Synthesis of Oligodeoxynucleotides Containing Analogs of O^6-Methylguanine and Reaction with O^6-Alkylguanine–DNA Alkyltransferase†

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Received February 2, 1994; Revised Manuscript Received May 25, 1994®

ABSTRACT: O^6-Alkylguanine–DNA alkyltransferase (AGT) repairs the mutagenic O^6-methylguanine (O^6-mG) lesion by transferring a methyl group from the 6-position of guanine to a cysteine residue on the protein. The simplest possible mechanism is an S_N2 process in which the cysteine displaces the methyl group off of the guanine in a concerted reaction. To probe the interactions between the protein and guanine leaving group, oligodeoxynucleotide duplexes containing analogs of O^6mG were synthesized and then reacted with AGT. The analogs, which were incorporated into deoxynucleotides include O^6-methylhypoxanthine (O^6-mH), S^6-methyl-6-thioguanine (S^6mG), S^6-methyl-6-thiohypoxanthine (S^6mH), Se^6-methyl-6-selenoguanine (Se^6mG), Se^6-methyl-6-selenohypoxanthine (Se^6mH), O^6-methyl-1-deazaguanine (O^6m1DG), O^6-methyl-3-deazaguanine (O^6m3DG), and O^6-methyl-7-deazaguanine (O^6m7DG), differ from O^6mG in that the heteroatoms have been replaced so that they are poorer hydrogen bond participants and proton acceptors. AGT was reacted with oligonucleotide duplexes of the sequence 5′-GGC GCT XGA GGC GTG-3′ in which X was O^6mG or an analog in which X was paired with C. The reactions in 50 mM Tris-HCl and 1 mM EDTA, pH 7.6 and 37 °C, were followed by anion-exchange HPLC in 10 mM NaOH with a NaCl gradient. All detected reactions were demethylations of the oligodeoxynucleotides except for O^6m3DG, which reacted in an unknown manner. The second-order rate constants obtained are as follows (M^{-1} s^{-1}): O^6mG, $(7.7 \pm 0.9) \times 10^5$; O^6mH, $(5.4 \pm 0.7) \times 10^6$; S^6mG, $(1.7 \pm 0.2) \times 10^3$; S^6mH, not detected; Se^6mG, $(4.1 \pm 1.6) \times 10^3$; Se^6mH, not detected; O^6m1DG, $(2.2 \pm 0.9) \times 10^3$; O^6m3DG, $(7.7 \pm 2.5) \times 10^4$; O^6m7DG, $(3.1 \pm 1.0) \times 10^4$. The large decreases in rate observed for changing the oxygen at the 6-position and the ring nitrogen at the 1-position suggest that these sites are hydrogen bond acceptors and/or proton acceptors during the reaction. The potential hydrogen bond from the protein to the 1-position of O^6mG as well as the increase in rate observed for O^6mH suggests that the duplex opens up in order for the reaction to occur.

O^6-Methylguanine (O^6mG)[1] is a potent mutagenic lesion that may be formed in DNA following reaction with a methylating carcinogen. This lesion can be repaired by the action of the protein O^6-alkylguanine–DNA alkyltransferase (EC 2.1.1.63) (AGT). This protein repairs the lesion by transferring the methyl group to a cysteine residue on the protein (Foote *et al.*, 1980; Olsson & Lindahl, 1980; Demple *et al.*, 1985; Rydberg *et al.*, 1990). This action restores the DNA, but the free cysteine is not regenerated and the protein is inactivated. The crystal structure of the Ada protein shows that the reactive cysteine is burried within the protein (Moore *et al.*, 1994). A conformational change appears to be needed to allow the cysteine to come into contact with the DNA. This conclusion is consistent with circular dichroism and fluorescence anisotropy studies, which have indicated that the protein undergoes a conformational change upon binding DNA (Takahashi *et al.*, 1990; Chan *et al.*, 1993). Studies with the mammalian protein suggest that a hydrophobic pocket might be retained during reaction with O^6-benzylguanine derivatives. O^6-Benzylguanine derivatives with hydrophobic substituents on the 9-position are better substrates than hydrophilic substituents (Moschel *et al.*, 1992; Chae *et al.*, 1994).

The mechanism by which AGT repairs O^6mG is unknown. The simplest mechanism is an S_N2 process in which the cysteine displaces the methyl group from the guanine in a concerted reaction. An S_N2 mechanism is supported by the relative rates of dealkylation of O^6-alkylguanine by AGT. The protein was found to react with O^6-alkylguanines as the free base and in oligonucleotides in the following order: methyl > ethyl > isopropyl (Morimoto *et al.*, 1985; Yarosh *et al.*, 1986; Graves *et al.*, 1989). Since in an S_N1 mechanism the alkyl chain must stabilize a positive charge, this reactive order is indicative of an S_N2 mechanism.

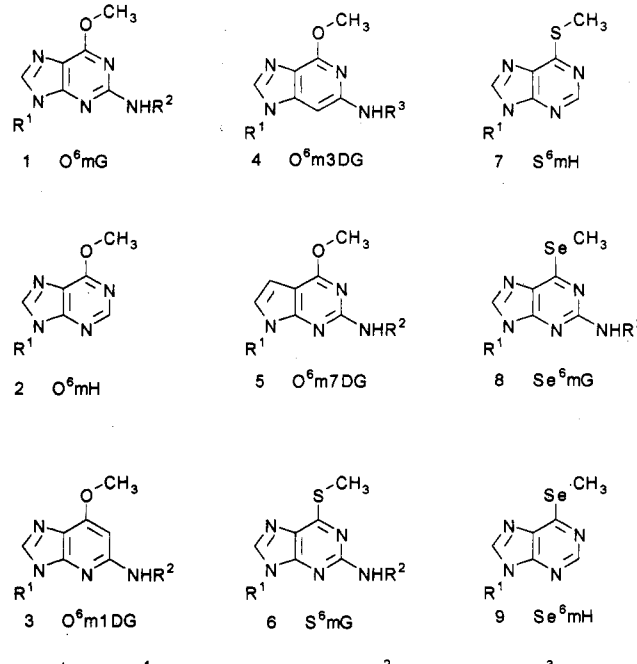
To test whether AGT protonates a heteroatom on O^6mG to neutralize the negative charge and thereby accelerate the reaction, we previously reacted AGT with analogs (see Figure 1) of the free base O^6mG. Analysis of the kinetics of inactivation of AGT suggested that the nitrogen at the 1-position and the exocyclic amino groups are involved in binding of the substrate to the protein and that a proton is transferred to the oxygen to neutralize the negative charge on the guanine leaving group (Spratt & de los Santos, 1992). The free base, however, is not the *in vivo* substrate for this protein. The reaction with the methylated free base is much slower than with double-stranded oligonucleotides and methylated DNA. It is possible that a slightly different mechanism operates in the repair of the bases. In order to more effectively mimic the native substrate, the O^6mG analogs were incorporated into oligodeoxynucleotides and reacted with AGT.

† This work was funded by NCI Grant 53625.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: AGT, O^6-alkylguanine–DNA alkyltransferase; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; O^6mG, O^6-methylguanine; O^6mH, O^6-methylhypoxanthine; S^6mG, S^6-methyl-6-thioguanine; S^6mH, S^6-methyl-6-thiohypoxanthine; Se^6mG, Se^6-methyl-6-selenoguanine; Se^6mH, Se^6-methyl-6-selenohypoxanthine; O^6m1DG, O^6-methyl-1-deazaguanine; O^6m3DG, O^6-methyl-3-deazaguanine; O^6m7DG; O^6-methyl-7-deazaguanine.

0006-2960/94/0433-11364$04.50/0



	R^1	R^2	R^3
a	H	H	H
b	2'-deoxyribose	H	H
c	2'-deoxyribose	phenoxyacetyl	iso-butyryl
d	2'-deoxy-5'-DMT-ribose	phenoxyacetyl	iso-butyryl
e	2'-deoxy-5'-DMT-ribose-3'-phosphoramidite	phenoxyacetyl	iso-butyryl

FIGURE 1: Structures of the O⁶mG analogs.

EXPERIMENTAL PROCEDURES

General. Pyridine and acetonitrile were dried by distillation from CaH_2. THF was dried by distillation from sodium in the presence of benzophenone. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham. T_4 polynucleotide kinase was purchased from USB. Calf intestine alkaline phosphatase and snake venom phosphodiesterase (*Croatalus durissus*) were purchased from Boehringer Mannheim. Nucleoside phosphorylase and thymidine phosphorylase were obtained from Sigma. ^{1}H NMR spectra were obtained on a Bruker AM360WB NMR with TMS as an internal standard for $CDCl_3$, DMSO-d_6, and CD_3OD and DDS for D_2O. Mass spectra were recorded on a Hewlett-Packard Model 5988 spectrometer. Scintillation spectroscopy was performed on a Beckman LS 9800 scintilation counter using Pico-Fluor 40 (Packard) as the cocktail. The AGT was purified from *E. coli* as the 24-kDa N-terminal fragment as previously described (Spratt & de los Santos, 1992).

Nucleobases. O⁶mH, S⁶mG, S⁶mH, Se⁶mG, Se⁶mH, O⁶m1DG, O⁶m7DG, and 3DG were synthesized as previously described (Spratt & de los Santos, 1992). 2'-Deoxy-*O*⁶-methylguanosine (**1b**) was prepared from 2'-deoxyguanosine by the procedure described by Fathi *et al.* (1990).

Enzymatic Glycosylations. O⁶mH, S⁶mG, S⁶mH, Se⁶mG, Se⁶mH, O⁶m1DG, and 3DG were ribosylated by the enzymatic procedure described by Krenistky *et al.* (1981, 1986). In a typical procedure, Se⁶mG (0.50 g, 2.2 mmol) and thymidine (1.0 g, 3.8 mmol) were suspended in 400 mL of 20 mM sodium phosphate, and the pH was adjusted to 7.0. Purine nucleoside phosphorylase (1000 units) and thymidine phosphorylase (1000 units) were added, and the mixtures were shaken for 24–72 h at 37 °C. The reactions could be monitored by TLC on silica gel with elution by 1-butanol/acetic acid/water (8/1/1). The nucleosides, except for O⁶m1DG (**3b**), were purified by adding a 50-mL slurry of Dowex-50 X8 (H form) to the reaction mixture and stirring slowly for 30 min. The solution was decanted, and the Dowex was poured into a 2.5 × 10 cm column. Thymine and thymidine were eluted with water. The product was eluted with a 5% NH_4OH solution and evaporated to an oil, in 50–80% yield.

Treatment of **3b** by acidic resin resulted in depurination. **3b** was purified by first concentrating to about 30 mL by rotary evaporation. The thymine which precipitated was filtered, and 10 mL of the filtrate was applied to a Sephadex LH-20 (1.5 × 100 cm) which was equilibrated in water. The column was eluted with water at 1 mL/min, and 10-mL fractions were collected. The product was eluted after thymine and thymidine in 75% yield from three separate chromatographies. The ^{1}H NMR spectra for the products are listed in Table 1, and the mass spectra are presented in Table 5.

3',5'-O-Di-p-toluoyl-2'-deoxy-O⁶-methyl-7-deazaguanosine. O⁶m7DG (1.8 g, 12.5 mmol) (dried by high vacuum in the presence of P_2O_5) was suspended in 250 mL of anhydrous acetonitrile containing 0.5 g of NaH (60% in mineral oil, 12.5 mmol) and stirred for 1 h. 1-Chloro-3,5-*O*-di-*p*-toluoyl-2-deoxy-D-ribose (4.9 g, 13 mmol) (Hoffer, 1960) was added, and the reaction mixture was stirred for 24 h. The reaction was monitored by TLC on silica gel with development by 10% ethyl acetate/methylene chloride. The product has a R_f of 0.5, just above 1-chloro-3,5-*O*-di-*p*-toluoyl-D-ribose. The reaction mixture was poured into 200 mL of 5% $NaHCO_3$ and extracted twice with methylene chloride. The product was purified by flash chromatography on silica gel. The column was first eluted with methylene chloride, and the product is eluted off with 2% ethyl acetate/methylene chloride in 60% yield.

*2'-Deoxy-O⁶-methyl-7-deazaguanosine (**5b**).* 3',5'-*O*-Di-*p*-toluoyl-2'-deoxy-*O*⁶-methyl-7-deazaguanosine (1 g, 1.9 mmol) was dissolved in 20 mL of 0.1 N sodium methoxide in methanol and stirred for 3 h at room temperature. The base was neutralized with acetic acid, and the solvent was evaporated. Methyl benzoate was dissolved by stirring with hexane, and the product was purified from the resulting oil by flash chromatography by elution with 5% methanol/methylene chloride. The ^{1}H NMR spectrum of the product is listed in Table 1.

O³',O⁵',N²-Triisobutyryl-2'-deoxy-3-deazaguanosine. 2'-Deoxy-3-deazaguanosine (1.2 g, 4.5 mmol), which was dried by evaporation of anhydrous pyridine, was suspended in 20 mL of pyridine. Isobutyryl anhydride (7.5 mL, 45 mmol) was added dropwise, and the suspension was heated at reflux for 1 h. After being cooled in an ice bath, the solution was poured into 100 mL of cold 10% $NaHCO_3$. After being stirred for 20 min, the solution was extracted with ethyl acetate. The ethyl acetate was dried over $MgSO_4$, filtered, and evaporated. The product was purified by flash chromatography on silica gel with elution by ethyl acetate in 40% yield.

^{1}H NMR (DMSO-d_6): 11.85 (1H, s, NH), 10.8 (1H, s, NH), 7.84 (1H, 2, 8), 7.10 (1H, s, 3), 6.03 (1H, d/d, 1'), 5.21 (1H, m, 3'), 4.25 (2H, m, 5'), 4.00 (1H, m, 4'), 2.82 (1H, m, 2'), 2.50 [4H, m, 2' and C*H*$(CH_3)_2$], 1.10 (18H, m, CH_3's).

*O⁶-Methyl-2'-deoxy-3-deazaguanosine (**4b**) and N²-Isobutyryl-O⁶-methyl-2'-deoxy-3-deazaguanosine (**4c**).* 2'-Deoxy-3-deazaguanosine was methylated by a modification of the Mitsunobu reaction (Himmelsbach *et al.*, 1984). *O³',O⁵',N²*-Triisobutyryl-2'-deoxy-3-deazaguanosine (0.40 g, 0.84 mmol) was dissolved in 5 mL of anhydrous THF. Eight equivalents of triphenylphosphine (3.52 g), methanol (0.15 mL), and

Table 1: ^{1}H NMR Spectra for 2′-Deoxyribonucleotides

	position	O^{6}mG (**1b**) DMSO-d_6	O^{6}mH (**2b**) DMSO-d_6	O^{6}m1DG (**3b**) DMSO-d_6	3DG DMSO-d_6	O^{6}m3DG (**4b**) DMSO-d_6	O^{6}m7DG (**5b**) D_2O	S^{6}mG (**6b**) DMSO-d_6	S^{6}mH (**7b**) DMSO-d_6	Se^{6}mG (**8b**) CD_3OD	Se^{6}mH (**9b**) CD_3OD
NH					10.31 s						
2	1H		8.59 s						8.74 s		8.75 s
NH_2	2H	7.20 s		5.87 s		6.39 s		6.52 s			
8	1H	8.11 s	8.55 s	8.01 s	7.88 s	8.06 s	6.84 d	8.17 s	8.66 s	8.17 s	8.37 s
1, 3, or 7	1H			5.94	5.42 s	7.10 s	6.15 d				
CH_3	3H	4.11 s	4.10 s	3.90 s		4.03 s	3.81 s	2.48 s	2.66 s	2.54 s	2.51 s
1′	1H	6.21 d/d	6.43 t	6.27 d/d	5.94 d/d	6.27 d/d	6.62 d/d	6.62 t	6.43 t	6.37 d/d	6.47 d/d
2′	1H	2.61 m	2.73 m	2.58 m	2.42 m	2.58 m	2.50 m	2.48 m	2.75 m	2.83 m	2.82 m
	1H	2.24 m	2.32 m	2.18 m	2.22 m	2.41 m	2.27 m	2.23 m	2.35 m	2.39 m	2.42 m
3′	1H	3.85 m	3.88 m	3.83 m	3.80 m	4.10 m	3.95 m	3.81 m	3.78 m	4.08 m	4.03 m
4′	1H	4.34 m	4.43 m	4.35 m	4.31 m	4.78 m	4.43 m	4.38 m	4.43 m	4.61 m	4.61 m
5′	1H	3.58 d/d	3.55 m	3.57 d/d	3.50 m	3.98 d/d	3.63 m	3.50 m	3.88 m	3.57 m	3.52 m
	1H	3.50 d/d	3.55 m	3.51 d/d	3.50 m	3.88 d/d	3.63 m	3.50 m	3.78 m	3.51 m	3.48 m
3′-OH	1H		5.35 t	5.25 d	5.38 d	5.35 t		5.30 d	5.32 d		
5′-OH	1H		5.02 t	5.17 t	4.91 t	4.97 d		4.97 t	5.01 t		

Table 2: ^{1}H NMR Spectra for NH_2 Protected 2′-Deoxyribonucleotides

	position	O^{6}mG (**1c**) DMSO-d_6	O^{6}m1DG (**3c**) DMSO-d_6	O^{6}m3DG (**4c**) $CDCl_3$	O^{6}m7DG (**5c**) DMSDO-d_6	S^{6}mG (**6c**) $CDCl_3$	Se^{6}mG (**8c**) DMSO-d_6
NH	2H	11.84 s	9.15 s		11.71 s	9.08 s	
8	1H	8.26 s	8.02 s	8.07 s	6.82 m	8.00 s	8.56 s
1, 3, or 7	1H		7.92 s	8.04 s	6.10 m		
CH_3	3H	4.06 s	4.02 s	4.02 s	4.02 s	2.70 s	2.59 s
1′	1H	6.22 d/d	6.39 m	6.27 d/d	6.22 d/d	6.51 d/d	6.38 d/d
2′	1H	2.58 m	3.01 m	2.59 m	2.58 m	3.02 m	2.75 m
	1H	2.28 m	2.32 m	2.42 m	2.22 m	2.39 m	2.32 m
3′	1H	3.85 m	4.31 m	4.11 m	3.82 m	4.13 m	3.90 m
4′	1H	4.39 m	4.81 m	4.74 m	4.41 m	4.99 m	4.45 m
5′	1H	3.56 m	4.02 d/d	3.97 d/d	3.56 m	3.97 d/d	3.60 m
	1H		3.89 d/d	3.87 d/d		3.85 d/d	3.51 m
3′-OH	1H	5.35 d			5.32 d		5.35 d
5′-OH	1H	4.98 t			4.97 t		4.95 t
CH_2	2H	4.87 s	4.65 s		4.82 s	4.71 s	5.05 s
phenyl	2H	7.32 m	7.38 m		7.32 m	7.35 t	7.31 m
	3H	6.99 m	7.09 m		6.99 m	7.05 m	6.98 m
CH	1H			2.59 m			
$(CH_3)_2$	3H			1.28 d			
	3H			1.26 d			

diethyl azodicarboxylate (1.06 mL) were added over the course of 2 h in four portions, and the reaction mixture was stirred under a nitrogen atmosphere for an additional 30 min. The progress of the reaction was monitored by TLC on silica gel with development by 5% methanol in methylene chloride. The product has an R_f of 0.6, just above that of triphenylphosphine oxide. The starting nucleoside has an R_f of 0.25. The crude methylated product was partially purified by flash chromatography on silica gel with elution by 1% methanol/methylene chloride. The product was contaminated with triphenylphosphine oxide and diethyl hydrazinodicarboxylate. The resulting oil was dissolved in 10 mL of 0.1 N sodium methoxide in methanol and stirred at room temperature for 2 h. The methanolic solution was neutralized with acetic acid and evaporated. The residue was dissolved in ethyl acetate, and the product was purified by flash chromatography on silica gel. The triphenylphosphine oxide was eluted with ethyl acetate and the nucleoside with 10% methanol/ethyl acetate in 50% overall yield. The ^{1}H NMR spectrum of the product is listed in Table 2, and the mass spectrum is presented in Table 5.

The deprotected nucleoside **4b** was prepared by heating 100 mg of **4c** to reflux in 10 mL of 0.5 N sodium methoxide in methanol for 2 h. The solution was neutralized with acetic acid and evaporated, and the product was purified by preparative reverse-phase HPLC on a Optisil 10 ODS (Phenomenex) (9.4 × 500 mm) column employing a gradient of 0–30% methanol in water at 4 mL/min in 50% yield. The ^{1}H NMR spectrum is listed in Table 1, and the mass spectrum is in Table 5.

Protection of the N^2-Amino with Phenoxyacetyl Chloride. The exocyclic amino groups of the nucleosides of O^{6}mG (**1b**), S^{6}mG (**6b**), Se^{6}mG (**8b**), O^{6}m1DG (**3b**), and O^{6}m7DG (**5b**) were protected with phenoxyacetyl chloride by the transient protection scheme procedure described by Schulhof *et al.* (1987). The products were purified by chromatography on silica gel with elution by 10% methanol in ethyl acetate in 40–70% yields. The ^{1}H NMR spectra of the products are listed in Table 2, and the mass spectra are presented in Table 5.

Protection of the 5′-Hydroxyl with 4,4′-Dimethoxytrityl Chloride. The 5′-hydroxyl of all nucleosides was protected by the procedure described by Jones (1984). The products were purified by flash chromatography on silica gel by elution first with 1% triethylamine/ethyl acetate followed by 1% triethylamine/10% methanol/ethyl acetate in 60–90% yields. The ^{1}H NMR spectra of the products are listed in Table 3, and the mass spectra are presented in Table 5.

Preparation of 3′-O-(2-Cyanoethyl Diisopropylphosphoramidites). The 3′-*O*-(2-cyanoethyl diisopropylphosphoramidites) were prepared by the procedure described by Smith *et al.* (1990) in approximately 60% yields. The ^{1}H NMR spectra of the products are listed in Table 4.

Table 3: ^{1}H NMR Spectra of 5′-*O*-(4,4′-Dimethyltrityl)-2′-deoxyribonucleotides in $CDCl_3$

	position	O^6mG (1d)	O^6mH (2d)	O^6m^{D}G (3d)	O^6m3DG (4d)	O^6m7DG (5d)	S^6mG (6d)	S^6mH (7d)	Se^6mG (8d)	Se^6mH (9d)
2	1H		7.86 s					8.70 s		8.75 s
NH	1H	8.79 s		8.88 s	7.77 s	8.91 s	8.90 s		8.89 s	
8	1H	8.00 s	7.84 s	7.96 s	7.98 s	6.82 m	8.05 s	8.05 s	8.04 s	8.37 s
1, 3 or 7	1H			7.89 s	7.93 s	6.10 m				
CH_3	3H	4.19 s	3.77 s		4.03 s	4.07 s	2.72 s	2.72 s	2.81 s	2.55 s
1′	1H	6.51 m	6.38 d/d	6.50 d/d	6.24 d/d	6.22 d/d	6.40 d/d	6.39 d/d	6.40 m	6.47 d/d
2′	1H	2.76 m	2.23 m	2.64 m	3.30 m	2.58 m	2.80 m	2.32 m	2.80 m	2.80 m
	1H	2.61 m	1.88 m	2.50 m	2.53 m	2.22 m	2.51 m		2.61 m	2.49 m
3′	1H	4.19 m	4.15 m	4.16 m	4.14 m	3.82 m	4.13 m	4.21 m	4.20 m	4.05 m
4′	1H	4.79 m	4.40 m	4.62 m	4.55 m	4.46 m	4.71 m	4.82 m	4.82 m	4.61 m
5′	1H	3.46 d/d	3.18 m	3.39 m	3.39 m	3.50 m	3.43 d/d	3.43 d/d	3.44 d/d	3.46 m
	1H	3.33 d/d	3.04 m	3.30 m			3.32 d/d	3.32 d/d	3.36 d/d	3.40 m
CH_2	2H	4.69 s		4.59 s		4.81 s	4.62 s		4.05 s	
phenyl and trityl		7.3 m, 12 H	7.2 m, 6H	7.25 m, 10H	7.37 m, 4H	7.3 m, 13H	7.3 m, 14H	7.25 m 9H	7.3 m, 14H	7.3 m, 9H
		6.75 d/d, 6H	6.75 m, 7H	7.0 m, 4H	7.25 m, 7H	6.99 m, 3H	6.75 d/d, 4H	6.85 m, 4H	6.78 d, 4H	6.76 d, 4H
				6.75 d, 4H	6.76 d, 2H	6.7 m, 2H				
trityl	6H	3.76 s	3.76 s		3.75 s	3.75 s	3.76 s	3.80 s	3.78 s	3.72 s
OCH_3			3.75 s							
CH	1H				2.53 m					
$(CH_3)_2$	3H				1.27 d					
	3H				1.25 d					

Table 4: ^{1}H NMR Spectra of 2′-Deoxyribonucleotide 3′-*O*-(2-Cyanoethyl *N*,*N*-Diisopropylphosphoramidite) Analogs in $CDCl_3$

	position	O^6mG (1e)	O^6mH (2e)	O^6m1DG (3e)	O^6m3DG (4e)	O^6m7DG (5e)	S^6mG (6e)	S^6mH (7e)	Se^6mG (8e)	Se^6mH (9e)
2	1H		7.86 s					8.70 s		8.75 s
NH	1H				7.77 s		8.90 s		8.89 s	
8	1H	8.00 d	7.85 s	7.96 s	7.98 s	6.85 m	8.05 s	8.05 s	8.04 s	8.37 s
1, 3, or 7	1H			7.99 s	7.93 s	6.21 m				
CH_3	3H	4.15 s	3.81 s	4.06 s	4.03 s	4.07 s	2.72 s	2.72 s	2.81 s	2.55 s
1′	1H	6.39 m	6.4 m	6.5 m	6.25 m	6.62 m	6.40 m	6.39 m	6.40 m	6.45 m
2′	2H	2.8–2.5 m	2.2–1.9 m	2.65–2.5 m	2.7–2.5 m	2.6–2.3 m	2.8–2.5 m	2.3–2.4 m	2.8–2.6 m	2.8–2.5 m
3′	1H	4.30 m	4.2 m	4.2 m	4.13 m	3.8 m	4.15 m	4.2 m	4.2 m	4.0 m
4′	1H	4.75 m	4.5 m	4.6 m	4.6 m	4.45 m	4.75 m	4.85 m	4.8 m	4.65 m
5′	2H	3.5–3.3 m	3.2–3.0 m	3.4–3.3 m	3.4 m	3.50 m	3.4–3.3 m	3.4–3.3 m	3.5–3.3 m	3.5–3.4 m
CH_2	2H	4.8–4.6 m		4.6 s		4.8 s	4.62 s		4.05 s	
phenyl and trityl		7.4–7.2 m,	7.2 m, 6H	7.2 m, 10H	7.3 m, 4H	7.3 m, 13H	7.3 m, 14H	7.25 m, 9H	7.3 m, 14H	7.3 m, 9H
		12H 6.7 m, 6H	6.7 m, 7H	7.0 m, 4H	7.2 m, 7H	7.0 m, 3H	6.75 d/d, 4H	6.85 m, 4H	6.78 d, 4H	6.76 d, 4H
				6.75 d, 4H	6.75 d, 2H	6.7 m, 2H				
trityl	6H	3.8 br s	3.75 s	3.75 s	3.75 s	3.75 s	3.76 s	3.80 s	3.78 s	3.72 s
OCH_3										
CHN	2H	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m
CHCO	1H				2.5 m					
CH_3		1.3–1.1 m, 12H	1.3–1.1 m, 12H	1.3–1.1 m, 12H	1.3–1.1 m, 18H	1.3–1.1 m, 12H	1.3–1.1 m, 12H	1.3–1.1 m, 12H	1.3–1.1 m, 12H	1.3–1.1 m, 12H
CH_2CN	2H	2.5–2.5 m	2.6–2.5 m	2.65–2.5 m	2.7–2.5 m	2.7–2.5 m	2.8–2.5 m	2.7–2.5 m	2.8–2.6 m	2.8–2.5 m
CH_2O	2H	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m	3.5–3.3 m

Table 5: Mass Spectral Data for O^6mG Analogs

compd	deoxyriboside	N^2-protected	5′-DMT
O^6mH	**2b**, 151 (100) base + 1, 267 (50) M + 1	NA[a]	**2d**, 151 (100) base + 1, 267 (35) nucleoside + 1, 303 (33) DMT
O^6m1DG	**3b**, 165 (75) base + 1, 281 (100) M + 1	**3c**, 117 (100) sugar + 1, 415 (19) M + 1	**3d**, 303 (100) DMT, 415 (9) protected nucleoside + 1
O^6m3DG	**4b**, 165 (72) base + 1, 281 (100) M + 1	**4c**, 235 (95) base + 1, 351 (100) M + 1	**4d**, 303 (100) DMT, 351 (6) protected nucleoside + 1
O^6m7DG	**5b**, 163 (17) base − 1, 279 (13) M − 1	**5c**, 279 (5) nucleoside − 1	**5d**, 279 (10) nucleoside − 1, 303 (100) DMT
S^6mG	**6b**, 182 (100) base + 1, 298 (45) M + 1	**6c**, 117 (60) sugar + 1, 182 (50) base + 1, 432 (1) M + 1	**6d**, 303 (100) DMT, 432 (1) protected nucleoside + 1
S^6mH	**7b**, 167 (15) base + 1, 283 (100) M + 1	NA	**7d**, 283 (100) nucleoside + 1, 585 (1) M + 1
Se^6mG	**8b**, 346 (5) M, 344 (8) M + 2	**8b**, 344 (50) base, 346 (100) base + 2, 478 (5) M, 480 (10) M + 2	**8c**, 303 (100) DMT, 478 (2) protected nucleoside, 480 (5) protected nucleoside + 2
Se^6mH	**9b**, 329 (2) M, 331 (4) M + 2	NA	**9c**, 303 (100) DMT, 630 (1) M, 632 (2) M + 2

[a] NA, not applicable.

Oligodeoxynucleotide Synthesis. Oligodeoxynucleotides of the sequence 5′-GGC GCT XGA GGC GTG-3′ were synthesized on a Millipore Cyclone Plus DNA synthesizer in the 0.2-μmol scale. The vial for X base was filled with anhydrous acetonitrile. During the automated synthesis when it was time for the modified nucleoside to be reacted with the growing oligomers, the machine was stopped and the column disconnected. With two syringes, one on each end of the column, 100 μL of 0.4 M tetrazole in acetonitrile and a 20-μg activated monomer in 100 μL of acetonitrile were added. The

solution was mixed with the resin by slowly forcing the solution from one syringe to the other over 5 min. The column was then attached to the DNA synthesizer, and the synthesis resumed. The complementary strand of the sequence 5′-CAC GCC TCC AGC GCC-3′ was synthesized in the normal manner.

The oligomer was removed from the resin by treating the column with concentrated ammonium hydroxide for 2 h. The oligodeoxynucleotide (except for X = O^6m3DG) was deprotected by heating the ammonium hydroxide solution at 55 °C for 12 h. The oligodeoxynucleotide containing O^6m3DG was treated with 10% DBU in methanol for 2 weeks at room temperature. The methanol was evaporated, and the residue was dissolved in water. The oligodeoxynucleotide was then loaded onto a SepPak C-18 cartridge, which was equilibrated with 100 mM triethylammonium acetate, pH 7. The SepPak was washed with water and then with 1% trifluoroacetic acid to remove the final dimethoxytrityl group. The oligomer was eluted off the SepPak with 20% acetonitrile usually in greater than 90% purity was determined by HPLC. The oligodeoxynucleotide was purified by reverse-phase HPLC using a 3.9 × 300 mm Bondclone C18 (Phenomenex, Torrence, CA) column. A gradient of 0–30% methanol in 100 mM triethylammonium acetate, pH 7.0, over 30 min at 1 mL/min was used. By this method, 5–30 μg of oligonucleotide was obtained. This yield is approximately 20% of that obtained with unmodified oligomers. The purity of the oligomer was rechecked on the above reverse-phase system in addition to the ion-exchange system used to monitor the methyl transfer reaction.

Enzymatic Hydrolysis. The oligodeoxynucleotides were hydrolyzed by reaction of approximately 0.1 A_{260} unit of oligomer in 1 mL of 50 mM Tris-HCl and 5 mM $MgCl_2$, pH 7.8 at 37 °C, with 2 units of snake venom phosphodiesterase for 30 min followed by 50 units of alkaline phosphatase for 30 min. The compositions of the oligomers were analyzed by reverse-phase HPLC with a 3.9 × 300 mm Phenomenex Bondclone-C18 column. The gradient was initially isocratic for 16 min at 95% 20 mM sodium phosphate, pH 6.8, and 5% acetonitrile, followed by a linear gradient to 50% acetonitrile over 10 min. The eluant was monitored at 254 nm. The retention times (min) were as follows: dC, 5.9; dG, 18.7; dT, 19.5; dA, 26.8; **1b**, 31.7; **2b**, 32.4; **3b**, 30.4; **4b**, 34.8; **5b**, 32.6; **6b**, 34.8; **7b**, 36.1; **8b**, 35.7; **9b**, 37.4. The integration of the peaks was not linear, but when a chromatogram of the hydrolyzed oligodeoxynucleotide was compared with that of a mixture of standards containing the appropriate amount of each nucleoside, then the integrations agreed to with 10%.

[5′-^{32}P]-Labeling of Oligodeoxynucleotides. A 50–200-pmol sample of O^6mG or an analog containing oligodeoxynucleotide, 10 pmol [γ-^{32}P]ATP (6000 Ci/mmol), and 50 units of T_4 polynucleotide kinase was incubated in 20 μL of 50 mM Bicine, 10 mM DTT, 1 mM spermidine, and 10 mM $MgCl_2$, pH 9.0 at 37 °C. After 30 min, 2.5 μL of 10 mM ATP was added and incubated for 5 min. The oligomer was separated from the ATP by loading the solution on to a C-18 SepPak that was equilibrated with water. The cartridge was washed with 5% acetonitrile, and the oligodeoxynucleotide was eluted off in 30% acetonitrile. HPLC of the labeled oligonucleotide showed that more than 95% of the radioactivity was associated with the oligonucleotide peak. The samples were evaporated under vacuum with a Speed-vac and resuspended in 20 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT, pH 7.6 with a 20% excess of the complementary strand.

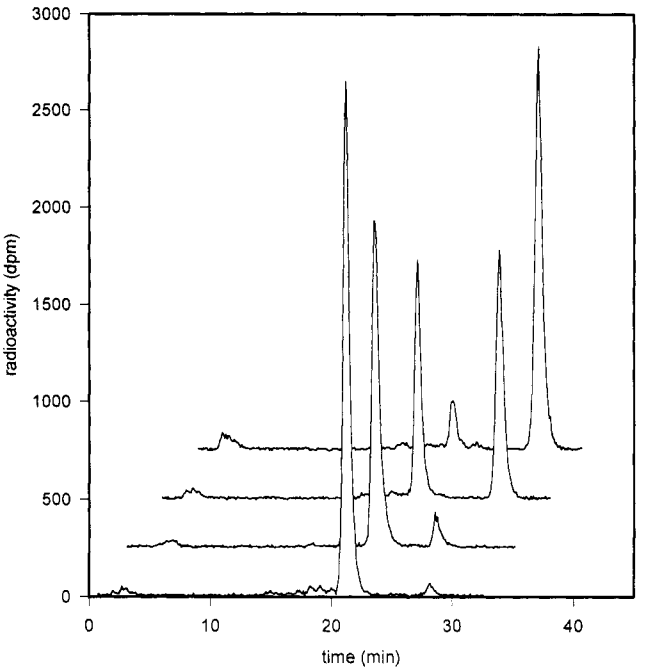


FIGURE 2: Anion-exchange HPLC chromatograms of the reaction between the oligodeoxynucleotide duplex containing O^6m1DG (2.77 nM) and AGT (139 nM) at 15 s, 2 min, 31 min, and 131 min.

The solution containing both strands was heated at 80 °C for 2 min and allowed to cool slowly to room temperature.

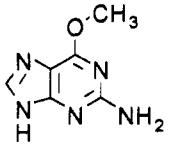
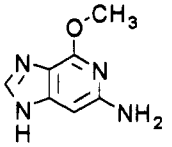
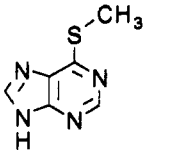
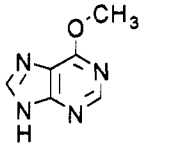
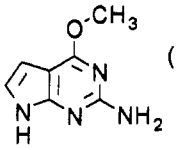
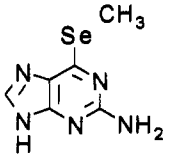
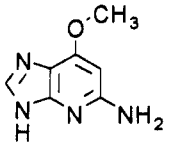
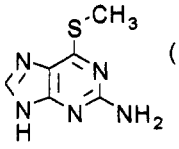
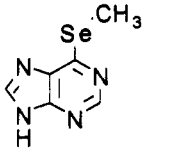
Enzyme–Substrate Reaction. The reaction was initiated by the addition of AGT to a 1-mL solution of the ^{32}P-labeled oligodeoxynucleotide duplex in 20 mM Tris-HCl, 1 mM EDTA, and 5% glycerol, pH 7.6 at 37 °C. The concentration of oligomer was 1 nM, and the AGT concentration ranged from 1 to 50 nM. Aliquots (100 μL) were removed at various times and quenched by the addition of 500 μL of 0.1 N NaOH. The progress of the reaction was analyzed by HPLC with a strong anion-exchange column Nucleopac Pa-100 (4.6 × 250 mm) (Dionex) in a method developed by Xu and Swann (1992). The strong anion-exchange column was eluted at 1 mL/min with buffer A (0.01 N NaOH, 0.4 M NaCl, 0.5% acetonitrile) and buffer B (0.01 N NaOH, 1.2 M NaCl 0.5% acetonitrile). The gradient was isocratic at 0% B for 5 min and went to 14% B at 10 min, to 17% B at 20 min, to 25% B at 30 min, and to 100% B at 40 min. The demethylated oligomer eluted later than the starting material as illustrated in Figure 2. The progress of the demethylation was analyzed according to second-order kinetics.

RESULTS

Similar synthetic schemes were employed for incorporating each of the O^6mG analogs into an oligodeoxynucleotide. The starting point for the syntheses were the free bases synthesized previously (Spratt & de los Santos, 1991). In order to synthesize oligodeoxynucleotides containing these analogs of O^6mG, the deoxyribose group must be attached to the 9-position first. Second, the exocyclic amino group must be protected. Third, the 4,4′-dimethoxytrityl group must be attached to the 5′-hydroxyl group. Finally, the 3′-hydroxyl must be activated with the phosphorylating agent.

O^6-Methyl-7-deazaguanine (**5a**) was glycosylated by reacting the sodium salt with 1-chloro-3,5-*O*-di-*p*-toluoyl-2-deoxy-D-ribose in a procedure based on the reaction described by Kazimierczuk *et al.* (1984). The other bases were enzymatically glycosylated with thymidine, thymidine phosphorylase,

Table 6: Reaction of Oligonucleotides Containing Analogs of O^6-Methylguanine with AGT[a]

X	k_2[b] / k_{inact}/K_{IN}[c]	X	k_2[b] / k_{inact}/K_{IN}[c]	X	k_2[b] / k_{inact}/K_{IN}[c]
	$(7.7\pm0.9)\times10^5$ 0.31 ± 0.07		$(7.7\pm2.5)\times10^4$ 0.21 ± 0.04		nd[d] nd
	$(5.4\pm0.7)\times10^6$ 0.028 ± 0.012		$(3.1\pm1.0)\times10^4$ 0.22 ± 0.07		$(4.1\pm1.6)\times10^3$ 0.046 ± 0.023
	$(2.2\pm0.9)\times10^3$ 0.032 ± 0.004		$(1.7\pm0.2)\times10^3$ 0.15 ± 0.02		nd 0.044 ± 0.021

[a] 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA, pH 7.6, 37 °C. [b] Second-order rate constant using the oligonucleotide duplex as substrate (M^{-1} s^{-1}). [c] Composite rate constant from Spratt and de los Santos (1991) (M^{-1} s^{-1}). [d] Not detected.

and purine nucleoside phosphorylase in reactions initially described by Krenitsky *et al.* (1981, 1986) for the glycosylations of guanine and 3-deazaguanine and expanded by Chapeau and Marnett (1991) to include modified guanines. The bases with the exocyclic amino groups were more readily glycosylated than the hypoxanthine analogs. Therefore an additional amount of thymidine was added to the glycosylation reactions of O^6mH, S^6mH, and Se^6mH in order to force the reaction to completion.

As shown by Borowy-Borowski and Chambers (1987), the 6-methoxy group is labile in concentrated ammonia at 55 °C. They solved the problem by hydrolyzing the protecting groups in methanol with DBU. Li and Swann (1989) have shown that the lability of the 6-position was due to the presence of the N^2-protecting group. The labile phenylacetyl protecting group (Schulhof *et al.*, 1987) was cleaved very quickly during the deprotection with ammonium hydroxide at 55 °C. The 6-methoxy group was not cleaved under these conditions. To test the stability of these O^6mG analogs, the free bases were incubated in concentrated ammonium hydroxide at 55 °C for 12 h. Analysis by HPLC did not show any decomposition (data not shown). In accordance with this method, we have used the labile phenoxyacetyl group (Schulhof *et al.*, 1987) to protect the exocyclic amino group except in the case of O^6-methyl-3-deazaguanine (**4**) when the isobutyryl group was used as described below.

Synthesis of the O^6-methyl-3-deaza-2′-deoxyguanosine (**4b**) began with the glycosylation of 3-deazaguanine (**4a**). The original synthesis of O^6-methyl-3-deazaguanine (**4a**) by the Mitsunobu reaction required a HPLC purification step and was accomplished in low yield (Spratt & de los Santos, 1992). Performing this reaction on the protected nucleoside, which is soluble in organic solvents and allows flash chromatography purification, increased the yield of the reaction. The reaction was done with the isobutryl group which was used by Himmelsbach *et al.* (1984), and no attempt was made to use the phenoxyacetyl group.

The 5′-hydroxyl groups were protected as the 4,4′-dimethoxytrityl group. The 3′-hydroxyl was phosphoramidated in the usual manner. The syntheses of the oligodeoxynucleotide of the sequence 5′-GGC GCT XGA GGC GTG-3′ in which X was the various analogs were done according to the standard methods. Each oligonucleotide showed a single peak on reverse-phase HPLC. The compositions of the oligodeoxynucleotides were checked by enzymatic hydrolysis and analyzed by RP-HPLC. Integration of the peaks was within 10% of a standard solution. The oligonucleotides were also ^{32}P-labeled and chromatographed on an ion-exchange HPLC system. In this system, the starting material was a single peak, and for the more reactive reactive analogs, the starting material was completely converted into the product. On this evidence, the oligodeoxynucleotides were judged to be successfully synthesized and purified.

The second-order rate constants for the reaction between AGT and the oligodeoxynucleotides duplexes containing the O^6mG analogs are presented in Table 6. The reactions were monitored by strong anion-exchange HPLC with elution in 10 mM NaOH. At this pH, the demethylated product has an extra negative charge and elutes after the starting material (Xu & Swann, 1992). For the oligodeoxynucleotides containing O^6mG, O^6mH, O^6m1DG, O^6m7DG, S^6mG, and Se^6mG, the reaction with AGT led to a product which eluted approximately 2 min after the starting material. There was no reaction detected with the oligodeoxynucleotides containing S^6mH and Se^6mH. Reaction of AGT with the oligodeoxynucleotide containing O^6m3DG led to two products which eluted much earlier than the starting material.

Each duplex was reacted at three different AGT concentrations over a 10-fold range. The second-order rate constant was constant over this range. The substrates obeyed second-order kinetics as illustrated by eq 1 in which A is AGT, B is the methylated oligonucleotide, C is the demethylated oligonucleotide, k is the second-order rate constant, and t is the incubation time. The subscripts indicate the time that the concentrations are measured.

$$kt = \frac{1}{[B]_0 - [A]_0} \ln \left(\frac{[A]_0([B]_0 - [C]_t)}{[B]_0([A]_0 - [C]_t)} \right) \quad (1)$$

DISCUSSION

The potential interactions between O^{6}mG and the protein were examined by reacting oligodeoxynucleotide duplexes containing analogs of O^{6}mG in which the heteroatoms were changed. The analogs of O^{6}mG are illustrated in Table 6. Each analog has only one change from O^{6}mG or O^{6}mH. Replacement or removal of the nitrogens would eliminate the possibility of a proton transfer or a hydrogen bond to that position. Replacement of oxygen with sulfur and selenium would reduce the ability of the protein to donate a proton to that site and decrease the strength of any hydrogen bond. Sulfur and selenium are larger than oxygen with covalent radii of 1.16, 1.03, and 0.64 Å, respectively (Pauling, 1960). If AGT is optimized to donate a proton to oxygen, then the distances in the active site would be suboptimal for the sulfur and selenium analogs making the proton transfer less efficient. Thus, if the heteroatom was involved in a hydrogen bond or a proton transfer prior to the rate-determining step, then replacement of that heteroatom should produce a poor substrate.

The second-order rate constants for the demethylation of the oligodeoxynucleotide duplexes containing the O^{6}mG analogs are shown in Table 6. Substitution of the ring nitrogens at the 7-position decreased the rate by a factor of 20. Replacement of the oxygen at the 6-position and the nitrogen at the 1-position decreased the rate 200–500-fold. Replacement of the nitrogen at the 3-position led to a substrate which reacts with AGT in a way other than simple demethylation. This reaction is currently being investigated.

The large decrease in rate with the substrates in which the 1- and 6-positions were modified suggests that the protein makes critical interactions with these positions to bring about the methyl transfer. Likely interactions would be hydrogen bonds and/or proton donations to these positions. The smaller decrease in rate observed when the 7-position was altered also suggests that there is an interaction between AGT and the N^7 of O^{6}mG but that this interaction is not as critical as those to the 1- and 6-positions. Suprisingly, removal of the exocyclic amino group increased the rate. This moiety is involved in the hydrogen bonding between strands. If weakening of the interaction between strands increases the rate, then perhaps the strands must open up in order for the reaction to occur.

Comparison of k_2 with k_{inact}/K_{IN} of the previous study (Spratt & de los Santos, 1992) reveals that the modifications of O^{6}mG produce similar results for the reaction of AGT with the free bases and the oligodeoxynucleotides with the exception of O^{6}mH. Replacements of the oxygen and the nitrogen at the 1-position decrease both k_2 and k_{inact}/K_{IN} while the replacements of the nitrogen at the 7-position leaves the rates relatively unchanged. Removal of the exocyclic amino group reduces k_{inact}/K_{IN} but increases k_2.

The proposed mechanism for the methyl transfer is illustrated in Figure 3. The major conclusion is that there are hydrogen bonds or proton transfers to the N^1- and O^6-positions of O^{6}mG. The interactions could be with either the protein or the complementary strand. Scicchitano *et al.* (1986) demonstrated that the identity of the nucleotide opposite O^6-mG did not significantly affect the rate of methyl transfer. It is therefore unlikely that the critical interaction that we have found can be with the cytidine on the opposite strand.

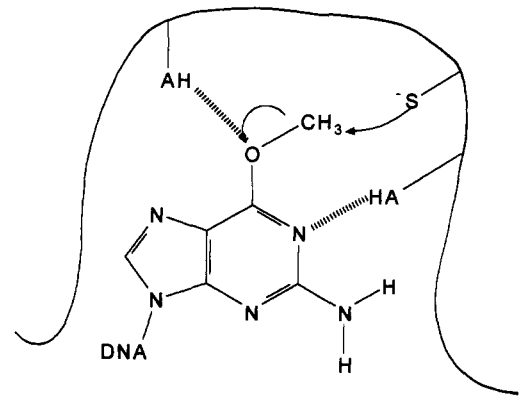


FIGURE 3: Proposed mechanism for methyl transfer.

An interaction with the protein would require the protein to open up the helix. The increase in rate due to the removal of the NH_2 group is consistent with this mechanism. The NH_2 group would be involved in hydrogen bonding to the opposite strand (Patel *et al.*, 1986; Kalnik *et al.*, 1989), and removal of this group would allow the DNA to open up more readily. If this step was part of the rate-determining step, then it would contribute to the 10-fold increase in rate.

The mechanism by which O^{6}mG is acted upon by AGT might be similar to the manner in which HhaI cytosine methyltransferase interacts with cytosine (Verdine, 1994). The crystal structure of HhaI cytosine methyltransferase bound to an oligodeoxynucleotide duplex containing 5-fluorocytosine shows the protein bound to duplex DNA with a disrupted guanine-5-fluorocytosine base pair. The fluorocytosine is flipped out of the helix and is in the active site (Klimasauskas *et al.*, 1994). By this method, a protein can perform complex chemistry on a nucleotide that is an integal part of the DNA. The possibility that this mechanism operates with AGT is under investigation.

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