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Site Specific Synthesis and Polymerase Bypass of Oligonucleotides Containing a 6-Hydroxy-3,5,6,7-tetrahydro-9H-imidazo[1,2-a]purin-9-one Base, an Intermediate in the Formation of 1N²-Etheno-2'-deoxyguanosine

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Abstract

The reaction of DNA with certain bis-electrophiles such as chlorooxirane and chloroacetaldehyde produces etheno adducts. These lesions are highly miscoding, and some of the chemical agents that produce them have been shown to be carcinogenic in laboratory animals and in humans. An intermediate in the formation of 1,N²-ethenoguanine is 6-hydroxy-3,5,6,7-tetrahydro-9H-imidazo[1,2-a]purin-9-one (6-hydroxyethanoguanine), which undergoes conversion to the etheno adduct. The chemical properties and miscoding potential of the hydroxyethano adduct have not been previously studied. A synthesis of the hydroxyethano-adducted nucleoside was developed, and it was site specifically incorporated into oligonucleotides. This adduct had a half-life of between 24 and 48 h at neutral pH and 25 °C at the nucleoside and oligonucleotide levels. The miscoding potential of the hydroxyethano adduct was examined by primer extension reactions with the DNA polymerases Dpo4 and pol T7[−], and the results were compared to the corresponding etheno-adducted oligonucleotide. Dpo4 preferentially incorporated dATP opposite the hydroxyethano adduct and dGTP opposite the etheno adduct; pol T7[−] preferentially incorporated dATP opposite the etheno adduct while dGTP and dATP were incorporated opposite the hydroxyethano adduct with nearly equal catalytic efficiencies. Collectively, these results indicate that the hydroxyethano adduct has a sufficient lifetime and miscoding properties to contribute to the mutagenic spectrum of chlorooxirane and related genotoxic species.

Introduction

In the early 1970s, epidemiological studies revealed a correlation between a unique tumor, hepatic angiosarcoma, and vinyl chloride exposure (1–3). Confirmatory evidence was obtained from animal studies (2), and it is now incontrovertible that vinyl chloride is a human carcinogen.

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Supporting Information **Available:** Copies of ¹H, ¹³C, COSY, and HMBC NMR spectra, MALDI-TOF mass spectra, CGE traces, HPLC traces of enzymatic digestions, and raw data from steady state kinetics experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Vinyl chloride (**1**) shares its mechanism of action with other vinyl monomers, e.g., acrylonitrile and vinyl carbamate, and requires metabolic activation prior to reaction with DNA (Scheme 1) (4–7). The DNA modifying species is the corresponding epoxide (**2**) (**8**). Chlorooxirane rapidly rearranges to chloroacetaldehyde (**3**), which can also react with DNA. However, several lines of evidence point to the chlorooxirane being the main source of the observed point mutations (5, 9). Nevertheless, much of the study of oxoethyl damage to DNA has been done with chloroacetaldehyde rather than with chlorooxirane.

Chlorooxirane and related genotoxins react with DNA bases at the N1-, N²-, N3-, and N7-positions of dGuo, the N1-position of dAdo, and the N3-position of dCyd to give the corresponding 2-oxoethyl adducts (**10**). With the exception of the N7-Gua adduct (**9**), the oxoethyl adducts are not directly observed because they undergo immediate cyclization with an adjacent nitrogen to give hydroxyethano derivatives. The hydroxyethano adducts derived from the initial reaction at a N1-Ade, N3-Cyt, and N1- and N3-Gua dehydrate readily to imines (or iminium ions) that, in turn, tautomerize to the 1,N⁶-etheno-Ade (**4**), 3,N⁴-etheno-Cyt (**5**), and N²,3- and 1,N²-etheno-Gua (**6** and **7**) adducts, respectively (Figure 1). The sequence is illustrated in Scheme 2 for the formation of 1,N²-etheno-Gua, and the other etheno adducts are formed similarly. The product resulting from initial reaction at the N²-position of dGuo and subsequent cyclization onto N1,7-hydroxy-3,5,6,7-tetrahydro-9H-imidazo[1,2-*a*]purin-9-one (**8**) is highly resistant to dehydration to the 1,N²-etheno derivative (**10**).

Sensitive assays have been developed that make it possible to study adduct burden in humans and experimental animals (11, 12). These studies show that exposure to vinyl chloride leads to formation of etheno adducts. In addition, background levels of etheno adducts have been found in unexposed populations (13). The endogenous C₂ donors are believed to arise from oxidative damage to unsaturated fatty acids, namely, 2,3-epoxy-aldehydes, although other lipid peroxidation products have also been shown to form etheno adducts (14–19).

The mechanism by which etheno adducts are formed has been studied by several laboratories including those of Wiewiorowski (20–22), Singer (23), and Bartsch (24). Hydroxyethano derivatives of Cyt and Ade accumulate during the reaction of chloroacetaldehyde with nucleosides and with nucleic acids and are gradually converted to the etheno derivatives over time. The hydroxyethano derivative of Cyt is more stable than that of Ade (*t*_{1/2} = 13 vs 1.4 h, both at pH 7.25 and 37 °C) (25). The dehydration/tautomerization reactions are acid-catalyzed; Kusmierek and Singer (26) examined the hydroxyethano derivatives in poly(Ado) and poly(Cyd) and found that their stability was enhanced at pH 8.1. No evidence was found for base-catalyzed formation of etheno derivatives or decomposition of the hydroxyethano species.

Syntheses of oligodeoxynucleotides containing the etheno adducts have allowed their mutagenicity to be evaluated (4, 27–29). Some of the etheno adducts are strongly miscoding, in contrast to the N7-(2-oxoethyl) adduct of Gua, which was not miscoding in one report (30). The point substitutions observed in vinyl chloride-induced tumors are consistent with the etheno adducts being the initiating lesions (24). Site specific mutagenesis of the 7-hydroxy-3,5,6,7-tetrahydro-9H-imidazo[1,2-*a*]purin-9-one (**8**) adduct has been reported, and this adduct had modest miscoding potential in COS-7 cells and was weakly mutagenic in bacteria (31, 32).

Although etheno adducts can explain the point mutations seen in cells of exposed animals, examinations of other types of DNA damage raise questions as to whether the etheno adducts are the only genotoxic lesions formed by chlorooxirane and related electrophiles. In

particular, the hydroxyethano intermediates are likely to have sufficient lifetime to cause replication errors apart from those of the etheno adducts. Kusmierek and Singer (25, 33) have prepared an oligonucleotide containing the hydroxyethano adduct of Cyt; this adduct showed differences in the relative proportions of point mutations relative to those of the etheno species; that is, the hydroxyethano adduct caused misincorporation of Ado > Urd >> Cyd during transcription, whereas the effect of ethenocytosine was Urd > Ado >> Cyd. A confounding factor was that they were unable to prepare DNA containing the adduct free of ethenocytosine.

To our knowledge, the hydroxyethano adducts of dGuo, 6-hydroxy-3,5,6,7-tetrahydro-9*H*-imidazo[1,2-*a*]purin-9-one base (HO-EdGuo, **11**),¹ have not been studied. We describe here the site specific synthesis of oligonucleotides containing the HO-EdGuo adduct, and the evaluation of its stability. In the course of this work, we developed an improved synthesis of the phosphoramidite reagent of 1,*N*²-etheno-2'-dGuo (ϵ -dGuo, **7**). In vitro replication studies of the ϵ -dGuo and HO-EdGuo-adducted oligonucleotides with a replicative bacteriophage T7 DNA polymerase (exonuclease⁻) (pol T7⁻) and a lesion bypass *Sulfolobus solfataricus* S2 DNA polymerase IV (Dpo4) DNA polymerase were also carried out.

Experimental Procedures

General Methods and Instrumentation

General procedures and instrumentation were similar to previous work, and details are provided in the Supporting Information (34–38).

HPLC Analyses

Reactions were analyzed with a 250 mm × 4.6 mm i.d. reversed phase column (YMC ODS-AQ column) at a flow rate of 1.5 mL/min using gradients 1–5 (vide infra). Preparative separations were carried out with a 250 mm × 10 mm i.d. reversed phase column (YMC ODS-AQ column) at a flow rate of 5 mL/min using gradients 1–5. In both cases, H₂O (A) and CH₃CN (B) mixtures were used for nucleosides and 0.1 M aqueous ammonium formate (A) and CH₃CN (B) for oligonucleotides.

1. Gradient 1—One percent B initial mixture, 15 min linear gradient to 10% B, 5 min linear gradient to 20% B, 5 min at 20% B, 3 min linear gradient to 100% B, 2 min at 100% B, followed by 3 min linear gradient to the initial conditions.

2. Gradient 2—One percent B initial mixture, 2 min linear gradient to 5% B, 25 min linear gradient to 10% B, 2 min linear gradient to 80% B, 1 min at 80% B, followed by 3 min linear gradient to the initial conditions.

3. Gradient 3—One percent B initial mixture, 5 min linear gradient to 7% B, 20 min linear gradient to 12% B, 2 min linear gradient to 80% B, 1 min at 80% B, 2 min linear gradient to initial conditions.

¹ Abbreviations: HO-EdGuo, 3-(2-deoxy- β -D-erythro-pentofuranosyl)-6-hydroxy-3,5,6,7-tetrahydro-9*H*-imidazo[1,2-*a*]purin-9-one; ϵ -dGuo, 1,*N*²-etheno-2'-deoxyguanosine; pol T7⁻, bacteriophage T7 DNA polymerase (exonuclease⁻); Dpo4, *Sulfolobus solfataricus* S2 DNA polymerase IV; LC/ES-MS, liquid chromatography/electrospray-mass spectrometry, MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; HRMS, high-resolution mass spectrometry; FAB, fast atom bombardment; HMBC, heteronuclear multiple bond correlation; CGE, capillary gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; DMA, dimethylacetamide; DMF, dimethylformamide; NMO, *N*-methylmorpholine-*N*-oxide; Et₃N, triethylamine; MeOH, methanol; THF, tetrahydrofuran; DMAP, 4-(dimethylamino)pyridine; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-morpholinopropanesulfonic acid.

4. Gradient 4—One percent B initial mixture, 5 min linear gradient to 7% B, 15 min linear gradient to 9% B, 2 min linear gradient to 80% B, 1 min at 80% B, 2 min linear gradient to initial conditions.

5. Gradient 5—One percent B initial mixture, 5 min linear gradient to 8% B, 15 min linear gradient to 11% B, 2 min linear gradient to 80% B, 1 min at 80% B, 2 min linear gradient to initial conditions.

Synthesis. 1-(2-Propenyl)-2'-deoxyguanosine (**13**) (**34**)

Crushed NaOH (154 mg, 3.85 mmol) was added to anhydrous dimethylacetamide (DMA) (25 mL) and stirred at room temperature until all of the NaOH had dissolved (~30 min, slight browning of the basic DMA solution may occur). dGuo·H₂O (1.0 g, 3.5 mmol) was added and stirred at room temperature until most of it was dissolved (~10 min). Allyl bromide (303 μ L, 423 mg, 3.5 mmol) was added in one portion. The mixture was stirred at room temperature for 1 h and then at 60 °C overnight. The solvent was removed in vacuo, and the residue was purified by silica gel column chromatography (CH₃CN:H₂O:concentrated NH₄OH 90:5:5, v/v/v) to give **13** (711 mg, 66%). ¹H NMR (DMSO-*d*₆): δ 7.94 (s, 1H, H-8), 6.97 (bs, 2H, NH₂), 6.13 (dd, 1H, *J*₁ = 6.0 Hz, *J*₂ = 7.9 Hz, H-1'), 5.84 (m, 1H, CH=CH₂), 5.25 (d, 1H, *J* = 3.9 Hz, 3'-OH), 5.12–4.99 (ddd, 2H, *J*₁ = 1.4 Hz, *J*₂ = 10.4 Hz, *J*₃ = 17.2 Hz, CH=CH₂), 4.92 (t, 1H, *J* = 5.5 Hz, 5'-OH), 4.60 (d, 2H, *J* = 4.8 Hz, CH₂–CH=CH₂), 4.34 (m, 1H, H-3'), 3.80 (m, 1H, H-4'), 3.58–3.47 (m, 2H, H-5', H-5''), 2.51 (m, 1H, H-2'), 2.20 (m, 1H, H-2''). ¹³C NMR (DMSO-*d*₆): δ 156.5 (C6), 153.9 (C2), 149.6 (C4), 135.9 (C8), 132.6 (CH₂–CH=CH₂), 116.1 (CH₂–CH=CH₂), 115.9 (C5), 87.9 (C4'), 82.6 (C1'), 71.1 (C3'), 62.1 (C5'), 42.6 (CH₂–CH=CH₂). High-resolution mass spectrometry (HRMS)—fast atom bombardment (FAB)⁺: calcd for C₁₃H₁₈N₅O₄ [M + H], *m/z* 308.1359; found, *m/z* 308.1353.

1-(2,3-Dihydroxypropyl)-2'-deoxyguanosine (**14**). From **13**

A solution of **13** (760 mg, 2.47 mmol) in H₂O (~10 mL) was stirred until completely dissolved, and then, *N*-methylmorpholine-*N*-oxide (NMO) (319 mg, 2.72 mmol) and OsO₄ (~1 mg) were added. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated, and the crude product was purified by silica gel column chromatography (CH₃CN:H₂O: concentrated NH₄OH 85:10:5) to give **14** (795 mg, 94%) as an inseparable mixture of diastereomers.

From dGuo (**34**)

Crushed NaOH (6.5 mg, 0.16 mmol) was added to anhydrous DMA (500 μ L), and the reaction was stirred until most of the NaOH had dissolved. dGuo·H₂O (35 mg, 0.12 mmol) was added, and the reaction was stirred until most of it was dissolved. (*R*)-(+)-Glycidol (8.1 μ L, 9.1 mg, 0.12 mmol) was added, and the mixture was stirred overnight at 60 °C. The DMA was removed in vacuo (centrifugal evaporator), and the residue was dissolved in H₂O. Purification by HPLC using gradient 1 gave **14** (25 mg, 59%). ¹H NMR (DMSO-*d*₆): δ 7.94 (s, 1H, H-8), 6.86 (bs, 2H, NH₂), 6.13 (dd, 1H, *J*₁ = 6.1 Hz, *J*₂ = 7.7 Hz, H-1'), 5.36 (d, 1H, *J* = 3.9 Hz, CHOH), 5.27 (d, 1H, *J* = 3.6 Hz, 3'-OH), 4.94 (t, 1H, *J* = 5.4 Hz, 5'-OH), 4.84 (t, 1H, *J* = 5.5 Hz, CH₂OH), 4.33 (m, 1H, H-3'), 4.10 (m, 1H, CH₂–CHOH), 3.81 (m, 3H, H-4', CH₂–CHOH, CH₂–CHOH), 3.59–3.44 (m, 2H, H-5', H-5''), 3.40 (m, 2H, CH₂OH), 2.53 (m, 1H, H-2'), 2.18 (m, 1H, H-2''). ¹³C NMR (DMSO-*d*₆): δ 157.3, 155.4, 149.3, 136.1, 116.5, 87.9, 82.7, 71.1, 70.4, 63.9, 62.1, 45.4. HRMS-FAB⁺: calcd for C₁₃H₁₉N₅O₆ [M + H], *m/z* 342.1414; found, *m/z* 342.1430.

3-(2-Deoxy- β -D-erythro-pentofuranosyl)-5,6,7,9-tetrahydro-6-hydroxy-9H-oxoimidazo[1,2-a]purin-9-one (HO-EdGuo, **11**)

To a stirred solution of **14** (5.4 mg, 0.016 mmol) in 50 mM, pH 7.0, potassium phosphate buffer (1 mL) was added NaIO (as a 20 mM solution in potassium phosphate buffer pH 7.0, 50 mM, 2 equiv), and the reaction was stirred for 15 min at room temperature. The product mixture was purified by HPLC using gradient 1 as a mixture of two diastereomers, which equilibrated too rapidly to be isolated individually. The fractions were immediately frozen after collection to prevent significant dehydration to the ϵ -dGuo (**7**) and lyophilized to give **11** (4.5 mg, 93%) as a mixture of diastereomers. The sample contained a mixture of **11** and **7** in an approximate ratio of 80:20. Compound **11**: ^1H NMR (DMSO- d_6): δ 8.72 (bs, 1H, 5-NH), 7.96 (s, 1H, H-2), 6.45 (d, 1H, J = 7.2 Hz, 6'-OH), 6.13 (t, 1H, J = 6.1 Hz, H-1'), 5.39 (m, 1H, H-6), 5.28 (m, 1H, 3'-OH), 4.94 (m, 1H, 5'-OH), 4.34 (m, 1H, H-3'), 4.07 (m, 1H, H-7), 3.83 (m, 2H, H-4', H-7), 3.61–3.32 (m, 2H, H-5', H-5''), 2.55 (m, 1H, H-2'), 2.22 (m, 1H, H-2''). HRMS-FAB $^+$: calcd for $\text{C}_{12}\text{H}_{16}\text{N}_5\text{O}_5$ [$\text{M} + \text{H}$], m/z 310.1151; found, m/z 310.1145.

1, N^2 -Etheno-2'-deoxyguanosine (ϵ -dGuo, **7**). From **14**

To a solution of **14** (55.0 mg, 0.16 mmol) in potassium phosphate buffer, pH 7.0 (50 mM), was added NaIO $_4$ (as a 20 mM solution in potassium phosphate buffer, pH 7.0, 50 mM, 1.5 equiv), and the reaction was stirred at room temperature for 7 days, giving three products. The reaction mixture was purified by HPLC using gradient 2 to give **7** (9.0 mg, 19%), **15** (7.9 mg, 15%), and **16** (7.1 mg, 7%). Compound **7**: ^1H NMR (DMSO- d_6): δ 12.46 (bs, 1H, 5-NH), 8.14 (s, 1H, H-2), 7.62 (d, 1H, J = 2.6 Hz, H-7), 7.44 (d, 1H, J = 2.6 Hz, H-6), 6.26 (dd, 1H, J_1 = 6.2 Hz, J_2 = 7.5 Hz, H-1'), 5.31 (d, 1H, J = 4.1 Hz, 3'-OH), 4.97 (t, 1H, J = 5.5 Hz, 5'-OH), 4.38 (m, 1H, H-3'), 3.85 (m, 1H, H-4'), 3.56 (m, 2H, H-5', H-5''), 2.60 (m, 1H, H-2'), 2.26 (m, 1H, H-2''). Compound **15**: ^1H NMR (DMSO- d_6): δ 8.10 (s, 1H, H-2), 7.20 (s, 1H, H-6), 6.23 (dd, 1H, J_1 = 7.5 Hz, J_2 = 6.3 Hz, H-1'), 5.28 (bs, 1H, 3'-OH), 4.99 (bs, 2H, 5'-OH, CH_2 -OH), 4.83 (s, 2H, CH_2 -OH), 4.37 (m, 1H, H-3'), 3.84 (m, 1H, H-4'), 3.60–3.50 (m, 2H, H-5', H-5''), 2.61 (m, 1H, H-2'), 2.25 (m, 1H, H-2''). ES-MS calcd for $\text{C}_{13}\text{H}_{16}\text{N}_5\text{O}_5$ [$\text{M} + \text{H}$], m/z 322.1; found, m/z 322.0. Compound **16**: ^1H NMR (DMSO- d_6): δ 8.09 (s, 2H, H-2), 7.00 (d, 2H, J = 1.5 Hz, H-6), 6.22 (t, 2H, J = 6.8 Hz, H-1'), 5.28 (bs, 2H, 3'-OH, 3''-OH), 5.05 (s, 2H, C- CH_2 -C), 4.95 (bs, 2H, 5'-OH, 5''-OH), 4.37 (m, 2H, H-3', H-3''), 3.84 (m, 2H, H-4', H-4''), 3.61–3.50 (m, 4H, H-5', H-5''), 2.60 (m, 2H, H-2'), 2.24 (m, 2H, H-2''). ES-MS calcd for $\text{C}_{25}\text{H}_{26}\text{N}_{10}\text{O}_8$ [$\text{M} + \text{H}$], m/z 595.2; found, m/z 595.2.

Reduction of 3-(2-Deoxy- β -D-erythro-pentofuranosyl)-5,6,7,9-tetrahydro-6-hydroxy-9H-oxoimidazo[1,2-a]purin-9-one (**11**)

A solution of **11** (1.5 mg, 0.0048 mmol) in 2 mL of phosphate buffer (pH 7.0, 50 mM) was treated with solid NaBH $_4$ (7.3 mg, 0.19 mmol), and the reaction was stirred at room temperature overnight. HPLC analysis (gradient 1) of an acidified aliquot indicated complete conversion of the starting material to two major products with retention times of $\sim$ 15.6 and $\sim$ 17.7 min in an $\sim$ 1:2.5 ratio. The peak at 15.6 min was identified as 1, N^2 -ethano-dGuo (**17**) based on NMR and MS spectral analysis; the peak at 17.7 min was identified as **7** based on NMR and UV spectral analysis and comparison to those from an authentic sample. Similar results were obtained when NaB-(CN)H $_3$ was used as the reductant. Compound **17**: ^1H NMR (DMSO- d_6): δ 7.91 (s, 1H, H-2), 7.81 (bs, 1H, 5-NH), 6.10 (dd, 1H, J_1 = 6.1 Hz, J_2 = 7.6 Hz, H-1'), 5.27 (bs, 1H, 3'-OH), 4.95 (bs, 1H, 5'-OH), 4.33 (m, 1H, H-3'), 4.05 (m, 2H, H-7), 3.81 (m, 1H, H-4'), 3.64 (m, 2H, H-6), 3.52 (m, 2H, H-5', H-5''), 2.50 (m, 1H, H-2'), 2.19 (m, 1H, H-2''). ES-MS calcd for $\text{C}_{12}\text{H}_{16}\text{N}_5\text{O}_4$ [$\text{M} + \text{H}$], m/z 294.1; found, m/z 294.1.

1-(2-Propenyl)-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)guanosine (23)

A mixture of **13** (815 mg, 2.7 mmol) and 1*H*-imidazole (903 mg, 13.3 mmol) was evaporated from anhydrous dimethylformamide (DMF) (3 × 30 mL). The residue was dissolved in anhydrous DMF (10 mL), and the solution was cooled to 0 °C. 1,3-Dichloro-1,1,3,3-tetraisopropyl-1,3-disiloxane (1.02 mL, 1.0 g, 3.2 mmol) was added dropwise with stirring under Ar. The reaction was allowed to stir overnight at room temperature. The reaction mixture was poured over ice (80 g), and the resulting mixture was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic extracts were washed with water (2 × 40 mL), dried (Na₂SO₄), filtered, and concentrated in vacuo. The residue was dissolved in methanol (MeOH) (40 mL) and heated in a hot water (~50 °C) bath for ~5 min. The MeOH was removed in vacuo, and the residue was purified by flash column chromatography on silica gel (25% EtOAc in hexanes) to give **23** (956 mg, 66%). ¹H NMR (DMSO-*d*₆): δ 7.85 (s, 1H, H-8), 6.97 (bs, 2H, NH₂), 6.07 (dd, 1H, *J*₁ = 4.1 Hz, *J*₂ = 7.3 Hz, H-1'), 5.83 (m, 1H, CH=CH₂), 5.13–4.99 (ddd, 2H, *J*₁ = 1.3 Hz, *J*₂ = 10.5 Hz, *J*₃ = 17.2 Hz, CH=CH₂), 4.67 (m, 1H, H-3'), 4.60 (d, 2H, *J* = 4.6 Hz, CH₂–CH=CH₂), 3.92 (m, 2H, H-5', H-5''), 3.79 (m, 1H, H-4'), 2.70 (m, 1H, H-2'), 2.50 (m, 1H, H-2''), 1.05 (m, 28H, *i*-Pr–Si). HRMS-FAB⁺: calcd for C₂₅H₄₄N₅O₅Si₂ [M + H], *m/z* 550.2881; found, *m/z* 550.2886.

1-(2,3-Dihydroxypropyl)-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)guanosine (24)

Protected nucleoside **23** (395 mg, 0.72 mmol) was dissolved in tetrahydrofuran (THF) (10 mL), and then H₂O (10 mL), NMO (92.5 mg, 0.79 mmol), and OsO₄ (~1 mg) were sequentially added. The mixture was stirred overnight at room temperature. The solvents were removed in vacuo, and the crude product was purified by flash column chromatography on silica (CH₃CN:H₂O: concentrated NH₄OH 90:5:5, v/v/v) to give **24** as a mixture of diastereomers (393 mg, 94%). ¹H NMR (DMSO-*d*₆ at 313° K): δ 7.84 (s, 1H, H-8), 6.80 (bs, 2H, diastereomeric NH₂), 6.08 (dd, 1H, *J*₁ = 4.1 Hz, *J*₂ = 7.3 Hz, H-1'), 5.29 and 5.26 (d, 1H, *J*₁ = 4.5 Hz, *J*₂ = 4.7 Hz, diastereomeric CHOH), 4.75 (m, 1H, diastereomeric CH₂OH), 4.68 (m, 1H, H-3'), 4.14 (m, 1H, CH₂–CHOH), 3.90 (m, 2H, H-5', H-5''), 3.82 (m, 3H, H-4', CH₂–CHO H, CHOH), 3.40 (m, 2H, CH₂OH), 2.71 (m, 1H, H-2'), 2.50 (m, 1H, H-2''), 1.03 (m, 28H, *i*-Pr–Si). HRMS-FAB⁺: calcd for C₂₅H₄₆N₅O₇Si₂ [M + H], *m/z* 584.2936; found, *m/z* 584.2933.

1-(2,3-Dibenzoyloxypropyl)-N²-benzoyl-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)guanosine (25)

Nucleoside **24** (50 mg, 0.086 mmol) was evaporated from anhydrous pyridine (3 × 5 mL) and then dissolved in anhydrous pyridine (5 mL). Benzoyl chloride (148 μL, 181 mg, 1.29 mmol), triethylamine (Et₃N) (240 μL), and 4-(dimethylamino)pyridine (DMAP) (3.2 mg) were sequentially added to the stirred solution, and the reaction mixture was stirred overnight at 55 °C under Ar. The mixture was cooled in an ice bath, and MeOH (10 mL) was added. After 15 min at room temperature, the solvents were removed in vacuo. The residue was dissolved in CH₂Cl₂ (35 mL) and washed with saturated NaHCO₃ (2 × 15 mL) followed by water (2 × 15 mL). The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo. Purification by flash chromatography on silica gel (50% EtOAc in hexanes) gave **25** as a mixture of diastereomers (55 mg, 71%). ¹H NMR (DMSO-*d*₆): δ 11.23 (bs, 1H, NH), 8.24 and 8.23 (s, 1H, diastereomeric H-8), 7.93 (m, 2H, aromatic), 7.80 (m, 2H, aromatic), 7.75 (m, 2H, aromatic), 7.65–7.33 (m, 9H, aromatic), 6.17 (m, 1H, H-1'), 5.69 (m, 1H, CHOBz), 4.77 (m, 1H, H-3'), 4.65 (m, 2H, CH₂–CHOBz, CH₂OBz), 4.51 (m, 2H, CH₂–CHOBz, CH₂OBz), 3.88 (m, 2H, H-5', H-5''), 3.74 (m, 1H, H-4'), 2.76 (m, 1H, H-2'), 2.49 (m, 1H, H-2''), 0.94 (m, 28H, *i*-Pr–Si). HRMS-FAB⁺: calcd for C₄₆H₅₈N₅O₁₀Si₂ [M + H], *m/z* 896.3722; found, *m/z* 896.3730.

1-(2,3-Dibenzoyloxypropyl)-*N*²-benzoyl-2'-deoxyguanosine (26)

To a stirred solution of nucleoside **25** (234 mg, 0.26 mmol) in THF (10 mL) was added tetrabutylammonium fluoride (TBAF) (1.3 mL, 1.0 M in THF). The reaction was stirred for 2 h at room temperature under Ar, and the solvent was removed in vacuo. Purification by flash column chromatography (5–10% MeOH in CH₂Cl₂) gave **26** as a mixture of diastereomers (149 mg, 87%). ¹H NMR (DMSO-*d*₆): δ 11.35 (bs, 1H, NH), 8.35 and 8.34 (s, 1H, diastereomeric H-8), 7.94 (m, 2H, aromatic), 7.83 (m, 2H, aromatic), 7.76 (m, 2H, aromatic), 7.61 (m, 3H, aromatic), 7.47 (m, 4H, aromatic), 7.35 (m, 2H, aromatic), 6.20 (m, 1H, H-1'), 5.72 (m, 1H, CHOBz), 5.27 (d, 1H, *J* = 4.2 Hz, 3'-OH), 4.91 (t, 1H, *J* = 5.5 Hz, 5'-OH), 4.67 (m, 2H, CH₂-CHOBz, CH₂OBz), 4.53 (m, 2H, CH₂-CHOBz, CH₂OBz), 4.33 (m, 1H, H-3'), 3.80 (m, 1H, H-4'), 3.57–3.43 (m, 2H, H-5', H-5''), 2.52 (m, 1H, H-2'), 2.24 (m, 1H, H-2''). HRMS-FAB⁺: calcd for C₃₄H₃₂N₅O₉ [M + H], *m/z* 654.2200; found, *m/z* 654.2188.

5'-O-(4,4'-Dimethoxytrityl)-1-(2,3-dibenzoyloxypropyl)-*N*²-benzoyl-2'-deoxyguanosine (27)

Nucleoside **26** (100 mg, 0.15 mmol) was evaporated with anhydrous pyridine (3 × 5 mL) and dried overnight under vacuum. Nucleoside **26** was dissolved in anhydrous pyridine (10 mL), and 4,4'-dimethoxytrityl chloride (64.8 mg, 0.19 mmol) was added. The mixture was stirred for 5 h at room temperature under Ar. The solvent was evaporated in vacuo, and the residue was purified by silica gel chromatography (CH₂Cl₂:MeOH + 1% pyridine 99.5:0.5 to 95:5, v/v) to give **27** as a mixture of diastereomers (117 mg, 80%). ¹H NMR (DMSO-*d*₆): δ 11.33 (bs, 1H, NH), 8.19 (s, 1H, diastereomeric H-8), 7.92 (m, 2H, aromatic), 7.79 (m, 4H, aromatic), 7.65–7.30 (m, 12H, aromatic), 7.20 (m, 6H, aromatic), 6.80 (m, 4H, aromatic), 6.23 (m, 1H, H-1'), 5.73 (m, 1H, CHOBz), 5.33 and 5.32 (d, 1H, *J*₁ = 4.2 Hz, *J*₂ = 4.4 Hz, 3'-OH), 4.67 (m, 2H, CH₂-CHOBz, CH₂OBz), 4.52 (m, 2H, CH₂-CHOBz, CH₂OBz), 4.32 (m, 1H, H-3'), 3.90 (m, 1H, H-4'), 3.72 (s, 6H, CH₃O), 3.11 (m, 2H, H-5', H-5''), 2.57 (m, 1H, H-2'), 2.28 (m, 1H, H-2''). HRMS-FAB⁺: calcd for C₅₅H₅₀N₅O₁₁ [M + H], *m/z* 956.3507; found, *m/z* 956.3501.

3'-O-[(*N,N*-Diisopropylamino)-2-cyanoethoxyphosphinyl]-5'-O-(4,4'-dimethoxytrityl)-1-(2,3-dibenzoyloxypropyl)-*N*²-benzoyl-2'-deoxyguanosine (28)

Compound **27** (35 mg, 0.037 mmol) was evaporated from anhydrous pyridine (3 × 5 mL) and dried overnight under vacuum. The oily residue was dissolved in freshly distilled CH₂Cl₂ (3 mL), and a solution of anhydrous 1*H*-tetrazole (122 μL of a 0.45 M solution in CH₃CN, 3.8 mg, 0.055 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphoramidite (18 μL, 16.6 mg, 0.05 mmol) was added. The mixture was stirred for 2 h at room temperature under Ar. The solvent was removed in vacuo, and the residue was purified by flash column chromatography on silica gel (CH₂Cl₂:MeOH + 1% pyridine 99.5:0.5 to 95:5, v/v) to give **28** as a mixture of diastereomers (23 mg, 54%). ¹H NMR (DMSO-*d*₆): δ 11.34 (bs, 1H, NH), 8.20 (s, 1H, H-8), 7.92 (m, 2H, aromatic), 7.80 (m, 4H, aromatic), 7.60 (m, 4H, aromatic), 7.49–7.29 (m, 8H, aromatic), 7.19 (m, 6H, aromatic), 6.79 (m, 4H, aromatic), 6.23 (m, 1H, H-1'), 5.73 (m, 1H, CHOBz), 4.63 (m, 1H, H-3'), 4.68 (m, 2H, CH₂-CHOBz, CH₂OBz), 4.59 (m, 3H, H-3', CH₂-CHOBz, CH₂OBz), 3.99 (m, 1H, H-4'), 3.71 (s, 7H, POCH₂, CH₃O), 3.59 (m, 1H, POCH₂), 3.48 (m, 2H, CH(CH₃)₂), 3.18 (m, 2H, H-5', H-5''), 2.73 (m, 2H, H-2', CH₂CN), 2.61 (t, 1H, *J* = 5.9 Hz, CH₂CN), 2.38 (m, 1H, H-2''), 1.10 and 1.07 (m, 12H, CH(CH₃)₂). ³¹P NMR (DMSO-*d*₆, 121 MHz): δ 149.2, 148.5.

5,5'-*N,O*-bis(4,4'-Dimethoxytrityl)-1,*N*²-etheno-2'-deoxyguanosine (29)

Nucleoside **7** (140 mg, 0.48 mmol), prepared as previously described (39), was evaporated from anhydrous pyridine (3 × 5 mL) and then dissolved in anhydrous pyridine (5 mL). To the stirred solution were added 4,4'-dimethoxytrityl chloride (350 mg, 0.99 mmol) and

diisopropylethylamine (167 μ L, 124 mg, 0.96 mmol). The reaction mixture was stirred for 5 h at room temperature under Ar. The solvent was removed in vacuo, and the residue was purified by silica gel chromatography (CH_2Cl_2 :MeOH:pyridine 98:1:1 to 93:6:1, v/v/v) to give bis-tritylated nucleoside **29** (301 mg, 70%). ^1H NMR ($\text{DMSO}-d_6$): δ 7.86 (s, 1H, H-2), 7.17 (d, 1H, $J = 2.9$ Hz, H-7), 7.25–7.10 (m, 19H, H-6, aromatic), 6.83 (m, 8H, aromatic), 5.71 (dd, 1H, $J_1 = 6.1$ Hz, $J_2 = 6.5$ Hz, H-1'), 5.08 (d, 1H, $J = 5.1$ Hz, 3'-OH), 3.88 (m, 1H, H-3'), 3.82 (m, 1H, H-4'), 3.71 (s, 3H, CH_3O), 3.70 (s, 3H, CH_3O), 3.69 (s, 6H, CH_3O), 3.04 (m, 2H, H-5', H-5''), 2.18 (m, 1H, H-2'), 1.85 (m, 1H, H-2''). HRMS-FAB⁺: calcd for $\text{C}_{54}\text{H}_{50}\text{N}_5\text{O}_8$ [M + H], m/z 896.3659; found, m/z 896.3672.

5'-O-(4,4'-Dimethoxytrityl)-1,*N*²-etheno-2'-deoxyguanosine (**30**)

Nucleoside **29** (301 mg, 0.34 mmol) was boiled in MeOH for ~10 min after which time the solvent was removed in vacuo. The residue was purified by silica gel chromatography (CH_2Cl_2 :MeOH:pyridine 98:1:1 to 93:6:1, v/v/v) to give 5'-O-tritylated nucleoside **30** (170 mg, 85%). ^1H NMR ($\text{DMSO}-d_6$): δ 8.00 (s, 1H, H-2), 7.61 (d, 1H, $J = 2.6$ Hz, H-7), 7.45 (d, 1H, $J = 2.6$ Hz, H-6), 7.32 (m, 2H, aromatic), 7.19 (m, 7H, aromatic), 6.78 (m, 4H, aromatic), 6.30 (dd, 1H, $J = 6.4$ Hz, H-1'), 5.34 (d, 1H, $J = 4.6$ Hz, 3'-OH), 4.41 (m, 1H, H-3'), 3.95 (m, 1H, H-4'), 3.71 (s, 3H, CH_3O), 3.70 (s, 3H, CH_3O), 3.20 (m, 1H, H-5'), 3.19 (m, 1H, H-5''), 2.74 (m, 1H, H-2'), 2.33 (m, 1H, H-2''). HRMS-FAB⁺: calcd for $\text{C}_{33}\text{H}_{32}\text{N}_5\text{O}_6$ [M + H], m/z 594.2352; found, m/z 594.2356.

3'-O-[(*N,N*-Diisopropylamino)-2-cyanoethoxyphosphinyl]-5'-O-(4,4'-dimethoxytrityl)-1,*N*²-etheno-2'-deoxyguanosine (**31**)

Compound **30** (35 mg, 0.059 mmol) was evaporated from anhydrous pyridine (3 $\times$ 3 mL) and dried overnight under vacuum. The oily residue was dissolved in freshly distilled CH_2Cl_2 (5 mL), and a solution of anhydrous 1*H*-tetrazole (165 μ L of a 0.45 M solution in CH_3CN , 5.4 mg, 0.077 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphoramidite (28 μ L, 27 mg, 0.088 mmol) was added. The mixture was stirred for 2 h at room temperature under Ar. The solvent was removed in vacuo, and the crude mixture of mono- and bis-phosphatidylated product was dissolved in MeOH (5 mL) and stirred for 10 min at room temperature. The solvent was removed in vacuo, and the residue was purified by flash column chromatography on silica gel (CH_2Cl_2 :MeOH + 1% pyridine 99:1 to 95:5, v/v) to give **31** (30 mg, 64%). ^1H NMR ($\text{DMSO}-d_6$): Δ 8.03 and 8.02 (s, 1H, diastereomeric H-2), 7.66 (d, 1H, $J = 2.6$ Hz, H-7), 7.43 and 7.41 (m, 1H, diastereomeric H-6), 7.32 (m, 2H, aromatic), 7.20 (m, 7H, aromatic), 6.78 (m, 4H, aromatic), 6.31 and 6.30 (m, 1H, H-1'), 4.64 (m, 1H, H-3'), 4.10 and 4.05 (m, 1H, diastereomeric H-4'), 3.71 (s, 7H, CH_3O , POCH_2), 3.65 (m, 1H, POCH_2), 3.55 (m, 2H, $\text{CH}(\text{CH}_3)_2$), 3.21 (m, 2H, H-5', H-5''), 2.89 (m, 1H, H-2'), 2.77 (t, 1H, $J = 5.9$ Hz, CH_2CN), 2.65 (t, 1H, $J = 5.9$ Hz, CH_2CN), 2.55 (m, 1H, H-2''), 1.11 and 1.03 (m, 12H, $\text{CH}(\text{CH}_3)_2$). HRMS-FAB⁺: calcd for $\text{C}_{42}\text{H}_{49}\text{N}_7\text{O}_7\text{P}$ [M + H], m/z 794.3431; found, m/z 794.3440.

Oligonucleotide Synthesis and Purification

Oligonucleotides were synthesized and deprotected as previously described (34). Unmodified oligonucleotides were purchased from Midland Certified Reagent Co. Inc. Oligonucleotides were purified by HPLC and/or polyacrylamide gel electrophoresis (PAGE). Following HPLC and/or PAGE purification, all oligonucleotides were desalted via a Sephadex G-25 column using a BioRad FPLC system and analyzed by capillary gel electrophoresis (CGE), matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS, and enzymatic digestion. Oligonucleotides containing the hydroxyethano-adducted oligonucleotides (**32b** and **33b**) were desalted on either a Biospin column (BioRad) or a C_{18} zip tip (Millipore) after NaIO_4 treatment and prior to analyses or use in polymerase reactions. Further details are provided in the Supporting Information.

5'-d(GCT-AGC-XAG-TCC)-3', X = 14 (32a)

Oligonucleotide **32a** was purified by HPLC using gradient 3. MALDI-TOF-MS: calcd for $[M - H]^-$, m/z 3718.7; found, m/z 3718.7.

5'-d(GCT-AGC-XAG-TCC)-3', X = 11 (32b)

To a solution of oligonucleotide **32a** in potassium phosphate buffer (50 mM, pH 7.0) was added an aqueous solution of NaIO_4 (20 mM solution in 50 mM, pH 7.0, potassium phosphate buffer). The overall NaIO_4 concentration of the reaction was 7.1 mM. The reaction was allowed to stand at room temperature for 30 min after which time the sample was desalted in a C18 zip tip. The conversion to the etheno-adducted oligonucleotide (**32c**) was monitored in 50 mM, pH 7.0, potassium phosphate buffer by removing aliquots, desalting on a C18 zip tip, and CGE analysis. MALDI-TOF-MS: calcd for $[M - H]^-$, m/z 3686.7; found, m/z 3686.8.

5'-d(GCT-AGC-XAG-TCC)-3' (X = 11) 3'-d(CGA-TCG-CTC-AGG-ATA)-5' (32b:34)

A solution of oligonucleotide **32b** was annealed to oligonucleotide **34** (molar ratio of 1:1.3) by heating the two oligonucleotides together in 50 mM, pH 7.0, potassium phosphate buffer at 90 °C for 3 min. The solution was then allowed to cool slowly to 25 °C. Aqueous NaIO_4 (20 mM solution in 50 mM, pH 7.0, potassium phosphate buffer) was added giving an overall NaIO_4 concentration of 30.8 mM. The reaction was allowed to stand at room temperature for 30 min after which time the sample was desalted using a C18 zip tip. The conversion to the etheno adduct was monitored in 50 mM, pH 7.0, phosphate buffer by removing aliquots, desalting with a C18 zip tip, and CGE analysis.

5'-d(GCT-AGC-XAG-TCC)-3', X = 7 (32c)

Oligonucleotide **32c** was purified by HPLC using gradient 4. MALDI-TOF-MS: calcd for $[M - H]^-$, m/z 3668.6; found, m/z 3669.2.

5'-d(TCA-CXG-AAT-CCT-TAC-GAG-CCC-CC)-3', X = 14 (33a)

Oligonucleotide **33a** could not be adequately purified by HPLC and was therefore purified by PAGE. MALDI-TOF-MS: calcd for $[M - H]^-$, m/z 7000.2; found, m/z 7000.9.

5'-d(TCA-CXG-AAT-CCT-TAC-GAG-CCC-CC)-3', X = 11 (33b)

A solution of oligonucleotide **33a** was stirred in 50 mM, pH 7.0, potassium phosphate buffer. Aqueous NaIO_4 (100 mM solution in 50 mM, pH 7.0, potassium phosphate buffer) was added giving an overall NaIO_4 concentration of 25 mM. The reaction was allowed to stand at room temperature for 30 min after which time the sample was desalted using a C18 zip tip or Biospin column. The desalted sample was analyzed by CGE and enzyme digest.

5'-d(TCA-CXG-AAT-CCT-TAC-GAG-CCC-CC)-3' (X = 11) 3'-d(C-TTA-GGA-ATG-CTC-GGG-GG)-5' (33b:35)

Oligonucleotide **33a** was annealed to primer **35** (molar ratio 1:1.5) in 50 mM, pH 7.0, potassium phosphate buffer by heating the two oligonucleotides at 90 °C for 3 min and then slowly cooling the solution to 25 °C. Aqueous NaIO_4 (100 mM solution in 50 mM, pH 7.0, potassium phosphate buffer) was added giving an overall NaIO_4 concentration of ~25 mM. The reaction was allowed to stand at room temperature for 1 h after which time the sample was desalted using either a C18 zip tip or a Biospin column. An aliquot was subjected to enzymatic digestion.

5'-d(TCA-CXG-AAT-CCT-TAC-GAG-CCC-CC)-3' (X = 11) · 3'-d(NC-TTA-GGA-ATG-CTC-GGG-GG)-5' (33b:36)

Oligonucleotide **33a** was annealed to primer **36** (molar ratio 1:1.25) in 50 mM, pH 7.0, potassium phosphate buffer by heating the two oligonucleotides at 90 °C for 3 min and then slowly cooling the solution to 25 °C. Aqueous NaIO₄ (100 mM solution in 50 mM, pH 7.0, potassium phosphate buffer) was added giving an overall NaIO₄ concentration of 50 mM. The reaction was allowed to sit at room temperature for 2 h after which time the sample was desalted using a Biospin column. The desalted sample was subjected to enzymatic digestion.

5'-d(TCA-CXG-AAT-CCT-TAC-GAG-CCC-CC)-3', X = 7 (33c)

Oligonucleotide **33c** was purified by HPLC using gradient 5. The purity of the oligonucleotide after HPLC purification was insufficient for use in the polymerase extension reactions, and it was further purified by PAGE for these studies. MALDI-TOF-MS: calcd for [M - H]⁻, *m/z* 6950.2; found, *m/z* 6951.4.

Enzymatic Digestion

Enzymatic hydrolysis and HPLC analysis of the hydrolysate for all modified oligonucleotides were performed as previously described (36). Full details are included in the Supporting Information.

Melting Studies (*T_m*)

Melting temperature determinations were performed as previously described (38). Full details are included in the Supporting Information.

Polymerase Extension Assays

Dpo4 and pol T7⁻ were expressed and purified as previously described (40, 41). Extension assays and gel visualization were performed as previously described (40). Because of the potential electrophilic nature of the hydroxyethano adduct, the polymerase extension reactions were run in the nonnucleophilic buffer 3-morpholinopropanesulfonic acid (MOPS) (50 mM, pH 7.5), rather than tris-(hydroxymethyl)aminomethane (Tris), which contains nucleophilic groups. Otherwise, the full details are included in the Supporting Information.

Steady State Kinetics

Steady state kinetics and data analysis were performed as previously described (40). The molar ratio of DNA:enzyme varied from 20:1 to 1:1. While it is preferred to have a molar ratio of primer-template:enzyme of ≥ 10:1, precedent does exist for ratios from this to 1:1 and was found to be necessary in some cases (42). Full details are included in the Supporting Information.

Results and Discussion**Synthesis and Stability of the HO-EdGuo Nucleoside**

The synthesis of the title adduct (**11**) is largely based on our previously reported synthesis of 6-hydroxy-5,6,7,8-tetrahydropyrimido-[1,2-*a*]purin-10(3*H*)-one, a homologue of the desired adduct derived from the reaction of dGuo with acrolein (34, 43). Periodate oxidation of a vicinal diol is a common strategy for the incorporation of aldehyde functionality into oligonucleotides under mild and neutral conditions (35-37, 43-51). dGuo was alkylated at the N1-position with allyl bromide under alkaline conditions to give the N1-propenyl derivative (**13**) in 66% yield (Scheme 3) (34, 43, 52, 53). HPLC analysis of the reaction mixture showed minor byproducts whose UV spectra were consistent with *O*⁶- and *N*²-

alkylation; these minor products were not isolated. Confirmation of the regiochemistry of the alkylation came from the heteronuclear multiple bond correlation (HMBC) spectrum of **13**, which showed a three-bond correlation between the methylene protons of the N1-propenyl group (δ 4.60 ppm) and the C2 and C6 carbon resonances (δ 153.9 and 156.5 ppm). Dihydroxylation of the olefin gave the corresponding vicinal diol (**14**) in high yield. Alternatively, dGuo could be alkylated at N1 with (*R*)-glycidol to give the same N1-(2,3-dihydroxypropyl)-dGuo (**14**) as a single diastereomer. Periodate cleavage of the vicinal diol created the aldehyde, which was not observed because it spontaneously cyclized to HO-EdGuo (**11**). NMR analysis of the product mixture showed the presence of approximately 20% of the ϵ -dGuo (**7**). High-resolution FAB mass spectrometric analysis was also consistent with the HO-EdGuo structure **11**.

If the dehydration of **11** was allowed to proceed by continued stirring of the reaction mixture from the periodate oxidation, ϵ -dGuo was obtained along with two unexpected products (**15** and **16**, Scheme 4) in 15 and 7% yield, respectively. Adduct **15** has been previously characterized by Golding from the reaction of glycidaldehyde and dGuo (54). In our case, **15** arises from the nucleophilic addition of ϵ -dGuo to formaldehyde, a byproduct of the periodate oxidation of **14**. The nucleophilicity of the C7-position of ϵ -dGuo is consistent with the earlier observation that the C7-proton will exchange in D₂O (10). The dimeric product **16** arises from the reaction of **15** with ϵ -dGuo and was also previously observed and characterized by Golding (54, 55).

HO-EdGuo could be purified by reversed phase HPLC using CH₃CN:H₂O as the mobile phase. The individual stereoisomers of HO-EdGuo were easily separated; however, they rapidly equilibrated to a 1:1 mixture. The mechanism of the stereochemical scrambling could either involve an opening of the hydroxyethano ring to **10** (Scheme 2) followed by reclosure or dehydration to the N5–C6 imine **12** (or the corresponding iminium ion) followed by rehydration. In the case of the later processes, the tautomerization of the imine to ϵ -dGuo would be slow relative to the dehydration/hydration reaction. The HO-EdGuo nucleoside (**11**) was quantitatively consumed over several days upon treatment with either NaBH₄ or NaB(CN)H₃ in pH 7.0 potassium phosphate buffer; it should be noted that the pH of the reaction could not be maintained when NaBH₄ was added (final pH ~11.5). For both of these reactions, the reduced 1,*N*²-ethano-dGuo (**17**) product was obtained in about 30% yield, with the remainder being ϵ -dGuo (**7**). The N1-(2-hydroxyethyl) product (**18**) derived from reduction of the open chain aldehyde (**10**) was not observed. These results suggest that the scrambling at C6 involves the dehydration/ hydration mechanism through an imine (or iminium ion) intermediate, although it is possible that the ring opening and closure reactions are much faster than reduction of the intermediate aldehyde. Further support of the dehydration/hydration pathway comes from previous work on the related 1,*N*²-glyoxal adduct of Gua. The C6-hydroxyl group of this adduct (**19**) was shown to quantitatively exchange with alcohol solvent to give the 6-alkoxy derivative (**20**), a process that would most likely occur through an imine or iminium ion intermediate (Scheme 5) (56). When the glyoxal or 6-alkoxy derivative was treated with NaB(CN)H₃, reduction at the C6-position occurred to give the 7-hydroxyethano or the *N*²-(2-hydroxyethyl) derivatives (**21** and **22**, Scheme 5). These later results are also consistent with an imine intermediate. Similar results have recently been published at the nucleoside level (57).

The dehydration of purified HO-EdGuo (**11**) to ϵ -dGuo (**7**) in 50 mM, pH 7.0, potassium phosphate buffer was followed by HPLC (Figure 2). The estimated half-life of the HO-dGuo nucleoside is 2 days. We found that both acidic and basic conditions increased the propensity of HO-EdGuo to form ϵ -dGuo and that HO-EdGuo was most stable around neutral pH. Heating also greatly accelerated the conversion to ϵ -dGuo.

Phosphoramidite Synthesis

Our plan for the site specific incorporation of the HO-EdGuo into oligonucleotides relied on the generation of the aldehyde by periodate oxidation after assembly and deprotection of an oligonucleotide containing the diol precursor **14**. The synthesis of the necessary phosphoramidite reagent (**28**) is shown in Scheme 6. N1-Alkylation of a 3',5'-silyl protected dGuo with (*R*)-glycidol under the conditions described in Scheme 3 also removed the ribose protecting groups. To avoid the need to differentiate the diol units, we protected the ribose hydroxyl groups of 1-(2-propenyl)-dGuo (**13**) and installed the vicinal diol by treatment of the olefin with OsO₄ (**24**). The vicinal diol was protected as benzoates with simultaneous protection of the *N*²-amino group and the silyl groups were removed with fluoride ion to give **26**. Nucleoside **26** was converted to phosphoramidite **28** in the usual way.²

Previous syntheses of the phosphoramidite reagent of ϵ -dGuo (**31**) gave an impure, unstable product that resulted in low coupling yields during oligonucleotide synthesis (58). We believe these difficulties were due to the reactivity of the N5-position. The adducted nucleoside (**7**) was readily prepared by the reaction of dGuo with 2,3-epoxybutanal (Scheme 7) (39). We found that attempted protection of the 5'-hydroxyl group with dimethoxytrityl chloride gave a mixture of 5'- and N5-derivatized products. If excess dimethoxytrityl chloride was used, the bis-tritylated product **29** was obtained. The N5-dimethoxytrityl group was selectively removed by briefly heating **29** in MeOH. Phosphitylation also gave the bis-derivative, which was not fully characterized. The N5-phosphoramidite group was removed by briefly stirring in MeOH at room temperature to provide the desired product **31** in 64% yield. The improved synthesis of phosphoramidite **31** resulted in significantly higher coupling yield during oligonucleotide synthesis.

Oligonucleotide Synthesis and Analysis

With phosphoramidites **28** and **31** in hand, oligonucleotides **32–33a,c** (Figure 3) were synthesized using standard solid phase synthesis. We have previously examined a number of related dGuo adducts in sequence **32** including **8**, thus providing us a basis for comparing the influence of the adduct structure on the physical properties of the oligonucleotide. The sequence of **33** is based on that of a similar 18-mer previously used in crystallographic studies of modified oligonucleotides bound to the lesion bypass polymerase Dpo4 (59, 60). Oligonucleotide **33** will be used for in vitro polymerase extension studies to assess the influence of these adducts on replication. These results will support our ongoing crystallographic studies (40).

Oligonucleotides **32–33a,c** were purified by HPLC and/or PAGE and characterized by MALDI-TOF mass spectrometry and enzymatic digestion followed by HPLC analysis of the nucleosides (see Supporting Information). The diol moiety of oligonucleotide **32a** could be cleaved in near quantitative yield by treatment with excess NaIO₄ (final concentration, ~7 mM) for 30 min to give the corresponding oligonucleotide containing the HO-EdGuo (**32b**). Similarly, the longer oligonucleotide **33a** could be cleaved to **33b** but required a final concentration of ~25 mM NaIO₄ to affect the oxidation in 30 min. In the case of the 12-mer oligonucleotides, the three species (**32a–c**) could be resolved by CGE. The resolution of CGE was not as good for the longer oligonucleotides **33a–c**, and we relied on enzymatic digestion and HPLC for product analysis. These analyses indicated that the purity of the

² When acetyl protecting groups were used for *N*² and the N1 side chain hydroxyl groups, the final phosphoramidite reagent could not be purified from hydrolyzed phosphitylating agent resulting in a diminished coupling yield during oligonucleotide synthesis. Phosphoramidite **28**, having benzoyl protecting groups, had sufficiently different chromatographic properties to allow for convenient purification by column chromatography. The purified reagent gave significantly improved coupling yields during solid phase oligonucleotide synthesis.

oligonucleotide containing the modified HO-EdGuo nucleotide was ~92% with the remaining 8% being the ϵ -dGuo adduct.

We planned to examine the miscoding potential of the HO-EdGuo adduct using in vitro extension reactions with two model polymerases. For polymerase extension assays, mixtures of the adducted template and the complementary primer oligonucleotides are usually heated and then slowly cooled to ensure proper annealing. Because heating the HO-EdGuo adduct results in extensive dehydration to the ϵ -dGuo, we examined the diol cleavage of oligonucleotide **33a** after it was annealed with its complementary -1 primer (**35**, Figure 3). The mixture of HO-EdGuo and ϵ -dGuo adducted oligonucleotides was not resolved from each other using CGE and product analysis, and optimization of the periodate reaction was therefore accomplished using HPLC analysis of the enzyme digests of the reactions. The periodate concentration used to cleave the diol in double-stranded DNA was the same as with single-stranded DNA (~25 mM), but the reaction required 60 vs 30 min. Analysis of the enzyme digest showed that the starting diol had been consumed, and a 90:10 ratio of HO-EdGuo and ϵ -dGuo adducts was present.

We next examined the formation of the HO-EdGuo adduct in a 23-mer oligonucleotide **33b** hybridized to a 19-mer complementary strand (**36**, 0-primer). In this case, the diol was paired with each of the four nucleotide bases. Periodate cleavage was significantly retarded in this double-stranded construct. Even with a periodate concentration twice that employed with a single-stranded oligonucleotide (50 vs 25 mM), the cleavage reaction required 2 h to approach completion vs 30 min for single strand. HPLC analysis of the enzyme digest indicated that ~2% of the starting diol (**33a**) remained. The ratio of HO-EdGuo and ϵ -dGuo adducted oligonucleotides (**33b**: **33c**) was 89:9. Interestingly, only one of the diastereomeric diols was observed after the periodate treatment, indicating that the diol stereochemistry influences the rates of periodate oxidation. Because the periodate reaction went to completion in other cases, we have no way of telling if this stereoselectivity is general.

During the course of our work, Johnson and co-workers reported a new synthesis of oligonucleotides containing ϵ -dGuo (**7**) through the incorporation of a phosphoramidite very similar to **28** and subsequent periodate cleavage and dehydration (50). Their synthesis of the phosphoramidite was similar to that described in Scheme 6. In their study, two oligonucleotides were obtained upon periodate cleavage of a heptamer containing diol **14** (5'-TTCXCCTT-3'), which had masses consistent with the HO-EdGuo adducted oligonucleotides and were assigned as diastereomers at the C6-position. It was reported that the HO-EdGuo adduct dehydrated to the ϵ -dGuo adducted oligonucleotide upon drying the purified sample in vacuo.

Stability of the HO-EdGuo Adduct in Oligonucleotides

The HO-EdGuo adducted oligonucleotide (**32b**) was purified by HPLC, and the dehydration to **32c** was followed in 50 mM, pH 7.0, potassium phosphate buffer at 25 °C using CGE analysis. The estimated half-life of the HO-EdGuo adduct in single strand was approximately 1 day (Figure 4).

The stability of the HO-EdGuo adduct was also examined in double-stranded DNA in which **32b** was hybridized to a complementary 15-mer (**34**) (Figure 5). A three-base 5'-overhang on the complementary oligonucleotide was necessary to ensure that it was well-separated from the oligonucleotides of interest (**32b** and **32c**) during CGE analysis (Figure 5). The stability of the HO-EdGuo adduct was slightly improved in duplex DNA (**32b**), and it dehydrated to the ϵ -dGuo adduct (**32c**) with an approximate half-life of ~48 h in 50 mM, pH 7.0, potassium phosphate buffer at 25 °C.

Melting Temperature Studies of Adducted Oligonucleotides

The influence of the modified base on the stability of 12-mer oligonucleotide **32** was examined by thermal melting analysis (T_m), and these results are summarized in Table 1. Oligonucleotides containing adducts **14**, **11**, and **7** were significantly destabilized relative to the unmodified duplex ($T_m = 59^\circ\text{C}$). Interestingly, there is a significant difference in the T_m values for the regioisomeric hydroxyethano adducts **11** and **8** (35 and 44 $^\circ\text{C}$, respectively). We believe that the 7-hydroxy regioisomer (**8**) exists, at least partly, in the ring-opened form in duplex DNA based on previous studies with the corresponding six-member ring homologue (**37**). The ring opening allows for Watson–Crick base pairing between the adducted Gua and the complementary Cyt and has been unambiguously established for the six-member ring homologue (**37**) in duplex DNA (61, 62). The melting temperatures of the identical 12-mer oligonucleotides adducted with regioisomeric hydroxypropano adducts were determined for comparison. The T_m of the 8-hydroxypropano adduct (**37**), which can undergo ring opening in duplex DNA, was 47 $^\circ\text{C}$ and is similar to the corresponding regioisomer of the hydroxyethano adduct **8** (44 $^\circ\text{C}$). The 6-hydroxypropano adduct (**38**), which we believe does not undergo ring opening, was 7 $^\circ\text{C}$ more destabilizing than its regioisomer **37**. This difference in T_m between the hydroxypropano regioisomers is similar to that of the hydroxyethanos ($\Delta T_m = 9^\circ\text{C}$). The T_m of the ϵ -dGuo adduct (**7**, 41 $^\circ\text{C}$) was intermediate between those of the two regioisomeric hydroxyethano adducts. Although this adduct cannot undergo ring opening and in this regard should be more similar to the 6-hydroxyethano adduct, the flat aromatic nature of the exocyclic ring can potentially provide a stabilizing π -stacking interaction in duplex DNA.

Polymerase Extension Reactions

The HO-EdGuo adduct is not a viable candidate for site specific mutagenesis studies due to its sensitivity toward dehydration. Extension past the modified bases in oligonucleotides **33a–c** was examined using pol T7[−], a model replicative DNA polymerase, and Dpo4, a model for a lesion bypass polymerase. Given that a small amount of the starting diol (**33a**) from incomplete periodate oxidation and varying amounts of ϵ -dGuo adduct (**33c**) was present, we examined the polymerase extension profiles of all three modified bases in parallel assays. Dpo4 was able to extend the primer-template **35:33** to yield full-length products when all four dNTPs were present, for all three of the adducts examined (Figure 6). Although not particularly efficient for any of the adducts, Dpo4 appeared to extend diol **14** slightly better than the HO-EdGuo (**11**) and ϵ -dGuo (**7**) adducted oligonucleotides. Extension products of varying length were observed owing to the low processivity of Dpo4, which we attribute more to the slow incorporation rates rather than altered k_{off} values (40). All three adducts were very strong blocks to replication for pol T7[−] (Figure 6); a single nucleotide was inserted opposite the adducted base, but further extension was inhibited. These results are in accord with previous studies of ϵ -dGuo adducted oligonucleotides with pol T7[−] (40, 58).

Results from the extension of **35:33** in the presence of a single dNTP are shown in Figures 7 and 8 for Dpo4 and pol T7[−], respectively. With pol T7[−], there was a strong preference for the incorporation of dATP across from all three adducts, although a modest amount of dGTP incorporation was observed for diol **33a** and HO-EdGuo **33b** at higher enzyme concentrations (Figure 8). Dpo4, on the other hand, incorporated dATP and dGTP and to a much lesser extent dCTP and TTP for all three adducts (Figure 7). Incorporation of multiple nucleotides was observed for dATP and dGTP with Dpo4; however, we believe that the incorporation of the nucleotides past the adduct site is largely an artifact of the single dNTP experiment and does not necessarily reflect the results obtained when all four dNTPs are present (40).

The steady state rates for dNTP incorporation were determined and are summarized in Tables 2 and 3 for Dpo4 and pol T7⁻, respectively. We found that the apparent catalytic efficiency for incorporation of any nucleotide opposite the three lesions by Dpo4 was 1800–29000-fold less efficient than incorporation of dCTP opposite dGuo and 10⁴–10⁶ less efficient for pol T7⁻. Nonmutagenic bypass by insertion of dCTP opposite the three lesions was the least efficient of the reactions with the three dNTPs tested for Dpo4. Although the misincorporation profiles for the HO-EdGuo and ϵ -dGuo adducts appeared similar by simple gel analysis, significant differences were observed in the efficiencies of dNTP incorporation. For example, Dpo4 incorporated a dATP across from the HO-EdGuo adduct 2.5-fold more efficiently than dGTP (Table 2), while dGTP was favored for the ϵ -dGuo adduct. Incorporation of dATP was favored for both the HO-EdGuo and the ϵ -dGuo adducts by pol T7⁻; however, the catalytic efficiency was approximately 6-fold better for the ϵ -dGuo adduct.

Insight into the extension reaction by Dpo4 can be gleaned from our recently completed crystallographic study of a Dpo4 ternary complex with the ϵ -dGuo adducted template-primer oligonucleotides **39:40** and ddGTP (Scheme 8) (40). The structure showed that the ϵ -dGuo adduct and the 5'-adjacent deoxycytidine were accommodated in the active site of Dpo4 and that the incoming ddGTP base paired to the 5'-adjacent deoxycytidine in the template strand. The ϵ -Guo was positioned in the base stack of the duplex and appeared to provide a platform for interstrand stacking with the incoming ddGTP. This arrangement predicts that the ϵ -dGuo adduct would lead to a one-base deletion when replicated by Dpo4. Two major products were observed from the extension of the primer that was paired with the ϵ -dGuo adducted template. The major product (84%) from the extension reaction was a one-base deletion as predicted by the crystal structure (Scheme 8). The minor product (16%) was derived from incorporation of dATP opposite the ϵ -dGuo adduct followed by accurate extension. The interstrand stacking interaction of the ϵ -dGuo adduct and the incoming ddGTP are likely to play an important role in stabilizing the ternary complex leading to a one-base deletion. The stacking arrangement is likely to be lost with a saturated adduct such as the HO-EdGuo adduct. As a result, incorporation of dATP rather than dGTP is favored with the HO-EdGuo adduct, which we believe is incorporated opposite the HO-EdGuo adduct rather than the 5'-adjacent dCyd, although we have no direct evidence of this.

The ability of the DNA polymerases to extend the primer strand with A, G, and C paired with the adducts was also examined (Figure 9). As expected from the full-length extension of the -1 primer, pol T7⁻ was unable to extend any of the primers (**36**) when hybridized to the modified templates (**33a–c**) (data not shown). For Dpo4, the primers with Ade and Gua opposite the lesions were extended while those with a Cyt are extended inefficiently. Extension past the modified base appeared to be significantly more efficient for the ϵ -dGuo adduct than for the HO-EdGuo adduct. Interestingly, the extension efficiency is noticeably different when the ϵ -dGuo is paired with an Ade vs that of a Gua. In the case where a Gua is positioned opposite the ϵ -dGuo adduct, none of the expected full-length extension product was observed and the major product appears to be incorporation of one and two bases past the adduct. This may reflect a misalignment of the template and primer (**33:36**) with the Gua at the terminus of the primer strand base pairing with the Cyt on the 5'-side of the ϵ -dGuo adduct. The distortion imposed by this misalignment may significantly slow the rate of further extension of the primer.

Conclusion

HO-EdGuo nucleoside (**11**) was synthesized, and a strategy was devised for its site specific incorporation into oligonucleotides. This DNA lesion dehydrates and tautomerizes to ϵ -dGuo (**7**) with an approximate half-life between 24 and 48 h in nucleoside and single- and

double-stranded oligonucleotides at neutral pH and room temperature. In addition, an improved synthesis of the phosphoramidite reagent of the ϵ -dGuo was developed providing significantly more convenient access to oligonucleotides containing this adduct. The miscoding potential of the HO-EdGuo and ϵ -dGuo adducts was examined by in vitro primer extension reactions with pol T7⁺ and Dpo4. The miscoding properties and rates of nucleotide incorporation of the HO-EdGuo adduct were significantly different from that of the ϵ -dGuo adduct. For the replicative polymerase T7⁺, dATP was preferentially incorporated opposite the ϵ -dGuo adduct while dATP and dGTP were incorporated opposite the HO-EdGuo adduct with similar catalytic efficiencies; both adducts were very strong blocks to further extension. The lesion bypass polymerase Dpo4 preferentially incorporated dATP opposite the HO-EdGuo adduct and dGTP opposite the ϵ -dGuo adduct. In the later case, a one-base deletion resulted through base pairing of the incoming dGTP with a Cyt on the 5'-side of the ϵ -dGuo adduct. The results would imply that the ability of the ϵ -dGuo adduct to induce frameshifts with Dpo4 would be sequence dependent. Collectively, these results indicate that the HO-EdGuo adduct has a sufficient lifetime in DNA to contribute to the genotoxic spectrum of vinyl chloride and related ϵ -dGuo forming bis-electrophiles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Modified nucleosides from the reaction of chlorooxirane.

Figure 2.
HPLC analysis of the dehydration of hydroxyethano nucleoside **11** to etheno **7** in 50 mM, pH 7.0, potassium phosphate buffer.

32: 5' -GCT AGC **X**AG TCC-3'

33: 5' -TCA **CXG** AAT CCT TAC GAG CCC CC-3'

a: X = 14, b: X = 11, c: X = 7

32: 5' -GCT AGC **X**AG TCC-3'

34: 3' -CGA TCG CTC AGG ATA-5'

35: 5' -GG GGG CTC GTA AGG ATT C

33: 3' -CC CCC GAG CAT TCC TAA **GXC** ACT-5'

36: 5' -GG GGG CTC GTA AGG ATT CN

33: 3' -CC CCC GAG CAT TCC TAA **GXC** ACT-5'

Figure 3.
Modified oligonucleotides for stability studies and polymerase extension reactions.

Figure 4. CGE analysis of the dehydration of hydroxyethano-adducted oligonucleotide **32b** in 50 mM, pH 7.0, potassium phosphate buffer.

Figure 5.
CGE analysis of the dehydration of the hydroxy-ethano adduct oligonucleotide **32b** in duplex DNA.

Figure 6.

Extension of template:primer **33a-c:35** in the presence of all four dNTPs with Dpo4 and pol T7⁻.

Figure 7.
Extension of template:primer 33a-c:35 in the presence of a single dNTP by Dpo4.

Figure 8.
Extension of template:primer 33a-c:35 in the presence of a single dNTP by pol T7.

Figure 9.
 Extension of template:primer **33a–c:36** by Dpo4 in the presence of all four dNTPs.

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 6.

Scheme 7.

Table 1
Melting Temperatures (T_m) of Oligonucleotides 32^a

X=	T_m	ΔT_m
dG	59 °C	
 14	37 °C	22 °C
 11	35 °C	24 °C
 7	41 °C	18 °C
 8	44 °C	15 °C
 37	47 °C	12 °C
 38	40 °C	19 °C

^a Conditions for T_m : 100 mM NaCl, 10 mM sodium phosphate (pH 7), 50 μ M EDTA, 0.5 A₂₆₀/mL of each oligonucleotide. The temperature was raised 1 °C/min.

Table 2

Steady State Kinetic Parameters for Dpo4

template	dNTP	K_m (μ M)	k_{cat} ($s^{-1} \times 10^{-3}$)	k_{cat}/K_m ($\mu M^{-1} s^{-1} \times 10^{-3}$)	f^a
dGuo	C	0.028 ± 0.003	8.9 ± 0.3	320	1
	A	230 ± 42	1.5 ± 0.1	0.0065	2.0×10^{-5}
	G	78 ± 6	0.89 ± 0.02	0.011	3.4×10^{-5}
diol dGuo (14)	C	89 ± 18	0.38 ± 0.03	0.0043	1
	A	61 ± 14	1.9 ± 0.2	0.031	7.2
	G	48 ± 5	0.74 ± 0.03	0.015	3.5
HO-EdGuo (11)	C	77 ± 8.0	0.36 ± 0.01	0.0047	1
	A	7.2 ± 1.1	1.2 ± 0.1	0.17	36
	G	9.1 ± 0.7	0.62 ± 0.02	0.068	14
ϵ -dGuo (7)	C	430 ± 94	0.47 ± 0.04	0.0011	1
	A	270 ± 46	0.94 ± 0.07	0.0035	3.2
	G	49 ± 11	1.2 ± 0.1	0.024	22

^a f = misincorporation frequency = $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

Table 3

Steady State Kinetic Parameters for Pol T7⁻

template	dNTP	K_m (μ M)	$k_{cat}^{-1} \times 10^{-13}$	k_{cat}/K_m (μ M ⁻¹ s ⁻¹ $\times 10^{-3}$)	f^a
dGuo	C	0.046 $\pm$ 0.009	110 $\pm$ 6	2400	1
	A	380 $\pm$ 100	6.7 $\pm$ 0.8	0.018	3.8 $\times 10^{-6}$
	G	97 $\pm$ 17	12 $\pm$ 1	0.12	2.5 $\times 10^{-5}$
diol dG (14)	C	920 $\pm$ 87	2.2 $\pm$ 0.1	0.0024	1
	A	200 $\pm$ 20	6.5 $\pm$ 0.4	0.033	14
	G	500 $\pm$ 55	3.9 $\pm$ 0.2	0.0078	3.3
HO-EdGuo (11)	C	240 $\pm$ 41	0.57 $\pm$ 0.03	0.0024	1
	A	400 $\pm$ 55	3.3 $\pm$ 0.2	0.0083	3.5
	G	110 $\pm$ 27	0.65 $\pm$ 0.05	0.0059	2.5
ϵ -dGuo (7)	C	430 $\pm$ 42	2.5 $\pm$ 0.1	0.0058	1
	A	190 $\pm$ 14	9.5 $\pm$ 0.3	0.050	8.6
	G	970 $\pm$ 160	2.0 $\pm$ 0.1	0.0021	0.36

a_f = misincorporation frequency = $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.