5′-Me-DNA—A New Oligonucleotide Analog: Synthesis and Biochemical Properties†

Ashis K. Saha,*,‡ Thomas J. Caulfield,‡
Cheryl Hobbs,§ Donald A. Upson,‡
Cheryl Waychunas,‡ and Anne M. Yawman§

Sterling Winthrop Pharmaceuticals Research Division, 1250 South Collegeville Road, Collegeville, Pennsylvania 19426

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Analogs of antisense or antigene oligodeoxyribonucleotides are of interest as potential antiviral, antibacterial, and anticancer agents.[1–4] Much recent research has shown that though the selective modulation of gene expression is certainly feasible, the exact mechanistic role of interfering oligonucleotides remains uncertain.[5–7] Both research and potential therapeutic applications, however, will likely benefit from discoveries of new modified oligonucleotides. Mechanistic doubts notwithstanding, the most critical criteria for success are stability to hydrolytic degradation by 3′-exonucleases and retention of hybridization strength. Additional requirements include proper transport characteristics and sensitivity of RNA/DNA hybrids toward cleavage by RNAse H.

The chemical approaches to this problem have concentrated mainly on modification or replacement of the natural phosphodiester linkage. The most promising to date have been phosphorothioates,[8] in which sulfur replaces one of the nonbridging oxygen atoms of phosphodiester, and methylphosphonates,[9] in which a methyl group replaces the same oxygen. Other modifications such as α-oligonucleotides[10] and 2′-methoxy oligonucleotides[11] have shown limited experimental promise.

Significant recent research has also focused on the development of neutral nonphosphate linkers.[5,12] However, new problems of aqueous solubility and the significant synthetic challenges such linkers pose have hampered full evaluation of this approach. We reevaluated our own such strategy[13] and desired to develop new linkers that may allow combination of the most desirable properties. The 5′-Me-DNA (Figure 1), in which a methyl group is introduced adjacent to the natural phosphodiester linkage of DNA, is a result of such thought. We believed that methyl groups at the 5′-position of deoxyribose would reduce recognition by nucleases[14] and yet

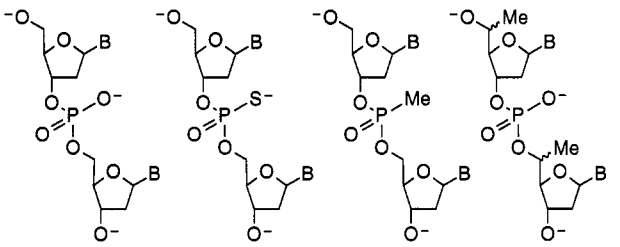

Figure 1.

be small enough to conserve hybrid stability. Cellular transport may be facilitated by lipophilic balance provided by the methyl groups while adequate aqueous solubility is retained. Conjecturing further, we also felt that hybrids of 5′-Me-DNA and RNA may retain susceptibility to RNAse H due to the negative charge in 5′-Me-DNA.[15] In this paper, we describe the synthesis of representative monomers, their incorporation into oligonucleotides using solid phase synthesis, and biochemical properties of such oligomers.

The monomers required for solid phase incorporation were synthesized as described in Scheme 1. We selected, for this preliminary study, one pyrimidine and one purine nucleoside. Thus, one-step Swern oxidation/Grignard reaction on readily prepared 2′-deoxy 3′-((*tert*-butyldimethylsilyl)oxy) nucleosides **1** gave the 5′-*R*/*S* methyl compounds **3** in 65–80% yields.[16] Stepwise Dess–Martin oxidation[17] followed by isolation of intermediate aldehyde **2** and Grignard reaction was also used as an alternative. Spectroscopic analysis revealed that the ratio of epimers at the 5′-position was approximately 1:1. Protection of the 5′-secondary hydroxyl with 4,4′-dimethoxytrityl chloride followed by removal of the 3′-silyloxy group and phosphoramidylation with chloro cyanoethyl *N*,*N* diisopropyl phosphite gave the monomers **6**, ready for utilization in solid phase synthesis in good overall yield.

For demonstration that solid phase incorporation of these monomers into oligonucleotides can be achieved, we prepared the sequences, 5′-TTTTTTTTT *TT -3′ and 5′-AAAAAAAAA*AA-3′ (oligomers 1 and 3, Table 1).[18] The average coupling yield ranged from 89 to 92%, and step yield for incorporation ranged from 78 to 86%, based on trityl assay. To assess the effect of incorporation of the 5′-Me monomers on stability against nuclease digestion, anion exchange HPLC was conducted for oligonucleotide 1 following exposure to 10% FBS (fetal bovine serum), which serves as a source of 3′-exonucleases. Retention times of control standards run in parallel were used to identify fragment lengths. As compared to the control strand (oligomer 2, Table 1), which was completely digested in 3 min, the terminal nucleotide of oligomer 1 was rapidly cleaved, but the resulting 10-mer remained undigested for 120 min. This result suggests that the 5′-Me group interferes with hydrolysis of the phosphodiester bond by 3′-exonucleases as compared to unmodified PDE.

* Address correspondence to this author at Janssen Research Foundation, Welsh and McKean Rds., Spring House, PA 19477.

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‡ Department of Medicinal Chemistry.
§ Department of Molecular Biology and Pharmacology.

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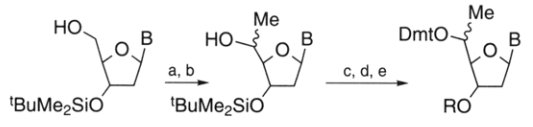
Scheme 1. Synthesis of 5′-Me Nucleosides[a,b]



1 3 4 (R = ^{t}BuMe$_2$Si)
5 (R = H)
6 (R = OP(OCH$_2$CH$_2$CN)(N-^{i}Pr$_2$))

[a] (a: B = T; b: B = A^{Bz}). [b] Key: (a) $(COCl)_2$, DMSO, Et_3N, −78 °C; (b) CH_3MgBr, 1 M in Et_2O, −78 °C; 65−80% from **1**; (c) DmtCl, 4-DMAP, Et_3N, Pyr/CH_2Cl_2; (d) ^{n}Bu$_4$NF (1 M in THF); 55−65% from **3**; (e) ClP(OCH$_2$CH$_2$CN)(^{i}Pr$_2$N), 4-DMAP, ^{i}Pr$_2$NEt, THF; 65−70%.

Table 1. Oligonucleotide Sequences

oligomer no.	sequence (asterisk indicates position of 5′-methyl linkage)
1	5′-TTTTTTTTT*TT-3′
2	5′-TTTTTTTTTTT-3′
3	5′-AAAAAAAAA*AA-3′
4	5′-GGGTGTGTGT*TAGCGGG-3′
5	5′-GGGTGTGTGTT*AGCGGG-3′
6	5′-GGGTGTGTGTTAGCGGG-3′
7	5′-GGGTGTGTG*T*T*AGCGGG-3′
8	5′-CCCGC*T*A*ACACACACCC-3′

We next investigated the synthesis of heterogeneous sequences containing single and multiple incorporation using monomers **6a** and **6b**. The oligomers synthesized are listed in Table 1. The degree of resistance to degradation by nucleases of the heterogeneous sequences containing the 5′-Me modification was assessed by polyacrylamide gel electrophoresis (PAGE). Intact oligonucleotides ran as a single band as expected (Figure 2; lanes 2, 5, and 9). Only trace amounts of the unmodified phosphodiester control oligonucleotide (oligomer 6, Table 1) were observed after 2.5 and 15 h of incubation in media containing FBS (Figure 2; lanes 3 and 4). In contrast, incorporation of 5′-Me-dA (oligomer 5, Table 1) resulted in accumulation of an oligonucleotide composed of 12 nucleotides after 2.5 h exposure to serum nucleases (Figure 2; lane 6). This 12-mer corresponds to the site of the modification and suggests that nucleases did not effectively cleave that linkage and proceed to the next phosphodiester bridge. Even after 15 h, approximately 35% of the original oligonucleotide remained as a 12-mer, as compared to only 1% for the control phosphodiester sequence. A similar result was obtained for incorporation of 5′-Me-T (oligomer 4, Table 1). Exposure to 3′-exonucleases for 2.5 and 15 h resulted in a buildup of an 11 nucleotide sequence correlating with the site of incorporation of the modified nucleotide and representing 46 and 37% of the original oligonucleotide, respectively (Figure 2; lanes 10 and 11). For both oligomers, the unmodified phosphodiester bonds downstream toward the 3′-end from the site of modification also appear to be somewhat protected from cleavage by nucleases.

Hybridization properties were studied by thermodynamic melting (T_m) experiments, and results are summarized in Table 2.[19] Oligomers 4 and 5 containing single incorporation of the 5′-Me phosphodiester linkage hybridized with their complementary DNA with no change in the T_m value. Oligomer 7, containing three tandem incorporation, remarkably, showed a drop of only 0.2 °C in T_m value. When oligomers 7 and 8, both

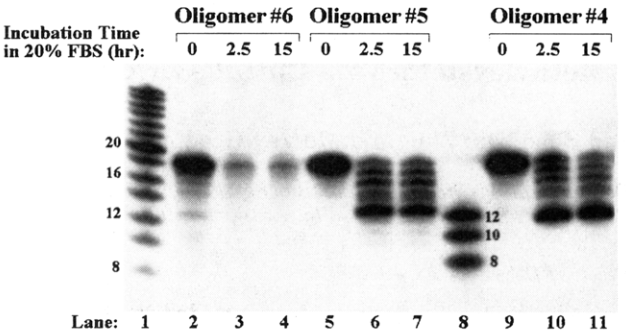


Figure 2. PAGE analysis of nuclease digestion. Lane 1 contains size markers d(GACT)$_n$ and d(GACT)$_n$GT oligomers.

Table 2. Hybridization Data

no.	sequence	T_m (°C)	ΔT_m
1	5′-GGGTGTGTGTTAGCGGG-3′ 3′-CCCACACACAATCGCCC-5′	68	
2	5′-GGGTGTGTGT*TAGCGGG-3′ 3′-CCCACACACAATCGCCC-5′	68 68	0
3	5′-GGGTGTGTGTT*AGCGGG-3′ 3′-CCCACACACAATCGCCC-5′	68	0
4	5′-GGGTGTGTG*T*T*AGCGGG-3′ 3′-CCCACACACAATCGCCC-5′	67.8	−0.2
5	5′-GGGTGTGTG*T*T*AGCGGG-3′ 3′-CCCACACACA*A*T*CGCCC-5′	66.8	−1.2

containing three tandem incorporation each were hybridized together, a drop of only 1.2 °C was observed. These results are in sharp contrast to findings with the widely studied phosphorothioate and methylphosphonate linkages, which generally result in 1−3 °C drop in T_m per incorporation.[20] The melting curve slopes for experiments involving 5′-Me-DNA were comparable to that for unmodified DNA indicating no major impact of the 5′-methine stereocenters in hybridization.

In conclusion, we have discovered a new class of nucleic acid analog with superior properties suitable for use in antisense and related nucleic acid based technology. The synthesis described is rapid and simple, yet applicable to all nucleosides relevant to such research. In this preliminary study, we purposely chose to study the epimeric mixture of 5′-methyl sugars, with the anticipation that configuration at the 5′-methine stereocenter may not severely impact hybridization.[21] Unlike phosphorothioates and methylphosphonates, where the source of chirality is in the phosphorus atom, the new 5′-Me phosphodiester linkage contains a chiral carbon atom. It is our belief that synthesis of pure epimers of 5′-Me nucleotides will be feasible given the current intense activity in asymmetric synthesis of secondary alcohols.[22] Our results on hybridization properties as well as stability toward exonucleases warrant significant further study in this new class of internucleotide linkage. Thermal melting properties of 5′-Me-DNA/RNA hybrids and the susceptibility of such hybrids toward cleavage by RNAse H will be the subject of future work.

Supplementary Material Available: Experimental procedures and physical data (6 pages).

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(19) Thermal melting data were acquired in a Varian Cary 1 spectrophotometer equipped with a Peltier temperature controller. The oligomers were mixed in equimolar amounts at a concentration of 2 μM each in a phosphate buffer containing 0.1 M NaCl for 24 h. The UV absorbance at 260 nm was measured every 0.25 °C while the temperature was ramped from 25 to 80 °C. Raw absorbance data was processed by RS/1 software.

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