# Replacement of the Negative Phosphodiester Linkages of DNA by Positive *S*-Methylthiourea Linkers: A Novel Approach to Putative Antisense Agents

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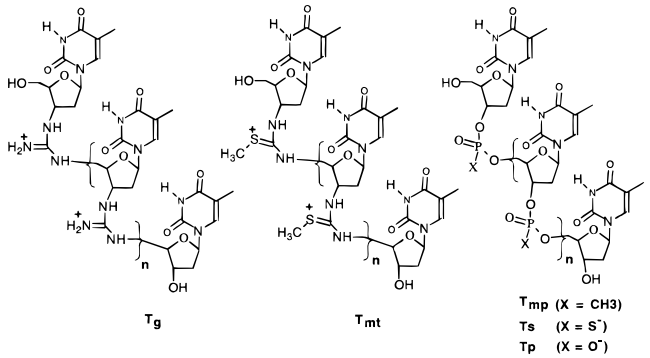
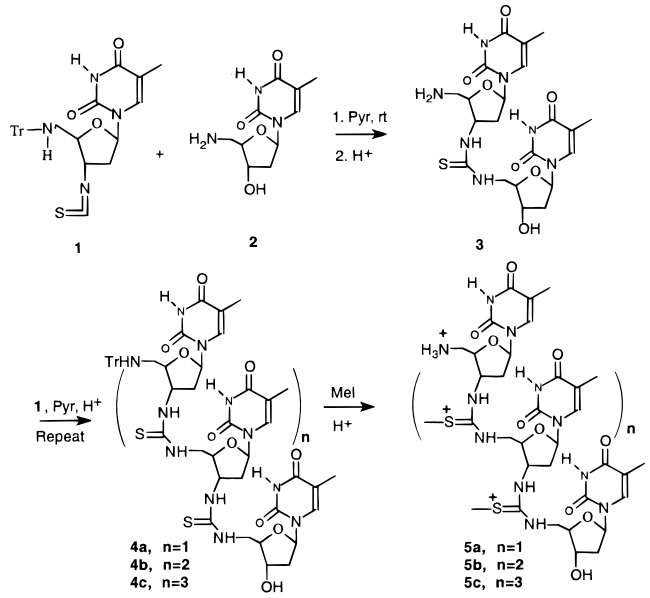
Antisense oligonucleotides with various backbone modifications have attracted considerable attention.[1−4] Among other requirements, successful development of antisense therapeutics presupposes[5−7] the oligos to (a) be stable in vivo, (b) have improved permeability and cellular uptake, and (c) have greater binding affinity with high specificity. Peptide (PNA-neutral),[8] methyl phosphonate (DNAmp-neutral),[9,10] phosphorothioate (DNAs-anionic),[11,12] and guanido (DNG-positive)[13−15] are, among others,[3,4,7,16] a few examples of backbone-modified oligos with different electrostatic attractions. Small positively charged oligos (DNGs) show unprecedented binding[13−15] to nucleic acids with retention of specificity. The nonionic oligo(DNAmp) exhibits the ability to be transported into cells by passive diffusion/fluid-phase endocytosis[17] and is more resistant to degradation than DNA. Both DNAmp and DNAs, however, have individual limited drawbacks of stereoisomeric complexity,[18] solubility (DNAmp), and toxicity (DNAs).[3,19] These findings have led recently to the development of mixed-backbone oligonucleotides (MBOs)[19,20] where the phosphorothioates and methyl phosphonates have been alternated in an oligo backbone to produce improved antisense properties. To investigate the effects on binding and specificity of a positively charged backbone with hydrophobicity, we designed the polycationic nucleotide linkage with methylisothiouronium salts,[21] or methylated thioureas, abbreviated as DNmt (Scheme 1). This linkage, while retaining the positive charge of DNG, also combines some structural backbone features of phosphorothioate and methyl phosphonate oligonucleotides.

**Scheme 1**




**Scheme 2**




We report herein the synthesis of the pentameric thymidyl deoxyribonucleic methylthiourea **5c** (Scheme 1), in which the phosphodiester linkages of DNA {−O($PO_2^-$)O−} are replaced by methylated thioureas {−NHC(=$SMe^+$)NH−}. Preliminary results indicate that this DNmt model compound exhibits complementary base pair recognition toward both RNA and DNA, and the double- and triple-helical structures composed of DNmt with DNA demonstrate remarkable stability.

The synthesis of **5c** (Scheme 2) was accomplished via a cyclic process starting with a condensation reaction between 3′-isothiocyano-5′-*N*-trityl-3′,5′-deoxythymidine (**1**) and 5′-amino-5′-deoxythymidine (**2**), affording the 3′→5′ thiourea-linked dimer **3**.[14,15,21] The synthesis of **1** was carried out by protection of 5′-amino-3′-azido-3′,5′-dideoxythymidine with trityl chloride followed by hydrogenation of the azido group. The reaction of the resulting 3′-amino-5′-*N*-trityl-3′,5′-deoxythymidine with excess thiopyridone[22] at room temperature followed by flash chromatography gave compound **1** in 65% overall yield. Chain extension from the dimer followed a cyclic two-step process involving deprotection of the 5′-amino group with acetic acid. The resulting amine was then condensed, in quantitative yields, with another 1 equiv of **1** in pyridine for 4−8 h depending upon the length of

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(21) The methyl isothiouronium salts could be drawn with the positive charge on nitrogen.
(22) Complete synthetic details will be provided in our full paper.

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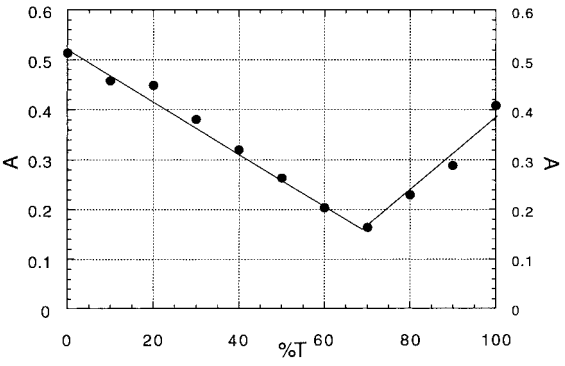


**Figure 1.** Job plot of poly(rA) ($5.3 \times 10^{-5}$ M) and **5c** in 0.15 mM $K_2HPO_4$ and 0.15 M KCl, pH 7.5.

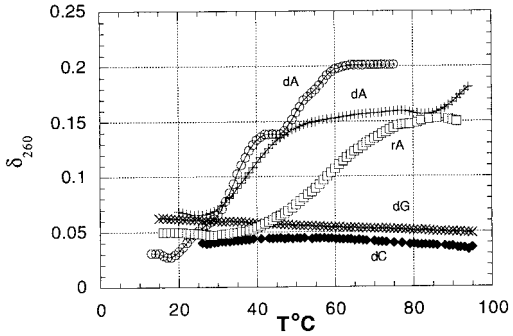


**Figure 2.** Plots of $A_{260}$ vs $T$ (°C) for (**5c**) annealed to poly(dA) (+) at $\mu = 0.05$ (KCl), poly(dA) (○) at $\mu = 0.25$ (KCl), poly(rA) (□), poly(dC) (◆), and poly(dG) (×) at pH 7.5 (0.001 M $K_2HPO_4$ buffer) and an ionic strength of 0.25 (KCl). The concentration of each of the oligonucleotides was $2.17 \times 10^{-5}$ M in bases. The ratio of **5c** to polynucleotides was 2:1. The change in absorbance (260 nm) with increasing temperature was monitored using a Cary 1-E spectrophotometer.

the oligomer. The thiourea-linked thymidyl oligomers **4a**−**c** were successfully converted to compounds **5a**−**c** by methylation of the thiourea linkages to methylisothiouronium salts in excess methyl iodide followed by deprotection with acetic acid and purified on a preparative Alltech SCX cation exchange column employing 1.50 M ammonium acetate buffer, pH 7.0, as the mobile phase. The purity of the sample was further confirmed by running it on an analytical cation exchange column with 1.5 M guanidine HCl as the eluant (see the Supporting Information). Compounds **5a, 5b,** and **5c** have retention times of 9.8, 14, and 18 min, respectively, consistent with the presence of three, four, and five positive charges.

To investigate the interaction of **5c** with polynucleotides, we constructed UV continuous variation plots at different ionic strengths, wavelengths, and temperatures. The plots (Figure 1) for interaction of **5c** with poly(rA) and poly(dA) always show maximum hypochromicity at an approximate 1:2::A:Tmt ratio which indicates triple-helix formation in a TmtApTmt triad, consistent with mixing curves obtained with other positively charged oligonucleotides.[13,22−24]

The slow thermal melting and cooling profiles (0.2 deg/min) of **5c** bound to poly(dA) show pronounced hysteresis at pH 7.0 and at an ionic strength of 0.15 (see the Supporting Information), further evidence of the triple-helical binding of positively charged **5c** to polynucleotides.[23,24] In the thermal denaturation analysis of **5c** bound to poly(dA), plots of absorbance at 260 nm ($A_{260}$) vs temperature exhibit two distinct inflections (Figure 2). On this basis, we assign the plots in Figure 2 to represent denaturation curves of triple- and double-helical structures of **5c** with ssDNA {**5c**$_2$·poly(dA) and **5c**·poly(dA)}. Analysis of Figure 2 also indicates that the DNmt triplex with RNA is more stable than the corresponding DNmt·DNA complex [triplex mp = 61 °C for poly(rA) vs mp = 32 °C for poly(dA)]. Under identical conditions, for solutions which contained **5c** and either poly(dG) or poly(dC), no hyperchromic shift at 260 nm was observed between ca. 5−93 °C (Figure 2).[25] From these preliminary results, DNmt appears to bind with DNA and RNA with specificity in forming hybrid duplex and triplex structures.

**5c** has a significantly greater affinity for poly(dA) and poly(rA) than does thymidyl DNA,[26] and the effect of ionic strength on duplex stability is quite pronounced. As expected,[13,23,24,27] we find that the ionic strength has an opposite effect on the $T_m$ values of DNmt hybrids with DNA (Figure 2) or RNA as compared to DNA complexes with DNA or RNA since electrostatic interactions are attenuated by increasing salt concentration. DNmt compounds are stable under the acidic and basic conditions required for DNA synthesis[14,28−31] and would be expected to be stable in vivo due to the absence of phosphodiester linkages.

In conclusion, we show in these preliminary investigations that the attractive forces between negatively charged DNA or RNA and positively charged DNmt contribute significantly to the stability of heteroduplex and -triplex structures formed between these species. DNmt, with positively charged {−NHC(═SMe$^+$)-NH−} linkages, has a lessened electrostatic attraction to DNA and RNA when compared to the positively charged DNG with guanido {−NHC(═NH$_2^+$)NH−} linkages. DNmt duplex and triplex structures with DNA or RNA, however, are much more stable at physiological ionic strength than the corresponding structures with DNA and RNA as the sole components while less stable than the guanido-linked thymidines that form even stronger duplex and triplex structures. Further studies, involving the use of mismatch sequences and complementary binding studies with mixed sequences would help us define the line between the binding enhancement due to electrostatic attractions of the backbones vs the specificity of the sequence due to hydrogen bonding. The increased hydrophobicity due to the alkyl group should considerably alter the cell diffusion and uptake of these oligos. Combined with the fact that these oligonucleotides can be synthesized with relative ease, have an achiral backbone linkage, would be stable to enzymatic hydrolysis due to the lack of a phosphodiester linkage, have increased binding due to the oppositely charged backbone and a alkyl group, which can be altered to control the hydrophobic/electrostatic interactions, DNmts should serve as a promising lead in the development of new antisense therapeutics.

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**Supporting Information Available:** Hysteresis curves of **5c** and poly(dA) with specific conditions for melting and annealing, HPLC chromatogram, mass spectrum, and UV absorption spectrum of compound **5c** (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(25) Little hyperchromic shift was observed with polyribonucleosides when melted with **5c** under similar conditions.
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