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## Synthesis and Thermodynamic Studies of Oligodeoxyribonucleotides Containing Tandem Lesions of Thymidine Glycol and 8-oxo-2'-Deoxyguanosine

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### Abstract

Thymidine glycol (Tg), which is also known as 5,6-dihydroxy-5,6-dihydrothymidine, and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) are two major types of DNA damage products induced by reactive oxygen species (ROS). Here we report the synthesis of oligodeoxyribonucleotides (ODNs) containing both Tg and 8-oxodG. The dual incorporation of the two single-base lesions was achieved by using a phosphoramidite building block of 8-oxodG with ultramild base protecting group and a building block of Tg whose nucleobase hydroxyl groups were protected with acetyl functionality. The availability of ODNs carrying neighboring 8-oxodG and Tg provided authentic substrates for assessing the formation and examining the replication and repair of this kind of tandem lesions. In addition, thermodynamic parameters derived from melting temperature data revealed that tandem lesions destabilized the double helix to a greater extent than either of the two single-base lesions alone. The thermodynamic results could offer a basis for understanding the repair of the tandem base lesions.

### Introduction

Reactive oxygen species (ROS) can lead to a plethora of modifications to DNA, which yield nucleobase and deoxyribose lesions, strand breaks, and DNA-protein cross-links (1,2). These oxidative DNA lesions may have implications in a variety of pathological conditions, including diseases associated with aging and cancer (1,3). Thymidine glycol (or 5,6-dihydroxy-5,6-dihydrothymidine, Tg) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) are two common monomeric base lesions induced by ROS. Thymidine glycol lacks mutagenicity under most conditions (4-7). However, it effectively blocks DNA replication (8,9). By contrast, 8-oxodG is a major mutagenic lesion and can result in G→T transversion mutation (10,11).

In recent years, several types of tandem DNA lesions (12-20), where damage involves two adjacent nucleosides, have been isolated and characterized. A first example of tandem lesions, with a 8-oxodG and a formamido (dβF) residue being neighboring to each other, was reported by Box et al. (12,21). Recently Bourdat et al. (15) showed that the amount of these tandem lesions, dβF-8-oxodG and its isomeric 8-oxodG-dβF, formed from  $\gamma$  radiation cannot account for the total amount of tandem lesions involving 8-oxodG. It was, therefore, postulated that other tandem lesions involving 8-oxodG may exist. Since thymidine glycol and 8-oxodG are major types of single-nucleobase lesions induced by ROS (2), we reason that tandem lesions with a 8-oxodG being adjacent to a Tg might be induced by ROS.

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Clustered DNA damages, in which two or more lesions within one or two helical turns of the DNA helix, can be initiated from a single ionizing radiation track (22-24). It has been shown that such damaged sites were difficult to be repaired with purified repair enzymes and in mammalian cells (25-31). To understand the biological significances of tandem lesions with a Tg being neighboring to an 8-oxodG, it is crucial to assess their formation and to examine the replication and repair of this type of lesions. The lack of procedures for preparing pure and sufficient tandem lesion-bearing substrates, however, hampers such studies. This motivates us to design synthetic approaches for the incorporation of both types of lesions into oligodeoxyribonucleotides (ODNs).

Solid-phase synthesis of ODNs by using phosphoramidite building blocks is advantageous over the post-synthetic methods for the preparation of modified or damaged ODNs because the chain length and the sequence are not restricted and large-scale preparation is available (32). In this regard, Iwai (33,34) described the synthesis of a building block of thymidine glycol where the nucleobase hydroxyl group(s) were protected with *tert*-butyldimethylsilyl (TBDMS) group. Very recently, Cadet et al. (35) reported the synthesis of a Tg building block where the nucleobase hydroxyl group(s) was protected with a base-labile levulinyl functionality. On the other hand, the deprotection of ODNs synthesized from the commercially available building block of 8-oxodG requires prolonged treatment with concentrated ammonium hydroxide; during such treatment, thymidine glycol, however, was shown to be significantly degraded (33). Therefore, dual insertion of 8-oxodG and thymidine glycol necessitates alternative synthetic strategies.

Here in this paper we reported the successful preparation of ODN substrates bearing both 8-oxodG and Tg, which provides substrates for examining the formation of the tandem lesions with neighboring 8-oxodG and Tg induced by ROS. The availability of this type of substrates also facilitates the investigation of the replication and repair of this type of lesions both *in vitro* and *in vivo*.

## Experimental Section

### Materials

Common reagents for solid-phase DNA synthesis were obtained from Glen Research Co. (Sterling, VA). Unmodified ODNs used in this study were purchased from Integrated DNA Technologies (Coraville, IA). Silica gel and TLC plates were obtained from EM Science (Gibbstown, NJ). All chemicals unless otherwise specified were from Sigma-Aldrich (St. Louis, MO).

### Mass Spectrometry

Electrospray ionization-mass spectrometry (ESI-MS) and tandem MS (MS/MS) experiments were carried out on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). An equal-volume solvent mixture of acetonitrile and water was used as solvent for electrospray, and a 2- $\mu$ L aliquot of  $\sim$ 5  $\mu$ M sample solution was injected in each run. The spray voltage was 3.4 kV and the instrument was operated in the negative-ion mode.

### HPLC

A 4.6 $\times$ 250 mm Apollo C18 column (5  $\mu$ m in particle size, 300  $\text{\AA}$  in pore size, Alltech Associate Inc., Deerfield, IL) was used for the separation of synthetic ODNs. A solution of 50 mM triethylammonium acetate (TEAA, solution A) and a mixture of 50 mM TEAA and acetonitrile (70/30, v/v, solution B) were used as mobile phases. The flow rate was 0.8 mL/min, and a gradient of 5 min 0-20% B, 45 min 20-50% B and 5 min 50-100% B was employed.

## Synthesis and Characterization of Compounds (Scheme 1)

**3',5'-O-(4,4'-dimethoxytrityl)thymidine (1)**—The title compound was synthesized according to the procedures reported by Sekine et al. (36). In this respect, it is worth mentioning that the incorporation of the bulky 4,4'-dimethoxytrityl (DMTr) functionality to the 3'-hydroxyl group required a reaction temperature of 70 °C (36), though the traditional DMTr protection of the 5'-hydroxyl group is typically carried out at room temperature. Yield: 6.79 g (97%). R<sub>f</sub>: 0.38 (EtOAc-hexane, 1:1). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 11.30 (br, 1H), 6.75-7.37 (m, 26H), 6.15 (m, 1H), 4.21 (m, 1H), 3.94 (m, 1H), 3.70 (s, 6H), 3.68 (d, *J* = 3.2 Hz, 6H), 3.00 (m, 1H), 2.92 (m, 1H), 1.77 (m, 1H), 1.61 (m, 1H), 1.36 (s, 3H). MALDI-MS: *m/z* 869.1 [M+Na]<sup>+</sup>.

**(5R,6S)-3',5'-O-(4,4'-dimethoxytrityl)thymidine glycol (2)**—Compound **1** (1.67 g, 1.97 mmol) and OsO<sub>4</sub> (0.5 g, 1.97 mmol) were dissolved in pyridine (7.5 mL), and the mixture was stirred at room temperature for 2 h. Sodium hydrogen sulfite (1.8 g), dissolved in a mixture of water (30 mL) and pyridine (20 mL), was then added to the reaction mixture. The mixture was stirred for another 2 h. The product was extracted twice with CHCl<sub>3</sub> (150 mL) and the organic layer was dried with Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure. The reaction mixture was then loaded onto a silica gel column and eluted with a CH<sub>2</sub>Cl<sub>2</sub> solution containing 0.5% MeOH and 0.1% Et<sub>3</sub>N. Yield: 1.17 g (67%). R<sub>f</sub>: 0.24 (CH<sub>3</sub>OH-CH<sub>2</sub>Cl<sub>2</sub>, 1:19). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 6.77-7.50 (m, 26H), 6.29 (m, 1H), 5.09 (s, 1H), 4.44 (m, 1H), 3.92 (m, 1H), 3.79 (m, 12H), 3.02 (m, 2H), 2.05 (m, 1H), 1.77 (m, 1H), 1.28 (s, 3H). MALDI-MS: *m/z* 903.4 [M+Na]<sup>+</sup>.

**(5R,6S)-3',5'-O-(4,4'-dimethoxytrityl)-5,6-O-acetyl-thymidine glycol (3)**—Compound **2** (281 mg, 0.32 mmol) was coevaporated twice with dry pyridine (10 mL) and the dried residue was dissolved again in dry pyridine (2 mL). DMAP (4-dimethylaminopyridine, 7.38 mg, 0.06 mmol) and acetic anhydride (0.36 mL, 3.84 mmol) were added into the solution under argon atmosphere. After 15 h, the solution was dried under reduced pressure, and the dried residue was redissolved in EtOAc (80 mL). The organic layer was washed with water (twice, 40 mL) and brine (40 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Glycol **3** (262 mg, 82%) was isolated with a silica gel column using a solvent gradient of 0-15% EtOAc in CH<sub>2</sub>Cl<sub>2</sub>. R<sub>f</sub>: 0.58 (EtOAc-CH<sub>2</sub>Cl<sub>2</sub>, 15:85). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 8.64 (s, 1H), 7.15-7.40 (m, 18H), 6.69-6.81 (m, 8H), 6.35 (s, 1H), 6.03 (dd, 1H, *J* = 5.8, 8.7 Hz), 4.26 (m, 1H), 4.10 (m, 3H), 3.76 (s, 6H), 3.72 (d, *J* = 2.3 Hz, 6H), 3.22 (m, 2H), 2.02 (s, 3H), 2.00 (s, 3H), 1.90 (s, 3H). MALDI-MS: *m/z* 987.4 [M+Na]<sup>+</sup>.

**(5R,6S)-5,6-O-acetyl-thymidine glycol (4)**—Compound **3** (262 mg, 0.27 mmol) was treated with 70% acetic acid at room temperature for 6 h. The resulting aqueous solution was dried under reduced pressure. The dried residue was dispersed in ether (50 mL) and extracted twice with water (50 mL each). The aqueous solutions were combined and dried. Yield: 93 mg (95%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 8.86 (s, 1H), 6.91 (s, 1H), 6.09 (t, 1H, *J* = 7.3 Hz), 4.48 (m, 1H), 3.96 (m, 1H), 3.81 (m, 2H), 2.142 (m, 2H), 2.10 (s, 3H), 2.08 (s, 3H), 1.86 (s, 3H). ESI-MS: *m/z* 383.1 [M+Na]<sup>+</sup>.

**(5R,6S)-5'-O-(4,4'-dimethoxytrityl)-5,6-O-acetyl-thymidine glycol (5)**—Compound **4** (90 mg, 0.26 mmol) was coevaporated twice with dry pyridine (5 mL). The dried residue was redissolved in dry pyridine (2 mL), and to the resulting solution was added 4,4'-dimethoxytrityl chloride (105 mg, 0.31 mmol) under argon atmosphere. After 4 h, the solvent was removed under reduced pressure. The product was loaded onto a silica gel column and eluted with 0-2% methanol in a solvent mixture of Et<sub>3</sub>N and CH<sub>2</sub>Cl<sub>2</sub> (1:99, v/v). Yield: 102 mg (61%). R<sub>f</sub>: 0.17 (CH<sub>2</sub>Cl<sub>2</sub>-CH<sub>3</sub>OH, 19:1). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 8.30 (s, 1H), 7.10-7.46 (m, 9H), 6.81-6.84 (m, 4H), 6.56 (s, 1H), 6.08 (t, 1H, *J* = 7.2 Hz), 5.27 (s, 1H), 4.35

(m, 1H), 3.42 (dd, 1H,  $J = 5.5, 9.8$  Hz), 3.29 (dd, 1H,  $J = 5.5, 9.8$  Hz), 2.32 (m, 1H), 2.06 (m, 1H), 2.05 (s, 3H), 2.03 (s, 3H), 1.78 (s, 3H). ESI-MS:  $m/z$  685.1 [M+Na]<sup>+</sup>.

**(5R,6S)-5'-O-(4,4'-dimethoxytrityl)-5,6-O-acetyl-thymidine glycol-3'-O-[(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite] (6)**—To a flask, which was suspended in an ice bath and contained a solution of compound **5** (150 mg, 226  $\mu$ mol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1.8 mL), was added *N,N*-diisopropylethylamine (DIEA, 100  $\mu$ L, 573  $\mu$ mol) followed by dropwise addition of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (78  $\mu$ L, 348  $\mu$ mol). The mixture was stirred at room temperature for 15 min. A second portion of DIEA (100  $\mu$ L, 573  $\mu$ mol) was added to the mixture and the solution was stirred for another 15 min. Workup was carried out by cooling down the mixture in an ice bath followed by addition of CH<sub>3</sub>OH (0.36 mL). The solution was quickly extracted with EtOAc (10 mL) and the organic layer was washed with NaHCO<sub>3</sub> (4 mL) and brine (4 mL) and dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated off to give **6** in white foam. The product was eluted with 0-5% methanol in a solvent mixture of Et<sub>3</sub>N and CH<sub>2</sub>Cl<sub>2</sub> (0.1:99.9, v/v). Yield: 133 mg (68%). R<sub>f</sub>: 0.64, 0.73 (CH<sub>2</sub>Cl<sub>2</sub>-CH<sub>3</sub>OH, 19:1); <sup>31</sup>P-NMR (CDCl<sub>3</sub>):  $\delta$  149.5, 149.2. ESI-MS:  $m/z$  863.1 [M+H]<sup>+</sup>.

**5'-O-(4,4'-dimethoxytrityl)-2-N-(phenoxyacetyl)-8-oxo-7,8-dihydro-2'-deoxyguanosine-3'-O-[(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite] (7)**—Compound **7** was prepared following the previously published procedures (Details shown in the Supporting Information) (37-39).

### ODN Synthesis

ODNs (sequences shown in Table 1) were synthesized on a Beckman Oligo 1000S DNA synthesizer (Fullerton, CA) at 1- $\mu$ mol scale. The phosphoramidite building blocks **6** and **7** were dissolved in anhydrous acetonitrile at a concentration of 0.06 M, ultramild phosphoramidite building blocks (Glen Research Inc., Sterling, VA) of dG, dC and dA were employed, and the factory-installed ODN assembly protocol was used without any modification. After synthesis, the products were cleaved from the controlled-pore glass (CPG) support and deprotected with 30% NH<sub>4</sub>OH at room temperature for 1.5 h. The ODNs were then purified by reversed-phase HPLC.

### Thermodynamic Studies

UV absorbance-versus-temperature profiles were recorded on a Varian Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA), and the ODNs were dissolved in a 1.2-mL solution containing 250 mM NaCl, 10 mM sodium cacodylate, and 0.1 mM EDTA (pH 7.0) at a total ODN concentration ( $C_t$ ) of 2.0, 3.4, 5.6, 9.5, or 16  $\mu$ M. The absorbance was recorded in the reverse and forward directions for a temperature range of 80-10°C at a rate of 1°C/min, and the melting temperature ( $T_m$ ) value was obtained by the derivative method.

The thermodynamic parameters were obtained from the van't Hoff plot (40), in which the

reciprocal of  $T_m$  was plotted against  $\ln \frac{C_t}{4}$ :

$$\frac{1}{T_m} = \left( \frac{R}{\Delta H^\circ} \right) \ln \frac{C_t}{4} + \frac{\Delta S^\circ}{\Delta H^\circ}$$

and

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

where R is the ideal gas constant (= 1.987 cal mol<sup>-1</sup> K<sup>-1</sup>). The error limits for  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$  derived from fitted parameters were calculated by using previously described equations (41-43).

## Results

### Synthesis of Phosphoramidite Building Blocks for the Dual Incorporation of Tg and 8-oxodG into ODNs

Phosphoramidite building blocks of both Tg and 8-oxodG are commercially available (Glen Research). The labile nature of thymidine glycol, which was shown to be decomposed upon treatment with concentrated ammonia at room temperature for extended period of time (33), prevents the use of the commercially available building block of 8-oxodG, which necessitates deprotection with concentrated ammonia at room temperature for 24-48 h, for the dual insertion of the two oxidatively damaged products into ODNs. In addition, the 5- and 6-hydroxyl groups in the commercially available Tg building block were protected with TBDMS. The nucleobase deprotection of those ODNs that are synthesized with the Tg building block, therefore, requires treatments in two steps, one with ammonium hydroxide and the other with tetrabutyl ammonium fluoride (TBAF).

With the above analysis in mind, we decided to prepare the phosphoramidite building blocks of 8-oxo-dG, whose N2 is protected with a phenoxyacetyl group (37-39), and thymidine glycol, whose 5- and 6-dihydroxyl groups were protected with acetyl functionality. The latter protection of thymidine glycol obviates the needs for the deprotection with TBAF.

To synthesize the thymidine glycol phosphoramidite building block, we first protected the 3' and 5' hydroxyl groups of thymidine with DMTr. The DMTr protecting group was chosen for its easy removal upon acid treatment and its strong UV absorbance, which allows for the monitoring of the elution of the poorly UV-absorptive Tg during flash column chromatography separation. The DMTr-protected compound was then oxidized with OsO<sub>4</sub> to offer the desired thymidine glycol (33). We then protected both hydroxyl groups in thymine glycol with acetyl groups, which was achieved by using DMAP as a catalyst (44). It is worth noting that the acetylation of the tertiary hydroxyl group was not complete without the addition of DMAP. This result is in line with what Gasparutto et al. (35) observed, where they showed that the protection of the 5-hydroxyl group of thymidine glycol with levulinyl functionality was incomplete. The resulting compound was treated with 70% acetic acid to give the 5,6-*O*-acetyl-thymidine glycol, which was converted to the phosphoramidite building block using published procedures (45).

With these two building blocks, we synthesized several ODNs (sequences listed in Table 1). We found that the acetyl protecting group of thymidine glycol can be readily removed upon treatment with 30% NH<sub>4</sub>OH at room temperature for 1.5 h and thymidine glycol remains intact after such treatment. The ODNs were then purified by reversed-phase HPLC (HPLC traces are shown in the Supporting Information). It is worth noting that the C6 position of the thymidine glycol functionality undergoes epimerization during the deprotection step (34,46). Therefore, we expect that both the (5R,6S) and the (5R,6R) isomers are present in the ODN, though only the major isomer of the thymidine glycol, i.e., the (5R,6S) isomer, from the OsO<sub>4</sub> oxidation was employed for phosphoramidite building block synthesis.

### ESI-MS and MS/MS Characterization of ODNs Containing Both 8-oxodG and Tg

We next characterized the above ODNs by ESI-MS and MS/MS (Table 1, Figures 1-2 and Supporting Information). Here we began our discussion with the characterizations of d(ATG\*GCGTgGCTAT) (ODN5, G\* represents 8-oxodG). In this regard, ESI-MS shows that the deconvoluted mass of the ODN is 50 Da higher than the calculated mass of the unmodified d(ATGGCGTGCTAT) (Figure 1a), which is consistent with the presence of both Tg and 8-oxo-dG in this substrate.

The product-ion spectrum of the  $[M - 3H]^{3-}$  ion ( $m/z$  1240.7, Figure 1b) of the ODN showed the formation of  $w_n$  ions, i.e.,  $w_3$ ,  $w_4$ ,  $w_5^{2-}$ ,  $w_6^{2-}$ ,  $w_7^{2-}$ ,  $w_8^{2-}$ , and  $w_{11}^{3-}$ , and  $[a_n - \text{Base}]$  ions, i.e.,  $[a_4 - \text{Gua}]$ ,  $[a_5 - \text{Cyt}]$ ,  $[a_6 - \text{Gua}]$ ,  $[a_8 - \text{Gua}]^{2-}$ ,  $[a_9 - \text{Cyt}]^{2-}$  and  $[a_{11} - \text{Ade}]^{2-}$  ions [nomenclature for fragment ions follows that reported by McLuckey et al. (47); “Ade”, “Cyt” and “Gua” represent adenine, cytosine and guanine, respectively]. Whereas the measured masses for the  $w_3$ ,  $w_4$ , and  $w_5^{2-}$  ions are the same as the calculated masses for the corresponding ions of the unmodified ODN, the  $w_6^{2-}$ ,  $w_7^{2-}$  and  $w_8^{2-}$  ions exhibited 34 Da higher, and the  $w_{11}^{3-}$  ion showed 50 Da higher, in mass than the corresponding fragments formed from the unmodified d(ATGGCGTGCTAT). These results are consistent with the presence of 8-oxodG and Tg at the third and seventh positions in the ODN, respectively. The above conclusion is further substantiated by the observed masses for the  $[a_n - \text{Base}]$  ions. In this respect, the measured masses for the  $[a_4 - \text{Gua}]$ ,  $[a_5 - \text{Cyt}]$  and  $[a_6 - \text{Gua}]$  ions are 16 Da higher, whereas the measured masses of the  $[a_8 - \text{Gua}]^{2-}$ ,  $[a_9 - \text{Cyt}]^{2-}$  and  $[a_{11} - \text{Ade}]^{2-}$  ions are 50 Da higher, than the calculated masses of the corresponding fragments from the unmodified d(ATGGCGTGCTAT). It is worth noting that we did not observe the  $[a_3 - \text{Base}]$  and  $w_9$  ion (Figure 1b), which is consistent with previous findings about the fragmentations of ODNs containing a 8-oxodG (48).

The sequences of ODN3 and ODN4 were also confirmed by the similar ESI-MS and MS/MS analysis (Figure 2 shows the MS and MS/MS for ODN3, and the mass spectrometric results for ODN4 are shown in the Supporting Information). First of all, the measured masses of these two ODNs are consistent with their calculated masses (Table 1). In addition, the production spectrum of the  $[M - 3H]^{3-}$  ion of ODN3 (Figure 2b) showed that the  $w_7^{2-}$ ,  $w_8^{2-}$ ,  $w_9^{3-}$ ,  $w_{11}^{3-}$ ,  $[a_8 - \text{Gua}]^{2-}$ ,  $[a_9 - \text{Cyt}]^{2-}$  and  $[a_{11} - \text{Ade}]^{2-}$  ions are 50 Da higher in mass than the calculated masses for the corresponding ions of the unmodified ODN. The measured masses of  $w_3$ ,  $w_4$ ,  $w_5$ ,  $[a_3 - \text{Gua}]$ ,  $[a_4 - \text{Gua}]$  and  $[a_5 - \text{Cyt}]$  ions, however, are the same as the calculated masses for the respective ions of the unmodified ODN. The product-ion spectrum of the  $[M - 3H]^{3-}$  ion of ODN4 is very similar to that of ODN3 except that the  $[a_6 - 143]$ , a characteristic ion for short ODNs carrying a Tg (49), and  $w_6^{2-}$  ions are found in the product-ion spectrum of ODN4 (Figure S15b), but undetectable in that of ODN3 (Figure 2b). Furthermore, the  $w_5$  ion is observed for ODN3 (Figure 2b), but not for ODN4 (Figure S15b).

### Thermodynamic Studies

We next measured the thermodynamic parameters for duplex formation with the synthesized lesion-bearing substrates. The specific nucleotide sequences were: strand 1, 5'-ATGGCXYGCTAT-3'; strand 2, 5'-ATAGCMNGCCAT-3', where XY/MN represents GT/AC, GT/AA, TG/CA, G\*T/AC, G\*T/AA, GTg/AC, G\*Tg/AC, G\*Tg/AA or TgG\*/CA (G\* represents 8-oxodG). We refer to the duplexes by abbreviations of the form of XY/MN (Table 2). We determined the  $\Delta H$  and  $\Delta S$  by fitting  $T_m^{-1}$  versus  $\ln(C_t/4)$  as shown in Figure 3 (Data for unmodified and single base lesion-bearing ODNs are shown in the Supporting Information). The  $\Delta H$ ,  $\Delta S$ ,  $\Delta G_{25^\circ\text{C}}$ , and the  $T_m$  values at  $C_t = 16.0 \mu\text{M}$  are listed in Table 2.

Our data on thermodynamic parameters (Table 2) showed that the free energy change associated with the formation of duplex at 25 °C ( $\Delta G_{25^\circ\text{C}}$ ) with 8-oxodG/dA base pair (-15.1 kcal/mol) is very similar to that for the formation of the duplex with 8-oxodG/dC base pair (-15.3 kcal/mol). These results are consistent with previous thermodynamic results as well as NMR and X-ray crystal structure studies (50-52), which showed that 8-oxodG can form base pairs with both dC and dA, depending on whether the damaged nucleoside adopts an *anti* or *syn* configuration about the glycosidic bond (50,51).

Consistent with previous thermodynamic studies (34), our results showed that the presence of thymine glycol decreases markedly the duplex stability and it preferentially forms base pairing with adenine. In addition, duplex ODN with tandem lesion is less stable than the corresponding

duplex with either of the two single-base lesions alone (Table 2). Moreover, there is no obvious difference in thermal stability of duplex ODNs with the two types of tandem lesions, i.e., 5'-G\*Tg-3' and 5'-TgG\*-3' (Duplexes 7 and 9, Table 2).

## Discussion

In the present work, we successfully synthesized phosphoramidite building blocks of thymidine glycol and 8-oxodG, which facilitate the simultaneous insertion of the two lesions into ODNs. Additionally, with the use of acetyl group for the protection of the hydroxyl functionalities on the thymine glycol moiety, post-synthesis deprotection can be achieved in one step. The availability of authentic ODN substrates enables the assessment of the formation of this type of tandem lesions under various oxidative stress conditions. In this respect, the tandem lesion-carrying ODN substrates will facilitate the design and optimization of enzymatic digestion procedures for the successful release of this type of tandem lesion from duplex DNA.

Methylation of cytosine at CpG sites plays an important role in the epigenetic silencing of genes in mammalian cells (53,54). Damage occurring at CpG dinucleotide and the resulting mutation at these sites may perturb the binding of DNA to methyl-CpG binding proteins (55). We reason that the type of tandem lesion that we synthesized here may have implications in CpG mutagenesis. In this context, under oxidative stress, mC could be damaged to form 5-methylcytosine glycol (56), which is susceptible to deamination to yield thymine glycol. On the other hand, the dG at CpG site can be oxidized to give a 8-oxodG (Scheme 2). Thymidine glycol is mostly a blocking lesion. It, however, can be bypassed by translesion synthesis DNA polymerases including yeast DNA polymerases  $\eta$  and  $\zeta$  as well as human DNA polymerase  $\kappa$ , and all three polymerases preferentially insert a dA opposite the lesion (5,57,58). On the other hand, 8-oxodG can be bypassed by various DNA polymerases and both dC and dA can be inserted opposite the lesion (59,60). Therefore, the tandem lesions with a pyrimidine glycol being neighboring to 8-oxodG may contribute to the previously observed ROS-induced mCG $\rightarrow$ TT mutation (61). The availability of ODN substrates bearing structurally defined tandem lesions will allow for the replication studies of these lesions both *in vitro* and *in vivo*, which may offer important insights into the roles of this type of lesions in CpG mutagenesis.

Recent repair studies on cluster lesions showed that the repair of one lesion can be hampered by the presence of another lesion nearby (25-28,31). Our thermodynamic measurement results clearly showed that tandem lesions, where an 8-oxodG is adjacent to a Tg, can cause more destabilization to DNA duplexes than either of the two lesions alone. In this regard, the presence of Tg and 8-oxodG neighboring to each other destabilizes the duplex DNA by  $\sim$ 5.1 kcal/mol (Table 2). Although either Tg or 8-oxodG, when present by itself, can be efficiently repaired by base excision repair (BER) enzymes (62), the elevated structural distortion caused by the vicinal single-base lesions may result in the compromised repair of these two lesions by BER machinery. In addition, both Tg and 8-oxodG could be excised from DNA by NER enzymes *in vitro* (63), the formation of these two lesions in tandem, due to the increased structural distortion, may also render the tandem lesions to be excised more efficiently by NER than while the two lesions are not proximal to each other. The availability of authentic ODN substrates will again enable us to examine such possibilities.

Taken together, we reported here for the first time the dual incorporation of Tg and 8-oxodG into ODNs. The availability of ODNs carrying neighboring 8-oxodG and Tg builds a solid foundation for examining the replication and repair of the tandem oxidative lesions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

ROS, reactive oxygen species; ODN, oligodeoxynucleotides; BER, base excision repair; NER, nucleotide excision repair; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

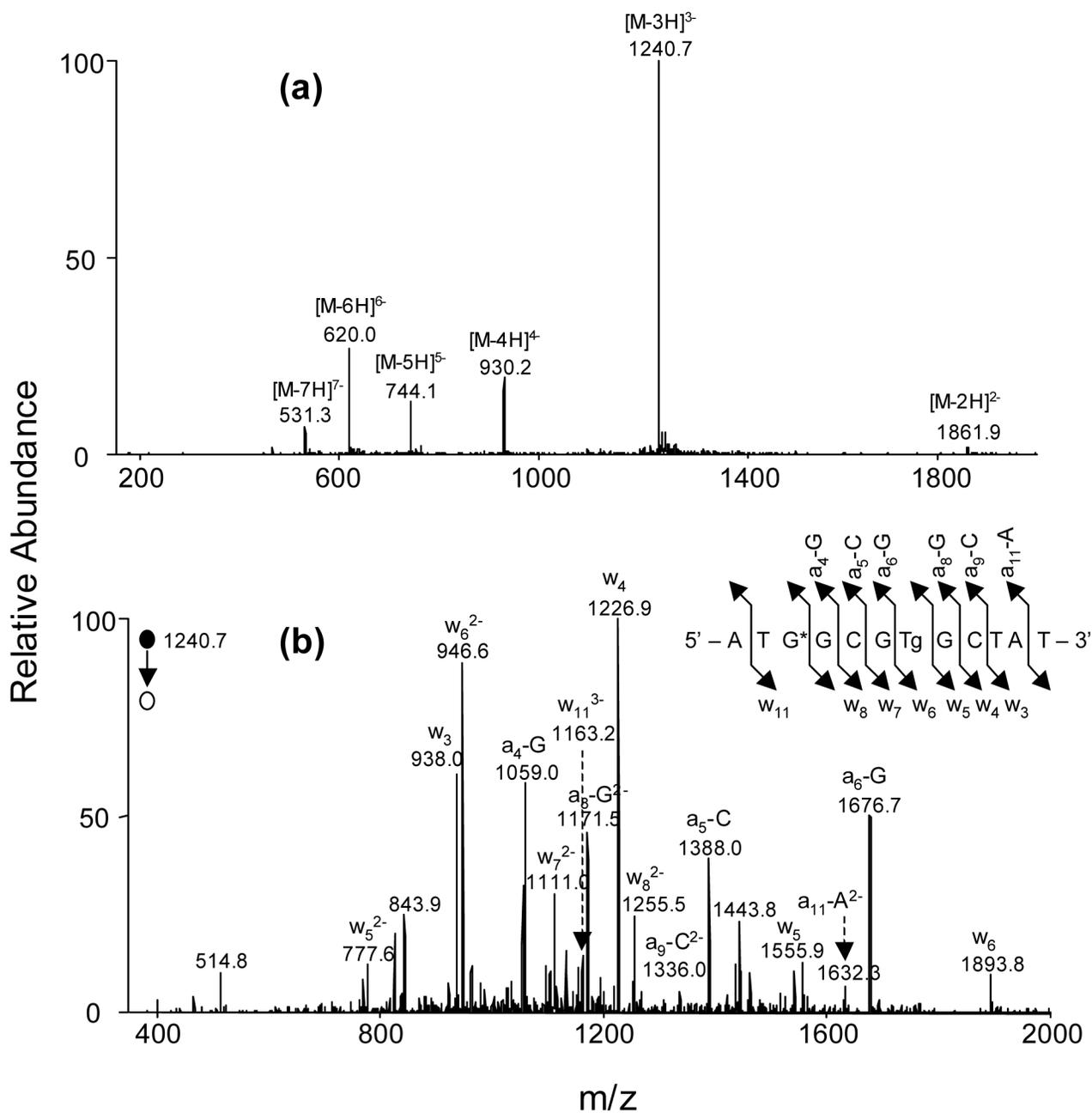
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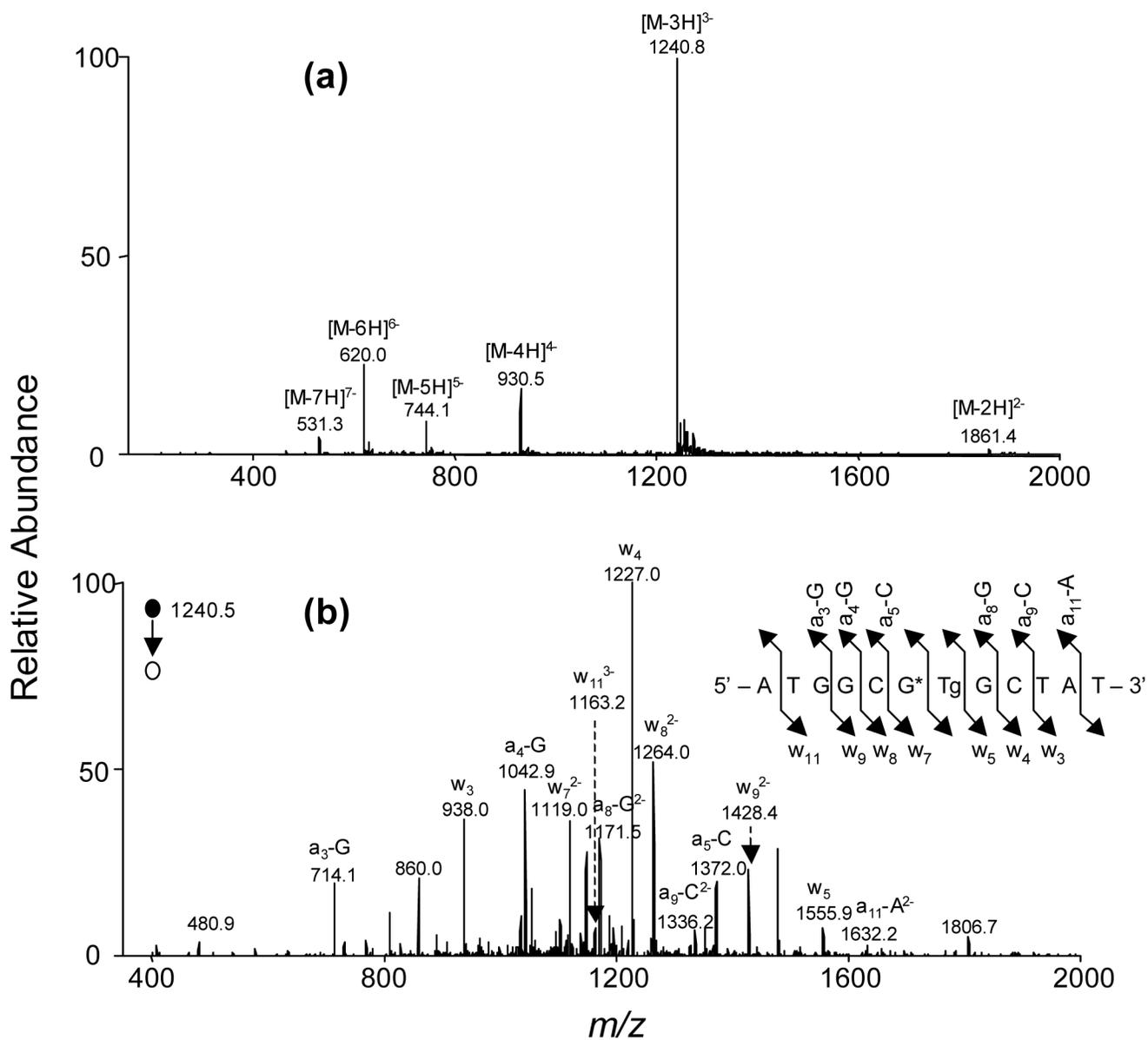
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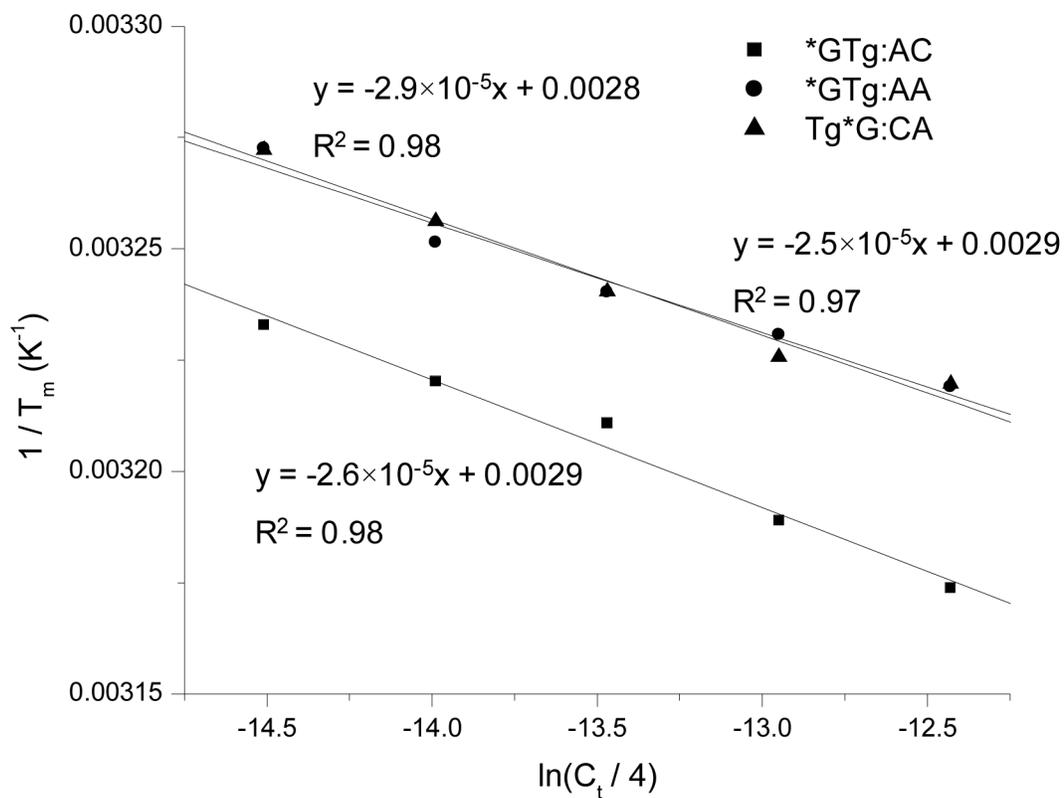
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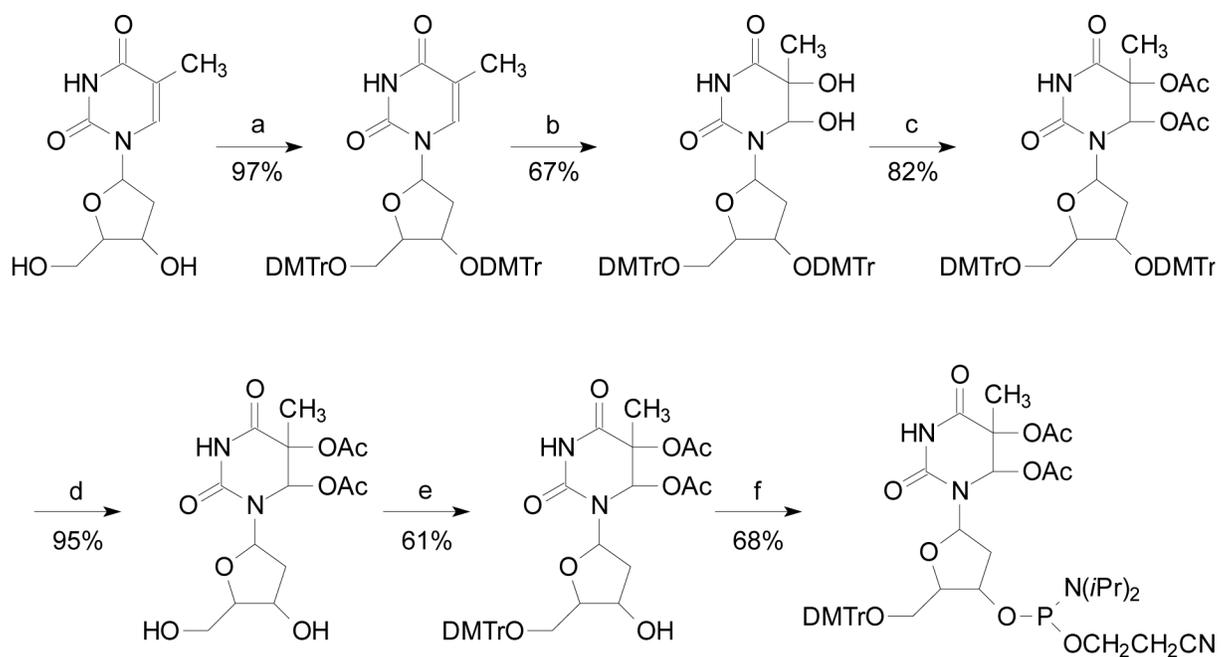
**Figure 1.** ESI-MS & MS/MS characterizations of d(ATG\*GCGTgGCTAT): (a) Negative-ion ESI-MS; (b) product-ion spectrum of the  $[M-3H]^{3-}$  ion ( $m/z$  1241).



**Figure 2.**  
ESI-MS & MS/MS characterizations of d(ATGGCG\*TgGCTAT): (a) Negative-ion ESI-MS; (b) product-ion spectrum of  $[M-3H]^{3-}$  ion ( $m/z$  1241).

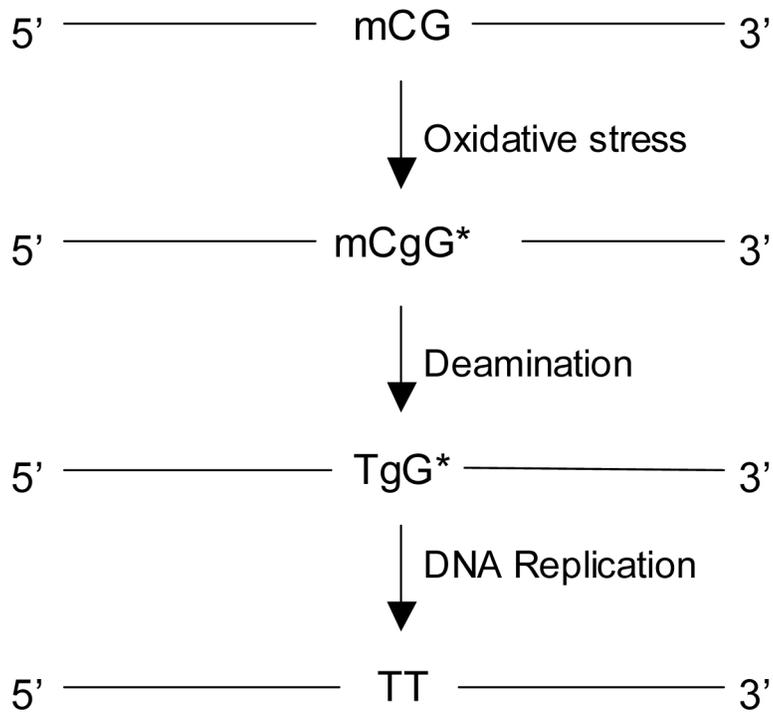
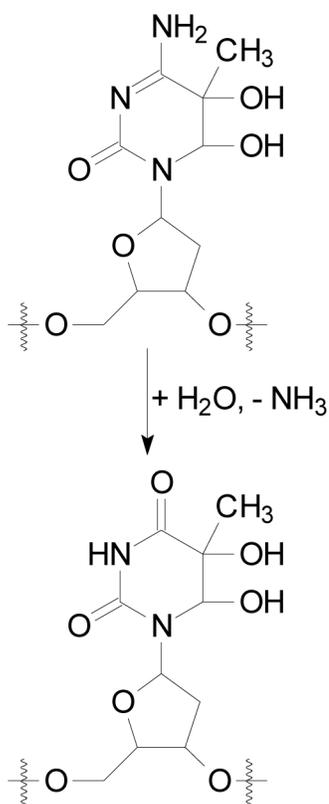
**Figure 3.**

Plots of  $1/T_m$  vs.  $\ln(C_t/4)$  for the duplexes containing thymidine glycol and 8-oxodG tandem lesions. The duplex is  $d(ATGGCXYGCTAT)/d(ATAGCMNGCCAT)$ , where XY/MN represents  $G^*Tg/AC$  (■),  $G^*Tg/AA$  (●),  $TgG^*/CA$  (▲).

**Scheme 1.**

Synthesis of phosphoramidite building block of Tg.

**Reagents:** (a) DMTrCl/Pyridine/70°C; (b) OsO<sub>4</sub>/Pyridine; (c) Ac<sub>2</sub>O/DMAP/Pyridine; (d) 70% acetic acid; (e) DMTrCl/Pyridine; (f) NC(CH<sub>2</sub>)<sub>2</sub>OP(Cl)N(*i*Pr)<sub>2</sub>/DIEA/CH<sub>2</sub>Cl<sub>2</sub>



**Scheme 2.**  
Deamination of 5-methylcytosine glycol and the implications of tandem lesions in mCG  $\rightarrow$  TT mutation.

**Table 1**  
Sequences of Synthesized Oligodeoxynucleotides

ODNs	Sequence	Observed $m/z$	Theoretical $m/z$
ODN1	5'-ATG GC <sup>G*</sup> TGC TAT-3'	3691.2	3691.6
ODN2	5'-ATG GCG T <sub>g</sub> GC TAT-3'	3709.2	3709.6
ODN3	5'-ATG GC <sup>G*</sup> T <sub>g</sub> GC TAT-3'	3725.2	3725.6
ODN4	5'-ATG GCT <sub>g</sub> G <sup>G*</sup> GC TAT-3'	3724.9	3725.6
ODN5	5'-AT <sup>G*</sup> GCG T <sub>g</sub> GC TAT-3'	3725.2	3725.6

<sup>G\*</sup>  
8-oxodG

Table 2

Thermodynamic Parameters for Duplex Formation

Duplex	$T_m$ [a] (°C)	$\Delta H^0$ (kcal/mol)	$\Delta S^0$ (cal mol <sup>-1</sup> K <sup>-1</sup> )	$\Delta G_{25^\circ\text{C}}$ (kcal/mol)	$\Delta\Delta G_{25^\circ\text{C}}$ (kcal/mol)
5'-ATGGCXYGCTAT-3' 3'-TACCGNMCGATA-5'					
1 (XY/MN: GT/AC)	60.1	-83±4.8	-220±13	-16.2±0.48	4.6
2 (XY/MN: GT/AA)	43.1	-72±4.5	-200±13	-11.6±0.21	0.6
3 (XY/MN: TG/CA)	60.1	-77±1.3	-207±4	-15.6±0.13	0.9/[b]
4 (XY/MN: G*/T/AC)	56.4	-83±2.4	-230±7	-15.3±0.21	-3.5/[b]
5 (XY/MN: G*/T/AA)	52.4	-90±7.2	-250±20	-15.1±0.58	4.0/[b]
6 (XY/MN: GTg/AC)	44.7	-78±1.5	-222±4	-12.2±0.08	5.1/[b]
7 (XY/MN: G*/Tg/AC)	40.9	-69±2.0	-195±6	-11.1±0.08	0.9/[b]
8 (XY/MN: G*/Tg/AA)	37.5	-81±5.7	-240±17	-10.7±0.21	5.1/[b]
9 (XY/MN: TgC*/CA)	37.4	-76±5.0	-220±15	-10.5±0.15	5.1/[b]

[a]  $C_t = 16 \mu\text{M}$ [b]  $\Delta\Delta G_{25^\circ\text{C}}$  (modified ODN) -  $\Delta G_{25^\circ\text{C}}$  (corresponding unmodified ODN).