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Solid-phase synthesis and hybridization properties of DNA containing sulfide-linked dinucleosides

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ABSTRACT

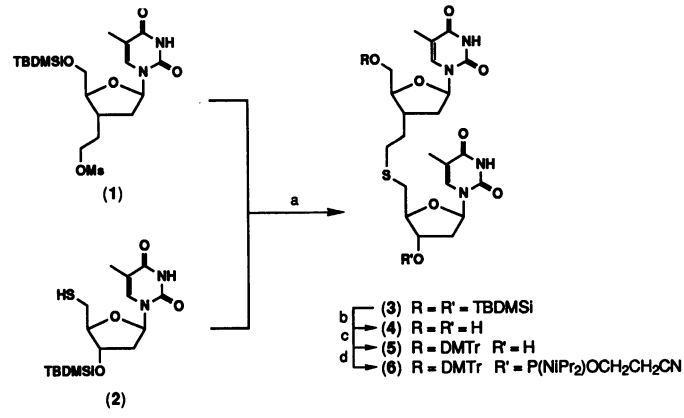
Oligodeoxyribonucleotides incorporating non-hydrolyzable dialkyl sulfide linked thymidine dimers (TsT) were synthesized chemically by the solid-phase approach. The sulfide dimer TsT was stable to degradation by snake-venom phosphodiesterase, calf spleen phosphodiesterase, Nuclease P1 and Nuclease S1. Thermal denaturation analysis indicated that the incorporation of TsT dimers into DNA weakened, but did not prevent, binding to complementary DNA and RNA over a wide range of salt concentrations (10 mM to 2 M NaCl).

INTRODUCTION

The use of anti-sense oligonucleotides represents a powerful new strategy in the development of biological tools and therapeutic agents (1,2). In response to the shortcomings of natural ssDNA and RNA, primarily poor cellular permeability and instability towards cellular nucleases, there has arisen a great interest in modified nucleic acid analogues (1–4). Alterations to the natural structure can be divided into those involving changes in the structure of the sugar moiety (6–12), modification of the internucleoside phosphate (13–20), and complete replacement of the phosphodiester group with with a non-phosphorus-containing linkage (21–27).

The desire for high chemical stability led us to choose a thioether-linked system in which the 3′- and 4′-carbons of adjacent sugars are joined by an alkane chain containing a single sulfur atom (Scheme 1). The corresponding sulfone and sulfoxides could also be easily obtained. The straightforward formation of sulfides from a thiol and an appropriately activated alcohol was another factor making this group an attractive choice. The decision to place the sulfur at the position shown in Scheme 1 was based on both synthetic and structural considerations. To familiarize ourselves with the latter, a scale model of a double helix was constructed (28). Careful study of the molecular models did not reveal any obvious unfavourable steric interactions upon replacement of the phosphate with the thioethylene group.

In this report we describe an efficient synthesis of a dimer of thymidine bearing a dialkyl sulfide linkage, its incorporation into DNA by automated techniques, and the binding properties of the resultant hybrid oligodeoxynucleotides.



(a) Cs_2CO_3/DMF; **(b)** nBu$_4$NF/THF; **(c)** DMTrCl/Et_3N/pyridine; **(d)** iPr$_2$NP(Cl)OCH_2CH_2CN/Et_3N/CH_2Cl_2.

EXPERIMENTAL

General methods

^{1}H-NMR spectra were recorded on either Varian XL200 or Varian XL300 spectrometers and the assignments are based on homonuclear decoupling and/or COSY experiments. When deuterochloroform was employed as solvent, internal tetramethylsilane (TMS) was used as the reference. The residual proton signal of methanol, assigned a value of 3.30 ppm, was used as reference in this case. The multiplicities are recorded using the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; m^n, symmetical signal of n lines; br, broad. ^{13}C-NMR spectra were all obtained at 75.4 MHz using a Varian XL300 spectrometer. The $^{13}CDCl_3$ and $^{13}CD_3OD$ signals, assigned values of δ 77.00 and 49.00 respectively, were used as references in these solvents. Peak assigments were, in some cases, made with the aid of APT or HETCOR experiments. Superscripted 3 or 5 appearing before signal assignments refer to the relevant nucleoside unit of the dimers. ^{31}P-NMR spectra were also obtained on a Varian XL300 instrument at 121.44 MHz and chemical shifts are given with respect to 85% phosphoric acid. Fast-atom bombardment (FAB) mass spectra were obtained

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on a VG ZAB-HS sector mass spectrometer in the direct-inlet mode. All compounds were shown to be homogeneous by t.l.c. and high-field NMR.

Tetrahydrofuran was distilled from sodium benzophenone ketyl. Dichloromethane was distilled from P_2O_5. Pyridine and triethylamine were distilled from calcium hydride. *N,N*-dimethylformamide was dried by shaking with KOH followed by distillation at reduced pressure from BaO. Thin-layer chromatography (t.l.c.) was performed using Kieselgel 60 F_{254} aluminium-backed plates (0.2 mm thickness) and visualized by UV and/or dipping in a solution of ammonium molybdate (2.5 g) and ceric sulfate (1 g) in 10% v/v aqueous sulphuric acid (100 mL), followed by heating. Kieselgel 60 (Merck 230–400 mesh) silica gel was employed for column chromatography.

Synthesis of sulfide linked dimer (6)

Dimer (3)

Mesylate **1** and thiol **2** were prepared according to Kawai *et al.* (30). Cesium carbonate (547 mg, 1.68 mmol), previously flame dried *in vacuo*, was suspended in dry *N,N*-dimethylformamide (7 mL) and a solution of mesylate **1** (517 mg, 1.12 mmol) and thiol **2** (458 mg, 1.23 mmol) in dry *N,N*-dimethylformamide (12 mL) was then added resulting in a yellow solution. After 3 h of stirring at ambient temperature under a nitrogen atmosphere, the solvent was removed *in vacuo* and the product was extracted with dichloromethane (200 + 100 mL) and washed with aqueous sodium bicarbonate (5% w/v, 200 mL) and water (200 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated *in vacuo* yielding a yellow foam. Chromatography over silica gel (4:1 ethyl acetate/hexanes, v/v) afforded the sulfide **3** as a colorless solid (725 mg, 88% yield): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.092; 0.096; 0.115 and 0.120 (four s, 12H, $SiMe_2$), 0.90 and 0.93 (two s, 18H, *t*-butyl), 1.53–1.67 (m, 1H, ^{5}H1″$_A$), 1.75–1.88 (m, 1H, ^{5}H1″$_B$), 1.92 and 1.93 (two fine d, 6H, J = 1.1 Hz, 5-Me's), 2.05–2.33 (two overlapping AB portions of ABXY, 4H, ^{5}H2′$_{AB}$ and ^{3}H2′$_{AB}$), 2.31–2,45 (m, 1H, ^{5}H3′), 2.53–2.70 (m, 2H, ^{5}H2″$_{AB}$), 2.77 (A of ABX, 1H, ^{3}H5′$_A$), 2.83 (B of ABX, 1H, ^{3}H5′$_B$), 3.70–3.76 (m, 2H, ^{5}H4′ and ^{5}H5′$_A$), 3.95–4.02 (m, 2H, ^{3}H4′ and ^{5}H5′$_B$), 4.33 (dt, 1H, ^{3}H3′), 6.08 (dd, 1H, ^{5}H1′), 6.21 (t, 1H, ^{3}H1′), 7.30 and 7.56 (two fine q, 2H, J = 1.1 Hz, H6's), 8.96 and 9.01 (two br s, 2H, NH), $J_{(3)H1'-(3)H2'A} = J_{(3)H1'-(3)H2'B}$ = 6.6 Hz, $J_{(5)H1'-(5)H2'A}$ = 6.7, $J_{(5)H1'-(5)H2'B}$ = 4.3, $J_{(3)H2'A-(3)H3'}$ = 6.7, $J_{(3)H2'B-(3)H3'} = J_{(3)H3'-(3)H4'}$ = 4.5, $J_{(3)H4'-(3)H5'A}$ = 5.1, $J_{(3)H4'-(3)H5'B}$ = 5.3, $^2J_{(3)H5'A-(3)H5'B}$ = −13.8; ^{13}C-NMR (75.4 MHz, $CDCl_3$) δ 12.58 (2C, 5-Me's), 17.80 and 18.40 ($SiCMe_3$), 25.61; 25.73 and 25.88 ($SiMe_2$ and $SiCMe_3$), 31.40; 32.15; 34.23 (3×CH_2), 36.60 (^{5}C3′), 38.76 and 40.19 (2×CH_2), 62.90 (^{5}C5′), 73.25 (^{5}C3′), 84.84 (2C); 85.34 and 85.90 (2×H1′ and 2×H4′), 110.22 and 111.08 (2×C5), 135.43 and 135.54 (2×C6), 150.35 and 150.57 (2×C2), 163.96 and 164.18 (2×C4); MS (FAB–glycerol/HFBA) m/e 739 ([MH$^+$], 3.7%), 613 ([MH$^+$–ThyH], 22), 355 (11), 157 (100), 127 ([ThyH + H$^+$], 85). Anal. calcd. for $C_{34}H_{58}N_4O_8S_1Si_2$: C, 55.26; H, 7.92; N, 7.59; S, 4.33. Found: C, 55.16; H, 8.00; N, 7.48; S, 4.44.

Diol (4)

A solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran (1 M, 947 μL, 0.947 mmol) was added to a stirred solution of sulfide **3** (280 mg, 0.379 mmol) in dry tetrahydrofuran (10 mL). After 2 h the solution was evaporated *in vacuo* and the resulting glass was chromatographed over silica gel (10:1 dichloromethane/methanol, v/v) to give the diol **4** as a colorless glass in quantitative yield: ^{1}H-NMR (200 MHz, CD_3OD) δ 1.35–1.47 (m, 1H, ^{5}H1″$_A$), 1.53–1.71 (m, 1H, ^{5}H1″$_B$), 1.86 and 1.88 (two fine d, 6H, J = 1.0 Hz, 5-Me's), 2.05–2.30 (two overlapping AB portions of ABXY, 4H, ^{5}H2′$_{AB}$ and ^{3}H2′$_{AB}$), 2.33–2.46 (m, 1H, ^{5}H3′), 2.55–2.77 (m, 2H, ^{5}H2″$_{AB}$), 2.78–2.94 (m, 2H, ^{3}H5′), 3.67–3.77 (m, 2H, ^{5}H4′ and ^{5}H5′$_A$), 3.85–4.05 (m, 2H, ^{3}H4′ and ^{5}H5′$_B$), 4.30–4.42 (m, 1H, ^{3}H3′), 6.04 (dd, 1H, ^{5}H1′), 6.22 (t, 1H, ^{3}H1′), 7.56 and 7.97 (two s, 2H, H6's), $J_{(3)H1'-(3)H2'A} = J_{(3)H1'-(3)H2'B}$ = 6.7 Hz, $J_{(5)H1'-(5)H2'A}$ = 6.9, $J_{(5)H1'-(5)H2'B}$ = 2.9; ^{13}C-NMR (75.4 MHz, CD_3OD) δ 12.57 (2C, 5-Me's), 32.08; 33.15; 35.26 (3×CH_2), 37.53 (^{5}C3′), 39.85 and 39.98 (2×CH_2), 62.13 (^{5}C5′), 74.10 (^{3}C3′), 86.20; 86.39; 87.28 and 87.89 (2×C1′ and 2×C4′), 110.72 and 111.76 (2×C5), 137.90 and 138.40 (2×C6), 152.22 and 152.28 (2×C2), 166.23 and 166.50 (2×C4); MS (FAB–glycerol/NBA) m/e 511 ([MH$^+$], 25%), 385 ([MH$^+$–ThyH], 67), 259 ([MH$^+$–2×ThyH], 21). Anal. calcd. for $C_{22}H_{30}N_4O_8S_1$: C, 51.75; H, 5.93; N, 10.98; S, 6.27. Found: C, 52.12; H, 6.22; N, 10.98; S, 6.27.

Dimethoxytritylation of (4)

4,4′-Dimethoxytrityl chloride (83 mg, 0.244 mmol) was added to a stirred solution of dimer **4** (104 mg, 0.204 mmol) in dry pyridine (2.5 mL) containing 4-dimethylaminopyridine (2 mg, 0.01 mmol) and triethylamine (0.041 mL). After stirring for 8 h, the reaction was poured into water (25 mL) and extracted with dichloromethane (3×15 mL). The organic phases were dried over Na_2SO_4, filtered and evaporated *in vacuo* to give a syrup which was chromatographed over silica gel (100:5:1 CH2Cl2/MeOH/Et3N, v/v) yielding **5** as a white foam (158 mg, 85% yield): ^{1}H-NMR (200 Hz, CD_3OD) δ 1.28–1.40 (m, 1H, ^{5}H1″$_A$), 1.46–1.75 (m, 1H, ^{5}H1″$_B$), 1.34 and 1.76 (two s, 6H, 5-Me's), 2.00–2.31 (m, 4H, ^{5}H2′$_{AB}$ and ^{3}H2′$_{AB}$), 2.32–2.41 (m, 1H, ^{5}H3′), 2.42–2.63 (m, 2H, ^{5}H2″$_{AB}$), 2.69–2.90 (m, 2H, ^{3}H5′), 3.09–3.28 (m, 2H, ^{5}H5′), 3.73 (s, 6H, 2×CH_3), 3.65–3.81 (m, 1H, ^{5}H4′), 3.85–3.98 (m, 1H, ^{3}H4′), 4.22–4.34 (m, 1H, ^{3}H3′), 6.02 (dd, 1H, ^{5}H1′), 6.17 (t, 1H, ^{3}H1′), 7.41 and 7.80 (two s, 2H, H6's), $J_{(3)H1'-(3)H2'A} = J_{(3)H1'-(3)H2'B}$ = 6.8 Hz, $J_{(5)H1'-(5)H2'A}$ = 4.4, $J_{(5)H1'-(5)H2'B}$ =2.6; ^{13}C-NMR (75.4 MHz, CD_3OD) δ 10.26 (2C, 5-Me's), 32.03; 33.12 and 35.31 (3×CH_2), 37.73 (^{5}C3′), 40.08 (2×CH_2), 47.39 (Ph_3C), 55.77 (2×OCH_3), 63.80 (^{5}C5′), 74.19 (^{3}C3′), 86.22; 86.54; 86.70 and 87.21 (2×C1′ and 2×C4′), 110.99 and 111.76 (2×C5), 137.80 and 137.84 (2×C6), 152.23 and 152.28 (2×C2), 166.25 and 166.52 (2×C4), 114.22 (CH of MeOPhO-), 160.27 and 160.30 (4 of MeOPhO-); MS (FAB–glycerol/NBA) m/e 813 ([MH$^+$], 9.7%), 687 ([MH$^+$–ThyH], 3.7), 509 ([MH$^+$–DMTrH], 4.0), 304 ([DMTrH + H$^+$], 100).

Phosphoramidite (6)

N,N-diisopropyl-β-cyanoethylchlorophosphonamidic chloride (55 μL, 0.246 mmol) was slowly added to a stirred solution of tritylated dimer **5** (100 mg, 0.123 mmol) in dry dichloromethane (1.5 mL) containing triethylamine (68 μL, 0.492 mmol). After 18 h of stirring at ambient temperature under a nitrogen atmosphere, the solution was diluted with ethyl acetate (35 mL) and washed with brine (4×70 mL). The organic phase was then dried (Na_2SO_4), filtered and evaporated *in vacuo* yielding a pale yellow foam. This crude material was dissolved in

dichloromethane (0.6 mL) and precipitated at −78°C with hexanes (~5 mL). The solvents were decanted off and the residue redissolved in dichoromethane containing ethyl ether and carefully evaporated (rotovap) to afford the phosphoramidite **6** as a colorless foam (120 mg, 96% yield) which was used as such in the subsequent solid-phase syntheses: ^{31}P-NMR (CD_2Cl_2) 148.94 and 149.31 ppm; MS (FAB−nitrobenzyl alcohol) m/e 1013 ([MH^+], 75%), 1011 ([MH^+-H_2], 100) 942 ([$MH^+-HOCH_2CH_2CN$],16), 912 ([MH^+-iPr_2NH], 10), 887 ([MH^+−ThyH], 82), 795 ([$MH^+-iPr_2NP(OH)OCH_2CH_2CN$], 21), 709 ([$MH^+$−DMTrH], 45).

Preparation of oligonucleotides (7−13)

An Applied Biosystems DNA/RNA synthesizer model 381A was used for the solid-phase synthesis of oligonucleotides. Reagents for the synthesizer were used as obtained from Applied Biosystems except for acetonitrile which was prepared by stirring over phosphorus pentoxide and distilling over calcium hydride. Deoxyribonucleoside phosphoramidites bearing the ammonia labile dimethylformamidine protecting group for exocyclic bases (FOD™ amidites, Applied Biosystems) were obtained from Applied Biosystems and were used as 0.1 M CH_3CN solutions. The activated sulfide dimer **6** was prepared as described above and used as a 0.08 M CH_3CN solution in an extra port of the synthesizer. Ribonucleoside (β-cyanoethyl)phosphoramidites bearing the benzoyl protecting group on adenosine and cytidine and isobutyryl on guanosine were obtained from Dalton Chemical Laboratories Inc. (Toronto) and used as 0.1 M CH_3CN solutions. LCAA-CPG bearing 3′-terminus deoxyribonucleosides used in the preparation of oligomers **7**−**12** were obtained from Applied Biosystems while N^4-benzoyl-5′-O-monomethoxytrityl-cytidine LCAA-CPG used in the synthesis of **13** was prepared as described previously (29). Oligomers **7**−**12** were prepared on a 1.0 μmol scale utilizing the standard DNA synthesis cycle provided by Applied Biosystems with minor modifications as follows: i) phosphoramidite coupling wait time was 60 sec, ii) capping wait time was 45 sec, iii) oxidation wait time was 20 sec and iv) 5% TCA addition time was extended to 100 sec. The final trityl group of each synthesis was left on the oligomer to aid in purification (see below). Oligomer **13** was prepared on a 1.0 mmol scale utilizing the same synthesis cycle described except that the phosphoramidite coupling time was extended to 10 min (as apposed to 1 min for DNA synthesis).

Cleavage, deprotection, and purification of oligonucleotides 7−13

All the DNA oligomers (**7**−**12**) prepared were treated identically. Each oligomer was cleaved from the support by treating the LCAA-CPG bound oligonucleotide, still in the synthesis column, with 29% ammonium hydroxide solution (2×30 min) and collecting the eluent in a 2.5 mL polypropylene screw-capped test tube. The protecting groups on the heterocyclic bases of oligomers **7**−**11** were removed by continued incubation at room temperature for an additional 6 h. Oligomers **12** and **13** were heated for 8 h (due to the 3′-benzoylcytidine residue) and 12 h at 55°C, respectively. The resultant DNA oligomers (**7**−**12**) were concomitantly detritylated and purified by reversed-phase column chromatography utilizing oligonucleotide purification cartridges (OPC®, Applied Biosystems). In each case, the entire (1.0 mmol) synthesis product was applied to the cartridge for purification. The eluting solvent was removed and the oligomers dissolved in 1 mL of water. The purified DNA oligomers were obtained as aqueous solutions in the following amounts (in OD units): **7**, 10.6; **8**, 9.3; **9**, 10.4; **10**, 17.4; **11**, 8.5; **12**, 8.5; as quantified at 260 nm. Approximately one half of the deprotected RNA oligomer **13** was purified on a 16% polyacrylamide gel (PAGE) and desalted using reversed-phase chromatography (C_{18} Sep-Pack®, Waters) to yield 9.4 OD units (260 nm) of pure product. All oligomers were stored as aqueous solutions at the concentrations indicated above at −20°C between use.

Enzyme digests

Enzymes were obtained from Boehringer Mannheim (Quebec) and incubations were carried out as follows. Snake-venom phosphodiesterase (SVPDE) from *Crotalus durissus* was obtained as a solution of 2 mg/ml in 50% v/v glycerol at *ca.* pH 6 with an indicated activity of 1.5 units/0.5 mL. Incubations were carried out using 2 μL SVPDE in 18 μL buffer (50 mM Tris−HCl, 10 mM $MgCl_2$ pH 8.0). Calf-spleen phosphodiesterase (CSPDE) was obtained as a suspension (2 mg/mL) in 3.2 M ammonium sulfate *ca.* pH 6 with an activity of 4 units/ml. Incubations were carried out using 5 μL of CSPDE in 25 μL buffer (0.5M ammonium acetate pH 6.5). Nuclease S_1 from *Aspergillus oryzae* was obtained as a solution in 50% glycerol (v/v), 20 mM Tris-HCL, 50 mM NaCl, 0.1 mM zinc chloride, *ca* pH 7.5 with an activity of 100 units/μL. Incubations were carried out using 0.2 μL enzyme in 19.8 μL buffer (50 mM sodium acetate, 1 mM zinc sulphate, 250 mM sodium chloride, 50 μg/mL BSA adjusted to pH 4.6 with acetic acid). Nuclease P_1 from *Penicillium citrinum* was obtained as a lyophilized powder and prepared as a 1 mg enzyme/mL solution in 30 mM ammonium acetate, *ca.* pH 5.3. The activity was 0.3 units/1 μL. Incubations were carried out using 5 μL Nuclease P1 in 15 μL buffer (50 mM ammonium acetate pH 5.3). Oligonucleotide sequences (typically 0.3 OD units) were treated with an enzyme for a specified time period at 37°C and the digests were analyzed by PAGE or HPLC as indicated. For PAGE analysis, the entire amount was applied to a 24% polyacrylamide gel (without urea) and run for 2−3 h (as required) at a current of between 25 and 40 mA. Following electrophoresis, the gels were wrapped in plastic wrap (Saran Wrap®), placed over a fluorecent TLC plate and iluminated with a UV lamp (254 nm). The gels were photographed using Polaroid PolaPlan® 4×5 cm Instant Sheet Film (#52, medium contrast, ISO 400/27°) through a Kodak Wratten gelatin filter(#58 green). For analyses by HPLC, 7 μL (normaly 0.1 OD units) of each digest was injected into a Waters HPLC system (Millipore, MA) equipped with a 254 nm detector and a Waters 740 Data Module with gradient control capabilities. The column used was a reversed-phase (4.6 mm×250 mm) Whatman Patrisil 5-C_8 from Chromatographic Specialties. The conditions used were a flow rate of 1.2 mL/min, solvent A: 50 mM triethylammonium acetate adjusted to pH 5.5, solvent B: acetonitrile, and gradient 0−40% solvent B over 20 min and then maintained at A/B (60:40) for a further 5 min. Under these conditions, the dimer TsT (**4**) exhibited a retention time of 20.7 min. Independent incubations (48 h, 37°C) of (**4**) with SVPDE, CSPDE, Nuclease P1, and Nuclease S1 exhibited peaks with retention times of 20.8, 21.0, 21.0, and 21.0 min, respectively.

Thermal denaturation measurements

Extinction coefficients for the sequences **7**, **12**, and **13** were 1.07, 1.28, and 1.28×10^5 L $mol^{-1}cm^{-1}$, respectively, as calculated using the nearest-neighbor approximation (30). For oligomer **8** which contains three sulfide links we assumed the same extinction

coefficient as that of its natural analogue **7** (**i.e.**, 1.07×10^5 L $mol^{-1}cm^{-1}$). Samples for thermal denaturation analysis were prepared by mixing 3.0 mmols of each complementary strand, lyophilizing the solution to dryness, and dissolving in 1.0 mL of sodium phosphate buffer containing 10 mM NaCl [prepared by adding NaCl crystals (5.8 mg) to 10 mM NaH_2PO_4/Na_2HPO_4 in deionized/autoclaved water and adjusted to pH 7.0]. Thermal denaturations were carried out using a Varian-Cary 1 UV/VIS spectrophotometer equipped with the Peltier thermal unit accesory available from the manufacturer. The oligonucleotide mixture (3 μmol in each strand) in 10 mM NaCl buffer (20 mM in total Na^+) was added to a 1-cm path length 1.5 mL quartz cuvet and placed in the spectrophotometer which was set on the dual beam optical mode to reduce optical drift. An optically matched cuvet containing 10 mM sodium chloride buffer was used as the blank. The cells were equilibrated at 40°C for 10 min and cooled to the start temperature over a 20 min period. Thermal denaturation curves were obtained by recording the absorbance (average of 30 readings) at 260 nm at 1 min intervals as the temperature was ramped to 80°C at 0.5°C/min. This resulted in approximately 140 points per thermal curve. Upon completion of the first thermal melt, the temperature was slowly reduced (over 20 min) to 40°C where upon the solution was made to the next sodium chloride concentration (210 mM NaCl) by the addition of sodium chloride crystals (5.8 mg/100 mM increase in NaCl). The blank cuvet was treated likewise. No correction factor for the increase in volume due to the additional sodium chloride was made. The second thermal melt was then obtained as described above. This process was repeated to obtain thermal denaturation curves at 410 mM, 610 mM 910 mM and 1910 mM NaCl concentrations. Denaturation curves for 100 mM NaCl were obtained independently in different runs.

T_m values are reported as the intersection of the absorbance versus temperature curve (as displayed by the Cary software) and the midpoint between the upper and lower linear sloping base lines. The lower base line was well defined in all melt curves except those obtained at 100 mM NaCl. In these cases the lower base line was approximated visually. Hypochromicities (%H) were calculated using the relationship $\%H = (A_{HT}-A_{LT})/A_{HT}$ where A_{HT} = the absorbance at the highest temperature for the curve, and A_{LT} = the absorbance at the initial temperature. For visualization purposes the thermal denaturation data was transferred to the spreadsheet program Quattro Pro® where it is presented as fraction absorbance change (defined as $(A_T-A_{LT})/(A_{HT}-A_{LT})$ where A_T is the absorbance at temperature T versus temperature. The denaturation curves for single strands are presented as normalized absorbance (defined as A_T/A_{LT}) versus temperature.

Native-gel hybridization

A 24% polyacrylamide gel containing no urea was pre-incubated in 0.09 Tris-borate-EDTA running buffer (pH 8.3) for 1 hour at 4°C using 1 mA applied current. Mixtures containing oligomer **8** (3.0 μmols) with either 1, 2, 3, or 4 μmols of oligomer **12** were each dissolved in 7 mL of 30% sucrose/10 mM $MgCl_2$ and incubated initially at 40°C for 30 min and then at 4°C for 20 min. Each sample was applied to a separate lane of the gel. Mixtures of oligomer **7** (3.0 μmols) with either 1, 2, or 3 μmols of oligomer **12** were treated similarly and applied on the same gel in separate lanes. Electrophoresis was conducted at 4°C for 18 h at a constant current of 5 mA. Following electrophoresis, the gel was photographed as described above.

RESULTS AND DISCUSSION

Synthesis of sulfide dimer (6)

Full details of the preparation of mesylate 1 and thiol **2** have been published (31). The appropriately protected and activated sulfide dinucleoside required for incorporation into DNA was prepared according to the method outlined in Scheme 1. The coupling (32) of the two units employing a 10% excess of the thiol with respect to **1** was carried out in a dimethylformamide (DMF) solution containing 1.5 equivalents of cesium carbonate (33). The sulfide-linked dimer was obtained in 88% yield. Removal of the silyl protecting groups with 2.5 equivalents of tetra-*n*-butylammonium fluoride in tetrahydrofuran (THF) gave, quantitatively, the diol **4**. Dimethoxytritylation and phosphoramidite formation by standard means (34) provided the sulfide dimer **6** in 75% yield for the two steps after precipitation from dichloromethane with hexanes at −78°C. The ^{31}P-NMR spectrum of **6** showed the anticipated signals at 148.9 and 149.3 ppm and the FAB mass spectrum exhibited a parent ion at m/e 1013.

Since we planned to incorporate the sulfide dimers into natural DNA by standard phosphoramidite chemistry, the possibility of oxidation of the sulfur atoms during the iodine oxidation step of the coupling cycle was of some concern. Thus, a sample of the disilylated sulfide dimer **3** was dissolved in the iodine containing reagent (I_2/pyridine/THF/H_2O) and stirred for 15 min, *i.e.*, 30 times longer than the oxidation time in the synthesis cycle. Thin-layer chromatography demonstrated that no oxidation had occurred when compared to the chromatogram of the reaction product involving the treatment of **3** with buffered oxone reagent (35).

Oligonucleotide preparations (7–13)

Oligonucleotides **7** to **12** were synthesized by standard solid-support methodology employing dimethylformamidine-protected β-cyanoethylphosphoramidites (36) on a Applied Biosystems automated DNA synthesizer to which was attached a fifth bottle containing a 0.08 M acetonitrile solution of the sulfide dimer phosphoramidite **6**. The coupling efficiency of the sulfide dimer units was routinely >98% as monitored by the release of the dimethoxytrityl (DMT) cation. This coupling efficiency is similar to that usually observed in the condensation of normal deoxyribonucleoside-β-cyanoethylphosphoramidites indicating that nucleoside 'blocks' (in this case dimers) can be coupled as efficiently as normal nucleosides. We utilized the amidine protected deoxyribonucleoside in order to limit the exposure time of the oligomers to ammonia in the deprotection step. Whereas the benzoyl protected nucleosides require usually 12–18 hours at 55°C for complete removal of the amide protecting moiety, the amidine group is readily removed within 8 hours at room

Table 1. Oligonucleotides synthesized

Oligonucleotide Sequences	
5'-d(GpCpGp TpTpTpTpTpTp GpCpT)-3'	**7**
5'-d(GpCpGp TsTpTsTpTsTp GpCpT)-3'	**8**
5'- d(TsTpTsTpTsTp GpCpT)-3'	**9**
5'- d(TsTpTpTpTpTp GpCpT)-3'	**10**
5'- d(TsTpTsTpTsT)-3'	**11**
5'-d(ApGpCp ApApApApApAp CpGpC)-3'	**12**
5'-r(ApGpCp ApApApApApAp CpGpC)-3'	**13**

's' sulfide link, 'p' phosphodiester link.

temperature (36). After cleavage from the support and deprotection, the oligonucleotides were easily purified by reversed-phase chromatography. On a polyacrylamide gel, the sulfide-containing oligomers **8**, **9**, and **10** appeared as single bands of strong intensity indicating their high purity. The relative mobility of these oligomers is in agreement with expectation based on qualitative analysis of their mass to charge ratios. For example, oligomers **7** and **8** have identical base composition differing only by the replacement of three phosphate linkages in **7** with three sulfide linkages resulting in **8**. This leads to an increase in the mass/charge ratio of oligomer **8** relative to oligomer **7** and, consequently, **8** exhibits a retarded mobility relative to **7** (lanes 2 and 3, Figure 1a). Similarly, oligomer **9** which contains two additional sulfide linkages relative to **10**, exhibits a retarded mobility on the polyacrylamide gel (lanes 4 and 5, Figure 1a).

Enzyme digests

Characterization of the oligonucleotides was carried out by subjecting the oligomers to various enzymes of known substrate specificity and subsequently analyzing the digests by HPLC and PAGE. The potential susceptibility to cleavage of the sulfide linkage by several nucleases commonly used in the characterization of natural oligonucleotides was first determined. As expected, we did not observe any degradation of the sulfide linkage with any of the hydrolytic enzymes used. For example, SVPDE (a 3′-exonuclease), CSPDE (a 5′-exonuclease), Nuclease S1 and Nuclease P1 had no action on the TsT dimer **4** after incubation at 48 h at 37°C (HPLC analysis). The hexamer **11** which contains alternating sulfide and phosphodiester bonds (5′-$T_sT_pT_sT_pT_sT$-3′) was degraded to the T_sT dimer **4** as the only product upon 60 min treatment with SVPDE. This observation was at first surprising since SVPDE normally functions as a 3′-exonuclease and the 3′-terminal sulfide linkage of **11** is SVPDE resistant as noted above. SVPDE is therefore behaving as an endonuclease cleaving normal phosphodiester bonds flanked immediately on either side by sulfide linkages. The endonuclease activity of SVPDE has been recognized in earlier studies on nucleotide phosphotriesters (37), phosphorothioates (38), methyl phosphonates (15), and more recently on oligonucleotides containing 1,3-propanediol (39), and 1,3-butanediol (40) sugar moieties.

The presence of internucleotide sulfide linkages at the 5′-end protects the oligonucleotide chain against degradation by the

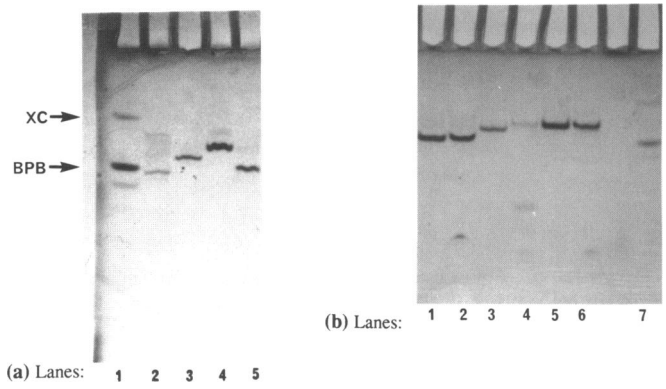

Figure 1. Electrophoresis on a 24% polyacrylamide gel. **(a)** lanes: 1) Xylene Cyanol and Bromophenol Blue dyes; 2) **7**; 3) **8**; 4) **9**; 5) **10**. **(b)** lanes: 1), 3) and 5) contain oligomers 10, 8 and 9 without CSPDE and lanes 2), 4) and 6) with CSPDE, respectively; lane 7) **8**; 2) **9**; 3) incubation of **7** with CSPDE; 4) incubation of **8** with CSPDE; 5) incubation of **9** with CSPDE; and 6) Xylene Cyanol and Bromophenol Blue dyes.

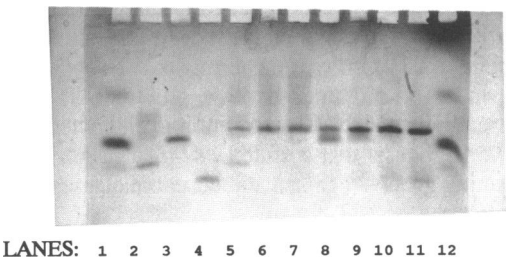

Figure 2. Native gel hybridizations on 24% polyacrylamide gel of complexes **7/12** and **8/12**. lanes: 1) 2% Xylene Cyanol and 2% Bromophenol Blue; 2) **7**; 3) **8**; 4) **12**; 5) 3 μmol **7** and 1 μmol **12**; 6) 3 μmol **7** and 2 μmol **12**; 7) 3 μmol **7** and 3 μmol **12**; 8) 3 μmol **8** and 1 μmol **12**; 9) 3 μmol **8** and 2 μmol **12**; 10) 3 μmol **8** and 3 μmol **12**; 11) 3 μmol **8** and 4 μmol **12**; 12) 2% Xylene Cyanol and 2% Bromophenol Blue. See experimental section for conditions.

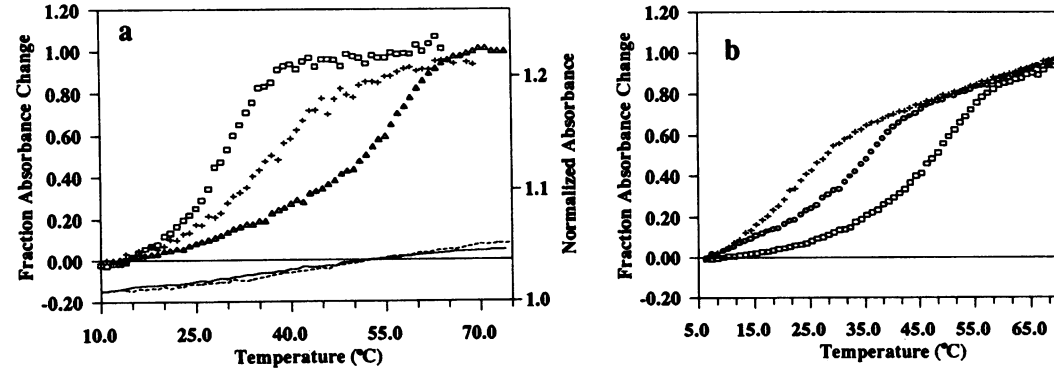

Figure 3. a) Thermal denaturation curves of oligomer **8** with complementary DNA and RNA. **(a)** Complex **8/12** in 210 mM (□), 910 mM (+) NaCl, and complex **7/12** in 210 mM NaCl (△). The single strands **12** (----) and **8** (——) in 1 M NaCl are presented as normalized absorbance using the right hand axis. **(b)** Melts curves for complex **8/13** in 100 mM (+) and 910 mM (○) NaCl, and complex **7/13** (□) in 210 mM NaCl.

Table 2. Effect of sodium chloride concentration on Tm

[NaCl] (mM)	8/12		7/12			8/13		7/13		
	T_m (°C)	%H	Tm (°C)	%H	ΔTm/s-link*	T_m (°C)	%H	T_m (°C)	%H	ΔTm/s-link*
10	21.3	15	47.3	15	8.6	15.9	10	40.1	18	11.4
100	30.5	15	55.0	17	8.2	25.0	10	47.0	18	7.3
210	33.8	17	58.5	20	8.2	33.0	11	53.6	18	6.9
410	36.8	17			8.4	37.2	10	55.4	16	6.2
610	36.3	18			8.8	36.7	10	57.2	18	6.8
710			64.0	22	9.2					7.4
910	37.16	18	65.0	24	9.3	36.0	10	58.9	18	7.6
1910	35.6	18				34.7	10			

* the change in Tm per sulfide link is reported as the interpolated value from the curves in figure 4.

5'-exonuclease CSPDE. For example, oligomers **9** and **10** were found to be completely stable to CSPDE after incubation at 37°C for 60 min (Figure 1b) whereas the unmodified strand **7** was completely degraded under these conditions. Furthermore, CSPDE treatment of oligomer **8** resulted in complete conversion of **8** to a new band which migrated identically with oligomer **9** (Figure 1b). This observation indicates that CSPDE cleaves the phosphodiesters on 5'-end until the first sulfide linkage is reached. At this point, the presence of the terminal TsT fragment 'protects' the remainder of oligomer **8** (now oligomer **9**) from further degradation by the enzyme. In addition to characterization, these observation suggests that sulfide links may be very effective as '5'-protecting groups' against cellular exonucleases for antisense oligonucleotides.

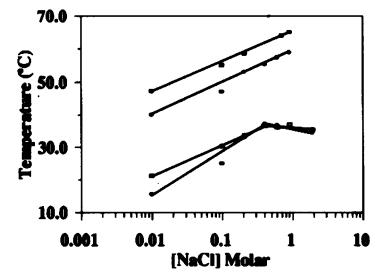

Figure 4. The effect of increasing NaCl concentration on the Tm of complexes **8/12** (□), **8/13** (○), **7/12** (■), and **7/13** (●).

Binding properties of complex 8/12

Native Gel hybridization. It is often possible to establish the formation of a complex between two complementary oligomers by comparing the mobility of the separate component strands with that of the mixture of the two oligomers on native gels. Indeed, we observed hybridization between the sulfide containing oligomer **8** and its complementary DNA oligomer **12**. As shown in Figure 2, each oligomer on its own appears as an intense band with a distinct mobility (lanes 2–4). Mixing oligomer **8** with increasing concentrations of oligomer **12** resulted in a new band migrating slower than either **8** or **12** (lanes 8–11). The characteristic retarded mobility of this new band and the 'titration' effect observed suggested duplex formation. A similar interaction between oligomers **7** and **12** was also observed (lanes 5–7). Based on the charge to mass ratio of these duplexes, **8/12** is expected to migrate more slowly on a gel relative to **7/12**. However, it is clear from Figure 2 that both duplexes exhibited nearly identical electrophoretic mobilities. The unexpected relative mobilities of these complexes may be attributed to slight differences in their three-dimensional structure. This hypothesis is substantiated by the T_m versus ionic strength studies described below.

Thermal denaturation (8/DNA 12 and 8/RNA 13). Thermal denaturation studies indicated that the replacement of the phosphodiester group with the dialkyl sulfide linkages weakened but did not prevent binding to either complementary DNA or RNA. T_m values for the association between oligomer **8** and its complementary DNA strand (**12**) are listed in Table 2 and representative thermal denaturation curves are shown in Figure 3a. The modified complex **8/12** exhibited a sharp cooperative transition with a T_m of 33.8°C whereas the cooperative transition for the unmodified complex **7/12** was broad with a T_m of 58.5°C at 210 mM sodium chloride concentration (Figure 3a, Table 2). This represents a reduction in the T_m of 8.2°C per phosphodiester bond replacement with a sulfide linkage. The destabilization of complex **8/12** relative to complex **7/12** persisted from low salt (10 mM NaCl) to high salt (910 mM NaCl) concentrations (~ 8.7 ± 0.5°C/sulfide link, Table 2). However, as illustrated in Figure 4, an inflection point was observed in the plot of T_m against sodium chloride concentration for complex **8/12** but not for the DNA/DNA complex **7/12**. This change in slope occurred at approximately 400 mM [NaCl] and may suggest a change in the nature of the complex **8/12** as the salt concentration is increased. In normal phosphodiester linked nucleic acids, the T_m of a complex is linear in the logarithm of the monovalent cation concentration. This is largely due to the electrostatic shielding of negative charges along the phosphodiester backbone of the duplex by the counterions and on the direct binding of the counterions on the nucleic acid.(41). Since oligomer **8** contains both phosphodiester and sulfide links, the inflection point is certainly due to the influence of the sulfide links on the stability of the complex. The increased instability and insensitivity to NaCl concentration of **8/12** above 400 mM

NaCl concentration may be attributed to a conformational change of the complex resulting from an altered hydration sphere around the backbone of the complex. However, we did not observed a significant change in the hypochromicity of **8/12** over this range of NaCl concentration that would indicate a change in the nature of the base-base stacking interactions. We are currently investigating this observation more quantitatively.

A cooperative interaction between the sulfide containing sequence **8** and its RNA complement was also observed. Representative thermal melts are shown in Figure 3b. The association of **8** with RNA resulted in lower T_m values than for the control DNA/RNA complex **7/13** over the entire sodium chloride concentration investigated (Table 2). In the case of **8/13**, unlike that of **8/12**, the destabilization resulting from the three TsT insert was more pronounced at low ionic conditions (10 mM NaCl, 11.4°C/sulfide-link) than at higher salt concentrations (>100 mM NaCl, ~ 6.5°C/sulfide link). The unusual dependence of T_m on the salt concentration previously observed for the complex **8/12** was also observed for complex **8/13** but not for the unmodified DNA/RNA complex **7/13** (Figure 4). As for the complex **8/12**, an inflection point was observed for complex **8/13** in the curve of T_m versus log [NaCl] at approximately 400 mM NaCl concentration. The slope of **8/13** was somewhat steeper than that of **8/12** at NaCl concentrations in the range 10 mM−400 mM indicating a stronger dependence on ionic strength for complex **8/13**. However, at NaCl concentration above 400 mM, both **8/13** and **8/12** exhibited similar slopes. These results indicate that DNA oligomers containing sulfide linkages are capable of forming stable complexes with complementary DNA and RNA oligomers. However, the stability of these complexes is lower than that of the normal DNA/DNA and DNA/RNA complexes and is highly dependent on the ionic strength of the hybridization buffer.

CONCLUSION

A non-hydrolyzable dialkyl sulfide containing analogue of a thymidine dinucleoside has been synthesized and shown to be easily incorporated into DNA by standard automated methodology. Preliminary studies have shown that the sulfur containing oligomer **8** is capable of binding to complementary single stranded DNA and RNA and exhibits increased stability towards nuclease degradation *in vitro*. Further studies involving the sulfide and sulfone-linked systems, for both the deoxyribo- and the ribo-series, are ongoing and will be described in due course.

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