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Incorporation of Positively Charged Deoxynucleic *S*-Methylthiourea Linkages into Oligodeoxyribonucleotides

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Abstract—Oligodeoxyribonucleic acids (15- and 18-mers) containing both negatively charged phosphate and positively charged *S*-methyl thiourea internucleoside linkages (DNmt/DNA chimera) have been synthesized. DNA binding characteristics and nuclease resistance of DNmt/DNA chimera have been evaluated.

The ability to introduce reporter groups into oligonucleotides confers additional functionality and unique properties to the oligonucleotides.[1,2] These functionalized oligonucleotides can be used to study the structure and hybridization properties of nucleic acids. Alternatively, they can be used to alter the solubility and stability of the nucleic acids by choosing an appropriate reporter group. While using covalently attached reporter groups to study the hybridization properties of nucleic acids, it is desirable not to change the natural hybridization properties of the nucleic acid under study. Thus, the presence of the reporter group on the backbone is less likely to disrupt the structure, unless the probe is designed to achieve this. Of the several modified DNA/RNA backbones, three have been employed for the attachment of reporter groups, viz., phosphorothioates,[3] phosphoramidates,[4] and phosphotriesters.[5] The attachment of reporter groups to these backbones is limited by stereoisomeric complexity during synthesis. To overcome this drawback, we incorporated neutral thiourea linkages into otherwise negatively charged DNA. The thio functionality now allows for introduction of any reporter group as a post-synthetic modification devoid of stereoisomers. Preliminary study involving the incorporation of *S*-methylated thiourea into DNA to produce DNmt/DNA chimera (Fig. 1) is reported. The hybridization properties of the DNmt/DNA chimera with complementary DNA were evaluated using spectroscopic techniques. The stability of the DNmt/DNA chimera towards nucleolytic cleavage was also investigated.

To facilitate solid-phase synthesis of oligonucleotide chimera containing both the standard phosphate and *S*-methyl thiourea linkages,[6] phosphoramidite **6** was synthesized (Scheme 1). The synthesis of **6** was accomplished via the dimer **5**, which involves coupling of a 3′-isothiocyanate **3**[7] with a 5′-amino group of 5′-amino-5′-deoxythymidine **4**. Dimer **5**[8] was activated for use in solid-phase synthesis by phosphitylation using [chloro-(diisopropylamino)-β-cyanoethoxyphosphine] to give the desired phosphoramidite **6**.[9] Both **3** and **4** were readily obtained starting from thymidine. Isothiocyanate **3** was synthesized in six steps involving selective hydroxyl protection, formation of 3′-azidothymidine through a 2,3′-anhydrothymidine intermediate, hydrogenation of

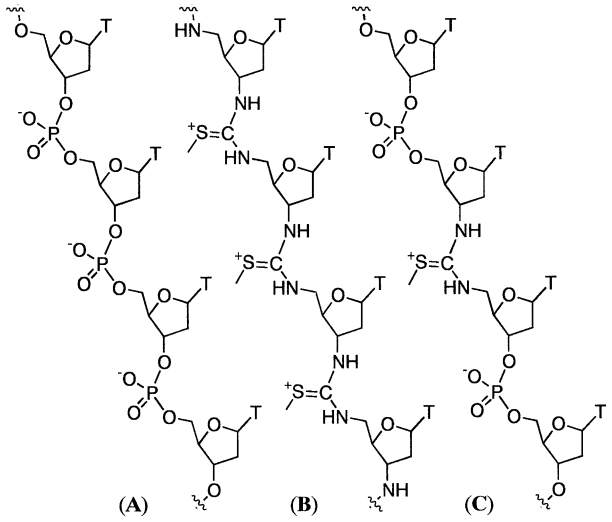

Figure 1. Structures of DNA (A), DNmt (B), and DNmt/DNA chimera (C).

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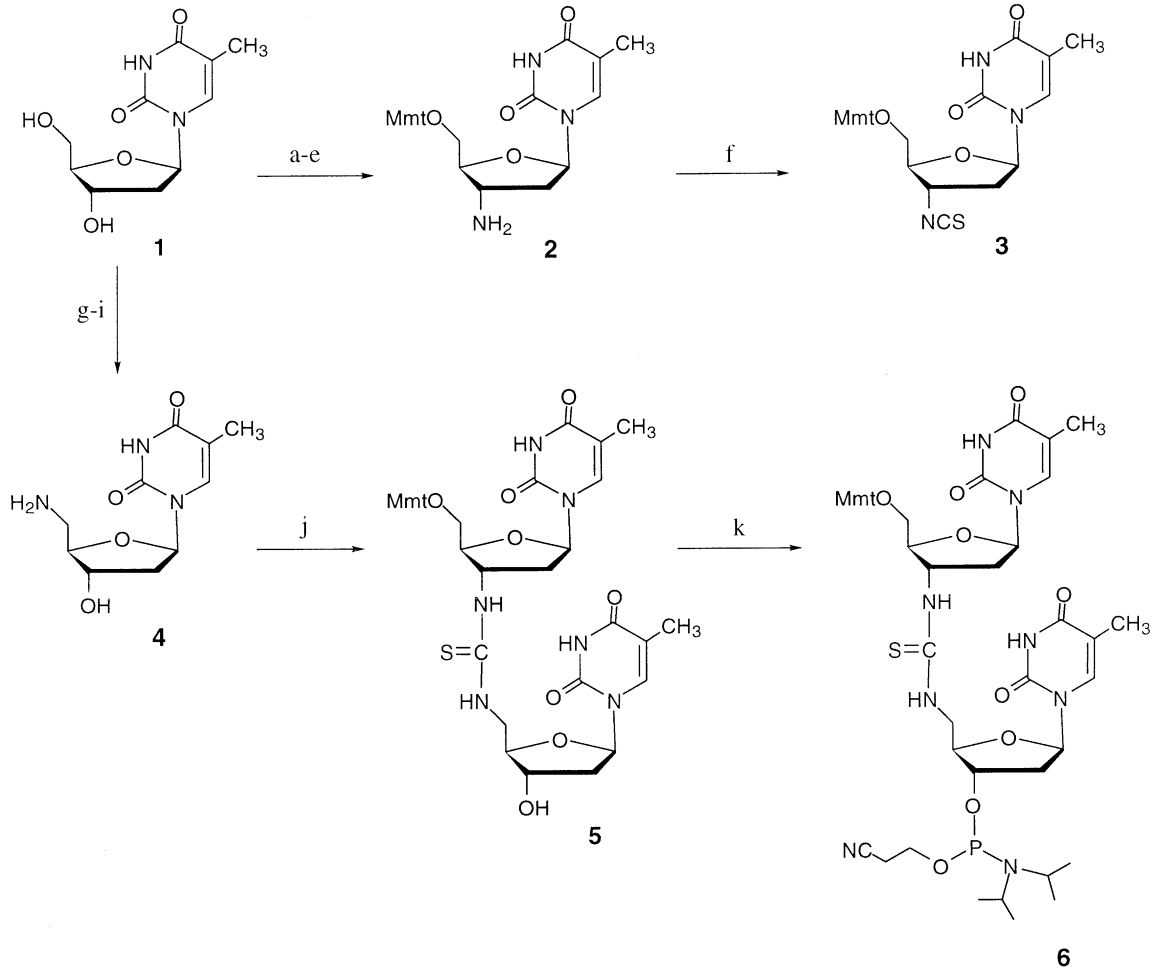
the 3′-azide, and, finally reacting the corresponding 3′-amino-5′-*O*-(diphenylmethoxy)trityldideoxythymidine **2**[10] with thiopyridone at room temperature in pyridine. Monomer **4** was obtained in three steps as previously reported.[11]

Phosphoramidite **6** was used as a building block to introduce thiourea linkages at desired positions in the chimeric oligonucleotides.[12] The chimeras were synthesized with the 5′-trityl group on to allow for HPLC purification. The thiourea linkages in the oligonucleotide were methylated while on solid support (CPG). In order to insure methylation occurred only on the thiourea linkages and not on the nucleotide bases, short pentamer sequences (5′-TT [T/A/C/G] TT-3′) were synthesized and treated under the employed methylation conditions. Both HPLC and mass spectrometry (ESI) indicated that the methylation condition used does not methylate the nucleobases. The final detritylated and HPLC purified oligonucleotides were analyzed by mass spectrometry (ESI) to be the desired chimeric products: **1** [$m/z = 4484.0$, 4506.0, 4522.0 $(M+H/Na/K)^+$; calculated for $\{M+H\}^+$ 4483.8], **2** [$m/z = 4492.0$, 4514.0, 4530.0 $(M+H/Na/K)^+$; calculated for $\{M+H\}^+$ 4491.8], **3** [$m/z = 5465.5$, 5487.5, 5503.5 $(M+H/Na/K)^+$; calculated for $\{M+H\}^+$ 5465.0]. Thus, DNmt/DNA chimeras were successfully synthesized using automated solid-phase synthesis methods.

Two 15-mer single base sequences, **1** and **2**, and an 18-mer mixed base sequence, **3**, were used to evaluate the hybridization properties of DNmt/DNA chimera (Fig. 2).[13] Sequences **1** and **3** contain two *S*-methyl thiourea linkages, one at each end of the sequence; while sequence **2** has a single *S*-methyl thiourea linkage positioned at the center of the strand. All the three sequences were hybridized with their respective complementary strands in a 1:1 mol ratio in phosphate buffer (10 mM Na_2HPO_4, pH 7.1, 0/10/100 mM NaCl). The CD spectra (Fig. 3)[14] of the DNmt/DNA hybrids (**1**•**7** and **2**•**7**) are very similar to the CD spectrum reported[15] for dT_{19}•dA_{19} duplex indicating that both **1** and **2** form duplexes. UV–thermal denaturation studies were performed to observe the effect of the *S*-methyl thiourea linkage on the formation of duplex. All of the temperature versus absorbance curves were sigmoidal, indicating that double helix formation is cooperative (Fig. 4). It is observed that incorporation of one or two *S*-methyl thiourea linkages (DNmt/DNA sequence **1** or **2**) has no effect on the hybridization properties with com-



Scheme 1. Preparation of dimer synthon (**6**) for DNmt/DNA synthesis. (a) Mmt-Cl, DMAP/TEA, anhyd pyridine, rt, 73%; (b) methanesulfonyl chloride, TEA, dichloromethane, 82%; (c) phthalimide, DMF/H_2O, ~100 °C, 86%; (d) LiN_3/DMF, ~100 °C, 70%; (e) Pd/C, EtOH/H_2O, 86%; (f) thiocarbonylpyridone, dichloromethane, rt, 90%; (g) *p*-toluene-sulfonyl chloride, pyridine, 0 °C–rt, 65%; (h) LiN_3/DMF, ~100 °C; (i) Pd/C, EtOH/H_2O, 99%; (j) **3**, DMAP, pyridine, 96%; (k) [chloro-(diisopropylamino)-β-cyanoethoxyphosphine], DIEA, dichloromethane.

plementary DNA sequence **7** at all salt concentrations (Table 1). A slight destabilization of the DNmt/DNA·DNA duplex occurs when compared with the control DNA·DNA duplex, as reported for the DNG/DNA chimera.[16] For the duplex formed with the mixed base DNmt/DNA chimera, **3·5**, no apparent change in *T*m occurs between the control duplex (**4·5**) and **3·5** duplex at all (0, 10, and 100 mM) salt concentrations. The stability of the DNmt/DNA·DNA duplexes (**1·7**, **2·7**, and, **3·5**) increases with the increase in salt concentration (0–100 mM NaCl) as seen for DNA·DNA duplexes (**6·7** and **4·5**). This indicates that the incorporation of one or two *S*-methyl thiourea linkages in place of the phosphodiester linkages of the normal DNA does not affect the overall electrostatics of duplex formation with complementary DNA strand.

1 5'-TTmT TTT TTT TTT TmTT-3'
2 5'-TTT TTT TmTT TTT TTT-3'
3 5'-TmTG TTA GTT TTC TTG TmTT-3'
4 5'-TTG TTA GTT TTC TTG TTT-3'
5 5'-AAA CAA GAA AAC TAA CAA-3'
6 5'-TTT TTT TTT TTT TTT-3'
7 5'-AAA AAA AAA AAA AAA-3'
8 5'-AAA AAA AAA AAA TAA-3'
9 5'-AAT AAA AAA AAA AAA-3'
10 5'-AAA AAA TAA AAA AAA-3'

Figure 2. Sequences used for DNmt/DNA characterization. *S*-Methyl thiourea linkage is indicated by 'm'.

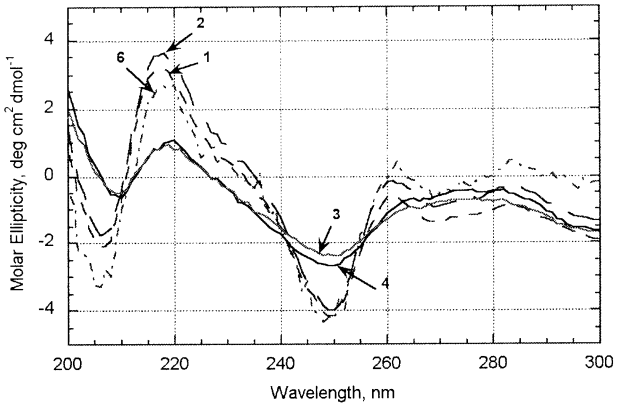


Figure 3. CD spectra of duplexes formed by annealing 1:1 mol ratio of **1**, **2**, **3**, **4**, and **6** with their respective complementary strands (**7** or **5**) in phosphate buffer (10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1), at 15 °C.

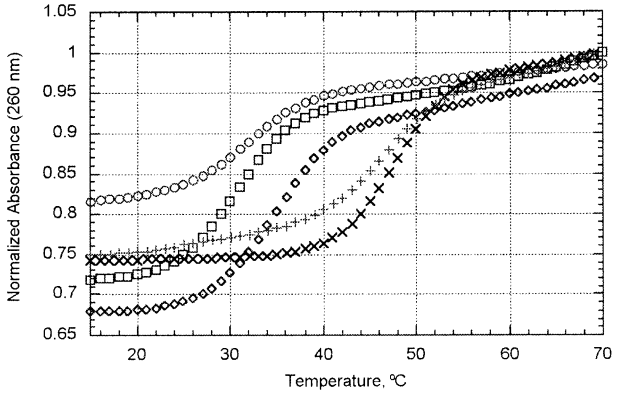


Figure 4. Thermal denaturation curves for **6·7** (◇), **1·7** (○), **2·7** (□), **4·5** (×), and, **3·5** (+) duplexes. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1.

The ΔG values (at 25 °C) calculated using the van't Hoff enthalpy values for transitions involving DNmt/DNAz·DNA and DNA·DNA duplexes, indicate that the free energy for formation of DNmt/DNA·DNA duplex is about the same as for DNA·DNA duplex.[17–19] This may support that little structural difference exists between DNA containing one or two *S*-methyl thiourea linkages and DNA composed entirely of phosphodiester linkages.

In order to investigate the sequence specificity of binding of DNmt/DNA chimera with complementary DNA, duplexes were formed between DNmt/DNA chimera (**1** and **2**) and DNA sequences containing single base mismatches at 3′-end (**8**), 5′-end (**9**), or in the center (**10**). The stability of the duplex was monitored by thermal denaturation studies. In general (Table 2), the central mismatch destabilizes the duplex (DNA·DNA or

Table 1. Melting temperatures and thermodynamic parameters for helix–coil transitions of DNmt/DNA chimeras with complementary DNA

	0 mM NaCl		10 mM NaCl		100 mM NaCl	
Duplex[a]	T_m[b] °C	$-\Delta G_{25}$[c] kJ/mol	T_m[b] °C	$-\Delta G_{25}$[c] kJ/mol	T_m[b] °C	$-\Delta G_{25}$[c] kJ/mol
6·7	19.8	27.3	23.6	30.8	33.4	38.4
1·7	17.6	24.6	20.3	28.3	30.0	35.7
2·7	17.8	25.0	20.2	28.0	29.9	36.1
4·5	31.7	38.6	36.0	43.5	47.0	50.9
3·5	32.1	38.8	36.1	43.0	46.1	49.6

[a]Absorbance was measured at 260 nm in phosphate buffer; the concentration of each strand was 6 μM.
[b]The maximum of the derivative plot (T_{max}) was multiplied by 0.971 to obtain the T_m.[18] The reported T_m values are an average of three experiments (±0.2).
[c]Thermodynamic parameters were calculated by the method of Gralla and Crothers.[17,19]

Table 2. Melting temperatures for hybrids formed between DNmt/DNA chimera and sequences containing single base-pair mismatches

	X·Y Duplex[a] T_m[b] (°C)			
X/Y	**7**	**8**	**9**	**10**
6	33.4	28.7	30.0	24.2
1	30.0	26.5	28.5	19.8
2	29.9	24.4	26.2	22.2

[a]Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1.
[b]The maximum of the derivative plot (T_{max}) was multiplied by 0.971 to obtain the T_m.[18] The reported T_m values are an average of three experiments (±0.2).

DNmt/DNA·DNA) to the maximum extent (9.2 °C for **6·10**, 10.2 °C for **1·10**, and 7.7 °C for **2·10**). There is a ΔT_m of 1.3 °C–2.0 °C between the 3′end and 5′end mismatch for duplexes **6·10**, **1·10**, and **2·10**. In all cases, the 3′-end mismatch is more tolerated than the 5′-end mismatch. Thus, the DNmt/DNA chimeras clearly bind to their complementary DNA with great sequence specificity.

Exonuclease I digests single-stranded DNA catalyzing the hydrolysis of phosphosdiester linkages from the 3′-terminus to 5′-terminus. Thus, it is assumed that upon modifying the phosphodiester linkage at the 3′-terminus, the oligonucleotide could be resistant to exonuclease digestion. To investigate this, DNmt/DNA oligonucleotides **1**–**3** were subjected to nucleolytic cleavage by exonuclease I and the hydrolyzate was analyzed by RP-HPLC.[20] Natural unmodified oligonucleotides of the same sequence, **4** and **5**, were used as controls. Under the conditions employed, the control oligonucleotides were readily hydrolyzed to shorter length products within 1 h of incubation; however, the DNmt/DNA chimera **1** and **3** were unaltered even after 12 h of incubation. The DNmt/DNA chimera **2** (rt 20.8 min), which contains only one methyl thiourea linkage at the center of the oligonucleotide, was partially hydrolyzed after 1 h (rt 19.5 and 20.8 min) of incubation and remained further unaltered even after 12 h (rt 19.5 and 20.8 min). These observations clearly indicate that the DNmt/DNA oligonucleotides with positively charged methyl thiourea linkages at 3′-terminus, **1** and **3**, are totally stable to cleavage by nucleolytic enzyme exonuclease I.

In conclusion, a method for the incorporation of a positively charged internucleoside linkage, *S*-methyl thiourea, into otherwise negatively charged DNA has been demonstrated. The insertion of the methyl thiourea linkage was accomplished using the standard DNA phosphoramidite chemistry and automated solid-phase synthesis techniques. The binding of the DNmt/DNA chimera to its complementary DNA strand occurs as with the unmodified DNA·DNA duplex exhibiting similar stability trends with increasing salt conditions. Also, a high degree of sequence specificity is displayed during the binding of DNmt/DNA chimera to its complementary DNA. Incorporation of positively charged *S*-methyl thiourea linkages at the 3′-terminus of the DNA confers resistance towards digestion by exonuclease I. Thus, DNmt/DNA is a molecule with binding characteristics similar to DNA but with enhanced nuclease resistance. The nucleophilic nature of the thio functionality in the molecule may enable one to introduce fluorescent molecules into DNA by alkylation for developing molecular biology tools or insert groups that may enhance cellular uptake of oligonucleotides.

Acknowledgements

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7. **3**: To a solution of 1.10 g (2.14 mmol) of 3′-amino-5′-*O*-(4-methoxyphenyl)-diphenylmethyl-3′-deoxythymidine in 75 mL of dichloromethane was added 0.51 g (2.20 mmol) of thiocarbonylpyridone. The resulting solution was stirred at rt for 6 h. The reaction was monitored by TLC to completion and the solvent was evaporated to dryness under reduced pressure. The crude residue was subjected to column chromatography using EtOAc/hexanes (1:1) solvent system containing 0.5% Et_3N to give the pure product as a brittle white foam. Yield was 0.92 g (90%). R_f 0.38 (EtOAc/hexanes, 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.2 (s, br, 1H), 7.6 (s, 1H), 7.2–7.4 (m, 12H), 6.9 (d, 2H), 6.3 (t, 1H), 4.6 (q, 1H), 4.2 (m, 1H), 3.8 (s, 3H), 3.4–3.6 (2×d, 2H), 2.5–2.7 (m, 2H), 1.5 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.0, 150.0, 144.0, 136.1, 131.3, 129.0, 128.0, 114.2, 85.0, 84.0, 63.0, 56.1, 40.0, 12.7. *m*/*z* (HRESI) 578.1703, calcd for $C_{31}H_{29}N_3O_5S$ $(M+Na)^+$ 578.1726.
8. **5**: To a solution of 0.30 g (0.55 mmol) of 3′-isothiocyanato-5′-*O*-(4-methoxyphenyl)-diphenylmethyl-3′-deoxythymidine (**3**) in 30 mL of anhydrous pyridine was added 0.31 g (1.29 mmol) of 5′-amino-5′-deoxythymidine (**4**) followed by 15.0 mg (0.12 mmol) of (dimethylamino)pyridine. The resulting solution was stirred for 2 h at rt. The solvent was evaporated at reduced pressure, and 30 mL of water was added to the residue to precipitate the product. The product was extracted into 2×30 mL of $CHCl_3$ and washed with water to remove the excess amine. The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give a white flaky product. Yield was 0.42 g (96%). R_f 0.43 (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (400 MHz $CDCl_3$) δ 7.9 (s, br, 1H), 7.6 (s, 1H), 7.2–7.5 (m, 12H), 7.18 (s, 1H), 6.9 (d, 2H), 6.41 (m, 1H), 6.05 (m, 1H), 5.35 (m, 1H), 4.5 (m, 1H), 4.2 (m, 1H), 3.9 (m, 1H), 3.8 (s, 3H), 3.7 (m, 1H), 2.4–3.4 (m, 4H), 1.9 (s, br, 2H), 1.45 (s, 3H), 1.15 (s, 3H). *m*/*z* (HRESI) 819.2784, calcd for $C_{41}H_{44}N_6O_9S$ $(M+Na)^+$ 819.2788.
9. **6**: *m*/*z* (FAB) 997.0, calcd for $C_{50}H_{61}N_8O_{10}SP$ $(M+H)^+$ 997.1.
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12. Oligonucleotide synthesis and purification: All modified oligonucleotides were synthesized on 1.3 μmol scale on a Pharmacia Gene Assembler Plus DNA synthesizer. Standard DNA synthesis conditions were employed, viz., CPG support and base protected 5′-*O*-(4,4′-dimethoxytrityl)deoxyribonucleoside-3-[*O*-(diisopropylamino)-β-cyanoethylphosphoramidite] monomers. The phosphoramidite activated dimer **6** was used, at an extra monomer position, in order to introduce a thiourea linkage into the oligonucleotide. The standard synthesis cycle was modified to perform an extended coupling (15 min) during the coupling of the modified phosphoramidite dimer **6**; a coupling efficiency of >95% was observed for this step. The resin containing the fully protected oligonucleotide was treated with MeI/EtOH (1:1) for 1 h at rt in a peptide synthesis vessel to

methylate the thiourea linkages. Cleavage and deprotection of the oligonucleotides was achieved by subjecting the resin to a solution of K_2CO_3 in methanol (0.05 M) for 2 h at rt. All oligonucleotides were desalted on polypak catridges prior to purification on RP-HPLC. A semipreparative C-8 RP column (Altech, 7 μ, 10×250 mm) with 0.1 M TEAA, pH 7.0 as solvent A and CH_3CN as solvent B was used for all HPLC purifications with a gradient of 0.2% B and 3 mL/min flow rate. The HPLC-purified trityl-on oligonucleotides were then detritylated using 1 mL of 80% acetic acid (1 h, rt), lyophilized, redissolved in double distilled water, and further purified by RP-HPLC.

13. TtT and TmT refer to thymidyl dinucleotide with a thiourea linkage and methylated thiourea linkage, respectively. All nucleotide solution concentrations were determined using the extinction coefficients (per mole of nucleotide) calculated according to nearest neighbor approximation for the DNA (Gray, D. M.; Hung, S.-H.; Johnson, K. H. In *Methods in Enzymology*; Academic: New York, 1995; Vol. 246, p 19). All standard unmodified DNA oligomers were obtained from Integrated DNA Technologies (Coralville, IA).

14. Spectroscopy: CD spectra were obtained using AVIV circular dichroism spectrophotometer. Scans were run from 300 nm to 200 nm taking a measurement every 1 nm. Melting curves were determined using a Cary 100 UV–vis spectrophotometer equipped with a temperature programmable cell block. Absorbance measurements were made at 260 nm using 1 cm path length quartz cuvettes at a heating rate of 0.2 °C/min over the range of 8–70 °C.

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20. Exonuclease I digestion studies: Oligonucleotides **1**–**4** and **6**, with phosphodiester or *S*-methyl thiourea linkages, were treated with exonuclease I (USB). A typical reaction mixture contained the following in 200 μL: 67 mM Tris·HCl (pH 8.5), 6.7 mM $MgCl_2$, 20 mM 2-mercaptoethanol, ~0.2 OD of oligonucleotide, and ~20 units of exonuclease I. The reactions were incubated at 37 °C. Aliquots (40 μL) were taken at different time intervals (0, 1, 3, 6, and 12 h), quenched by rapid freezing in dry ice–2-propanol bath, and stored frozen until HPLC analysis. Reaction mixtures were analyzed on C-8 RP-HPLC column (Altech, 7 μ, 4.6×250 mm) using a gradient of 0.5%/min of CH_3CN in 0.1 M TEAA, pH 7.0, for 50 min at a flow rate of 1 mL/min. Reactions without enzyme were run for each oligonucleotide and analyzed by HPLC as controls.