Incorporation of 2′-Deoxy-6-thioguanosine into G-Rich Oligodeoxyribonucleotides Inhibits G-Tetrad Formation and Facilitates Triplex Formation

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ABSTRACT: An efficient and expeditious method for the synthesis of S^6-(cyanoethyl)-N^2-isobutyryl(or trifluoroacetyl)-2′-deoxy-6-thioguanosine (**7** and **2**) from 2′-deoxyguanosine (G) has been developed. Compound **7** has been incorporated into several G-rich triple-helix-forming oligonucleotides (TFOs) using solid-support, phosphoramidite chemistry. The purified oligonucleotides containing 2′-deoxy-6-thioguanosine (S^6-dG) residues in the place of G have been characterized by nucleoside composition analysis. These modified TFOs have been shown to be stable in aqueous, as well as buffered, solutions normally used to assay triple-helix formation. It has also been demonstrated that partial incorporation of S^6-dG is effective in inhibiting the formation of G tetrads in G-rich oligodeoxyribonucleotides, thus facilitating triple-helix formation in potassium-containing buffers.

Soon after the classical discovery of the double-helical structure of DNA (Watson & Crick, 1953), the existence of triple-helical DNA was recognized [Felsenfeld et al., 1957; for comments, see Moffat (1991)]. Subsequently, it was shown (Moser & Dervan, 1987; Cooney et al., 1988; Praseuth et al., 1988; Lyamichev et al., 1988; Pilch et al., 1990; Beal & Dervan, 1991; Durland et al., 1991) that, under suitable conditions, short oligonucleotides will bind in a sequence-specific manner to a duplex target and form a local triple-helical structure, or triplex. Since triplex-forming oligonucleotides (TFOs)[1] bind to duplex DNA in the major groove, they have the potential to interfere with the binding of various proteins. Formation of a triplex at such a site would block the access of the protein to the DNA, thus preventing binding (Maher et al., 1989; Duval-Valentin et al., 1992; Blume et al., 1992). Gene expression is known to be regulated by the actions of a variety of proteins, many of which act by binding to DNA sequences. It has been well documented that the expression of certain genes is critical for the progression of many diseases, especially viral and malignant diseases. The ability to design an oligonucleotide that would bind to a specific sequence and turn off (or turn on) a particular gene could have enormous benefit for the treatment of such diseases.

Two major triplet motifs are known. In one, T (thymidine) in the third strand H-bonds to A (2′-deoxyadenosine) in the duplex, and protonated 2′-deoxycytidine (C^+) in the third strand bonds to G (2′-deoxyguanosine) in the duplex (Povsic & Dervan, 1989; Maher et al., 1992; Strobel et al., 1991; Grigoriev et al., 1992). A major drawback of this motif is that protonation of the third-strand C, which is required for binding to G in the duplex, optimally requires a pH of 5−6, which is well below physiological range. The other motif, which is the focus of this article, involves T in the third strand bonding to A in the duplex, and G in the third strand bonding to G in the duplex (the G/T motif). Figure 1 shows the H-bonding schemes involved for this type of triplex. All of the bases involved in H-bonding are in their normal, uncharged tautomeric forms; thus pH is not a significant factor in triplex formation. It has been shown that, under appropriate conditions, TFOs utilizing a G/T motif can bind with high sequence selectivity and essentially no pH dependence (Beal & Dervan, 1991; Durland et al., 1991). Such TFOs generally consist entirely of G and T residues and are often biased in favor of G.

Although these oligonucleotides hold significant promise as first-generation TFO therapeutics, it is apparent that the high G content is problematic. It is documented that G-rich oligonucleotides can form self-associated structures (Guschlbauer et al., 1990; Jin et al., 1990; Kim et al., 1991; Sen & Gilbert, 1992; Il'icheva & Florent'ev, 1992; Lu et al., 1993), all of which involve the formation of G tetrads (Figure 2) by the formation of eight H-bonds, by coordination of the four O^6 atoms of guanine with alkali cations believed to bind to the center of the quadruplex, and by strong stacking interactions. A number of such structures have been identified, including intra- and intermolecular associations between oligonucleotides. Such complexes are known to be stabilized by monovalent cations such as Na^+, and K^+. As both ions normally are present in significant amounts in the physiological environment, it is likely that G-rich oligonucleotides may self-associate to some degree *in vivo*. Formation of such structures reduces the ability of the TFO to bind to its intended target duplex, thus reducing its efficacy for inhibiting gene expression. Chemical modification of the guanine moiety that disrupts the multiple H-bonding of the G tetrad can reduce/eliminate its formation (Rao et al., 1993). Replacement of all or some of the G residues in G-rich oligonucleotides with 2′-deoxy-6-thioguanosine (S^6-dG) is likely to inhibit self-association by interfering with the coordination of the alkali cation and by reducing the strength of the H-bonds to that position. In addition, the larger van der Waals radius of sulfur, relative to oxygen,

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[1] Abbreviations: S^6-dG, 2′-deoxy-6-thioguanosine; TFO, triplex-forming oligonucleotide; DMS, dimethyl sulfate.

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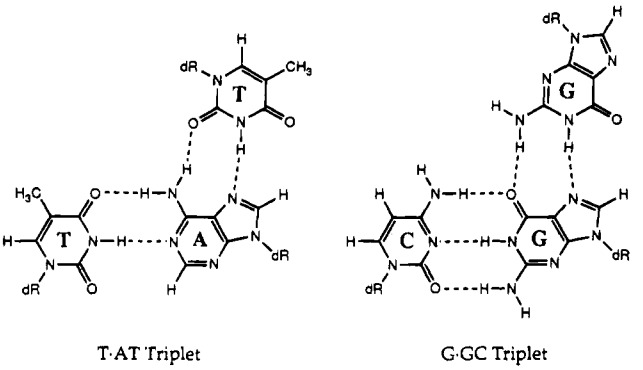


T·AT Triplet G·GC Triplet

FIGURE 1: Hydrogen-bonding pattern for reverse Hoogsteen T·AT and G·GC base triplets in antiparallel triplexes.

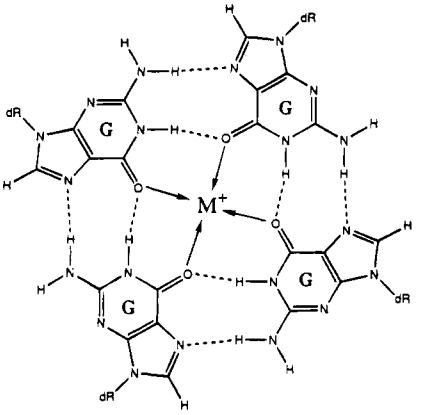


FIGURE 2: Structure of a hydrogen-bonded G tetrad, with an internally coordinated monovalent cation (M^+).

may lead to significant steric repulsion in the tetrad. However, this subtle change is not expected to affect the groups believed to be involved in the H-bonding of GGC triplets. We now report that partial incorporation of S^6-dG is effective in inhibiting the formation of G tetrads in G-rich oligonucleotides and in facilitating triplex formation in potassium-containing buffers.

MATERIALS AND METHODS

General Procedures. Melting points (uncorrected) were determined in a Thomas-Hoover capillary melting point apparatus. Elemental analyses were performed by Quantitative Technologies Inc. (Whitehouse, NJ). The presence of solvent as indicated by elemental analysis was verified by ^{1}H NMR spectroscopy. Thin-layer chromatography (TLC) was performed on aluminum plates coated (0.2 mm) with silica gel 60 F_{254} (EM Science). The components were visualized by ultraviolet absorbance and 10% H_2SO_4 in MeOH spray followed by heating. Silica gel (EM Science, 230−400 mesh) was used for flash column chromatography. All solvents and chemicals used were reagent grade and were not further dried/purified unless otherwise noted. Evaporations were carried out at a temperature ≤30 °C and under diminished pressure for solvents with bp < 80 °C or under high vacuum for higher boiling solvents. Infrared (IR) spectra were recorded in KBr or Nujol with a Perkin-Elmer 1420 IR spectrophotometer, and ultraviolet spectra (UV) were recorded with a Beckman DU-50 spectrophotometer or Hewlett-Packard 8452 diode array spectrophotometer. Nuclear magnetic resonance (^{1}H NMR and ^{31}P NMR) spectra were recorded at 400 MHz with a Brücker AM400 wide bore NMR spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane (internal) for ^{1}H spectra or relative to 80% polyphosphoric acid (external) for ^{31}P spectra (key: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad).

S^6-(Cyanoethyl)-N^2-(trifluoroacetyl)-2′-deoxy-6-thioguanosine (2). N^2-Isobutyryl-2′-deoxyguanosine (Ti et al., 1992) (**1**, 2.02 g, 5.99 mmol) was dried by repeated coevaporation with anhydrous pyridine (4 × 10 mL) and then dissolved in dry pyridine (distilled from CaH_2, 30 mL). The solution was protected from moisture and cooled in an ice bath (0−5 °C), and trifluoroacetic anhydride (4.23 mL, 29.2 mmol) was added dropwise with stirring. After the mixture was stirred for 45 min, 2-mercaptopropionitrile (6.0 mL) was added and the new mixture was stirred at ambient temperature for 5 h. The mixture was diluted with CH_2Cl_2 (150 mL), and the organic layer was washed with water (25 mL). The aqueous layer was separated and extracted with CH_2Cl_2 (2 × 50 mL), and the CH_2Cl_2 solutions were combined and dried (Na_2SO_4). After evaporation of the solvent, the product was purified by silica gel column chromatography using progressively increasing concentrations of MeOH in CH_2Cl_2 (0−3%) as the eluent. Evaporation of the eluate gave a homogeneous solid, which was dried *in vacuo* for 16 h to yield 2.20 g (85%) of **2**: mp 160−162 °C (dec) (no visible melting); IR 1760 (C=O), 2220 (C≡N), 3340−3600 (NH, OH) cm^{-1}; UV (MeOH) λ_{max} 248 (ε, 29 500), 292 (23 600), 300 (20 900) nm; ^{1}H NMR (DMSO-d_6) δ 2.27−2.52 (m, 1 H, $C_{2''}H$), 2.69−2.84 (m, 1 H, $C_{2'}H$), 3.15 (t, J = 6.6 Hz, 2 H, CH_2CN), 3.58 (m, 4 H, $C_{5'}H_2$ and SCH_2), 3.85 (m, 1 H, $C_{4'}H$), 4.44 (m, 1 H, $C_{3'}H$), 4.84 (br s, 1 H, $C_{5'}OH$), 5.33 (br s, 1 H, $C_{3'}OH$), 6.38 (t, J = 6.4 Hz, 1 H, $C_{1'}H$), 8.69 (s, 1H, C_8H), 12.17 (s, 1 H, CONH). Anal. Calcd for ($C_{15}H_{15}F_3N_6O_4S$·0.25H_2O): C, 41.24; H, 3.56; N, 19.26; F, 13.06. Found: C, 41.08; H. 3.43; N, 18.94; F, 13.05.

5′-O-(4,4′-Dimethoxytrityl)-S^6-(cyanoethyl)-N^2-(trifluoroacetyl)-2′-deoxy-6-thioguanosine (3). Compound **2** (2.0 g, 4.62 mmol) was dried by repeated coevaporation with anhydrous pyridine (3 × 25 mL) and then dissolved in dry pyridine (30 mL). The solution was protected from moisture and cooled in an ice bath (0−5 °C), and [bis(4-methoxyphenyl)phenyl]methyl chloride (4,4′-dimethoxytrityl chloride, 1.83 g, 5.4 mmol) was added with stirring in three portions at 30 min intervals. The mixture was stirred at 0−5 °C for 15 h and MeOH (2 mL) was added. After 10 min, the mixture was diluted with CH_2Cl_2 (100 mL), and the solution was washed with 2% aqueous $NaHCO_3$ solution (75 mL). The aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL), and the CH_2Cl_2 solutions were combined and dried (Na_2SO_4). The solution was evaporated, and the residue was coevaporated (<35 °C) with toluene (3 × 50 mL) to remove residual pyridine. The residue was dissolved in CH_2Cl_2 (30 mL) and the solution was applied to a silica gel column (2.5 × 20 cm). The column was flash eluted with progressively increasing concentrations of MeOH in CH_2Cl_2 (0−2.5% MeOH). Eluate containing the homogeneous product was evaporated to dryness, and the residue was dried *in vacuo* for 16 h to yield 2.6 g (76%) of **3**: mp 104−106 °C; IR 1765 (C=O), 2210 (C≡N), 3200−3600 (NH, OH) cm^{-1}; UV (MeOH) λ_{max} 246 (ε, 34 100), 292 (20 800), 300 (sh) (18 200) nm; ^{1}H NMR (DMSO-d_6) δ 2.30−2.49 (m, 1 H, $C_{2''}H$), 2.80−3.10 (m, 1 H, $C_{2'}H$), 3.15 (t, 2 H, CH_2CN), 3.25−3.45 (m, 2 H, $C_{5'}H_2$), 3.60 (t, 2 H, SCH_2), 3.70 (s, 6 H, 2 OCH_3), 4.00 (m, 1 H, $C_{4'}H$), 4.54 (m, 1 H, $C_{3'}H$), 5.34 (d, 1 H, $C_{3'}OH$), 6.42 (t, J = 5.6 Hz, 1 H, $C_{1'}H$), 6.67−7.35

(m, 13 H, DMT), 8.57 (s, 1 H, C_8H), 12.05 (s, 1 H, CONH). Anal. Calcd for ($C_{36}H_{33}F_3N_6O_6S$): C, 58.84; H, 4.52; N, 11.44; F, 7.76; S, 4.36. Found: C, 58.74; H, 4.69; N, 11.14; F, 7.49; S, 4.50.

5′-O-(4,4′-Dimethoxytrityl)-S⁶-(cyanoethyl)-N²-(trifluoroacetyl)-2′-deoxy-6-thioguanosine 3′-O-(2-Cyanoethyl) N,N-Diisopropylphosphoramidite (4). Compound **3** (0.71 g, 0.96 mmol) was placed in a septum-sealed flask, and the flask was flushed with dry argon. Diisopropylamine (70 μL, 0.5 mmol, distilled from CaH_2 and stored under argon), tetrazole (37.5 mg, 0.5 mmol), and anhydrous CH_2Cl_2 (5 mL) were added. The mixture was stirred at ambient temperature for 15 min under an argon atmosphere, after which 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (0.37 mL, 1.15 mmol) was added dropwise *via* a syringe. The course of the reaction was monitored by TLC. After the reaction mixture was stirred for 45 min, EtOAc (50 mL) was added and the organic phase was washed with a cold 5% aqueous $NaHCO_3$ solution (75 mL). The EtOAc solution was dried (Na_2SO_4) and evaporated. The residue was dissolved in a CH_2Cl_2/EtOAc/Et_3N (70:29:1) mixture (5 mL), and the solution was applied to a silica gel (washed thoroughly with 70:29:1 CH_2Cl_2/EtOAc/Et_3N) column (2.5 × 15 cm). The column was eluted with the same solvent mixture, and the eluate containing the homogeneous product was evaporated to give a colorless foam. The foam was dissolved in CH_2Cl_2 (5 mL), and the solution was added slowly to rapidly stirred pentane (300 mL). The supernatant was decanted from the precipitated solid. The solid was dried *in vacuo* until free of residual solvents to give 0.75 g (83%) of **4**: ^{1}H NMR (CD_3CN) δ 1.00−1.35 (m, 6 H, CH(CH_3)$_2$), 1.95 (m, 6 H, CH(CH_3)$_2$), 2.40−2.80 (m, 2 H, $C_{2'}H$ and $C_{2''}H$), 3.03 (t, 2 H, CH_2), 3.10−3.70 (m, 4 H, $C_{5'}H_2$ and CH_2), 3.73 (s, 6 H, 2 OCH_3), 4.20 (m, 1 H, $C_{4'}H$), 4.85 (m, 1 H, $C_{3'}H$), 6.37 (t, J = 6.4 Hz, 1 H, $C_{1'}H$), 6.60−7.40 (m, 13 H, DMT), 8.18 (s, 1 H, C_8H); ^{31}P NMR (CD_3CN) δ 149.38. Anal. Calcd for ($C_{45}H_{50}F_3N_8O_7SP$): C, 57.80; H, 5.39; N, 11.98; S, 3.43. Found: C, 57.51; H, 5.74; N, 12.01; S, 3.30.

S⁶-(Cyanoethyl)-N²-isobutyryl-3′,5′-O-bis(tert-butyldimethylsilyl)-2′-deoxy-6-thioguanosine (6). To an ice-cooled (0−5 °C) solution of *N*²-isobutyryl-6-*O*-[(triisopropylphenyl)sulfonyl]-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyguanosine (**5**, 1.28 g, 1.44 mmol) (Gaffney et al., 1984) in anhydrous CH_2Cl_2 (20 mL) was added 1-methylpyrrolidine (1.48 mL, 14.4 mmol). The solution was protected from moisture and stirred at 0−5 °C for 30 min before 2-mercaptopropionitrile (1.25 g, 14.4 mmol) was added. The reaction mixture was stirred for 2 h, during which time it was allowed to warm to room temperature. The mixture was evaporated to dryness, and the residue was dissolved in CH_2Cl_2 (100 mL). The CH_2Cl_2 solution was washed with water (2 × 25 mL), and the organic layer was dried (Na_2SO_4). The solvent was evaporated and the residue was purified on a flash silica gel column (2.5 × 25 cm) using progressively increasing concentrations of MeOH in CH_2Cl_2 (0−2% MeOH) as the eluent. Eluate containing the homogeneous product was evaporated to dryness, and the residue was dried *in vacuo* for 24 h to yield 0.82 g (89.6%) of **6**: mp 60−62 °C (foams, no visible melting); IR 1740 (C=O), 2210 (C≡N), 3320 (NH) cm^{-1}; UV (MeOH) λ_{max} 246 (ε, 24 200), 294 (15 400), 300 (sh) (14 200) nm; ^{1}H NMR ($CDCl_3$) δ 0.07(s, 3 H, CH_3), 0.10 (s, 3 H, CH_3), 0.90 and 0.91 (2 s, 18 H, *t*-Bu), 1.28 (d, 6 H, COCH(CH_3)$_2$), 2.25−2.65 (m, 2 H, $C_{2'}H$ and $C_{2''}H$), 2.94 (m, 1 H, COC*H*), 3.03 (t, 2 H, CH_2CN), 3.40−3.60 (m, 2 H, $C_{5'}H_2$), 3.70−3.85 (m, 2 H, SCH_2), 3.99 (m, 1 H, $C_{4'}H$), 4.59 (m, 1 H, $C_{3'}H$), 6.37 (t, J = 6.48 Hz, 1 H, $C_{1'}H$), 7.90 (s, 1 H, NH), 8.20 (s, 1 H, C_8H). Anal. Calcd for ($C_{29}H_{50}N_6O_4SSi_2$): C, 54.85; H, 7.94; N, 13.24. Found: C, 54.55; H, 8.00; N, 12.88.

S⁶-(Cyanoethyl)-N²-isobutyryl-2′-deoxy-6-thioguanosine (7). To a solution of **6** (1.05 g, 1.58 mmol) in anhydrous pyridine (3 mL) was added a solution of HF/tetrabutylammonium fluoride in dry pyridine (1.58 mL, 1.58 mmol; the reagent was prepared as 1 M tetrabutylammonium fluoride solution in 2 M HF in pyridine). The mixture was stirred at ambient temperature for 24 h with the exclusion of moisture, after which the solvent was evaporated to dryness. The residue was purified on a flash silica gel column (2 × 15 cm) eluting with progressively increasing concentrations of MeOH in CH_2Cl_2 (0−5% MeOH). Eluate containing the homogeneous product was evaporated to dryness, and the residue was dried *in vacuo* for 15 h to give 0.41 g (64%) of compound **7** as a colorless solid: mp 164 °C; IR 1740 (C=O), 2220 (C≡N), 3150−3600 (NH, OH) cm^{-1}; UV (MeOH) λ_{max} 246 (ε, 27 100), 294 (17 300), 302 (sh) (15 400) nm; ^{1}H NMR (DMSO-d_6) δ 1.11 (d, 6 H, COCH(CH_3)$_2$], 2.67−2.74 (m, 1 H, $C_{2''}H$), 2.28−2.33 (m, 1 H, $C_{2'}H$), 2.84 (m, 1 H, COC*H*), 3.17 (t, 2 H, CH_2CN), 3.50−3.62 (m, 4 H, $C_{5'}H_2$ and SCH_2), 3.87 (m, 1 H, $C_{4'}H$), 4.43 (m, 1 H, $C_{3'}H$), 4.84 (t, 1 H, $C_{5'}OH$), 5.05 (d, 1 H, $C_{3'}OH$), 6.34 (s, J = 6.4 Hz, 1 H, $C_{1'}H$), 8.52 (s, 1 H, C_8H), 10.43 (br s, 1 H, NH). Anal. Calcd for ($C_{17}H_{22}N_6O_4S$·0.25MeOH): C, 49.98; H, 5.47; N, 20.28; S, 7.74. Found: C, 50.26; H, 5.52; N, 19.90; S, 7.59.

5′-O-(4,4′-Dimethoxytrityl)-S⁶-(cyanoethyl)-N²-isobutyryl-2′-deoxy-6-thioguanosine (8). In a manner similar to that described for the preparation of **3**, tritylation of **7** (0.25 g, 0.615 mmol) with [bis(4-methoxyphenyl)phenyl]methyl chloride (0.25 g, 0.74 mmol) in dry pyridine (8 mL) gave impure **8**, which was purified by flash chromatography on a silica gel column (1.5 × 10 cm) using 0−1% MeOH in CH_2Cl_2 as the eluent. The pure product was dissolved in a small volume of CH_2Cl_2 and added to hexanes to give a white precipitate, which was collected by filtration and dried *in vacuo* for 15 h to give compound **8** (yield, 0.37 g (85%): mp 144−146 °C; IR 1740 (C=O), 2220 (C≡N), 3200−3600 (NH, OH) cm^{-1}; UV (MeOH) λ_{max} 238 (ε, 33 400), 294 (15 000) nm; ^{1}H NMR (DMSO-d_6) δ 1.10 (d, 6 H, COCH(CH_3)$_2$), 2.25−2.95 (m, 2 H, $C_{2'}H$ and $C_{2''}H$), 3.05−3.65 (m, 6 H, $C_{5'}H_2$ and SCH_2CH_2CN), 3.70 (s, 3 H, OCH_3), 3.71 (s, 3 H, OCH_3), 3.97 (m, 1 H, $C_{4'}H$), 4.50 (m, 1 H, $C_{3'}H$), 5.33 (d, 1 H, $C_{3'}OH$), 6.37 (t, J = 5.8 Hz, 1 H, $C_{1'}H$), 6.70−7.30 (m, 13 H, DMT), 8.44 (s, 1 H, C_8H), 10.5 (s, 1 H, NH). Anal. Calcd for ($C_{38}H_{40}N_6O_5S$·0.5hexane): C, 65.49; H, 6.29; N, 11.18. Found: C, 65.31; H, 6.03; N, 10.89.

5′-O-(4,4′-Dimethoxytrityl)-S⁶-(cyanoethyl)-N²-isobutyryl-2′-deoxy-6-thioguanosine 3′-O-(2-Cyanoethyl) N,N-Diisopropylphosphoramidite (9). In a manner similar to that described for the preparation of **4**, phosphitylation of **8** (1.0 g, 1.41 mmol) in dry CH_2Cl_2 (10 mL) containing diisopropylamine (99 μL, 0.71 mmol) and tetrazole (53 mg, 0.71 mmol) with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (0.55 mL, 1.69 mol) gave impure **9**. Purification of crude **9** on a silica gel column (2.5 × 25 cm) using CH_2Cl_2/EtOAc/Et_3N (69:30:1) as the eluent and subsequent precipitation into pentane gave pure compound **9** (1.12 g (87.5%)): ^{1}H NMR (CD_3CN) δ 1.15 (m, 18 H, isopropyl and isobutyryl protons), 1.95 (m, 1 H, COC*H*), 2.40−2.90

(m, 2 H, $C_{2'}H$ and $C_{2''}H$), 3.00−3.90 (m, 6 H, $C_{5'}H_2$ and CH_2CH_2), 3.73 (s, 6 H, 2 OCH_3), 4.20 (m, 1 H, $C_{4'}H$), 4.89 (m, 1 H, $C_{3'}H$), 6.34 (t, J = 6.40 Hz, 1 H, $C_{1'}H$), 6.67−7.38 (m, 13 H, DMT), 8.10 (s, 1 H, C_8H), 8.75 (br s, 1 H, NH); ^{31}P NMR (CD_3CN) δ 149.4, 149.64. Anal. Calcd for ($C_{47}H_{57}N_8O_7SP{\cdot}0.25H_2O$): C, 61.79; H, 6.34; N, 12.27; S, 3.51. Found: C, 61.50; H, 6.49; N, 12.35; S, 3.28.

Nucleoside Composition Analysis of Oligonucleotides (Gehrke et al., 1984). To a solution of the oligonucleotide (8 μg) in 50 μL of Milli-Q water were added, sequentially, 100 μL of a 0.03 M solution of NaOAc (pH ~5.3), 5 μL of 0.02 M $ZnSO_4$, 10 μL (2 units) of nuclease P1, and 1.5 μL (1.5 units) of bacterial alkaline phosphatase, and the mixture was incubated at 37 °C for 2 h. The pH of the mixture was adjusted to 8.5 by the addition of Tris (20 μL, 0.5 M) and reincubated for an additional 2 h at 37 °C. The deoxynucleoside products were analyzed on a Waters reversed-phase HPLC (Supelcosil column, 25 × 4.6 mm) equipped with a photodiode array (PDA) detector (Model 996) [HPLC conditions: elution with buffer A, 0.5 M NaCl in 10 mM NaOH; buffer B, 1.5 M NaCl in 10 mM NaOH; flow rate, 2.5 mL/min; initial conditions, A:B = 90:10 (5 min), then A:B = 40:60 (45 min), and finally A:B = 0.10 (60 min)]. Prior to the analysis of TFOs, a mixture of monomers (dG + S^6-dG + T) was analyzed (Figure 3) as a control to correlate the data generated by digested samples. The calculated relative ratios of the nucleosides were in good agreement with the experimental ratios, indicating that S^6-dG was successfully incorporated using conventional phosphoramidite chemistry.

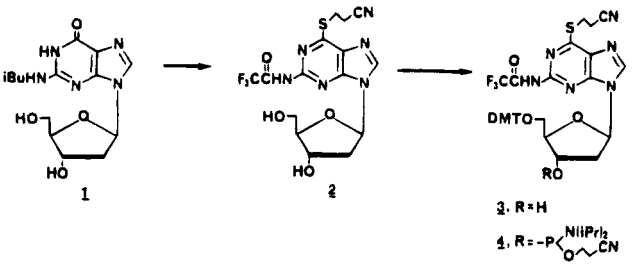
Assay for Triplex Formation. In vitro triplex formation was assessed using the gel shift assay, essentially as described previously (Cooney et al., 1988; Durland et al., 1991). Briefly, trace concentrations ($\leq 10^{-11}$ M) of end-labeled (^{32}P) duplex were incubated with increasing concentrations of third strand in a buffer consisting of 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 10% sucrose (to make the sample dense enough to load on the gel). Incubations were at 37 °C for 20−24 h (Durland et al., 1994). Samples were then separated on 12% polyacrylamide gels buffered in 89 mM Tris, 89 mM boric acid, and 10 mM $MgCl_2$ (TBM_{10}). Electrophoresis was at 80 V for 2−3 h at room temperature. Gels were dried and autoradiographed.

Triplex formation was detected by the appearance of a discrete band migrating more slowly than the duplex in samples containing added third strand. The apparent dissociation constant for triplex formation was estimated by the concentration of the TFO leading to 50% triplex, as previously described (Durland et al., 1991).

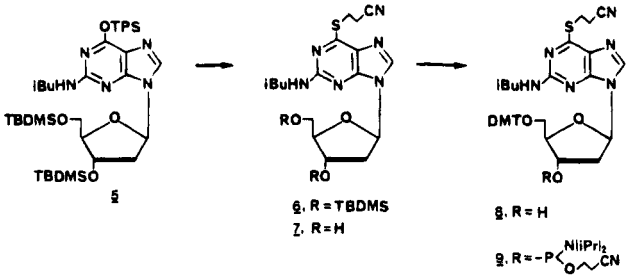
RESULTS AND DISCUSSION

Synthesis of 2′-Deoxy-6-thioguanosine Phosphoramidites. Although a few reports have appeared in the literature describing the synthesis of S^6-dG-containing oligonucleotides (Bronstein et al., 1988; Rappaport, 1988; Richardson & Bronstein, 1989), none of these syntheses considered protecting the thione group of S^6-dG, which is of paramount importance in order to mask its nucleophilicity and prevent oxidative hydrolysis. The formation of the dimeric disulfides can also be expected in sulfur-containing nucleotides. Our attempts to use 5′-*O*-(dimethoxytrityl)-2′-deoxy-6-thioguanosine 3′-phosphoramidite in a solid-support oligonucleotide synthesis resulted not only in lower coupling yields but also in the formation of multiple products. Therefore, we decided to protect the thione functionality of S^6-dG with a cyanoethyl group (Coleman & Siedlecki, 1991, 1992; Milton et al., 1993), since it can be readily removed under mild alkaline conditions. During the course of the present work, two papers have appeared (Christopherson & Broom, 1991; Waters & Connolly, 1992), in which the syntheses of certain oligonucleotides containing S^6-dG have been reported employing the cyanoethyl group for the protection of the thione moiety. However, these approaches involve the synthesis of S-protected synthons from the preformed S^6-dG itself.

Scheme 1



Scheme 2



In our first approach (Scheme 1), N^2-isobutyryl-2′-deoxyguanosine (Ti et al., 1982) (**1**) was transformed into the corresponding 6-pyridyl intermediate using trifluoroacetic anhydride in pyridine (Fathi et al., 1990). Since the pyridyl group attached to the 6-position of the purine ring is very susceptible to nucleophilic displacement (Kung & Jones, 1991), the 6-pyridyl intermediate was allowed to react with 2-mercaptopropionitrile. This one-flask, two-step procedure gave a homogeneous product (85% yield), which was identified as S^6-(cyanoethyl)-N^2-(trifluoroacetyl)-2′-deoxy-6-thioguanosine (**2**). The 5′-hydroxyl group of **2** was selectively protected as the 4,4′-dimethoxytrityl ether by treatment with dimethoxytrityl chloride in pyridine. The pure product **3** was isolated in 76% yield after silica gel column chromatography. Compound **3** was conveniently converted into the corresponding 3′-phosphoramidite (**4**) by reaction with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite in the presence of tetrazole and diisopropylamine in CH_2Cl_2. The yield of pure **4** after silica gel column chromatography was 83%. In this approach, during the preparation of **2**, the N^2-isobutyryl group of **1** was transamidated to give the trifluoroacetyl derivative due to the high reactivity of trifluoroacetic anhydride, which is reacting with the basic NH functionality to eliminate the isobutyryl group.

Although the phosphoramidite **4** was found to be suitable for solid-support DNA synthesis, this methodology limits the possibility of using a very labile protecting group on the exocyclic amino function. In view of this, we have also synthesized the fully protected S^6-dG synthon *via* a 6-*O*-[(triisopropylphenyl)sulfonyl] intermediate (Scheme 2). Thus, treatment of N^2-isobutyryl-6-*O*-[(triisopropylphenyl)sulfo-

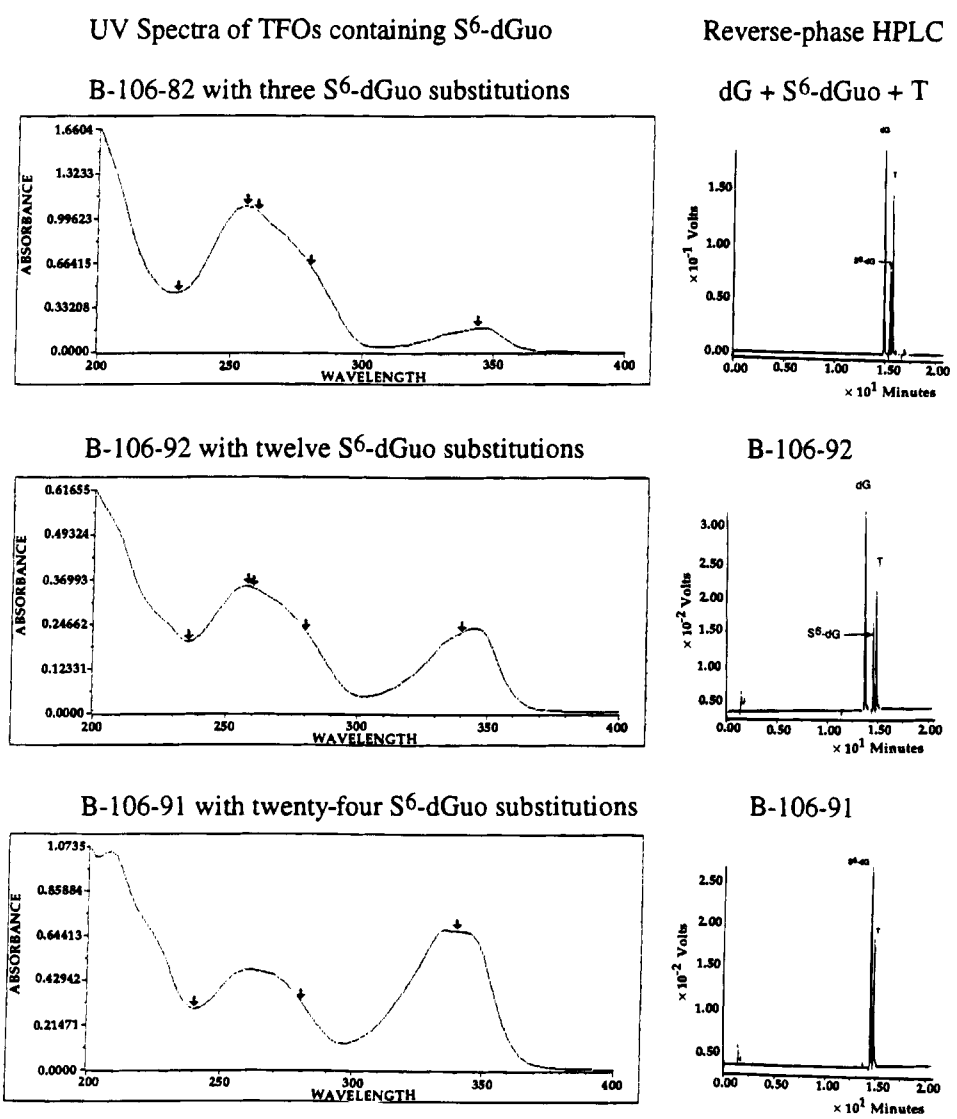


FIGURE 3: Ultraviolet absorption spectra and reverse-phase HPLC profiles of the nucleoside composition analysis of TFOs containing S^6-dG. In addition to the absorption of 256 nm in the UV spectrum, due to the presence of natural bases, a strong absorption is also visible at 340 nm due to the presence of S^6-dG.

Table 1: Sequences of Triplex-Forming Oligonucleotides (TFOs) Used in This Study[a]

TFO Name	TFO Sequence
Z100-50	5'-ggggttgggggttgggggttgggg-3'
Z102-52	5'-ggggttggg*s*ggttgg*s*ggttg*s*ggg-3'
Z100-54	5'-g*s*g*s*tt*s*g*s*g*s*gtt*s*g*s*g*s*tt*s*g*s*g*s*-3'
Z100-55	5'-*ssss*tt*sssssss*tt*sssss*tt*sssss*-3
B106-62	5'-gtggtggtggtgttggtggtggtttgggggggtgggg-3
B106-82	5'-gtg*s*tggtggtgttg*s*tggtggtttgg*s*gggtgggg-3'
B106-83	5'-gtg*s*tggtg*s*tgttg*s*tggt*s*gtttgg*s*gggtg*s*gg-3'
B106-91	5'-*s*t*ss*t*ss*t*ss*ttt*ss*t*ss*t*ss*ttt*sssssss*tssss-3'
B106-92	5'-*s*tg*s*tg*s*tg*s*tgttg*s*tg*s*t*s*gtttt*s*g*s*g*s*gtg*s*g*s*-3'

[a] *s*, S^6-dG.

nyl]-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyguanosine (Gaffney et al., 1984) (**5**) with 1-methylpyrrolidine (10 equiv) and 2-mercaptopropionitrile (10 equiv) in CH_2Cl_2 for 2 h at room temperature gave the S^6-(2-cyanoethyl) thioether (**6**) in an 89% yield. Attempted desilylation of **6** using $[H_3C(CH_2)_3]_4NF$ in THF resulted in the removal of the cyanoethyl group along with the silyl protecting groups, yielding N^2-isobutyryl-2′-deoxy-6-thioguanosine. However, selective removal of the sugar protecting groups using the buffered desilylating reagent HF/$[H_3C(CH_2)_3]_4NF$ in dry pyridine (Jones et al., 1978; Gaffney & Jones, 1982) afforded N^2-isobutyryl-S^6-(cyanoethyl)-2′-deoxy-6-thioguanosine (**7**). Although this approach allows the usage of any type of protecting group for the amino function, it is demonstrated only using an isobutyryl group, as we reported earlier (Rao et al., 1992). Compound **7** was converted to the corresponding 5′-*O*-(dimethoxytrityl) derivative (**8**) by the conventional procedure, which upon treatment with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite in the presence of tetrazole and diisopropylamine gave the target building block (**9**).

Synthesis, Purification, and Characterization of TFOs Containing S^6-dG. As we planned to incorporate the monomeric synthon **9** into a natural oligonucleotide by standard phosphoramidite methodology, the possibility of oxidative hydrolysis (by iodine), nucleophilic substitution with NH_3 to form the 2,6-diaminopurine derivative, and the formation of dimeric disulfides were of concern. Therefore, in a preliminary study, samples of S^6-dG and **7** were treated separately with 35% aqueous NH_3 (concentrated NH_4OH, at ambient temperature, as well as at 55 °C) for 15 h. No hydrolysis or amination products were detected by TLC and HPLC procedures. Similarly, S^6-dG and **7** were dissolved separately in the oxidizing agent (I_2/pyridine/THF/H_2O),

Table 2: Effect of Salts on the Apparent K_d of Triplex Formation by 36-Base Oligomers[a]

TFO	sequence	apparent K_d (M) TBB[b]	+KCl[c]	+LiCl[c]
BRY106-1 (duplex target)	5'-gatcgctcctcctccgcgtcctcctctttccccccgccccgagcg-3' 3'-ctagcgaggaggaggcgcaggaggaggaaaggggggcggggctcgc-5'			
B106-62	5'-gtggtggtggtgttggtggtggtttgggggggtgggg-3'	6×10^{-9}	1×10^{-6}	6×10^{-9}
B106-82	5'-gtg*s*tggtggtgttg*s*tggtggtttgg*s*gggtgggg-3'	6×10^{-9}	3×10^{-8}	6×10^{-9}
B106-92	5'-*s*tg*s*tg*s*tg*s*tgttg*s*tg*s*t*s*gttt*s*g*s*g*s*gtg*s*g*s*-3'	3×10^{-8}	3×10^{-8}	3×10^{-8}
B106-91	5'-*s*t*ss*t*ss*t*ss*t*s*tt*ss*t*ss*t*ss*ttt*ssssss*t*ssss*-3'	1×10^{-6}	1×10^{-6}	1×10^{-6}

[a] *s*, S^6-dG. All binding reactions were performed at 37 °C for 24 h using 1×10^{-10} M labeled duplex (BRY106-1). [b] Binding reactions were performed in TBB buffer: 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 10% sucrose. [c] Binding reactions were performed in TBB buffer plus 50 mM indicated salt.

Table 3: Effect of Salts on the Apparent K_d of Triplex Formation by 26-Base Oligomers[a]

TFO	sequence	apparent K_d (M) TBB	+KCl
ZRY100-0 (duplex target)	5'-ccccttcccccctttcccccttcccccc-3' 3'-ggggaagggggggaagggggaagggggg-5'		
Z100-50	5'-ggggttgggggggttgggggttgggggg-3'	2×10^{-10}	5×10^{-8}
Z102-52	5'-ggggttggg*s*ggttgg*s*ggttg*s*ggg-3'	5×10^{-10}	3×10^{-8}
Z100-54	5'-g*s*g*s*tt*s*g*s*g*s*gtt*s*g*s*g*s*tt*s*g*s*g*s*-3'	2×10^{-7}	6×10^{-7}

[a] *s*, S^6-dG. Conditions were as described for Table 2, except that the concentration of duplex (ZRY100-0) was $\sim2.5\times10^{-11}$ M.

normally used during the oxidation step of the oligonucleotide synthesis cycle, and stirred at ambient temperature for 10 min. TLC and HPLC analyses indicated that no oxidation occurred.

Based on these preliminary studies, we further evaluated the standard deprotection conditions, *viz.*, concentrated NH_4OH at 55 °C for 15 h, with an oligonucleotide containing S^6-dG. A 36-base oligomer (B106-91) containing only S^6-dG and T was synthesized on an automated DNA synthesizer. The oligomer was deprotected with concentrated NH_4OH at 55 °C for 15 h and purified by HPLC. The purified oligonucleotide was subsequently hydrolyzed enzymatically (Gehrke et al., 1984) to the corresponding free nucleosides in order to determine the nucleoside composition and to check whether or not S^6-dG was converted to G during the deprotection step. The HPLC analysis (Figure 3) showed only S^6-dG and T in the expected ratio, indicating that standard deprotection conditions can be applied to other TFOs containing S^6-dG.

Once the deprotection conditions were established, a series of TFOs (Table 1) was prepared by the standard solid-support, phosphoramidite method on a 0.2−1 μmol scale on an ABI 380B automated DNA synthesizer. The coupling efficiency of **9** was optimized to 98% (as monitored by trityl cation release) by increasing the coupling time to 15 min. All base-labile protecting groups on the oligonucleotides were removed by treatment with concentrated NH_4OH at 55 °C for 15 h, and the TFOs were purified by HPLC using an ion-exchange Q-Sepharose (Pharmacia) column and the solvent systems described previously (Murphy et al., 1993). The purified product was desalted by passage through a C_{18} Sep-Pak (Waters) cartridge and analyzed on a 20% denaturing polyacrylamide gel after labeling with [^{32}P]ATP using polynucleotide kinase (Sambrook et al., 1989). Unmodified oligonucleotide was used as the standard for a comparison of mobility and purity. The modified TFOs were found to be >95% pure and with the expected lengths.

A.

TFO	sequence
Z400-50	5'-ggttggtgtggttgg-3'
Z400-51	5'-g*s*tt*s*gtgtg*s*tt*s*g-3'
Z400-52	5'-*ss*tt*ss*tgt*ss*tt*ss*-3'

s = S^6-dG

B.

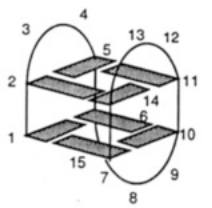


FIGURE 4: (A) Sequences of 15-base oligomers Z400-50, Z400-51, and Z400-52. The sequence of Z400-50 is identical to the one previously demonstrated to bind to thrombin (Bock et al., 1992). (B) Cartoon diagram of the folded structure previously described for the sequence of Z400-50 in the presence of potassium (Macaya et al., 1993; Wang, et al., 1993). The continuous line represents the oligonucleotide backbone, and the shaded rectangles represent guanines participating in G tetrads. The upper tetrad consists of g2, g5, g11, and g14, while the lower tetrad consists of g1, g6, g10, and g15. All T residues, as well as g8, are in the loops. Individual bases are numbered from the 5′-end.

The effect of S^6-dG on triplex formation in the absence and presence of monovalent cations were evaluated using an electrophoretic band shift assay (Durland et al., 1991). Apparent dissociation constants for B106 TFOs binding to a synthetic duplex, BRY106-1, are described in Table 2. The unmodified TFO, B106-62, binds with high affinity in the absence of added monovalent cations. The addition of 50 mM KCl reduced binding affinity by more than 100-fold. In contrast, 50 mM LiCl had little or no effect on binding. It has been shown previously (Durland et al., 1991) that potassium strongly promotes the formation of G-tetrad structures, while lithium generally does not. This suggests that, in the case of B106-62, G-tetrad formation is interfering with triplex formation in the presence of KCl.

Increasing substitutions of S^6-dG for G led to a weaker binding affinity relative to B106-62. Complete substitution of S^6-dG for G (B106-91) reduced binding affinity by more than 100-fold. At the same time, however, the effect of KCl on the affinity of a given TFO is reduced. In the case of

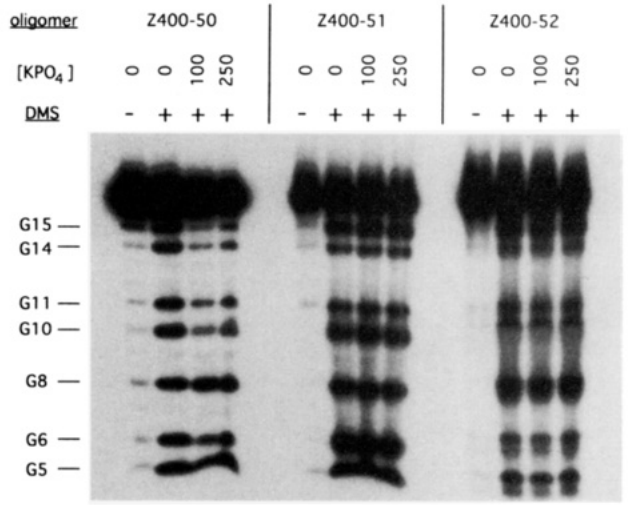


FIGURE 5: Dimethyl sulfate (DMS) protection studies of G-tetrad formation in oligomers Z400-50 (lanes 1−4), Z400-51 (lanes 5−8), and Z400-52 (lanes 9−12). End-labeled oligonucleotides were untreated (lanes 1, 5, and 9), or treated for 5 min with 1% DMS in either water (lanes 2, 6, and 10), 100 mM potassium phosphate (pH 6.1) (lanes 3, 7, and 11), or 250 mM potassium phosphate (lanes 4, 8, and 12). Subsequently, all samples were precipitated and treated with 2 M pyrrolidine at 90 °C for 20 min. After repeated drying to remove pyrrolidine, samples were electrophoresed through a 20% polyacrylamide/7 M urea gel, dried, and autoradiographed. Oligonucleotide fragments corresponding to cleavage at specific G residues are labeled (numbering from the 5′-end).

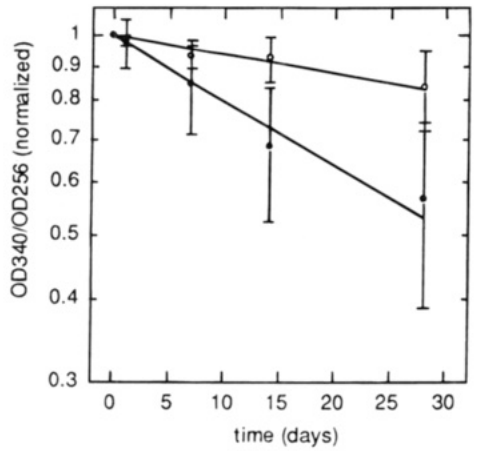


FIGURE 6: Kinetics of S^6-dG decay in aqueous solutions. TFOs containing S^6-dG (B106-82, B106-91, and B106-92) were incubated at 37 °C in water (○) or in aqueous solutions buffered to pH 7.6 (●). UV absorbance was periodically measured and expressed as the ratio OD_{340}/OD_{256}, normalized to the starting ratio for each TFO. Data are graphed as mean ± standard deviation (error bars) at each time. For samples in water, data were averaged for all TFOs tested. For samples in buffered solutions, data were averaged for all TFOs in five different buffers: TBB; TBB + 50 mM KCl; TBB + 50 mM NaCl; TBB + 50 mM LiCl; and 20 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 10 mM NaCl, 140 mM KCl, and 10% sucrose. TBB is 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 10% sucrose. No significant differences were observed when each of the five buffers was analyzed separately. Best-fit lines were calculated by fitting the data to the theoretical exponential decay curve, OD_{340}/OD_{256}-(norm) = e^{-kt}, where t is time and k is the decay constant. In water, $k = 0.006\,52\ s^{-1}$ and $t_{1/2} = 107$ days. At pH 7.6, $k = 0.0227\ s^{-1}$ and $t_{1/2} = 30.6$ days.

B106-91 and B106-92, the apparent dissociation constant was essentially identical in the absence and presence of 50 mM KCl. This suggests that the presence of S^6-dG is preventing G-tetrad formation and that triple-helix formation is no longer sensitive to potassium.

These data indicate that S^6-dG has opposing effects on triplex formation. The overall binding affinity is reduced. This suggests that S^6-dG•GC triplets are weaker than G•GC triplets, for reasons that are not presently understood. On the other hand, S^6-dG appears to reduce G-tetrad formation, probably by interfering with the previously proposed coordination of potassium cations. As a result, triplex binding affinities in the presence of potassium show an optimum with respect to percent substitution with S^6-dG. Binding of B106-82 and B106-92 in the presence of 50 mM KCl is better than binding of the fully substituted TFO, B106-91, or the parent TFO, B106-62.

We extended our studies to include a second triplex system, as described in Table 3, with similar results. In this system, the parent TFO, Z100-50, binds with high affinity to its target duplex in the absence of potassium, but binding is substantially reduced by the addition of 50 mM KCl. Substitution of S^6-dG for G reduces triplex formation in the absence of potassium. As before, however, binding in the presence of KCl passed through an optimum when some of the G's were replaced by S^6-dG.

To confirm directly that S^6-dG inhibits the formation of G-tetrad structures, we synthesized oligonucleotides having a sequence known to fold into an intramolecular complex under appropriate conditions. This compound (oligomer Z400-50, Figure 4A) was originally identified as a sequence capable of binding to thrombin (Bock et al., 1992) and was later shown to form a specific structure containing two stacked G tetrads (Figure 4B) (Wang et al., 1993; Macaya et al., 1993). We substituted some or all of the G residues known to participate in G-tetrad formation with S^6-dG and examined the effects on folding. Dimethyl sulfate (DMS) protection studies of the unmodified oligomer Z400-50 in H_2O showed approximately equal modification of all G residues (Figure 5). However, the addition of increasing concentrations of potassium phosphate buffer led to partial protection of the G residues known to participate in G tetrads. These include g5, g6, g10, g11, g14, and g15 (numbered from the 5′-end). Complete protection of these residues was observed in 100 mM KH_2PO_4. In contrast, g8, which is in a looped out region of the folded oligomer, was not protected by added K^+. Substitution of some or all of the G residues in oligomer Z400-50 with S^6-dG (oligomers Z400-51 and Z400-52; Figure 4A) abolished methylation protection in the presence of K^+. These data support the conclusion that S^6-dG destabilizes the formation of G tetrads.

This conclusion is contingent on the assumption that S^6-dG is stable in oligomers during extended incubation in aqueous solution. It has been suggested (Waters & Connolly, 1992) that S^6-dG incorporated into oligonucleotides is rapidly oxidized to G. In order to evaluate this possibility, we took advantage of the fact that S^6-dG absorbs strongly at ~340 nm, while G has no ultraviolet absorbance near that wavelength. We incubated several of the 36-base S^6-dG-containing oligomers in water or in various buffers at 37 °C and monitored the ratio of OD_{340}/OD_{256}. Oxidation of S^6-dG to dG would be manifested as a reduction of this ratio. This was clearly observed (Figure 6). In water, we observed an exponential decay in the OD_{340}/OD_{256} with a $t_{1/2}$ of about 107 days. Incubation in a number of different buffered solutions (such as those used to assay triplex formation) resulted in a somewhat faster decay rate, with an average $t_{1/2}$ of 30.6 days. These data indicate that S^6-dG is gradually oxidized to (presumably) G. However, the rate of oxidation

is very slow, with half-times of at least 1 month under the conditions employed. Thus, during a standard incubation at 37 °C for 24 h (the conditions normally used in assaying triplex formation), very little oxidation of S^6-dG takes place. Moreover, it was observed that the storage of S^6-dG-containing oligomers in water at 4 °C led to no detectable change in the OD_{340}/OD_{256} ratio even after *1 year*.

These data indicate that S^6-dG destabilizes G tetrads and reduces the tendency of G-rich oligomers to self-associate. The molecular basis for this effect is not known, but the existing hypotheses about G-tetrad formation provide a simple rationalization. As shown in Figure 2, G tetrads consist of a planar array of four G residues mutually H-bonded such that the oxygen atoms at position six of G surround a cavity in the center of the tetrad. Substitution of sulfur for these oxygens (as in S^6-dG) may disrupt the tetrad because of the significantly larger van der Waals radius of sulfur, leading to steric hindrance. In addition, it is believed that monovalent cations stabilize G tetrads by forming coordination complexes with the central oxygen atoms of one or two tetrads. Replacement of these oxygens with sulfur may reduce the strength of this interaction and destabilize the tetrad.

Regardless of the molecular basis for the destabilization, we have shown that it occurs in at least three systems. This provides us with a potential approach to increase the efficacy of TFOs *in vitro* and *in vivo*. Because Na^+ and especially K^+ are believed to be present in considerable concentrations within cells, it is reasonable to speculate that a substantial amount of the TFO molecules introduced into a cell are involved in G tetrads. This will reduce or prevent their ability to bind to the target DNA sequence. If G-tetrad formation can be reduced or eliminated, the apparent concentration of free TFO will increase considerably, leading to increased biological efficacy at lower TFO concentrations.

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