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Comparison of the In Vitro Replication of the 7-(2-Oxoheptyl)-1,*N*²-etheno-2'-deoxyguanosine and 1,*N*²-Etheno-2'-deoxyguanosine Lesions by *Sulfolobus solfataricus* P2 DNA Polymerase IV (Dpo4)

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Abstract

Oligonucleotides were synthesized containing the 7-(2-oxoheptyl)-etheno-dGuo adduct, which is derived from the reaction of dGuo and the lipid peroxidation product 4-oxo-2-nonenal. The in vitro replication of 7-(2-oxoheptyl)-etheno-dGuo by the model Y-family polymerase *Sulfolobus solfataricus* P2 DNA Polymerase IV (Dpo4) was examined in two sequences. The extension products were sequenced using an improved LC-ESI-MS/MS protocol developed in our laboratories and the results were compared to that of the 1,*N*²-etheno-dGuo adduct in the same sequence contexts. Both etheno adducts were highly miscoding when situated in a 5'-TXXG-3' local sequence contexts with <4% of the extension products being derived from error-free bypass. The major extension products resulted from the misinsertion of Ade opposite the adduct and a one-base deletion. The major extension products from replication of the etheno lesions in a 5'-CXXG-3' local sequence context were the result of misinsertion of Ade, a one-base deletion, and error-free bypass. Other minor extension products were also identified. The 7-(2-oxoheptyl)-etheno-dGuo lesion resulted in a larger frequency of misinsertion of Ade, whereas the 1,*N*²-etheno-dGuo gave more of the one-base deletion product. Conformational studies of duplex DNA containing the 7-(2-oxoheptyl)-etheno-dGuo in a 5'-TXXG-3' sequence context by NMR indicated the presence of a pH-dependent conformational transition, likely involving the glycosyl bond at the adducted guanosine; the *pK_a* for this transition was lower than that observed for the 1,*N*²-ε-dGuo lesion. However, the 7-(2-oxoheptyl)-etheno-dGuo lesion, the complementary Cyt, and both flanking base pairs remained disordered at all pH values, which is attributed to the presence of the hydrophobic heptyl group of the 7-(2-oxoheptyl)-etheno-dGuo lesion. The altered *pK_a* value and the structural disorder at the 7-(2-oxoheptyl)-etheno-dGuo lesion site, as compared to the same sequence containing the 1,*N*²-etheno-dGuo, may contribute to higher frequency of misinsertion of Ade.

Introduction

Etheno (ε) adducts of dGuo, dAdo, and dCyd result from the reaction of DNA with epoxides of vinyl monomers and have been implicated, at least in part, in the hepatocarcinogenicity of

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Supporting Information Available: ¹H, ¹³C, and ³¹P NMR spectra, MS and CGE characterization of oligonucleotides, sequences of oligonucleotides and primers, gels of extension reactions, reconstructed ion profiles of the extension reactions, CID mass spectra of extension products and standards, tables of the observed and expected CID fragment ions for the extension products, and calibration curves. This material is available free of charge via the Internet at <http://pubs.acs.org>.

vinyl chloride (1,2). Subsequent studies identified background levels of etheno adducts in unexposed test animals (3). Lipid peroxidation products are believed to be the source of the endogenous etheno adducts (4-6). In a series of studies, Blair and co-workers characterized the substituted etheno adducts 7-(2-oxoheptyl)- ϵ -dGuo (**1**) (7), 7-(2-oxoheptyl)- ϵ -dAdo (**2**) (8,9), 3-(2-oxoheptyl)- ϵ -dCyd (**3**) (10,11), and 7-(2-oxoheptyl)- ϵ -Guo (**12**) from the reaction of the lipid peroxidation product 4-oxo-2-nonenal (ONE) with the appropriate nucleoside or nucleic acid (Figure 1); the 2-oxoheptyl-substituted etheno adducts are uniquely derived from oxidative stress. 7-(2-Oxoheptyl)- ϵ -dGuo and 3-(2-oxoheptyl)- ϵ -dCyd were detected from the small intestines of min mice, which are a colorectal cancer mouse model that over expresses cyclooxygenase-2 (13). Structurally related substituted etheno adducts have been characterized from 4-oxo-2-hexenal (14-17), a peroxidation product from ω 3-fatty acids, buten-1,4-dial (18-21), which is derived from the metabolism of furan (22), and 4-oxo-2-pentenal, which is derived from the metabolism of *N*-nitrosopyrrolidine (23-25), methylfuran (26), and the β,δ -elimination of an abasic site in DNA (27-29). The $N^2,3$ - ϵ -dGuo adduct has been identified from the reaction of dGuo with chloroacetaldehydes and chlorooxirane (30,31), but analogous etheno adducts from ONE and related 4-oxo-alkenal have not been characterized to date.

The 3-(2-oxoheptyl)- ϵ -dCyd (**3**) adduct has been site-specifically incorporated into a 13mer oligonucleotide using the corresponding phosphoramidite reagent (32). The modified oligonucleotide was then ligated into a shuttle vector and its mutagenicity was evaluated in *Escherichia coli* and three human cell lines (XPA, XPV, and repair-proficient GM637 cells). The 3-(2-oxoheptyl)- ϵ -dCyd adduct was found to be a strong block to replication in all cell lines examined; however, the adduct was highly miscoding when bypassed. In bacteria, the miscoding frequency was up to 48% and consisted mostly of C $\rightarrow$ G transversions. The miscoding frequency was >90% in the human cell lines, with C $\rightarrow$ A transversions as the major mutation followed by C $\rightarrow$ T transitions. Similar results were observed when the parent unsubstituted ϵ -dCyd adduct was replicated in simian kidney cells (33). The marked differences in the miscoding properties of 3-(2-oxoheptyl)- ϵ -dCyd between the human and bacterial cells were attributed to the different DNA polymerases involved in translesion synthesis (32). The observation that the mutational spectrum and frequency was nearly identical for wild type GM637 and pol η -deficient XPV cells suggests a limited role for pol η in the bypass of 3-(2-oxoheptyl)- ϵ -dCyd. Replication of the 3-(2-oxoheptyl)- ϵ -dCyd adduct in mouse fibroblast cells in which the genes encoding pol η , ι , or κ were individually knocked out did not qualitatively effect translesion synthesis (34). Interestingly, insertion of dAde was abolished when the lesion was replicated in cells in which the *Rev3* or *Rev1* genes were inactivated, but dThy insertion was not affected. These studies suggested that pol ζ /REV1 was involved in mutagenic bypass of the 3-(2-oxoheptyl)- ϵ -dCyd adduct leading to C $\rightarrow$ T transitions, while bypass by Y-family polymerases are responsible for C $\rightarrow$ A transversions.

We report here the site-specific synthesis of oligonucleotides containing the 7-(2-oxoheptyl)- ϵ -dGuo lesion and the in vitro replication of this lesion by the model Y-family polymerase Dpo4 in two sequences. As we previously reported with the 1, N^2 - ϵ -dGuo in the same sequence contexts, the misincorporation profile was dependent on the identity of the 5'-neighboring base to the lesion (35). The products of translesion synthesis were sequenced by an improved LC-ESI-MS-MS method previously described by our laboratories (36); the distribution of products from translesion synthesis of the 7-(2-oxoheptyl)- ϵ -dGuo was similar to that of the 1, N^2 - ϵ -dGuo lesion. NMR studies of the 7-(2-oxoheptyl)- ϵ -dGuo in the 5'-TXXG-3' sequence indicated that the lesion underwent a pH-dependent conformational transition, similar to the 1, N^2 - ϵ -dGuo lesion, but with a more acidic pK_a . The nature of this conformational transition remains to be determined because the complementary Cyt, and

both flanking base pairs remained disordered at all pH values examined, but likely involves rotation about the glycosyl bond as observed for the 1,*N*²- ϵ -dGuo lesion (37,38).

Experimental Procedures

7-(2-Oxoheptyl)-1,*N*²-etheno-2'-deoxyguanosine (1)

dGuo•H₂O (114 mg, 0.40 mmol) and K₂CO₃ (100.0 mg, 0.72 mmol) were suspended in DMF (5 mL) and stirred at room temperature for 15 min. A solution of ONE (39) (125 mg, 0.80 mmol) in DMF (5 mL) was prepared; a portion of this solution (2.5 mL, 0.40 mmol) was added to the reaction, and a second portion (2.5 mL, 0.40 mmol) was added after 60 min. The reaction was stirred for an additional 6 h with the progress of the reaction monitored by HPLC (gradient B). The reaction was poured over crushed ice (50 mL). After neutralization with 1 % HCl (w/v), the mixture was allowed to stand at room temperature overnight. The precipitated solid was removed by centrifugation, rinsed with water, frozen on dry ice, and dried on a lyophilizer to afford **1** (61.0 mg). The filtrate was concentrated in vacuo and the resulting solid isolated as just described afforded additional **1** (27.0 mg). The purity of **1** (88.0 mg, 55% yield) was judged to be > 95% by HPLC (gradient B) and used without further purification. ¹H-NMR (600 MHz, DMSO-*d*₆) δ 12.22 (br s, 1H, NH), 8.06 (s, 1H, H-2), 7.13 (s, 1H, H-6), 6.20 (dd, 1H, *J* = 7.2, 0.9 Hz, H-1'), 5.28 (d, 1H, *J* = 2.7 Hz, 3'-OH), 4.95 (s, 1H, 5'-OH), 4.36 (m, 1H, H-3'), 4.10 (dd, 2H, *J* = 17.9, 21.3 Hz, H-10), 3.83 (m, 1H, H-4'), 3.57 (m, 1H, H-5'), 3.51 (m, 1H, H-5''), 2.59 (m, 1H, H-2'), 2.54 (m, 2H, H-12), 2.23 (m, 1H, H-2''), 1.50 (m, 2H, H-13), 1.25 (m, 4H, H-14, H-15), 0.85 (t, 3H, *J* = 7.1 Hz, H-16); ¹³C NMR (150 MHz, DMSO-*d*₆) δ : 205.6, 153.6, 149.7, 146.7, 136.8, 117.8, 115.9, 115.4, 87.6, 82.9, 70.7, 61.7, 41.1, 39.5, 39.4, 30.8, 22.7, 22.0, 13.8.

FAB HRMS⁺ *m/z* calcd for C₁₉H₂₆N₅O₅ [M+H]⁺, 404.1934; found, 404.1917.

5,5'-*N,O*-bis(4,4'-Dimethoxytrityl)-7-(2-oxoheptyl)-1,*N*²-etheno-2'-deoxyguanosine (4)

Nucleoside **1** (65 mg, 0.16 mmol) was dried by dissolving in anhydrous pyridine (5 mL) and evaporating the solvent in vacuo on a rotary evaporator; the procedure was repeated two additional times. To a stirred solution of **1** in anhydrous pyridine (3 mL) were added 4,4'-dimethoxytrityl chloride (164 mg, 0.48 mmol) and diisopropylethylamine (85 μ L, 62 mg, 0.48 mmol). The reaction mixture was stirred for 5 h at room temperature under an argon atmosphere. The solvent was removed in vacuo and the residue was purified by flash column chromatography on silica gel, eluting with a gradient of CH₂Cl₂:pyridine (98:2, v/v) to CH₂Cl₂:CH₃OH:pyridine (98:1:1) to afford bis-tritylated nucleoside **4** (107 mg, 66 %). ¹H-NMR (400 MHz, DMSO-*d*₆) δ 7.78 (s, 1H, H-2), 7.32 (m, 2H, aromatic), 7.31-7.04 (m, 18H, aromatic), 6.94 (s, 1H, H-6), 6.86-6.81 (m, 8H, aromatic), 5.64 (t, 1H, *J* = 6.2 Hz, H-1'), 5.09 (d, 1H, *J* = 4.9 Hz, 3'-OH), 4.14 (s, 2H, H-10), 3.82 (m, 2H, H-3', H-4'), 3.71 and 3.68 (s, 12H, 4OCH₃), 3.02 (m, 2H, H-5', H-5''), 2.5 (m, 2H, H-12), 2.10 (m, 1H, H-2'), 1.78 (m, 1H, H-2''), 1.48 (m, 2H, H-13), 1.25 (m, 4H, H-14, H-15), 0.84 (t, 3H, *J* = 6.8 Hz, H-16).

TOF ES MS⁺ *m/z* calc. for C₆₁H₆₂N₅O₉ [M+H]⁺, 1008.4548; found, 1008.4548

5'-*O*-(4,4'-Dimethoxytrityl)-7-(2-oxoheptyl)-1,*N*²-etheno-2'-deoxyguanosine (5)

A solution of **4** (107 mg, 0.11 mmol) in CH₃OH (10 mL) was heated at reflux for 15 min after which time the solvent was removed in vacuo. The residue was purified by flash column chromatography on silica gel, eluting with a gradient of CH₂Cl₂:CH₃OH:pyridine (95:3:2 to 94:4:2, v/v/v) to afford 5'-*O*-tritylated nucleoside **5** (67 mg, 90 %). ¹H-NMR (400 MHz, DMSO-*d*₆) δ 7.93 (s, 1H, H-2), 7.32 (m, 2H, aromatic), 7.19 (m, 7H, aromatic), 7.13 (s, 1H, H-6), 6.78 (m, 4H, aromatic), 6.24 (t, 1H, *J* = 6.4 Hz, H-1'), 5.32 (d, 1H, *J* = 4.6 Hz, 3'-OH), 4.37 (m, 1H, H-3'), 4.12 (s, 2H, H-10), 3.94 (m, 1H, H-4'), 3.70 and 3.69 (s, 6H,

2OCH₃), 3.19 (m, 1H, H-5'), 3.11 (m, 1H, H-5''), 2.68 (m, 1H, H-2'), 2.55 (t, 2H, *J* = 7.4 Hz, H-12), 2.31 (m, 1H, H-2''), 1.5 (m, 2H, H-13), 1.32 (m, 4H, H-14, H-15), 0.86 (t, 3H, *J* = 6.9 Hz, H-16); ¹³C NMR (150 MHz, DMSO-*d*₆) δ: 206.9, 167.5, 158.7, 154.2, 147.1, 144.6, 135.5, 135.4, 130.8, 129.0, 128.7, 128.0, 127.7, 127.0, 126.8, 118.3, 115.7, 113.0, 86.4, 86.3, 83.1, 72.0, 68.0, 64.1, 55.1, 42.2, 39.7, 38.8, 38.7, 31.3, 30.3, 28.9, 23.7, 23.2, 23.0, 22.5, 13.8, 13.7, 10.7.

FAB HRMS⁺ *m/z* calcd for C₄₀H₄₄N₅O₇ [M+H]⁺, 705.3241; found, 705.3273.

3'-O-[*N,N*-Diisopropylamino]-2-cyanoethoxyphosphinyl]-5'-O-(4,4'-dimethoxytrityl)-7-(2-oxoheptyl)-1,*N*²-etheno-2'-deoxyguanosine (6)

Nucleoside **5** (82 mg, 0.12 mmol) was evaporated from anhydrous pyridine (3 × 5 mL) and dried overnight under vacuum. The oily residue was dissolved in freshly distilled CH₂Cl₂ (25 mL) and a 0.45 M solution of 1*H*-tetrazole in CH₃CN (330 μL, 11 mg, 0.15 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl-phosphoramidite (55 μL, 52 mg, 0.174 mmol) were added. The reaction mixture was stirred for 3 h at room temperature under an argon atmosphere. The solvent was removed in vacuo and the crude mixture of mono- and bis-phosphatidylated product was dissolved in CH₃OH (15 mL) and stirred for 30 min at room temperature. The solvent was removed in vacuo and the residue was purified by flash column chromatography on silica gel, eluting with a gradient of CH₂Cl₂:CH₃OH:pyridine (97:1:2 to 94:4:2, v/v/v) to afford phosphoramidite **6** (56 mg, 54% yield). ¹H-NMR (400 MHz, DMSO-*d*₆) δ 7.96 and 7.95 (s, 1H, diastereomeric H-2), 7.32 (m, 2H, aromatic), 7.20 (m, 7H, aromatic), 7.16 and 7.14 (s, 1H, diastereomeric H-6), 6.78 (m, 4H, aromatic), 6.26 and 6.25 (m, 1H, diastereomeric H-1'), 4.11 (s, 2H, H-10), 4.07 and 4.02 (s, 1H, diastereomeric H-4'), 3.73 and 3.62 (m, 2H, POCH₂), 3.70 and 3.69 (s, 6H, 2OCH₃), 3.52 (m, 2H, CH(CH₃)₂), 3.21 (m, 2H, H-5', H-5''), 2.84 (m, 1H, H-2'), 2.75 (t, 1H, *J* = 5.9 Hz, CH₂CN), 2.65 (t, 1H, *J* = 5.9 Hz, CH₂CN), 2.55 (t, 2H, *J* = 7.3 Hz, H-12), 2.42 (m, 1H, H-2''), 1.50 (m, 2H, H-13), 1.25 (m, 4H, H-14, H-15), 1.12 and 1.02 (m, 12H, 2CH(CH₃)₂), 0.85 (t, 3H, *J* = 6.8 Hz, H-16); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 206.0, 158.5, 154.1, 150.3, 150.0, 147.2, 145.1, 137.0, 135.9, 135.8, 135.7, 130.1, 128.1, 128.0, 127.1, 124.3, 119.4, 119.2, 118.2, 116.4, 113.5, 86.1, 85.2, 84.9, 83.1, 83.0, 73.9, 73.4, 64.0, 58.9, 58.8, 58.7, 58.6, 55.4, 43.1, 41.6, 31.2, 24.8, 24.8, 24.7, 24.7, 24.6, 24.6, 23.1, 22.4, 20.3, 20.2, 20.1, 14.3; ³¹P NMR (400MHz, DMSO-*d*₆) δ 149.15, 148.5.

FAB HRMS⁺ *m/z* calc. for C₄₉H₆₁N₇O₈P [M+H]⁺, 906.4319; found, 906.4309.

Oligonucleotide synthesis

Modified oligonucleotides were synthesized on a Perseptive Biosystems Model 8909 DNA synthesizer using Expedite reagents (Glen Research, Sterling, VA) with the standard synthetic protocol on a 1-μmol scale. Oligonucleotides containing the 7-(2-oxoheptyl)-ε-dGuo were prepared using a standard DNA synthesizer cycle with p-(*t*-butylphenoxy)acetyl-protected phosphoramidites. The modified oligodeoxynucleotides were cleaved from the solid support and the exocyclic amino groups were deprotected in a single step using 0.1 M aq NaOH; the cleavage reaction was neutralized and the oligonucleotides purified by HPLC (*gradient A*). All unmodified oligonucleotides were purchased from Midland Certified Reagents (Midland, TX).

5'-TCA TXG AAT CCT TAC GAG CCC CC-3' (7)

Solid-phase synthesis on 1-μmol scale afforded 31 A₂₆₀ units (135 nmol). MALDI-TOF-MS (HPA) *m/z* calcd for [M-H]⁻ 7080.7, found 7080.5.

5'-TCA CXG AAT CCT TAC GAG CCC CC-3' (8)

Solid-phase synthesis on 1- μ mol scale afforded 36 A_{260} units (158 nmol). MALDI-TOF-MS (HPA) m/z calcd for $[M-H]^-$ 7065.7, found 7065.6.

5'-CGC ATX GAA TCC-3' (9)

Solid-phase synthesis on 1- μ mol scale afforded 20 A_{260} units (158 nmols). MALDI-TOF-MS (HPA) m/z calcd for $[M-H]^-$ 3766.6, found 3766.5.

HPLC Separations

The purification of the oligonucleotides and the analyses of reaction mixtures were performed on a Beckman HPLC system (32 Karat software version 7.0, pump module 125) with a diode array UV detector (module 168) monitoring at 260 nm using a Waters YMC ODS-AQ columns (250 mm $\times$ 4.6 mm, 1.5 mL/min for analysis and 250 mm $\times$ 10 mm, 5 mL/min for purification). The following solvent gradients (v/v) were used:

Gradient A (CH_3CN :0.1 M $NH_4 HCO_2$)—Initially 1 % CH_3CN ; a linear gradient from 1-8% CH_3CN over 5 min; a linear gradient from 8-12% CH_3CN over 15 min; a linear gradient from 12-80% CH_3CN over 2 min; isocratic at 80% CH_3CN for 1 min; then a linear gradient to 1% CH_3CN over 2 min.

Gradient B (CH_3CN : H_2O)—Initially 10 % CH_3CN ; a linear gradient to 80% CH_3CN over 25 min; then a linear gradient to the initial conditions over 5 min.

Capillary Gel Electrophoresis (CGE)

Electrophoretic analyses were carried out using a Beckman P/ACE MDQ instrument system (using 32 Karat software, version 5.0) monitored at 260 nm on a 31.2 cm $\times$ 100 μ m eCAP capillary with samples applied at 10 kV and run at 9 kV. The capillary was packed with their 100-R gel (for ss-DNA) using the Tris-borate buffer system containing 7 M urea. The purity of the oligonucleotides were judged to be > 99% (see Figures S4-S6 of the Supporting Information).

Oligonucleotide labeling and annealing

The labeling and annealing of the oligonucleotides was performed as previously described (40).

Single-nucleotide Incorporation Assays

These assays were performed as previously described with the following modifications (40). The reactions with Dpo4 (35) were initiated by the addition of the dNTP with final concentrations of 30, 60, and 120 μ M. The final concentration of Dpo4 was 100 nM and the reactions were run at 37 $^{\circ}C$ for 30 min. The reactions with the primer and unmodified template were run under the same reaction conditions but with half the enzyme concentration. Reactions were quenched with 70 μ L of 20 mM EDTA in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes, and heated at 95 $^{\circ}C$ for 10 min. Aliquots (6 μ L) were separated by electrophoresis on a denaturing gel.

Steady-state Kinetics

These assays were performed according to a published procedure with the following modifications (40). All reactions (10 μ L final volume, 100 nM DNA, 100 nM Dpo4) were run at nine dNTP concentrations. The reactions were quenched and analyzed by gel electrophoresis as described above.

Full-length Polymerase Extension Assays

These assays were performed as previously described with the following modifications (40). The reactions were run with 100 nM DNA and 100 nM Dpo4 at 37 °C for 30 min. The final dNTP concentrations were 30, 60, and 120 μ M each. The reactions with the primer and unmodified template were run using the same reaction conditions but with half the enzymes concentration. The reactions were quenched and analyzed by gel electrophoresis as described above.

LC-ESI-MS-MS Sequencing of the Extension Reactions

Extension of templates **7** and **8** and identification of the products by LC-ESI-MS-MS was accomplished as previously described (36). Mass spectrometric analyses was performed in the Vanderbilt University facility on a Waters Acquity UPLC system (Waters, Milford, MA) connected to a Finnigan LTQ mass spectrometer (ThermoElectron) equipped with an Ion Max API source and a standard electrospray probe using an Acquity UPLC BEH C18 column (1 μ m, 1.0 mm $\times$ 100 mm). These analyses were performed as previously described (36). The Mongo Oligo Mass Calculator (v. 2.6) from the University of Utah (<http://library.med.utah.edu/masspec/>) was used to calculate the theoretical CID spectra of the candidate oligonucleotide sequences. The proposed oligonucleotide sequences were then purchased from Midland Certified Reagents (Midland, TX) and subjected to the same LC-ESI-MS-MS analysis in order to compare the CID spectra.

Construction of the Calibration Curves

Standard calibration curves were constructed using 5 – 7 different concentrations (from 0.02 to 0.40 nmol) of the corresponding oligonucleotide (analyte) and a constant amount of the 5'-pCTTCACGAGCCCC-3' standard (0.10 nmol) (36). The amount of the standard corresponded to ~50% yield for the extension reaction. The yield of formation of the corresponding analyte was calculated based on the ratio (nmol) of the extension product and the biotinylated primer (nmol) used for the full-length extension reaction. The correlation coefficients (r^2) were typically > 0.98. The calibration curves were constructed in full scan mode using the sum of the extracted $[M-2H]^{-2}$ and $[M-3H]^{-3}$ ions for the analyte oligonucleotides and the sum of the $[M-3H]^{-3}$ and $[M-4H]^{-4}$ ions for the standard. Each peak consisted of at least 15 scans. Calibration curves are shown in Figures S23 and S24 of the Supporting Information.

To determine the accuracy of the calibration curves, a mixture of the 5'-pCTTACGAGCCCC-3' standard (0.10 nmol) was added to known amounts of the 5'-pTCCGTGA-3' (0.06 nmol), 5'-pTCAGTGA-3' (0.18 nmol), 5'-pTCTGTGA-3' (0.40 nmol), and 5'-pTCGTGA-3' (0.08 nmol) analyte oligonucleotides and the mixture volume was brought to 20 μ L; 10 μ L of this solution was analyzed. The amounts of the analyte oligonucleotides were calculated based on the ratio of the area of analyte to standard (5'-pCTTACGAGCCCC-3') and the corresponding calibration curve. The calculated amount of the analyte oligonucleotides was generally within 10% of the actual amount.

UV Melting Studies

Experiments were conducted on a Cary 100 Bio spectrophotometer (Varian Associates, Palo Alto, CA) with ~ 0.3 A_{260} units of duplex sample in 1 mL of 10 mM NaH_2PO_4 , 100 mM NaCl, and 5 μ M Na_2EDTA (pH 7.0) in a 1.0 cm cuvette. Experiments were also conducted at pH 5.2 and pH 8.6. Samples were heated at a rate of 2 °C/min and data were collected at a rate of 1 point/min.

NMR Experiments

The modified duplex, 5'-d(CGCATXGAATCC)-3' (**9**) and 5'-d(GGATTCCATGCG)-3' where X= 7-(2-oxoheptyl)- ϵ -dGuo, was prepared as previously described for the 1, N^2 - ϵ -dGuo containing duplex (37,38). Briefly, samples for NMR measurements were dissolved to a duplex concentration of 0.3 mM in 500 μ L of 10 mM NaH₂PO₄, 100 mM NaCl, and 5 μ M Na₂EDTA (pH 7.0). Samples for the observation of non-exchangeable protons were exchanged with D₂O and suspended in 500 μ L of 99.99% D₂O. Samples for the observation of exchangeable protons were dissolved to a concentration of 0.3 mM in 500 μ L of 10 mM NaH₂PO₄, 100 mM NaCl, and 5 μ M Na₂EDTA containing 9:1 H₂O:D₂O. The desired pH values of the NMR samples were obtained by titration with concentrated DCl or NaOD. The temperature was 20 °C for observation of non-exchangeable protons and 5 °C for observation of exchangeable protons. Chemical shifts of the proton resonances were referenced to the water resonance at 4.82 ppm at 20 °C and 4.96 ppm at 5 °C. NMR experiments and analysis were carried out as described before (37).

Results

Phosphoramidite and Oligonucleotide Synthesis

We have reported an improved synthesis of the phosphoramidite reagent of 1, N^2 - ϵ -dGuo (41). A key to this work was the recognition that the N5-position was reactive (42). 7-(2-Oxoheptyl)- ϵ -dGuo (**1**) was prepared by the reaction of ONE with dGuo by a slightly modified procedure in 55% yield (7). Modified nucleotides containing a ketone have been incorporated into oligonucleotides previously using the phosphoramidite approach indicating that this functional group is compatible with solid-phase synthesis (32,43). Reaction of **1** with excess dimethoxytrityl chloride (DMTr-Cl) afforded 5',N5-bis-dimethoxytritylated product **4** (Scheme 1). The N5-DMTr group was selectively removed by briefly heating **4** in CH₃OH at reflux. Phosphitylation of **5** gave the 3',N5-bis-phosphoramidite reagent, which was not characterized. The crude product was stirred in methanol at room temperature to afford the desired phosphoramidite reagent **6**. The synthesis of **6** required 5 steps from dGuo and ONE and proceeded in ~18% overall yield.

Phosphoramidite **6** was used for the syntheses of three oligonucleotides (**7–9**); standard solid-phase protocols were used and the oligonucleotides characterized by MALDI-TOF-MS. The modified oligonucleotides were purified by HPLC and their purity was >99% based on capillary gel electrophoretic analysis (see Figures S4 and S5 of the Supporting Information). The 23mer oligonucleotides (**7** and **8**, see Figure 2 for sequences) were synthesized for in vitro replication studies with Dpo4, and the 12mer oligonucleotide, 5'-CGCATXGAATCC-3' (**9**), was synthesized for structural studies.

Insertion Opposite 7-(2-Oxoheptyl)- ϵ -dGuo by Dpo4 in the Presence of a Single dNTP

The 7-(2-oxoheptyl)- ϵ -dGuo adduct was incorporated at position 5 of the 23mer oligonucleotides (**7** and **8**); these substrates were then annealed to complementary 5'-³²P-labeled 18mer (-1) primer strand for single nucleotide incorporation studies. Oligonucleotides **7** and **8** differ in the identity of the base on the 5'-side of the lesion (Thy vs Cyt). In general, the efficiency for dNTP insertion opposite the 7-(2-oxoheptyl)- ϵ -dGuo lesions was low and the pattern of dNTP insertion was similar to that of 1, N^2 - ϵ -dGuo (see Figure S9 of the Supporting Information). Insertion of dATP appears to be favored for both etheno adducts in both sequences and this was confirmed by steady-state kinetic analysis (Table 1). The successive insertion of three dAdo nucleotides was observed for the 5'-TXG-3' (**7**) template, which was also reported for the 1, N^2 - ϵ -dGuo in the same sequence context (35).

Only dCTP was inserted opposite dGuo of the unmodified control templates. Insertion of dCTP opposite the lesion was $\geq 10^4$ less efficient (k_{cat}/K_m) than for the unmodified template in both sequences. Incorporation of dATP opposite the 7-(2-oxoheptyl)- ϵ -dGuo was most efficient with k_{cat}/K_m values 18- and 32-fold greater than the insertion of dCTP for the 5'-TXG-3' (**7**) and 5'-CXG-3' (**8**) templates, respectively. Insertion of dTTP and dGTP were 16 and 41-fold less efficient than insertion of dATP for the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-TXG-3' (**7**) template and 17 and 4-fold less efficient in the 5'-CXG-3' (**8**) template; the catalytic efficiencies were similar to misinsertion opposite an unmodified template (X-dGuo).

Translesion Synthesis Across 7-(2-Oxoheptyl)- ϵ -dGuo by Dpo4

The 5'-TXG-3' (**7**) and 5'-CXG-3' (**8**) templates were annealed to a complementary 16mer (-3) primer strand and the ability of Dpo4 to extend past the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the presence of all four dNTPs was examined (Figure 2). Full-length products were observed in both sequences after a 30 min reaction time indicating that Dpo4 could efficiently bypass the lesion.

Characterization of Extension Products by MS

We previously characterized extension products from in vitro translesion synthesis by LC-ESI-MS-MS (35) and an improvement of this method was recently reported (36). Briefly, a 5'-biotinylated, -1 primer with a dUrd at the -3 position was hybridized to the templates (see Figure S7 of the Supporting Information for primer sequence). Immobilization of the extended primers on streptavidin-coated beads allowed for the partial purification and enrichment of the extension products, which translated into greater sensitivity during the MS analysis. The immobilized extension products were then cleaved from the beads by treatment with uridine DNA glycosylase followed by treatment with piperidine (see Figure S8 of the Supporting Information). The resulting oligonucleotides, which were 7mers for full-length extension products, were sequenced by LC-ESI-MS-MS. A 13mer oligonucleotide standard was added just prior to MS analysis in order to better estimate the chemical yield of the extension products. We assumed that the remainder of the mass balance was unextended primer or truncated extension products, which were not observed.

We used this method to sequence the extension products from bypass of the 7-(2-oxoheptyl)- ϵ -dGuo lesion, and the 1, N^2 - ϵ -dGuo adduct was reexamined in the same sequences for comparison. The full scan product ion mass spectra from translesion synthesis of the modified 5'-TXG-3' (**7**) and 5'-CXG-3' (**8**) templates are shown in Figure 3 and the sequence assignments of the extension products are summarized in Table 2.

The same seven extension products were identified for the bypass of both etheno lesions in the 5'-TXG-3' (**7**) template, although their distribution was slightly different. Misinsertion of Ade (5'-pTCAATGA-3', ~35%) and a one-base deletion (5'-pTC-ATGA-3', ~20%) were the major extension products when the 7-(2-oxoheptyl)- ϵ -dGuo lesion was bypassed. A minor product (5'-pTC-ATG-3', ~4%) was identified as also arising from a one-base deletion, but incomplete extension (*vide infra*). Other minor products (<5% each) were derived from error-free bypass (5'-pTCCATGA-3'), and the misinsertion of Gua (5'-pTCGATGA-3').

Analysis of the CID spectrum of the m/z 1086.2 $[M-2H]^{-2}$ ion indicated that two products of the same nucleotide composition were present, one resulting from the misinsertion of Thy (5'-pTCTATGA-3'), and a tandem misinsertion (5'-pTCATTGA-3') involving incorporation of Ade opposite the lesion followed by misinsertion of the Thy opposite the template Thy that is 3' to the lesion. The CID spectra from the two sequences are predicted to be very

similar (Figure 4). The only differences are the a_4 -b and w_4 $[M-H]^-$ fragment ions, which are predicted to give m/z 1074.1 and 1276.2 for the 5'-pTCTATGA-3' oligonucleotide and m/z 1083.1 and 1267.2 for the 5'-pTCATTGA-3' sequence (Figure 4). A prominent fragment ion at m/z 1074.6 was observed consistent with the a_4 -b fragment ion of the 5'-pTCTATGA-3' product (see Figure S14 of the Supporting Information); however, the w_4 fragment ion at m/z 1276.2 was not observed. Less abundant ions were observed at m/z 1268.0 $[M-H]^-$ and m/z 634.1 $[M-2H]^{-2}$ and are consistent with the w_4 fragment ions of the 5'-pTCATTGA-3' product. The CID spectrum of an authentic standard of the 5'-pTCTATGA-3' oligonucleotide showed the a_4 -b fragment ion at m/z 1074.1, but not the w_4 fragment ion at m/z 1276.2, similar to the spectrum from the extension reaction. Additionally, the fragment ions at m/z 1083.1 and 1267.2 were not observed. The w_4 fragment ions at m/z 1267.1 and 633.1 ($[M-2H]^{-2}$) were observed in the CID spectrum of an authentic standard of the 5'-pTCATTGA-3' sequence, while the a_4 -b fragment ion was not observed (m/z 1083.1); as expected, no fragment ions were observed at m/z 1074.1 and 1276.2.

Two major products from translesion synthesis of the 1, N^2 - ϵ -dGuo lesion derived from misinsertion of Ade (16%) and a one-base deletion (31%) were identified. The minor products (<5%) were identified as the error-free bypass product, misinsertion of Gua, misinsertion of Thy, and a tandem misinsertion (5'-pTCATTGA-3'). The later two were observed as a mixture, as discussed above.

We previously reported that the 1, N^2 - ϵ -dGuo lesion in the 5'-TXXG-3' sequence resulted in four products including the misinsertion of Ade (5'-pTCAATGA-3'), a tandem misinsertion (5'-pTCATTGA-3'), a one-base deletion (5'-pTC-ATGA-3'), and a two-base deletion (5'-pTC- -TGA-3') (35). The products arising from misinsertion of Ade and a one-base deletion were observed in the current analysis. As discussed above, the tandem misinsertion product was a mixture with the isomeric misinsertion of Thy (5'-pTCTATGA-3'). Upon closer examination of the CID spectrum, the sequence assignment for the two-base deletion product was revised to the isomeric 5'-pTC-ATG- -3' (35), which arises from a one-base deletion opposite the etheno-lesion and truncation of the extension reaction one-base short of full-length. The proposed two-base deletion product is expected to have w_1 and w_2 fragment ions at m/z 330.1 and 659.1, respectively, and an a_4 -B fragment ion at m/z 1074.1 in the CID mass spectrum, whereas these same fragment ions for the 5'-pTC-ATG-3' product are predicted for m/z 346.1, 650.1, and 1083.1, respectively (Figure 5). The observed CID spectrum of the extension product (m/z 777.3, $[M-2H]^{-2}$) showed a prominent fragment ion at m/z 650.0 and a less abundant ion at m/z 1083.2, consistent with the one-base deletion product and incomplete extension (5'-pTC-ATG-3'); fragment ions unique to the two-base deletion product were not observed (see Figure S16 in the Supporting Information).

Five extension products were observed for the Dpo4 bypass of the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-CXXG-3' template (8). The major products were derived from misinsertion of Ade (5'-pTCAGTGA-3', 26%), a one-base deletion (5'-pTC-GTGA-3', 20%), and error-free bypass (5'-pTCCGTGA-3', 14%). Minor products (< 5%) resulted from the misinsertion of Gua (5'-pTCGGTGA-3') and Thy (5'-pTCTGTGA-3'). Four extension products were characterized from the bypass of the 1, N^2 - ϵ -dGuo lesion in this sequence. The major products were error-free bypass (~33%) and a one-base deletion (24%). The two minor products resulted from the misinsertion of Ade (~8%) and Gua (~3%). The product derived from the misinsertion of Thy was below the limit of MS detection and not observed.

Quantitation of the extension products by MS was based on calibration curves developed for the response of a single extension product analyte versus a 13mer standard.

Chromatographic separation of the extension products was not achieved. Quantitation errors were therefore introduced since the ionization efficiency of the co-eluting analytes is expected to be different than that of a single component. A sample containing known quantities of the extension products from the 5'-CXXG-3' template and the 13mer standard was analyzed by LC-ESI-MS-MS to estimate the quantitation error. The quantitation was generally within 10% error (36).

Characterization of the 7-(2-Oxoheptyl)- ϵ -dGuo Adduct in Duplex DNA Opposite dCyd

The UV thermal melting temperatures (T_m) of the 5'-CGCATXXGAATCC-3' (9) • 5'-GGATTCCATGCG-3' duplexes where X is the 7-(2-oxoheptyl)- ϵ -dGuo, 1, N^2 - ϵ -dGuo, and dGuo at pH 5.2, 7.0, and 8.6 are listed in Table 3. The local sequence of the modified base is related to oligonucleotide 7. The presence of the 7-(2-oxoheptyl)- ϵ -dGuo and 1, N^2 - ϵ -dGuo lesions lowered the T_m of the duplex by 13 – 20 °C compared to the unmodified duplex. The 7-(2-oxoheptyl)- ϵ -dGuo was more destabilizing than the 1, N^2 - ϵ -dGuo lesions by 1-7 °C depending on the pH. The T_m of the unmodified duplex (52 – 53 °C) and 7-(2-oxoheptyl)- ϵ -dGuo containing duplex (33 – 34 °C) were not sensitive to pH. The T_m of the duplex containing the 1, N^2 - ϵ -dGuo lesion was 4 – 5° C less stable at basic pH (35 °C) than under acidic (40 °C) or neutral (39 °C) conditions. The 7-(2-oxoheptyl)- ϵ -dGuo lesion decreased the thermal stability of the duplex at acidic and neutral pH compared to that of 1, N^2 - ϵ -dGuo lesion (ΔT_m 7 °C and 5 °C, respectively). Interestingly, the T_m of the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex remained when the base opposite the lesion was Cyt, Ade, or a one-base deletion. This is in contrast to the 1, N^2 - ϵ -dGuo containing duplex, which is slightly more stable when Ade or a one-base lesion was opposite the lesion.

Temperature dependent one-dimensional proton NMR spectra of the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex showed that all the imino proton resonances were broadened between 40-45 °C under acidic and neutral pH, whereas all the imino resonances were broadened between 35-40 °C under basic pH (Figure S25 of the Supporting Information). These results suggest that the neighboring base pairing to 7-(2-oxoheptyl)- ϵ -dGuo was less stable at neutral and acidic pH compared to basic pH.

NMR Analysis of 7-(2-Oxoheptyl)- ϵ -dGuo Opposite dCyd in Duplex DNA

At 5° C, the far downfield region of the proton NMR spectra of the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex obtained either at pH 7 or pH 8.6 appeared similar (Figure 6). The 7-(2-oxoheptyl)- ϵ -dGuo base lacks an imino proton due to its alkylation at the N8 position (this is N1 in guanine numbering). The imino proton resonances of the unmodified Thy and Gua bases were assigned from NOESY spectra (*vide infra*). The imino resonance of the 5'-neighbor base pair T⁵:A²⁰ was not observed, and that of the 3'-neighbor base pair G⁷:C¹⁸ was broad, indicating increased exchange with water (Figure S25 in the Supporting Information). At pH 7, all of the imino proton resonances broadened between 40 – 45 °C, whereas at pH 8.6 all of the imino proton resonances broadened between 35 – 40 °C. The lack of dependency of the T_m of the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex on pH, which was also observed for the unmodified duplex, was attributed to base-catalyzed exchange in the presence of buffer (Figure S26 in the Supporting Information). Figure 6 shows contour plots of expanded regions of the NOESY spectrum showing NOE connectivities between the imino protons (bottom) and between the imino and amino or Ade H2 (top) protons. At pH 7 and pH 8.6, the expected pattern of NOE cross-peaks were observed for the G²:C²³, C³:G²², and A⁴:T²¹ base pairs, located in the 5' direction from the 7-(2-oxoheptyl)- ϵ -dGuo adduct, except between A⁴:T²¹ and T⁵:A²⁰ (Figure 7A-C). Likewise, NOE connectivities were observed between base pairs G⁷:C¹⁸, A⁸:T¹⁷, A⁹:T¹⁶, T¹⁰:A¹⁵, and C¹¹:G¹⁴, located in the 3'-direction from the 7-(2-oxoheptyl)- ϵ -dGuo adduct. The N5H amino proton of 7-(2-oxoheptyl)- ϵ -dGuo was not observed, presumably because of

exchange. Other aspects of the NOESY spectra of the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex acquired at pH 7.0 and pH 8.6 were also similar (Figure 7B and 7C). Sequential nucleotide assignments were made using standard protocols (44,45). The sequential NOE connectivity in the modified strand was observed from C¹ through T⁵ H1'. No NOEs between 7-(2-oxoheptyl)- ϵ -dGuo (X⁶) and its 3' neighbor G⁷ were observed, indicative of disorder. The entire sequential NOE connectivity for the complementary strand was observed from G¹³→G²⁴. However, the C¹⁸ H1'→C¹⁹ H6 (observed at pH 8.6), C¹⁹ H6→C¹⁹ H1', and C¹⁹ H1'→A²⁰ H8 NOEs were weak. The C¹⁹ H6→C¹⁹ H5 cross-peak was broad. The heptyl chain proton resonances were assigned at neutral and basic pH values. No NOEs arising between the heptyl protons and DNA were observed.

The proton NMR spectrum of the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex differed at 5 °C and pH 5.2. The G⁷ N1H proton resonance remained broadened (Figure S25 in the Supporting Information). Additional line broadening involving the imino proton resonances at base pairs A⁸:T¹⁷ and A⁹:T¹⁶ was attributed to increased exchange with water at these two base pairs at the lower pH value (Figure S25 in the Supporting Information). The NOE connectivity between the G⁷•C¹⁸ and A⁸•T¹⁷ base pairs was missing. (Figure 6, labeled as G⁷). Very weak NOE cross-peaks were observed between the G⁷ N1H and the amino protons from the complementary Cyt (C¹⁸). The resonances for the C¹⁹ amino protons were not observed, suggesting rapid exchange with water. The anticipated pattern of sequential base aromatic proton to deoxyribose H1' NOEs was identified from nucleotide C¹ through nucleotide A⁴ (Figure 7A). The A⁴ H1'→T⁵ H6, and T⁵ H6→T⁵ H1' NOEs were weak and not observed at the threshold plotted in Figure 7A. The anticipated NOEs involving the lesion, T⁵ H1'→X⁶ H2 (H2 is the imidazole proton of 7-(2-oxoheptyl)- ϵ -dG, see Figure 1 for atom numbering), X⁶ H2→X⁶ H1', and X⁶ H1'→G⁷ H8, were missing and the NOE connectivity was interrupted. The G⁷ H8→G⁷ H1' NOE was weak. Proceeding in the 3'-direction from the G⁷ H8→G⁷ H1' NOE, the NOESY connectivities continued uninterrupted to the 3'-terminus of the modified strand. The NOE connectivities for the complementary strand were continuous from G¹³ through C¹⁹, which is the complementary nucleotide to the lesion X⁶ (Figure 7A). However, the C¹⁹ resonances were broad. The C¹⁹ H6→C¹⁹ H1' and C¹⁹ H1'→A²⁰ H8 NOEs were missing. Proceeding in the 3'-direction from the A²⁰ H8→A²⁰ H1' NOE, the NOESY connectivities continued uninterrupted to the 3'-terminus of the modified strand. The pattern of the NOEs between the base aromatic protons and the deoxyribose H2' and H2'' protons was similar to that observed for the NOEs between the aromatic protons and deoxyribose H1' protons, in both the modified and complementary strands (data not shown). Due to the line broadening, it was not possible to assign the heptyl chain proton resonances at acidic pH.

Discussion

Sulfolobus solfataricus P2 DNA polymerase IV (Dpo4) is a member of the Din B family of DNA polymerases. Din B polymerases are found in all kingdoms of life and include *Escherichia coli* pol IV and human pol κ . The specialized function of Din B polymerases is to replicate through damaged DNA (46,47). Dpo4 was noted to have lesion bypass properties similar to human pol η , which is part of the Rad30 family of lesion bypass polymerases (48). Recombinant Dpo4 can be expressed at high levels in *E. coli* and has been crystallized with DNA substrates containing the 1,N²- ϵ -dGuo (35), 8-oxo-dGuo (49-52), *cis-syn* thymine dimer (53), O⁶-alkyl-dGuo (54,55), the tetrahydrofuran analogue of an abasic site (56), a malondialdehyde adduct of dGuo (M₁dGuo) (57), and N²-dGuo and N⁶-dAdo adducts of benzo[a]pyrene diol epoxide (BPDE) adducts (58,59).

We reported the in vitro replication of 1,N²- ϵ -dGuo by the Dpo4 (35). The misincorporation profile was dependent on the 5'-neighboring base relative to the lesion. Replication of 1,N²-

ϵ -dGuo by Dpo4 resulted in largely a one-base deletion when a dCyd was 5' to the modified base (template **8**); a minor product derived from misincorporation of dATP was also observed. In the current work, we identified two additional products from the bypass of the 1, N^2 - ϵ -dGuo adduct in the 5'-C**X**G-3' template (**8**) by Dpo4. These were derived from the misinsertion of Gua (5'-pTC**G**GTGA-3', 3%) and error-free bypass (5'-pTC**C**GTGA-3', 33%). Replication of the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the same sequence resulted in five extension products. In addition to the same four products from the bypass of the 1, N^2 - ϵ -dGuo lesion, a minor product identified as the misinsertion of Thy resulted from the replication of the 7-(2-oxoheptyl)- ϵ -dGuo adduct (5'-pTC**T**GTGA-3', 4%). The overall yield for the bypass of the two etheno lesions was very similar in the 5'-C**X**G-3' template sequence, but the distribution of products differed. Replication of the 7-(2-oxoheptyl)- ϵ -dGuo lesion resulted in a lower frequency of the error-free product than did 1, N^2 - ϵ -dGuo (14% vs 33%), and a significantly larger proportion of the product distribution resulted from misinsertion of Ade (26% vs. 8%). Replication of both etheno lesions by Dpo4 resulted in a one-base deletion product.

Replication of the 1, N^2 - ϵ -dGuo and 7-(2-oxoheptyl)- ϵ -dGuo lesions in the 5'-T**X**G-3' template sequence (**7**) resulted in the same seven extension products and the total yield of extension products was similar for the two adducts. Replication of the etheno adducts was considerably more error-prone in the 5'-T**X**G-3' template than the 5'-C**X**G-3' sequence with <5% of the extension products resulting from error-free bypass. The major extension products from bypass of both etheno lesions in the 5'-T**X**G-3' template (**7**) were derived from a one-base deletion (5'-pTC-ATGA-3' and 5'-pTC-ATG-3') and misinsertion of Ade (5'-pTC**A**ATGA-3'). In the case of the 1, N^2 - ϵ -dGuo lesion, the one-base deletion product predominated by ~2:1 ratio, whereas bypass of the 7-(2-oxoheptyl)- ϵ -dGuo lesion resulted in a higher proportion of misinsertion of Ade (~1:1.5); this same trend was also observed for the 5'-C**X**G-3' sequence. Two additional minor products were identified (< 5%), which arise from the misinsertion of Gua (5'-TC**G**ATGA-3') and error-free bypass (5'-pTC**C**ATGA-3').

The catalytic efficiency for dATP insertion opposite the 7-(2-oxoheptyl)- ϵ -dGuo lesions of the 5'-T**X**G-3' template was 16 – 40 fold higher than the other dNTP. The mechanism can either involve the instructive insertion of dATP opposite the Thy situated on the 5'-side of the lesion through a dNTP-stabilized misincorporation mechanism (35,60,61) or the non-instructive insertion of dATP opposite the 7-(2-oxoheptyl)- ϵ -Gua (Scheme 2). Extension of the misaligned or aligned primer-template complex leads to the one-base deletion or misinsertion of Ade products, respectively. It is possible that the aligned and misaligned primer-template can interconvert. The catalytic efficiency for dATP insertion opposite the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-C**X**G-3' template was slightly higher than the 5'-T**X**G-3' sequence. The modest sensitivity to the 5'-neighboring base (Thy vs Cyt) suggests a non-instructive insertion of dATP opposite the 7-(2-oxoheptyl)- ϵ -dGuo lesion, since it is unlikely that dATP will template with the 5'-Cyt of the 5'-C**X**G-3' sequence. This indicates that the one-base deletion product may arise from a misincorporation–misalignment mechanism for the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-T**X**G-3' template (60).

Support for the dNTP-stabilized misincorporation mechanism come from the crystallographic analysis of the ternary complex of Dpo4 bound to the 1, N^2 - ϵ -dGuo containing template paired with a -1 primer, which showed dATP involved in nascent Watson-Crick pairing with the 5'-Thy (35); a related structure was also reported for the 5'-C**X**G-3' template and dGTP. The template strand was relatively unperturbed and the 1, N^2 - ϵ -Gua provided an important π -stacking platform for the incoming dNTP in both structures (60). The post-insertion complex of the 5'-T**X**G-3' template showed the Ade at the primer terminus was paired with the 5'-Thy; once again the 1, N^2 - ϵ -Gua was well stacked and

stabilized the one-base deletion product. Similar crystal structures were reported for the major malondaldehyde adduct of dGuo (M_1 dGuo) bound to Dpo4 in the same sequence contexts (57). The catalytic efficiency for dATP insertion opposite the $1,N^2$ - ϵ -dGuo and M_1 dGuo by Dpo4 were 4.0 and 2.5 higher for the 5'-TXXG-3' template than the 5'-CXXG-3' template, respectively; K_m values that were 8- and 4-fold higher for the 5'-CXXG-3' were the major contribution to the differences. Insertion of dGTP was most efficient for the $1,N^2$ - ϵ -dGuo lesion in the 5'-CXXG-3'. Insertion of dGTP opposite the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-CXXG-3' template was 4 fold less efficient than misinsertion of dATP; however, the one-base deletion product (5'-pTC-GTGA-3') was nearly as abundant as the misinsertion of Ade (5'-pTCAGTGA-3') indicating that extension of misaligned primer with the Gua terminus paired with the 5'-Cyt was very favorable. These results support a dNTP-stabilized misincorporation mechanism for the one-base deletion product involving instructive insertion of the dNTP opposite the 5'-base to the lesion (60).

The Streisinger slippage models represents a third possible mechanism leading to the one-base deletion product. The misalignment of the template and primer is caused by the extrusion of the modified base into an extra-helical loop and this model is often applied to deletions in repetitive sequence. NMR structures of DNA duplexes containing C8-aromatic amines and N^2 -BPDE-dGuo show that intercalation of the adduct moiety into the DNA stabilizes the slippage intermediate (62,63). Crystal structures of the Dpo4 bound to DNA templates containing the non-coding the tetrahydrofuran model of an abasic site and the N^2 -BPDE-dGuo adduct show the modified nucleotide in an extrahelical loop resulted in misalignment of the template and primer (56,59). In the case of C8-aromatic amines, the intercalation of the adduct into the base stack stabilizes the slippage intermediate when situated in a repeat sequence. This feature was not present in the structure of Dpo4•BPDE-adduct structures.

Nearly 90% of the extension products that were characterized from the bypass of the 7-(2-oxoheptyl)- ϵ -dGuo and 80% from the $1,N^2$ - ϵ -dGuo lesion in the 5'-TXXG-3' template (7) were derived from an initial insertion of dATP (one-base deletion or misinsertion of Ade). This result indicates that Dpo4 can efficiently extend either the aligned or misaligned primer in which Ade was opposite the lesion or the 5'-Thy. Misinsertion of Ade was preferred over the one-base deletion product (35% vs 24%) for bypass of the 7-(2-oxoheptyl)- ϵ -dGuo lesion, whereas the preference was reversed for the $1,N^2$ - ϵ -dGuo lesion (16% vs 31%). This result may reflect disrupted stacking due to the disordered conformation of the 7-(2-oxoheptyl)- ϵ -Gua base making the one-base deletion pathway less favorable. Interestingly, the T_m values for the 7-(2-oxoheptyl)- ϵ -dGuo duplex (9) were insensitive to the nature of the complementary base (Cyt, Ade, or a one-base deletion), suggesting that the 7-(2-oxoheptyl)- ϵ -Gua base may be disordered in all three duplexes. This result is in contrast to the same duplex containing the $1,N^2$ - ϵ -Gua base, in which the T_m was 6° C higher when opposite a one-base deletion and 4° C higher when paired with Ade as compared to $1,N^2$ - ϵ -Gua•Cyt pair (Table 3). A full structure of the $1,N^2$ - ϵ -Gua•Ade duplex is currently in progress.

The catalytic efficiency for insertion of dCTP, dTTP, and dGTP opposite the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-TXXG-3' template (7) was 32-, 16-, and 41-fold lower than dATP insertion and this difference is reflected in the distribution of the extension products. Insertion of dCTP opposite either etheno lesion was the least efficient in both sequences and the 5'-neighboring base had a minor influence in that relative efficiencies. Interestingly, the error free extension product was observed in 14% and 33% in the CXXG-3' template indicating that extension from the ϵ -Gua•Cyt template-primer terminus by Dpo4 was efficient in this sequence. A possible explanation is that during translesion synthesis the ϵ -Gua is involved in a Hoogsteen pairing interaction with the complementary Cyt, as observed for $1,N^2$ - ϵ -dGuo in duplex DNA (38).

We recently reported the structure of 1,*N*²- ϵ -dGuo lesion in the same duplex, 5'-(CGCAT $\underline{\text{X}}$ GAATCC)-3'•5'-(GGATTCCATGCG)-3' (where X= 1,*N*²- ϵ -dGuo). We established that the 1,*N*²- ϵ -dGua base existed as a mixture of *anti* and *syn* conformations about the glycosyl bond in the 5'-T $\underline{\text{X}}$ G-3' (**9**) sequence context at neutral pH (37,38). Increasing the pH to 8.6 shifts the conformational equilibrium toward the *anti* conformation (37), whereas decreasing the pH to 5.2 shifts the equilibrium toward the *syn* conformation, accompanied by the formation of a Hoogsteen base pair between 1,*N*²- ϵ -dGuo and the complementary protonated cytosine (38). A similar conformational transition was observed for 1,*N*²- ϵ -dGuo lesion in the 5'-C $\underline{\text{X}}$ C-3' sequence context in DNA (64).

The NMR spectra of duplexes containing the 7-(2-oxoheptyl)- ϵ -dGuo lesion also depend upon pH, indicative of a pH-mediated conformational equilibrium. The likely explanation is a *syn-anti* conformational equilibrium involving the glycosyl bond, similar to that observed for 1,*N*²- ϵ -dGuo, although this cannot be established with certainty. However, the *pK_a* is reduced as compared to the duplex containing the 1,*N*²- ϵ -dGuo lesion, which exhibited a *pK_a* value near neutrality. As detailed in Table 3, the *T_m* of the 1,*N*²- ϵ -dGuo adduct in the same sequence increased at pH 5.2 concomitant with the stabilization of a Hoogsteen-like base pair under acidic conditions. In the present case, the spectra are similar at pH 7 and pH 8.6, but differ at pH 5.2, suggesting that for the duplex containing the 7-(2-oxoheptyl)- ϵ -dGuo lesion the *pK_a* of the transition is shifted to the more acidic pH. This probably accounts for the observation that the *T_m* of 7-(2-oxoheptyl)- ϵ -dGuo containing duplex remains constant over the range of pH 5.2 to pH 8.6. Unfortunately, the 7-(2-oxoheptyl)- ϵ -dGuo lesion remains in a disordered conformation throughout the pH range from pH 5.2 – 8.6. The structural disorder likely involves thermodynamic destabilization attributed to the hydrophobic heptyl side chain at the lesion site. This is consistent with thermal melting studies showing that the 7-(2-oxoheptyl)- ϵ -dGuo containing duplex is less stable than the duplex containing the 1,*N*²- ϵ -dGuo lesion.

Conclusions

Oligonucleotides containing the 7-(2-oxoheptyl)- ϵ -dGuo lesion, an etheno adduct uniquely derived from lipid peroxidation, have been synthesized using the phosphoramidite approach. Translesion synthesis of the 7-(2-oxoheptyl)- ϵ -dGuo lesion by Dpo4 in 5'-T $\underline{\text{X}}$ G-3' (**7**) and 5'-C $\underline{\text{X}}$ G-3' (**8**) local sequence contexts was examined and compared to 1,*N*²- ϵ -dGuo. The extension products were sequenced by tandem mass spectrometry using an improved protocol that enhances sensitivity. This approach allowed us to identify extension products from the bypass of the 1,*N*²- ϵ -dGuo lesion that were not previously observed, as well as revise the sequence of one of the extension products. The major extension products for the 7-(2-oxoheptyl)- ϵ -dGuo adduct was misinsertion of Ade followed by a one-base deletion, whereas replication of the 1,*N*²- ϵ -dGuo lesion resulted in more of the one-base deletion products. The 7-(2-oxoheptyl)- ϵ -dGuo adduct was more miscoding than the 1,*N*²- ϵ -dGuo; replication of the lesions in the 5'-T $\underline{\text{X}}$ G-3' (**8**) sequence was more miscoding than in the 5'-C $\underline{\text{X}}$ G-3' (**7**) sequence. The one-base deletion product arises from either a dNTP-stabilized misincorporation or misincorporation–misalignment mechanism and may be different depending on the lesion. NMR analysis indicated that the structure of the 7-(2-oxoheptyl)- ϵ -dGuo lesion in DNA is dependent upon pH. In the 5'-T $\underline{\text{X}}$ G-3' (**9**) sequence context it showed similar spectra at pH 7 and pH 8.6, as compared to pH 5.2, but the adduct remained disordered across this pH range. The source of the pH-dependent conformational transition remains to be determined, but likely involves interconversion between *syn* and *anti* conformations about the glycosyl bond, similar to the duplex containing the 1,*N*²- ϵ -dGuo lesion, but with a lower *pK_a* value. This may contribute to the differences in the translesion synthesis between the 7-(2-oxoheptyl)- ϵ -dGuo and 1,*N*²- ϵ -dGuo lesions by the Dpo4 polymerase.

Supplementary Material

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Abbreviations

7-(2-oxoheptyl)-ϵ-dGuo	3-(2-deoxy- β -D- <i>erythro</i> -pentofuranosyl)-2,4-dihydro-7-(2-oxoheptyl)-9 <i>H</i> -imidazo[1,2- <i>a</i>]purin-9-one
7-(2-oxoheptyl)-ϵ-dAdo	3-(2-deoxy- β -D- <i>erythro</i> -pentofuranosyl)-3-(2-oxoheptyl)-3 <i>H</i> -imidazo[2,1- <i>i</i>]-purine
3-(2-oxoheptyl)-ϵ-dCyd	6-(2-deoxy- β -D- <i>erythro</i> -pentofuranosyl)-3-(2-oxoheptyl)-imidazo[1,2- <i>c</i>]pyrimidin-5(6 <i>H</i>)-one
7-(2-oxoheptyl)-ϵ-Guo	3-(β -D- <i>erythro</i> -pentofuranosyl)-2,4-dihydro-7-(2-oxoheptyl)-9 <i>H</i> -imidazo[1,2- <i>a</i>]purin-9-one
ϵ	etheno
M₁dGuo	3-(2-deoxy- β -D- <i>erythro</i> -pentofuranosyl)pyrimido[1,2- <i>a</i>]purin-10(3 <i>H</i>)-one
ONE	4-oxo-2-nonenal

UDG	uracil DNA glycosylase
DMTr	dimethoxytrityl
pol	DNA polymerase
CID	collision induced dissociation
UPLC	ultra performance liquid chromatography
ESI	electrospray ionization
MALDI-TOF-MS	matrix assisted laser desorption ionization time-of-flight mass spectrometry
T_m	thermal melting temperature
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser enhancement spectroscopy

Figure 1.
Structures of the nucleoside adducts of 4-oxo-2-nonenal (1-3).

Figure 2. Full-length extension of the 7-(2-oxoheptyl)-ε-dGuo lesion in sequences **7** (top) and **8** (bottom). Reaction times were 30 min for the modified oligonucleotides (X = 7-(2-oxoheptyl)-ε-dGuo) and 5 min for the unmodified control (X = dGuo).

Figure 3.

Full scan product ion mass spectra for the bypass and extension of the 7-(2-oxoheptyl)- ϵ -dGuo lesion in the 5'-TXXG-3' (7, top) and 5'-CXXG-3' (8, bottom) templates. Sequence assignments are listed in Table 2. The CID mass spectra of each extension product can be found in the Supporting Information (Figures S10-S22).

Figure 4.

Predicted CID fragment $[M-H]^-$ ions for the 5'-pTCTATGA-3' and 5'-pTCATTGA-3' extension products (m/z 1086.2, $[M-2H]^{-2}$). The experimental CID spectra are found in Figure S14 of the Supporting Information.

Figure 5.
Predicted CID fragment $[M-H]^-$ ions for the 5'-pTC-ATG-3' and 5'-pTC-TGA-3' extension products (m/z 777.3, $[M-2H]^{-2}$)

Figure 6.

^1H NOESY spectra showing the NOE connectivity from anomeric H1' to 3'-neighbor aromatic protons of the 7-(2-oxoheptyl)- ϵ -dGuo•dC duplex at A) pH 5.2, B) 7.0, and C) 8.6 (20 °C). Asterisks indicate the cytosine H5-H6 cross-peaks. The dotted line indicates that the NOE cross peak between C¹⁸ and C¹⁹ was observed at lower threshold level.

Figure 7.

^1H NOESY spectra showing the sequential NOE connectivity between the imino protons (bottom) and NOEs between imino and the amino and base protons (Top) of the 7-(2-oxoheptyl)- ϵ -dGuo•dCyt duplex at A) pH 5.2, B) pH 7.0, and C) pH 8.6. Labeled peaks are a, T¹⁷ N3H→A⁹ H2; b, T¹⁶ N3H→A⁸ H2; c, T¹⁰ N3H→A¹⁵ H2; d, T²¹ N3H→A⁴ H2; e,e', G² N1H→C²³ N⁴H, h/n; f,f', G¹⁴ N1H→C¹¹ N⁴H, h/n; g,g', G²² N1H→C³ N⁴H, h/n; h,h', G⁷ N1H→C¹⁸ N⁴H, h/n. The symbols h and n stand for hydrogen bonded and non-hydrogen bonded amino protons of cytosine.

Scheme 1.

Scheme 2.

Steady-state kinetic parameters for single-nucleotide incorporation opposite the 7-(2-oxoheptyl)- ϵ -dGuo adduct in oligonucleotides **7** and **8** by Dpo4

Table 1

Template	dNTP	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\cdot\text{min}^{-1}$)	f^a
5'---T-[7-(2-oxoheptyl)- ϵ -dGuo]-G---3' (7)	C	85 $\pm$ 12	0.010 $\pm$ 0.004	1.2 $\times 10^{-4}$	1.0
	T	62 $\pm$ 10	0.014 $\pm$ 0.09	2.3 $\times 10^{-4}$	1.9
	G	120 $\pm$ 23	0.011 $\pm$ 0.001	9.2 $\times 10^{-5}$	0.8
	A	7.6 $\pm$ 1.2	0.029 $\pm$ 0.008	3.8 $\times 10^{-3}$	32
	C	4.7 $\pm$ 0.4	6.4 $\pm$ 0.13	1.4	1.0
5'---T-G-G---3'	T	22 $\pm$ 4	0.069 $\pm$ 0.001	3.1 $\times 10^{-3}$	2.2 $\times 10^{-3}$
	G	80 $\pm$ 17	0.042 $\pm$ 0.003	5.3 $\times 10^{-4}$	3.7 $\times 10^{-4}$
	A	33 $\pm$ 8	0.12 $\pm$ 0.01	3.6 $\times 10^{-3}$	2.6 $\times 10^{-3}$
	C	135 $\pm$ 25	0.043 $\pm$ 0.002	3.2 $\times 10^{-4}$	1.0
	T	18 $\pm$ 3	0.025 $\pm$ 0.001	1.4 $\times 10^{-3}$	4.4
5'---C-[7-(2-oxoheptyl)- ϵ -dGuo]-G---3' (8)	G	35 $\pm$ 8	0.050 $\pm$ 0.003	1.4 $\times 10^{-3}$	4.4
	A	16 $\pm$ 3	0.090 $\pm$ 0.006	5.6 $\times 10^{-3}$	18
	C	0.028 $\pm$ 0.003	0.053 $\pm$ 0.02	19	1.0
	T	61 $\pm$ 9	0.099 $\pm$ 0.003	1.6 $\times 10^{-3}$	8.4 $\times 10^{-5}$
	G	78 $\pm$ 6	0.053 $\pm$ 0.001	6.8 $\times 10^{-4}$	3.6 $\times 10^{-5}$
5'---C-G-G---3'	A	230 $\pm$ 42	0.090 $\pm$ 0.006	3.9 $\times 10^{-4}$	2.1 $\times 10^{-5}$

^a f = misincorporation frequency = $(k_{cat}/K_m)_{\text{incorrect dNTP}} / (k_{cat}/K_m)_{\text{correct dNTP}}$ (dCTP)

Table 2

Summary of the bypass and extension of the 7-(2-oxoheptyl)- ϵ -dGuo and ϵ -dGuo lesions in 5'-TXG-3' (**7**) and 5'-CXG-3' (**8**) templates by Dpo4 as determined by LC-ESI-MS-MS sequencing

Extension products and their observed masses ([M-2H] ⁻² and [M-3H] ⁻³)	<u>X</u> = 7-(2-oxoheptyl)- ϵ -dGuo	<u>X</u> = 1,N ² - ϵ -dGuo	comments
3' ----- AG <u>X</u> TACT-5' (template 7)			
5'-pTCCATGA-3' (<i>m/z</i> 1078.8, 719.0)	2 %	4 %	error-free
5'-pTCTATGA-3' + 5'-pTCATTGA-3' (<i>m/z</i> 1086.2, 723.5)	4 %	4 %	misinsertion of Thy tandem misinsertion
5'-pTCGATGA-3' (<i>m/z</i> 1099.4, 731.3)	1 %	3 %	misinsertion of Gua
5'-pTCATGA-3' (<i>m/z</i> 1090.2, 727.0)	35 %	16 %	misinsertion of Ade
5'-pTC-ATGA-3' (<i>m/z</i> 933.1, 622.5)	20 %	17 %	one-base deletion
5'-pTC-ATG- -3' (<i>m/z</i> 777.3, 518.1)	4 %	14 %	one-base deletion
3' ----- AG <u>X</u> CACT-5' (template 8)			
5'-pTCCGTGA-3' (<i>m/z</i> 1086.6, 724.1)	14 %	33 %	error-free
5'-pTCTGTGA-3' (<i>m/z</i> 1094.5, 729.1)	4 %		misinsertion of Thy
5'-pTCGGTGA-3' (<i>m/z</i> 1106.4, 737.8)	1 %	3 %	misinsertion of Gua
5'-pTC-GTGA-3' (<i>m/z</i> 941.8, 627.8)	20 %	24 %	one-base deletion
5'-pTCAGTGA-3' (<i>m/z</i> 1098.3, 732.1)	26 %	8 %	misinsertion of Ade

Table 3

Melting temperatures (T_m , expressed in °C) of duplex DNA containing the 7-(2-oxoheptyl)- ϵ -dGuo and 1, N^2 - ϵ -dGuo lesions, and the unmodified dGuo in the 5'-CGCATXGAATCC-3' (**9**) • 5'-GGATTCTYATGCG-3' DNA duplexes at acidic, neutral, and basic pH values (10 mM NaHPO₄, 100 mM NaCl, 5 μ M Na₂EDTA)

lesion (X)	Y	pH 5.2	pH 7.0	pH 8.6
7-(2-oxoheptyl)- ϵ -Gua	C	33±1	34±1	34±1
	A		34±1	
	–		34±1	
1, N^2 - ϵ -Gua ^a	C	40±1	39±1	35±1
	A		43±1	
	–		45±1	
Gua ^a	C	53±1	53±1	52±1

^aThe melting temperatures were previously reported (37,38).