Synthesis of oligonucleotides containing 2'-deoxy-6-thioguanosine at a predetermined site

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ABSTRACT

A new approach has been devised for the synthesis of oligonucleotides containing 2'-deoxy-6-thioguanosine [d(s⁶G)]. The synthesis of oligonucleotides containing d(s⁶G) requires special protection and deprotection strategies to prevent the thione functionality from undergoing oxidation and hydrolysis. Previous attempted syntheses have neglected to address this problem. By using the cyanoethyl protecting group for the thione and phenoxyacetyl for the exocyclic amino group, it was possible to deprotect oligonucleotides with a mixture of sodium hydroxide and sodium hydrogen sulfide without any significant conversion of d(s⁶G) to deoxyguanosine. Application of this strategy will allow investigation of the structural as well as biological activity of d(s⁶G)-containing oligonucleotides.

INTRODUCTION

The purine antimetabolite 6-thioguanine (s⁶G) has been used for more than thirty years for the treatment of human malignancies, especially leukemias (1). Despite the widespread clinical use of thioguanine, the precise molecular basis of its action is not entirely clear. Evidence from a large number of research groups over many years has rather firmly identified incorporation of thioguanine into DNA as the primary locus of action of the mercaptopurine antimetabolites (2, 3, 4, 5, 6, 7). These correlations between incorporation into DNA and cytotoxicity have found some physicochemical basis from studies describing the overall effect of incorporating thioguanine into nucleic acids in place of guanine (8, 9, 10). It has also been established that NH---S hydrogen bond lengths are greater and hydrogen bond strengths are less than is the case with the NH---O hydrogen bonds (11). More specifically, Bugg has proposed (12) that significant distortions in nucleic acid helices would probably result from the alterations in C⁶-S bond lengths and NH---S versus NH---O hydrogen bond lengths in s⁶G-C base pairs. To date, however, no one has precisely defined what the nature of such distortions might be or how they might exert such a profound effect upon cell replication.

An appropriate way to study this problem is to create oligonucleotides containing s⁶G-C base pairs at uniquely defined positions, and then to carry out appropriate physicochemical studies to define the geometry of these base pairs vis-à-vis the normal G-C base pair. Although several studies have appeared describing incorporation of thioguanine into nucleic acids (8–10, 13), we are aware of only three reports describing approaches to the specific incorporations of 6-thioguanine into chemically synthesized oligonucleotides.

The first of these reports was a proceedings abstract (14). This brief report described the synthesis of a DNA octamer containing s⁶G. Few data were provided and there was no indication as to the percentage of s⁶G as opposed to G in the final product.

The second was a report by Rappaport claiming the synthesis of dodecamers containing 2'-deoxy-6-thioguanosine. His procedure was laborious and time-consuming and little evidence was presented to provide assurance that oxidative loss of the thione chromophore from the deprotected oligonucleotides had not taken place. Rappaport also stated the method was not suited for preparing sufficient amounts of material for NMR studies (15).

The third report was a patent application published under the patent cooperation treaty (16). This document describes standard machine chemistry without special protection of the thione moiety of d(s⁶G). In our hands, the deprotection procedures required to liberate the free oligonucleotide as described in this patent lead to the formation of substantial amounts of guanine from 6-thioguanine.

None of the syntheses reported to date have involved the use of protecting groups for the thione functionality present in 2'-deoxy-6-thioguanosine. The protection of this functionality is critical to prevent oxidation and hydrolysis that is common in sulfur-containing nucleotides.

We have developed a novel protection/deprotection strategy that allows for the synthesis of large amounts of DNA containing d(s⁶G) by modifying the automated phosphoramidite approach to DNA synthesis (17). This paper describes how such a strategy was conceived, the results of an illustrative oligonucleotide synthesis and preliminary characterization data which reveal that NMR spectroscopy will be a valuable technique in assessing the consequences of replacing G with s⁶G in oligodeoxynucleotides.

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EXPERIMENTAL

The ^1H -NMR spectra were recorded on either an IBM AF-200 MHz FT-NMR spectrometer or a Varian Unity 500 MHz NMR spectrometer. NMR samples were prepared using $(\text{CD}_3)_2\text{SO}$ or CDCl_3 with tetramethylsilane (TMS) as the internal standard, or D_2O with DSS (2,2-dimethyl-2-silapentanesulfonic acid). The UV spectra were recorded on a Beckman DU-8 spectrophotometer. Low resolution and high resolution fast atom bombardment (FAB) mass spectra were obtained on a Varian MAT 731 mass spectrometer using a glycerol matrix. HPLC analysis and purification were performed on a Hitachi HPLC system equipped with an L-3000 photodiode array detector. Thin layer chromatography (TLC) was performed on Kieselgel 60 F254 aluminum-backed silica gel sheets.

The enzymatic digestions were performed using snake venom phosphodiesterase (*Crotalus adamanteus*) (SVP) and bacterial alkaline phosphatase (BAP), both purchased from Sigma Chemical Company. Purified dimer or octamer (5–10 μg) was incubated with SVP (0.018 U) and BAP (2 U) overnight at 37°C in total volume of 78.2 μL containing 32 mM tris buffer pH 7.5 and 15 mM MgCl_2 . Nucleosides were recovered by the addition of 10 μL of 2.5 M sodium acetate and 234 μL of 95% ethanol, mixing and cooling to -70°C for 30 minutes, followed by centrifugation at 12,000 rpm for 15 minutes. The supernatant was collected and diluted to 1 mL with 95% ethanol, chilled, recentrifuged, recollected and evaporated to dryness. The material was then dissolved in water and subjected to HPLC using a 5 μm Supelcosil LC-18S column at a flow rate of 1.2 mL/min. Solvent A: 50 mM potassium phosphate pH 7.6, solvent B: acetonitrile. Gradient: 0 minutes, 2% B; 10 minutes, start linear gradient to 25% B over 18 minutes; 30 minutes, 25% B for 5 minutes.

 N^2 -Phenoxyacetyl-2'-deoxy-6-thioguanosine (II)

2'-Deoxy-6-thioguanosine (18) (0.283 g, 1 mmol) (**I**) was coevaporated with dry pyridine (5 $\times$ 5 mL) then suspended in pyridine (6 mL). Trimethylsilyl chloride (0.63 mL, 5 mmol) was added via a dry syringe and the reaction was stirred for 1 hour. Phenoxyacetyl chloride (0.166 mL, 1.2 mmol) was added via a dry syringe and the reaction was allowed to stir at room temperature for 4 hours. The reaction vessel was cooled in an ice bath and water (1 mL) was added with stirring. After 15 minutes concentrated ammonium hydroxide (1 mL) was added and the slurry was stirred for an additional 15 minutes. The slurry was concentrated under vacuum and poured into a separatory funnel containing water (35 mL). The aqueous layer was collected and concentrated to one-half its original volume. After one night at 5°C the title compound (**II**) had crystallized and was collected to give 0.268 g (65%). $R_f = 0.57$ in chloroform/methanol 70/30 (v/v); ^1H -NMR, δ , [$(\text{CD}_3)_2\text{SO}$] 13.07 (s, 1H, NH-CO), 12.08 (s, 1H, N¹-H), 8.42 (s, 1H, H8), 7.3 (m, 5H, phenoxy), 6.21 (t, 1H, H1'), 5.27 (s, br, 1H, OH), 5.0 (s, br, 1H, OH), 4.8 (s, 2H, Pac-CH₂), 4.37 (m, br, 1H, H3'), 3.84 (m, 1H, H4'), 3.51 (m, 1H, H5' + 5''), 2.26–2.66 (m, 2H, H2' + 2''); UV λ_{max} : pH 11.0, 313 nm, 253 nm; FAB/MS (MH)⁺, obsd 418. The purity of compound **II** was assessed by HPLC and high resolution mass spectrometry. In an isocratic solvent system of 65% 0.1 M TEAA/35% CH₃CN with a Supelco LC-18S analytical column at 1 mL/min compound **II** eluted as a single species (98% purity at 320 nm detection). High resolution mass spectrometry; mass calculated (MH⁺) 418.1187, mass found (MH⁺) 418.1210.

 S^6 -Cyanoethyl- N^2 -phenoxyacetyl-2'-deoxy-6-thioguanosine (III)

To a solution of dry N^2 -phenoxyacetyl-2'-deoxy-6-thioguanosine (**II**) (0.25 g, 0.6 mmol) in dry DMF (6 mL) was added powdered anhydrous potassium carbonate (0.166 g, 1.2 mmol). This was followed by the addition of 3-bromopropionitrile (50 μL , 0.6 mmol) via a dry syringe. After stirring at room temperature for 3 hours the DMF was removed by coevaporation with xylenes and the residue was recrystallized from hot absolute ethanol. After storage overnight at 0°C the title compound (**III**) was collected to give 0.225 g (80%). $R_f = 0.68$ in chloroform/methanol 70/30 (v/v). ^1H -NMR, δ , [$(\text{CD}_3)_2\text{SO}$] 10.83 (s, 1H, NH-CO), 8.57 (s, 1H, H8), 7.3 (m, 2H, phenoxy), 6.95 (m, 3H, phenoxy), 6.35 (t, 1H, H1'), 5.3 (d, 1H, OH), 5.0 (s, 2H, Pac-CH₂), 4.9 (s, 1H, OH), 4.4 (m, br, 1H, H3'), 3.85 (m, 1H, H4'), 3.45–3.65 (m, 4H, H5' + H5'' + CH₂-S), 3.10 (t, 2H, CH₂-CN), 2.26–2.80 (m, 2H, H2' + H2''); UV λ_{max} MeOH; 300 nm sh, 293 nm, 267 nm, 245 nm; FAB/MS (MH)⁺, obsd 471. The purity of compound **III** was assessed by HPLC and high resolution mass spectrometry. In an isocratic solvent system of 65% 0.1 M TEAA/35% CH₃CN with a Supelco LC-18S analytical column at 1 mL/min compound **III** eluted as a single species (95% purity at 290 nm). High resolution mass spectrometry; mass calculated (MH⁺) 471.1452, mass found (MH⁺) 471.1432.

 S^6 -Cyanoethyl-5'-O-dimethoxytrityl- N^2 -phenoxyacetyl-2'-deoxy-6-thioguanosine (IV)

S^6 -Cyanoethyl- N^2 -phenoxyacetyl-2'-deoxy-6-thioguanosine (**III**) (0.41 g, 0.87 mmol) was dried by coevaporation with dry pyridine (2 $\times$ 10 mL) and then dissolved in pyridine (5 mL). The solution was cooled in an ice bath and 4,4'-dimethoxytrityl chloride (0.325 g, 0.96 mmol) was added to the solution. The reaction was allowed to stir overnight at 5°C and then examined for completion by TLC (chloroform/methanol 90/10). Methanol (2 mL) was then added to the reaction mixture. After stirring for 10 minutes the solvents were removed under vacuum. The residue was dissolved in ethyl acetate, washed with aqueous sodium bicarbonate (3 $\times$ 25 mL), water (1 $\times$ 25 mL) and dried over anhydrous sodium sulfate. The product was then purified by silica gel column chromatography using a 0–3% methanol/chloroform gradient to yield pure compound (**IV**) 0.60 g (89%). $R_f = 0.71$ in chloroform/methanol 90/10 (v/v). ^1H -NMR, δ , (CDCl_3); 8.93 (s, 1H, NH-CO), 8.06 (s, 1H, H8), 6.9–7.5 (m, 13H, DMTr), 6.7–6.9 (m, 5H, phenoxy), 6.5 (t, 1H, H1'), 4.80 (s, br, 1H, OH), 4.62 (s, 2H, Pac-CH₂), 4.16 (d, 1H, H3'), 3.75 (s, 6H, 2OCH₃) + (m, 1H, H4'), 3.57 (t, 2H, CH₂-S), 3.2–3.4 (m, 2H, H5' + H5''), 3.06 (t, 2H, CH₂-CN), 2.5–2.9 (m, 2H, H2' + H2''); UV λ_{max} abs EtOH; 300 nm sh, 289 nm, 265 nm, 240 nm sh.

 S^6 -Cyanoethyl-5'-O-dimethoxytrityl- N^2 -phenoxyacetyl-2'-deoxy-6-thioguanosine 3'-O-diisopropylaminocynoethyl phosphoramidite (V)

S^6 -cyanoethyl-5'-O-dimethoxytrityl- N^2 -phenoxyacetyl-2'-deoxy-6-thioguanosine (**IV**) (0.30 g, 0.39 mmol) was dried by coevaporation with dry dichloromethane and pyridine (2 $\times$ 1 mL each) and then dissolved in dry dichloromethane (1 mL) and placed under nitrogen. To this stirred solution was added via a dry syringe under nitrogen, tetrazole (0.014 g, 0.2 mmol) and diisopropylamine (28 μL , 0.2 mmol) dissolved in dichloromethane (1 mL) followed by addition of 2-cyanoethyl

N, N, N', N'-tetraisopropylphosphorodiamidite (0.13 g, 0.43 mmol) dropwise via a dry syringe under nitrogen. The reaction was allowed to stir at room temperature for 35 minutes. TLC (chloroform/methanol 90/10) indicated the reaction had proceeded to completion. The reaction mixture was filtered and the solvent was removed under vacuum. The residue was dissolved in argon-saturated ethyl acetate, washed with ice cold 10% sodium carbonate (2 × 15 mL), dried over anhydrous sodium sulfate, and the solvent was removed under vacuum. TLC indicated several impurities were present. The product was then purified by silica gel column chromatography using a 1%-triethylamine, 5%-hexanes, 94% chloroform elution mixture to yield the pure compound (V) 0.3 g (79%). $R_f = 0.85$ in chloroform/methanol 90/10 (v/v). $^1\text{H-NMR}$, δ , [(CD₃)₂SO]; 10.78 (s, 1H, NH-CO), 8.49 (s, 1H, H8), 6.7–7.3 (m, 18H, phenoxy + DMTr), 6.42 (t, 1H, H1'), 4.95 (s, CH₂-Pac), 4.7 (m, br, 1H, H3'), 4.1 (m, 1H, H4'), 3.7 (s, 6H, 2 OCH₃), 3.6 (m, 2H, H5' + H5''), 3.15 (t, 2H, -CH₂-), 2.90 (t, 2H, -CH₂-), 2.75 (t, 2H, -CH₂-), 2.60 (t, 2H, -CH₂-), 2.4–2.6 (m, 2H, H2' + H2''), 0.90–1.10 (m, 12H, diisopropyl). $^{31}\text{P-NMR}$ spectrum (CH₂Cl₂, external capillary standard of 85% H₃PO₄) 149.27 and 149.43 ppm.

d[[DMTr-S⁶CE(s⁶G^{Pac})]p^{CE}TAc]

S⁶-Cyanoethyl-5'-O-dimethoxytrityl-N²-phenoxyacetyl-2'-deoxy-6-thioguanosine-3'-O-diisopropylaminocycanoethyl phosphoramidite (V) (0.15 g, 0.16 mmol) and 3'-O-acetylthymidine (0.045 g, 0.18 mmol) were placed in a flask and dried overnight on a vacuum pump at room temperature. Dry tetrazole (0.070 g, 0.80 mmol) was dissolved in freshly distilled acetonitrile (1.5 mL) and added to the nucleoside containing flask with a dry syringe under nitrogen. After being allowed to stir for 10 minutes a stock 0.2 M I₂ solution (1 mL) was added and the solution was stirred for an additional 10 minutes. The solvent was removed under vacuum and the residue was dissolved in chloroform (10 mL) and washed with saturated sodium thiosulfate. The organic phase was collected, dried over anhydrous sodium sulfate and evaporated to dryness. The product was purified by silica gel column chromatography using a 0.5–4.0% gradient of methanol/chloroform to yield the pure product 0.11 g (63%). UV and NMR data confirmed the structure of the dinucleotide.

Deprotection and purification of d[[DMTr-S⁶CE(s⁶G^{Pac})]p^{CE}TAc]

An aliquot of the protected dinucleotide (20 mg) was dissolved in a 2% solution of trichloroacetic acid in dichloromethane (3 mL) and allowed to stand at room temperature for 10 minutes. A bright orange color formed immediately. Chloroform (20 mL) was added to the reaction mixture and the solution was washed with 5% sodium bicarbonate (2 × 20 mL) and water (2 × 20 mL). The organic layer was collected, evaporated to dryness and dissolved in a 1 M NaOH/0.01 M NaSH solution (10 mL). After 4 hours at room temperature the solution was neutralized by the addition of concentrated HCl. The deprotected dinucleotide was chromatographed on a Whatman Partisil 10 PAC (90 mm × 50 cm) magnum column at 1.5 mL/min with an isocratic mobile phase consisting of 18% acetonitrile in 50 mM aqueous ammonium bicarbonate (pH 7.6). A small amount of pure material (5–10 µg) was collected, lyophilized and subjected to enzymatic digestion and HPLC analysis.

Synthesis and purification of 5'-d(ApApApCps⁶GpTpTpT)-3'-S⁶-Cyanoethyl-5'-O-dimethoxytrityl-N²-phenoxyacetyl-2'-deoxy-6-thioguanosine-3'-O-diisopropylaminocycanoethyl phosphor-

amidite (V) (0.3 g, 0.31 mmol) was dissolved in anhydrous acetonitrile (3 mL) and placed into position on an ABI 380B DNA synthesizer. The DNA synthesis was carried out on 10 µm ABI column containing thymidine at the 3' end with FOD phosphoramidites purchased from ABI. It was found through the use of the trityl assay that the coupling of (V) was lower than expected for a DNA synthesizer. After the synthesis was complete the column was treated with 15 mL of 1 M NaOH/0.01 M NaSH for 3 hours at room temperature. The solution was neutralized to pH 7 and the product of the synthesis was purified by HPLC. The oligomer was chromatographed on a Keystone ODS/H (10 × 250 mm) column at 1 mL/min with an isocratic mobile phase consisting of 15% acetonitrile in 50 mM ammonium bicarbonate (pH 7.6). Use of a diode array detector allowed us to easily identify the peak that contained the 8-mer due to the characteristic long wavelength absorbance of d(s⁶G). A small amount of octamer (20 µg) was collected and lyophilized. The homogeneity of the octamer was then assessed by high performance gel electrophoresis on an ABI 23A HPEC system. An 8% PAGE-7M urea gel was poured in a 3.5 × 100 mm tube and pre-electrophoresed in a 0.5–1.1 mA current gradient. Elution of the gel and UV detection at 254 nm showed the octamer existed as a pure single species.

Electrospray mass spectrometry

The 8-mer was purified by reverse-phase HPLC using a Supelco LC-18S column and a gradient solvent system consisting of 25 mM Et₃NH HCO₃⁻, pH 7, containing 2% CH₃CN (A) and 40% aqueous CH₃CN (B). The linear gradient proceeded from 5–40% B over 70 minutes. The octamer peak was collected, evaporated to dryness and dissolved in 1 mL of 95% aqueous MeOH. An electrospray ionization negative ion mass spectrum was obtained with a Vestec 201 ionization instrument and a quadrupole detector operating in the negative ion mode. The mass spectrum was obtained at a flow rate of 3 µL/min. with the entire experiment consuming a total of 1.4 µg of sample.

NMR experiments

Approximately 6 mg of the 8-mer were purified by reverse-phase HPLC as previously described. The material was collected and lyophilized twice from 99.9% D₂O and then dissolved in 400 µL of 99.996% D₂O containing 100 mM NaCl and 20 mM potassium phosphate buffer, pH 6. One and two-dimensional proton data sets were collected on a Varian Unity 500 NMR spectrometer. Two-dimensional phase-sensitive NOESY spectra of the 8-mer were collected at 10°C (to assure complete duplex formation) using a 150 ms mixing time and a spectral width of 5000 Hz. The data sets were acquired in hypercomplex mode with 800 t_1 increments, 32 scans per fid, and 2048 complex points in t_2 . The t_2 dimension was processed with a gaussian filter and 1 Hz of line broadening. The t_1 increments were zero filled to 2048 points and transformed with a gaussian apodization function.

RESULTS AND DISCUSSION

Our approach to the synthesis of an oligonucleotide containing d(s⁶G) involves the synthesis of a protected d(s⁶G) phosphoramidite species which was inserted into a DNA oligomer through the use of an automated DNA synthesizer. It was important to develop a protection strategy for d(s⁶G) that would protect it from degradation and side reactions throughout the

Figure 1. Reaction scheme for the synthesis of the protected d(s⁶G) phosphoramidite. Reagents: i. trimethylsilyl chloride, phenoxyacetyl chloride in pyridine, conc. ammonium hydroxide in water; ii, 3-bromopropionitrile and potassium carbonate in DMF; iii, 4,4'-dimethoxytrityl chloride in pyridine; iv, 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite, tetrazole and diisopropylamine in dichloromethane.

DNA synthesis cycle. It was equally important to ensure that the protecting groups chosen would be removed quantitatively at the end of the oligonucleotide synthesis.

Several issues made the choice of protecting groups for d(s⁶G) more complicated than standard bases. In a variety of model deprotection experiments, it was found that 6-thioguanosine was not stable to the deprotection conditions used in standard DNA synthesis; namely, concentrated ammonium hydroxide at 55°C for 12 hours in sealed vials. The instability of d(s⁶G) under these conditions has been reported previously (15). It was also found that prolonged exposure of 6-thioguanosine to I₂, the agent used to oxidize the phosphite to the phosphate in automated DNA synthesis, led to sulfur oxidation. These results indicate earlier workers attempting to synthesize oligonucleotides containing d(s⁶G) without the use of thione protection probably had some d(s⁶G) degradation and, if so, did not synthesize pure oligonucleotides.

The results of these preliminary experiments suggest the requirement for novel protection strategies for both the exocyclic amino and thione functional groups of d(s⁶G). Since cleavage of the oligonucleotide from the solid support at the end of the automated DNA synthesis requires basic conditions, we set out to incorporate protecting groups that would be removed under

Figure 2. A, Reverse-phase HPLC profile of d(AAAs⁶GTTT) in 15% acetonitrile/50 mM ammonium bicarbonate at 1 ml/min. B, Diode array detected UV spectrum of d(AAAs⁶GTTT).

mild basic conditions. A protection strategy was sought that would allow all the protecting groups, including those used to protect the internucleotide linkage, to be removed in a single step.

The phenoxyacetyl (Pac) protecting group was chosen for protection of the exocyclic amino group of d(s⁶G). It has been shown to be removed very quickly in concentrated ammonium hydroxide at room temperature and yet be stable enough to be used in automated DNA synthesis (19, 20). The choice of a protecting group for the thione functionality of d(s⁶G) proved to be more challenging.

A thorough review of the literature led to examination of the use of the cyanoethyl group (CE) to protect the S⁶ position of d(s⁶G). The cyanoethyl group has been previously reported for protection of the O⁶ position of guanosine (21), and thione function of 4-thiouridine (22). Synthesis of S⁶-cyanoethyl-N₂-phenoxyacetyl-6-thioguanosine was carried out to examine suitable deprotection conditions. HPLC analysis revealed that four hours in concentrated ammonium hydroxide at room temperature led to complete deprotection and quantitative production of 6-thioguanosine. These data encouraged attempts at the synthesis of dinucleotides containing protected d(s⁶G) and examination of similar deprotection conditions.

The route to the fully protected d(s⁶G) phosphoramidite is illustrated in Figure 1. The synthesis of d(s⁶G), compound I, was carried out as previously described by Robins and Revankar (18). Phenoxyacetylation of the exocyclic amino group of d(s⁶G) was achieved with phenoxyacetyl chloride in dry pyridine by modifying a procedure previously described for guanosine (20). The transient protection method using trimethylsilyl chloride was used to block the hydroxyl positions during acylation. Protection of d(s⁶G^{Pac}), compound II, at the S⁶-position involved deprotonation at N¹ followed by alkylation at S⁶ with 3-bromopropionitrile in DMF to yield dS⁶CE(s⁶G^{Pac}), compound III (23). Treatment of compound III with

Figure 3. Expanded contour plot of the phase sensitive NOESY spectrum (mixing time 150 ms) of d(AAAs⁶GTTT)₂ in D₂O buffer, pH 6.0 at 10°C. The sequential connectives from A1 to T8 through H8/H6-H1' cross-peaks are shown by continuous lines. The intraresidue cross-peaks are labeled.

dimethoxytrityl chloride protected the 5'-OH with the acid labile, 4, 4'-dimethoxytrityl (DMTr) group. The final step in the synthesis involved activation of the 3'-OH by treating d[DMTr-S⁶CE(s⁶G^{Pac})], compound IV, with 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite in the presence of diisopropylamine and tetrazole in dichloromethane (19) to produce d[DMTr-S⁶CE(s⁶G^{Pac})]-3'-diisopropylaminocynoethyl phosphoramidite, compound V. Coupling compound V to 3'-O-acetylthymidine in the presence of tetrazole in acetonitrile followed by oxidation of the phosphite to the phosphate with I₂ led to the synthesis of the dinucleotide d[[DMTr-S⁶CE(s⁶G^{Pac})]p^{CE}TAc]. This dinucleotide was then used to evaluate the effectiveness of the deprotection conditions developed.

Deprotection of the synthesized dinucleotide in concentrated ammonium hydroxide at room temperature for four hours led to the production of two products as judged by reverse-phase HPLC. Enzymatic digestion (24) and HPLC analysis of the products revealed approximately 25% of the d(s⁶G) had been converted to deoxyguanosine during deprotection. The labile nature of the thione moiety had been encountered previously in the synthesis of polyribonucleotides containing thiopurines (10, 13). The mechanism of the d(s⁶G) to d(G) conversion is assumed to involve oxidation of sulfur followed by displacement by hydroxide. It has been shown that 6-thioinosine may be oxidized to the corresponding 6-sulfonate in the presence of oxygen; this oxidation product then undergoes hydrolysis to inosine (25). A similar mechanism of degradation takes place in the conversion of 6-thioguanosine to guanosine. This conversion is especially prevalent at elevated temperature or pH (10, 13).

These data led to the investigation of several alternative sets of deprotection conditions. Strongly basic conditions were required to induce B-elimination to remove the cyanoethyl group present at S⁶ of d(s⁶G) and the phosphate linkage. Yet prevention of the displacement of the oxidized thione species by hydroxide, which converts d(s⁶G) into dG during deprotection, was needed. It was reasoned the addition of a highly nucleophilic species in the deprotection solution would compete for and prevent displacement by hydroxide, thereby preventing formation

Table 1. Nonexchangeable ¹H Chemical Shifts (ppm) of d(AAAs⁶GTTT)₂ in D₂O containing 0.1 M NaCl, 20 mM potassium phosphate buffer (pH 6.0) at 10°C.

	H8	H6	H5/CH3	H1'	H2', H2''	H3'	H4'
A1	7.70			5.53	2.12, 2.35	4.53	3.90
A2	7.84			5.43	2.38, 2.47	4.72	4.08
A3	7.78			5.83	2.26, 2.52	4.69	4.16
C4		6.86	4.87	5.20	1.70, 2.20	4.44	3.87
s ⁶ G5	7.56			5.63	2.28, 2.44	4.61	4.06
T6		7.01	0.90	5.82	1.87, 2.27	4.57	3.95
T7		7.24	1.35	5.91	1.94, 2.28	4.58	3.91
T8		7.15	1.30	5.98	2.00, 2.00	4.30	3.74

of dG. The use of thiolate proved ideal because the displacement of any oxidized thione species by thiolate would produce d(s⁶G). It is also likely the thiolate possesses anti-oxidant properties. It was found that a 1 M solution of NaOH containing 0.01 M NaSH could deprotect d(s⁶G) without detectable formation of d(G). Deprotection experiment conducted on the protected dinucleotide d[[DMTr-S⁶CE(s⁶G^{Pac})]p^{CE}TAc] in 1 M NaOH/0.01 M NaSH at room temperature for four hours led to a single product by reverse-phase HPLC. Enzymatic digestion (23) of the deprotected dinucleotide and HPLC analysis of the products indicated d(s⁶G) and thymidine were present. No detectable amount of d(G) had been formed during deprotection.

These results encouraged implementation of this newly-developed protection/deprotection strategy in the synthesis of an oligonucleotide (8-mer) containing d(s⁶G) with the use of an automated DNA synthesizer. However, an HPLC experiment revealed the normal exocyclic amino protecting groups employed in automated DNA synthesis; namely, isobutryl for guanine and benzoyl for cytosine and adenine, were not removed under the deprotection conditions employed.

The recently reported FOD phosphoramidites developed by Applied Biosystems were designed to contain protecting groups that are removed under mildly basic conditions. Their use was ideal for this synthesis. A deprotection experiment revealed the FOD phosphoramidites were deprotected in three hours at room temperature when subjected to 1 M NaOH/0.01 M NaSH.

The automated DNA synthesis of a self-complementary oligonucleotide, 5'-d(ApApApCps⁶GpTpTpT)-3' was carried out on a 10 μmol scale with a 380-B Applied Biosystems Automated DNA synthesizer. Deprotection of the synthesized 8-mer with 1 M NaOH/0.01 M NaSH for three hours at room temperature led to one major product by HPLC as shown in Figure 2. The UV spectrum of the product was consistent with the bases contained in the 8-mer.

It has been previously reported that the enzymatic digestion of oligonucleotides containing d(s⁶G) at an internal site presented some difficulty (15). However, enzymatic degradation of the synthesized 8-mer followed by reverse-phase HPLC of the constituent nucleosides proceeded smoothly to give a ratio of 1:1:3.3:3.3, (dC:d(s⁶G):dT:dA) based on the extinction coefficients at 260 nm and the peak areas (23).

The 8-mer was further characterized by electrospray mass spectrometry. Since the quadrupole detector has a mass limit of 2000 amu, it was impossible to detect the (M-H) ion because it was outside the mass range. However, the (M-2H), (M-3H) and (M-4H) were all observed and measured. Based on these mass measurements, it was possible to obtain a Mr for the

d(s⁶G)-containing octamer of 2424.8 amu, in excellent agreement with the calculated Mr of 2424.7 amu.

The octamer was also characterized by high field two-dimensional NMR experiments. NMR evidence indicated the structure of the double-stranded octamer was generally consistent with normal B-DNA. Figure 3 represents an expanded contour plot of the phase-sensitive 500 MHz NOESY spectrum of the octamer in D₂O buffer, apparent pH 6.0 at 10°C. All of the expected aromatic H1' crosspeaks were visible which made assignments possible based on sequence-specific assignment techniques (26).

Assignment of the A1-H1' is straightforward since the H8 on A1 can only give a crosspeak to its own H1'. However, the H1' of A1 gives a crosspeak to the H8 of A1 as well as the H8 of A2, thereby linking A1 to A2. This process was then used to assign all of the aromatic and H1' resonances in the octamer. The H2', 2'' resonances were assigned by evaluation of the crosspeaks between the assigned aromatic protons and the H2', 2'' protons. These assignments were confirmed by the NOE between the assigned H1' protons and the H2', 2'' protons. The H3' protons were then assigned based on the NOE generated between the assigned aromatic protons and the H3' protons. The H3' assignments were confirmed by the NOE between the H3' and the assigned H2', 2'' protons.

Assignments of the H4' protons were made based on NOE between the assigned H1' protons and the H4' protons. These assignments were confirmed by the NOE generated between the H4' protons and the assigned H3' resonances. A summary of the assignments, both the base and sugar protons, is shown in Table 1.

It is interesting to note the low intensity of the C4-H6 to C4-H1' crosspeak. This could represent a structural perturbation caused by the d(s⁶G) which would be expected to hydrogen bond to C4. The significance and implications of this structural effect of d(s⁶G) are under investigation using distance geometry and molecular graphics techniques.

Application of the synthetic strategy described in this paper will lead to the synthesis of oligonucleotides containing d(s⁶G) without the possibility of degradation to dG. Investigations into the structural as well as biological effects of d(s⁶G)-containing oligonucleotides will be aided by the availability of this technology. Work is currently under way in this laboratory to investigate further the effects that the insertion of d(s⁶G) for d(G) has on the overall structure of DNA and how these structural changes are involved in mechanism of action of 6-TG.

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