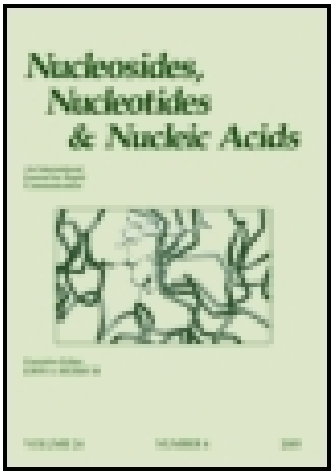

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Synthesis and Thermodynamic Stabilities of Damaged DNA Involving 8-Hydroxyguanine (7,8-Dihydro-8-Oxoguanine) in a ras-Gene Fragments

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SYNTHESIS AND THERMODYNAMIC STABILITIES OF DAMAGED DNA INVOLVING 8-HYDROXYGUANINE (7,8-DIHYDRO-8-OXOGUANINE) IN A *ras*-GENE FRAGMENT§

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ABSTRACT: A new building block of 8-hydroxyguanine (7,8-dihydro-8-oxoguanine, oh^{8}Gua) with a N^2-(*N*,*N*-dimethylaminomethylene) group was prepared for oligonucleotide synthesis. Oligodeoxyribonucleotides corresponding to a part of the human c-Ha-*ras* gene and containing oh^{8}Gua in codon 12 were synthesized using the phosphoramidite monomer. The oligonucleotides were used for thermal denaturation studies and circular dichroism measurements. Thermodynamic parameters showed that, other than oh^{8}Gua:C and oh^{8}Gua:A pairs, oh^{8}Gua at the first and second positions of codon 12 forms stable pairs with G and T, respectively. These results may explain the different spectra of mutations induced by the base at the first and second positions of codon 12.

INTRODUCTION

8-Hydroxyguanine (7,8-dihydro-8-oxoguanine, oh^{8}Gua) is generated by a variety of reagents that produce oxygen radicals.[1-5] The DNA lesion may be involved in mutagenesis, carcinogenesis, and aging.[6,7]

The modified base has been studied as a pre-mutagenic lesion. Eukaryotic and prokaryotic DNA polymerases inserted dCMP and dAMP opposite oh^{8}Gua *in vitro*.[8,9] A G to T transversion was detected when templates with oh^{8}Gua were replicated in

§ This paper is dedicated to Professor M. Ikehara on the occasion of his 70th birthday.

Escherichia coli.[10-12] These results suggested that oh^{8}Gua induced G to T transversions in organisms. However, we detected various point mutations at the modified site and the 5'-adjacent position when we transfected a vector bearing the modified base at the second position of codon 12 (the 35th position) of the c-Ha-*ras* gene into NIH3T3 cells.[13] Reports on mutations by oh^{8}Gua in mammalian cells revealed that the base induced mainly G to T transversions, although other types of mutations and flanking mutations were detected.[14,15] Also, we observed the G to T mutation almost exclusively when we transfected a vector bearing the modified base at the first position of codon 12 (the 34th position) of the c-Ha-*ras* gene into NIH3T3 cells (Kamiya *et al.*, unpublished results).

To clarify the reasons for the different mutation-spectra of oh^{8}Gua at the 34th and 35th positions of the c-Ha-*ras* gene, we measured the melting temperatures (T_m values) and calculated the thermodynamic parameters of oligodeoxyribonucleotide duplexes with base pairs of oh^{8}Gua at the sites corresponding to the 34th and 35th positions (hereafter abbreviated as 34-oh^{8}Gua and 35-oh^{8}Gua, respectively). The oh^{8}Gua residues at both sites could pair with C and A, which is consistent with the previous data.[8,9] In addition, we found that oligonucleotide duplexes with the 35-oh^{8}Gua:T and 34-oh^{8}Gua:G pairs were thermodynamically more stable than the corresponding G:T and G:G pairs. These results may explain the different mutation-spectra of the oh^{8}Gua at the first and second positions of codon 12.

MATERIALS AND METHODS

General

^{1}H-NMR spectra were recorded on a JEOL FX-270 spectrometer with tetramethylsilane as an internal standard. ^{31}P-NMR spectra were recorded on a JEOL FX-90Q spectrometer with trimethyl phosphate as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad). UV absorption spectra were recorded with a Shimadzu UV-240 spectrophotometer and a Beckman DU-65 spectrophotometer. Mass spectra (MS) were measured on a JEOL JMX-DX303 spectrometer. Thin layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F_{254} plates (No. 5715). The silica gel for column chromatography was Wako-gel C-300.

8-Methoxy-2'-deoxyguanosine (3). 8-Bromo-2'-deoxyguanosine (**2**, 2.3 g, 6.6 mmol), prepared according to the reported method,[16] was suspended in 20 ml of methanol, and 3 N $NaOCH_3$/MeOH (16ml) was added. The reaction mixture was stirred for 48 hr at 75°C and then 20 ml of methanol was added to the mixture. Dowex 50W (pyridinium form) was added to neutralize the reaction mixture, and the resin was filtered.

The filtrate was concentrated to dryness *in vacuo*. A portion of water was added to the residue and the precipitates were collected (1.54 g, 79%). λ_{max} (MeOH) 250 and 285 nm. ^{1}H-NMR (DMSO-d_6): 2.02 and 2.86 (each m, 1 H, 2'-H), 3.48 (m, 2 H, 5'-H), 3.75 (m, 1 H, 3'-H), 3.96 (s, 3 H, 8-OMe), 4.34 (m, 1 H, 4'-H), 4.80 (br, 1 H, 5'-OH), 5.18 (br, 1 H, 3'-OH), 6.08 (t, 1 H, 1'-H), 6.32 (s ,2 H, 2-NH_2), 10.13 (s, 1 H, 1-NH).

5'-*O*-Monomethoxytrityl-N^2-(*N*,*N*-dimethylaminomethylene)-8-methoxy-2'-deoxyguanosine (5). Compound **3** (0.59 g, 2 mmol) was co-evaporated with pyridine three times and once with toluene, and suspended in DMF (9 ml). To the stirred mixture, *N*,*N*-dimethylformamide dimethylacetal (0.8 ml, 7.4 mmol) was added and stirring was continued for 4 hr at room temperature. The mixture was concentrated, and the residue was co-evaporated with pyridine, and dissolved in pyridine (10 ml). Monomethoxytrityl chloride (0.93 g, 3 mmol) was added to the solution and the reaction mixture was stirred for 14 hr at room temperature. The reaction was quenched with 2 ml of methanol and the mixture was partitioned between $CHCl_3$ and water. The separated organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography with a 0 to 4% gradient of MeOH in $CHCl_3$ containing 0.1% pyridine. The main UV-absorbing fractions were combined and concentrated to afford the title compound as a foam (0.82 g, 66%). λ_{max} (MeOH) 240, 280, 303, and 310 nm. ^{1}H-NMR (DMSO-d_6): 2.16 and 2.84 (each m, 1 H, 2'-H), 2.94-3.12 (2s, 6 H, -NMe_2), 3.14 (m, 2 H, 5'-H), 3.76 (s, 3 H, OMe, monomethoxytrityl), 3.82 (s, 3 H, 8-OMe), 3.90 (m, 1 H, 4'-H), 4.42 (m, 1 H, 3'-H), 5.35 (d, 1 H, 3'-OH), 6.72 (t, 1 H, 1'-H), 6.81 (m, 2 H, monomethoxytrityl), 7.25 (m, 12 H, monomethoxytrityl), 8.58 (d, 1 H, =N-CH=), 10.52 (s, 1 H, 1-NH).

5'-*O*-Monomethoxytrityl-N^2-(*N*,*N*-dimethylaminomethylene)-7,8-dihydro-8-oxo-2'-deoxyguanosine (6). Thiophenol (2.56 ml, 24.4 mmol) and triethylamine (1.56 ml, 11.1 mmol) were added to a solution of **5** (0.69 g, 1.11 mmol) in DMF (13.3 ml). The mixture was heated at 50°C for 18 hr with stirring. After removal of solvent *in vacuo*, the residue was partitioned between $CHCl_3$ and water. The separated organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography with a 0 to 6% gradient of MeOH in $CHCl_3$ containing 0.1% pyridine. The main UV-absorbing fractions were combined and concentrated to afford the title compound as a foam (0.60 g, 89%). λ_{max} (MeOH) 235, 285, and 315 nm. ^{1}H-NMR (DMSO-d_6): 2.10 and 2.82 (each m, 1 H, 2'-H), 3.04-3.8 (2s, 6 H, -NMe_2), 3.18 (m, 2 H, 5'-H), 3.74 (s, 3 H, OMe, monomethoxytrityl), 3.82 (m, 1 H, 4'-H), 4.42 (m,

1 H, 3'-H), 5.18 (d, 1 H, 3'-OH), 6.12 (t, 1 H, 1'-H), 6.75 (m, 2 H, monomethoxytrityl), 7.24 (m, 12 H, monomethoxytrityl), 8.54 (d, 1 H, =N-CH=), 10.54 (s, 1 H, 1-NH), and 11.26 (s, 1 H, 7-NH). FAB/MS *m/z* 611 $(M+H)^+$ 19%, 273 (monomethoxytrityl)$^+$ 100%, 222 (heterocyclic base +H)$^+$ 34%. *Anal.* Calcd for $C_{33}H_{34}N_6O_6 \cdot 1/2H_2O$: C, 63.96; H, 5.53; N, 13.56. Found: C, 64.18; H, 5.56; N, 13.42. Rf ($CHCl_3$-MeOH, 10:1 v/v) 0.33.

5'-*O*-Monomethoxytrityl-N^2-(*N*,*N*-dimethylaminomethylene)-7,8-dihydro-8-oxo-2'-deoxyguanosine-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropyl-phosphoramidite] (7). Compound **6** (165 mg, 0.27 mmol) was co-evaporated with pyridine three times and dissolved in dichloromethane (2 ml). Diisopropylethylamine (103 µl, 0.59 mmol) and 2-cyanoethyl *N*,*N*-diisopropyl chlorophosphoramidite (73 µl, 0.32 mmol) were added to the solution with stirring and the stirring was continued for 1 hr under an Ar atmosphere at room temperature. The mixture was concentrated *in vacuo*, and the residue was purified by column chromatography at 4°C (in a cold room). After elution with a 0 to 2 % gradient of MeOH in $CHCl_3$ containing 0.1% pyridine, the main UV-absorbing fractions were collected and evaporated. The residue was dissolved in $CHCl_3$ (1.4 ml) and precipitated with hexane (22 ml). The precipitate was collected by centrifugation (3000 rpm, 3 min) and dissolved in $CHCl_3$. After concentration *in vacuo*, the title compound was obtained as a foam (183 mg, 84%). ^{1}H-NMR (DMSO-d_6): 1.10 (m, 12 H, *i*Pr), 2.30 (m, 1 H, 2'-H), 2.55 (t, 2 H, -CH_2CN), 2.72 (m, 1 H, 2'-H), 2.84 (m, 2 H, 5'-H), 3.05 (2s, 6 H, -NMe_2), 3.72 (s, 3 H, OMe, monomethoxytrityl), 3.84-4.20 (m, 3 H, 4'-H and -OCH_2-), 4.82 (m, 1 H, 3'-H), 6.14 (m, 1 H, 1'-H, two diastereomers), 6.78 (m, 2 H, monomethoxytrityl), 7.25 (m, 12 H, monomethoxytrityl), 10.88 (m, 1 H, 1-NH), and 11.45 (m, 1 H, 7-NH). ^{31}P-NMR ($CDCl_3$): 146.06 and 146.33 (two diastereomers). Rf ($CHCl_3$-MeOH, 10:1 v/v) 0.48.

Solid phase synthesis of oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer, using the 1 µmol scale. In the oh^{8}Gua cycle, the coupling and detritylation procedures were modified . The coupling and detritylation times were increased from 25 sec to 10 min and from 35 sec to 1 min, respectively. Phosphoramidite derivatives other than oh^{8}Gua were commercially available as benzoyl- (A and C) or isobutyryl- (G) protected compounds. The oligonucleotide was cleaved from the support by 2 ml of concentrated ammonia containing 0.1 M 2-mercaptoethanol and kept in a sealed flask for 6 hr at 55°C. The reaction mixture was concentrated *in vacuo* and then loaded onto a C-18 open column preequilibrated with 50 mM triethylammonium acetate (TEAA). The oligonucleotide was eluted with a linear

gradient of 5 to 40% acetonitrile in 50 mM TEAA. After detritylation with 80% AcOH, the oligonucleotide was purified by reverse phase and anion exchange HPLCs, and subsequent gel filtration by Sephadex G-25 as described.[17] The approximate yields of the oligonucleotides were about 40% for 12mers from 1 μmol support.

The nucleoside compositions of the oligonucleotides were analyzed by reverse phase HPLC after complete digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase.[18]

Temperature - UV absorbance and CD spectra. Oligonucleotides were dissolved in a buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, and 1 mM EDTA. Oligonucleotide concentrations were 1, 2, 3.3, 5, and 6 μM for the 12mer duplexes and 1.3, 2.3, 3.3, 5, and 6 μM for the other duplexes. Each sample was heated at 80°C for 10 min and then cooled gradually to an appropriate temperature. Thermal denaturation curves were recorded at 260 nm on a Gilford Response II UV-VIS Spectrophotometer using the temperature programming, and the UV-melting temperatures were evaluated as described previously.[19] van't Hoff transition enthalpies($\Delta H°$), entropies($\Delta S°$), and free energies($\Delta G°$) were determined by calculations based on the slope of a $1/T_m$ versus $\ln(C_t/4)$ plot and the following equations.[20]

$$1/T_m = (R/\Delta H°)\ln(C_t/4) + \Delta S°/\Delta H°$$

(C_t = total concentration of single strands)

$R/\Delta H°$ = slope $\Delta S°/\Delta H°$ = intercept

$$\Delta G° = \Delta H° - T\Delta S°$$

Samples for CD measurement were prepared by the same procedure used in the thermal denaturation study, except that the oligonucleotide concentrations were 6 μM for the 12mer duplexes and 7 μM for the other duplexes. CD spectra were measured by a Jasco J720 Spectropolarimeter at 20°C.

RESULTS

Synthesis of an oh^{8}Gua phosphoramidite derivative (7)

We previously synthesized oligonucleotides with oh^{8}Gua using an 8-methoxy-2'-deoxyguanosine monomer unit and by demethylation of an 8-methoxyguanine residue in oligonucleotides with thiophenol.[13,21] To improve the overall yield of oligonucleotides with oh^{8}Gua, we prepared a new derivative of oh^{8}Gua (**7**) for incorporation of the oxidative damage into the DNA (Figure 1).

8-Bromodeoxyguanosine (**2**) was treated with NaOMe/MeOH. 8-Methoxydeoxyguanosine (**3**), obtained in a 79% yield, was then treated with *N*,*N*-dimethylformamide dimethylacetal to protect the 2-amino group selectively.[22] The

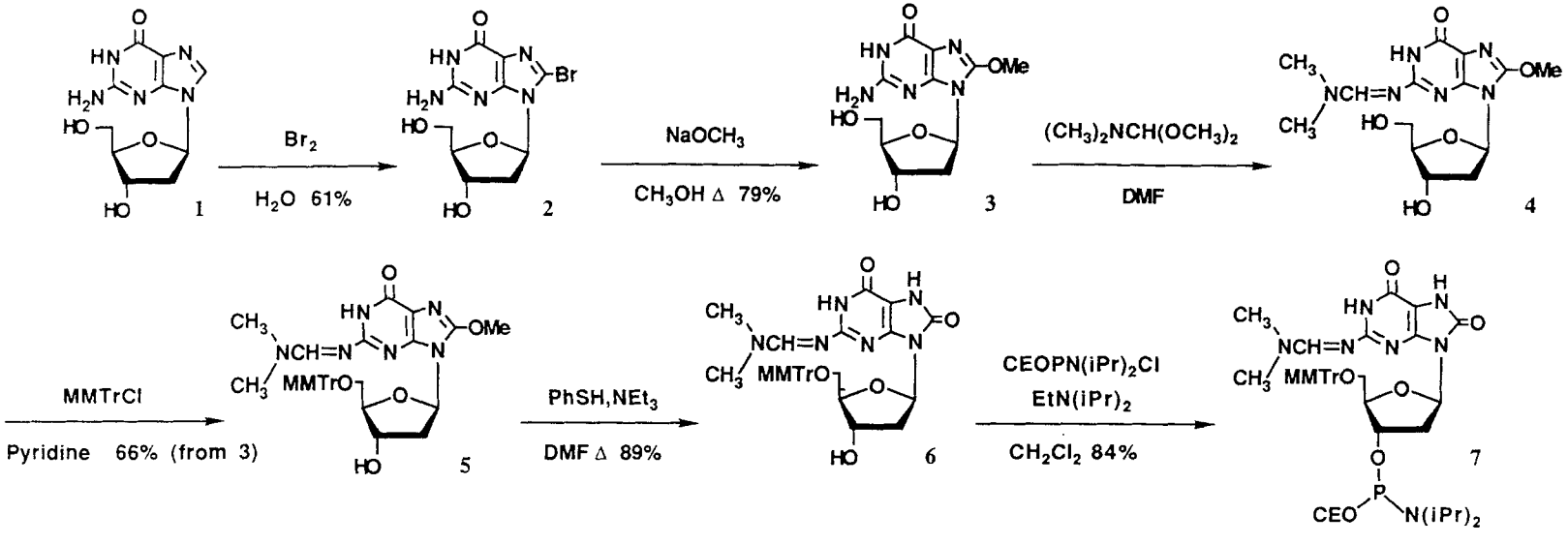

FIG. 1. Scheme for synthesis of the oh^{8}Gua building unit.

protected compound was monomethoxytritylated without isolation. The protected nucleoside of 8-methoxyguanine (**5**) was obtained in a 66% yield from **3**. We used a monomethoxytrityl group for 5'-protection due to the lability of 5'- dimethoxytrityl group of the 8-substituted guanine derivative.[21] Conversion of **5** to the oh^{8}Gua derivative (**6**) was achieved with thiophenol in the presence of triethylamine, with an 89% yield. The desired phosphoramidite (**7**) was obtained, with some modifications (see MATERIALS AND METHODS), by the usual method[23] with an 84% yield. The phosphoramidite derivative was purified by column chromatography at 4°C (in a cold room). When the purification was carried out at room temperature, decomposition of the compound was observed. A similar result was reported by Bodepudi *et al.* [24]

Oligonucleotide synthesis

Oligonucleotides with oh^{8}Gua were synthesized by an automated DNA synthesizer with slight modification of the supplier's program. Namely, the coupling time was increased to 10 min and a trichloroacetic acid treatment was carried out for 1 min during the oh^{8}Gua cycle. The coupling yield of the oh^{8}Gua unit was estimated by quantitation of the dimethoxytrityl cation in the cycles before and after the oh^{8}Gua cycle. The coupling yield was estimated as 97-99%.

The oligonucleotide was cleaved from the support by concentrated ammonia containing 2-mercaptoethanol, as described.[8] The dimethylformamidine group was removed together with the acyl groups when the ammonia solution of oligonucleotide was heated at 55°C for 6 hr.

The oligonucleotide with oh^{8}Gua was purified by reverse phase column chromatography before and after detritylation and by subsequent anion exchange chromatography, according to procedures used for unmodified oligomers. Yields of oligonucleotides (12 mers) with oh^{8}Gua were about 40%, which are comparable to those of unmodified oligonucleotides.

Effects of base pairs involving oh^{8}Gua in the middle of 12mers on thermodynamic stability

We first measured the T_ms at various concentrations and calculated the thermodynamic parameters (ΔH°, ΔS°, and ΔG°) of duplex DNA with a base pair involving G or oh^{8}Gua in the middle of the 12 mers (duplexes I and II in Figure 2). Plots of $1/T_m$ versus $\ln(C_t/4)$ are shown in Figure 3, in which duplex I is used as an example. Table 1 shows the T_ms and the parameters of the duplex oligonucleotides. At both positions, oh^{8}Gua:C pairs were more stable than other pairs with the modified base,

34 5'GCGCC**G**GCGGTG3' 3'CGCGG**N**CGCCAC5'	(I)	34 5'C**G**GCGGTGTG3' 3'**N**CGCCACAC5'	(IV)
35 5'GCGCCG**G**CGGTG3' 3'CGCGGC**N**GCCAC5'	(II)	35 5'G**G**CGGTGTGG3' 3'**N**GCCACACC5'	(V)
34 35 5'GCGCC**GG**CGGTG3' 3'CGCGG**N**CGCCAC5'	(III)	34 35 5'C**GG**CGGTGTG3' 3'**N**CGCCACAC5'	(VI)

FIG. 2. Duplex oligonucleotides used in this study. **G** = oh[8]Gua or G. **N** = A, G, C, or T.

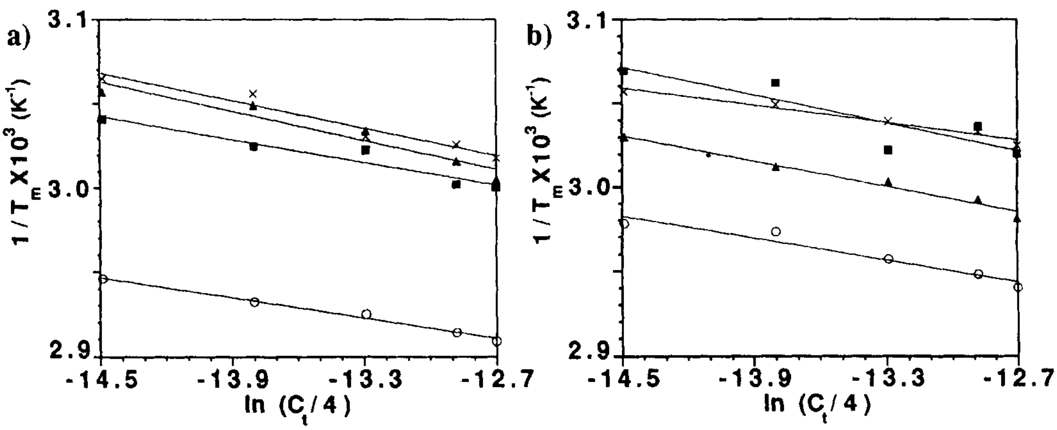

FIG. 3. Plots of $1/T_m$ vs. $\ln(C_t/4)$ for duplex I containing **N** = C (O), A (▲), T (■), G (X), where **G** are G (a) and oh[8]Gua (b), respectively.

although the oh[8]Gua:C pairs were less stable than the matched G:C pairs. The modified base probably formed three hydrogen bonds with C in a Watson-Crick manner, as described previously.[25] Also, oh[8]Gua:A pairs showed higher T_m and smaller ΔG° values than mismatched G:A pairs, indicating that the modified base and A formed hydrogen bonds, as reported previously.[26]

The duplex with the 35-oh[8]Gua:T pair showed a smaller ΔG° value (-17.6 kcal/mol at 298K) than the G:T pair. This value was comparable to that of the oh[8]Gua:A pair at the same position (-17.7 kcal/mol at 298K). On the other hand, the 34-oh[8]Gua:T pair was less stable than the 34-oh[8]Gua:A and G:T pairs. In contrast, the oh[8]Gua:G pairs

TABLE 1. Melting temperatures (T_m) and thermodynamic parameters of duplex oligonucleotides (duplexes I and II in Figure 2)

	Base[a] pair	T_m[b] (°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/kmol)	$\Delta G°_{298}$ (kcal/mol)
	GC	68.9	-98.9	-263	-20.6
	GA	56.6	-69.0	-183	-14.6
	GT	57.8	-87.1	-236	-16.7
	GG	56.9	-73.1	-196	-14.8
34					
	G̲C	65.2	-92.4	-247	-18.9
	G̲A	60.0	-77.6	-206	-16.1
	G̲T	57.9	-72.3	-193	-14.7
	G̲G	56.1	-116	-325	-18.8
	GC	68.9	-98.9	-263	-20.6
	GA	62.6	-77.0	-203	-16.4
	GT	59.3	-80.4	-217	-16.3
	GG	62.0	-92.4	-249	-18.2
35					
	G̲C	67.0	-87.1	-230	-18.6
	G̲A	64.0	-85.6	-228	-17.7
	G̲T	59.0	-96.4	-264	-17.6
	G̲G	59.2	-63.9	-166	-14.4

[a] G̲ = oh^{8}Gua. [b] The concentration of the duplexes was 3.3 μM.

had the reverse tendency. Namely, the pair at the 34th position showed a small $\Delta G°$ value (-18.8 kcal/mol at 298K), comparable with that of the oh^{8}Gua:C pair, while the 35-oh^{8}Gua:G pair showed the largest $\Delta G°$ value (Table 1). Thus, the 35-oh^{8}Gua:T and 34-oh^{8}Gua:G pairs are *thermodynamically* more stable than the corresponding pairs with G. These findings support the results that G to A transitions were found in NIH3T3 cells at the 35th position (discussed below).[13]

Thermodynamic stability effects of base pairs involving oh^{8}Gua at the end of a duplex DNA

When a deoxyribonucleotide is incorporated into the 3'-end of a growing chain, and the chain is then elongated during DNA replication, the stability of this new base pair must be important. We investigated the thermodynamic stabilities of base pairs involving oh^{8}Gua at the ends of duplex DNAs (duplexes IV and V in Figure 2). In these duplexes the modified base was located at the second position from the 5'-end, to consider cross-

stacking between the 5'-flanking base and the base opposite oh^{8}Gua. To confirm that a matched or mismatched base pair at the end affected the stability of a duplex, we first calculated the thermodynamic parameters of oligonucleotides with a base pair with G (Table 2). At the 34th position, mismatched base pairs destabilized the duplex DNA by *ca.* 2 kcal/mol of ΔG° as compared to the G:C pair. At the 35th position, the degree of destabilization was *ca.* 3 kcal/mol of ΔG°. The fact that a base pair at the end of a duplex influenced the stability of the duplex encouraged us to investigate the thermodynamic parameters of DNA with a terminal base pair involving oh^{8}Gua. The duplex with the oh^{8}Gua:C pair at the 34th position was most stable in the series of oh^{8}Gua-duplexes, although the duplex was less stable than the fully matched DNA (Table 2). The oh^{8}Gua:A pair stabilized the DNA more than the G:A pair, suggesting the formation of hydrogen bonds. Interestingly, the duplexes with the oh^{8}Gua:T and oh^{8}Gua:G pairs showed smaller ΔG° values than the G:T and G:G pairs, respectively. Thus, the "mismatched" base pairs with oh^{8}Gua were more stable than the mismatched base pairs with G. A similar tendency was observed at the 35th position. The duplexes with the oh^{8}Gua:T and oh^{8}Gua:G pairs, however, had smaller ΔG° values than the "matched" oh^{8}Gua:C pair. When the ΔG° values at 310K were calculated, the duplexes with the oh^{8}Gua:T and oh^{8}Gua:G pairs had larger ΔG° values (-10.3 and -10.1 kcal/mol, respectively) than the oh^{8}Gua:C pair (-10.5 kcal/mol). However, the pairs of oh^{8}Gua with A, T, and G were still more stable than the corresponding G-pairs at 310K (data not shown).

Thermodynamic parameters of duplex oligonucleotides with a mismatch base pair flanking oh^{8}Gua

We and other investigators have observed a point mutation at the 5'-flanking sites of oh^{8}Gua when a vector with oh^{8}Gua was replicated in mammalian cells.[13-15] We then studied the thermodynamic stabilities of oligonucleotides with a mismatched base pair at the 5'-flanking site of oh^{8}Gua. We introduced mismatched pairs and oxidation damage into the 34th and 35th positions, respectively, according to the finding that c-Ha-*ras* genes with a point mutation at the 34th position were detected when the gene with the 35-oh^{8}Gua was transfected into NIH3T3 cells.[13]

Tables 3 and 4 show the T_ms and thermodynamic parameters of duplexes in which the mismatched base pairs were located in the middle (duplex III in Figure 2) and at an end (duplex VI in Figure 2). The differences between the ΔG° values of matched and mismatched oligonucleotides (hereafter $\Delta\Delta G^\circ$) were calculated. As shown in Table 3, the $\Delta\Delta G^\circ$ values of the oligonucleotides were similar in duplexes with the oh^{8}Gua:C and G:C pairs at the 35th position. A slight stabilization of the mismatch base pair by the oh^{8}Gua:C pair was observed in the case of the G:T pair at the 34th position (3.9 kcal/mol

TABLE 2. Melting temperatures (T_m) and thermodynamic parameters of duplex oligonucleotides (duplexes IV and V in Figure 2)

	Base[a] pair	T_m[b] (°C)	ΔH ° (kcal/mol)	ΔS ° (cal/kmol)	ΔG °$_{298}$ (kcal/mol)
	GC	52.6	-68.3	-183	-13.7
	GA	47.9	-48.7	-125	-11.4
	GT	47.8	-57.8	-154	-12.0
	GG	48.7	-51.9	-135	-11.7
34					
	G̲C	50.5	-65.6	-176	-13.2
	G̲A	49.2	-59.3	-158	-12.3
	G̲T	48.3	-64.9	-176	-12.5
	G̲G	49.3	-56.0	-148	-12.0
	GC	53.7	-70.5	-190	-14.0
	GA	44.3	-52.4	-139	-11.0
	GT	44.3	-51.6	-136	-11.0
	GG	44.6	-55.8	-150	-11.2
35					
	G̲C	50.8	-54.6	-142	-12.3
	G̲A	46.2	-61.1	-165	-11.9
	G̲T	44.7	-83.1	-235	-13.1
	G̲G	45.0	-73.6	-205	-12.6

[a] G̲ = oh^{8}Gua. [b] The concentration of the duplexes was 3.3 μM.

TABLE 3. Melting temperatures (T_m) and thermodynamic parameters of duplex oligonucleotides (duplex III in Figure 2)

Base at 35	Base pair[a] at 34	T_m[b] (°C)	ΔH ° (kcal/mol)	ΔS ° (cal/kmol)	ΔG °$_{298}$ (kcal/mol)	$\Delta\Delta G$ °$_{298}$[c] (kcal/mol)
	GC	68.9	-98.9	-263	-20.6	
G	GA	56.6	-69.0	-183	-14.6	+6.0
	GT	57.8	-87.1	-236	-16.7	+3.9
	GG	56.9	-73.1	-196	-14.8	+5.8
	GC	67.0	-87.1	-230	-18.6	
G̲	GA	53.8	-52.8	-135	-12.5	+6.1
	GT	55.5	-76.7	-207	-15.0	+3.6
	GG	53.9	-56.6	-147	-12.7	+5.9

[a] G̲ = oh^{8}Gua. [b] The concentration of the duplexes was 3.3 μM. [c] $\Delta\Delta G$ °$_{298}$ = ΔG °$_{298}$ - ΔG °$_{298}$(GC).

TABLE 4. Melting temperatures (T_m) and thermodynamic parameters of duplex oligonucleotides (duplex VI in Figure 2)

Base at 35	Base pair[a] at 34	T_m[b] (°C)	ΔH ° (kcal/mol)	ΔS ° (cal/kmol)	ΔG °$_{298}$ (kcal/mol)	$\Delta\Delta G$ °$_{298}$[c] (kcal/mol)
	GC	52.6	-68.3	-183	-13.7	
G	GA	47.9	-48.7	-125	-11.4	+2.3
	GT	47.8	-57.8	-154	-12.0	+1.7
	GG	48.7	-51.9	-135	-11.7	+2.0
	GC	50.4	-84.2	-234	-14.4	
G	GA	45.9	-51.7	-136	-11.2	+3.2
	GT	45.1	-58.6	-158	-11.6	+2.8
	GG	46.4	-60.0	-161	-12.0	+2.4

[a] G = oh^{8}Gua. [b] The concentration of the duplexes was 3.3 μM. [c] $\Delta\Delta G$ °$_{298}$ = ΔG °$_{298}$ - ΔG °$_{298}$(GC).

versus 3.6 kcal/mol at 298K). However, stabilization of a mismatch pair by oh^{8}Gua was not observed when the mismatches were located at the ends of the duplexes (Table 4). Therefore, it seemed that oh^{8}Gua did not stabilize the 5'-mismatches, and that the 5'-adjacent mutations were not dependent on the thermodynamic stability of the mismatches.

Circular dichroism measurements of oligonucleotide duplexes with oh^{8}Gua (I,II,IV,V)

CD spectra of oligonucleotide duplexes with various base pairs of oh^{8}Gua or G were measured. All the duplexes, which are highly GC-rich, showed patterns corresponding to an A-like B or A structure[27] (Figure 4). The CD spectra of oligonucleotides with oh^{8}Gua in the middle of the 12mer (duplexes I and II in Figure 2, N=C) and the corresponding G-duplexes were similar (Figure 4a). On the other hand, duplex oligonucleotides with an oh^{8}Gua:C pair at an end showed a characteristic pattern. A broad peak between 280-320 nm was observed in the case of the duplex with the 35-oh^{8}Gua (Figure 4b). Such a broad peak was observed when the base opposite oh^{8}Gua was A (data not shown). Also, a wide-ranged broad peak was observed in the cases of the 34-oh^{8}Gua paired with C (Figure 4c), and A and G (data not shown).

DISCUSSION

We observed previously that a c-Ha-*ras* gene was activated by a point mutation when a vector with oh^{8}Gua in the c-Ha-*ras* gene was transfected into NIH3T3 cells. The

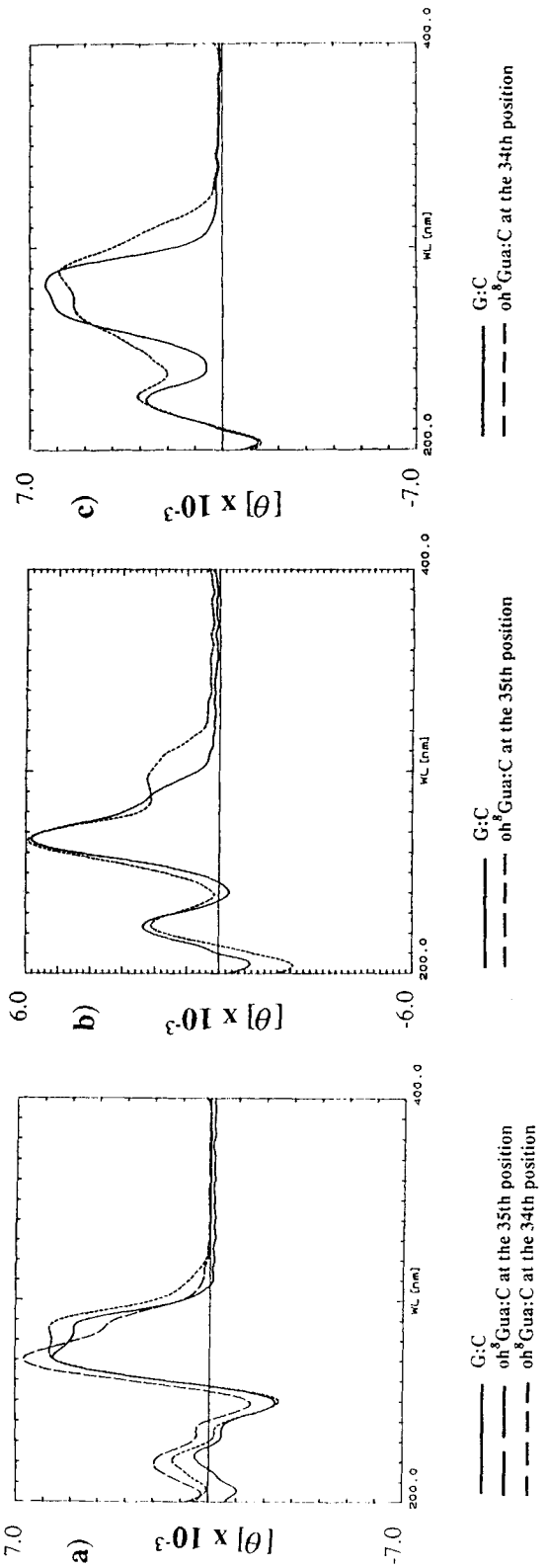

FIG. 4. CD spectra of a) duplex 12mers with an oh^{8}Gua:C or a G:C pair in the middle, and b) at the 35th position and c) at the 34th position at an end.

point mutations consisted of G to T, A, and C mutations at the modified site (the 35th position) and the 5'-flanking site (the 34th position) in the case of the *ras* gene with the 35-oh^{8}Gua.[13] On the other hand, a G to T transversion at the modified position was almost exclusively detected in the case of the *ras* gene with the 34-oh^{8}Gua (Kamiya *et al.*, unpublished results). To study the different mutation-spectra in terms of the thermodynamic stabilities of base pairs with oh^{8}Gua, we first synthesized oligodeoxynucleotides with oh^{8}Gua.

We previously synthesized an 8-methoxy-2'-deoxyguanosine phosphoramidite or phosphotriester derivative and prepared oligonucleotides with oh^{8}Gua by demethylation of an 8-methoxyguanine residue in oligonucleotides with thiophenol.[13,21] The conversion of 8-methoxyguanine to oh^{8}Gua required a long reaction period (2 days) and careful separation of the product from the remaining precursor by HPLC was needed in some cases, especially in the case of long oligonucleotides.

To obtain oligodeoxynucleotides with oh^{8}Gua more easily and with an improved yield, we prepared a new oh^{8}Gua phosphoramidite monomer (**7**). The monomer unit was synthesized via five steps from deoxyguanosine, and the yield of each step was high (Figure 1).

Two kinds of oh^{8}Gua-phosphoramidite monomers have been reported by other groups.[28] Bodepudi *et al.* synthesized the phosphoramidite with an isobutyryl group.[24] Their scheme started from 8-*O*-benzyloxy-2'-deoxyguanosine, obtained from 8-bromo-2'-deoxyguanosine in a low yield.[29] The phosphoramidite was not purified by column chromatography. Also, the oligonucleotides were treated in aqueous ammonia containing 2-mercaptoethanol for 16 hr at 55°C for deprotection of the isobutyryl group. On the other hand, Roelen *et al.* reported an oh^{8}Gua building block with acetyl and diphenylcarbamoyl groups for the N^2- and N^7,O^6-positions, respectively.[30] They prepared the phosphoramidite by two synthetic routes. The first one included the conversion of 8-bromo-2'-deoxyguanosine to an acetylated oh^{8}Gua derivative, with a very low yield. The alternative method started from 8-bromoguanosine and contained many steps, although an overall yield was fairly good. Deprotection of the modified oligonucleotides took 48 hr at 50°C with dry methanolic ammonia. Our new strategy provides a good overall yield of oh^{8}Gua-containing oligonucleotides and a shorter period for base-deprotection in the synthesis.

The thermodynamic parameters of the present duplexes were interesting in various ways. In the case of the modification at the middle of duplexes, the T_m values of the duplexes with the oh^{8}Gua:T or oh^{8}Gua:G pair at the 34th and 35th positions, respectively, were lower than those for the oh^{8}Gua:C and oh^{8}Gua:A pairs (Table 1). On the other hand, the ΔG° values showed that the 35-oh^{8}Gua:T pair was as stable as the

oh^{8}Gua:A pair at the same position and that the 34-oh^{8}Gua:G pair was as stable as the oh^{8}Gua:C pair (Table 1). That is, the 35-oh^{8}Gua:T and 34-oh^{8}Gua:G pairs were stable *thermodynamically*, but not thermally. These results suggested that, to understand the misincorporation of nucleotides opposite the modified base, it is necessary to compare thermodynamic parameters in addition to T_m values, as mentioned in the report by Plum *et al.*[31]

More surprising results were obtained from the thermodynamic parameters of duplexes with a base pair of oh^{8}Gua at the end (IV,V). The duplex oligonucleotides with the oh^{8}Gua:A, oh^{8}Gua:T, and oh^{8}Gua:G pairs showed higher T_ms and lower ΔG° values, as compared to the counterparts with G (Table 2). These results indicated that the stabilities of "mismatched" base pairs of oh^{8}Gua were high. This implies that oh^{8}Gua can adopt both *syn* and *anti* conformations, and that the conformational conversion can more easily occur near an end than in the middle of the oligonucleotides. The flexibility of the backbone structures at the end may facilitate the hydrogen bonding of the unusual base pair. In addition, the base pairs of the 35-oh^{8}Gua with T and G appeared to be more stable than the "matched" oh^{8}Gua:C pair (Table 2).

When a deoxyribonucleotide is incorporated into the 3'-end of a growing chain, the stability of this new base pair must be important. The stability probably affects the phosphodiester bond-formation by DNA polymerase(s) and the recognition by enzymes during the editing process, although other factors, such as the "shape" of a base pair, must be important in these events. The present results on duplex DNAs with a variety of base pairs at the end predict the stabilities of the newly-form*ed* pairs and, possibly, the newly-form*ing* base pairs. Therefore, the results suggest that "incorrect" nucleotides may be incorporated opposite oh^{8}Gua more frequently than opposite G, and that subsequent elongation may occur more easily. Furthermore, the editing machinery in cells may not recognize base pairs that are thermodynamically stable. The results shown in Table 2 indicate the possibility of incorporation of G and T, in addition to C and A, opposite oh^{8}Gua, especially at the 35th position. In addition, the stabilities of base pairs of oh^{8}Gua may be related to the repair of the modified and opposite bases. The results shown in Table 1 suggest that the 35-oh^{8}Gua:T and 34-oh^{8}Gua:G pairs may "elude" the repair system in cells.[32-35]

The oh^{8}Gua:T pairs at the 34th and 35th positions may assume different conformations because the relative thermodynamic stabilities of these pairs are different (Table 1). Similarly, the oh^{8}Gua residues may pair with G in different ways. Figure 5 shows postulated base pairs involving oh^{8}Gua. G to A transitions induced by the 35-oh^{8}Gua were detected,[13] while no G to C mutations at the 34th position were found in NIH3T3 cells (Kamiya *et al.* unpublished results). The hydrogen bond pattern or the

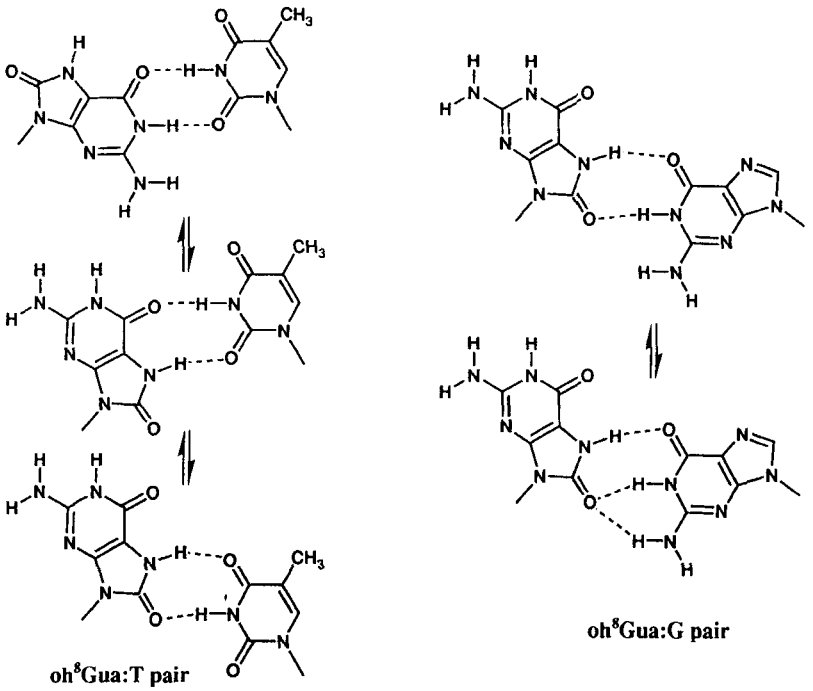

FIG. 5. Postulated base pairs of oh^{8}Gua with T, and with G.

shape of the 34-oh^{8}Gua:G pair might not be favored by DNA polymerase(s) involved in replication.

Mismatches at the 5'-flanking site of oh^{8}Gua do not seem to be stabilized by the modified base (Tables 3 and 4). Mutations detected at the flanking 5'-position to oh^{8}Gua in mammalian cells[13] are not explained by the stabilization by oh^{8}Gua.

The results described in this paper, together with further *in vitro* studies, will reveal the reasons for the different mutation-spectra by oh^{8}Gua at the first and second positions of codon 12 in the c-Ha-*ras* gene. Structual studies will provide more insight into these mutations.

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