Synthesis and Characterization of Oligodeoxynucleotides Containing O^6-Methyl-, O^6-Ethyl-, and O^6-Isopropylguanine†

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ABSTRACT: The carcinogenicity and mutagenicity of *N*-nitroso compounds is believed to result primarily from alkylation of DNA on O^6 of guanine. To study the base-pairing properties of O^6-alkylguanines and the structural changes produced in DNA by their presence, self-complementary dodecanucleotides were synthesized of the general structure CGCXAGCTYGCG (where X was O^6-methylguanine, O^6-ethylguanine, or O^6-isopropylguanine and Y one of the natural bases). The O^6-alkyldeoxyguanosines used as building blocks for the synthesis of the oligomers were prepared from the 6-(2-mesitylenesulfonyl) derivative by successive displacement with *N*-methylpyrrolidine and then alkoxide ions. The difficulties previously encountered in the synthesis of oligomers containing O^6-alkylguanine [Borowsy-Borowski, H., & Chambers, R. W. (1987) *Biochemistry 26*, 2465–2471] were overcome by use of phenylacetyl as N^2-protection for the O^6-alkylguanine residue. The lability of the phenylacetyl group allowed very mild treatment with ammonia to be used for deprotection, and there was no detectable contamination of the product with 2,6-diaminopurine. Depurination of O^6-alkylguanine residues with a free 5′-OH, which can occur if a protic acid is used to remove the 5′-protecting group from the alkylated nucleoside during oligonucleotide synthesis, was avoided by using $ZnBr_2$. The oligonucleotide sequences that would form double helices containing O^6-alkylG·C base pairs had lower optical melting temperatures (T_m) than the parent with G·C pairs, but only the isopropylG·C oligomer had significantly less hypochromicity. But the sequences giving helices with O^6-alkylG·T base pairs had less hypochromicity than the parent and had biphasic melting profiles that began at a significantly lower temperature than the sequences with O^6-alkylG·C base pairs.

O^6-Alkylguanine residues formed in DNA by alkylation are believed to play a crucial role in the carcinogenic action of *N*-nitroso compounds and alkylating agents [reviewed by Saffhill et al. (1985)]. The original suggestion by Loveless (1969) that this is related to the GC → AT transition mutations occurring on replication of DNA in which some of the guanine had been alkylated on the O^6-position had been supported by more recent experiments showing the activation of the *H ras* oncogene in *N*-methyl-*N*-nitrosourea-induced tumors by a GC → AT transition (Sukumar et al., 1983; Zarbl et al., 1985). In this paper we report the synthesis of dodecadeoxynucleotides containing O^6-methylguanine (meG), O^6-ethylguanine (etG), and O^6-isopropylguanine (iprG) in the quantity (c50 mg) and purity needed for structural studies using 2D NMR and X-ray crystallography. Preliminary NMR structural studies on these oligonucleotides have been published (Li et al., 1988), and we have also used these oligomers to investigate the kinetics and specificity of the *Escherichia coli* DNA repair enzyme O^6-alkylguanine DNA alkyltransferase (Graves et al., 1987). Their availability opens the way to further studies of the biological properties of alkylated bases in DNA including the consequences of their insertion into specific sites in the genome.

We wished particularly to prepare DNA with the O^6-position of guanine bearing a branched alkyl chain, because *N*-alkyl-*N*-nitroso compounds alkylate through an S_N1 mechanism in which a carbonium ion is generated by the decomposition of the *N*-alkyl diazohydroxide [reviewed by Lawley (1984)]. So if the alkyl carbonium ions generated in the S_N1 alkylation are ≥3 carbon atoms, they will rearrange spontaneously to the secondary and tertiary carbonium ion. For example, alkylation of DNA by *N*-(*n*-propyl)-*N*-nitrosourea gave O^6-isopropylguanine (iprG) as well as O^6-*n*-propylguanine (Morimoto et al., 1983), and alkylation by *N*-(*n*-butyl)-*N*-nitrosourea gave O^6-isobutylguanine (Saffhill, 1984). These secondary alkyl carbonium ions preferentially attack the O^6 of guanine residues in DNA. When DNA was alkylated by *N*-(*n*-propyl)-*N*-nitrosourea, the ratio of O^6-alkylation to N^7-alkylation was 0.63 for *n*-propylation but 1.6 for isopropylation; with *N*-(*n*-butyl)-*N*-nitrosourea the ratio was 0.67 for *n*-butylation but 1.75 for isobutylation (Saffhill, 1984). Peculiarities in the structure or repair of DNA containing guanine residues with longer or branched alkyl chains on O^6 may be one of the reasons for the relatively greater carcinogenicity that has been observed (Druckrey et al., 1967) for long-chain *N*-alkylnitroso compounds.

O^6 of guanine is susceptible to phosphorylating, silylating, sulfonating (Daskalov et al., 1981), and some acylating agents (Bridson et al., 1977). The sulfonated derivatives are sufficiently stable to be isolated but reactive enough to be displaced by nucleophiles to form 6-substituted guanine. This route has been used for the synthesis of O^6-methyl-2′-deoxyguanosine (Gaffney & Jones, 1982), and we have followed a similar route (Scheme I).

There are difficulties in the synthesis of oligonucleotides containing O^6-alkylguanine, and recently Borowy-Borowski and Chambers (1987) reported that "in most cases side products cannot be avoided with current methodology". The first problem arises from the fact that the groups currently

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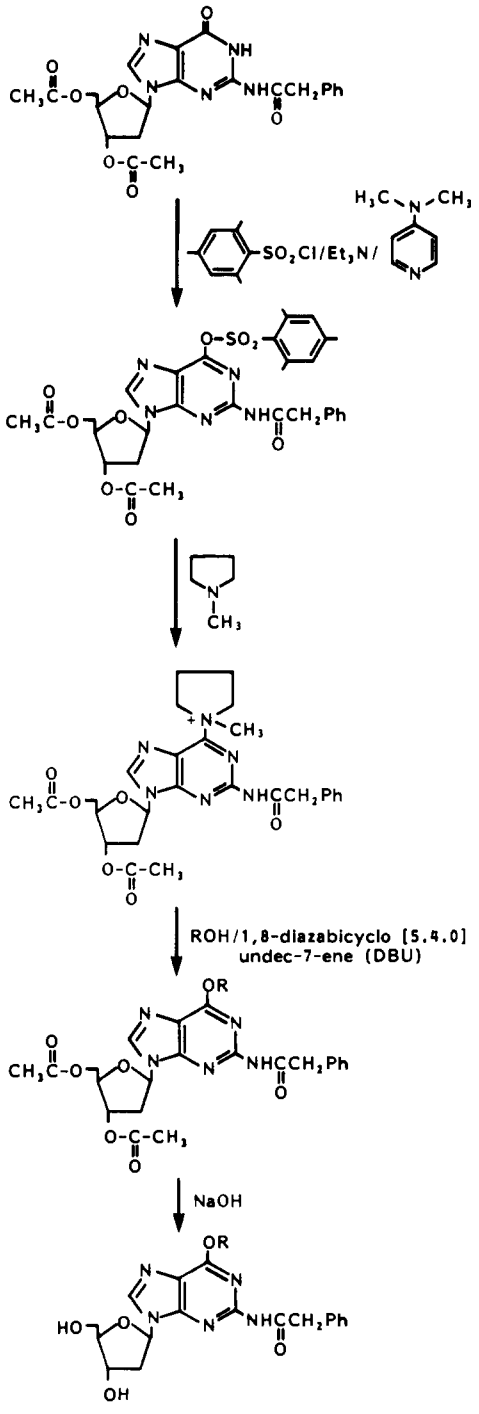
Scheme I



used to protect N^2 of O^6-alkylguanine during oligonucleotide synthesis are resistant to removal by ammonia. This leads to contamination of the final product by incompletely deprotected material as well as contamination by 2,6-diaminopurine formed by displacement of the O^6-alkyl function by ammonia during the prolonged exposure at high temperature that is required to remove the N^2 protection from the N^2-acyl-O^6-alkylguanine (Fowler et al., 1982; Kuzmich et al., 1983; Chambers et al., 1985). We have overcome this problem by using the phenylacetyl group for the N^2 protection of the O^6-alkylguanine. Phenylacetyl has previously been used for N^2 protection of guanine (Kohli & Kumar, 1979; Reese & Skone, 1984).

The second problem is the susceptibility of N^2-acyl-O^6-alkyl-2′-deoxyguanosine residue with a free 5′-OH at the 5′ terminus of the oligomer to depurination by protic acids. This was overcome by the use of $ZnBr_2$ to remove the 9-phenylxanthen-9-yl group protecting the 5′ function during the condensation reactions.

Experimental Procedures

Chemicals. 3-Nitro-1,2,4-triazole, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), 9-chloro-9-phenylxanthene, and 2-chlorophenyl phosphorodichloridate were prepared according to published procedures (Chattopadhyaya & Reese, 1980). Pyridine and triethylamine were purified by distillation, first over 2-mesitylenesulfonyl chloride and then over KOH. Tetrahydrofuran was distilled over calcium hydride and acetonitrile over phosphorus pentoxide.

Protected Deoxynucleotides Containing the Naturally Occurring Bases. Protected deoxynucleotides containing adenine, cytosine, guanine, and thymine were prepared as before (Li et al., 1987).

Enzymes. Snake venom phosphodiesterase (*Crotalus durissus* 2 mg/mL) and calf intestine alkaline phosphatase (2000 units/mL) were obtained from Boehringer Mannheim.

Synthesis of N^2-(Phenylacetyl)-O^6-alkyl-2′-deoxyguanosine. 2′-Deoxyguanosine (6 g, 21 mmol) was suspended in pyridine (68 mL), dimethylformamide (108 mL), and acetic anhydride (12.5 mL) at room temperature for 48 h. Ethanol (10 mL) was then added, and the solution was stirred for 20 min. After evaporation of the solvent under reduced pressure, the white residue, 3′,5′-bis(*O*-acetyl)-2′-deoxyguanosine, was recrystallized from ethanol/water (100 mL, 9:1 v/v), yield 7.4 g (93%).

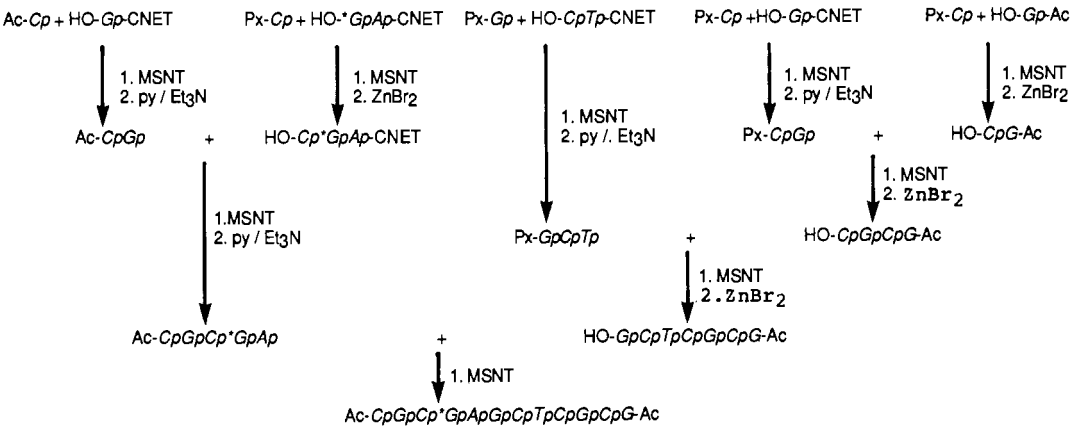
3′,5′-Bis(*O*-acetyl)-2′-deoxyguanosine (5 g, 14.2 nmol) was dried by evaporation of a pyridine solution (30 mL). Phenylacetic anhydride (20 g, 85 mmol) in pyridine (70 mL) was then added and the solution stirred (120 °C, 30 min). After cooling to room temperature, the solution was poured into saturated aqueous $NaHCO_3$ (200 mL). The bicarbonate solution was extracted once with CH_2Cl_2 (150 mL). The organic layer was dried (Mg_2SO_4) and evaporated under reduced pressure to a thick gum. The product, 3′,5′-bis(*O*-acetyl)-N^2-(phenylacetyl)-2′-deoxyguanosine, was purified by column chromatography on Kieselgel 60H (Merck) eluted with a gradient of increasing ethanol concentration in chloroform. The product eluted at 3–4% ethanol in chloroform was then evaporated under reduced pressure to a white foam. The foam was then dissolved in the minimum amount of chloroform and precipitated into diethyl ether, yield 6.7 g (90%).

3′,5′-Bis(*O*-acetyl)-N^2-(phenylacetyl)-2′-deoxyguanosine (0.94 g, 2 mmol) was dissolved in anhydrous CH_2Cl_2 (14 mL). Triethylamine (1.12 mL), 2-mesitylenesulfonyl chloride (0.88 g), and 4-(dimethylamino)pyridine (10 mg) were added to the solution. The reaction was followed by TLC until the starting material was entirely converted to the O^6-sulfonated derivative, which has a higher R_f. This took about 30 min. The solution was cooled to 0 °C, *N*-methylpyrrolidine (2 mL) was added, and the solution was left for 20 min at 0 °C until TLC showed complete conversion to the O^6-(*N*-methylpyrrolidinium) derivative with $R_f = 0$. The appropriate alcohol (methanol, ethanol, or propan-2-ol) (14 mmol) and then 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (6 mmol) were added to the solution. The solution was then allowed to warm to room temperature and stirred for 40 min for the O^6-methyl-, 120 min for the O^6-ethyl-, and 200 min for the O^6-isopropylguanine derivative. The reaction mixture was then poured into aqueous KH_2PO_4 solution (25 mL, 1 M, pH 6.5). The organic layer was dried and evaporated to a foam. The foam was dissolved in a diethyl ether/$CHCl_3$ mixture (9:1 v/v) and purified by column chromatography on Kieselgel 60H using the diethyl ether/$CHCl_3$ mixture as eluant. The product was eluted at

Table I: NMR Spectral Data for N^2-(Phenylacetyl)-O^6-alkyl-2′-deoxyguanosine (alkyldG^{pA}) in DMSO-d_6

	chemical shift (ppm) and no. of protons		
	methyldG^{pA}	ethyldG^{pA}	isopropyldG^{pA}
$\underline{CH_3}O$	4.063 (s) (3 H)		
$\underline{CH_3}CH_2O$		1.361–1.416 (t) (3 H)	
$\underline{(CH_3)_2}CHO$			1.351–1.372 (2) (6 H)
$CH_3\underline{CH_2}O$		4.515–4.599 (m) (2 H)	
$(CH_3)_2\underline{CH}O$			5.504–5.566 (m) (1 H)
H1′	6.298–6.353 (m) (1 H)	6.298–6.351 (m) (1 H)	6.283–6.317 (m) (1 H)
H2′	2.228–2.318 (1 H)	2.227–2.316 (m) (1 H)	2.223–2.279 (m) (1 H)
H2″	2.645–2.751 (m) (1 H)	2.641–2.748 (m) (1 H)	2.636–2.702 (m) (1 H)
H3′, $\underline{CH_2}Ph$	3.835–3.869 (m) (3 H)	3.826–3.870 (m) (3 H)	3.802–3.841 (m) (3 H)
H4′	4.382–4.432 (m) (1 H)	4.395–4.419 (m) (1 H)	4.371–4.397 (m) (1 H)
H5′, H5″	3.460–3.636 (m) (2 H)	3.463–3.637 (m) (2 H)	3.462–3.584 (m) (2 H)
OH	4.886–4.938 (t) (1 H)	4.900–4.944 (t) (1 H)	4.904 (t) (1 H)
OH	5.318–5.375 (d) (1 H)	5.318–5.335 (d) (1 H)	5.304–5.314 (d) (1 H)
$CH_2\underline{Ph}$	7.197–7.355 (m) (5 H)	7.199–7.333 (m) (5 H)	7.216–7.324 (m) (5 H)
H8	8.446 (s) (1 H)	8.443 (s) (1 H)	8.409–8.415 (s) (1 H)
$N\underline{H}$	10.682 (s) (1 H)	10.653 (s) (1 H)	10.614 (s) (1 H)

Scheme II: Strategy for the Synthesis of Oligodeoxynucleotides Containing O^6-Alkylguanine (*G)[a]



[a] When, after deprotection, two molecules of this dodecanucleotide anneal to form a double helix, it will contain two *G·C pairs. The unmodified oligomer containing G·C and G·T pairs and the oligomer containing *G·T pairs were obtained by substituting appropriate monomers in the scheme. Abbreviations: py, pyridine; MSNT, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; Px, 9-phenylxanthen-9-yl; Ac, acetyl. Protected residues are printed in italic: *G, N^2-phenylacetyl-O^6-alkyl-2′-deoxyguanosine; *A*, N^6-benzoyl-2′-deoxyadenosine; *C*, N^4-benzoyl-2′-deoxycytidine; *G*, N^2-acetyl-O^6-(3-chlorophenyl)-2′-deoxyguanosine; *T*, O^4-phenylthymidine; *p*, phosphate residues protected by 2-chlorophenyl; *p*-CNET, phosphate protected by both 2-chlorophenyl and 2-cyanoethyl groups.

100% $CHCl_3$. The fractions containing the product were combined and evaporated to a white foam. The product, 3′,5′-bis(*O*-acetyl)-N^2-(phenylacetyl)-O^6-alkyl-2′-deoxyguanosine, was dissolved in pyridine (3 mL) and evaporated to a small volume under reduced pressure. Pyridine (9 mL) and NaOH solution (2.5 M, 3 mL) were then added to the residue, and the solution was vigorously stirred at room temperature for 6 min. Dowex 50-X8, pyridinium form (8 mL), was then added to neutralize the solution. The Dowex was filtered and washed with pyridine (5 mL). The filtrate was evaporated under reduced pressure to a white powder [N^2-(phenylacetyl)-O^6-alkyl-2′-deoxyguanosine]. The O^6-methyl and O^6-ethyl derivatives were purified by column chromatography on Kieselgel 60H using a gradient of increasing ethanol concentration in chloroform. The product eluted from the column at 7% ethanol in chloroform for the methyl derivative and at 5–6% ethanol for the ethyl derivative. However, the column chromatography on Kieselgel 60H does not separate the isopropyl derivative from impurities. In this case the crude product, N^2-(phenylacetyl)-O^6-isopropyl-2′-deoxyguanosine, was dissolved in a minimum amount of acetonitrile and diluted with water to 10% acetonitrile. This solution was purified by reverse-phase liquid chromatography on a Nova-Pak C18 cartridge at 25 °C (Waters Associates; 3 mL/min with a gradient from water to 48% aqueous acetonitrile over 20 min, retention time 17 min). Each N^2-(phenylacetyl)-O^6-alkyl-2′-deoxyguanosine was dissolved in the minimum amount of $CHCl_3$ and precipitated into 30–40 °C petroleum ether. The NMR spectral data are summarized in Table I, and the microanalysis, UV spectral characteristics, and yield were as follows.

Anal. Calcd for medG^{pA}: C, 57.1; H, 5.3; N, 17.5; O, 20.0. Found: C, 56.1; H, 5.1; N, 17.0; O, 20.3. λ_{max} (H_2O) 217, 268 nm. Yield 71%.

Anal. Calcd for etdG^{pA}: C, 58.1; H, 5.6; N, 16.9; O, 19.4. Found: C, 57.5; H, 5.6; N, 16.5; O, 20.0. λ_{max} (H_2O) 218, 267 nm. Yield 52%.

Anal. Calcd for iprdG^{pA}: C, 59.0; H, 5.8; N, 16.4; O, 18.7. Found: C, 58.6; H, 5.9; N, 16.1; O, 19.1. λ_{max} (H_2O) 220, 267 nm. Yield 25%.

Synthesis of 5′-O-(9-Phenylxanthen-9-yl)-N-acyl-2′-deoxynucleoside 3′-(2-Chlorophenyl phosphates) and 3′-(2-Chlorophenyl 2-cyanoethyl phosphates). The nucleosides of the normally occurring and alkylated bases were phosphorylated and protected on the 5′ position and the phosphate as described before (Li et al., 1987).

Oligonucleotide Synthesis. Oligodeoxynucleotides were synthesized by the phosphotriester approach in solution as

Table II: Conditions and Yields of Final Coupling Reactions Used for the Preparation of Fully Protected Dodecamers[a]

3′-components (mmol)	5′-OH components (mmol)	MSNT (mmol)	pyridine (mL)	reaction time (min)	product	isolated yield (%)
Ac-*CpGpCpGpAp* (0.058)	HO-*GpCpTpCpGpCpG-Ac* (0.055)	0.49	1.5	110	Ac-*CpGpCpGpApGpCpTpCpGpCpG*-Ac	91
Ac-*CpGpCpGpAp* (0.052)	HO-*GpCpTpTpGpCpG*-Ac (0.055)	0.44	1.5	80	Ac-*CpGpCpGpApGpCpTpTpGpCpG*-Ac	90
Ac-*CpGpCpmeGp* (0.047)	HO-*ApGpCpTpCpGpCpG*-Ac (0.042)	0.44	1.2	110	Ac-*CpGpCpmeGpApGpCpTpCpGpCpG*-Ac	73
Ac-*CpGpCpmeGp* (0.044)	HO-*ApGpCpTpTpGpCpG*-Ac (0.038)	0.35	1.0	100	Ac-*CpGpCpmeGpApGpCpTpTpGpCpG*-Ac	66
Ac-*CpGpCpetGp* (0.046)	HO-*ApGpCpTpCpGpCpG*-Ac (0.041)	0.37	1.0	90	Ac-*CpGpCpetGpApGpCpTpCpGpCpG*-Ac	61
Ac-*CpGpCpetGp* (0.042)	HO-*ApGpCpTpTpGpCpG*-Ac (0.039)	0.38	1.0	90	Ac-*CpGpCpetGpApGpCpTpTpGpCpG*-Ac	89
Ac-*CpGpCpiprGpAp* (0.066)	HO-*GpCpTpCpGpCpG*-Ac (0.058)	0.31	1.0	60	Ac-*CpGpCpiprGpApGpCpTpCpGpCpG*-Ac	86
Ac-*CpGpCpiprGpAp* (0.054)	HO-*GpCpTpTpGpCpG*-Ac (0.047)	0.31	1.0	60	Ac-*CpGpCpiprGpApGpCpTpTpGpCpG*-Ac	91

[a] Abbreviations: MSNT, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; Ac, acetyl; A, N^6-benzoyl-2′-deoxyadenosine; C, N^4-benzoyl-2′-deoxycytidine; G, N^2-acetyl-O^6-(3-chlorophenyl)deoxyguanosine; T, O^4-phenylthymidine; meG, N^2-(phenylacetyl)-O^6-methyl-2′-deoxyguanosine; etG, N^2-(phenylacetyl)-O^6-ethyl-2′-deoxyguanosine; iprG, N^2-(phenylacetyl)-O^6-isopropyl-2′-deoxyguanosine; p, phosphate residues protected by 2-chlorophenyl groups. All bases and phosphate groups in these reactions were protected. The protected bases and the protected phosphate groups are italicized.

previously described (Li et al., 1987). The synthetic strategy is shown in Scheme II. The final coupling reactions to produce the fully protected dodecamers are shown in Table II. $ZnBr_2$, rather than a protic acid, was used for the removal of the 5′-(9-phenylxanthen-9-yl) protecting group because 5′-terminal N^2-protected O^6-alkyldeoxyguanosine residues with a free 5′-OH depurinate rapidly in acid. To avoid the possibility of side reactions on O^6 of guanine residues and O^4 of thymine residues, these were protected with 3-chlorophenyl groups and phenyl groups, respectively. (Reese & Skone, 1984; Li et al., 1987).

Kinetics of Ammonolysis of N-Acylated Nucleosides. (1) Removal of N^2-(Phenylacetyl)-O^6-alkyl-2′-deoxyguanosine (alkyldG^{pA}). Samples of alkyldG^{pA} (0.15 mg in 0.15 mL of H_2O) were treated with aqueous ammonia (3 mL; d = 0.88) in sealed flasks at 22 °C. After 0.5, 1, 2, 4, 6, and 30 h, the contents of a flask were evaporated under reduced pressure, and the residue was redissolved in water (1 mL) and analyzed by reverse-phase liquid chromatography (Figure 1A). The amount of O^6-alkyl-2′-deoxyguanosine and alkyldG^{pA} was measured from the absorbance at 260 nm of each chromatographic peak, and the pseudo-first-order rate constants were calculated (Table III). A similar reaction in which medG^{pA} was treated with 0.15 M (*E*)-2-nitrobenzaldoxime/0.135 M N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) in ammonia (d = 0.88) for 1 and 30 h at 25 °C showed that no detectable 2,6-diaminopurine or guanine had been formed (Figure 1A).

(2) Removal of the Benzoyl Group from N^6-Benzoyl-2′-deoxyadenosine (dA^{Bz}). dA^{Bz} (100 μg in 100 μL of solution) was put into 1.5-mL screw-cap Eppendorf tubes containing 0, 50 or 100 μL of 0.3 M (*E*)-2-nitrobenzaldoxime/0.27 M TMG in acetonitrile/H_2O (80:20 v/v). The solvent was removed in vacuo and the residue redissolved in aqueous ammonia (d = 0.88) and incubated at 25 °C. After 0, 1, 2, 4, 6.5, 8.5, 11, and 14 h, samples that had contained the different amounts of oxime/TMG were dried in vacuo. The residue was redissolved in H_2O and analyzed by reverse-phase liquid chromatography. The amount of dA^{Bz} remaining was assessed from the absorbance of its chromatographic peak at 260 nm. The rate constants for removal of the benzoyl protecting group are given in Table III.

Deprotection and Purification of Oligodeoxynucleotides. The same deprotection procedure was used for the alkylated and nonalkylated dodecamers. This involves two stages:

Table III: Pseudo-First-Order Rate Constants at 22 °C for Removal of the Phenylacetyl Group from N^2-(Phenylacetyl)-O^6-alkyl-2′-deoxyguanosine (alkyldG^{pA}) and for Removal of the Benzoyl Group from N^6-Benzoyl-2′-deoxyadenosine (dA^{Bz})

substrate	conditions	rate constant (k)	$t_{1/2}$
methyldG^{pA}	aq ammonia (d = 0.88)	1.42×10^{-2}/min	48 min
ethyldG^{pA}	aq ammonia (d = 0.88)	1.41×10^{-2}/min	49 min
isopropyldG^{pA}	aq ammonia (d = 0.88)	1.27×10^{-2}/min	55 min
dA^{Bz}	aq ammonia (d = 0.88)	0.72×10^{-2} h	9.6 h
dA^{Bz}	0.03 M oxime/0.027 M TMG in aq ammonia (d = 0.88)	1.93×10^{-1} h	3.6 h
dA^{Bz}	0.06 M oxime/0.054 M TMG in aq ammonia (d = 0.88)	3.79×10^{-1}/h	1.8 h

(1) The fully protected dodecamers were treated with a solution of (*E*)-2-nitrobenzaldoxime (0.3 M) and TMG (0.27 M) in acetonitrile/water (80:20 v/v) at room temperature for 20 h. This removes the 2-chlorophenyl protecting groups from the internucleotide linkage, the phenyl group from O^4-phenylthymine residues, and the 3-chlorophenyl group from N^2-acetyl-O^6-(3-chlorophenyl)guanine residues. Ten molecular equivalents of oxime and 9 molecular equivalents of TMG were used for each protecting group.

(2) The above solution was evaporated under reduced pressure to a small volume and aqueous ammonia added (d = 0.88, twice the volume of oxime/TMG solution used). After 30 h at room temperature, the ammonia was evaporated under reduced pressure. The residue was suspended in water (20 mL) and the pH adjusted to 6.5 with acetic acid. The solution was extracted with ether (3 × 20 mL), CH_2Cl_2 (3 × 20 mL), and then ether (20 mL). The aqueous phase was then concentrated under reduced pressure and desalted (Sephadex G-25) by using ethanol/water (1:4 v/v) as eluting solvent. The oligomers were purified by reverse-phase liquid chromatography (conditions are given in the legend to Figure 2).

The completeness of the deprotection, the correct base analysis, and the absence of unwanted products of side reactions were checked by nucleoside analysis. Dodecamers (approximately 1 A_{260} unit in 50 μL of H_2O) were added to 100 μL of Tris-HCl (50 mM, pH 8.3)/$MgCl_2$ (5 mM). After equilibration at 37 °C for 10 min, phosphodiesterase (10 μL) was added. After 1 h, alkaline phosphatase (5 μL) was added.

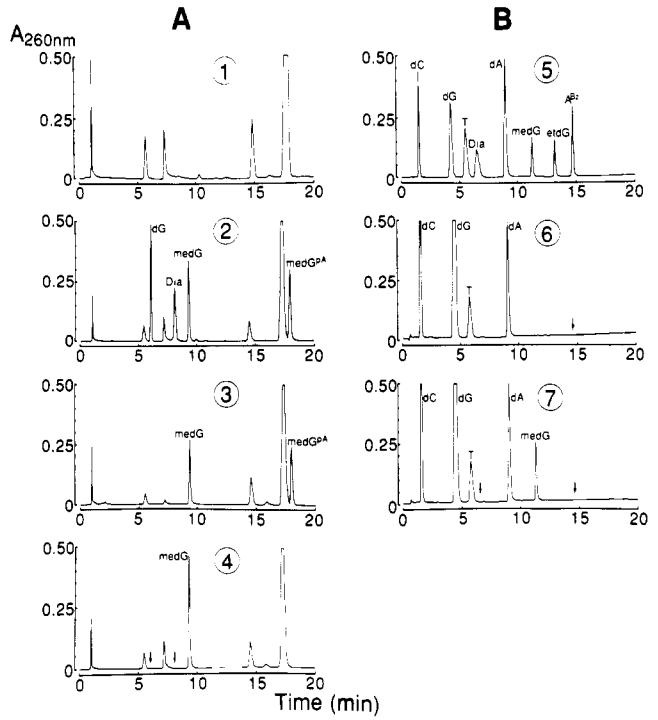


FIGURE 1: (A) Reverse-phase chromatographic analysis to detect possible side reaction products formed during the removal of the N^2-phenylacetyl group from N^2-(phenylacetyl)-O^6-methyl-2′-deoxyguanosine (medG^{pA}). medG^{pA} was treated with 0.15 M (*E*)-2-nitrobenzaldoxime/0.135 M TMG in ammonia (d = 0.88) at 20 °C. Chromatogram 1 is a reagent blank showing the position of elution of constituents of the oxime/TMG/NH_4OH reagent; (2) and (5) show the position of elution of the nucleosides deoxyadenosine (dA), deoxycytidine (dC), deoxyguanosine (dG), thymidine (dT), (medG), 2,6-diaminopurine deoxyriboside (Dia), O^6-methyl-2′-deoxyguanosine (medG), and medG^{pA}, when injected as a solution in the reagent; (3) is a chromatogram of a mixture of medG^{pA} and the reagent after 1-h reaction (note that just over half the medG^{pA} has been converted to medG); (4) is a chromatogram of the same mixture after 30 h (note that there is no medG^{pA} remaining and that there is no detectable deoxyguanosine or 2,6-diaminopurine deoxyriboside). The expected elution times of these (6.2 and 8.1 min) are shown by arrows. (Conditions: Waters Nova-Pak C18 cartridge at room temperature eluted at 4 mL/min with a gradient from 0 to 28% acetonitrile over 20 min. Detection: absorbance at 260 nm.) (B) Reverse-phase analysis of the nucleosides derived from enzymic digestion of the dodecamers CGCGAGCTCGCG and CGC*meG*AGCTCGCG. Chromatogram 5 shows the position of elution of relevant nucleosides. dA^{Bz} is N^6-benzoyl-2′-deoxyadenosine; other abbreviations are as a (A). Chromatogram 6 is of a digest of CGCGAGCTCGCG and chromatogram 7 of a digest of CGC*meG*AGCTCGCG. The amount injected is 10 times greater than in Figure 2 so that any minor impurities could be seen. Note that there is no detectable 2,6-diaminopurine deoxyriboside or dA^{Bz}. The expected elution times of these (6.8 and 14.6 min) are shown by arrows. (Conditions: Dodecamers were digested as described under Experimental Procedures. Waters Nova-Pak C18 cartridge at 25 °C eluted at 4 mL/min; 1.6% acetonitrile in 50 mM KH_2PO_4 for the first 6 min, increased to 25% over the next 14 min. Detection: absorbance at 260 nm).

After a further 25 min, the reaction was stopped by heating at 80 °C for 5 min. The resultant nucleosides were separated by reverse-phase liquid chromatography (Figure 2). The amount of each nucleoside, obtained by comparing the integrated absorbance at 260 nm of each peak with the absorbance of standard solutions, is given under Results, and an expanded chromatogram showing that there was no measurable amount of 2,6-diaminopurine or residual protected A (dA^{Bz}) is shown in Figure 1B.

DNA Melting Study. Melting curves were obtained by measuring the changes in absorption at 260 nm in a Unicam SP500 spectrometer (Pye Unicam, Cambridge, U.K.) fitted with a Gilford 222 photometer and a Gilford 2527 thermo-

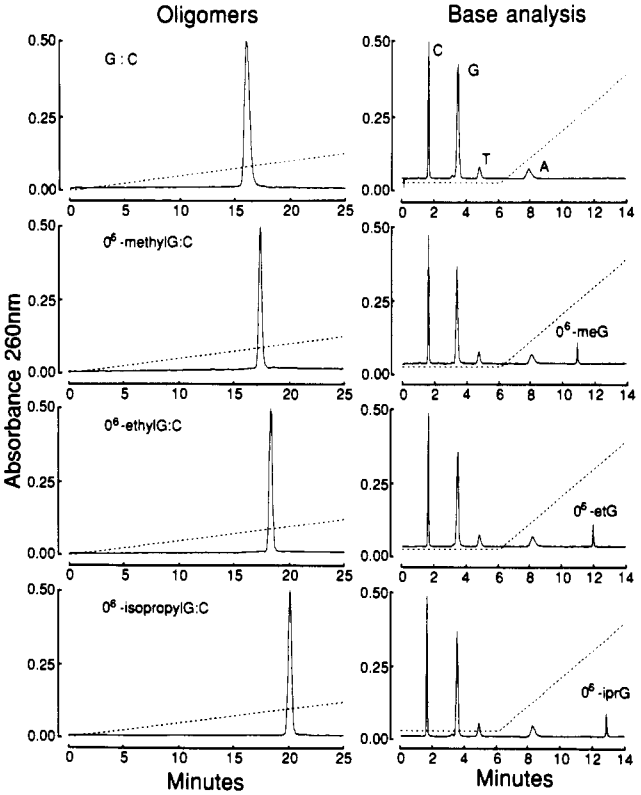


FIGURE 2: Reverse-phase liquid chromatograph analysis of dodecamers containing O^6-alkylguanine and their constituent nucleosides. Dodecamers were degraded to nucleosides by treatment with phosphodiesterase and alkaline phosphatase. Oligomers were chromatographed on a Waters Nova-Pak C18 cartridge at 45 °C and 3 mL/min with a gradient of 0–11% acetonitrile in 0.1 M aqueous KH_2PO_4 (pH 6.3) over 30 min. Nucleosides were chromatographed on a Nova-Pak C18 cartridge at 25 °C and 3 mL/min. The column was eluted for 7 min with 1.6% acetonitrile in 50 mM aqueous KH_2PO_4 (pH 4.5), and then the concentration of acetonitrile was increased to 25% over the following 10 min.

programmer (Gilford Instruments, Oberlin, OH). All melting curves were measured on solutions with an initial absorbance at 260 nm of 1.10–1.25 at 12 °C in 1 M NaCl, 10 mM KH_2PO_4, and 1 mM EDTA, pH 7. To make the melting curves directly comparable, the sensitivity of the instrument was set so that a 33% increase from the absorption at 12 °C would give a full-scale deflection of the recorder. The temperature was increased by 1 °C/min. Results are shown in Figure 3.

NMR Spectroscopy. ^1H NMR spectra of the dodecadeoxynucleotides were recorded on a Brüker AM500 500-MHz spectrometer. Oligonucleotides (400 A_{260} units) were dissolved in 0.4 mL of D_2O containing 0.1 M NaCl, 1.0 mM EDTA, and 10 mM KH_2PO_4 (pH 7). The results are shown in Figure 4. The NMR spectra of 2′-deoxyadenosine (dA), 2′-deoxyguanosine, O^6-methyl-2′-deoxyguanosine (medG), O^6-ethyl-2′-deoxyguanosine (etdG), and O^6-isopropyl-2′-deoxyguanosine (iprdG) were recorded on a Varian 400-MHz spectrometer. The nucleosides (1.5 mg) were dissolved in 0.6 mL of D_2O buffer containing 73 mM NaCl, 0.75 mM EDTA, and 75 mM KH_2PO_4 (pH 7). The spectral data are summarized in Table IV. For comparison this table also gives the equivalent chemical shifts for the O^6-methyl-2′-deoxyguanosine and O^6-ethyl-2′-deoxyguanosine in the dodecamer double helices in solution. The complete NMR data from these duplexes will be presented in detail elsewhere.

Results and Discussion

These experiments show that pure oligonucleotides con-

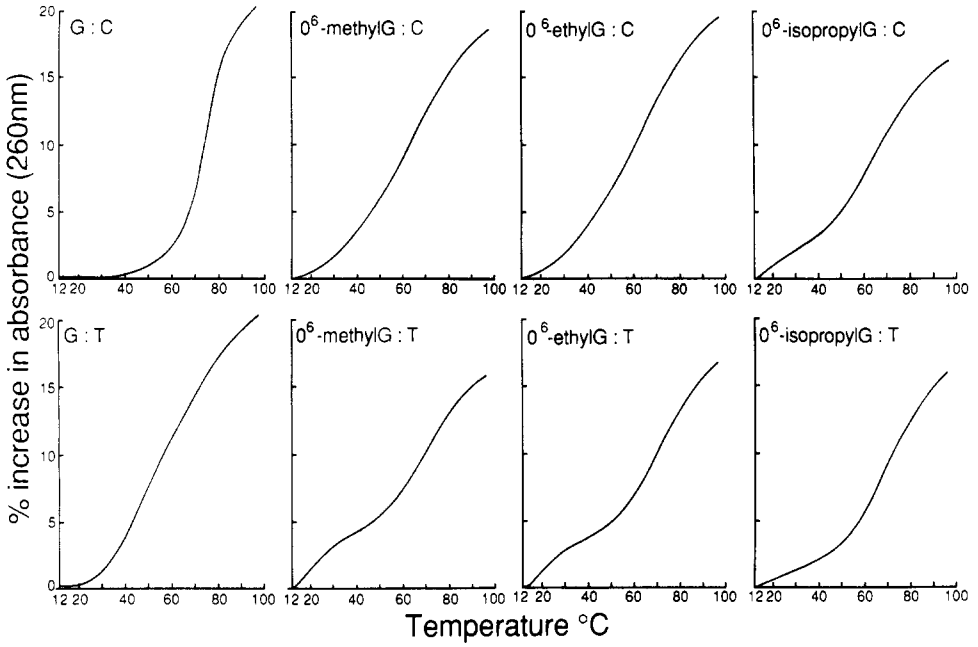


FIGURE 3: Thermal melting curves of oligodeoxynucleotide double helices containing O^6-alkylguanine base paired to C and to T compared to melting curves of parent DNA helices. All oligonucleotide solutions had an initial absorbance of 1.1–1.25 at 260 nm at 12 °C in buffer: 1 M NaCl, 10 mM KH_2PO_4, and 1 mM EDTA (pH 7). The span of the recorder was set at 33% of the absorbance at 12 °C. The cuvette was heated at 1 °C/min.

Table IV: NMR Chemical Shifts (ppm) of Nucleosides, Alkylated Nucleosides, and Alkylated Nucleosides in Dodecamers[a]

	dA	dG	medG	etdG	iprdG	CGCmeGAGCTCGCG	CGCetGAGCTCGCG
$(\underline{CH_3})_2CHO$					1.395–1.411		
$\underline{CH_3}CH_2O$				1.408–1.444			1.11
$\underline{CH_3}O$ (partially labeled with ^{13}C)			4.23 and 3.86			3.71 and 3.43	
$CH_3\underline{CH_2}O$				4.443–4.497			3.73
$(CH_3)_2\underline{CH}O$					5.436–5.483		
H1′	6.441–6.475	6.276–6.313	6.291–6.326	6.282–6.317	6.311–6.345		
H2′	2.531–2.590	2.474–2.538	2.481–2.540	2.478–2.537	2.470–2.521		
H2″	2.799–2.868	2.749–2.822	2.764–2.833	2.760–2.829	2.766–2.836		
H3′	4.176–4.201	4.110–4.142	4.135–4.163	4.130–4.156	4.133–4.159		
H4′	4.665–4.634	4.601–4.624	4.616–4.646	4.613–4.643	4.614–6.644		
H5′, H5″	3.758–3.864	3.730–3.837	3.743–3.850	3.739–3.846	3.740–3.846		
H(2)	8.194						
H(8)	8.301	7.979–7.983	8.030	8.019	8.039		

[a] Nucleoside NMR spectral data were obtained on a 400-MHz spectrometer; 1.5 mg of nucleosides were dissolved in 0.6 mL of D_2O containing 0.1 M NaCl, 1.0 mM EDTA, and 10 mM KH_2PO_4 (pH 7). The data on the dodecanucleotides were from solutions of 400 A_{260} units in 0.4 mL of D_2O (pH 7).

taining O^6-alkylguanine can be obtained if N^2-(phenylacetyl)-O^6-alkyl-2′-deoxyguanosine is used as the building block because of the facile removal of the phenylacetyl group by aqueous ammonia (Table III). The half-life of the phenylacetyl group in aqueous ammonia (d = 0.88) at 22 °C was only about 50 min (Table III) and unexpectedly the rate of removal was not influenced by the nature of the O^6-alkyl group, so that the different alkylated guanines could be deprotected with equal facility. This is an important advantage. Gaffney et al. (1984) successfully used methoxide ions (methanol/DBU) for 72 h to deprotect oligomers containing N^2-acetyl-O^6-methylguanine, and we have used the same method to deprotect oligomers containing O^4-methylthymine (Li et al., 1987). However, methoxide cannot be used to deprotect oligomers containing bases O6-alkylated with different alkyl groups because as the alkoxide exchanges with the O^6-alkyl group the appropriate alkoxide must be used (in this case ethoxide and isopropoxide would be necessary). We have attempted to use ethanol/DBU to deprotect oligomers containing O^4-ethylthymine without success. The reason for the failure was probably because higher alkoxides are difficult to generate and sterically hindered and, in addition, the partially deprotected oligomers precipitated from the ethanolic solution.

We cannot explain why the phenylacetyl group is so easily removed. One would expect that the rate of ammonolysis of N-acyl functions should be inversely related to the pK_a of the parent acid, but there is no correlation in this case. Phenylacetyl (phenylacetic acid pK_a = 4.21) is easily removed, while benzoyl (benzoic acid pK_a = 4.19) and isobutyryl (isobutyric acid pK_a = 4.84) are resistant to ammonolysis (Fowler et al., 1982; Kuzmich et al., 1983). The rapid removal of the phenylacetyl reduces the chance of formation of 2,6-diaminopurine because only short exposure to ammonia is needed for deprotection. In addition, the rapid removal of the phenylacetyl also inhibits formation of 2,6-diaminopurine because O^6-alkylguanine is less susceptible to substitution by ammonia than is N^2-protected O^6-alkylguanine. Previous workers have had to use prolonged exposure to concentrated ammonia at high temperature to deprotect oligomers containing N^2-isobutyryl-O^6-alkylguanine (for example, 48 h at 65 °C; Bo-

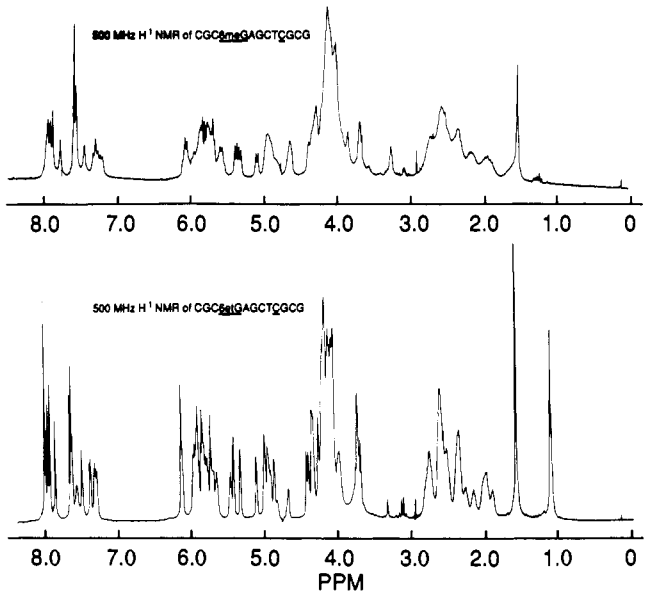


FIGURE 4: 500-MHz ^{1}H NMR spectra of oligodeoxynucleotides double helices each containing two O^6-meG·C and O^6-ethylG·C base pairs. Oligonucleotides (400 A_{260} units) were dissolved in 0.4 mL of D_2O containing 0.1 M NaCl, 1.0 mM EDTA, and 10 mM KH_2PO_4 (pH 7).

rowy-Borowski & Chambers, 1987) but the ease of removal of the phenylacetyl group allowed us to adopt a much milder deprotection procedure. The limitation now was not the removal of the phenylacetyl but the rate of removal of the protecting groups from the naturally occurring bases, for example, from N^6-benzoyladenine. This has a $t_{1/2}$ of 8 h in ammonia (Shuldolf et al., 1987), so that if ammonia alone is used for deprotection, a prolonged exposure is necessary. However, the benzoyl group can also be removed by equimolar (*E*)-2-nitrobenzaldoxime (oxime) and TMG (Patel et al., 1984), and a solution of oxime and TMG in ammonia rapidly removed the benzoyl group. The $t_{1/2}$ at 22 °C for the removal of the benzoyl group from N^6-benzoyl-2′-deoxyadenosine by ammonia was 9.6 h, but in a solution of 0.06 M oxime/0.054 M TMG in aqueous ammonia the $t_{1/2}$ for removal of the benzoyl group was only 1.8 h (Table III). We used 30 h at room temperature with 0.15 M oxime/0.135 M TMG in ammonia (d = 0.88) as the second stage of deprotection of the oligomers. Digestion of the oligomers to nucleosides, separation of them by chromatography, and quantitation of the amount of each by comparison of the absorbance at 260 nm with the absorbance of known amounts of each nucleoside showed that the oligomer had exactly the expected base ratio and that there was no remaining N^6-benzoyl-2′-deoxyadenosine (Figure 1B).

After deprotection, the dodecamers eluted as a single component on reverse-phase liquid chromatography (Figure 2). The oligomers containing O^6-alkylguanine were well separated from the nonalkylated parent sequence, with each oligomer having a slightly different retention time depending on the alkyl group. The oligomers were eluted in the order methyl, ethyl, isopropyl.

NMR data for the free nucleosides and for the same nucleosides in the DNA double helices formed from these oligomers in solution are given in Table IV. The NMR spectra of the oligonucleotides containing O^6-methylguanine and O^6-ethylguanine are shown in Figure 4. In the alkylated nucleosides the protons on C8 have resonance frequencies slightly downfield from that of 2′-deoxyguanosine toward that of 2′-deoxyadenosine, although this shift is not as great as the shift of the C6 proton of thymidine when the O^4 is methylated (Li et al., 1987). There is also a shift of the Cl′ sugar proton resonances. This downfield shift is the consequence of the aromatization of the purine ring in O^6-alkyl-2′-deoxyguanosine. This aromatization and the consequent increase in lipophilicity make it chemically resemble 2′-deoxyadenosine. This chemical similarity may explain why O^6-methyl-2′-deoxyguanosine is a substrate for adenosine deaminase (Pegg & Swann, 1979). The NMR spectra of the oligomers containing O^6-methylguanine and O^6-ethylguanine showed sharp peaks, and the resonances of the methyl group protons of the methylguanine sequence and the resonances of the methyl and methylene protons of the ethylguanine sequence are well resolved. As one might expect, the chemical shift of the alkyl protons is further upfield in the oligomers than in the free nucleoside. A preliminary report of two-dimensional nuclear Overhauser spectra has been published, and the broad outlines of the structure of the double helices formed by these oligomers in solution have been established (Li et al., 1988). The NMR shows that sequences containing O^6-methylguanine and O^6-ethylguanine produce B-form DNA duplexes in solution. However, the dodecamers containing isopropylguanine do not form stable double helices in solution, and a number of different conformations are present.

The melting profiles in Figure 3 show two patterns. In the alkylG·C series, the alkylated oligomers melt much earlier than the parent, with only the iprG·C oligomer having significantly lower hypochromicity than the G·C parent. However, in the alkylG·T series the melting profile is clearly biphasic and all sequences have lower hypochromicity than the parent sequence. The destabilizing effect of O^6-alkylguanine on the DNA helix, in particular the effect of O^6-alkylG·T base pairs (Figure 3), which has also been reported by Gaffney et al. (1984), seems inconsistent with the postulate that these two bases form a stable base pair [reviewed by Saffhill et al. (1985)] and with the report that the tetramer T*meG*CA can be hybridized to a ..TGTA.. sequence selectively and sufficiently strongly for it to be ligated into a plasmid (Green et al., 1984).

Chambers et al. (1985) reported the interaction of uvr A and Rec A on mutagenesis involving meG and butylG in the sequence AAAGTCGG*AAACAT (where G* = O^6-meG or O^6-butylG). As the meG or butylG in this pentadecamer is flanked on one side by three weak A·T base pairs, the helix might be expected to be substantially opened around the meG or butylG residue. The instability in the helix suggested by the melting curves of the DNA sequences containing O^6-alkylguanine may play an important role in the recognition by those enzymes that seem to recognize damaged DNA rather than a specific base modification. We are hopeful that the high-resolution NMR study under way will provide us with information about the extent of distortion of the helix produced by the presence of O^6-alkylguanine residues in DNA so that the way in which these repair proteins recognize their target can be more thoroughly understood.

ACKNOWLEDGMENTS

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Registry No. dG, 961-07-9; medG^{pA}, 120022-79-9; etdG^{pA}, 120022-80-2; iprdG^{pA}, 120022-81-3; dA^{Bz}, 4546-72-9; Ac-CpGpCpGpAp, 120032-87-3; Ac-CpGpCpmeGp, 120022-82-4; Ac-CpGpCpetGp, 120022-83-5; Ac-CpGpCpiprGpAp, 120032-88-4; HO-GpCpTpCpGpCpG-Ac, 120032-89-5; HO-GpCpTpTpGpCpG-Ac, 120022-84-6; HO-ApGpCpTpCpGpCpG-Ac, 120084-94-8; HO-ApGpCpTpTpGpCpG-Ac, 106266-25-5; Ac-CpGpCpGpApGpCpTpCpGpCpG-Ac, 120085-10-1; Ac-

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