amino group of guanine in the minor groove of the B-DNA helix was also effective at increasing the observed DNA curvature.[17a,b] It would appear from the endonuclease catalysis data, that curvature of the DNA helix axis is an inherent property of the

d(GAATTC) sequence which optimizes the interactions between *Eco*RI and its recognition sequence.[17a]

Hagerman has additionally demonstrated that the substitution of uracils for thymines in DNA containing short $(dA)_n(dT)_n$ sequences or the substitution of 5-methylcytosines for cytosines in $(dI)_n(dC)_n$ tracts (*via* the corresponding deoxyribonucleoside phosphoramidites) had a pronounced effect on the degree of stable curvature of the helix axis.[18] Such effects argued for a general structural perturbation due to the methyl group and it was thereby concluded that pyrimidine methyl groups could influence protein-DNA interactions not only through protein-methyl group contacts but also by locally altering DNA structure.[18] In spite of these findings, Diekmann *et al.* stated that the ability of a DNA sequence to adopt a B' structure with large propeller twist to confer intrinsic curvature on the helix axis was mainly determined by the stability of the stacking interactions between adjacent base pairs. Pyrimidine methyl groups can modulate the extent of curvature present but were not a dominant factor for the observed phenomenon.[19]

Phosphoramidites of 7-deaza-2'-deoxyadenosine d(c^7A) (**4a**, **12a-b**) have also been employed to replace deoxyadenosine within $(dA)_6$ tracts to evaluate the effect of the isosteric nucleobase on the bending of oligonucleotides.[20] A decreased bending was observed upon substitution of d(c^7A) for dA in these oligonucleotides. However, the decrease in bending was strongly dependent on the position of d(c^7A) within the $(dA)_6$ tracts. These observations are in agreement with those reported by Ono *et al.* who described the incorporation of the 2'-deoxydeazaadenosine phosphoramidite **4b** or **11** at selected locations into $(dA)_4$-$(dT)_4$ tracts.[21]

Table 1. Deoxyribonucleoside Phosphoramidite Derivatives in the Study of DNA Thermodynamics and Protein-DNA Interactions



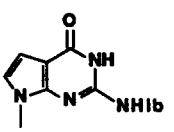
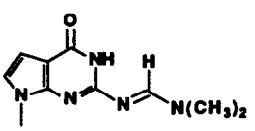
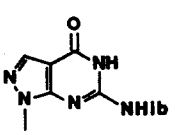
Compound	R	R'	B	References
1 a	CH_3—	—N(Pr-i)$_2$		3a-b,5a-b 8,35
b	$NCCH_2CH_2$—			
2 a	CH_3—	—N(Pr-i)$_2$		3b,68
b	$NCCH_2CH_2$—			
3 a	CH_3—	—N(Pr-i)$_2$		3b,7a,c
b	$NCCH_2CH_2$—			

Table 1. CONT'D

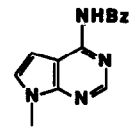
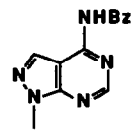
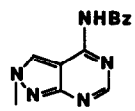
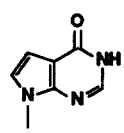
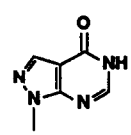
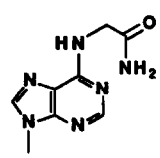
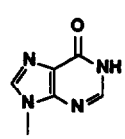
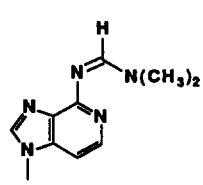
Compound	R	R'	B	References
4a b	CH_3— $NCCH_2CH_2$—	—N(Pr-i)$_2$		4a-b,5a,6a 7d,12a-b,20 21,34,69b
5a b	CH_3— $NCCH_2CH_2$—	—N(Pr-i)$_2$		4c,6a,7a
6	CH_3—	—N(Pr-i)$_2$		4c,7a
7a b	CH_3— $NCCH_2CH_2$—	—N(Pr-i)$_2$		5b,14 55a-b,68
8	CH_3—	—N(Pr-i)$_2$		7a,55a-b
9	CH_3—	—N(Pr-i)$_2$		15
10a b*	CH_3— $NCCH_2CH_2$—	—N(Pr-i)$_2$		13,14,17,19 34,35,55a-b
11	$NCCH_2CH_2$—	—N(Pr-i)$_2$		21

Table 1. CONT'D

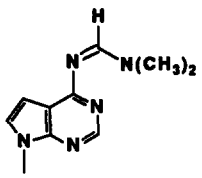
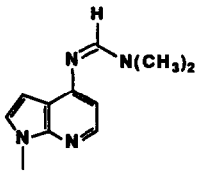
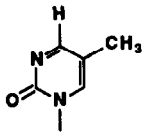
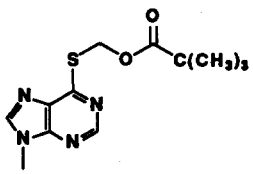
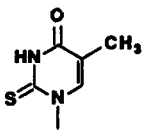
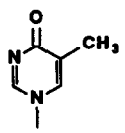
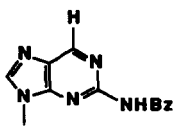
Compound	R	R'	B	References
12a	CH_3—	—$N(Pr\text{-}i)_2$		20
b	$NCCH_2CH_2$—			
13	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		27b
14*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12a-b,16a 34,69a,70
15a R= SCH_3	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12a,b,d,69a 70-72
b = Ph				
c = p-NO_2Ph				
d = CH_2CH_2CN				
e = CH_2OPiv				
16	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		72
17	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12a-b,16b 70
18	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		16b
19*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		6b,10 17b,69

Table 1. CONT'D

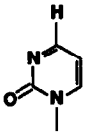
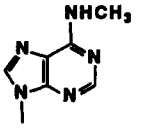
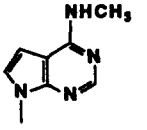
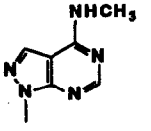
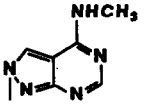
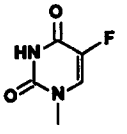
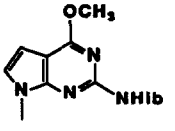
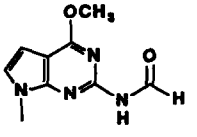
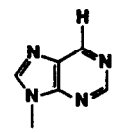
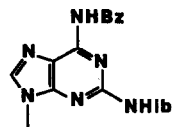
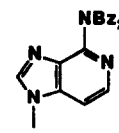
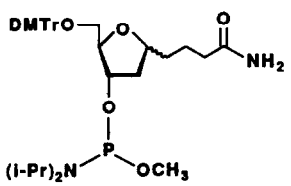
Compound	R	R'	B	References
20*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		16a,69b
21a	CH_3—	—N O		6d,73
b	CH_3—	—$N(Pr\text{-}i)_2$		
c	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		35
22	CH_3—	—$N(Pr\text{-}i)_2$		6d
23	CH_3—	—$N(Pr\text{-}i)_2$		6d
24	CH_3—	—$N(Pr\text{-}i)_2$		6d
25	CH_3—	—$N(Pr\text{-}i)_2$		8
26a	CH_3—	—$N(Pr\text{-}i)_2$		7b
b	$NCCH_2CH_2$—			
27a	CH_3—	—$N(Pr\text{-}i)_2$		7b
b	$NCCH_2CH_2$—			

Table 1. CONT'D

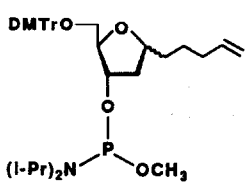
Compound	R	R'	B	References
28*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		9,12a,13,14 17,34,69a,74
29*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		17b
30	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12c

*** The (9-phenyl)xanthen-9-yl group was also used as a 5'-*O*-protecting group. ib= isobutyryl; Piv= pivaloyl; Bz= benzoyl**

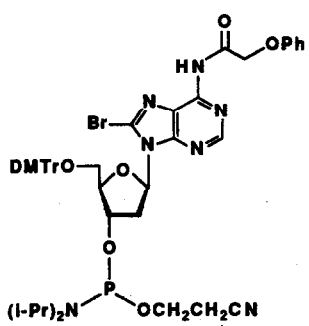
The 2-deoxy-D-ribofuranose phosphoramidite derivatives **31a-b** and **32** were prepared by François *et al.*[22] and applied to the synthesis of the oligodeoxyribonucleotide GTCGTGACYGGAAAAC, where Y represents the modified nucleotidic residue. Unexpectedly, a DNA duplex composed of the oligonucleotide analogue derived from the single insertion of **32** and its complementary sequence exhibited a higher T*m* than that of similar duplexes featuring either a pyrimidine-pyrimidine mismatch or a single incorporation of **31a** or **31b**.[22] It was speculated that the increased duplex stability resulted from an hydrophobic effect.



31a α-anomer
b β-anomer



32



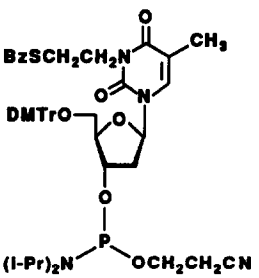
33

The 8-bromo-2'-deoxyadenosine phosphoramidite **33** has been synthesized and incorporated into oligonucleotides as a means to identify DNA nucleobase-amino acid contact pairs in protein-DNA complexes *via* photocross-linking techniques.[23a] Preliminary experiments showed that the presence of a

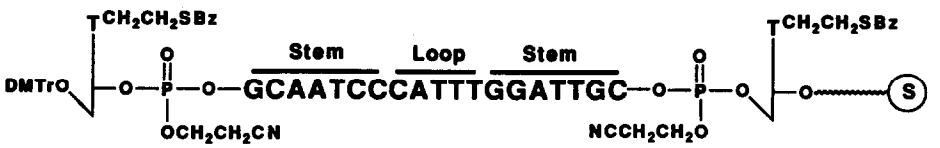
single photo-active 8-bromoadenine residue in oligoribonucleotides having a specific binding site for the transcription factor NF-κB has not significantly affected the stability of the corresponding DNA duplexes and their function as protein-binding sites.[23a] Consequently, 8-bromo-2'-deoxyadenosine-containing oligonucleotides may be useful in probing specific contacts in protein-DNA complexes.

A general method for the stabilization of the molecular architecture of DNA hairpins through disulfide bond formation has been proposed by Glick.[23b] The synthetic approach consisted of the consecutive preparation of N^3-benzoylmercaptoethyl-2'-deoxythymidine, the derivatization of a controlled-pore glass support with the modified nucleoside, and the incorporation of the modified phosphoramidite **34a** at the 5'-end of the oligonucleotide forming the hairpin structure (**34b**). Due to the unstability of the mercaptoalkylated thymine nucleobase to the harsh alkaline conditions employed for the removal of conventional base protecting groups, FOD-amidites[24] were used during solid-phase oligonucleotide synthesis. The purified mercaptoalkylated oligomer was aerobically stirred for 12 h to effect the predominant intramolecular formation of the disulfide link. The cross-linked hairpin DNA was isolated in 22% yield with a purity greater than 97%. The T*m* of the cross-linked hairpin was 21 °C higher than that of the unmodified hairpin.[23b] This synthetic methodology may be applicable to "trap" various conformations of the same sequence under controlled conditions.[23c]

It must be noted that the chemical synthesis of oligodeoxyribonucleotide dumbbells from deoxyribonucleoside phosphoramidites has also been undertaken to instigate detailed structural and physical studies of these macromolecules.[25]



34a

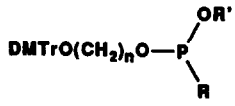


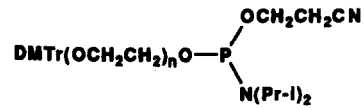
34b

A novel strategy for potential site-directed chemical reactions in DNA has been described by Asseline *et al.*[26] The approach involved the incorporation of a propanediol linkage (*via* **35a-b** or **36b**) at a defined location into an oligonucleotide and the subsequent generation of a DNA duplex with a complementary DNA strand having an extra nucleobase opposite the propanediol residue. Upon addition of an equimolar amount of a bis-acridine derivative, the stability of the duplex increased and NMR studies indicated that the bis-acridine intercalated the duplex at the site occupied by the propanediol residue.[26] These results suggest that this approach could target a specific chemical reaction, at a selected site in DNA, with a suitably derivatized bis-acridine derivative.

Seela and Kaiser[27a] employed the phosphoramidites **35a-b** to replace either dA or dT residues in the palindromic dodecamer d(CGCGAATTCGCG). The modified oligonucleotides exhibited a strong tendency to form hairpins presumably because of the highly flexible 1,3-propanediol linker.

The polyethylene glycol phosphoramidite **37b** has similarly been applied to the synthesis of the oligodeoxyribonucleotide d(GCTCACAAT-X-ATTGTGAGC), where X represents the hexaethylene





35a n = 3; R = N,N-diisopropylamino; R' = methyl
b = 3; = N,N-diisopropylamino; = 2-cyanoethyl
36a n = 2; R = morpholino; R' = 2-cyanoethyl
b = 3; = morpholino; = 2-cyanoethyl

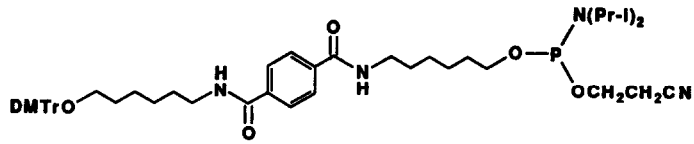
37a n = 2
b = 6
c = 10

glycol linker.[28a] Circular dichroism spectroscopy revealed that the oligonucleotide analogue existed as a bulged duplex and a hairpin structure in equilibrium with each other. Interestingly, the same oligonucleotide having X replaced with four thymines existed only as a hairpin structure. It was argued that the hairpin form was enthalpically stabilized by the stacking of the thymine residues of the loop region.[28a]

The incorporation of **37b** into the oligonucleotide $(dA)_{12}$-X-$(dT)_{12}$-X-$(dT)_{12}$ by solid-phase methods generated an oligonucleotide capable of folding back on itself twice to form a triple helix at low temperature.[28b] Thermal denaturation analysis revealed two cooperative transitions. At low-salt concentration (0.1 M sodium chloride), the transition of triplex to duplex with a dangling X-$(dT)_{12}$ extremity occurred at *ca.* 30 °C while the duplex to coil transition was observed at *ca.* 60 °C. CD spectroscopy indicated that the conformation of the triplex structure of $(dA)_{12}$-X-$(dT)_{12}$-X-$(dT)_{12}$ was essentially identical to that of a mixture composed of $(dA)_{12}$ + $2(dT)_{12}$.[28b] The facile formation of intramolecular triple-helical DNA structures relative to intermolecular triplexes provides a better opportunity to study the interaction of small molecules, such as intercalators, with these helices.

The terephthalamide phosphoramidite **38** has also been applied to the solid-phase synthesis of $(dT)_6$-X-$(dA)_6$ and $(dT)_6$-X-$(dT)_6$-X-$(dA)_6$, where X represents the terephthalamide linker.[29] The folding patterns displayed by these oligonucleotides were similar to those reported by Durand *et al.*[28a]

In this context, various ribonucleoside phosphoramidites have been used in the synthesis of oligoribonucleotides to investigate the structure and stabilizing factors of RNA hairpins[30] in addition to the thermodynamics of internal[31a] and bulge[31b] RNA loops.



38

Deoxy- and ribonucleoside phosphoramidites have been employed in the synthesis of chimeric DNA-RNA oligonucleotides.[32] These chimeras have shown subtle conformational effects resulting from the incorporation of a ribonucleotide into a DNA strand or the insertion of a deoxyribonucleotide in a RNA strand. To further study the conformational behavior of these modified oligonucleotides, chimeric duplexes have been synthesized and the structure of these duplexes has been analyzed by X-ray crystallographic techniques. These analyses revealed that [r(G)d(CGTATACGC)]$_2$, [d(GCGT)r(A)d(TACGC)]$_2$ and [r(GCG)d(TATACCC)/d(GGGTATACGC)] formed A-helices whereas [d(CG)r(CG)d(CG)]$_2$ formed a left-handed Z-helix.[32]

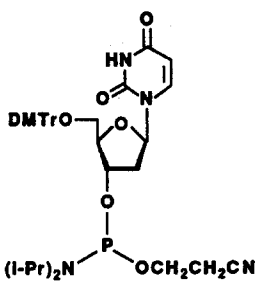
The insertion of modified nucleosides in oligonucleotides provided valuable insights toward a better understanding of the recognition of promoters by RNA polymerase in microorganisms. For example, Caruthers *et al.*[33] substituted uracil for either one or both adjacent thymine residues at position 34 and 35 relative to the transcription initiation site of the bacteriophage lambda P_R promoter. The loss of either methyl group reduced the rate of formation of transcriptionally competent complexes by 4-5 fold. The loss of the methyl group at both sites produced an inactive promoter. Consequently, the methyl group of these specific thymine residues appears critical for the interaction of RNA

polymerase with the lambda P_R promoter.[33b]

The interactions between the *trp* repressor and its operator sequence have similarly been studied by nucleobase analogue substitution.[34] The incorporation of the deoxyribonucleoside phosphoramidites **4b**, **14** and **28** at specific locations into oligonucleotides (20-mers) containing 18 base pairs of the *trp* operator was accomplished by standard solid-phase synthesis and afforded fourteen modified sequences. It was found that the carbonyl at dT_{+4} (the position of the center of symmetry of the doubled-stranded operator has been defined as 0) was critical for the formation of the high-affinity sequence-specific complex. In addition, the thymine methyl group at dT_{+4} and the *N*-7 of dA_{+5} appear necessary for high-affinity binding by the repressor. Interestingly, the deletion of the adenine amino group at dA_{-4} or dA_{+5} resulted in a sequence binding to the repressor with a higher affinity than that observed with an unmodified sequence.[34]

To better rationalize the high-affinity binding of HIV-1 *rev* protein to a bubble structure located within the *rev*-response element (RRE) RNA in stem-loop II, the incorporation of deoxyribonucleoside phosphoramidites (**1b**, **21c**, **28**, **39**) and ribonucleoside phosphoramidites (**40**, **41**) at selected positions into a minimal synthetic RNA duplex carrying the bubble was undertaken.[35] It has been speculated that high-affinity recognition of RRE RNA by *rev* requires hydrogen bonding to functional groups in the major groove of a distorted RNA structure.[35]

The insertion of 5-bromouridine (5-BrU) in oligoribonucleotides *via* the phosphoramidite derivative **40** has also been used to probe the interaction of MS2 coat protein with the translational operator of the MS2 replicase gene.[36] The increased stability of the complex formed between a specific 5-BrU operator and the coat protein relative to a wild-type operator presumably resulted from the formation of a transient covalent link between the modified operator and a cysteine side-chain of the protein *via* a Michael addition.[36]



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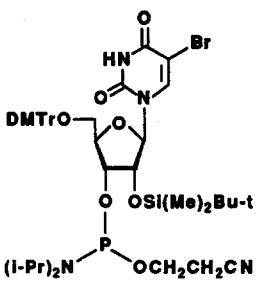

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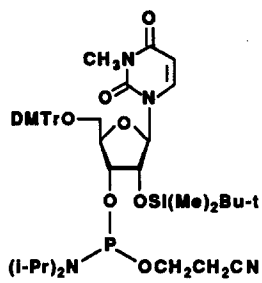
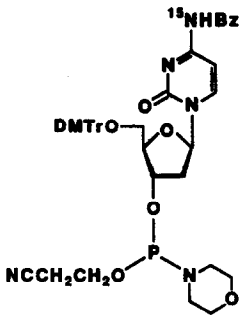


41

NMR studies of ^{15}N-labelled oligodeoxyribonucleotides have provided useful structural information about the nature of protein-DNA interactions.[37] ^{15}N-labelled phosphoramidites (**42** and **43**) were prepared and incorporated at specific locations into oligonucleotides corresponding to the symmetric 18 base pairs of the *lac* operator. The advantage of applying ^{15}N-edited NOE spectroscopy to map the individual environment of the protons coupled to the labelled nuclei was demonstrated and sequence-specific ^{15}N-chemical shifts were reported.[37a,b] Additionally, Massefski *et al.*[38] described the synthesis of the phosphoramidite **44** and its application to the solid-phase synthesis of a 17 base pair oligomer corresponding to the O_L operator of bacteriophage lambda. It was shown by "selective difference decoupling" that the three guanine residues of the oligonucleotide had different ^{15}N-chemical shifts.[38] Thus, the significant sequence dependence of ^{15}N-7 chemical shifts along with their sensitive detection through protons and facile assignments strongly support the utilization of $^{15}N7$-purine oligonucleotides in the study of DNA structure and dynamics.

A convenient synthesis of DNA segments having the exocyclic amino function of cytosine labelled with Nitrogen-15 has been reported by Kellenbach *et al.*[39] Their approach relied on the incorporation of the triazolo deoxyribonucleoside phosphoramidite **45a** into oligonucleotides and the subsequent



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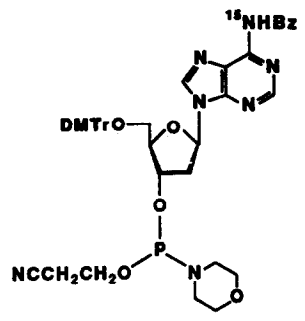

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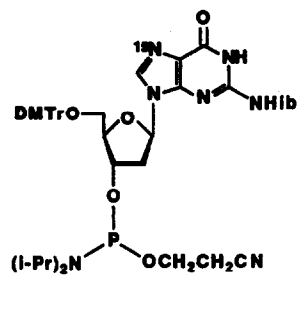
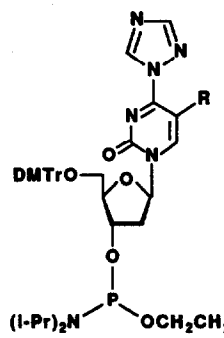


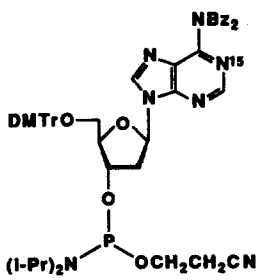
44

treatment of the modified oligomers with concentrated ^{15}N-ammonium hydroxide to generate $^{15}N^4$-cytosine residues. It must be noted that the ^{15}N-chemical shift of the exocyclic amino function of cytosine (*ca.* 98 ppm) is different than the ^{15}N-chemical shifts of proteins which usually resonate at 105-130 ppm.[39] The ^{15}N-labelling of the amino function of cytosines in oligonucleotides should consequently facilitate the study of protein-DNA interactions.



45a R= H
b = Me

^{15}N-NMR analysis of specifically labelled nucleic acids has been useful in probing local structural phenomena ranging from thermally induced local melting to the behavior of mismatched base pairs, and to the structural changes triggered by enzyme recognition or drug binding.[40] For example, the duplex formation of the self-complementary oligodeoxyribonucleotides d(CGT[$^{15}N^1$-A]CG) and d(CGT[$^{15}N^6$-A]CG) was reflected by an upfield shift of *ca.* 2.6 ppm for the N^1-resonance and a downfield shift of *ca.* 1.2 ppm for the N^6-resonance.[40] The T*m* and thermodynamic data obtained for the helix-to-coil transition from the ^{15}N-NMR chemical shifts agreed well with those reported by other methods. The labelled phosphoramidite **46** or **47** was used in the solid-phase synthesis of the above self-complementary hexamers.[40]



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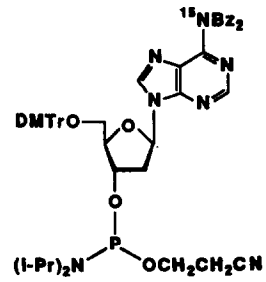

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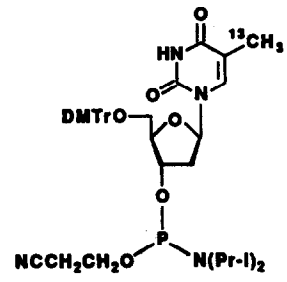
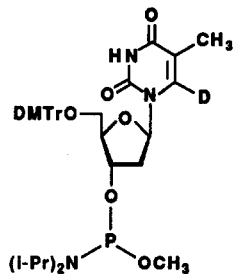


48

The deuterium-labelled deoxyribonucleoside phosphoramidites **49-51** have been incorporated at selected positions into the self-complementary dodecamer d(CGCGAATTCGCG). Such site-selective deuteration allowed the solid-state ^{2}H-NMR dynamic study of the nucleobases,[41a] methyl groups,[41b] and furanose rings[41c] in the DNA duplex.[41d] Similarly, the deuterium-labelled deoxyribonucleoside phosphoramidite **52** has been synthesized to enable the large scale preparation of a self complementary dodecaribonucleotide [r(CGCGAAU*U*CGCG) where U* is the deuterated residue] necessary for the study of RNA structure and dynamics.[41e]



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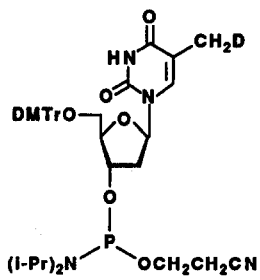

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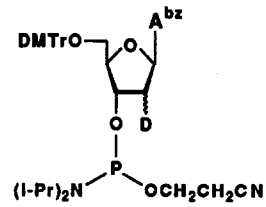
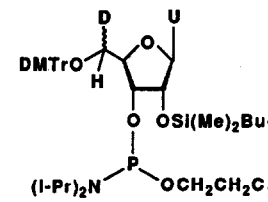


51 bz= benzoyl



52

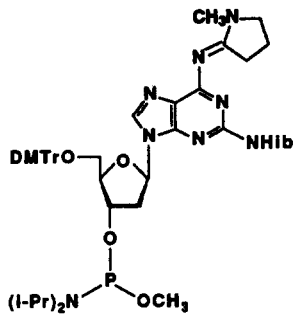
Carbon-13 relaxation is also a powerful probe for dynamic processes, as relaxation rates give accurate information about the motional characteristics of a particular C-H bond. A ^{13}C-6-labelled thymidine phosphoramidite has been synthesized by Williamson and Boxer[42a-b] and inserted in the oligonucleotide d(CGCGT*T*GT*T*CGCG) which adopted a hairpin conformation. NMR relaxation measurements indicated that subnanosecond internal motions were present in the loop region of the hairpin.[42b] Furthermore, the incorporation of the ^{13}C-labelled deoxyribonucleoside phosphoramidite **48** into an oligonucleotide corresponding to the stronger binding half-site of the consensus glucocorticoid response element has been reported.[43] The modified oligomer and its complementary sequence were interacted with the glucocorticoid receptor-DNA binding domain of the rat glucocorticoid receptor and the resulting complex was analyzed by NMR spectroscopy. The analysis revealed a hydrophobic contact between the labelled thymine methyl group and the methyl groups of a valine residue. These data demonstrated that stable isotope labelling of DNA functional groups in the major groove can simplify the NMR interpretation of protein-DNA complexes and provide more insight into the mechanisms underlying protein-DNA recognition.[43]

1.2. DNA and RNA Recognition.

Increased stabilization of DNA duplexes would permit more stringent hybridization conditions and should enhance probe specificity. In principle, the substitution of 2-aminoadenine for adenine would increase the stability of Watson-Crick base-pairing with thymine through the formation of additional hydrogen bonding between the 2-oxo group of the pyrimidine and the 2-amino function of the purine. Chollet *et al.*[44] reported the preparation of the protected 2,6-diaminopurine deoxyribonucleoside phosphoramidite **53** and its incorporation at selected positions into oligonucleotides by solid-phase synthesis. Tanaka *et al.*,[45] Brown *et al.*,[46] and Chazin *et al.*[47] also described the insertion of 2,6-diaminopurine in oligodeoxyribonucleotides by the phosphoramidite method. Hybridization probes containing 2-aminoadenine residues exhibited increased selectivity and strength in hybridization experiments with phage or genomic DNA. Each 2-aminoadenine contributed an additional 0.5-1 °C to the *Tm* of pentadecamer duplexes.[44a] In addition, ^{1}H-NMR analysis of a dodecanucleotide duplex having 2-aminoadenines instead of adenine nucleobases did not show disturbance of the global or local conformation of the modified DNA duplex relative to the native DNA duplex.[47]

Designing synthetic DNA probes from amino-acid sequences led Kong Too Lin and Brown[48a] to the development of the base-modified deoxyribonucleoside phosphoramidite **54** and its incorporation into oligonucleotides in an attempt to reduce probe multiplicity by removing the T/C degeneracy. The insertion of one or two such modified nucleobase(s) in two complementary 17-mers led to DNA duplexes as stable as the unmodified duplexes. However, the incorporation of three modified bases decreased the *Tm* of the corresponding duplex by 6 °C. By comparison, an equivalent duplex with three similarly located mismatches exhibited a *Tm* depression of 30 °C.

Oligonucleotides were also synthesized from the integration of the deoxyribonucleoside phosphoramidite **55**.[48b] The resulting DNA duplexes were not as stable (ΔTm = 6-13 °C) as those generated from the incorporation of **54**. It was speculated that the 5-methylene group or, perhaps, both methylene groups (in **54**) contributed to helix stability.[48a] Thus, deoxyribonucleosides derived from 6*H*,8*H*-3,4-dihydropyrimidino [4,5-*c*] [1,2] oxazin-7-one can base-pair with dG and dA and may find application in the preparation of hybridization probes and primers.[48c]



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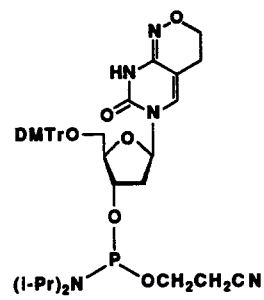

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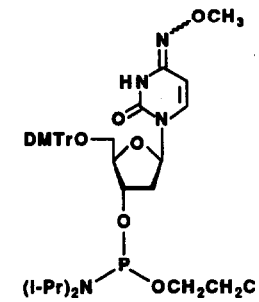
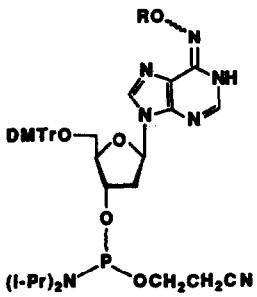


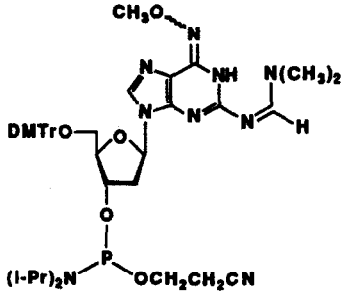
55

Subsequently, Brown and Kong Thoo Lin[49] described the synthesis of the deoxyribonucleoside phosphoramidites **56a** and **57** and their incorporation at selected sites into hepta-decaoligodeoxyribonucleotides. It was shown that the purine nucleobase originating from **57** could form the most stable base pairs with a thymine or a cytosine of the opposite strand.[48c,49] Additionally, the triple insertion of **57** in oligonucleotides yielded duplexes of greater stability than those obtained with three purine-pyrimidine mismatches (ΔTm = 14 °C).[49] Furthermore, the single insertion of the phosphoramidite **56a** in a pentadecanucleotide indicated that the modified nucleobase (M) formed a relatively stable base pair with either thymine (T) or cytosine (C) of the complementary strand. The difference in stability between the (M:T)- and (M:C)-duplexes was small (ΔTm = 4 °C). These duplexes were however less stable than (A:T)- and (G:C)-duplexes, respectively, but were more stable than (A:C)- and (G:T)-duplexes.[50a-b] Under these conditions, the insertion of a N^6-hydroxyadenine residue (H) *via* **56b** generated a duplex having a (H:T) or a (H:G) base pair which exhibited a stability similar to that of the duplex carrying a (M:T) or a (M:G) base pair (ΔTm = 1-2 °C).[50c] Collectively, the incorporation of methoxyamino or hydroxyamino purines (from either **56a-b** or **57**) as "degenerate" nucleobases into DNA sequences may improve the efficacy of oligonucleotidic probes and primers.

It must be noted that the insertion of the 5-fluorodeoxyribonucleoside phosphoramidite **58** in oligonucleotides led to the formation of duplexes containing A:5-FU and G:5-FU base pairs that were considerably more stable than the corresponding duplexes having A:T and G:T base pairs.[51] Consequently, the synthesis of mixed hybridization probes for the detection of specific gene sequences can be alleviated by designing single hybridization probes containing 5-fluorouracil to pair with adenine or guanine, hypoxanthine[52] to pair with adenine or cytosine, and guanine to pair with cytosine or thymine at positions of codon degeneracy without significantly sacrificing duplex stability.[51] The chemical consequences of the incorporation of 5-fluorouracil into DNA (*via* the deoxyribonucleoside phosphoramidite **25**) have also been examined by NMR spectroscopy.[53]

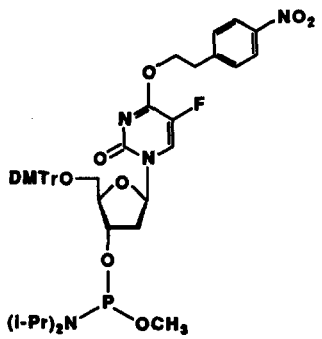


56a R= Me
b = $Si(Ph)_2Bu$-t



57

To evaluate the base-pairing abilities and mutagenicity of xanthine, the 2'-deoxyxanthosine phosphoramidites **59a-b** have been incorporated into oligodeoxyribonucleotides.[54] The thermal stability of each duplex containing xanthine base-paired with any of the four natural bases was determined and compared to that of similar duplexes carrying hypoxanthine. It was shown that xanthine-containing duplexes were as stable, at pH 5.5, as hypoxanthine-containing duplexes but were less stable than the latter at neutral pH.[54a] In addition, Seela and Kaiser[55a,b] reported the application of the isosteric 2'-deoxyinosine phosphoramidites **7a** and **8** in the synthesis of oligonucleotides to define the hybridization properties of these nucleobases. The single incorporation of the phosphoramidite **8** into the hexamer d(GCI*CGC) led to a DNA duplex having a T*m* lower (30 °C) than that of the unmodified duplex (T*m* = 46 °C) but higher than that of a similar duplex having hypoxanthine (T*m* = 27 °C). The phosphoramidite **8** may therefore become a useful synthon in the construction of hybridization probes containing an ambiguous base. Of interest, the purine nucleoside analogues corresponding to **7a** and **8** displayed an improved stability of the *N*-glycosidic bond relative to the unmodified purine nucleosides.[55b,c]



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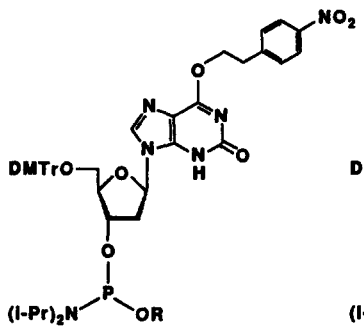
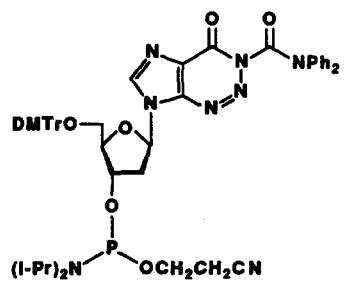


59a R= Me
b = CH_2CH_2CN

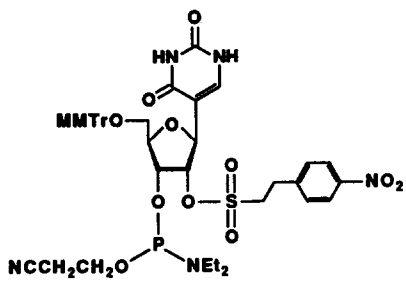


60

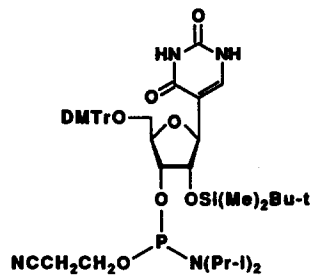
Fernandez-Forner *et al.*[56] disclosed the synthesis of the 2-aza-2'-deoxyinosine phosphoramidite **60** and its insertion in oligodeoxyribonucleotides to evaluate the stability of duplexes having this purine analogue base-paired with native nucleobases. Although the solid-phase synthesis of an oligonucleotide analogue proceeded well, the deprotection conditions (concentrated ammonium hydroxide, 40 °C, 2 days) promoted the decomposition of the 2-azahypoxanthine nucleobase and yielded an oligonucleotide containing a 5-amino-1-(ß-D-2'-deoxyribofuranosyl)imidazole-4-carboxamide (dAICA) residue.[56] Hybridization of the oligonucleotide analogue (19-mer) with a complementary DNA sequence carrying

a cytosine residue opposite the modified nucleobase produced a duplex having a depressed T*m* (48 °C) relative to that of an unmodified duplex under similar conditions (T*m* = 60 °C).[56] This approach represents, at least, an alternative to dAICA triphosphate and deoxyribonucleotidyltransferase for the incorporation of dAICA derivatives into oligonucleotides.

The pseudouridine phosphoramidites **61** and **62** have recently been applied to the automated synthesis of oligoribonucleotides.[57,58a,b] Specifically, two duplexes composed of AUAC Ψ Ψ ACCUG (Ψ represents pseudouridine), AUACUUACCUG and their complementary sequence CAGGUAAGUAU were prepared. These oligonucleotides corresponded to the 5'-end of human U1 snRNA paired to the mRNA consensus 5'-splice site. While the coupling efficiency of **61** or **62** was similar to that obtained with standard monomeric phosphoramidites, the free energy of duplex formation was found unchanged by the substitution of pseudouridine for uridine.[58a]

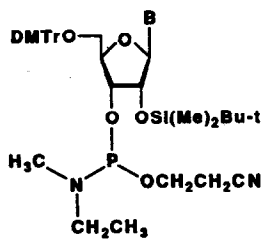


61



62

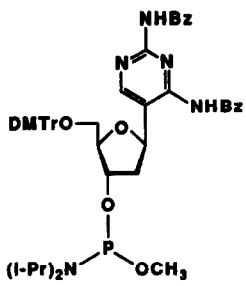
In a different context, the ribonucleoside phosphoramidites **63a-g** have been used in the total chemical synthesis of an *E. coli* $tRNA^{Ala}$ with its specific minor nucleosides (dihydrouridine, ribothymidine and pseudouridine). The phosphoramidites **63a-g** led to coupling yields greater than 98% within 2 min on a silica support.[58c,d] Triethylamine tris-hydrofluoride was found more effective than tetra-*n*-butylammonium fluoride for the complete removal of the 2'-*O*-silyl protecting groups. Following isolation and characterization, the full length tRNA exhibited a good aminoacyl acceptance activity.[58c,d]



63a B = **uracil-1-yl**
b = **uracil-5-yl**
c = **thymin-1-yl**
d = **5,6-dihydrouracil-1-yl**
e = **N^4-acetylcytosin-1-yl**
f = **N^6-phenoxyacetyladenosin-9-yl**
g = **N^2-phenoxyacetylguanin-9-yl**

Novel nucleobases have been designed to expand the genetic alphabet from four to six letters.[59] Early experiments with *iso*-C and *iso*-G demonstrated that these modified nucleobases having novel hydrogen-bonding patterns were substrates for DNA and RNA polymerases.[59b] However, the chemical stability of these nucleobases affected the fidelity with which *iso*-C or *iso*-G was incorporated into oligonucleotides containing A and T residues.[59a] To circumvent this problem, the deoxyribonucleoside phosphoramidite **64** has been prepared and inserted in an oligonucleotide which served as a template for the Klenow fragment of DNA polymerase I. It was shown that 2'-deoxyxanthosine 5'-triphosphate was incorporated with high fidelity opposite the modified nucleobase and a full-length product was obtained.[59a] When applied to RNA, an expanded genetic alphabet should provide a greater diversity in the functional groups available to these molecules and, perhaps, increase their catalytic activities.[59a]

The synthesis of the 2'-*O*-methylpseudoisocytidine phosphoramidite **65a** and its integration into an oligothymidylate at two specific sites opposite the guanine residues in the duplex d(AAGAAGAAGAA)/d(TTCTTCTTCTT) has been reported.[60a] A mixture of this duplex with the modified oligothymidylate showed two thermal transitions between pH 7-8.7. One transition (Tm = 42 °C) corresponded to the denaturation of the duplex, whereas the the second transition (Tm = 12 °C) pertained to the dissociation of the modified oligothymidylate from the duplex. Interestingly, the second transition was not observed when deoxycytidine or 2'-*O*-methyldeoxycytidine was substituted for 2'-*O*-methylpseudoisocytidine in the oligothymidylate.[60a] Extension of this work to a 2'-*O*-methylpseudoisocytidine-containing hexadecanucleotide complementary to the "polypurine tract" found in the genome of human T-cell leukemia virus (HTLV-III) has recently been reported.[60b] It has been demonstrated that 2'-*O*-methylpseudoisocytidine can advantageously replace 2'-deoxycytidine in the formation of a triplex structure, as it can base-pair with 2'-deoxyguanosine in the Hoogsteen scheme without being protonated.



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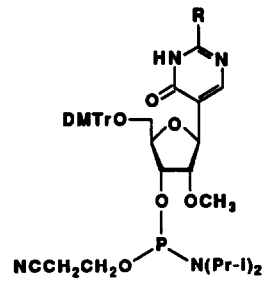
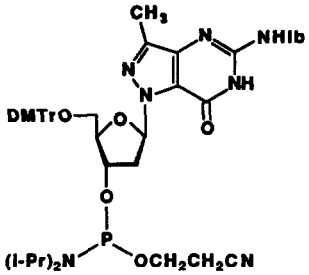


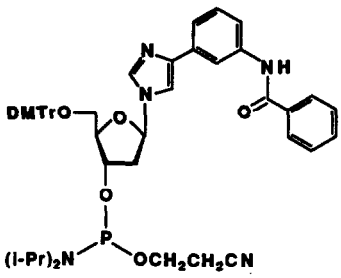
65a R= NHAc
b = N=CHNMe$_2$

In an attempt to design modified nucleobases that bind GC base pairs without protonation in a pyrimidine-motif triple-helical complex, Koh and Dervan[61a] reported the synthesis of the deoxyribonucleoside phosphoramidite **66** from ethyl(3-methyl-4-nitropyrazole-5-carboxylate) and 1-chloro-2-deoxy-2,5-di-*O*-*p*-toluyl-α-D-ribofuranose and its insertion in oligonucleotides at multiple interspaced or contiguous positions. It was shown by affinity cleavage analysis that an oligonucleotide carrying nucleobases derived from **66** was able to bind, at pH 7.8, a single fifteen base pair sequence of plasmid DNA containing five GC base pairs and, at pH 7.4, a single sixteen base pair sequence containing six contiguous GC base pairs. The nucleobase in **66** thus binds GC base pairs within a triple-helix motif with similar selectivity and strength as cytosine residues but over an extended pH range.[61a] This feature should increase the number of purine sequences amenable to oligonucleotide-directed triple-helix formation. Furthermore, the novel deoxyribonucleoside phosphoramidite **67** has been inserted in oligonucleotides to enable the selective binding of TA and CG Watson-Crick base pairs within a pyrimidine-purine-pyrimidine triple helix.[61b] When used in combination with the natural triplets TAT and C$^+$GC, this base-pairing specificity allowed oligonucleotide-directed sequence-specific recognition of double-helical DNA sequences containing all four base pairs at physiologically relevant pH and temperature without the need for an alternate strand crossover junction. It must however be noted that the sequence composition of target sites may influence the affinity of the modified nucleobase with TA and CG base pairs.[61b]

A strategy for the recognition of structured RNA has been proposed by Richardson and Schepartz.[62a] It was hypothesized that two oligodeoxyribonucleotides, linked by a tether, complementing two nonduplex and noncontiguous sites on *Leptomonas collosoma* SL RNA should cooperatively bind to the SL RNA with greater sequence and structure specificity than that of the two oligomers taken separately. The phosphoramidite **35b** was incorporated once or repeatedly (5 or 10 times) during solid-phase oligonucleotide synthesis to generate the tether between an heptamer (3'-



66



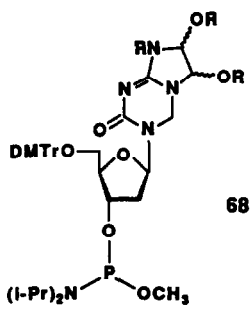
67

sequence) and a decamer (5'-sequence). The coupling efficiency of **35b** averaged 97%[62a] while, under similar conditions, the phosphoramidite **37c** exhibited a coupling efficiency of *ca.* 80%.[62b] Binding competition experiments with specific oligoribonucleotides and RNase H assays indicated that a tethered oligonucleotide probe interacted cooperatively and simultaneously with a single molecule of SL RNA. These probes are therefore attractive for the characterization and differentiation of tertiary structures in globular RNAs and ribonucleoproteins.[62a-b]

In a different context, two pyrimidine-rich oligodeoxyribonucleotides tethered with an hexaethylene glycol linker (derived from the phosphoramidite **37b**[28]) have been shown to form a stable triple-helix with a purine-rich single-stranded DNA sequence.[63] The formation of triple-stranded structures with single-stranded nucleic acids such as mRNAs, viral RNAs or DNAs may prove more efficient than double-helix formation at arresting translation, reverse transcription, or replication.[63]

The phosphoramidite **35b** has further served as a linker in the synthesis of homopyrimidine oligomers having opposite sugar-phosphate backbone polarities.[64] When appropriately linked, these oligonucleotide analogues can cooperatively form triple-helix structures with two or more homopurine segments located on the opposite strand of a target DNA duplex and stabilize the entire triple-helix relative to the corresponding unlinked homopyrimidine oligomers.[64]

Deoxyribonucleoside phosphoramidite derivatives have also been applied to the synthesis of circular oligonucleotides which have been found to form bimolecular triple-helical complexes with complementary DNA[65] and RNA[65b] oligonucleotides with high binding affinity relative to standard Watson-Crick complementary oligomers. Finally, several modified deoxyribonucleoside phosphoramidites have been incorporated into oligonucleotides to investigate either the base-pairing properties of an altered DNA template (as a mutagenesis model) with incoming deoxyribonucleoside triphosphates during DNA replication[54a] or the mechanism of mismatch repair in *E. coli*.[66] In another case, the deoxyribonucleoside phosphoramidite **68** led to the incorporation of 5-azacytosine residues at specific sites into oligonucleotides[67] in an effort to delineate the mechanism of DNA methyltransferase inhibition by triazines and its impact on the regulation of gene expression.

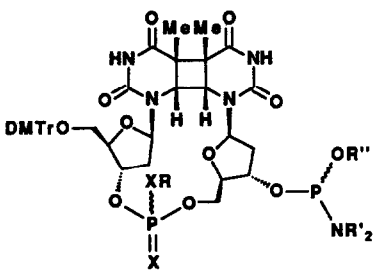


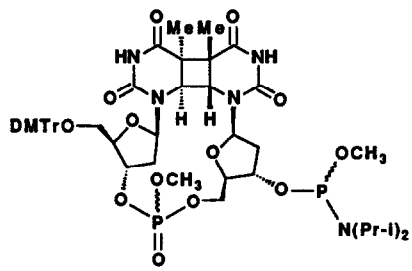
68 R= isobutyryl

2. DNA DAMAGE, DNA REPAIR, AND MUTAGENESIS

2.1. *DNA Photodamage*

It is well documented that exposure of DNA to ultraviolet light induces the formation of photoproducts at dipyrimidine sequences.[75a] Failure to repair such lesions has been associated with the genetic disease *Xeroderma pigmentosum* and skin cancer.[75b] Much of the work on mutagenesis by ultraviolet light and DNA repair has been hampered by the lack of well characterized DNA photolesions required for biological studies. To attenuate this limitation, Taylor *et al.*[76] and Murata *et al.*[77] described the synthesis of phosphoramidite building blocks composed of *cis-syn* (**69**,[76a] **70**,[77] and **71**[77]) and *trans-syn* thymine dimers (**72**[76b]). The sequence-specific incorporation of these dimeric phosphoramidites into oligonucleotides *via* solid-phase DNA synthesis was also described.





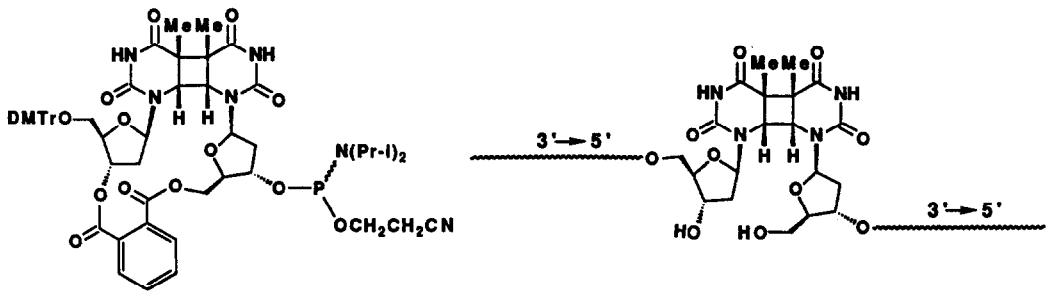
69 X= O; R= R"= Me; NR'_2= morpholino
70 = O; = CH_2CH_2CN; R'= i-Pr
71 = S; R= 2,4-dichlorobenzyl; = i-Pr
R"= CH_2CH_2CN

72

Two dodecamers derived from the insertion of **69** or **70** and **71**, respectively, were hybridized with their complementary sequences and each duplex was subjected to the endonuclease V of bacteriophage T4. It was shown that the phosphorodithioate link of the thymine dimer was cleaved by endonuclease V at a lower rate than that of the dimer having a natural phosphodiester linkage.[77] These data clearly indicated that the oligonucleotidic phosphoramidites **69**-**72** could be used in the preparation of unique photolesion-containing DNA duplexes for physical, enzymological, and mutagenesis studies.

In a different context, the phosphoramidite building block **73** has been prepared and centrally incorporated into an oligonucleotide (22-mer) by solid-phase synthesis.[78] Upon deprotection, the modified oligomer **74** was purified and submitted to photochemical and photoenzymatic cleavage experiments. Direct 254 nm photolysis of the modified 22-mer (**74**) led to the expected cleavage products (11-mers) in quantitative yield. Visible light photolysis in the presence of *E. coli* photolyase also generated the cleavage products to the same extent.[78] The ability to photochemically induce site-specific cleavage of nucleic acids may find application in the activation of prodrug forms of antisense oligonucleotides and ribosymes.

Fourrey *et al.*[79] reported the use of the triazolo deoxyribonucleoside phosphoramidite **45a** in the preparation of dimers containing thymine and 4-thiouracil residues. These dinucleotides were obtained by displacement of the 4-triazolyl function with hydrosulfide ion. The irradiation of the dimers with ultraviolet light resulted in the formation of (6-4)-pyrimidine-pyrimidone photoproducts. The mechanism of this DNA photodamage has been investigated[80] and the data supported the mechanism of (5-4)-bipyrimidine formation in tRNA containing 4-thiouridine. This information should prove useful in the study of the chemical behavior of related adducts in model systems and, ultimately, in double-stranded oligodeoxyribonucleotides.



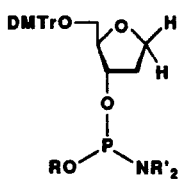
73 74

2.2. Apurinic/Apyrimidinic Lesions.

Biologically important lesions such as abasic (apurinic/apyrimidinic) sites in DNA arise spontaneously by a hydrolytic process which is accelerated by nucleobase modifications such as alkylation of purines and/or fragmentation of the heterocyclic rings.[75a,81a,b] The damaged bases are enzymatically excised by specific DNA glycosylases leading to the formation of abasic sites.[81a] It is well-known that these lesions represent a common structural intermediate in chemical mutagenesis, namely, a 2'-deoxyribose moiety linked through 3'- and 5'-phosphodiester bonds.[81b] In order to better understand the mechanisms involved in the repair of abasic sites in DNA, Takeshita *et al.*[82] inserted the tetrahydrofuranyl phosphoramidite **75** or the acyclic phosphoramidites **36a-b** at a defined position in an oligonucleotide to mimic the 2'-deoxyribose entity of abasic lesions. It was found that double-stranded oligodeoxyribonucleotides containing both types of abasic sites in one strand were endonucleolytically cleaved by *E. coli* endonuclease IV and exonuclease III. In contrast to exonuclease III, endonuclease IV did not cleave the DNA strand containing the abasic site generated from **36a**.

Synthetic oligonucleotides containing such abasic sites could also serve as templates for AMV reverse transcriptase, *E. coli* DNA polymerase I (Klenow fragment), *Drosophila* DNA polymerase-α, and calf thymus DNA polymerase-α.[82,83a] The extension of primer templates by these DNA polymerases led to the predominant incorporation of dAMP opposite any abasic site.[82,83a] These observations were consistent with those reported by Eritja *et al.*[83b] who employed the phosphoramidite **76a** for the incorporation of stable abasic sites into synthetic oligonucleotides.

In a different application, the insertion of the phosphoramidite **76b** in a synthetic c-Ha-*ras* gene demonstrated that abasic site analogues enhanced the transforming activity of the gene, relative to that of the unmodified oncogene, in transfected NIH3T3 cells.[84]

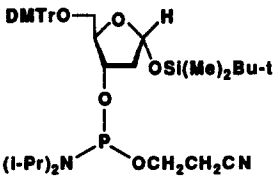


75 R= CH_2CH_2CN; NR'$_2$= morpholino
76a = Me; R'= i-Pr
b = CH_2CH_2CN; R'= i-Pr

The influence of the above abasic sites on the physicochemical properties of DNA duplexes[85] and triple-helical complexes[86] has been studied. Most of the structural aspects of these abasic site analogues appear relevant to the natural system except for the chemical reactivity of abasic DNA. In an attempt to synthesize oligodeoxyribonucleotides with genuine abasic sites, Groebke and Leumann[87] reported the preparation of the silylated 2-deoxy-D-ribofuranose phosphoramidite **77** in three steps from deoxy-D-ribose. The incorporation of **77** into oligonucleotides required a slightly longer reaction time than that of the natural deoxyribonucleoside phosphoramidites and occurred in yields greater than 95%. Although the abasic residue was stable throughout chain assembly, the removal of the nucleobase and phosphate protecting groups was performed with concentrated ammonium hydroxide in ethanol

(3:1) at ambient temperature. The purified oligonucleotide was then desilylated in a phosphate buffer (pH 2.0) within 1 h at 20 °C.[87] This easy protocol facilitated the synthesis of abasic DNA oligomers in large quantities regardless of the base sequence.

Subsequently, Vasseur *et al.*[88] and Péoc'h *et al.*[89] described the synthesis of the 2-deoxy-*D*-ribofuranose phosphoramidites **78a-b** and their application to solid-phase oligonucleotide synthesis. Typically, the ß-anomer **78b** led to coupling yields better than 98%. UV irradiation of the partially deprotected and purified oligomers in a 0.2 M ammonium formate buffer (pH 4) resulted in a rapid and complete cleavage of the *o*-nitrobenzyl protecting group. Oligomers up to 30 bases in length with natural abasic sites at selected positions have been synthesized according to this approach.[89] It must be noted, however, that the incorporation of the abasic phosphoramidite **78a** into oligonucleotides promoted the chemical cleavage of single-stranded DNA at specific sites under mild basic conditions.[90]



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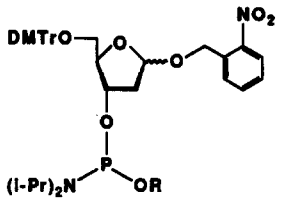


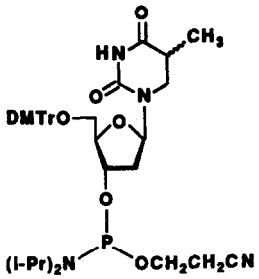
78a R= Me
b = CH_2CH_2CN

The 2-pyrimidinone phosphoramidites **14** and **20** (Table 1) have alternatively been used for the insertion of true abasic sites in oligonucleotides.[91] The coupling efficiency of **14** or **20** was similar to that obtained with the four common deoxyribonucleoside phosphoramidites.[16a] Incubation of a deprotected self-complementary dodecanucleotide containing a 2-pyrimidinone residue at pH 3 generated a DNA segment carrying an abasic site. The modified oligomer formed a DNA duplex exhibiting a much lower T*m* (42.3 °C) than that obtained with the native self-complementary dodecanucleotide (T*m* = 61.7 °C).[91] These data suggested that abasic sites may induce significant local destabilization in larger DNA macromolecules.

2.3. DNA Damage Caused by Ionizing Radiation.

It is well-known that gamma irradiation of HeLa cells with a cobalt-60 source in the absence of oxygen promotes the formation of 5,6-dihydrothymidine (DHT)[92] as the predominant 5*R*-diastereoisomer. However, when DNA is submitted to the action of gamma irradiation in aerated solution, the conversion of adenines to 7,8-dihydro-8-oxoadenines has been observed.[93] In order to investigate the mutagenic effects and the repair mechanism(s) of these DNA lesions, the synthesis of oligonucleotides carrying such defects at specific locations was necessary. The DHT phosphoramidite **79** was prepared in three steps from thymidine and was inserted in an oligonucleotide with a coupling yield of *ca.* 95%.[94a] Due to the sensitivity of 5,6-dihydrothymine to prolonged ammoniacal treatment, the phenoxyacetyl and isobutyryl groups were used for the protection of the amino function of purines and cytosines, respectively.[94b-d] The presence of DHT in the deprotected oligonucleotide was confirmed by partial chain cleavage occurring at the lesion site upon incubation of the oligomer with aqueous sodium hydroxide.[94a]

Similarly, the phosphoramidite **80a** and unmodified deoxyribonucleoside phosphoramidites carrying novel amino protecting groups[94b-d] have been applied to the synthesis of oligonucleotides ranging from 9-47 bases long.[95] The presence of modified adenines was verified by enzymatic digestion of the purified DNA segments.[95b,c] Oligodeoxyribonucleotides containing a single 7,8-dihydro-8-oxoadenine residue have been used as templates for *in vitro* transcription experiments with the Klenow fragment of *E. coli* DNA Polymerase I and the thermostable *Taq* DNA Polymerase from *Thermus aquaticus*. It was found that 7,8-dihydro-8-oxoadenine did not block replication, and thymine was incorporated opposite the damage.[95a]



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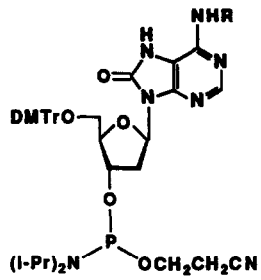
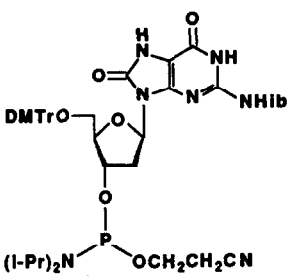


80a R= phenoxyacetyl
b = acetyl

The correlation between the formation of 7,8-dihydro-8-oxoguanine (8-oxoG) in DNA and carcinogenesis has been postulated.[96] To investigate the miscoding abilities of 7,8-dihydro-8-oxoguanine, the deoxyribonucleoside phosphoramidites **81**[97] and **82**[98] were prepared and introduced at defined positions in oligonucleotides. On primed templates, DNA polymerases directed the incorporation of either cytosine or adenine opposite the modified guanine residues.[99a] However, the frequency of mutations induced by 8-oxoG during replication *in vivo* was marginally above background in human cells. The most predominant mutation (1-2%) was a single G →T transversion. A higher frequency of this transversion (3-5 fold) was found in an excision repair deficient cell line. These data indicated that in contrast to the almost 100% mutagenicity generated by 8-oxoG with human polymerases *in vitro*,[99a] 8-oxoG was efficiently repaired *in vivo*.[99b] In addition, Wood *et al.*[99c] showed that the incorporation of 7,8-dihydro-8-oxoadenine into DNA *via* **80b** was at least an order of magnitude less mutagenic than that of 8-oxoG in *E. coli* cells with normal DNA repair capabilities.



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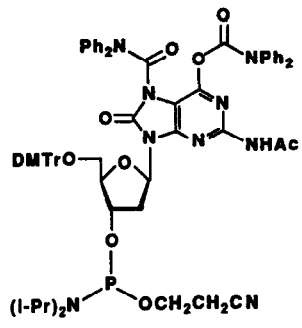
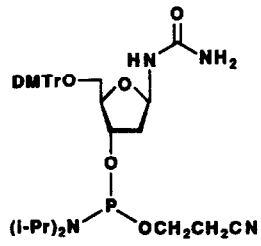


82

When DNA is submitted to ionizing radiation, deoxyribosylurea and deoxyribosylformamide were produced as fragmentation products of thymidine and 2'-deoxycytidine.[100a] The lack of oligonucleotides bearing one such lesion has made it difficult to study of the mutagenic activity of these defects and the repair mechanism(s) utilized by biological systems to correct these aberrations. Consequently, the synthesis of the deoxyribosylurea and deoxyribosylformamide phosphoramidites **83** and **84**, respectively, was performed and these monomers were integrated at specific locations in oligonucleotides ranging from 5 to 47 bases long.[100b-e] The instability of the ureido and formamido entities to alkaline conditions required phosphoramidites having phenoxyacetyl and isobutyryl protecting groups for the exocyclic amino function of the nucleobases during solid-phase synthesis of the modified oligonucleotides. The crude oligomers were purified by HPLC or preparative gel electrophoresis[100c] and the presence of the formylamino deoxyribosyl residue was confirmed by FAB mass spectrometry sequencing.[100d] Oligodeoxyribonucleotides having deoxyribosyl formylamine

moieties were used as templates for *in vitro* replication studies and it was demonstrated that the Klenow fragment of DNA Polymerase I directed the misincorporation of mainly guanine opposite the formylamino lesion.[100d]



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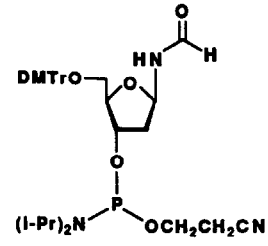


84

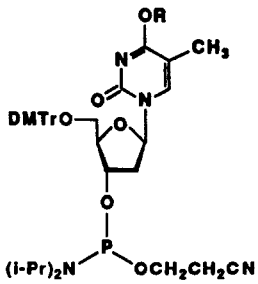
2.4. Alkylating Agents and Carcinogenesis.

The carcinogenicity of *N*-nitroso alkylating agents such as nitrosoureas and nitrosoamines is believed to be induced by O^6-alkylguanines and O^4-alkylthymines.[101a-b] Although, O^6-alkylguanines have attracted considerable attention in chemical carcinogenesis,[75a] the mutagenic potential of O^4-alkylthymines has been recognized[101c] and has been attributed to the very inefficient repair of O^4-alkylthymine residues in eukaryotic cells. To investigate the biological and structural role of O^4-alkylthymines in chemical carcinogenesis, the incorporation of these analogues into oligonucleotides has been undertaken. Because of the sensitivity of O^4-alkylthymines to acids, bases, and nucleophiles such as thiols or amines, the insertion of these modified nucleobases in synthetic DNA has been difficult. In spite of these drawbacks, Fernandez-Forner *et al.*[102a] developed a procedure for the solid-phase synthesis of oligonucleotides containing the mutagenic O^4-ethylthymine entity. Their approach entailed the preparation and utilization of the O^4-ethyl-2'-deoxyribonucleoside phosphoramidite **85a** in conjunction with deoxyribonucleoside phosphoramidites having nucleobase protecting groups similar to the *p*-nitrophenylethyl group.[102a,b] Due to the fragile O^4-ethylthymine residues, the modified oligomers were deprotected by treatment with 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine to remove nucleobase and phosphate protecting groups and then with 0.5 M DBU in ethanol/pyridine (1:1) to liberate the oligonucleotides from the solid support.[102a]

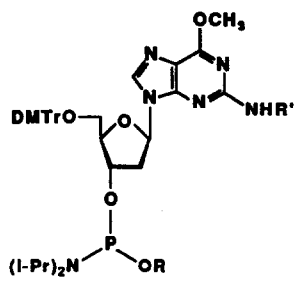
Along similar lines, Bhanot *et al.*,[103] Xu *et al.*,[104] Smith *et al.*,[105] Topal *et al.*,[106] Roelen *et al.*,[107a] and Pauly *et al.*[108a] reported the synthesis and application of deoxyribonucleoside phosphoramidite derivatives to the solid-phase synthesis of oligonucleotides containing O^4-alkylthymines[104] and/or O^6-alkylguanines.[103,104a,105,106,107a,c,108a] The O^4-alkylthymine phosphoramidite **85a** or **85b** was incorporated into oligonucleotides of up to 48 bases in length by routine solid-phase synthesis. The amino function of purines was protected with the phenoxyacetyl group while the amino group of cytosines was protected with the isobutyryl group to permit the complete deprotection of the oligomers with methanol/DBU or ethanol/DBU (9:1) and avoid the use of ammonia which is known to convert O^4-alkylthymines to 5-methylcytosines.[104b] Oligodeoxyribonucleotides containing O^6-methylguanines (from the incorporation of the phosphoramidite **86a** or **87a**) can, nonetheless, be deprotected by treatment with ammonia for two days at 20 °C without significant formation of 2,6-diaminopurines.[103,104a]

Roelen *et al.*[107b] and Xu *et al.*[107d] also reported the solid-phase synthesis of oligodeoxyribonucleotides containing O^4-alkylthymines. Their approaches consisted of the single incorporation of the triazolo deoxyribonucleoside phosphoramidite **45b** at a defined position into oligonucleotides. Treatment of the fully protected and solid-phase bound oligomers with methanol, ethanol or *n*-propanol at 50 °C in the presence of DBU, yielded the corresponding oligonucleotides carrying O^4-methyl-, ethyl-, or *n*-propylthymine residues.[107b]

The 3'-*O*-(*N,N*-diisopropylamino)methoxyphosphinyl derivative of 5'-*O*-DMTr-O^4(O^2)-alkylthymidines or appropriately blocked O^6-alkyl-2'-deoxyguanosines has also been applied to the incorporation of *O*-alkylated nucleosides at selected positions into synthetic oligonucleotides.[108b-c] This



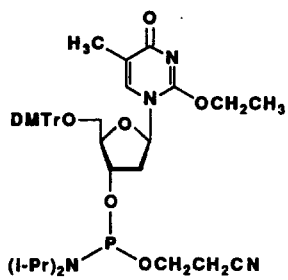
85a R= Et
b = Me



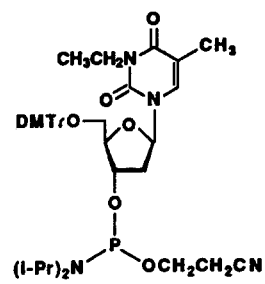
86a R= 2-cyanoethyl; R'= phenylacetyl
b = 2-cyanoethyl; = acetyl
c = 2-cyanoethyl; = isobutyryl
87a = methyl; = isobutyryl
b = methyl; = acetyl

type of monomeric phosphoramidites is not, however, recommended for the insertion of O^2-methylthymines in oligonucleotides, as the thiophenolate treatment required for the removal of the methyl phosphate protecting groups would also demethylate O^2-methylthymines. Interestingly, O^2-ethyl-, isopropyl-, and *n*-butylthymine derivatives were stable under similar conditions.[108b]

The O^2-ethylthymine deoxyribonucleoside phosphoramidite **88** has recently been prepared by Bhanot *et al.*[109] and inserted in an oligonucleotide by standard solid-phase synthesis with a coupling efficiency greater than 97%. The deprotection of the modified oligomer was performed using DBU/tetrahydrofuran/ethanol (14:43:43) for one week at ambient temperature. The purified oligomer served as a DNA template in the presence of T7 DNA polymerase and led to the incorporation of both dA and dT opposite the O^2-ethylthymine residue. While the incorporation of dA impeded DNA synthesis, the incorporation of dT resulted in efficient chain extension.[109] These data supported a molecular mechanism whereby an ethylating agent such as *N*-ethyl-*N*-nitrosourea can induce A→T transversion mutations contributing to cytotoxicity and, for example, to carcinogenic processes *via* the activation of proto-oncogenes. Similarly, the single insertion of the N^3-ethylthymine deoxyribonucleoside phosphoramidite **89** in a 17-mer produced a template for *in vitro* DNA replication experiments mediated by the Klenow fragment of *E. coli* DNA Polymerase I.[110] Like DNA lesions imparted by the incorporation of O^2-ethylthymines, DNA lesions incurred from the integration of N^3-ethylthymines contributed, in part, to the observed cytotoxicity and mutagenicity of ethylating agents.[110b]



88



89

The O^6-methylguanine deoxyribonucleoside phosphoramidites **86b** and **87b** have also been inserted in oligonucleotides by solid-phase synthesis.[111a,b] Typically, the coupling efficiency of **87b** averaged 91%.[111a] The random incorporation of a single O^6-methylguanine residue into self-complementary dodecanucleotides led to the formation of duplexes having T*m* values lower than that observed with the corresponding unmodified oligomers (ΔTm = 19-26 °C). These data indicated that

the O^6-methylation of guanine generated regions of localized instability in DNA regardless of the base opposite the lesion. Such instability may disrupt critical regulatory events and may be as responsible as the mutation itself for triggering oncogenic processes.[111a] Furthermore, the insertion of O^6-methylguanine phosphoramidites in DNA sequences corresponding to either the region of the *c-fos* promoter containing the binding site for serum response factor (SRF), the region of the HIV LTR containing two binding sites for the transcription factor NF-κB, or the region of the HIV LTR containing three binding sites for the cellular factor SP1, inhibited the binding of cellular factors to these DNA sequences. It was additionally discovered that the binding inhibition resulted from only certain modified guanines within a given binding site.[112] These findings suggest that the alkylation of O^6-guanines may seriously affect the regulation of gene expression at the transcription level. It must also be noted that, consistent with these results, the incorporation of O^6-methylguanine (*via* **86c**) into codon 12 of a synthetic c-Ha-*ras* gene led to significant induction of focus formation upon transfection of the modified *ras* gene into normal NIH 3T3 cells.[113]

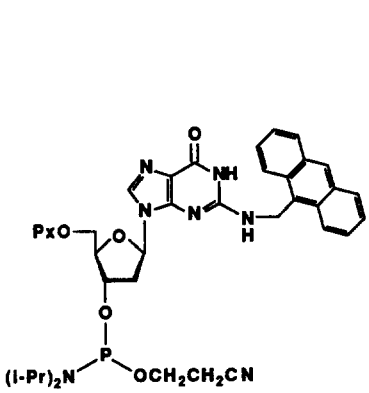
2.5. Polycyclic Aromatic Hydrocarbon-DNA Adducts.

The occurrence of carcinogenic or mutagenic effects associated with polycyclic aromatic hydrocarbons has been correlated with the ability of electrophilic metabolites to modify chromosomal DNA and interfere with the normal biochemical events involved in cell replication.[114a] Several lines of evidence suggest that these electrophiles preferentially react with the exocyclic amino group of guanine.[114b] Whether this preferential reaction occurs from optimal positioning of the aromatic electrophile *via* initial binding within one of the DNA grooves or by transient intercalation of the aromatic ring within the DNA double helix, is still unclear.[115] In order to provide a better understanding of the interactions of electrophilic polycyclic aromatic hydrocarbons with nucleic acids, the synthesis of oligonucleotides carrying well-characterized adducts, at selected sites, has been reported by Casale and McLaughlin.[115] The anthracenyl deoxyguanosine phosphoramidite derivative **90** was prepared from 2'-deoxy-4-desmethylwyosine and incorporated into oligonucleotides by solid-phase synthesis. The coupling efficiency of **90** was modest (65-70%) in spite of an extended condensation time (*ca.* 60 min). The circular dichroism spectrum of a DNA duplex (12-mer) having two anthracen-9-ylmethyl residues (one in each strand) failed to reveal a well-defined helical structure. Conversely, a duplex (13-mer) having only a single polycyclic aromatic residue existed essentially as a normal B-form DNA but exhibited a lower T*m* (50.6 °C) than that of an unmodified duplex (T*m* = 56.6 °C). The lack of substantial fluorescence quenching and the observed destabilization of the double-stranded 13-mer indicated that the covalent alkylation of DNA by electrophilic aromatic hydrocarbons may not result from the intercalation of the polycyclic aromatic electrophile. Exterior groove binding may be responsible for the initial association and/or positioning of the aromatic hydrocarbon prior to the alkylation of duplex DNA.[115]

The 3'-*O*-phosphoramidite derivative of N^6-(7-methylen-12-methylbenz-[*a*]-anthracenyl)-5'-*O*-DMTr-2'-deoxyadenosine has also been inserted in oligonucleotides in an attempt to determine the effect of the bulky polycyclic aromatic hydrocarbon group on the conformation and duplex stability of these oligomers.[116] A duplex composed of a pentadecamer carrying one aralkylated residue and a complementary unmodified sequence had a lower T*m* (46 °C) than that obtained with a native DNA duplex (T*m* = 55 °C) under the same conditions. The CD spectra of unmodified and aralkylated duplexes showed that the conformation of these duplexes was not significantly different from each other in solution.[116]

To further probe the mechanism(s) of cell transformation by polycyclic aromatic hydrocarbons, epoxide and diol epoxide adducts of these species at the exocyclic amino function of deoxyadenosine and deoxyguanosine have recently been synthesized and incorporated into oligonucleotides through the phosphoramidites **91a-d** and **92**.[117] The nucleosidic precursors to these phosphoramidite derivatives were 6-fluoro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)purine[117a,b] and 2-fluoro-2'-deoxyinosine.[117c] It has been shown that the interaction of the nonanucleotide d(GGTCA*CGAC), where A* represents the altered base (derived from **91d**), with the complementary strand d(CTCGTGACC) generated a duplex having a T*m* of 23 °C at low ionic strength. Under similar conditions, an unmodified DNA

duplex exhibited a T*m* of 43 °C.[117d] Surprisingly, the thermal stability of the modified duplex was unchanged when dG, instead of dT, was opposite the adducted-adenine residue. A less stable duplex (T*m* = 14 °C) was nevertheless obtained when dA was opposite the modified adenine.[117d] It would therefore be interesting to determine whether such preference for specific mismatches in these DNA duplexes is a good predictor of nucleotide misincorporation during replication of the modified DNA.



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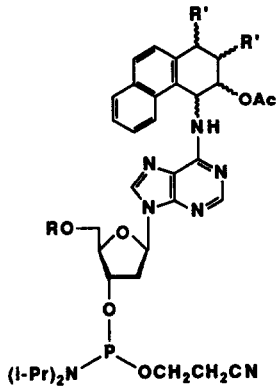
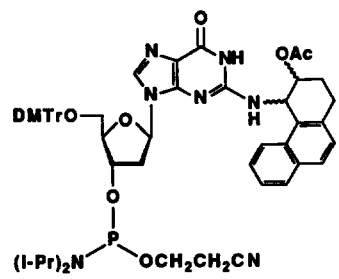
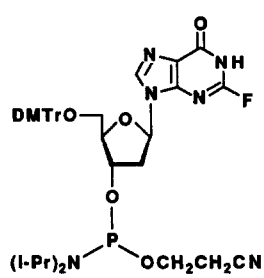


91 **a** (3*R*, 4*R*); R= Px; R'=H
b (3*S*, 4*S*); R= Px; R'= H
c (3*S*, 4*R*); R= DMTr; R'= H
d (1*R*, 2*S*, 3*R*, 4*S*); R= DMTr; R'= OAc



92 (3*S*, 4*S*)

Harris *et al.*[118] have reported the insertion of the halogenated deoxyribonucleoside phosphoramidites **93** and **94a-b** in oligonucleotides as an alternate approach to the preparation of oligonucleotides containing polycyclic aromatic hydrocarbon adducts. Their strategy consisted of the treatment of solid-phase bound halogenated oligonucleotides with amines derived from (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-8,9,10,11-tetrahydro-[*a*]-pyrene or (±)-*trans*-8,9-dihydroxy-*anti*-10,11-epoxy-8,9,10,11-tetrahydro-[*a*]-anthracene which afforded, under the recommended conditions, the corresponding adducted oligomers.[118]



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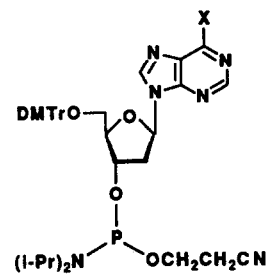
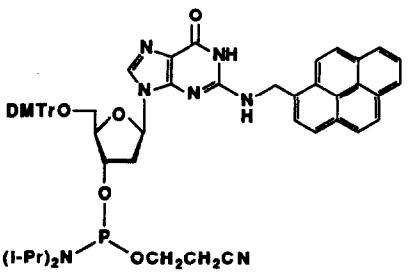


94 **a** X= Cl
b = F

Of additional interest, *N*-pyrenylmethyl deoxyribonucleoside phosphoramidites (**95**, **96**)[119a] and the 2'-*O*-(1-pyrenylmethyl)uridine phosphorodiamidite **97**[119b-d] have been prepared and incorporated at defined locations into oligonucleotides by conventional solid-phase synthesis. Particularly, the pyrenylmethyl-oligonucleotide U*TTTTTTTTT, derived from the single insertion of **97** (designated by

U*) at the 5'-terminus of an oligothymidylate, hybridized to poly rA and produced a complex having a slightly higher T*m* (25.1 °C) than that of the corresponding oligothymidylate-poly rA hybrid (T*m* = 23.5 °C) under the same conditions.[119b,c] Unexpectedly, the fluorescence intensity of the pyrenylmethyl-oligonucleotide increased upon complexation with poly rA. In fact, the fluorescence of the complexe U*TTTTTTTTT-poly rA was *ca.* 49 times larger than that of U*TTTTTTTTT. This feature should lead to increased sensitivity in the detection of sequence-specific gene probes.[119d]



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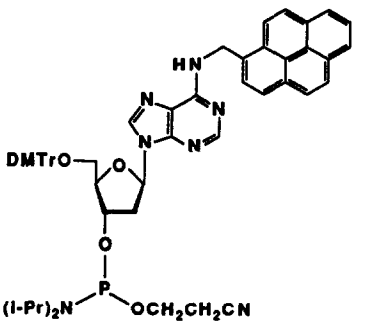
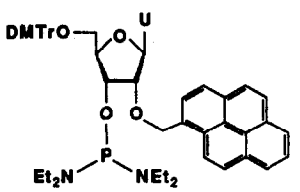


96

The synthesis of the anthraquinonylmethyl ribonucleoside phosphoramidite **98** from N^3-benzoyluridine has been reported by Yamana *et al.*[120] The incorporation of **98** into a self-complementary oligonucleotide (CCU*AGCTAGG) occurred with a coupling efficiency of *ca.* 87% according to a manual solid-phase synthesis protocol. The purified oligonucleotide formed a duplex exhibiting a T*m* of 57.4 °C in a buffer containing 0.1 M sodium chloride and 0.01 M sodium phosphate (pH 7.0). Under identical conditions, the unmodified DNA duplex had a T*m* of 40 °C.[120] It has been postulated that the increased stability of the modified DNA duplex resulted from the intercalation of the anthraquinone moiety between adjacent base pairs.



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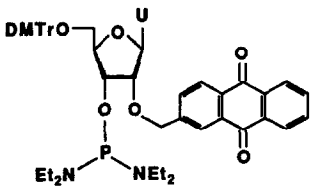


98

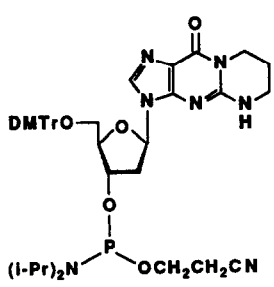
2.6. Acrolein-DNA Adducts.

Recent work has established that the mutagenicity of acrolein, a substance ubiquitous in the human environment, and other α, ß-unsaturated bifunctional carbonyl compounds could be due either to the formation of cross-links in DNA or to the formation of exocyclic adducts with one or more of the nucleic acid bases.[121] To better understand the effects created by acrolein-2'-deoxyguanosine adducts on replication *in vitro* and *in vivo*, the synthesis of oligonucleotides containing a specific exocyclic adduct was conducted *via* the incorporation of the 1,N^2-(1,3-propano)-2'-deoxyguanosine phosphoramidite **99** by standard solid-phase synthesis.[122] It is believed that the modified nucleobase of these oligonucleotides has preserved the major steric elements of both major and minor DNA-acrolein adducts and could, therefore, mimick these adducts in DNA replication studies.[122b]

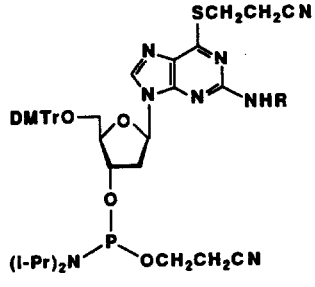
2.7. The Cytotoxicity of 6-Thioguanine and 5-Hydroxymethyl-2'-Deoxyuridine.

The cytotoxicity of 6-thioguanine has been exploited for years in the treatment of leukemias and

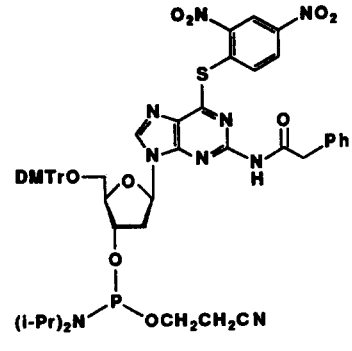
other human malignancies[123] but the molecular basis of its action is not clearly understood. It has, nonetheless, been argued that the incorporation of 6-thioguanine into DNA was responsible for its cytotoxicity. In an attempt to properly study this problem, Christopherson and Broom[124a] and others[69,124b,125a] devised the synthesis of 6-thioguanine-2'-deoxyguanosine phosphoramidites (**100a**,[124a] **100b**,[69,124b] **101**[125a]) and their incorporation into self-complementary oligonucleotides. It was shown that a dodecameric DNA duplex containing one or two 6-thioguanines (one in each strand) exhibited a significantly lower T*m* than that of the unmodified duplex (ΔTm = 6.9 °C or 12.8 °C, respectively).[125a,b] These findings prompted speculations about distorsions being created by 6-thioguanines in the DNA structure. Such distorsions could have been responsible for the observed decrease in stability of DNA duplexes and could, conceivably, account for the cytotoxicity of these drugs.[125a]



99



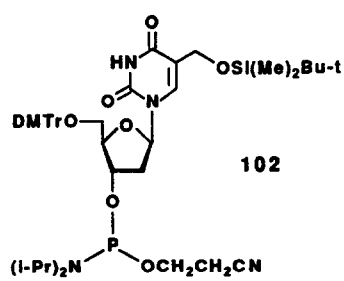
100a R= phenoxyacetyl
b = benzoyl
c = isobutyryl



101

In a different context, the incorporation of 6-thioguanines into short guanine-rich oligonucleotides may inhibit self-association and the formation of stable quadruplexes (G-tetrads) by interfering with hydrogen bonding and the coordination of alkali metal ions.[126] Given these properties, 6-thioguanine-containing oligonucleotides (*via* **100c**) may become more efficient in the formation of triple helices toward the inhibition of DNA replication. Interestingly, oligonucleotides containing 6-thioguanine residues exhibited a characteristic UV absorption at 345 nm dependent on the number of thioated nucleobases present.[126]

5-Hydroxymethyl-2'-deoxyuridine has demonstrated anti-leukemic and antiviral activities.[127] The insertion of this nucleoside in DNA may provide a better approach for studying its biological function. Conte *et al.*[128] described the preparation of the deoxyribonucleoside phosphoramidite **102** from 2'-deoxyuridine and its double and consecutive incorporation into Dickerson's dodecamer. The coupling efficiency of **102** was similar to that of the unmodified deoxyribonucleoside phosphoramidites. The dodecamer was characterized by ^{1}H-NMR spectroscopy and by enzymatic digestion with snake venom phosphodiesterase.[128] Further studies concerning the structure and properties of the modified oligomer are in progress.



102

3. INHIBITION OF GENE EXPRESSION

3.1. Oligonucleoside Phosphorothioates.

In the late seventies Burgers and Eckstein reported the synthesis of dinucleoside monophosphorothioates by the sulfurization of phosphite triesters with elemental sulfur.[129] Unlike dinucleoside monophosphates, the thiophosphate derivatives, being chiral at phosphorus, existed as pairs of diastereoisomers. Phosphorothioate analogues of ATP were particularly useful in the elucidation of the stereochemical course of enzymatic phosphoryl and nucleotidyl transfer reactions.[130] The chemical synthesis of dinucleoside phosphorothioates was easily accomplished by the phosphite triester approach[129,131] and by the phosphoramidite methodology.[130b,132] The configuration of the chromatographically resolved diastereoisomers was assigned by hydrolysis with snake venom phosphodiesterase (SVP) and nuclease P1 which exclusively cleaved the *Rp* or the *Sp* diastereoisomer, respectively.[130b] The incorporation of a phosphorothioate diester function into an octanucleotide (d[pGGpsAATTCC]) containing the *Eco*RI recognition sequence (GAATTC) showed that only the *Rp* diastereoisomer was recognized and cleaved, albeit slowly, by the endonuclease.[133a,b] The *Sp* diastereoisomer was not a substrate for *Eco*RI.[133b,134] Others[135a-o] have pursued this pioneering work and a wealth of information regarding the insertion of phosphorothioates,[135a-c,e,n,o] alkyl phosphonates,[135c,e,f,j] and alkyl phosphotriesters[135c-e,g-i,k,o] at specific locations throughout the *Eco*RI recognition sequence has been published in an attempt to rationalize the interactions of the endonuclease with the phosphate backbone of its recognition site. It has also been shown by Taylor *et al.*[136a] and others[136b] that many restriction enzymes did not cleave the DNA strand having a phosphorothioate diester link at the specified cleavage site but cleaved the unmodified complementary strand at the expected site.[136a-b] These data suggest that phosphorothioated DNA may find application in the sequence-specific preparation of nicked DNA duplexes.

Along similar lines, oligoribonucleotides containing a configurationally defined phosphorothioate function have been applied to probe the cleavage mechanism of hammerhead ribozymes.[137] It was found that, when located at the cleavage site, the *Rp* phosphorothioate isomer was cleaved very slowly in the presence of magnesium ion. In contrast, the cleavage of the *Sp* isomer was only slightly reduced from that of the natural phosphodiester. It has, therefore, been speculated that the magnesium ion was bound to the pro-*R* oxygen in the transition state of the hammerhead cleavage reaction.[137]

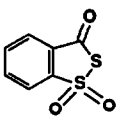
The incorporation of internucleotidic phosphorothioate linkages into oligonucleotides has considerably enhanced their resistance to hydrolysis by nucleases relative to unmodified oligonucleotides.[130a,134,138-143] As a consequence of this attribute, oligodeoxyribonucleoside phosphorothioates have demonstrated their usefulness as "antisense" molecules by inhibiting gene expression.[141c,144-154] In this context, the design, the synthesis, the application of oligodeoxyribonucleotides, oligoribonucleotides, and their analogues to the regulation of gene expression have been reviewed.[144a,d,151,155-163] For example, it has been demonstrated that antisense phosphorothioates complementary to the messenger RNA (the sense sequence) of the HIV-I *rev* gene, inhibited the cytopathic effect of the virus in chronically infected H9 cells.[164] The inhibition of HIV-LTR gene expression by oligonucleoside phosphorothioates targeted to the TAR element in a cell culture model has also been reported.[165] In addition, phosphorothioate oligomers exhibited antimalarial activities in strains of *Plasmodium falciparum*[166a] while antiviral activity against herpes simplex virus type 2,[166b-e] influenza A,[167] and influenza C[167] in cell cultures has also been recorded. Consequently, oligodeoxyribonucleoside phosphorothioates may represent a new class of experimental chemotherapeutic agents against AIDS,[168] hepatatis B,[169] and other infectious diseases.[141c,170,171a] The availability of these oligonucleotide analogues is therefore crucial for clinical investigations.[171]

The automated synthesis of oligonucleoside phosphorothioates can be performed according to the phosphoramidite approach.[142,144b,172a] The stepwise aqueous iodine oxidation responsible for the conversion of phosphite triesters to phosphotriesters was replaced with a relatively slow sulfurization reaction (7.5 min) requiring elemental sulfur (S_8).[142] Because of the poor solubility of S_8 in most organic solvents, the sulfurization reaction has been problematic and has often led to instrument failure. Four sulfur-transfer reagents were recently reported to eliminate the problems associated with the use

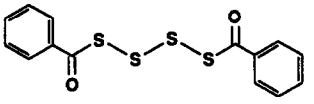
of elemental sulfur. Specifically, low concentrations (0.05 M-0.2 M) of the thiosulfonate **103** in acetonitrile converted a dinucleoside phosphite triester to the corresponding phosphorothioate dimer in yields better than 99% within 30 s at 20 °C.[173a-d] This reagent led to a rapid, efficient, and reliable automated synthesis of phosphorothioate oligomers carrying either exclusively or a predetermined number of P(S) links without detectable modification of the nucleobases.[141c,154,165,173a-c,e,174,175] The sulfur-transfer reagent **103** and its synthetic precursor, 3*H*-1,2-benzodithiol-3-one, have additionally been applied to the sulfurization of *H*-phosphonate and *H*-phosphonothioate diesters.[176]

Dibenzoyl tetrasulfide (**104**) has also demonstrated rapid sulfurization kinetics and satisfactory solubility properties during the solid-phase synthesis of a fully phosphorothioated nonadecadeoxyribonucleotide by the phosphoramidite approach. A solution of **104** (0.4 M) in tetrahydrofuran effected the sulfur-transfer reaction in near quantitative yields within 1 min.[177a] Phenylacetyl disulfide (**105**)[177b-c] and *N,N,N',N'*-tetraethylthiuram disulfide (**106**)[153b,178] were similarly applied to the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. Relative to **103**, the sulfurization kinetics provided by **105** were sluggish. A 5% solution of phenylacetyl disulfide in 1,2-dichloroethane/*sym*-collidine (4:1) enabled the sulfurization of a dinucleoside phosphite triester within 5 min at 20 °C.[177b-c] Even slower sulfurization rates were obtained with **106** in acetonitrile as it took 15 min for the reagent (0.5 M) to completely sulfurize phosphite triesters.[178]

The synthesis of oligonucleoside phosphorothioates containing exclusively P(S) linkages can alternatively be accomplished by the sulfurization of *H*-phosphonate oligomers with elemental sulfur[145a,179,180] but the preparation of oligonucleotides having P(S) links at defined positions could not be easily achieved by this approach.



103



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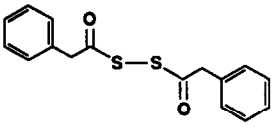
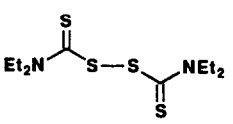


105

The efficiency of the sulfur-transfer reagent **103** has been further tested in the solid-phase synthesis of oligoribonucleoside phosphorothioates. Morvan *et al.*[181] described the preparation of such an oligoribonucleotide analogue from the ribonucleoside phosphoramidite **107**. The stepwise sulfurization reaction was complete within 30 s and the resulting oligoribonucleoside phosphorothioate exhibited enhanced stability to nucleases and satisfactory base-pairing abilities. These preliminary studies indicate that RNA phosphorothioates deserve consideration as potential antisense molecules.



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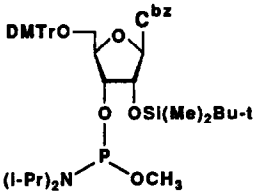
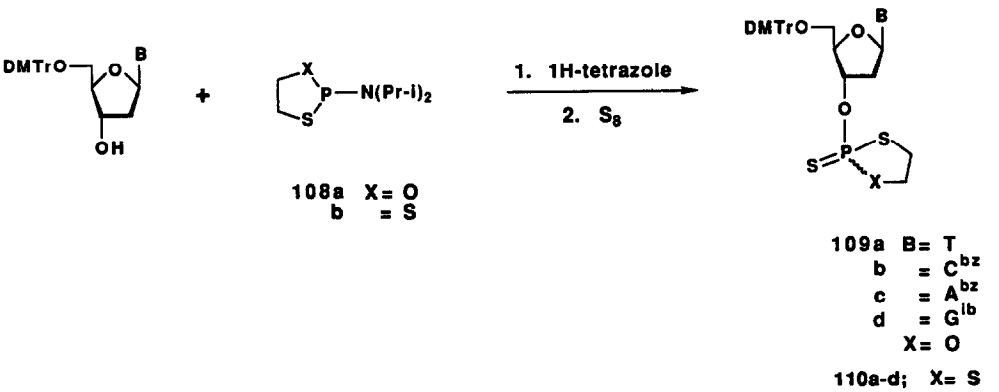


107

Gao *et al.*[174] reported that oligonucleoside phosphorothioates can inhibit human DNA polymerases and RNase H *in vitro*. It has been demonstrated that the phosphorothioate oligomer *S*-dC_{28} was a competitive inhibitor of DNA polymerase α and β with respect to DNA template. *S*-dC_{28} was also a competitive inhibitor of RNase H_1 and H_2 with respect to RNA-DNA duplex. These inhibitory effects were not sequence-specific and depended on the total number of thioate linkages rather than on the position of the linkages within the oligomer or the chain length itself. To minimize such inhibitory effects, it has been recommended to decrease the number of phosphorothioate functions to *ca.* 15-20 per oligonucleotide.[174]

In addition to providing chirality to the phosphate backbone of oligonucleotides, the presence of the larger sulfur atom led to subtle conformational changes and altered the charge distribution around the phosphate group.[182] Furthermore, the configuration of the phosphorothioate function has been shown to have a striking influence on the B-Z transition of d(GC) and d(CG) octamers containing alternating phosphorothioate links.[183] There are also lines of evidence suggesting that internucleotidic phosphorothioate linkages may induce sequence-specific conformational changes in DNA that affect duplex stability.[184]

In this regard, a significant advance in the stereospecific synthesis of oligodeoxyribonucleoside phosphorothioates has recently been published.[185] The approach entailed the preparation of the nucleoside 3'-*O*-(2-thio-1,3,2-oxathiaphospholanes) **109a-d** from the reaction of suitably protected nucleosides with the phosphoramidite **108a** and 1*H*-tetrazole followed by oxidation with elemental sulfur. Individual diastereoisomeric **109a-d** were separated by silica gel chromatography and the purity of each stereoisomer was assessed by ^{31}P-NMR spectroscopy. The reaction of diastereoisomerically pure **109a** with solid-phase bound thymidine and DBU gave dTpsT in 95% yield with a stereoselectivity greater than 99%.[185] The application of this methodology to the stereospecific synthesis of larger oligodeoxyribonucleoside phosphorothioates required a LCAA-CPG support having the leader nucleoside anchored through a succinyl-sarcosyl linker[186] to survive prolonged contact with DBU. The solid-phase synthesis of octamers has been achieved using diastereomerically pure **109a-d.** ^{31}P-NMR spectroscopy indicated that the content of phosphates was less than 1%. The stereoregular oligodeoxyribonucleoside phosphorothioates were characterized by enzymatic and electrophoretic methods.[185]



108a X= O
b = S

109a B= T
b = C^{bz}
c = A^{bz}
d = G^{ib}
X= O

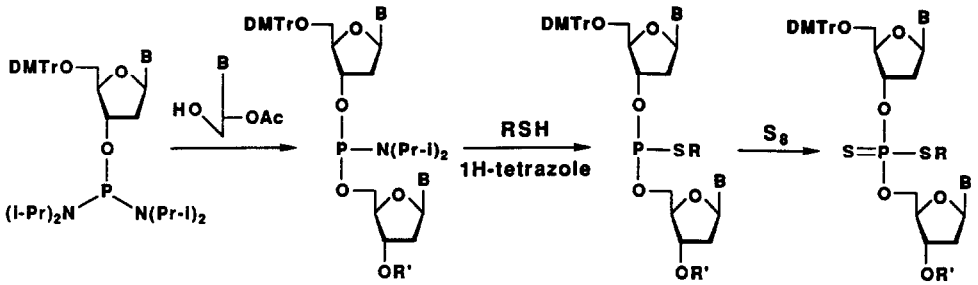
110a-d; X= S

The stereospecific synthesis of oligodeoxyribonucleoside phosphorothioates should provide valuable structural informations regarding the interactions of these oligonucleotide analogues with target macromolecules. Such stereocontrolled syntheses should additionally shed light on the cellular uptake, distribution, and metabolism of these sulfur-containing oligonucleotides.

3.2. Oligonucleoside Phosphorodithioates.

The chirality at phosphorus in phosphorothioate oligonucleotides can be abolished by substituting sulfur for the remaining non-bridging oxygen atom of the internucleotidic phosphodiester function. The synthesis of dinucleoside phosphorodithioates has been delineated by Nielsen, Brill and Caruthers.[187a-c] Typically, the deoxyribonucleoside phosphorodiamidite **111** was converted to the dinucleoside phosphoramidite **113** upon reaction with 3'-*O*-acetylthymidine. Activation of **113** with 1*H*-tetrazole and hydrogen sulfide afforded the dinucleoside *H*-phosphonothioate **115** in 90% yield. The sulfurization of **115** with S_8 generated the desired dinucleoside phosphorodithioate **117** in 70% isolated yield.[187a] Replacing hydrogen sulfide with 4-chlorobenzyl mercaptan produced the dinucleoside thiophosphite **116** from **114.** In spite of the sensitivity of **116** to air oxidation, its sulfurization with elemental sulfur afforded **118** in 68% yield.[187d] The purified phosphorodithioate dimer was characterized by FAB mass spectrometry, ^{31}P- and ^{1}H-NMR spectroscopies. The dimer was resistant to digestion with SVP under

conditions which completely hydrolysed the natural dinucleoside monophosphate. It was also shown that the phosphorodithioate analogue of d(TpT) was resistant to the nucleolytic activity of bovine spleen phosphodiesterase and nuclease P1.[188a]



111 B= T
112 = C^{tol}

113 B= T; R'= Ac
114 B= C^{tol}; R'= Phac

115 B= T; R= H; R'= Ac
116 B= C^{tol}; R= p-ClBn; R'= Phac

117 B= T; R= H; R'= Ac
118 B= C^{tol}; R= p-ClBn; R'= Phac

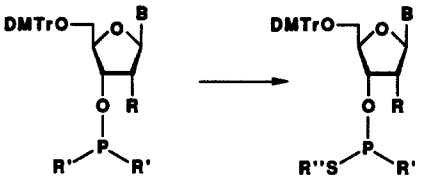
C^{tol}= N^4-(p-toluoyl)cytosin-1-yl; Phac= phenoxyacetyl; p-ClBn= p-chlorobenzyl

The preparation of oligodeoxyribonucleoside phosphorodithioates has alternatively been achieved *via* thioamidite intermediates.[187b,c,189] The preferred route for the synthesis of deoxyribonucleoside phosphorothioamidites involved the thiolysis of deoxyribonucleoside phosphorodiamidites with mercaptans.[189a,b,] Unexpectedly, the thioamidites **119** and **124** (Table 2) were relatively inert toward activation with 1*H*-tetrazole.[189a-b,190] It was rationalized that the steric bulk of the amidite *N*-substituents was significantly inhibiting the activation reaction.[189b,190] This rationale was found valid, as unlike **119** or **124**, the thioamidites **120-123**[189a] and **126**[189f] were readily activated with 1*H*-tetrazole and used in the solid-phase synthesis of oligodeoxyribonucleoside phosphorodithioates (20-mers). Each coupling step occurred with an efficiency of *ca.* 96-98%[189f] and was followed by oxidation with elemental sulfur in pyridine/carbon disulfide (1:1). The deprotected oligonucleotides were submitted to ^{31}P-NMR spectroscopy which revealed the presence of phosphorodithioate linkages at 113 ppm (D_2O).[189a] Of practical importance, reporter groups such as monobromobimane, 5-iodoacetamidofluorescein or 3-(2-iodoacetamido)-PROXYL have been inserted at specific sites in oligonucleotides having phosphorodithioate linkages[189k] in a manner similar to that reported for oligonucleoside phosphorothioates.[191]

Alternate routes to the synthesis of deoxyribonucleoside phosphorodithioates have recently been published. For example, the reaction of protected deoxyribonucleosides with 2-(*N,N*-diisopropylamino)-1,3,2-dithiaphospholane (**108b**) and 1*H*-tetrazole followed by oxidation with elemental sulfur, afforded the deoxyribonucleoside-3'-*O*-(2-thio-1,3,2-dithiaphospholanes) **110a-d** in 74-89% yield. The derivative **110b** led to the solid-phase synthesis of an oligodeoxycytidine phosphorodithioate (10-mer) in the presence of DBU with an average coupling yield of 96%.[192]

The conversion of the deoxyribonucleoside phosphoramidite **132** to the monomeric phosphorodithioate **133** has been applied to the large-scale preparation of phosphorodithioate oligomers *via* the phosphotriester approach.[189d-e,193] In addition, activated dithiophosphoric acid esters,[194] deoxyribonucleoside-*H*-phosphonothioates,[195] and deoxyribonucleoside-*H*-phosphonodithioates[188,196] have been employed as starting materials in the synthesis of oligonucleoside phosphorodithioates. Recent data showed that dithioated DNA was not digested by either snake venom phosphodiesterase,[187d] calf spleen phosphodiesterase,[187d] nuclease P1,[188a,b] the (3'→5')-exonuclease

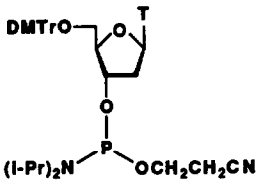
Table 2. Deoxyribo- and Ribonucleoside Phosphorothioamidite Derivatives.



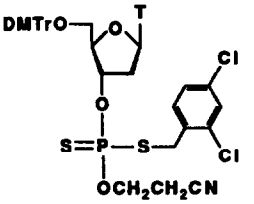
B= Protected Nucleobases

Compound	R	R'	R''	References
119	H—	—N(Pr-i)$_2$	Cl—C$_6$H$_4$—CH$_2$—	187b,c
120	H—	—N(CH$_3$)$_2$	Cl—C$_6$H$_4$—CH$_2$—	187c,189a
121	H—	—N(CH$_2$)$_4$	Cl—C$_6$H$_4$—CH$_2$—	187c,189a
122	H—	—N(CH$_3$)$_2$	Cl,Cl—C$_6$H$_3$—CH$_2$—	189a,e,190
123	H—	—N(CH$_2$)$_4$	Cl,Cl—C$_6$H$_3$—CH$_2$—	189a,d,193b
124	H—	—N(Pr-i)$_2$	NCCH$_2$CH$_2$—	189b,190
125	H—	—NEt$_2$	NCCH$_2$CH$_2$—	189b
126	H—	—N(CH$_3$)$_2$	NCCH$_2$CH$_2$—	189b,f,190
127	H—	—NEt$_2$	Cl,Cl—C$_6$H$_3$—CH$_2$—	189b
128	H—	—N(Pr-i)$_2$	CH$_3$—	189c
129	t-Bu(Me)$_2$SiO—	—N(CH$_3$)$_2$	NCCH$_2$CH$_2$—	198
130	t-Bu(Me)$_2$SiO—	—N(CH$_3$)$_2$	Cl,Cl—C$_6$H$_3$—CH$_2$—	198
131	t-Bu(Me)$_2$SiO—	—N(CH$_2$)$_4$	Cl,Cl—C$_6$H$_3$—CH$_2$—	198

activity of bacteriophage T4 DNA polymerase, or by the nucleases present in HeLa cell nuclear extracts.[189d,e] The hybridization of a fully dithioated DNA segment with a complementary unmodified DNA sequence resulted in the formation of an hybrid having a lower T*m* relative to an unmodified DNA duplex.[197] The reduction of 0.5-2 °C per dithioate linkage was found to be higher than that observed with monothioated DNA (ΔTm = 0.4-0.6 °C per monothioate linkage).[189f] Like the phosphorothioate homooligomer ß-*S*-$(dC)_{28}$,[172a-c] oligodeoxyribocytidine phosphorodithioates[172d] inhibited *de novo* infection of susceptible cells by HIV-1. These oligonucleotide analogues are very potent inhibitors of HIV-1 reverse transcriptase *in vitro*. An increasing inhibitory effect correlated with the number of internucleotidic phosphorodithioate linkages. Comparative experiments demonstrated that a deoxyribodeoxycytidine phosphorodithioate oligomer (14-mer) was as effective as a phosphorothioate oligomer (28-mer) at inhibiting *de novo* infection by HIV-1.[172d]



132



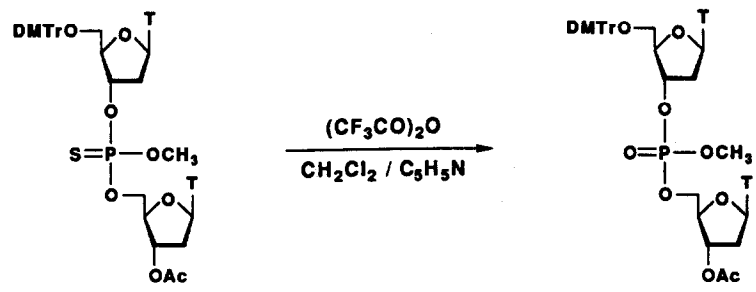
133

The synthesis of ribonucleotide dimers having internucleotidic phosphorodithioate linkages has been described by Petersen and Nielsen.[198] Similar to the preparation of deoxyribonucleoside phosphorothioamidites, the ribonucleoside phosphorothioamidites **129-131** (Table 2) were prepared from the thiolysis of the corresponding ribonucleoside phosphorodiamidites. The activation of **129** with 1*H*-tetrazole in the presence of a suitably protected ribonucleoside afforded, after sulfurization, the fully protected dinucleoside phosphorodithioate. The deprotected ribonucleotide analogue was resistant to hydrolysis with SVP and RNase A under conditions which totally hydrolysed the natural ribonucleotide. Moreover, the phosphorodithioate dimer was found virtually unchanged after treatment with concentrated ammonium hydroxide for 16 h at 5 °C. Collectively, the enhanced chemical stability of ribonucleoside phosphorodithioates and their resistance to nucleases should facilitate the study of biological processes involving RNA.[198]

Although the increased resistance of oligodeoxyribonucleoside phosphorothioates and dithioates to nucleases has been a desirable feature with respect to their application as potential therapeutics, it has nonetheless complicated the characterization of these oligonucleotides. To circumvent this problem, Burgers and Eckstein reported the desulfurization of an oligonucleotide containing internucleotidic phosphorothioate links with an excess of ethanolic iodine at 0 °C.[199] The desulfurized oligomer was then digested with SVP and bacterial alkaline phosphatase to the corresponding nucleosides. While aqueous iodine in pyridine[133b-c,183] or in 2,6-lutidine[133c] has also been applied to the desulfurization of oligodeoxyribonucleoside phosphorothioates, aqueous iodine in tetrahydrofuran/water/1-methylimidazole (16:3:1) led to the complete desulfurization of phosphorodithioated dimers and trimers.[188b] However, when applied to the desulfurization of a fully phosphorodithioated decanucleotide, these conditions produced extensive chain cleavage.[189f] In contrast, an aqueous solution of sodium metaperiodate has been effective in rapidly and cleanly desulfurizing oligonucleoside phosphorothioates (up to 20-mers).[133d]

An alternate approach to the desulfurization of thioated oligonucleotides may become available, as it has recently been reported that trifluoroacetic anhydride in pyridine effected the quantitative desulfurization of the dinucleoside phosphorothioate **134** to **135** within 24 h.[200] The desulfurization of model dithiophosphates such as $(RO)_2P(S)SR'$ under similar conditions yielded exclusively the corresponding monothiophosphates $(RO)_2P(O)SR'$.[200] This protocol may therefore be practically limited to the desulfurization of oligonucleoside phosphorothioates. It must, however, be noted that

dinucleoside phosphorodithioates have been converted to their corresponding dinucleoside monophosphates by treatment with butylene 1,2-oxide in ethanol-water for 120 min at 50 °C.[192]

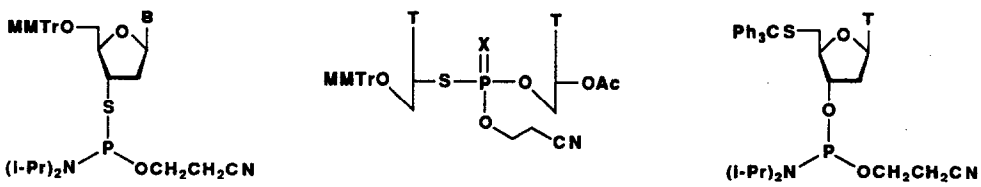


134 **135**

3.3. Oligodeoxyribonucleoside Phosphorothioates Achiral at Phosphorus.

Another class of sulfur-containing oligonucleotides emerged from the replacement of the bridging 3'-oxygen atom of the phosphodiester linkage with a sulfur atom.[201,202] The 3'-thio-2'-deoxyribonucleoside phosphoramidite **136a** was prepared from 5'-*O*-MMTr-3'-thiothymidine and chloro-(2-cyanoethoxy)-*N,N*-diisopropylaminophosphine.[201,202b] The insertion of **136a** in an oligothymidylate (d[TpTpTspTpT]) proceeded with a coupling efficiency of *ca.* 80% when 5-(*p*-nitrophenyl)-1*H*-tetrazole was used as an activator.[201] Because of the sensitivity of the P-S link to aqueous iodine, tetra-*n*-butylammonium oxone or, preferably, tetra-*n*-butylammonium periodate in dichloromethane was used as the oxidizing reagent in the automated synthetic cycle.[201,202b] The deprotected and purified oligomer was digested with nuclease P1 and alkaline phosphatase to thymidine and 3'-thiothymidine in a ratio of 4:1. Interestingly, the treatment of d(TpTpTspTpT) with 30 mM aqueous silver nitrate resulted in a clean and quantitative cleavage of the P-S linkage.[201]

In addition to investigating the physical and chemical properties of a dithymidine phosphate analogue containing 3'-thiothymidine (**137**), Cosstick and Vyle[202b] reported the synthesis of dithymidine-3'-*S*-phosphorodithioate (**138**) from the condensation of **136a** with 3'-*O*-acetylthymidine followed by oxidation of the resulting dinucleoside thiophosphite triester with elemental sulfur. After deprotection and separation of the two diastereoisomeric dimers, the relative rates of enzymatic hydrolysis of d(TspT) and both *Rp* and *Sp*-d[Tsp(s)T] were determined. d(TspT) was digested with SVP as rapidly as the natural dinucleotide d(TpT). However, d(TpT) was hydrolysed 100 times faster than d(TspT) with nuclease P1. *Rp*-d[Tsp(s)T] was resistant to SVP and was hydrolysed 250 times slower than d(TpT) with nuclease P1. Conversely, *Sp*-d[Tsp(s)T] was not hydrolysed with nuclease P1 but was digested with SVP at 64% the rate observed for d(TpT).[202b] This work has been extended to d(AspT) which was synthesized from the reaction of the phosphoramidite **136b** with 3'-*O*-acetylthymidine and 5-(*p*-nitrophenyl)-1*H*-tetrazole.[202c] It should be emphasized that the fragility of the bridging phosphorothioate link to iodine or silver ions may become a useful tool for the site-specific "nicking" of DNA.[202d]



136a B= T
b = A^{bz}

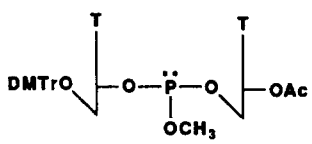
137 X= O
138 = S

139

Recently, Mag *et al.*[203] delineated the synthesis of the 5'-tritylmercapto-2'-deoxyribonucleoside phosphoramidite **139** and its incorporation into a dodecamer by routine solid-phase synthesis. Oligo-5'-thiothymidylates[204] were sluggishly hydrolysed by both SVP and spleen phosphodiesterases.[204c,d] Typically, SVP hydrolysed dT_9 and $d(Tps)_8T$ to the extent of 50% and 10%, respectively, within 2 min.[205] Furthermore, the digestion of dT_{13} and $d(Tps)_{12}T$ with S1 nuclease proceeded with a half-life of 5 min and 350 min, respectively.[205] As expected from the data reported by Cosstick and Vyle[201] the internucleotidic link of these oligonucleoside phosphorothioate analogues was also chemically cleaved by 50 mM aqueous silver nitrate or mercuric chloride without affecting the natural phosphodiester linkages.[203]

3.4. "Boronated" Oligodeoxyribonucleotides.

The synthesis of an interesting class of oligonucleotide analogues having "boronated" internucleotidic linkages has been described by Sood *et al.*[206a] The synthetic approach consisted of the condensation of 5'-*O*-DMTr-2'-deoxythymidine-3'-*O*-(*N,N*-diisopropylamino) methoxyphosphine with 3'-*O*-acetylthymidine and 1*H*-tetrazole yielding the dinucleoside methyl phosphite triester **140** which upon reaction with borane-dimethylsulfide generated the dinucleoside boranophosphate methyl ester **141**. The purified dinucleotide analogue **141** was characterized by NMR spectroscopy (^1H, ^{13}C, ^{11}B, ^{31}P), FAB mass spectrometry, and elemental analysis. Incidentally, a ^{31}P-NMR spectrum of **141** revealed a broad peak at 118.0 ppm corresponding to the boranophosphate function.[206a] Treatment of **141** with concentrated ammonium hydroxide at ambient temperature or at 55 °C led only to the dinucleoside boranophosphate **142** without cleavage of the internucleotidic link. Shaking **141** with 1 M hydrochloric acid in methanol (1:1) at 20 °C resulted in a minimal conversion (less than 10%) of the boranophosphate methyl ester to the corresponding phosphate methyl ester according to ^{11}B- and ^{31}P-NMR spectroscopies.[206a,b] The boranophosphotriester function in **141** was also stable to the conditions required for chain extension by the phosphoramidite approach. The synthesis of a trimer having two boranophosphate triesters has been reported.[206a,b] Finally, it has been demonstrated that the dinucleoside boranophosphate **142** was quite resistant to the nucleolytic activity of both SVP and calf spleen phosphodiesterase (CSP). For example, the dinucleotide analogue **142** exhibited only 8% hydrolysis under conditions causing the hydrolysis of the natural dithymidylate to greater than 97%.[206a,b] Given the stability of internucleotidic boronated bridges to basic or acidic conditions and to exonucleases, oligonucleotides with boronated phosphates should further be investigated as potential therapeutic agents.



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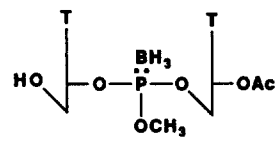

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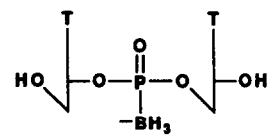
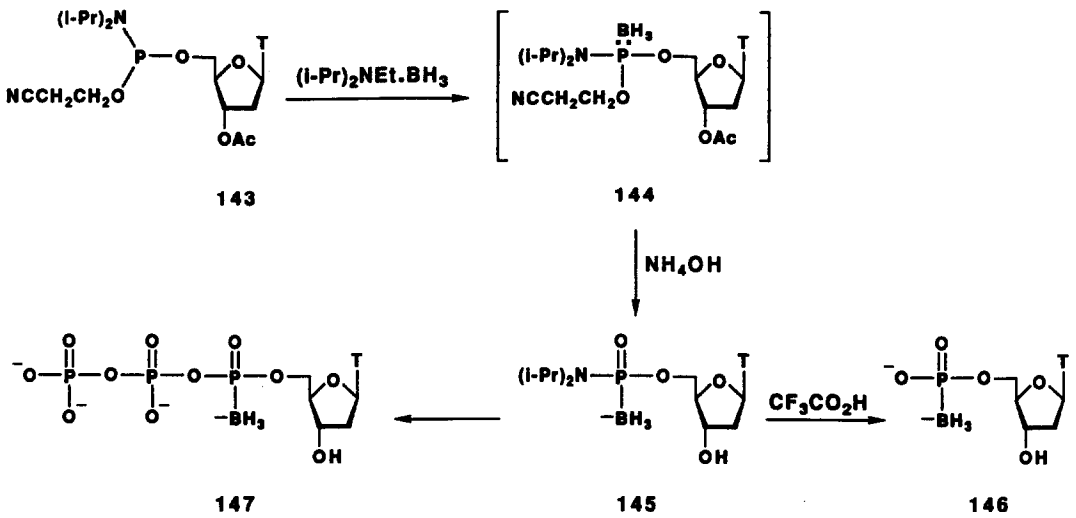


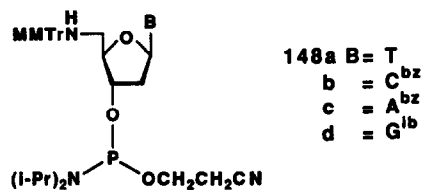
142

Of interest, the reaction of the deoxyribonucleoside phosphoramidite **143** with *N,N*-diisopropylethylamine-borane complex gave the intermediate **144** which upon treatment with ammonium hydroxide yielded the boranophosphoramidate **145**. Acid hydrolysis of **145** generated the novel thymidine 5'-boranophosphate **146**, whereas the reaction of **145** with bis-(tri-*n*-butylammonium)pyrophosphate yielded **147**.[206c] The thymidine 5'-boranophosphate **146** was hydrolyzed by snake venom phosphodiesterase but was a poor substrate for *E. coli* alkaline phosphatase. The thymidine 5'-triphosphate analogue **147** can substitute for natural thymidine 5'-triphosphate in the complete extension of an heptadecaoligodeoxyribonucleotide primer using Sequenase (a modified T7 DNA polymerase) and a template (25-mer) containing one deoxyadenosine residue.[206c]



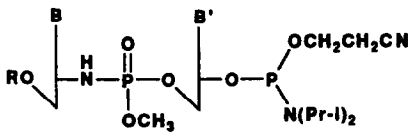
3.5. Oligodeoxyribonucleoside Phosphoramidates.

The properties of oligodeoxyribonucleotides having the bridging 5'-oxygen atom of the phosphodiester function replaced with a NH function have also been scrutinized. Although the synthesis of such achiral oligodeoxyribonucleoside phosphoramidates has been described earlier,[207] Mag,[208] Engels,[208] and Bannwarth[209] reported the preparation of the 5'-protected amino-2',5'-dideoxyribonucleoside phosphoramidites **148a-d** for the solid-phase synthesis of these oligonucleotide analogues. Particularly, the phosphoramidite **148c** was inserted in oligonucleotides at defined locations with an average coupling yield of 98-99%. The phosphoramidate link was stable to the acidic conditions required to remove the MMTr group but was completely cleaved upon incubation with 80% acetic acid for 5 h at ambient temperature.[208b,209] The primary amino function generated from this cleavage can be detected with great sensitivity and can thereby be used for diagnostic purposes.

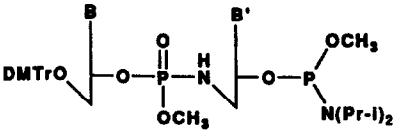


The phosphoramidite dimers **149a**[208c] and **150**[210] have also been synthesized to enable the incorporation of achiral phosphoramidate linkages at selected positions into oligonucleotides. Earlier studies indicated that oligonucleoside phosphoramidates having a 5'-terminal amino group were slowly hydrolysed by spleen phosphodiesterase under conditions which completely hydrolysed unmodified oligonucleotides.[207b] Phosphoramidate oligomers hybridized well to natural complementary sequences and acted as primers and templates in enzymatic reactions.[211a,b] Moreover, the multiple incorporation of the dinucleotidic phosphoramidite **149b** into oligothymidylates led to the formation of unusually stable complexes with poly (dA).[211c] Thus, these achiral oligonucleotide analogues deserve consideration as potential antisense molecules in the regulation of gene expression.

The synthesis of chiral oligonucleoside-*N*-alkylphosphoramidates has been described by Jäger *et al.*[212] Protected deoxyribonucleoside 3'-*O*-methyl-(*N,N*-diisopropyl)phosphoramidites were employed as building blocks in both solution and solid-phase oligonucleotide synthesis. The site-specific incorporation of an alkylphosphoramidate link was accomplished by replacing the usual aqueous iodine



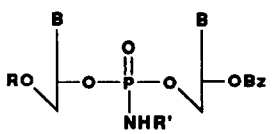
149a R= DMTr; B= T; B'= A^{bz}
b = Tr; B= B'= T

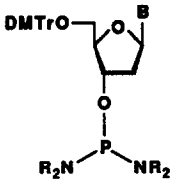


150 B= T, C^{bz}, A^{bz} or G^{Ib}
B'= T or C^{bz}

oxidation step with an iodine-alkylamine treatment. The phosphoramidate linkages in **151a-c** were resistant to the acidic conditions required for complete detritylation. In addition, a purified dinucleoside *N*-alkylphosphoramidate obtained from **151b** was resistant to digestion with nuclease P1 under conditions that would quantitatively hydrolyse d(TpT).[212] In this context, the synthesis of chiral (3'→5')-dithymidylyl phosphoranilidates from the reaction of a solid-phase bound (3'→5')-dithymidylyl methyl phosphite triester with iodine/aniline/2,6-lutidine has been reported.[135e] Following deprotection, the diastereoisomeric phosphoranilidates were separated and were shown to resist SVP-catalyzed hydrolysis.[135e]

The dinucleoside phosphoramidates derived from **152a-c** hybridized to poly-thymidylic acid and the T*m* of the resulting duplexes increased with the chain length of the *N*-alkylphosphoramidate substituent.[212] The insertion of phosphoramidate links in oligonucleotides has also been achieved *via* the deoxyribonucleoside phosphorodiamidites **153a-d** or **154a-d**.[213a-c] Typically, **153a** was coupled, in the presence of 5-(*p*-nitrophenyl)-1-*H*-tetrazole, to a trinucleotide prepared from ß-cyanoethyl deoxyribonucleoside phosphoramidites. A non-aqueous oxidation with *tert*-butyl hydroperoxide generated a 5'-terminal *N,N*-diethylphosphoramidate function resisting to the nucleolytic activity of SVP.[213a] Alternatively, the *N,N*-dialkylphosphoramidite or *N*-morpholinophosphoramidite linkages incorporated into oligonucleotides were hydrolysed under mildly acidic conditions to the corresponding *H*-phosphonate diesters[213a,c,214] which can potentially be converted to a variety of *P*-chiral analogues or natural phosphodiesters.[179]





151a R= MMTr; R'= $n\text{-}C_4H_9$; B= T
b = $n\text{-}C_8H_{17}$
c = $n\text{-}C_{12}H_{25}$

152a-c R= DMTr; R'= $n\text{-}C_4H_9$; B= A^{bz}
= $n\text{-}C_8H_{17}$
= $n\text{-}C_{12}H_{25}$

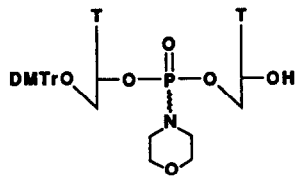
153a B= T; R= Et
b = C^{bz}
c = A^{bz}
d = G^{Ib}

154a-d NR_2= morpholino

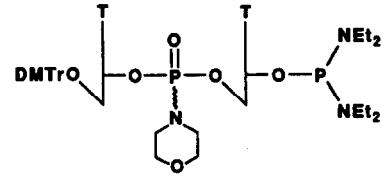
Of particular interest, Shimidzu *et al.*[215] reported the synthesis and properties of oligothymidylate analogues having alternating phosphodiester and stereodefined phosphormorpholidate linkages. These oligomers were prepared by inserting the dimeric bis-amidites **156a-b** in oligothymidylates. A coupling efficiency of *ca.* 89% was obtained when 5-(*p*-nitrophenyl)-1*H*-tetrazole was used as an activator. The internucleosidic phosphoramidite linkages were hydrolysed to *H*-phosphonate diesters upon treatment with 1*H*-tetrazole and water.[215a] Incidentally, **156a-b** were prepared from the phosphitylation of the diastereomerically pure dithymidylyl phosphormorpholidate derivatives **155a-b**[216a] with bis-(*N,N*-diethylamino)chlorophosphine. Incubation of the undecathymidylate analogues *Rp* and *Sp*-

d([$Tp_{morpholinyl}Tp$]$_5$T) with S1 nuclease resulted in the cleavage of a 5'-thymidine monophosphate at the 3'-terminus of the oligomers. The remaining d([$Tp_{morpholinyl}Tp$]$_4$$Tp_{morpholinyl}$T) was not hydrolysed further by the enzyme. Interestingly, the rate at which 5'-thymidine monophosphate was cleaved from the *R*p-undecathymidylate was faster than that of the *S*p-oligothymidylate. These data indicated that S1 nuclease recognized differences in configuration around the 3'-terminus of these oligonucleotide analogues.[215a] Phosphodiesterase I digested the above undecathymidylate analogues to 5'-thymidine monophosphate, dithymidylyl phosphormorpholidate, and 5'-phosphorylated dithymidylyl phosphormorpholidate. The *R*p-oligomer was also digested faster than the *S*p-oligomer with this enzyme. In contrast, micrococcal nuclease hydrolysed the oligothymidylate analogues at a much slower rate than that observed with phosphodiesterase I and, in this case, the *S*p-oligothymidylate was digested faster than the *R*p-congener.[215a]

Hybridization studies showed that the *S*p-oligothymidylate analogue formed an hybrid with poly dA having a T*m* of 28 °C which compared well with that of the native oligothymidylate dT_{11} under similar conditions (T*m* = 30 °C).[215a,d] These results indicate that the *S*p oligothymidylate analogue had a favorable configuration around the phosphormorpholidate linkage during complex formation. In this context, the *R*p-oligothymidylate analogue did not form a stable hybrid with poly dA. Thus, the incorporation of stereoregulated phosphormorpholidate linkages into oligonucleotides represents an effective way of enhancing the potential of these analogues as regulatory molecules in the control of gene expression.[215a]



155a ***R*p isomer**
b ***S*p isomer**

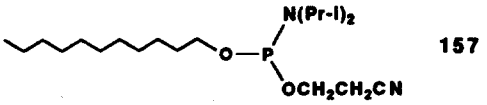


156a-b

Based on Letsinger's original procedure for rapid synthesis of oligodeoxyribonucleotides,[217] dichloro-(*N,N*-diethylamino)phosphine has been applied to the synthesis of oligonucleoside phosphoramidates.[218] However, chiral oligodeoxyribonucleoside phosphoramidates have predominantly been prepared by the *H*-phosphonate approach,[145a-b,179,219-222a] the nucleophilic substitution of internucleotidic phosphate triesters by alkylamines,[223] the activation of internucleotidic phosphodiesters with triphenyl phosphine and carbon tetrachloride,[224] the addition of alkyl azide to internucleotidic phosphite triesters[225] or by the oxidation of the latter with iodine in the presence of alkylamines.[226] Like oligodeoxyribonucleoside phosphorothioates, phosphoramidate analogues such as phosphormorpholidates, phosphorbutylamidates, and phosphorpiperazidates have exhibited anti-HIV activity in cell cultures.[145a-b]

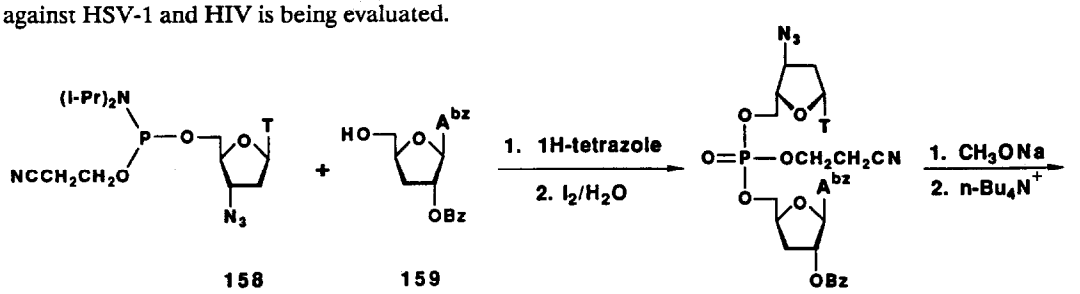
3.6. Hydrophobic Oligonucleotides and Oligodeoxyribonucleoside Methylphosphonates.

The application of oligonucleotides as potential therapeutic agents has been hampered by the low efficiency with which these molecules permeated intact cells. To increase the therapeutic potency of oligonucleotides, Kabanov *et al.*[227a] synthesized the phosphoramidite **157** as a means to introduce an hydrophobic group at the 5'-end of oligonucleotides with a coupling efficiency of 90-95%. 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.[227a-b] The attachment of hydrophobic groups to oligonucleotides has therefore shown its efficacy at improving the biological activity of theses biomolecules.

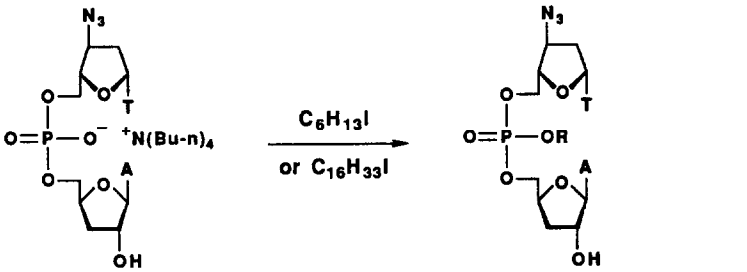


157

In an effort to synthesize lipophilic dinucleoside phosphate triesters as potential antiviral and antibiotic prodrugs, Meier and Huynh-Dinh[228] reported the preparation of the AZT phosphoramidite derivative **158**. Without isolation, **158** was reacted with N^6-benzoyl-2'-*O*-benzoyl-3'-deoxyadenosine (**159**) and 1*H*-tetrazole. Following aqueous iodine oxidation, the resulting (5'→5')-dinucleoside phosphotriester **160** was isolated by chromatography, deprotected, and ion-exchanged to the corresponding phosphodiester **161**. Alkylation of **161** with either 1-iodohexane or 1-iodohexadecane afforded the lipophilic phosphotriesters **162a-b** in 60% yield. These dimers were characterized by ^{1}H-, ^{13}C-, ^{31}P-NMR spectroscopies and by FAB-mass spectrometry.[228] The biological activity of **162a-b** against HSV-1 and HIV is being evaluated.



158 159 160



161

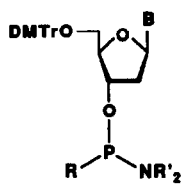
162a R= C_6H_{13}
b = $C_{16}H_{33}$

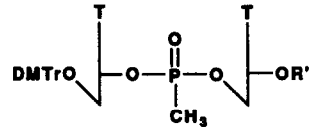
Nonionic oligomers such as oligodeoxyribonucleoside methylphosphonates are taken up by cells through passive diffusion[229] or, more likely, by a fluid phase/adsorbtive endocytic route.[230,231] This cellular uptake pathway is distinct from that of oligodeoxyribonucleoside phosphodiesters or phosphorothioates which seem to enter cells by endocytosis involving a saturable binding site.[230-232] Oligodeoxyribonucleoside methylphosphonates are totally resistant to nuclease hydrolysis[229] both in culture media and in cells.[231] By virtue of such attributes, these oligonucleotide analogues exemplify another category of antisense molecules with potential chemotherapeutic value as inhibitors of gene expression.[233]

The basic concepts stemming from the phosphoramidite approach led Dorman *et al.*[234] to the synthesis of the deoxyribonucleoside methyl phosphonamidite **163a**. Specifically, the reaction of 5'-*O*-DMTr-2'-deoxythymidine with chloro-(*N,N*-dimethylamino)methylphosphine or *N,N*-diisopropylamino-(*p*-nitrophenyloxy)methylphosphine and sodium hydride[235] afforded **163a** and **164a** respectively, in 95% isolated yield. Unexpectedly, the activation of **163a** with 1*H*-tetrazole generated several products as judged by ^{31}P-NMR spectroscopy. Nonetheless, the activation of **163a** with imidazole in the presence of

3'-*O*-DMTr-2'-deoxythymidine followed by routine iodine oxidation afforded the expected dinucleoside methylphosphonate **165a** in *ca.* 90% yield.[234] Jäger and Engels[236] independently reported the preparation of **163b** from the reaction of a suitably protected deoxyribonucleoside with bis-(*N,N*-dimethylamino)methylphosphine and catalytic amounts of 2,4,6-collidine hydrochloride. It was further demonstrated that deoxyribonucleoside methylphosphonamidites reacted with 3'-*O*-benzoylthymidine within 1 min upon activation with 1*H*-benzotriazole. The resulting dinucleoside methylphosphonites were oxidized with *tert*-butyl hydroperoxide affording the corresponding dinucleoside methylphosphonates in 81% yield. Consistent with the observations of Dorman *et al.*,[234] the use of 1*H*-tetrazole for the activation of deoxyribonucleoside methylphosphonamidites induced the disproportion of (3'→5')-dinucleoside methylphosphonite intermediates to symmetrical (3'→3')- and (5'→5')-dinucleoside methylphosphonites.[236] Paradoxically, 1*H*-tetrazole has been used for the activation of the phosphonamidites **164a-d** in the solid-phase synthesis of oligodeoxyribonucleoside methylphosphonates having contiguous[145b,222a,b,231,237a-g] or non-contiguous[222a,237d-f,h-j] methylphosphonate linkages. The incorporation of the methylphosphonamidites occurred within 30-120 s with a coupling yield of 96-97%,[237a,b] without apparent formation of side products.[237i] The removal of the base protecting groups was effected with ethylenediamine/ethanol (1:1) at ambient temperature. Under these conditions *ca.* 1% of each internucleotidic phosphonate linkage was cleaved.[237i]

In the same context, Roelen *et al.*[238] have reported the preparation of the deoxyribonucleoside phosphonamidites **164a** and **164e-f** from the reaction of 5'-*O*-DMTr-2'-deoxythymidine with the appropriate bis(*N,N*-diisopropylamino)alkylphosphine in the presence of 2,4,6-collidine hydrochloride. The phosphonamidites **164a,e,f** were incorporated at a defined location into oligonucleotides by solid-phase synthesis using 1*H*-tetrazole as an activator. The coupling efficiency of **164a,e,f** was *ca.* 97%. The resulting phosphonite function was oxidized or sulfurized to the corresponding phosphonate or phosphonothioate internucleotidic linkage.[238]





163a B= T; R= R'= Me
b = C^{bz}
c = A^{bz}
d = G^{ib}

164a-d R= Me; R'= i-propyl
e B= T; R= n-butyl; R'= i-propyl
f = T; = n-octyl; = i-propyl

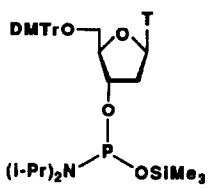
165a R'= DMTr
b = Ac

An interesting preparation of dinucleoside methylphosphonates has been described by Lebedev *et al.*[239a] Their approach is based on the reaction of the deoxyribonucleoside methylphosphonamidite **164a** with 5'-*O*-trifluoroacetyl-3'-*O*-acetylthymidine and 4-(*N,N*-dimethylamino)pyridine (DMAP).[240] The coupling reaction proceeded to 73% yield within 2.5 h while, in the absence of DMAP, the coupling rate became *ca.* 100 times slower. This condensation reaction was less sensitive to trace amounts of water than those mediated by 1*H*-tetrazole.[239a] It must also be noted that the *Rp* and *Sp* diastereoisomers of the deoxyribonucleoside methylphosphonamidites **164a-d** can be separated on silica gel pretreated with triethylamine.[239b]

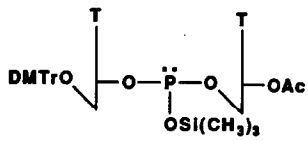
In a different application, the deoxyribonucleoside methylphosphonamidites **164a** and **164c-d** were incorporated at defined locations into an oligonucleotide containing the *Eco*RI recognition sequence. The *Rp* and *Sp* methylphosphonate diastereoisomers of each sequence were separated by HPLC. It was shown that *Rp*-*Rp* duplexes exhibited T*m* values similar to that of the parent unmodified duplex.

Conversely, *Sp*-*Sp* duplexes displayed lower T*m* values, probably because of the steric interactions imparted by the P-CH_3 group.[135j] In addition, methylphosphonate oligomers (larger than 8 bases) did not readily adopt a helical B geometry and hybridized poorly to natural DNA presumably because of conformational distorsions induced by the methyl group around the 3'- and 5'-*O*-*P*- linkages.[237c]

Of interest, Dabkowski *et al.*[241] reported the synthesis of the deoxyribonucleoside phosphoramidite **166** from 5'-*O*-DMTr-2'-deoxythymidine, bis-(*N,N*-diisopropylamino) trimethylsilyloxyphosphine, and *N,N*-diisopropylammonium tetrazolide. The condensation of **166** with 3'-*O*-acetylthymidine and *N,N*-diisopropylammonium tetrazolide afforded the dinucleoside trimethylsilyl phosphite triester **167** which upon treatment with methyl iodide yielded the dinucleoside methylphosphonotriester **168**.[241a] It must be pointed out that **167** could readily be hydrolysed to the corresponding *H*-phosphonate derivative[241a] or converted to either a dinucleoside phosphoroazolide[241a] or a dinucleoside phosphorofluoridate[242] which could all potentially be transformed into various *P*-chiral analogues.



166



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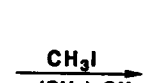
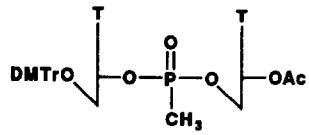
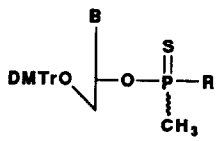


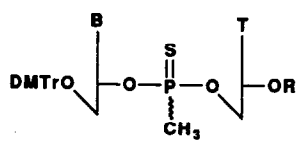


168

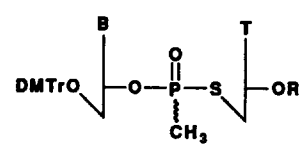
The synthesis of oligodeoxyribonucleoside methylphosphonothioates has also been investigated.[243] For example, the reaction of the phosphonamidite **164b** with hydrogen sulfide and 1*H*-tetrazole afforded the deoxyribonucleoside 3'-hydrogen methylphosphonothioate **169** in 94% yield. Treatment of **169** with iodine and 3'-*O*-acetylthymidine in pyridine generated the dinucleoside methylphosphonothioate **171** in 47% yield.[243a] Alternatively, the phosphonamidite **164a** was converted to the methylphosphonothioate **170** upon treatment with 3-hydroxypropionitrile, DMAP, and trifluoroacetic anhydride, followed by oxidation with elemental sulfur.[243c] After decyanoethylation of **170**, the lithium salt of each of the purified *Rp* and *Sp* diastereoisomers were condensed with 5'-deoxy-5'-iodo-3'-*O*-acetylthymidine affording the corresponding dinucleoside methylphosphonothioate **172**.[243c] Along similar lines, Brill and Caruthers[244] reported the synthesis of the phosphoramidite **173** and its congeners having any combination of the four nucleobases for the incorporation of stereospecific methylphosphonothioate linkages into synthetic oligomers.



169 B= C^{bz}; R= H
170 B= T;
R= OCH_2CH_2CN



171 B= C^{bz}; R= Ac



172 B= T; R= Ac
173 B= T;
R= $P[N(Pr\text{-}i)_2]OCH_2CH_2CN$

Of practical importance, the incorporation of two contiguous methylphosphonate linkages at each terminus of an oligonucleotide considerably increased its resistance to hydrolysis by exonucleases.[237f,i,245] Unlike natural oligodeoxyribonucleotides and their phosphorothioate analogues, oligodeoxyribonucleotides having exclusively or alternating methylphosphonate linkages were unable to form hybrids with RNA that were substrates for the RNase H activity of *E. coli*.[237f,246] However, methylphosphonate oligomers having three or more consecutive natural phosphodiesters elicited RNase H activity upon hybridization with complementary RNA.[237f,247,248] Thus, oligodeoxyribonucleoside

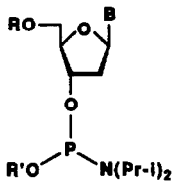
methylphosphonates with one or more regions having three successive phosphodiester functions provided a highly nuclease-resistant arrangement of methylphosphonate and phosphodiester linkages without loosing the ability to promote cleavage of the target RNA by RNase H.[237f,246,248] Oligodeoxyribonucleoside methylphosphonates have also demonstrated anti-HIV activity in infected cells.[145b,237h,249,250]

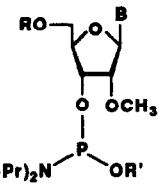
3.7. Oligonucleoside Phosphotriesters.

The phosphoramidites **174a-d** were applied to the synthesis of phosphate-methylated oligodeoxyribonucleotides to study the conformation of parallel mini-duplexes[251a-c] and to determine the stability of the hybrids formed between these oligonucleotide analogues and natural DNA or RNA.[251d] Buck *et al.*[251d,e] argued that phosphate-methylated oligonucleotides had an optimal combination of steric and stereoelectronic factors for the formation of the strongest hybrids with unmodified DNA.[251d,e] Such oligonucleotide analogues have been claimed to interrupt the life cycle of HIV-1, the causative agent of AIDS.[251f] Since then, this claim has been retracted.[251g] The notorious lability of methyl phosphotriesters required the development of procedures allowing the deprotection of the nucleobases without affecting the methyl phosphate protecting group. Kuijpers *et al.*[252] reported the synthesis of well-defined phosphate-methylated DNA segments from methyl and ß-cyanoethyl phosphoramidite derivatives of N^6-Fmoc-5'-*O*-DMTr-2'-deoxyadenosine and 5'-*O*-DMTr-2'-deoxythymidine, respectively. A 0.05 M solution of potassium carbonate in methanol was shown to remove both Fmoc and ß-cyanoethyl protecting groups while releasing oligomers from the solid support and leaving intact methyl phosphate protecting groups.[252]

The solid-phase synthesis of a phosphate-methylated oligonucleotide (d[Tp(OMe)]$_5$T) has also been achieved by Alul *et al.*[253] Using standard phosphoramidite chemistry on a Controlled-Pore Glass (CPG) support derivatized with an oxalyl linker, the formation of phosphodiesters was detected during the oxidation of methyl phosphite triesters with aqueous iodine. The search for an alternate oxidant led to a 0.5 M solution of *tert*-butyl hydroperoxide in dichloromethane which minimized unwanted demethylation. Upon completion of the synthesis, the phosphate-methylated oligomer was rapidly released from the support with 5% ammonium hydroxide in methanol. The oligomer was then isolated by HPLC and was characterized by FAB-mass spectrometry, migration on TLC, and by conversion to d([Tp]$_5$T) with thiophenol and triethylamine.[253]

Furthermore, the phosphoramidites **176a-b** have been applied to the synthesis of phosphate-methylated RNA dimers to instigate conformational analysis studies.[254]





174a-d R= MMTr; R'= Me
B= T, C^{fmoc}, A^{fmoc}, G^{fmoc}

175a-d R= DMTr; R'= Et
B= T, C^{bz}, A^{bz}, G^{ib}

fmoc= 9-fluorenylmethyloxycarbonyl

176a R= MMTr; R'= Me; B= C^{fmoc}
b = MMTr; = Me; = A^{fmoc}

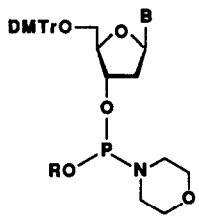
177a-d R= DMTr; R'= Me
178a-d = DMTr; = CH_2CH_2CN
B= U, C^{bz}, A^{bz}, G^{ib}

To evaluate the effectiveness of various oligonucleotide analogues as antisense molecules, the deoxyribonucleoside phosphoramidites **175a-d**,[135e,h] **179a-d**[135d] and the phosphonamidites **164a-b** were inserted at predetermined positions in oligonucleotides complementary to the translation initiation site of the mRNA encoding chloramphenicol acetyl transferase (CAT).[148] Oligonucleotides carrying ethyl phosphotriester functions led to 51% inhibition of plasmid-derived CAT gene expression in CV-1 cells.

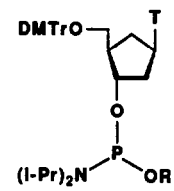
Under identical conditions, similar oligonucleotides having isopropyl phosphotriester, methylphosphonate, and natural phosphodiester linkages produced 0%, 65%, and 35% inhibition of CAT expression, respectively.[148] These findings suggest that the steric interactions created by either the ethyl or, most strikingly, by the isopropyl phosphate protecting group(s), may significantly affect the hybridization abilities of these oligonucleotide analogues with nucleic acid targets.

The deoxyribonucleoside phosphorodiamidites **153a-d** have particularly demonstrated their usefulness in the solid-phase synthesis of oligodeoxyribonucleoside isopropyl phosphotriesters.[255] Typically, **153a-d** were activated with 5-(*p*-nitrophenyl)-1*H*-tetrazole and incorporated at selected positions into oligonucleotides. A subsequent treatment with 5-(*p*-nitrophenyl)-1*H*-tetrazole in isopropyl alcohol:acetonitrile (1:4) followed by oxidation with *tert*-butyl hydroperoxide yielded each chiral isopropyl phosphotriester function with a coupling efficiency of *ca.* 99%. The *Rp* and *Sp* diastereoisomers of a tetradecamer were resolved by HPLC and it was shown that the *Sp* isomer was more potent than the *Rp* isomer in preventing primer chain elongation beyond the isopropyl phosphate protecting group.[255]

The deoxyribonucleoside phosphoramidite **180** has been employed in the synthesis of dinucleotide blocks toward the synthesis of octathymidylates having alternating neopentylphosphothionotriester/phosphodiester linkages of defined stereochemistry at phosphorus.[256] These oligothymidylate analogues were about 15 times more resistant to endonuclease P1 than unmodified oligonucleotides. No hybridization studies involving these oligonucleotides with complementary sequences were however presented.



179a-d B= T, C^{bz}, A^{bz}, G^{ib}
R= Isopropyl
180 B= T; R= neopentyl



181 R= Me
182 = CH_2CH_2CN

The phosphoramidites **175a-d** were also applied to the solid-phase synthesis of the self-complementary DNA sequence d(GGAA[Et]TTCC). Upon separation of the diastereoisomers, it was demonstrated that the *Rp*-*Rp* duplex had a lower T*m* than that of the *Sp*-*Sp* or the unmodified duplex (ΔTm = 11 °C).[135g] Similar modified DNA sequences were used to map the contact points between DNA and the endonuclease *Eco*RI.[135k,257,258]

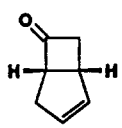
3.8. Oligonucleotides with Modified Carbohydrate Entities.

The search for novel synthetic oligonucleotides as potential inhibitors of gene expression has focused not only on the chemical modification of the atoms linked to the phosphate backbone (*vide supra*) but also on the chemical modification of the carbohydrate moieties. In this regard, the synthesis of carbocyclic oligothymidylates was initiated by the 1*H*-tetrazole-mediated coupling of the carbathymidine phosphoramidite **181** with (+)-carbathymidine (C-dT) linked to a solid support. C-dT_4 and C-dT_{12} were synthesized with a stepwise yield of *ca.* 90%. The stability of purified C-dT_4 and natural dT_4 to nucleolytic hydrolysis were compared. It was shown that calf spleen phosphodiesterase (CSP) hydrolyzed dT_4 *ca.* 9 times faster than C-dT_4. S1 nuclease did not hydrolyze C-dT_4 after 24 h of incubation, whereas dT_4 was 50% hydrolysed within 3.5 h under the same conditions. It was also found that C-dT_{12} formed a more stable hybrid with $d(C_2A_{12}C_2)$ relative to dT_{12} (ΔTm = 10 °C).[259]

Szembō *et al.*[260a] independently described the solid-phase synthesis of the carbocyclic

oligothymidylates C-dT_{10}, C-dT_{12} and C-dT_{20} using the phosphoramidite **182**. In agreement with the findings of Perbost *et al.*,[259] it was observed that the coupling efficiency of **182** was lower (95%) than that of the corresponding unmodified phosphoramidites (98%). Conformational and/or stereoelectronic differences between carbocyclic and natural nucleosides were invoked to explain the relative lack of reactivity of the phosphoramidite **182**. It has additionally been discovered that unlike dT_{10}, C-dT_{10} adopted a single-stranded helical structure in a solution of appropriate ionic strength.[260b] In spite of the stability of the duplex formed between a carbocyclic oligothymidylate and a complementary polynucleotide, the carbocyclic oligomer was inactive as a template or as a primer in DNA polymerase assays. It was postulated that $(C\text{-}dT)_n$ was strongly bound to the polymerase and thus inhibited the initiation of replication.[260b] The usefulness of carbocyclic oligomers as antisense molecules awaits further investigation.

Payne and Roberts[261] delineated the preparation of the (fluorocyclopentyl)thymine phosphoramidite **185** and its application to the synthesis of novel carbocyclic oligonucleotides. The precursor nucleoside **184** was obtained from the ketone **183** in 16% overall yield. The insertion of **185** in oligonucleotides proceeded with a coupling efficiency of 85% consistent with that observed with other carbocyclic phosphoramidites.[259,260a] Polyacrylamide gels provided evidence that fluorine-containing oligonucleotides can form hybrids with complementary strands. The susceptibility of these duplexes to cleavage by restriction enzymes is currently being studied. ^{19}F-NMR spectroscopy has been particularly helpful in detecting the presence of fluorine in these modified oligonucleotides.[261]



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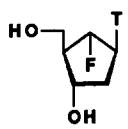

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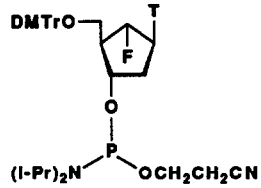

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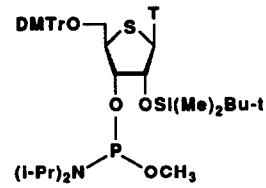


186

The synthesis of oligodeoxyribonucleotides containing 1-(4'-thio-ß-D-ribofuranosyl)thymine has recently been reported by Bellon *et al.*[262] The thioribofuranosyl phosphoramidite **186** was incorporated into dodecamers [$T^*(dT)_{11}$ and $(dT)_5T^*(dT)_6$, where T^* represents 1-(4'-thio-ß-D-ribofuranosyl)thymine] which were subjected to digestion with CSP and hybridization with complementary sequences. The 5'-exonuclease hydrolyzed $T^*(dT)_{11}$ and $(dT)_{12}$ to the extent of 50% within 43 min and 1 min, respectively, thus indicating the strong resistance of the modified oligonucleotide to the nucleolytic activity of CSP. The heteroduplex $d(C_2A_{12}C_2)/(dT)_5T^*(dT)_6$ and the parent duplex $d(C_2A_{12}C_2)/(dT)_{12}$ exhibited T*m* values of 40 °C and 45 °C, respectively.[262] 4-Thiooligonucleotides are being evaluated as antisense molecules inspite of their relatively low affinity for complementary sequences.

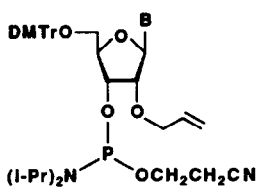
Chimeric oligonucleotides composed of deoxyribonucleotides and 2'-*O*-methylribonucleotides complementary to RNA fragments, induced site-specific cleavage of the RNA with *E. coli* RNase H.[263] The 2'-*O*-methylribonucleoside phosphoramidites **177a-d** and **178a-d** were used in the solid-phase synthesis of the ribonucleotidic part of the chimeric oligonucleotides, whereas standard deoxyribonucleoside phosphoramidites were utilized for the insertion of short DNA segments (3-5 bases) within the oligonucleotidic chains.[263a] The phosphoramidites **177a-d** and **178a-d** were also used to construct chimeric adapters containing double-stranded 2'-*O*-methyl RNA that would withstand the nucleolytic activity of Bal 31. It was shown that seven consecutive 2'-*O*-methyl ribonucleotide residues were sufficient to provide resistance to Bal 31 hydrolysis.[264] This feature allowed the unidirectional digestion of DNA with Bal 31. New synthetic routes to the synthesis of protected 2'-*O*-methyl- or 2'-*O*-ethylribonucleoside-3'-*O*-phosphoramidites have recently been developed by Wagner *et al.*[265] and Sproat *et al.*[266]

Oligomers composed of unmodified ribonucleotides and their 2'-*O*-methyl analogues have been prepared from the respective phosphoramidites to study RNA binding proteins and/or RNA processing enzymes.[267] The presence of 2'-*O*-methylribonucleotides conferred protection against nucleases and stability to alkaline hydrolysis. A highly efficient synthesis of these oligoribonucleotides on a urethane-linked aminopropylated CPG support using the phosphoramidites **178a-d** and 5-(*p*-nitrophenyl)-1*H*-tetrazole as an activator has been reported.[268] Given the steric hindrance caused by the neighbouring 2'-*O*-methyl group, a longer condensation time (6 min) was required to ensure high coupling efficiency (>99%). The enzymatic stability of 2'-*O*-methylribonucleotides were compared to identical DNA and RNA sequences under the same conditions. 2'-*O*-Methyl RNA oligomers were resistant to a variety of RNA and DNA nucleases but were sensitive to P1 nuclease, snake venom phosphodiesterase, and Bal 31 nuclease.[268] The sensitivity of single-stranded 2'-*O*-methyl RNA to Bal 31 is in sharp contrast with the remarkable stability of their double-stranded congeners to this nuclease.[264]

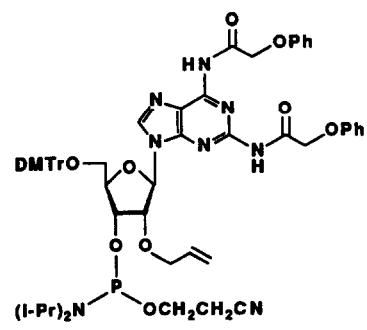
Of interest, the incorporation of N^4-benzoyl-5'-*O*-DMTr-2'-*O*-methylcytidine-3'-*O*-(*N,N*-diisopropylamino)methoxyphosphine (**177b**) at a defined location into an oligoribonucleotide confirmed the participation of the 2'-OH group of the substrate in a cleavage reaction catalysed by a synthetic ribosyme.[269a] Furthermore, 2'-*O*-methylribonucleotides prepared by the phosphoramidite approach were also used for probing the structure and function of U1,[270d] U2,[270a,c] U4,[270b,g] U5 and U6[270b,g] small nuclear ribonucleoprotein particles (snRNPs) which are subunits of functional spliceosomes.[270f]

The stability of heteroduplexes composed of 2'-*O*-methyl RNA and complementary RNA fragments[271] in addition to the decreased sensitivity of these analogues to several nucleases, have enhanced the suitability of these oligonucleotides as antisense molecules. Incidentally, 2'-*O*-methyl oligoribonucleotides have been designed to specifically bind to sequence flanking the HIV-1 *gag-pol* RNA hairpin in an attempt to alter -1 ribosomal frameshifting, and the expression of the *gag* and *pol* genes.[272] Oligonucleotides binding just downstream to the stem of the hairpin enhanced ribosomal frameshifting by 6-fold. Conversely, oligonucleotides binding upstream to the stem had no effect on frameshifting efficiency. The efficiency of -1 frameshifting of retroviral RNA determines the ratio of *gag-pol* gene products which is critical to viral assembly. Thus, inhibition or enhancement of ribosomal frameshifting may affect viral production and might disrupt the HIV life cycle.[272]

The preparation of the 2'-*O*-allyl-3'-*O*-phosphoramidites **187a-e** has been described by Sproat *et al.*[273a] and others.[273c] The solid-phase synthesis of oligoribonucleotides began with the activation of **187a-d** with 5-(*p*-nitrophenyl)-1*H*-tetrazole. The coupling time was extended to 8 min to ensure high condensation yields.[273a] It must be pointed out that RNA oligomers prepared from base-protected 5'-*O*-DMTr-2'-*O*-(3,3-dimethylallyl)ribonucleoside 3'-*O*-(ß-cyanoethoxy)-*N,N*-diisopropylaminophosphines were not as efficient as 2'-*O*-allylribonucleotides in providing a rapid and stable binding to complementary RNA sequences.[274] Consequently, 2'-*O*-allylribonucleotides may serve as antisense molecules in diverse areas of molecular biology.



187a B= uracil
b = N^4-benzoyl cytosin-1-yl
c = N^6-trimethylacetyl adenin-9-yl
d = N^2-dimethylformamidine guanin-9-yl
e = hypoxanthin-9-yl



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The synthesis of novel 2'-*O*-modified oligonucleotides from nucleoside phosphoramidite intermediates has also been reported by others.[275] Preliminary data regarding the biophysical and biological evaluation of these oligonucleotide analogues were also presented.

Lamm *et al.*[276a] reported the incorporation of the 2,6-diaminopurine-2'-*O*-allylribonucleoside-3'-*O*-phosphoramidite **188**[273a-b] into oligoribonucleotides in an attempt to specifically deplete U5 snRNP from HeLa cell nuclear splicing extracts. It was demonstrated that U5 snRNP was essential for spliceosome assembly. The absence of U5 snRNP prevented the stable association of U4/U6 but not that of U1 and U2 snRNPs with pre-mRNA.[276a] Biotinylated 2'-*O*-allyloligoribonucleotides having 2-aminoadenine nucleobases were also used to deplete U2 snRNA from HeLa nuclear extracts.[276b] These oligonucleotides were not significantly inhibiting spliceosome assembly because accumulation of splicing intermediates was observed. The modified oligonucleotides inhibited mRNA production *in vitro* by interfering with exon ligation. These results suggest that the functional requirement for U2 snRNP in the splicing mechanism occurred after spliceosome assembly.[276b]

Cotten *et al.*[277] described the synthesis of 2'-*O*-methyl- and 2'-*O*-ethylribonucleotides as inhibitors of the U7 snRNP-dependent mRNA processing event. These oligonucleotides were prepared by solid-phase techniques using appropriate (ß-cyanoethyl)-*N,N*-diisopropylphosphoramidites. An extended coupling reaction time (5 min) produced an average coupling efficiency of 98%. It was shown that 2'-*O*-ethylribonucleotides were *ca.* 5 times more effective than that of native antisense RNA molecules at inhibiting mRNA processing. Only a slight excess of inhibitor over target RNA was required for an 80% inhibition of the processing reaction.[277]

Of particular importance, Shibahara *et al.*[278] reported that oligo-(2'-*O*-methyl)ribonucleoside phosphorothioates inhibited the replication of the human immunodeficiency virus (HIV-1) in cultured MT-4 cells. Interestingly, ribonucleotides having five phosphorothioate linkages at the 3'- and 5'-termini exhibited almost as much anti-HIV activity as fully phosphorothioated oligomers. In contrast, both 2'-*O*-methylribo- and deoxyribonucleotides failed to inhibit the replication of the virus. These data indicated that fully thioated oligonucleotides were not necessary to demonstrate anti-HIV activity and that oligo-(2'-*O*-methyl)ribonucleotides were sensitive to the nucleases present in the cell line used for the study.[278] 2'-*O*-Methylribonucleoside phosphorothioate oligomers have also been useful in deciphering the mechanisms whereby the expression of Intercellular Adhesion Molecule 1 was inhibited by antisense oligonucleotides.[154] Finally, 2'-*O*-methylribonucleotides and their phosphorothioate analogues were effective in forming pseudo half-knot structures with the HIV TAR element. This element is a structural RNA that binds to *Tat*, a viral regulatory protein. The binding of *Tat* to TAR RNA is a critical step in the viral life cycle. Pseudo half-knot formation disrupts the structure of TAR in the region specifically recognized by the *Tat* protein and thus offers a new therapeutic target site for antisense oligonucleotides.[279]

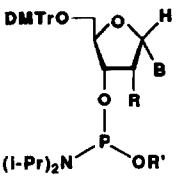
Almost two decades ago, dithymidylyl monophosphate composed of nucleosides having the sugar and the nucleobase moieties linked by an α-glycosidic bond, as opposed to the natural ß-glycosidic bond, was found to be a substrate for snake venom and bovine spleen phosphodiesterases.[280] The rate of enzymatic hydrolysis of α-dithymidylyl monophosphate was considerably lower than that of the corresponding ß-anomer.[280] This early observation led to the design of novel antisense molecules as potential therapeutics.[281]

Although the original preparation of α-oligodeoxyribonucleotides was accomplished by the phosphotriester methodology,[282a-c] the α-deoxyribonucleoside phosphoramidites **189a-d** and **190a-c** were prepared and applied to the solid-phase synthesis of α-oligonucleotides.[282c-e,283,284] Purified α- and ß-[d(CATGCG)] were subjected to hydrolysis with S1 nuclease, calf spleen phosphodiesterase (CSP), and snake venom phosphodiesterase (SVP).[282b] Under specific conditions, S1 nuclease and CSP hydrolysed the ß-oligomer to the extent of 100% and 89%, respectively, within 10 min. Under identical conditions, the α-oligomer was resistant to CSP and showed only 7% hydrolysis with S1 nuclease. SVP digested the α-oligomer at a rate 30 times slower than that of the ß-oligomer.[282b] In a similar study, Thuong *et al.*[285] pointed out that the stability of acridine-substituted α-oligothymidylates to CSP and SVP was roughly 300-500 times higher than that of similarly derivatized ß-oligothymidylates.

To evaluate the suitability of α-oligonucleotides as antisense molecules,[286] an α-

hexadecadeoxyribonucleotide was prepared from the phosphoramidites **189a-d**.[287] The half-life of the radiolabelled α-oligonucleotide in undiluted fetal bovine serum was *ca.* 24 h. In contrast, ß-oligomers were completely hydrolysed under these conditions within the first 15 min.[287] Furthermore, α-oligomers survived well in *Xenopus* oocytes with a half-life of over 8 h compared to that of only 10 min for ß-oligomers.[288] However, α-oligonucleotides exhibited poor stability in NIH3T3 cellular extracts. For example, an α-hexaadenylate was hydrolyzed to the extent of 50% within 132 min compared to that of 54 min for the corresponding ß-oligomer.[289]

NMR and UV absorption studies indicated that, unlike native DNA, α-oligodeoxyribonucleotides hybridized with complementary ß-strands in a parallel orientation.[282c,290] The hybrid composed of α-d(CATGCG) and ß-d(GTACGC) adopted a predominant right-handed B-conformation in aqueous solution.[290] The stability of the duplex depended on the base composition of the α-sequences; pyrimidine α-nucleosides provided more stability than purine α-nucleosides.[291] Interestingly, oligonucleotides composed of only α–thymidine have been shown to form antiparallel duplex-structures according to UV and CD spectroscopies.[292]



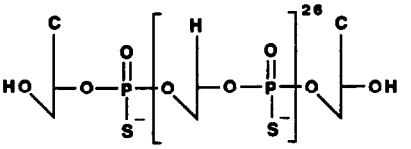
189a B= T; R= H; R'= Me
b = C^{bz}
c = A^{bz}
d = G^{pal} pal= palmitoyl
190a-c R= H; R'= CH_2CH_2CN
191a B= U; R= $OSi(Me)_2Bu\text{-}t$; R'= Me
b = C^{bz}
c = A^{bz}
d = G^{ac} ac= acetyl

α- and ß-Anomeric $d(G_2T_{12}G_2)$ oligodeoxyribonucleotides were prepared by the phosphoramidite approach and used in hybridization experiments with the riboadenylate rA_{12}. The ß-DNA/RNA and α-DNA/RNA hybrids exhibited a *Tm* of 27 °C and 53 °C, respectively.[284] The duplex ß-$d(G_2T_{12}G_2)/rA_{12}$ was a substrate for *E. coli* RNase H and resulted in the hydrolysis of rA_{12}. Conversely, α-$d(G_2T_{12}G_2)/rA_{12}$ did not lead to the degradation of rA_{12} with either *E. coli* RNase H or *Drosophila* embryo RNase H under identical conditions.[282c,284,293a] The parallel annealing of α-DNA to RNA was presumably responsible for the protection of RNA against RNase H-mediated hydrolysis.[293b] Such annealing may also account for the fact that the translation of rabbit ß-globin mRNA in cell-free systems and in micro-injected oocytes was not inhibited by an α-oligodeoxyribonucleotide targeted to the coding region of the message. However, the corresponding ß-oligodeoxyribonucleotides and ß-oligodeoxyribonucleoside phosphorothioates were inhibitory.[294] Paradoxically, α-oligodeoxyribonucleotides targeted against the 5'-cap region of rabbit ß-globin mRNA, specifically inhibited the translation of the message in rabbit reticulocyte lysates or wheat germ extracts.[295a] These experiments provided evidence that α-oligodeoxyribonucleotides can be potentially useful as inhibitors of translation. It has further been reported that an α-oligodeoxyribonucleotide (20-mer) targeted against the primer binding site of Friend murine leukemia virus, inhibited viral spreading.[295b] Moreover, Morvan *et al.*[173e] and Rayner *et al.*[296] described the solid-phase preparation of α-oligodeoxyribonucleoside phosphorothioates exhibiting anti-HIV activity. These included a dodecamer complementary to the splice acceptor site of the pre-mRNA encoding the *Tat* protein,[173e] and a 28-mer targeted against the translation initiation site of *rev* mRNA.[296]

The synthesis of α-oligothymidylyl phosphorothioates on a teflon-based solid support has been accomplished *via* the α-deoxyribonucleoside phosphoramidite **189a** with coupling yields averaging 98%. The stepwise sulfurization reaction was effected by elemental sulfur or *N,N,N',N'*-tetraethylthiuram disulfide.[297a] Like standard oligodeoxyribonucleoside phosphorothioates, the α-oligothymidylyl phosphorothioates displayed superior resistance to nucleases relative to α-oligothymidylates.[297a] Furthermore, the oligothymidylyl phosphorothioate α–$d([Tps]_7Tp)$ formed a more stable duplex with polyriboadenylic acid than that observed with ß-$d([Tps]_7Tp)$ (ΔTm = 14 °C).[297b]

It is known that the phosphorothioate homooligomer ß-*S*-dC_{28} blocked *de novo* infection of susceptible cells by HIV-1.[172a-c] The α-*S*-dC_{28} oligomers displayed an anti-HIV activity comparable to

that of the ß-*S*-dC_{28} oligomer, whereas both ß-dC_{28} and α-dC_{28} were inactive.[296] These observations were expected since the abasic oligodeoxyribonucleoside phosphorothioate **192** and the oligomeric phosphorothioate **193** demonstrated significant anti-HIV activities as well.[298] These results and those from others[299] suggest that the anti-HIV activity of phosphorothioate homooligomers is not entirely related to the enhanced resistance of these oligonucleotides to nucleases.



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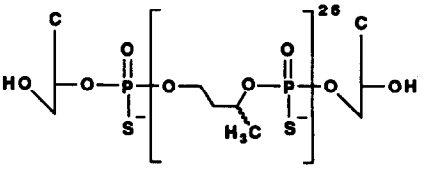

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The deoxyribonucleoside phosphoramidite **194** has been employed in the solid-phase synthesis of alternating α, ß-oligothymidylates with alternating (3'→3')- and (5'→5')-internucleotidic linkages (α, ß-dT_{28}).[300] These oligonucleotide analogues have been proposed as models for antisense oligodeoxyribonucleotides. The synthetic design was based on the assumption that oligonucleotides having alternating (3'→3')- and (5'→5')-internucleotidic phosphodiester links may not be as rapidly hydrolysed with nucleases as natural oligomers. However, this approach would reduce the number of hybridizing nucleobases by one half and, hence, seriously limit the application of these oligonucleotides as antisense molecules. It has been postulated that the alternate substitution of an α-monodeoxyribonucleotide for a ß-monodeoxyribonucleotide in the (3'→3')- and (5'→5')-internucleotidic linkage motif would minimize this limitation.[300]

The hybridization of the purified oligonucleotide analogues with complementary unmodified ß-dA_{28}, and polyriboadenylic acid (poly rA) led to hybrids having thermostability similar to that of duplexes composed of the ß-polythymidylyl phosphorothioate *S*-dT_{28} and ß-dA_{28} or poly rA. Furthermore, the α, ß-dT_{28} oligomer exhibited better resistance to the nucleolytic activity of SVP and CSP relative to that of unmodified oligothymidylates and α-oligothymidylates. Although α, ß-dT_{28} oligomers were less resistant than *S*-dT_{28} to SVP and CSP, the former oligonucleotide analogues were considerably more resistant to S1 nuclease.[300] These results indicate that alternating α, ß-oligothymidylates with alternating (3'→3')- and (5'→5')-internucleotidic phosphodiester linkages may represent a new class of antisense molecules achiral at phosphorus. Others[301,302] have also observed that oligonucleotides carrying terminal (3'→3')- and (5'→5')-internucleotidic phosphodiester linkages were stabilized against extracellular[301,302] and intracellular degradation.[302]

Debart *et al.*[303] reported the solid-phase synthesis of the α-anomeric uridylates α-rU_6, α-rU_{12}, and α-r(UCUUAACCCACA) from the α-ribonucleoside phosphoramidite **191a-c**. The duration of each condensation step was 15 min and resulted in an average coupling efficiency of 97%. Purified α-rU_6 was resistant to CSP, ribonuclease A, and S1 nuclease for at least 1 h, 35 min, and 5 h, respectively, at 37 °C. Conversely, ß-rU_6 was totally hydrolysed by ribonuclease A, and was digested to the extent of 86% with CSP and S1 nuclease under the same conditions. α-rU_6 was nonetheless 58% hydrolysed by SVP within 2 h at 37 °C. Under these conditions, SVP caused the near complete hydrolysis (94%) of ß-rU_6 within 2 min.[303a]

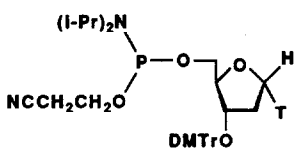
The dodecaribonucleotide α-r(UCUUAACCCACA) did not hybridize with ß-d(TGTGGGTTAAGA) above 0 °C. Nonetheless, this α-oligoribonucleotide led to the formation of a hybrid with ß-d(AGAATTGGGTGT) (Tm = 25.5 °C). Spectrophotometric evidence suggested the formation, at low temperature, of a triplex composed of two α-RNA and one ß-DNA strands.[303b] Interestingly, the α-dodecaribonucleotide being complementary, in parallel orientation, to the splice acceptor site of HIV-1 *tat* mRNA inhibited, with apparent lack of specificity, *de novo* HIV-1 infection of MT4 cells.[303b]

In contrast to α- and ß-D-oligodeoxyribonucleotides, very little is known about the properties of L-oligodeoxyribonucleotides. Morvan *et al.*[304] synthesized the ß-L- and α-L-deoxyribonucleoside phosphoramidites **195** and **200a** which were used in the solid-phase preparation of α- and ß-L-

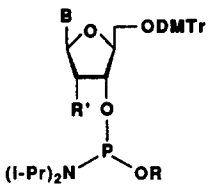
octathymidylates to evaluate the hybridization properties and stability of these oligonucleotide analogues to nuclease hydrolysis. Both α-L- and ß-L-octathymidylates were not digested by S1 nuclease or CSP within 24 h at 37 °C. Under identical conditions, a ß-D-octathymidylate was 50% hydrolysed within 69 min and 11 min, respectively, with these nucleases.[304] The annealing of either the α- or ß-L-octathymidylate with ß-D-octadeoxyriboadenylic acid (ß-D-dA_8) or poly rA failed at low ionic strength (0.1 M sodium chloride). Under these conditions, the natural ß-D-octathymidylate formed a duplex with ß-D-dA_8 (Tm = 5 °C) or poly rA (Tm = 10 °C).[304] It would, therefore, appear that α- or ß-L-oligodeoxyribonucleotides may not be effective antisense molecules.

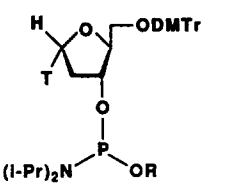
The synthesis of oligodeoxyribonucleotides containing ß-L-2'-deoxyribose has also been reported by Damha *et al.*[305] Strategically, ß-L-2'-deoxyuridine was converted to ß-L-2'-deoxycytidine and both of these nucleosides were transformed into the phosphoramidite derivatives **196** and **197**. ß-L-Hexadeoxycytidylic and ß-L-hexadeoxyuridylic acids were prepared by standard solid-phase chemistry. In agreement with the observations of Morvan *et al.*,[304] ß-L-oligomers were completely resistant to the nucleolytic activity of CSP, P1 and S1 nucleases. ß-L-hexadeoxycytidylic acid and ß-L-hexadeoxyuridylic acid were hydrolysed by SVP[305] at rates comparable to those reported for α-oligodeoxyribonucleotides.[282b] A trideca-ß-D-oligodeoxyribonucleotide, containing three consecutive ß-L-dC residues at the 5'-terminus, hybridized with a complementary and unmodified DNA sequence. The Tm of the hybrid was slightly lower than that of the native DNA duplex (ΔTm = 3 °C).[305] Given these properties, the application of mixed ß-(L)-D-oligodeoxyribonucleotides as potential antiviral agents should be further investigated.

In this context, the preparation of the four ß-L-deoxyribonucleoside phosphoramidites **198a-d** and the solid-phase synthesis of the hexadeoxyribonucleotide ß-L-d(CGCGCG) have independently been described by Urata *et al.*[306] Consistent with the observations reported by others,[304,305] the oligomer was totally resistant to the nucleolytic activity of nuclease P1.[306]



194





195 B= T; R= Me; R'= H
196 = U; = Me; = H
197 = C^{bz}; = Me; = H
198a = T; = CH_2CH_2CN; R'= H
b = C^{bz} = CH_2CH_2CN; = H
c = A^{bz} = CH_2CH_2CN; = H
d = G^{Ib} = CH_2CH_2CN; = H
199 = U = CH_2CH_2CN; = $OSi(Me)_2Bu$-t

200a R= Me
b = CH_2CH_2CN

The synthesis and physicochemical properties of α-L- and ß-L-oligodeoxyribonucleotides covalently linked to an acridine derivative have been investigated by Asseline *et al.*[307a] The ß-L- and α-L-phosphoramidites **198a** and **200b** were prepared and applied to the solid-phase synthesis of octathymidylates having a phosphorothioate function at the 3'-terminus. These were then coupled with 2-methoxy-6-chloro-(ω-bromopentylamino)acridine and purified. The resulting oligonucleotide analogues were resistant to the 3'-exonucleolytic activity of SVP by virtue of the protection provided by the acridine moiety at the 3' terminus.[307a] The oligomers did not show degradation upon incubation with calf thymus 5'-exonuclease or with nuclease P1 for 72 h. By comparison, similarly synthesized α- and ß-D-oligothymidylates were almost completely hydrolysed upon treatment with calf thymus 5'-nuclease within 40 h and 7 min, respectively. Additionally, nuclease P1 digested the α-D-oligomer within 42 h.[307a] Both ß-L- and α-L-3'-acridinyl oligothymidylates did not interact with poly dA or poly

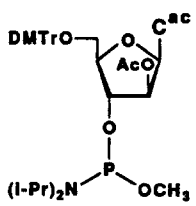
rA under various salt and temperature conditions. Conversely, ß-L- and α-L-tetradeoxyadenylates covalently attached to an acridine derivative, formed double and triple helices with poly rU or poly dT. These hybrids were considerably weaker than those obtained with the corresponding ß-D-tetradeoxyadenylate.[307a]

In contrast to ß-L-dU_{12}, the oligoribouridylic acid ß-L-rU_{12} prepared from the ribonucleoside phosphoramidite **199** strongly hybridized to poly rA (Tm = 38 °C in 1.0 M sodium chloride) relative to ß-D-rU_{12} (Tm = 40 °C).[307b] In addition, ß-L-rU_{12} was resistant to digestion with RNase A and whole cell extracts from L-cells for at least for 48 h and 4 h, respectively. Under similar conditions, ß-D-rU_{12} was completely hydrolyzed by RNase A within 30 sec while it took 2 h for the whole cell extracts to quantitatively hydrolyse the native polyribouridylate. Collectively, these findings indicate that ß-(L)-RNA can form reasonably stable heterochiral duplexes with ß-D-RNA in parallel-stranded A-like conformations and may prove suitable for antisense experiments.[307b]

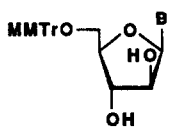
It is known that *ara*C is a potent antileukemic agent as well as an inhibitor of DNA replication.[308] It has been postulated that its cytotoxic effects resulted from its incorporation into DNA as a "fraudulent" nucleotide. The insertion of *ara*C at defined locations in synthetic oligonucleotides would therefore facilitate investigations regarding the molecular implication of *ara*C with DNA chain elongation, replication fidelity, and fragment ligation.

Beardsley *et al.*[309a] prepared the protected *ara*C phosphoramidite **201** which was incorporated at a specific location into a self-complementary oligonucleotide (d[CGCGAATT*ara*CGCG]) by usual solid-phase synthesis. The duplex resulting from the annealing of the modified oligonucleotide was slightly less stable than that of the duplex formed with the unmodified oligonucleotide (ΔTm = 4 °C) thereby indicating that the *ara*C-G base pairs did not significantly disturb the DNA duplex.[309a] The most striking effect was observed when *ara*C was located at the 3'-terminus of a DNA primer. All the polymerases tested (*E. coli* Pol I, T4 polymerase, AMV reverse transcriptase and HeLa cells Pol α_2) utilized *ara*C-terminated primer/template substrates very poorly.[309b] When compared to unmodified primers, the rate of incorporation of the next nucleotide was reduced by 100 fold, even though polymerases with associated (3'→5')-exonuclease activity preferentially excised *ara*CMP from the primer terminus prior to chain elongation.[309a,b] It is, therefore, difficult to argue that the inhibition of DNA synthesis was due to the slight instability observed with *ara*C-G base pairs. The mechanism whereby the incorporation of *ara*C into DNA inhibits DNA replication appears complex and remains to be elucidated.

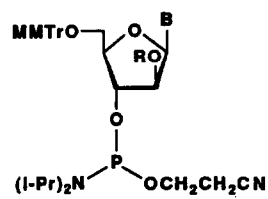
The synthesis of the arabinonucleoside phosphoramidites **203a-b** from the reaction of suitably protected arabinonucleosides (**202a-b**) with chloro-(2-cyanoethoxy)-*N,N*-diisopropylaminophosphine was reported by Damha *et al.*[310] It was pointed out that the phosphitylation reaction did not occur with complete regioselectivity, as 2'-*O*-phosphoramidites (*ca.* 3%) and 2',3'-bisphosphoramidites (5-10%) were also produced. The acetylation of **203a-b** led to the quantitative formation of **204a-b** which upon activation with 1*H*-tetrazole enabled the solid-phase synthesis of 5'-*ara*(UAUAUA).[310]



201



202a B= U
b = A^{bz}



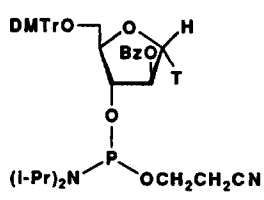
203a-b R= H
204a-b = Ac

Oligonucleotides containing 1'-α-D-arabinofuranosylthymines were synthesized by Adams *et al.*[311] The synthetic strategy consisted of the regioselective benzoylation of the 2'-OH function of 5'-*O*-DMTr-1'-α-D-arabinofuranosylthymine and the conversion of the resulting benzoate to the phosphoramidite

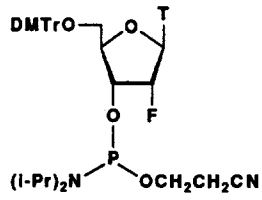
derivative **205**. The solid-phase synthesis of a pentadecathymidylate (α-*ara*T_{15}) from **205** was achieved according to a RNA synthesis protocol which led to coupling yields averaging 97%. The purified oligothymidylate was hybridized with d($C_3A_{15}C_3$) at near physiological conditions. The T*m* of the resulting hybrid was considerably lower than that observed with the native dT_{15} sequence (ΔTm = 25 °C). However, α-*ara*T_{15} and dT_{15} generated hybrids of similar stability with polyadenylic acid.[311] Thus, oligonucleotides containing 1'-α-D-arabinofuranosylthymines could be useful in antisense research.

Synthetic oligonucleotides having modified carbohydrates are of interest with respect to unusual conformational properties and stability to cellular nucleases. Particularly, the 2'-fluorothymidine phosphoramidite **206** has been applied to the solid-phase synthesis of complementary oligonucleotides (18-mers) containing the *Eco*RV recognition sequence GATATC.[312] The thermal denaturation of a modified duplex showed a significant decrease in stability relative to the unmodified duplex (ΔTm = 4.2 °C) when both thymidines of one strand of the recognition sequence were replaced with 2'-fluorothymidines. Such a duplex was cleaved by *Eco*RV at one-third the rate of the native hybrid, whereas a duplex having a 2'-fluorothymidine at the scissile linkage in each strand was digested at two-third the rate of an unmodified duplex. These data indicate that the increased resistance of these hybrids to cleavage by *Eco*RV emerged from the altered conformations of the duplexes resulting from the incorporation of 2'-fluorothymidines rather than the electronegative effect induced by the 2'-fluoro substituents.[312]

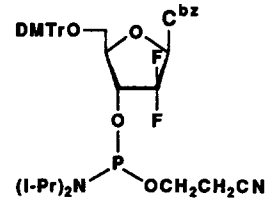
The incorporation of the 2',2'-difluoro-2'-deoxycytidine phosphoramidite **207** into oligodeoxyribonucleotides (19-mers) carrying recognition sequences for the restriction endonucleases *Kpn*I, *Bam*HI, *Hpa*II, and *Msp*I has also been reported.[313] It was shown that the insertion of 2',2'-difluoro-2'-deoxycytidine, at the scission sites, reduced the rates of cleavage with *Kpn*I and *Hpa*II by *ca.* 10% and 4%, respectively, relative to the rates obtained with unmodified duplexes. Furthermore, duplexes resulting from the single insertion of **207** exhibited lower thermostability than that of the unmodified duplexes (ΔTm = 2-4 °C). An even more pronounced thermal instability was observed, under similar conditions, with a duplex having one fluorinated deoxycytidine in each strand (ΔTm = 8 °C).[313] Altogether, the incorporation of 2',2'-difluoro-2'-deoxycytidine into DNA significantly affected duplex stability and, in few cases, slightly decreased the rate of endonucleolytic digestion.



205



206

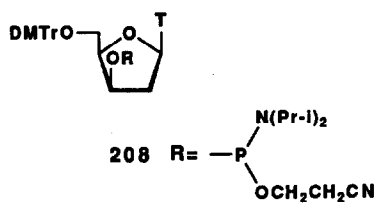


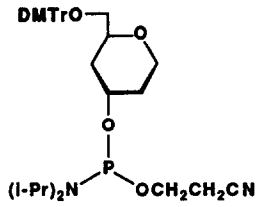
207

Rosemeyer and Seela[314] delineated the synthesis of oligonucleotides containing 1-(2'-deoxy-ß-D-xylofuranosyl)thymines. The deoxyxylonucleoside phosphoramidite **208** has been prepared and applied to the solid-phase synthesis of an oligothymidylate (13-mer) having twelve consecutive 1-(2'-deoxy-ß-D-xylofuranosyl)thymines. The modified oligothymidylate was resistant to CSP but was completely digested by SVP within 45 min at 35 °C.[314] The oligothymidylate analogue formed a stable complex with dA_{12} (T*m* = 36 °C) relative to that of the natural DNA duplex (T*m* = 43 °C). CD spectroscopy revealed that the modified oligothymidylate existed as a left-handed helical single-strand similar to the strands of Z-DNA.[314] In addition, the general pattern displayed by the CD spectrum of a complex composed of the oligodeoxyxylothymidylate and dA_{12} corresponded to that of Z-like DNA. These data implied that the hybridization of the single-stranded and right-handed helix dA_{12} with a left-handed and single-stranded oligodeoxyxylothymidylate resulted in a left-handed double-helix.[314]

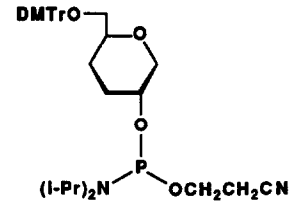
Oligonucleotides containing either 1-(2,4-dideoxy-ß-D-*erythro*-hexopyranosyl)thymines or 1-(3,4-dideoxy-ß-D-*erythro*-hexopyranosyl)thymines were synthesized from the respective phosphoramidites

209 and **210** on a solid support.[315] The double incorporation of **209** at each terminus of an oligothymidylate (13-mer) resulted in the formation of the most stable duplex with dA_{13} relative to the parent unmodified duplex. Furthermore, the modified oligothymidylate was slightly more resistant than the natural oligothymidylate to digestion with SVP. Conversely, modified oligothymidylates resulting from the double incorporation of the phosphoramidite **210** at each end of the oligomer provided strong resistance to degradation with SVP.[315a] Oligonucleotides modified by the incorporation of **209** are being further evaluated as antisense molecules.





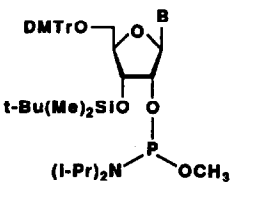
209



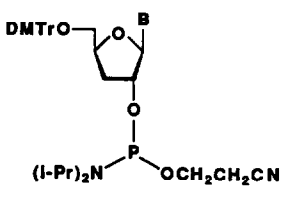
210

3.9. (2'→5')-Oligonucleotides.

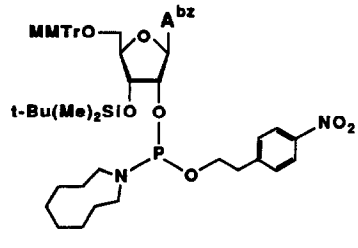
The ability of (2'→5')-oligoribonucleotides to form ordered structures is mostly unknown. To gain information about the association of these oligoribonucleotides, Kierzek *et al.*[316] described the preparation of the ribonucleoside phosphoramidites **211a-d** and the solid-phase synthesis of the self-complementary oligoribonucleotide (2'→5')-CGGCGCCG. The deprotected and purified oligomer produced, upon annealing, a complex having a *Tm* of 46 °C at high ionic strength (1.0 M sodium chloride). This *Tm* was considerably lower than that extrapolated for the same (3'→5')-oligonucleotidic sequence under similar conditions (*Tm* = 79 °C). NMR analysis of the complex indicated the formation of an antiparrallel duplex.[316] In this context, (2'→5')-oligodeoxyribonucleotides have also been prepared from deoxyribonucleoside phosphoramidite precursors (**212a-d**).[317,318] Consistent with the findings of Kierzek *et al.*[316] some association between (2'→5')-dA_{12} and (2'→5')-dU_{12} occurred at high salt concentration (1 M sodium chloride) (*Tm* = 22.8 °C). This association was, however, weaker than that observed with unmodified DNA strands under the same conditions (*Tm* = 40.8 °C).[318] It has nonetheless been reported that the self-complementary (2'→5')-$d(AU)_6$ formed a hairpin structure having a higher *Tm* value (39.3 °C) than that observed with the native $d(AU)_6$ oligomer (*Tm* = 29.9 °C).[318] It is difficult to predict the usefulness of (2'→5')-oligonucleotides as antisense molecules since no thermostability data pertaining to (2'→5')-DNA or RNA/(3'→5')-RNA hybrids under physiological conditions have, as yet, been published.



211a B= U
b = C^{ac}
c = A^{phac}
d = G^{phac}



212a B= T
b = U
c = A^{bz}
d = A^{phac}



213

phac= phenoxyacetyl

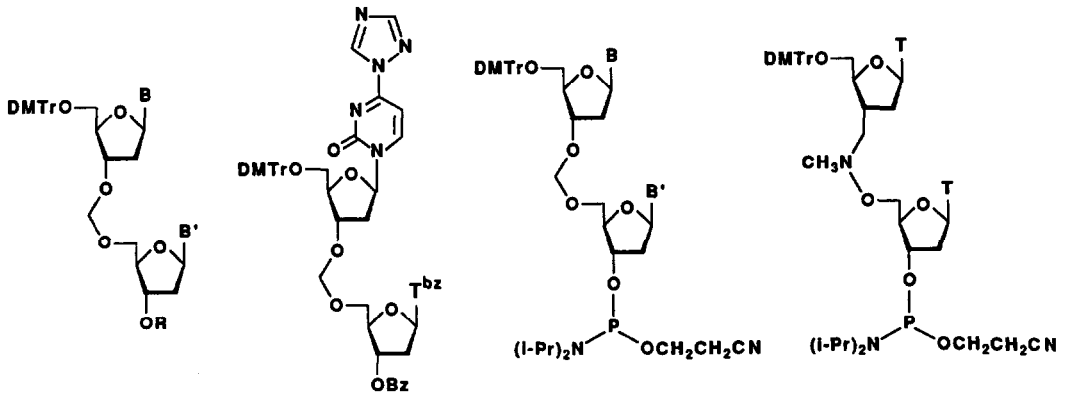
It has been known for some time that the (2'→5')-oligoadenylate pppA(2'→5')pA(2'→5')pA [2-5A] played an important role in the antiviral mechanism of interferon, and the regulation of cell growth and

differentiation.[319] In recent years, the synthesis of 2-5A analogues of has been undertaken to mechanistically probe its mode of action. The ribonucleoside phosphoramidite **213** has been particularly useful in the synthesis of oligoadenylates with (2'→5')-phosphorothioate linkages.[320a,b,g] In addition to providing resistance to nucleases, the inherent chirality of the phosphorothioate functions led, in the case of the trimeric 2-5A, to four diastereoisomers, which after separation, have demonstrated different abilities in binding to and activating RNase L from L929 extracts.[320c,d] In contrast, the diastereoisomers of the fully phosphorothioated (3'→5')-adenylate trimer did not bind to and did not activate RNase L, an enzyme involved in the interferon-induced antiviral and antiproliferative cascade.[320e] The phosphorothioate analogues of 2-5A also inhibited HIV-1 reverse transcriptase in viral lysates, and HIV-1 replication in MT-2 cells.[320f] These inhibitory effects represent an important function of (2'→5')-oligoadenylate analogues in the control of retrovirus replication.

3.10. Oligonucleotides Having Defined Unphosphorylated Internucleosidic Bridges.

In an effort to design oligonucleotide analogues as potential inhibitors of gene expresssion, the preparation of DNA segments having some of the native phosphodiester linkages replaced with enzymatically stable, achiral and uncharged methylene acetal bridges, has been reported.[321] Specifically, 5'-*O*-DMTr-N^3-benzoyl-3'-*O*-(4-pentenyloxymethyl)thymidine reacted with N^3-benzoyl-3'-*O*-methoxyacethyl thymidine and *N*-iodosuccinimide to give the dimer **214** in 80% yield.[321a,b] Following the selective removal of the methoxyacetyl group from **214** with catalytic amount of potassium *tert*-butoxide in dichloromethane-methanol (1:1), the 3'-OH function of the resulting dimer was phosphitylated with 2-cyanoethoxy-(*N,N*-diisopropylamino)chlorophosphine and *N,N*-diisopropyl-ethylamine (DIPEA) affording the dinucleoside phosphoramidite **218**. One or more T-CH_2T dimer(s) can be incorporated into an oligonucleotidic chain by the standard solid-phase phosphoramidite method. The coupling efficiency of the dinucleoside phosphoramidite **218** was *ca.* 95%.[321a,b]

Given the stability of N^3-unprotected thymidine to the iodonium-promoted coupling conditions, the synthesis of the dinucleoside (3'→5')-methylene acetal **215** was accomplished in excellent yields.[322] Treatment of **215** with excess phosphoryl tris-triazolide yielded the 4-triazolo derivative **217** which upon treatment with concentrated ammonium hydroxide and subsequent chemoselective benzoylation, afforded the dinucleoside (3'→5')-methylene acetal **216**. The phosphitylation of **216** with 2-cyanoethoxy-(*N,N*-diisopropylamino)chlorophosphine and DIPEA produced the dinucleoside phosphoramidite **219** in good yields.[322] This approach enabled the synthesis of DNA segments having (3'→5')-methylene acetal linked to cytosine nucleosides. Attempts to condense N^4-protected-3'-*O*-methylthiomethylene



214 B= B'= T^{bz}; R= methoxyacetyl
215 B= U; B'= T^{bz}; R= benzoyl
216 B= C^{bz}; B'= T; R= H

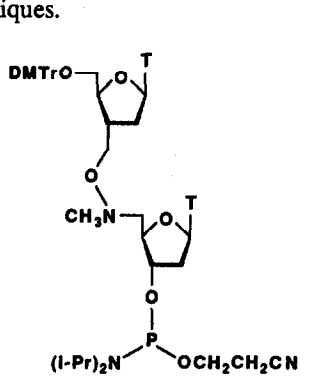
217

218 B= B'= T^{bz}
219 B= C^{bz}; B'= T

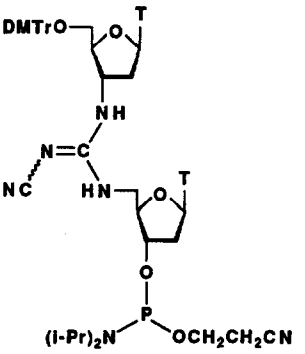
220

cytidine derivatives with an appropriate thymidine acceptor in the presence of *N*-iodosuccinimide and catalytic amounts of triflic acid, failed.[322]

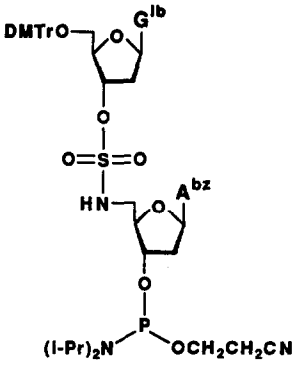
Along these lines, the oligodeoxyribonucleoside phosphoramidite **220** has been prepared and incorporated into oligonucleotides (15-18 mers) to assess the hybridization properties and nuclease resistance of the corresponding oligonucleotide analogues.[323a] The average coupling efficiency of **220** was *ca.* 99%. Hybridization studies revealed that the insertion of one to five modified internucleosidic linkages had little effect on the stability of duplexes composed of the oligonucleotide analogues and complementary RNA oligomers with respect to unmodified DNA/RNA duplexes. In addition, an hexadecaoligonucleotide containing five modified internucleosidic bridges had a full-length half-life of 16 h when incubated with HeLa cellular extracts. Under similar conditions, the unmodified oligomer exhibited a half-life of only 30 min.[323a] Interestingly, the incorporation of the dinucleoside phosphoramidite **221** into an hexadecaoligonucleotide produced an oligomer having a lower affinity for its complementary RNA sequence and increased sensitivity to nucleases relative to an oligonucleotide similarly modified by the insertion of **220**.[323b,c] Thus, oligonucleotides modified by the incorporation of **220** add to the repertoire of promising analogues for the inhibition of gene expression by antisense techniques.



221



222



223

The dimeric phosphoramidite **222** has been synthesized from 3'-amino-3'-deoxythymidine, *S,S*-dimethyl-*N*-cyanodithioiminocarbonate and 5'-amino-5'-deoxythymidine toward the development of uncharged, enzymatically stable, and achiral oligonucleotides capable of hybridizing with target sequences.[324] However, the incorporation of **222** into oligonucleotides and the physicochemical properties of these modified oligonucleotides have not, as yet, been reported.

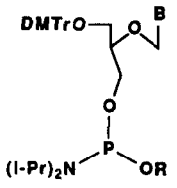
The sulfamate-linked dinucleoside phosphoramidite **223** has been prepared and singly incorporated into complementary oligonucleotides {d(GAGCTC[G*A]ATTCACTGGCCG) and d(CGGCCACT[G*A]ATTCGAGCTC)} containing an *Eco*RI recognition sequence.[325] Upon hybridization, the resulting duplex was slightly less stable than the parent unmodified duplex (ΔTm = 3 °C) indicating that the sulfamate linkage did not significantly affect the thermodynamic stability of the hybrid. It was also found that the sulfamate linkage was totally resistant to cleavage by both SVP and *Eco*RI.[325] Consequently, sulfamate-modified oligonucleotides may be useful for probing nuclease-DNA interactions or in antisense experiments for therapeutic applications.

3.11. Acyclic Oligonucleotide Analogues.

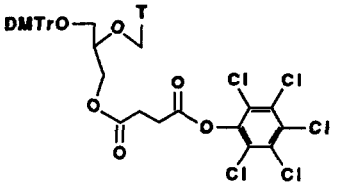
Acyclic analogues of naturally occurring deoxyribonucleosides derived from glycerol have been found active against a range of viral pathogens *in vitro* and *in vivo*.[326a,b] The incorporation of glyceronucleosides[326c] into oligonucleotides may be of interest, as these oligonucleotide analogues may also have antiviral activity. Usman *et al.*[326b] reported the synthesis of the glyceronucleoside

phosphoramidites **224a-b** and the reaction of LCAA-CPG support with **226**. The condensation of **224a-b** with the derivatized support yielded glycerooligonucleotides resisting to the nucleolytic activity of both SVP and CSP.[326b]

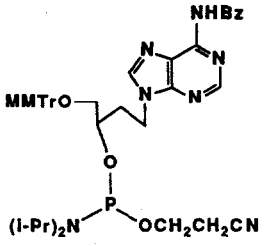
Oligonucleotide analogues containing glyceronucleoside residues at defined locations have been prepared by Schneider and Benner.[327] Typically, the glycerophosphoramidite **225** was inserted in oligonucleotides by solid-phase methods with a coupling efficiency of *ca.* 90%. Like a GT mismatch, each "flexible" nucleoside analogue decreased the T*m* of DNA duplexes by 9-15 °C.[327] It is, therefore, unlikely that oligonucleotides (*ca.* 15 bases) composed of such acyclic nucleosides could form stable duplex structures with complementary natural oligonucleotides in aqueous solution.



224a B= T; R= Me
b = A^{bz}; = Me
225 = T; = CH_2CH_2CN



226



227

Augustyns *et al.*[328] reported the incorporation of (*S*)-9-(3,4-dihydroxybutyl)adenine, as a nucleoside substitute, in the synthesis of dimers. The acyclic phosphoramidite **227** was coupled with 2'-deoxyadenosine to test the applicability of standard DNA synthesis protocols and the sensitivity of the modified dimers to nucleases. A*p(dA), where A* represents the acyclic nucleoside, was 50% hydrolysed by SVP within 129 min but was stable to S1 nuclease and bovine spleen phosphodiesterase (BSP). Alternatively, (dA)pA* was digested to the extent of 50% with S1 nuclease, SVP, and BSP within 8 min, 568 min, and 22 min, respectively. Under the same conditions and in the same order, the native d(ApA) was 50% degraded within 17 min, 26 min, and 4 min, respectively. Incidentally, the fully modified dimer A*pA* was stable to the nucleolytic activity of these enzymes.[328] The acyclic phosphoramidite **227** has also been incorporated at selected positions into oligonucleotides during solid-phase synthesis. An oligodeoxyriboadenylate ($A^*p[dAp]_{11}A^*$) having one acyclic analogue at each terminus was hydrolysed by SVP at a rate seventeen times slower than that of dA_{13}. The modified oligonucleotide formed a duplex with dT_{13} exhibiting a slightly lower T*m* than that of the natural duplex dA_{13}/dT_{13} (ΔTm = 3 °C).[328] Because of demonstrated stability to nucleases and retention of base-pairing properties, the utilization of similar oligonucleotide analogues should be considered when performing antisense studies.

CONCLUDING REMARKS

While nucleosidic phosphoramidite derivatives have tremendously facilitated the automated synthesis of oligonucleotides,[1] modified nucleosidic and non-nucleosidic phosphoramidites have enabled the functionalization of oligonucleotides for biomedical applications.[2] Furthermore, the synthesis of modified oligonucleotides *via* phosphoramidite precursors has played a strategic role in the study of protein-DNA interactions, DNA/RNA recognition, and the control of gene expression. These applications, among others, have been reviewed in this Report. Nucleosidic phosphoramidites have also

been applied to the synthesis of "branched" RNA oligonucleotides to gain insight into the chemical processing (splicing) of pre-mRNA, a prerequisite event in the proper expression of eukaryotic genes. Alternatively, phosphoramidite derivatives have served in the phosphorylation of biomolecules such as, for example, phosphopeptides and *myo*-inositols. It is generally accepted that protein phosphorylation and cell signalling constitute the cornerstone of an ubiquitous transduction mechanism known to regulate a large array of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation. These applications along with the synthesis of "branched" RNA oligonucleotides, and recent advances pertaining to the structure and function of catalytic RNA molecules will be the focus of a future Report.

Acknowledgements: We would like to thank Judith B. Regan and Dr. Kasturi Srinivasachar for proofreading sections of the Report.

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