Misincorporation of dNTPs Opposite 1,N^2-Ethenoguanine and 5,6,7,9-Tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*]purine in Oligonucleotides by *Escherichia coli* Polymerases I exo$^-$ and II exo$^-$, T7 Polymerase exo$^-$, Human Immunodeficiency Virus-1 Reverse Transcriptase, and Rat Polymerase β[†]

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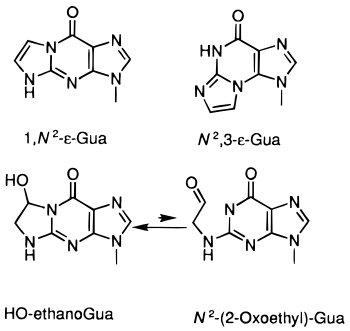
ABSTRACT: 1,N^2-Ethenoguanine (1,N^2-ϵ-Gua) and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*]purine (HO-ethanoGua) are two modified bases formed in the reaction of DNA with 2-chlorooxirane, the epoxide derivative of vinyl chloride. The oligonucleotides (19-mers), 5′-CAGTGGGTG*TCCGAATTGA-3′, were prepared, with each of these modified bases substituted for G at G*. HO-ethanodeoxyguanosine exists predominantly as a mixture of diastereomers of the closed cyclic hemiaminal form, 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*]purine, shown by $H_2{}^{18}O$ experiments to be in equilibrium with the open form, N^2-(2-oxoethyl)Gua. Both adducts retarded the 3′-extension of a complementary 10-mer primer by all of the polymerases examined, but in every case, some full-length product was obtained. Nucleotide sequence analysis indicated misincorporation of dGTP and dATP across from both 1,N^2-ϵ-Gua and HO-ethanoGua, with the extent varying considerably among the polymerases. Similar results were obtained when the abilities of the polymerases to incorporate a single dNTP were evaluated. In addition, −1 and −2 base frame shifts were detected with both 1,N^2-ϵ-Gua and HO-ethanoGua with some of the polymerases. Steady-state kinetic experiments with *Escherichia coli* polymerase I exo$^-$ and T7 polymerase exo$^-$/thioredoxin showed large decreases in k_{cat} for all dNTP incorporations compared to the normal G·dCTP pair and high misincorporation frequencies for dATP and dGTP with both adducts (compared to dCTP). Collectively, the results indicate that both of these adducts have considerable miscoding potential with some of these polymerases, that there are a number of differences between the 1,N^2-ϵ-Gua and HO-ethanoGua adducts (which formally differ only in the presence of the elements of water), and that misincorporation of dNTPs at a single modified base can vary considerably among different polymerases even in the absence of exonuclease activity.

The etheno (ϵ)[1] derivatives of purines and pyrimidines have an extra five-membered, unsaturated ring containing two added carbons (Scheme 1) (Singer & Bartsch, 1986). These ϵ compounds were originally discovered as modified tRNA bases in tRNAs (Nakanishi et al., 1970) and as products of RNA treated with 2-haloacetaldehydes (Kochetkov et al., 1971; Barrio et al., 1972). For instance, 6-methylimidazo[1,2-*a*]purine (6-methylwyosine or 6-methyl-1,N^2-ϵ-Gua) is a naturally occurring tRNA base (Agris, 1996). The strong fluorescence of ϵ-Ade has been utilized in studies of the interaction of $NADP^+$, ATP, and other derivatives with proteins (Leonard, 1984). The hepatocarcinogen vinyl chloride is oxidized to 2-chlorooxirane, which reacts with DNA to form 1,N^6-ϵ-Ade, 3,N^4-ϵ-Cyt, N^2,3-ϵ-Gua, 1,N^2-ϵ-Gua, and HO-ethanoGua (Guengerich et al., 1993, 1994; Müller et al., 1997). Other carcinogens [e.g., acrylonitrile and urethan (Guengerich et al., 1981a; Guenger-

Scheme 1: Structures of Five-Membered Ring Exocyclic Gua DNA Adducts



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[1] Abbreviations: ϵ, etheno; HO-ethanoGua, 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*]purine; HO-ethanodGuo, HO-ethanoGua deoxyribose; AcO-ethanoGua, 5,6,7,9-tetrahydro-7-acetoxy-9-oxoimidazo[1,2-*a*]purine; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Gua, guanine; Cyt, cytosine; Ade, adenine; dGuo, deoxyguanosine; dThd, thymidine; dCyd, deoxycytidine; dAdo, deoxyadenosine; propanoG, 1,N^2-propanoguanine; M_1G, pyrimido[1,2-*a*]purin-10(3*H*)-one; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography; CGE, capillary gel electrophoresis; MS, mass spectrometry; ES, electrospray; FAB, fast atom bombardment; Kf, Klenow fragment (of pol I) exo$^-$; RT, human immunodeficiency virus-1 reverse transcriptase; pol II, polymerase II exo$^-$; T7, polymerase T7 exo$^-$/thioredoxin mixture; pol β, rat polymerase β.

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ich & Kim, 1991)] can be oxidized to similar epoxides that react with DNA to form these same ϵ products. Of particular interest is the recent discovery that low but finite levels of 1,N^6-ϵ-Ade and 3,N^4-ϵ-Cyt have been found in DNA prepared from experimental animals and humans that were not exposed to any of the known precursors (Fedtke et al., 1990). The origin of these may be products of lipid peroxidation (El Ghissassi et al., 1995) or possibly halogenated drinking water contaminants (Kronberg et al., 1992).

Concern arises about the biological significance of the ϵ bases because of their presence in DNA and the carcinogenicity of chemicals that give rise to these. Because the reaction of DNA with appropriately functionalized two-carbon compounds yields all of these ϵ adducts, studies with individual bases incorporated in oligonucleotides and vectors are necessary to evaluate the ability of each base to cause misincorporation. 1,N^6-ϵ-Ade has been found to be weakly mutagenic in bacteria (Basu et al., 1993). 3,N^4-ϵ-Cyt was reported to be highly mutagenic in one bacterial study (Palejwala et al., 1993) but weakly mutagenic in others (Basu et al., 1993; Moriya et al., 1994). However, in the latter study, it was found to be considerably more mutagenic in a mammalian cell system (Moriya et al., 1994). N^2,3-ϵ-Gua appears to be rather mutagenic as judged by the results of two bacterial studies in which N^2,3-ϵ-dGTP was incorporated into plasmids (Cheng et al., 1991; Singer et al., 1987); however, the instability of the glycosidic bond (Khazanchi et al., 1993; Kusmierek et al., 1989) has precluded more systematic studies of the characterization of the miscoding properties of this base.

In the course of our studies on the reaction of 2-halooxiranes with DNA (Guengerich et al., 1979, 1981b; Guengerich & Raney, 1992), we characterized HO-ethanoGua as a major product of the reaction of 2-chlorooxirane with Gua derivatives (Guengerich et al., 1993). Recently, we have found that treatment of DNA with 2-chlorooxirane yields products in the concentration order N^7-(2-oxoethyl)Gua $\gg$ 1,N^6-ϵ-Ade > HO-ethanoGua > N^2,3-ϵ-Gua > 3,N^4-ϵ-Cyt > 1,N^2-ϵ-Gua (Müller et al., 1997). We were interested in the miscoding potential of HO-ethanoGua and comparison to 1,N^2-ϵ-Gua, which differs only in the absence of the elements of H_2O and, to our knowledge, has not been examined for miscoding properties. We report the synthesis of oligomers containing 1,N^2-ϵ-Gua and HO-ethanoGua at a defined position and misincorporation studies with five model polymerases. Some comparisons are made to studies done with the homologs containing six-membered rings, which are also found in DNA both before and after treatment of animals with carcinogens (Chaudhary et al., 1994; Nath & Chung, 1994).

EXPERIMENTAL PROCEDURES

Chemicals

Most chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,N^2-ϵ-dGuo was a gift of L. J. Marnett (Department of Biochemistry, Vanderbilt University School of Medicine). $H_2{}^{18}O$ was supplied by Cambridge Isotope Laboratories (Cambridge, MA). Reagents for oligonucleotide synthesis were purchased from PerSeptive Biosystems (Framingham, MA).

Instrumental and Chromatographic Analysis

TLC. TLC was done with silica gel F 254 (Merck, Gibbstown, NJ) as the adsorbent on glass plates. The separated compounds were visualized under UV light (254 nm) or by staining with an anisaldehyde/H_2SO_4 mixture and subsequent heating. Column chromatography was performed with silica gel 60 (70−230 mesh, Merck).

HPLC. HPLC was done with reversed-phase octadecylsilane columns: 10 × 250 mm Beckman Ultrasphere, 5 μm (Beckman, San Ramon, CA) for analysis and preparative isolation of base and nucleoside adducts; 4.6 × 250 mm and 10 × 250 mm YMC-Pack ODS-AQ, 5 μm (YMC, Wilmington, NC) for DNA oligomer purification and enzymatic digest analysis. The columns were connected to a Spectra-Physics 8700 pumping system (Thermo-Separation Products, Piscataway, NJ), with the effluent passing through a Hewlett-Packard 1040A diode array detector (Hewlett-Packard, Palo Alto, CA). The separation of the base and nucleoside adducts involved increasing gradients of CH_3OH (solvent B) in 50 mM NH_4HCO_2 at pH 5.0 (solvent A), usually increasing from 0 to 50% CH_3OH over 25 min, with a flow rate of 2.5 mL min^{-1}. Initial purification of the oligonucleotides with this solvent system was achieved with the following gradient: 0 min (99% A, 1% B), 50 min (70% A, 30% B), 55 min (10% A, 90% B), and 60 min (99% A, 1% B) at a flow rate of 2.5 mL min^{-1}. To increase purity to >99%, a second HPLC step was performed with the following gradient: 0 min (75% A, 25% B) and 50 min (70% A, 30% B), as well as a 20% (w/v) polyacrylamide gel electrophoresis purification. Enzymatic digests of the oligonucleotides (*vide infra*) were analyzed on the analytical YMC-Pack column with the above solvent system at a flow rate of 1.0 mL min^{-1} and the following gradients: unmodified and 1,N^2-ϵ-Gua-containing 19-mers, 0 min (99% A, 1% B), 30 min (80% A, 20% B), 45 min (50% A, 50% B), and 50 min (99% A, 1% B); 10-mer primer and HO-ethanoGua-containing 19-mer, 0 min (99% A, 1% B), 40 min (80% A, 20% B), 55 min (50% A, 50% B), and 60 min (99% A, 1% B).

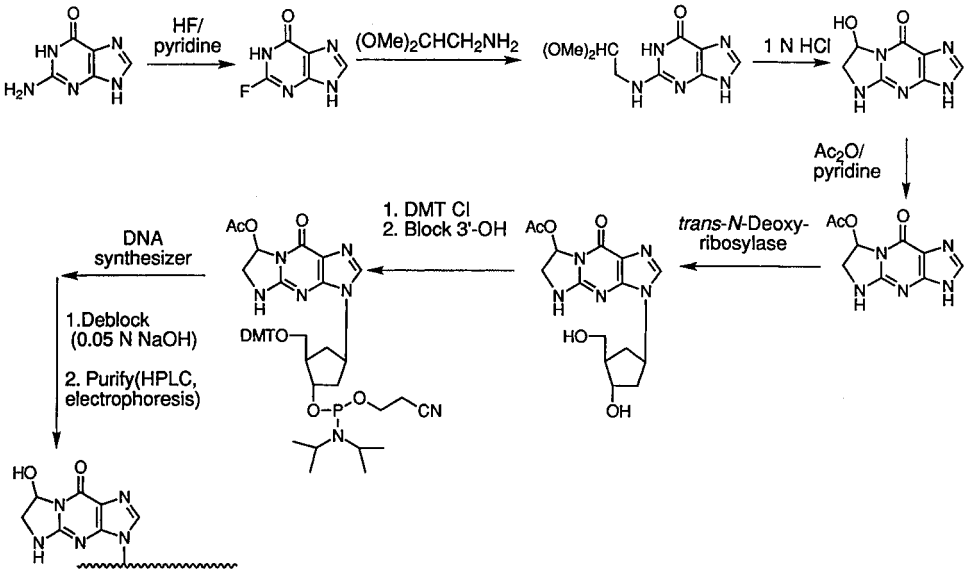
CGE. Oligonucleotide purity was evaluated using a Beckman P/ACE 2000 instrument (Beckman, Fullerton, CA) using the "ssDNA 100" gel capillary and "TRIS-borate-urea buffer" from the manufacturer. Samples were applied at −5 kV and run at −10 kV (30 °C).

UV and CD Spectroscopy. UV spectra were recorded using a a modified Cary 14/OLIS instrument (On-Line Instrument Systems, Bogart, GA). CD spectra were recorded using a JASCO J-720 spectropolarimeter (Japan Applied Spectroscopic Co., Tokyo, Japan).

NMR. ^{1}H-NMR spectra were recorded in $H_2O/{}^2H_2O$ mixtures or $(C^2H_3)_2SO$ using a Bruker AM 400 spectrometer (Bruker, Billerica, MA) in the Vanderbilt facility. ^{31}P-NMR spectra were acquired in C^2H_3CN on a Bruker AC 300 instrument with 85% H_3PO_4 as the external standard.

MS. Mass spectra of the base and nucleoside DNA adducts were collected on a Kratos Concept II HH instrument (Kratos, Manchester, U.K.) with FAB ionization and a mixture of glycerol, $(CH_3)_2SO$, and 3-nitrobenzyl alcohol as the matrix. Oligonucleotide ES mass spectra were obtained on a Finnigan TSQ 7000 ES instrument (Finnigan, San Jose, CA) equipped with a Unix computer system and deconvolution software.

Scheme 2: Synthesis of Oligonucleotides Containing HO-ethanoGua[a]



[a] $Ac_2O = (CH_3)_2CO$; DMT Cl = 4,4′-dimethoxytrityl chloride; Block 3′-OH indicates 2-cyanoethyl *N,N,N′,N′*-tetraisopropylchlorophosphoramidite.

HPLC/ES MS studies were conducted using the Finnigan TSQ 7000 triple-quadrupole mass spectrometer operating in the positive ion mode with an electrospray needle voltage of 4.5 kV. N_2 was used as the sheath gas (60 psi) to assist with nebulization and as the auxiliary gas (15 psi) to assist with desolvation. The stainless steel capillary was heated to 220 and 200 °C, respectively, to provide optimal desolvation, and the ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity (Müller et al., 1997). The tube lens and the heated capillary were operated at 74.5 and 20.0 V, respectively, and the electron multiplier was set at 1700 V. Selected reaction monitoring experiments were conducted by monitoring *m/z* for each protonated molecular ion. For the analysis of nucleosides, the oligomer was digested according to the method of Chaudhary et al. (1994). The digest was filtered through a 0.22 μm filter, and 250 μL aliquots were analyzed by HPLC/MS. HPLC involved the use of a Hewlett-Packard 1090 HPLC pumping system, connected to a Phenomenex Partisil ODS-3 reversed-phase column (3.2 × 250 mm, 5 μm, Phenomenex, Torrance, CA). The solvent system consisted of 10 mM $NH_4CH_3CO_2$ buffer at pH 5.5 (A) and 0.05% CH_3CO_2H in CH_3OH (v/v) (B). Separation of nucleosides involved the following gradient: 0 min (100% A, 0% B), 7 min (100% A, 0% B), 37 min (50%A, 50% B), 45 min (30% A, 70% B), 55 min (100% A, 0% B), and 70 min (100% A, 0% B). The flow rate was 0.25 mL min^{-1}. Precursor ions (the MH^+ ions of the DNA adducts) were generated in the ESI source and focused (quadrupole 1). These ions were dissociated in a collision cell (quadrupole 2), yielding defined product ions which were analyzed (quadrupole 3). The optimal collisional offset voltage to maximize the yields of HO-ethanodGuo product ions was −18 mV.

Synthesis of 1,N^2-ϵ-Gua-Modified 19-mer

5′-O-(4,4′-Dimethoxytrityl)-1,N^2-ϵ-dGuo. The dimethoxytritylation step was carried out as described in detail by DeCorte et al. (1996) starting with 50 mg (0.17 mmol) of 1,N^2-ϵ-dGuo. Flash column chromatography (CH_2Cl_2/CH_3OH/pyridine, 98:1:1, v/v/v, isocratic) yielded 76 mg (0.13 mmol, 74%) of 5′-*O*-(4,4′-dimethoxytrityl)-1,N^2-ϵ-dGuo: TLC $R_f = 0.70$ (CH_2Cl_2/CH_3OH, 9:1, v/v); MS *m/z* (assignment and relative abundance in parentheses) 594 (MH^+, 6), 303 (4,4′-dimethoxytrityl$^+$, 100), 176 [MH^+ − dimethoxytrityldeoxyribose (ϵ-Gua$^+$), 42]; ^{1}H-NMR [$(C^2H_3)_2SO$] δ 1.90 (m, 1 H, H-2′), 2.21 (m, 1 H, H-2″), 3.09 (m, 2 H, H-5′, H-5″), 3.40 (s, 1 H, H-4′), 3.67 (s, 3 H, CH_3O), 3.69 (s, 3 H, CH_3O), 3.90 (m, 1 H, H-3′), 5.75 (t, 1 H, H-1′), 6.79−6.89 (m, 4 H, aromatic), 7.09−7.26 (m, 7 H, aromatic), 7.33−7.37 (m, 2 H, aromatic), 7.75 (d, 1 H, H-6), 7.89 (d, 1 H, H-7), 8.57 (s, 1 H, H-2).

3′-O-[(N,N-Diisopropylamino)(2-cyanoethyl)phosphinyl]-5′-O-(4,4′-dimethoxytrityl)-1,N^2-ϵ-dGuo. The synthesis of the phosphoramidate derivative was achieved following established procedures (Decorte et al., 1996), which were modified by omitting the $NaHCO_3$ extraction step. The concentrated crude compound was instead directly subjected to flash column chromatography (CH_2Cl_2/ethyl acetate/pyridine, 69:30:1, v/v/v, isocratic), yielding 78 mg (0.098 mmol, 77%) of 3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethyl)-phosphinyl-5′-*O*-(4,4′-dimethoxytrityl)-1,N^2-ϵ-dGuo: TLC R_f = 0.72 (CH_2Cl_2/CH_3OH, 9:1, v/v); ^{31}P-NMR (C^2H_3CN) δ 149.42, 149.64. A signal at δ 15.52 (inorganic phosphorus) indicated ∼50% hydrolysis of the phosphoramidate. Due to this degradation, no further characterization of the product was performed and immediate oligonucleotide synthesis was carried out.

HO-ethanoGua-Modified 19-mer (Scheme 2)

HO-ethanodGuo. HO-ethanoGua was synthesized as described elsewhere (Guengerich et al., 1993), and an aliquot was enzymatically converted to the nucleoside with *trans-N*-deoxyribosylase (Müller et al., 1996) to serve as a chromatographic standard for the oligonucleotide digest assay.

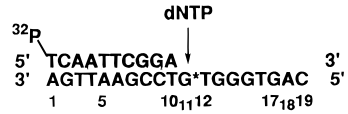
AcO-ethanoGua. HO-ethanoGua (25 mg, 0.13 mmol) was dried by repeated treatment with and evaporation of anhydrous pyridine *in vacuo* (3 × 2.5 mL). The compound was then redissolved in anhydrous pyridine (2 mL), and freshly distilled $(CH_3CO)_2O$ (0.061 mL, 0.65 mmol) was added and the mixture stirred for 3 h at room temperature under an Ar atmosphere. Most of the pyridine was removed *in vacuo*

without heating by repeated addition of hexane (3 × 2 mL). The reddish-brown residue was dissolved in CH_3OH and purified by HPLC. The peak eluting with a t_R of 19.0 min was collected, and the buffer salts were removed by repeated lyophilization to yield 29 mg (0.13 mmol, 95%) of AcO-ethanoGua: MS *m/z* 236 (MH^+, 58), 176 [$MH^+ - CH_3CO - H_2O$ (ϵ-Gua), 20]; ^{1}H-NMR [$(C^2H_3)_2SO$] δ 2.03 (s, 3 H, C*H*$_3$CO), 3.51 (d, 1 H, H-6), 3.91 (dd, 1 H, H-6), 6.91 (d, 1 H, H-7), 7.68 (s, 1 H, NH), 8.14 (s, 1 H, H-2), 12.53 (s, 1 H, NH). Formation of the desired product (as compared to potential amide formation) was further confirmed by an alkaline hydrolysis experiment. Treatment of the product with NaOH (pH 9−10) at room temperature resulted in immediate cleavage of the compound to the starting material as demonstrated by HPLC analysis.

Deoxyribosylation of AcO-ethanoGua. The attachment of the deoxyribose to the protected DNA base adduct was achieved by an enzymatic synthesis with *Lactobacillus helveticus trans-N*-deoxyribosylase following a recently described procedure (Müller et al., 1996). AcO-ethanoGua (29 mg, 0.13 mmol) was converted to tetrahydro-7-acetoxy-9-oxo-3-β-D-deoxyribofuranosylimidazo[1,2-*a*]purine (diastereomers **1** and **2**) (39 mg, 0.11 mmol, 90% combined yield). As expected, a set of two diastereomers due to the presence of two chiral centers in the molecule (H-7 and H-1′) was detected upon HPLC analysis, occurring in a 1:1 ratio. The two peaks eluting with a t_R of 20.2 min (diastereomer **1**) and 20.8 min (diastereomer **2**) were collected and characterized separately. Diastereomer **1**: MS *m/z* 352 (MH^+, 6), 337 ($MH^+ - CH_3$, 32), 236 (MH^+ − deoxyribose, 8), 176 [MH^+ − deoxyribose − CH_3CO − H_2O (ϵ-Gua^+), 6]; ^{1}H-NMR (^2H_2O) δ 2.14 (s, 3 H, C*H*$_3$CO), 2.51 (m, 1 H, H-2′), 2.77 (m, 1 H, H-2″), 3.78 (m, 3 H, H-5′, H-5″, H-6), 4.09 (m, 2 H, H-4′, H-6), 4.61 (m, 1 H, H-3′), 6.29 (t, 1 H, H-1′), 7.10 (d, 1 H, H-7), 7.98 (s, 1 H, H-2). Diastereomer **2**: MS *m/z* 352 (MH^+, 44), 236 (MH^+ − deoxyribose, 62); ^{1}H-NMR (^2H_2O) δ 2.12 (s, 3 H, C*H*$_3$CO), 2.49 (m, 1 H, H-2′), 2.73 (m, 1 H, H-2″), 3.75 (m, 3 H, H-5′, H-5″, H-6), 4.06 (m, 2 H, H-4′, H-6), 4.58 (m, 1 H, H-3′), 6.26 (t, 1 H, H-1′), 7.07 (d, 1 H, H-7), 7.96 (s, 1 H, H-2). While the above analytical data allowed no distinction between the two diastereomers, the antiphasic orientation of the CD spectra clearly demonstrated the postulated stereochemistry (*vide infra*).

*5′-O-(4,4′-Dimethoxytrityl)-5,6,7,9-tetrahydro-7-acetoxy-9-oxo-3-β-D-deoxyribofuranosylimidazo[1,2-a]purine (Diastereomers **1** and **2**).* The dimethoxytritylation was done with the combined materials from the previous step (39 mg, 0.11 mmol) following the established protocol (Decorte et al., 1996) with a modification in the extraction step. The 10% K_2CO_3 solution was replaced by H_2O to avoid hydrolysis of the acetyl ester. Flash column chromatography (ethyl acetate/CH_2Cl_2/CH_3OH/pyridine, 55:45:0.1:0.2, v/v/v/v, isocratic) yielded 54 mg (0.083 mmol, 75%) of 5′-*O*-(4,4′-dimethoxytrityl)-5,6,7,9-tetrahydro-7-acetoxy-9-oxo-3-β-D-deoxyribofuranosylimidazo[1,2-*a*]purine (diastereomers **1** and **2**): TLC R_f = 0.61 and 0.71 (ethyl acetate/CH_2Cl_2/CH_3OH, 50:50:0.2, v/v/v); MS *m/z* 654 (MH^+, 6), 303 (4,4′-dimethoxytrityl$^+$, 100); ^{1}H-NMR [$(C^2H_3)_2SO$] δ 1.71 (m, 1 H, H-2′), 1.87 (m, 1 H, H-2″), 2.13 (s, 3 H, C*H*$_3$CO), 2.79 (m, 3 H, H-5′, H-5″, H-6), 3.05 (m, 2 H, H-4′, H-6), 3.70 (s, 3 H, C*H*$_3$O), 3.72 (s, 3 H, C*H*$_3$O), 4.06 (m, 1 H, H-3′), 5.62 (t, 1 H, H-1′), 6.67−6.82 (m, 4 H, aromatic), 6.90 (d, 1 H, H-7), 7.12−7.30 (m, 7 H, aromatic), 7.31−7.44 (m, 2 H, aromatic), 7.50 (s, 1 H, H-2).

Scheme 3: Oligonucleotides[a]



[a] G* = Gua, 1,N^2-ϵ-Gua, or HO-ethanoGua. The site of the first incorporation is indicated with an arrow.

*3′-O-[(N,N-Diisopropylamino)(2-cyanoethyl)phosphinyl]-5′-O-(4,4′-dimethoxytrityl)-5,6,7,9-tetrahydro-7-acetoxy-9-oxo-3-β-D-deoxyribofuranosylimidazo[1,2-a]purine (Diastereomers **1** and **2**).* The phosphoramidation step followed the procedure used for the 1,N^2-ϵ-dGuo derivative. Direct application of the crude product to flash column chromatography (CH_2Cl_2/ethyl acetate/pyridine, 69:30:1, v/v/v, isocratic) yielded 56 mg (0.066 mmol, 80%) of 3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethyl)phosphinyl]-5′-*O*-(4,4′-dimethoxytrityl)-5,6,7,9-tetrahydro-7-acetoxy-9-oxo-3-β-D-deoxyribofuranosylimidazo[1,2-*a*]purine (diastereomers **1** and **2**): TLC R_f = 0.60 (ethyl acetate/CH_2Cl_2/CH_3OH, 50:49.8:0.2, v/v/v); ^{31}P-NMR (C^2H_3CN) δ 149.62, 149.72. Again, a signal at δ 15.53 (inorganic phosphorus) indicated ∼50% hydrolysis of the product. Thus further characterization was not done in favor of immediate oligonucleotide synthesis.

Oligonucleotides (Scheme 3)

Oligonucleotides were synthesized with an Expedite Nucleic Acid Synthesis System (Millipore Corp., Bedford, MA) on a 1 μmol scale using 4-*tert*-butylphenoxyacetyl protecting groups (PerSeptive Biosystems) according to the manufacturer's standard protocol. To compensate for the hydrolysis of the phosphoramidate, the DNA adduct nucleosides were dissolved, making them twice as concentrated (100 mg mL^{-1}) as the unmodified nucleosides (50 mg mL^{-1}). Nevertheless, a drop of 50% in overall coupling efficiency was noted after the insertion of the respective DNA adducts into the sequences in each synthesis. Following synthesis, the beads from two 1 μmol cassettes of each DNA oligomer were suspended in 50 mM NaOH (3 mL) and stirred slowly for 24 h at room temperature. After cautious neutralization with 50 mM CH_3CO_2H (1 mL), the solutions were filtered through a 0.22 μm filter and aliquots were subjected to HPLC analysis and preparative workup. The unmodified 19-mer and the 10-mer primer (Scheme 3) each showed one major peak with a t_R of 49.0 and 54.2 min, respectively, which were collected and repeatedly lyophilized to remove buffer salts. Yields after the initial purification were 39 and 70 A_{260} units [0.21 and 0.70 μmol, respectively, estimated by the method of Borer (1975)]. HPLC analysis of the 1,N^2-ϵ-Gua-modified 19-mer demonstrated two peaks with t_Rs of 49.7 and 50.2 min in a 1:1 ratio, which were purified separately. Upon enzymatic digestion, the second peak was identified to contain the DNA adduct, and a yield of 38 A_{260} units (∼0.2 μmol) was determined. The OH-ethanoGua-modified 19-mer was also a mixture of two product peaks, eluting with t_Rs of 50.5 and 51.5 min in a ratio of 3:7. HPLC analysis of the enzymatic digest of the oligomer revealed that the first product peak contained the modified nucleoside; its yield was 19 A_{260} units (∼0.1 μmol). All DNA oligomers were subjected to a second HPLC purification and further

polyacrylamide gel electrophoresis to obtain purities of >99% as evaluated by CGE (*vide infra*).

Characterization of Oligonucleotides

Direct injection electrospray mass spectrometry was used to verify the identities of three of the four oligomers used: 10-mer, calcd M_r of 3027.0, anal. 3026.9; unmodified 19-mer, calcd M_r of 5883.9, anal. 5884.9; and 1,N^2-ϵ-Gua 19-mer, calcd M_r of 5907.9, anal. 5908.3 (see Supporting Information). Attempts to analyze the intact HO-ethanoGua-containing 19-mer directly were unsuccessful.

Each oligonucleotide (1 A_{260} unit = ~5.4 nmol) was digested in a two-step protocol. The first step involved dissolving the DNA oligomer in 20 μL of 10 mM Tris-HCl buffer (pH 7.0) containing 10 mM $MgCl_2$ and the addition of 8 μg of nuclease P_1 (Sigma Chemical Co., St. Louis, MO), followed by a 3 h incubation at 37 °C. In the second step, 20 μL of 100 mM Tris-HCl buffer (pH 9.0), 9 μg of snake venom phosphodiesterase (Sigma), and 6 μg of alkaline phosphatase (Sigma) were added and the digest was kept for another 3 h at 37 °C. Each sample was diluted with 100 μL of H_2O and filtered through a 0.22 μm filter; a 50 μL aliquot was analyzed by HPLC, and the concentrations of the individual deoxyribonucleosides were estimated by comparisons made with external standards: 10-mer primer (Scheme 3), theoretical (relative ratios in parentheses) dCyd (2), dGuo (2), dThd (3), dAdo (3) and found dCyd (2.0), dGuo (2.4), dThd (3.3), dAdo (3.4); unmodified 19-mer template, theoretical dCyd (3), dGuo (7) dThd (5), dAdo (4) and found dCyd (3.0), dGuo (7.2), dThd (5.3), dAdo (4.2); 1,N^2-ϵ-Gua-containing 19-mer template, theoretical dCyd (3), dGuo (6), dThd (5), dAdo (4), 1,N^2-ϵ-dGuo (1) and found dCyd (3.0), dGuo (6.1), dThd (5.3), dAdo (4.2), 1,N^2-ϵ-dGuo (1.0). In the case of the HO-ethanoGua-containing 19-mer template, the OH-ethanodGuo and dThd were not resolved by HPLC, and the sample ($^1/_{10}$ the normal amount was digested) was analyzed by combined HPLC/ES MS/MS (Müller et al., 1997). HO-ethanodGuo was identified and confirmed by repeating the analysis in the presence of a known amount of 4,5,8-^{13}C-labeled compound (Müller et al., 1997) (*vide infra*).

Enzymes

trans-N-Deoxyribosylase was partially purified from *L. helveticus* (Müller et al., 1996) by L. K. Hutchinson in the Department of Biochemistry at the Vanderbilt University School of Medicine. Recombinant rat pol β was a gift of S. Wilson (University Texas, Galveston, TX). Other polymerases were purified by L. L. Furge from *Escherichia coli* (Lowe & Guengerich, 1996) using stock plasmids provided: Kf (C. Joyce, Yale University, New Haven, CT) (Derbyshire et al., 1988), pol II (M. F. Goodman, University of Southern California, Los Angeles, CA) (Cai et al., 1995), pol T7 and thioredoxin (K. A. Johnson, Pennsylvania State University, University Park, PA) (Kati et al., 1991; Lunn et al., 1984), and RT (S. Hughes, Frederick Cancer Facility, Frederick, MD) (Le Grice & Grüninger-Leitch, 1990).

Polymerase Assays

General. The 10-mer primer (2 μM) was 5′-end-labeled using T4 polynucleotide kinase and purified on a Biospin column (Bio-Rad, Hercules, CA). Template and labeled primer (2:1 molar ratio) were annealed in a buffer containing 50 mM sodium MOPS (pH 7.0), 50 μg of bovine serum albumin per milliliter, and 5 mM $MgCl_2$ by incubating at 90 °C for 10 min and slowly cooling to room temperature. The different assays were then performed as follows.

Primer Extension. All four dNTPs, at 100 μM each, were incubated in the presence of 50 or 100 nM primer/template mixture in 10 μL of 50 mM sodium MOPS buffer (pH 7.0) containing 8 mM $MgCl_2$, 4 mM dithiothreitol, 2 μg of bovine serum albumin per milliliter, and the particular polymerase, added at several different concentrations. These reactions were performed for 30 min at 25 °C with all polymerases, except for pol II which was incubated at 37 °C. Reactions were quenched by the addition of 10 mM EDTA in 90% formamide (v/v), and the reaction products were analyzed by electrophoresis on 20% (w/v) denaturating polyacrylamide gels using Sequagel (National Diagnostics, Atlanta, GA).

One-Base Incorporation. ^{32}P-labeled 10-mer primers were extended using unmodified or adducted 19-mer templates in the presence of single dNTPs (100 μM) with 50 nM Kf or pol II, 200 nM T7, or 4.6 μM pol β. The incubation times were 15 min for pol I and pol II, but in the case of T7 and pol β, the reaction time was extended to 30 min.

Steady-State Kinetics. The general approach of Boosalis et al. (1987) was used, as modified in this laboratory (Lowe & Guengerich, 1996). A Molecular Dynamics Model 400E Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA) was used to measure incorporation of radioactivity, and the results were analyzed using a k·cat computer program (Biometallics, Princeton, NJ) as described elsewhere (Lowe & Guengerich, 1996).

Nucleotide Sequence Analysis. The bands corresponding to 100 pmol of the primer extension products obtained with the several enzymes were extracted from the gel by shaking overnight at 4 °C in distilled H_2O. The products were recovered by C_2H_5OH precipitation and sequenced as described by Maxam and Gilbert (1980) except for the T-specific reaction, where the method described by Friedmann and Brown (1978) was used.

RESULTS

Synthesis of Protected HO-ethanodGuo. The preparation of an oligonucleotide substituted with HO-ethanoGua required development of an appropriate strategy for incorporation of a suitably substituted phosphoramidite (Scheme 2). The free hydroxyl group on the exocyclic ring should be blocked in order to prevent coupling in the phosphoramidite reaction of the DNA synthesizer, using a group that can be readily deprotected. The hydroxyl group was readily acetylated in pyridine. The deoxyribose moiety could be enzymatically added to HO-ethanoGua with purine nucleoside phosphorylase, but the acetylated derivative gave no nucleoside product with this enzyme. However, *L. helveticus trans-N*-deoxyribosylase catalyzed the reaction and was used in the synthesis (Müller et al., 1996).[2]

HPLC of the acetylated nucleoside revealed two closely eluting peaks with similar areas. These two compounds had identical UV, mass, and ^{1}H-NMR spectra. However, the CD spectra were mirror images of each other (see Supporting

[2] The strategy of inserting a 2-fluoroinosine group into the oligomer, adding 2-aminoacetaldehyde dimethyl acetal, and then closing the ring (Decorte et al., 1996; Guengerich et al., 1993) was considered but not attempted because studies with the nucleoside indicated that the glycosidic bond was hydrolyzed under all conditions used to cleave the acetal.

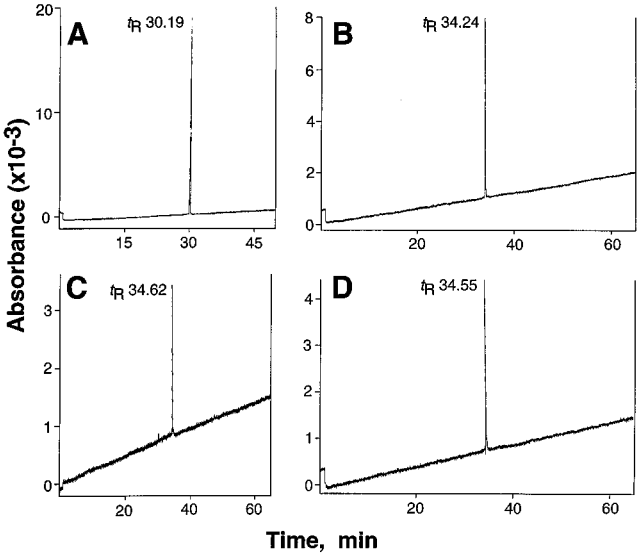


FIGURE 1: CGE traces of oligonucleotides: (A) primer (10-mer), (B) 19-mer template (unmodified), (C) 19-mer containing 1,N^2-ϵ-Gua, and (D) 19-mer containing HO-ethanoGua.

Information), supporting the conclusion that these are diastereomers and differ in their configuration at C7. The absolute configurations of these diastereomers were not determined. Treatment with 0.10 N NaOH led to the loss of the CD spectra at wavelengths of >250 nm, and a single HPLC peak was eluted.

The evidence for the diastereomers raised the issue of whether individual hydroxyl diastereomers exist and could be used separately. Alternatively, the closed and open forms, HO-ethanoGua and N^2-(2-oxoethyl)Gua, respectively, might be in equilibrium and the hydroxyl group would epimerize (Scheme 1). In order to test this possibility, the exchange of oxygen from $H_2{}^{18}O$ was examined with HO-ethanodGuo, using FAB MS and measuring the intensity of the ions at *m/z* 310 and 312. At neutral pH, 72% of the HO-ethanodGuo had incorporated one ^{18}O atom following overnight incubation at room temperature. When the experiment was repeated in 0.10 N NaOH, conditions to be used in deblocking after oligonucleotide synthesis, the nucleoside had completely incorporated one ^{18}O atom (>95% incorporation). Thus, HO-ethanoGua appears to be in relatively rapid equilibrium between the open and the two (stereochemically different) closed forms.

Synthesis and Characterization of Oligonucleotides. 1,N^2-ϵ-dGuo and the acetoxy-protected form of HO-ethanodGuo prepared above were treated to add 5′-dimethoxytrityl and 3′-phosphoramidite groups in the usual manner and used to prepare 19-mer oligonucleotides (Scheme 2). The coupling yields at the site of the derivative were 50% of those at the other steps, even with excess derivatized phosphoramidites. Deblocking of the HO-ethanoGua derivative was done in 0.05 N NaOH instead of NH_4OH to remove the acetoxy group. The 19-mer templates and the 10-mer primer were purified by a combination of reversed-phase HPLC and preparative polyacrylamide gel electrophoresis, taking care to slice only the middle portion of the gel band in the more demanding separations.

CGE electrophoretograms of the purified oligomers are shown (Figure 1). Purity in all cases was judged to be >99%.

The oligomers containing 1,N^2-ϵ-Gua and HO-ethanoGua were digested with nucleases and phosphatase, and the

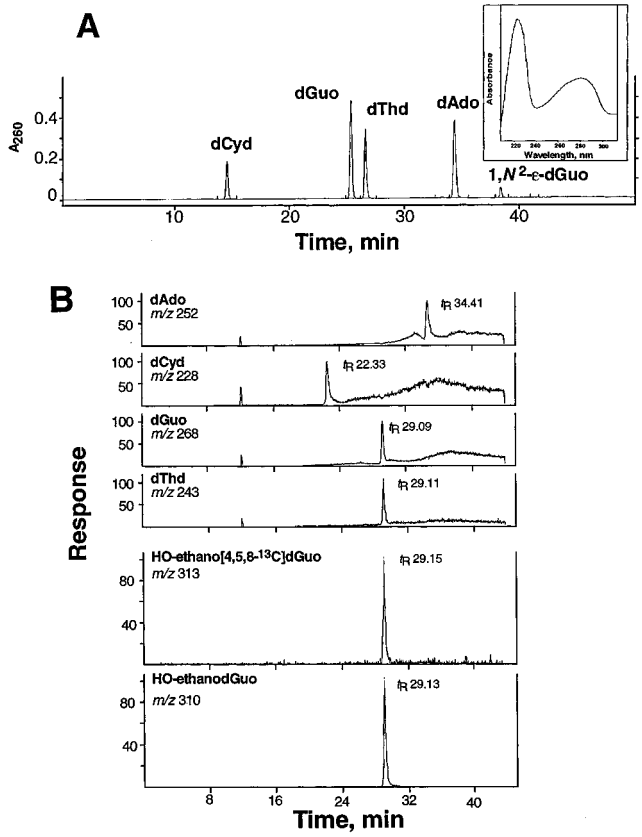


FIGURE 2: Analysis of digests of modified oligomers. See Experimental Procedures for description. (A) HPLC/UV of the digest of 1,N^2-ϵ-Gua-containing 19-mer, with the spectrum of 1,N^2-ϵ-dGuo in the inset. (B) HPLC/ES MS/MS of the digest of HO-ethanoGua-containing 19-mer, with *m/z* traces shown for the appropriate MS/MS transitions of dAdo, dCyd, dGuo, dThd, HO-ethanodGuo, and [4,5,8-^{13}C]HO-ethanodGuo (heavy atom derivative added to verify t_R) (Müller et al., 1997). HPLC/selected reaction monitoring/MS/MS was done monitoring the 310 to 194 or the 313 to 197 transition (loss of deoxyribose) in the latter two cases. No *m/z* 313 transition was seen in the absence of added ^{13}C material, but the same *m/z* 310 transition peak was seen.

products were analyzed by HPLC (Figure 2). The distinct UV spectra and t_R permitted positive identification of the former oligomer, using diode array spectroscopy, to characterize 1,N^2-ϵ-dGuo (Figure 2A). With HO-ethanodGuo, the similarity of the t_R to that of dThd and the lack of such a distinct spectrum precluded such analysis. The nucleoside/phosphatase digest of this oligomer was analyzed by HPLC/ES MS/MS as reported elsewhere (Müller et al., 1997). The presence of HO-ethanoGua was clearly verified by monitoring the appropriate transition (Figure 2B).

Polymerase Extension Assays. Five polymerases were studied because of their availability and existing literature regarding their properties. The initial experiments involved analysis of the extension of the 10-mer primer/19-mer template complexes in the presence of a mixture of all four dNTPs. With all five polymerases, both of the modified templates retarded extension but some full-length product was obtained (Figures 3−7). The results are qualitatively summarized in Table 1.

With all of the polymerases except pol β, extension of the HO-ethanoGua-containing 19-mer yielded a ladder of all the possible products differing in length by one base. Kf also yielded a large amount of 11-mer with both modified oligomers, corresponding to addition of only one base at the site of the modified Gua. Under these conditions, Kf often

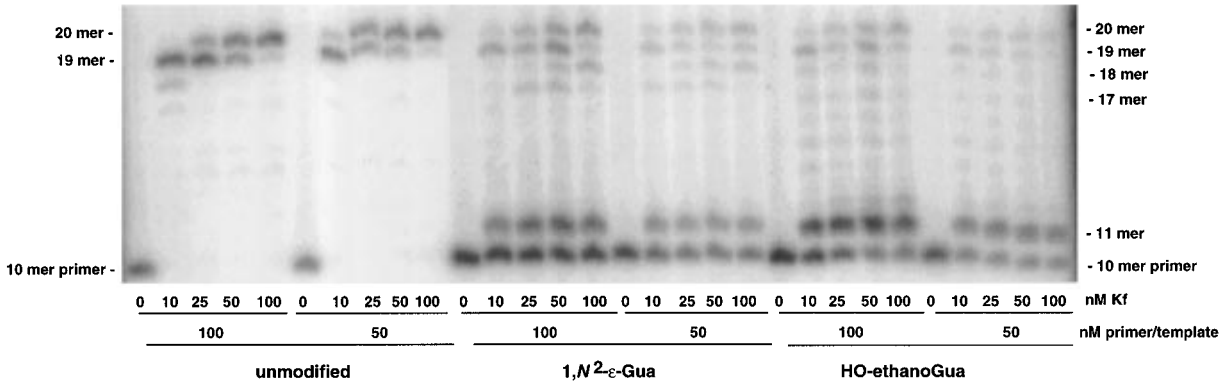


FIGURE 3: Extension of 10-mer primer by Kf in the presence of all four dNTPs.

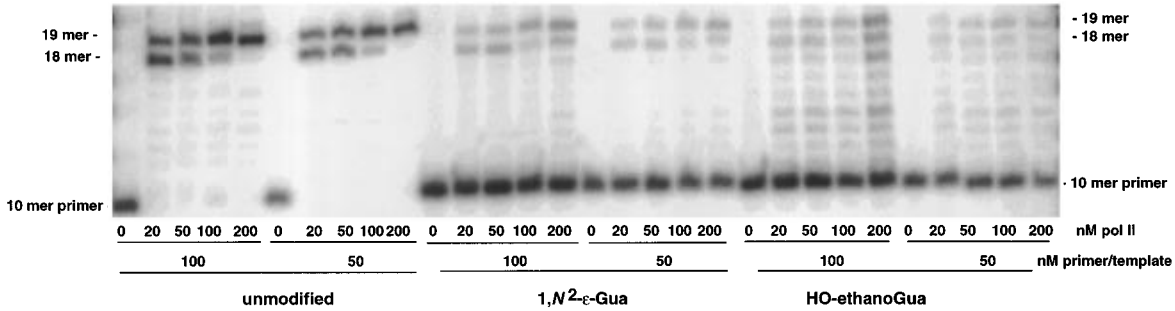


FIGURE 4: Extension of 10-mer primer by pol II in the presence of all four dNTPs.

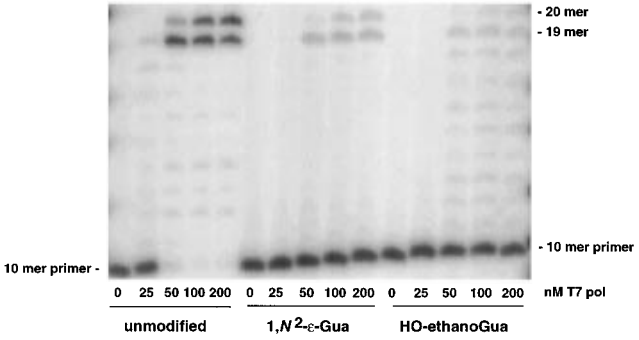


FIGURE 5: Extension of 10-mer primer by T7 pol in the presence of all four dNTPs.

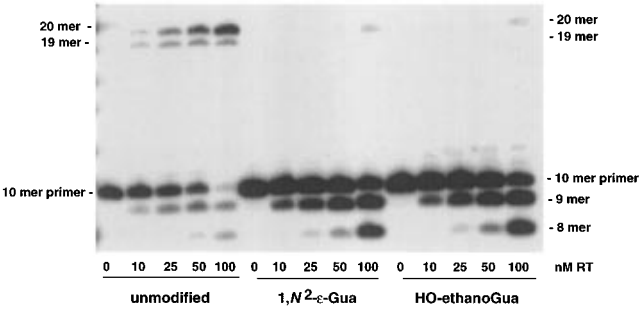


FIGURE 6: Extension of 10-mer primer by RT in the presence of all four dNTPs.

extends a primer one base beyond the length of the template (Clark et al., 1987; Clark, 1988), and this phenomenon was observed with all three templates with Kf, T7, and RT (Figures 3, 5, and 6). Although RT is usually considered to be devoid of exonuclease activity (Kornberg & Baker, 1992), digestion of this sequence was observed, and the extent was increased when the modified bases were present (Figure 6). This appears not to be a sequence-dependent activity, because we also observed such digestion with another oligomer used previously in other work (Lowe & Guengerich, 1996) and

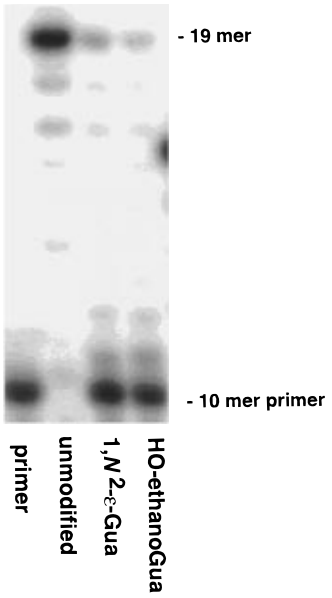


FIGURE 7: Extension of 10-mer primer by pol β in the presence of all four dNTPs.

inspection of other work indicates similar RT processing (Kati et al., 1992).

Nucleotide Sequence Analysis of Extended Primers. The products obtained by primer extension in the presence of all four dNTPs were analyzed using modifications of the Maxam−Gilbert method (Maxam & Gilbert, 1980), and the results are summarized in Table 2. The extent of misincorporation cannot be analyzed quantitatively in such assays, and the results must be considered qualitatively. Typical gels obtained in the analysis of the Kf products with the modified bases are shown in Figure 8. Products from the reactions with RT and pol β were not analyzed.

Kf and pol II yielded more misincorporation than T7. Differences between the products derived with the 1,N^2-ϵ-

Table 1: Products of Polymerase Extension Assays of 10-mer Primer in the Presence of All Four dNTPs and a 19-mer Template[a]

	products from each template (length)		
polymerase	Gua	1,N^2-ϵ-Gua	HO-ethanoGua
Kf	19, 20	11, 17−20	11, 19, 20 (plus 12−18)
pol II	18, 19	18, 19	18, 19 (plus 11−17)
T7	19, 20	18−20	19, 20 (plus 11−18)
RT	18, 19	20, 9−7	20, 9−7
pol β	19	19	19

[a] See Scheme 3 and Figures 3−7.

Table 2: Nucleotide Sequence Analysis of a 10-mer Primer Extended in the Presence of All Four dNTPs and 19-mer Primer

		bases incorporated opposite Gua or Gua adduct		
polymerase	length of product	Gua	1,N^2-ϵ-Gua	HO-ethanoGua
Kf	19,[a] 20	C	G, C (A) [b]	G, T (A)[b]
	18		−1 base frameshift	−1 base frameshift
	17[a]		−2 base frameshift	−2 base frameshift
	11		A, G	A, G
pol II	19	C	A, T (C)[b]	A, T (C)[b]
	18		−1 base frameshift	−1 base frameshift
T7	19, 20	C	C	C
	18		−1 base frameshift	−1 base frameshift

[a] See Figure 8. [b] Tentative assignment. See Figure 8 for data in the case of 1,N^2-ϵ-Gua and Kf. See Figure 9 and Tables 3 and 4 for single-base incorporation results.

Gua- and HO-ethanoGua-containing oligomers were seen. Also, there were differences in the bases misincorporated in the 11-mer (one-base incorporation) and the fully extended primer with Kf. In the case of the 11-mer product formed with Kf, both A and G were clearly present (Figure 8B). In the 19-mer Kf product, G was clearly inserted (Figure 8C) but the relative intensities of bands in the G and G + A lanes, compared with those of the standard oligomers (Figure 8A), did not allow an unambiguous call for A. Similarly, a definite call for C in the case of the 19-mer product of pol II was not possible because of the insertion of T and the relative band intensities in the T and T + C lanes.

Primer Extension Assays with Individual dNTPs. The incorporation assays were repeated to incorporate only a single base; i.e., only use one dNTP in the assay. In all cases, it was possible to force some misincorporation (Figure 9), even with the unmodified oligomer. Following incorporation of a single base, the next position in the template contains a T, so incorporation of an A is then favored. In most cases in which dATP was used, some 12-mer was found.

The results are summarized in Table 3 and are considered qualitative. Of interest is the observation of the 13-mer product in the reaction of the 1,N^2-ϵ-Gua template with Kf (Figure 9A). Incorporation of three Cs at sites 11−13 is highly unlikely, since pairing of dCTP opposite a T would have to occur after the incorporation of dCTP opposite 1,N^2-ϵ-Gua (Scheme 3). Moreover, further incorporation of more dCTP would have been expected. An alternative is that the 1,N^2-ϵ-Gua and the following T slip and the three Cs are incorporated opposite the G_3 triplet at positions 13−15 (Scheme 3). Sequence analysis indicated that this latter slippage event occurred (Figure 8D).

Steady-State Kinetic Assays of Single-Base Incorporation. Single dNTP incorporation assays were done under typical steady-state conditions (Boosalis et al., 1987) in order to provide more quantitative estimates of the tendency of polymerases to misincorporate bases. The results are summarized in Table 4.

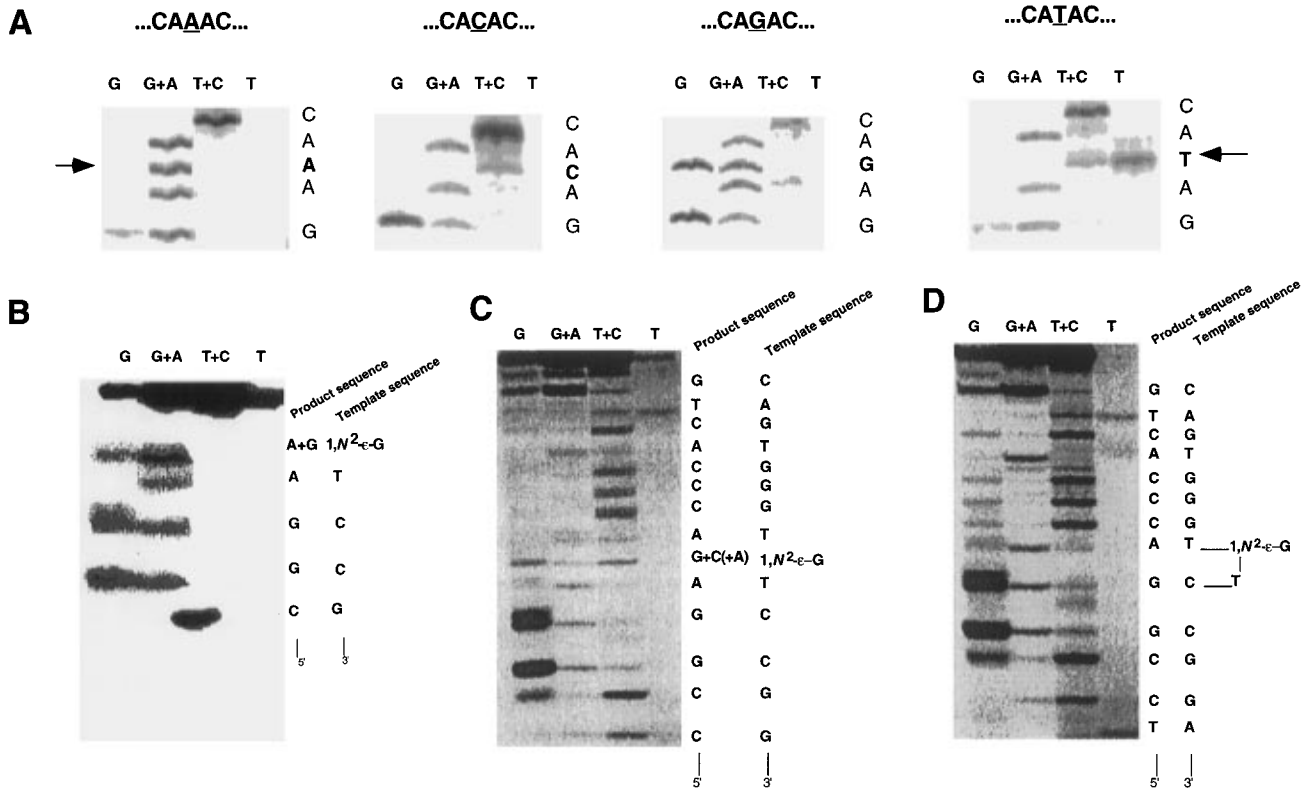


FIGURE 8: Nucleotide sequence analysis. (A) Residues 9−13 of standard 19-mers synthesized to contain A, C, G, and T at position 11. Also shown are the (B) 11-mer, (C) 19-mer, and (D) 17-mer formed by extension when paired to the 19-mer template containing 1,N^2-ϵ-Gua.

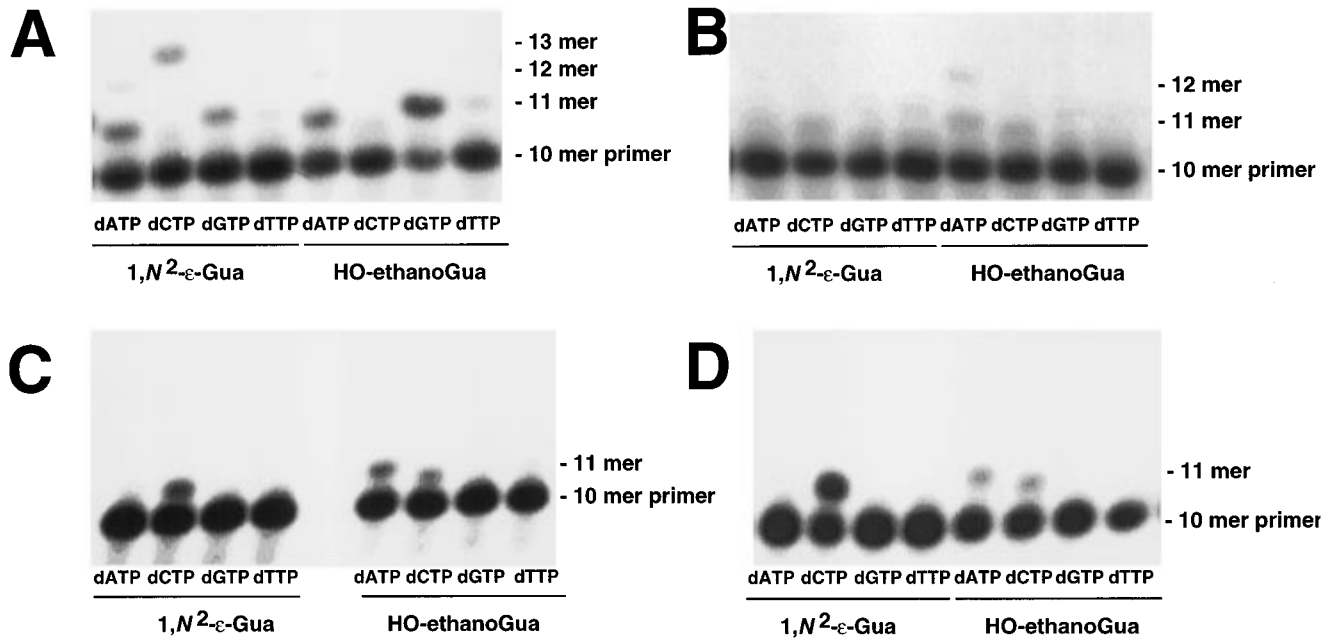


FIGURE 9: Extension of primer in the presence of a single dNTP when paired with modified 19-mers: (A) Kf, (B) pol II, (C) T7, and (D) pol β.

Table 3: Products of Polymerase Extension Assays of 10-mer Primers in the Presence of Modified 19-mers and a Single dNTP

	products from each template[a]		
polymerase	Gua	1,N^2-ϵ-Gua	HO-ethanoGua
Kf	C ≫ A, G, T	A > C > G	A > C > G
pol II	C ≫ A, G, T	A > C	A > C
T7	C	C	A > C
pol β	C	C	A > C

[a] See Figure 9.

Kf misincorporated both A and G opposite 1,N^2-ϵ-Gua and HO-ethanoGua. Compared to the normal incorporation of dCTP opposite Gua, k_{cat} was considerably lower in all cases and K_m was much higher. With the HO-ethanoGua-containing template, a higher misincorporation frequency was seen for dATP than for dGTP. With the 1,N^2-ϵ-Gua-containing template, the opposite pattern was observed. However, the misincorporation frequencies cannot be considered absolute because the C incorporation is considered to involve a slipped frameshift intermediate (*vide supra*).

With T7, k_{cat} values for all incorporations opposite modified Gs were considerably lower than those for dCTP opposite G, but the K_m values were not altered. Little misincorporation was seen with the 1,N^2-ϵ-Gua template. The misincorporation frequency measured with the HO-ethanoGua template indicated that the enzyme inserted dATP as readily as dCTP.

DISCUSSION

Procedures were developed for the incorporation of the DNA adducts 1,N^2-ϵ-Gua and HO-ethanoGua into defined oligonucleotides, which were purified, characterized, and used in *in vitro* misincorporation studies. The results are of interest in terms of understanding the potential genotoxic properties of these two adducts and their possible contributions in tumors caused by agents that yield these adducts.

Both 1,N^2-ϵ-Gua and HO-ethanoGua strongly blocked replication with all polymerases examined (Figures 4−8). This appears to be the result of a decreased rate of incorporation at the position opposite the modified base in most cases (Figures 3−7), although more detailed studies are in order to determine which step(s) in the incorporation is perturbed. With Kf, a strong block seems to occur after one base is incorporated. The purines A and G were readily incorporated opposite both 1,N^2-ϵ-Gua and HO-ethanoGua by several polymerases (Tables 2 and 4). We also found that pol I, pol II, and T7 are able to extend the primer after −1 or −2 base slippages at the adduct site (Figure 8D and Table 2), and we detected some 17- and 18-mer bands in addition to the full-length extension product.

Some differences between the effects of 1,N^2-ϵ-Gua and HO-ethanoGua were noted. dATP was inserted more readily opposite HO-ethanoGua with several of the polymerases. This result is of interest in that the level of HO-ethanoGua formed by treatment of DNA with 2-chlorooxirane is more than 1 order of magnitude higher than that of 1,N^2-ϵ-Gua (Müller et al., 1997). However, these results will need to be considered in the context of further work on rates of DNA repair and mutagenesis in cellular systems. The steady-state kinetic studies indicate a greater tendency for misincorporation opposite HO-ethanoGua than 1,N^2-ϵ-Gua with T7 (Table 4).[3] It is not possible to make a quantitative interpretation of the Kf misincorporation frequencies (Table 4) because of the apparent tendency of the template to slip rather than incorporate at the site of the adduct (Scheme 4 and Figure 8B).

We noted some similarities in comparing our results with those described recently by Hashim and Marnett (1996) on the effect of propanoG on the fidelity of Kf in the same sequence context. PropanoG and the derivatives shown in

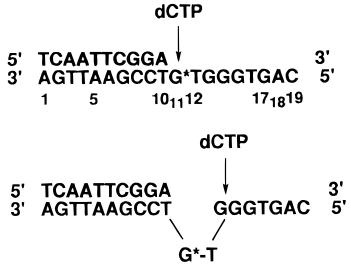
[3] Some comment is in order about the K_m values in Table 4. The K_m value found (for dCTP) for the unmodified oligonucleotide and Kf is the same as that reported by Hashim and Marnett (1996); a large increase was seen with all incorporated dNTPs, as also observed here. The physical meaning of K_m in polymerase assays is not clear (Johnson, 1993); K_m is certainly not a simple dissocation constant. K_d values must be obtained through more complex pre-steady-state experiments (Johnson, 1993; Lowe & Guengerich, 1996; Furge & Guengerich, 1997).

Table 4: Steady-State Kinetic Parameters for dNTP Incorporation

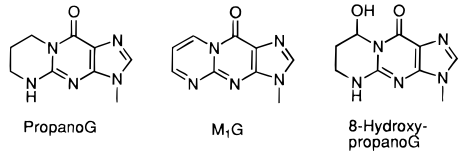
	Kf			T7		
pairing	k_{cat} (min^{-1})	K_m (μM)	misincorporation frequency[a]	k_{cat} (min^{-1})	K_m (μM)	misincorporation frequency
Gua·dCTP	81 ± 5	0.027 ± 0.008		0.15 ± 0.01	1.8 ± 0.7	
1,N^2-ϵ-Gua·dCTP	0.54 ± 0.02	67 ± 8		0.0046 ± 0.0002	1.1 ± 0.3	
1,N^2-ϵ-Gua·dGTP	0.27 ± 0.02	31 ± 8	1.1[b]			
1,N^2-ϵ-Gua·dATP	0.12 ± 0.01	75 ± 24	0.2[b]			
HO-ethanoGua·dCTP	0.28 ± 0.02	5 ± 2		0.0032 ± 0.002	0.48 ± 0.17	
HO-ethanoGua·dGTP	0.35 ± 0.01	41 ± 7	0.15			
HO-ethanoGua·dATP	0.17 ± 0.01	7 ± 3	0.45	0.0052 ± 0.0003	0.94 ± 0.33	1.2

[a] Misincorporation frequency = $(k_{cat}/K_m)_{dNTP}/(k_{cat}/K_m)_{dCTP}$ (where dNTP ≠ dCTP). [b] These are considered to be much higher because the values for dCTP incorporation reflect incorporation of three dCTPs. See the text for discussion of a possible frameshift.

Scheme 4: Postulated Template Slippage in Kf Misincorporation Events



Scheme 5: Structures of Related Six-Membered Exocyclic Ring Gua DNA Adducts



Scheme 5 differ from 1,N^2-ϵ-Gua and HO-ethanoGua in the size of the exocyclic ring. Hashim and Marnett (1996) reported primarily misincorporation of dATP opposite propanoG in the full-length extended product with Kf. Interestingly, we found that such miscoding is also detectable using pol II, T7, and pol β opposite HO-ethanoGua; with 1,N^2-ϵ-Gua, the same misincorporation is seen with pol II but not with T7 or pol β. As in the case of propanoG and Kf (Hashim & Marnett, 1996), the most common frame shift mutation corresponded to a one-base deletion at the adduct site with all of the enzymes studied (except with pol β, where only full-length extended product was detectable). With Kf in particular, we were able to detect substantial two-base deletion opposite 1,N^2-ϵ-Gua and HO-ethanoGua, to a greater extent than that detected opposite propanoG under the same conditions (Hashim & Marnett, 1996). The one-base deletion opposite propanoG was also explained in previous work (Shibutani & Grollman, 1993) by the slippage between A at the 3′-primer terminus and the T residue 5′ to the adduct site and can be extrapolated to our two adducts. In contrast, the two-base slippage (resulting in a two-base deletion at the adduct site) cannot be explained in our case by C incorporation opposite the adducts, since there was a negligible amount of C incorporation opposite 1,N^2-ϵ-Gua and no C incorporation opposite HO-ethanoGua. However, this two-base deletion was more pronounced in the template containing 1,N^2-ϵ-Gua than HO-ethanoGua, when extensions were performed with Kf under the same conditions. The similar structures of M_1G, the unsaturated form of propanoG [conjugation product of malondialdehyde (Seto et al., 1983; Basu et al., 1988), Scheme 5], and 1,N^2-ϵ-Gua should provide an interesting comparison of misincorporation, slippage, and extension by several polymerases and are the focus of further investigations.

Incorporation of T opposite the Gua adducts was observed only in the full-length product from the primer extension with Kf and the HO-ethanoGua-containing template (Table 2). The corresponding mutation was seen in an *in vivo* mutagenesis study with this adduct, along with mutations corresponding to incorporation of G and A.[4] However, a high level of mutations corresponding to insertion of T opposite 1,N^2-ϵ-Gua was seen in the study, regardless of whether *uvrA*$^+$ or *uvrA*$^-$ *E. coli* cells were used. This misincorporation, dominant in the bacterial study, was not seen with any of the polymerases examined here, except at a minor level with pol II (Table 2). Thus, it appears that both of the adducts studied here are able to direct the incorporation of *all four* dNTPs opposite the site (we assume that pol III is the polymerase producing the mutations *in vivo*). This pairing with all of the four dNTPs is not simply the result of a lack of information, since the different polymerases all have their own preferences. In the case of 1,N^2-ϵ-Gua, the adduct is relatively stable, unless an enamine tautomer exists (Guengerich et al., 1993). With HO-ethanoGua, the possibility that different forms of the adduct may be favored with individual polymerases (and also different sequence contexts) and favor different misincorporations exists, i.e., the ring-opened form N^2-(2-oxoethyl)Gua, its hydrate [$CH(OH)_2$], and the two isomers of the cyclic hemiaminal HO-ethanoGua (Scheme 1). Nevertheless, the case can be made that only very limited insight into mechanisms of mispairing can be obtained from studies with oligomers in the absence of polymerases.

In conclusion, 1,N^2-ϵ-Gua and HO-ethanoGua are both capable of blocking polymerization and miscoding when examined in defined oligomers. With several polymerases, there is a tendency to incorporate A and G opposite both of these adducts. Although the closed form of HO-ethanoGua differs from 1,N^2-ϵ-Gua only in the elements of H_2O, the former tends to be more likely to direct the misincorporation of A and to produce a ladder of products extended by one base at a time (Figures 3−6). This phenomenon may be the result of an increased k_{off} rate for the oligomer−polymerase complex, i.e., more distributive mechanism. Efforts to define alterations in rates of individual steps due to the presence of adducts are in progress. Another major conclusion from this work is that a single adduct can produce quite different mutations, not only quantitatively but also

[4] S. Langouët, S. P. Fink, M. Müller, L. J. Marnett, and F. P. Guengerich, unpublished results.

qualitatively, depending upon the polymerase. Previous studies on 8-oxo-7,8-dihydroGua have revealed differences among various polymerases in the extent to which A is inserted instead of C (Shibutani et al., 1991; Lowe & Guengerich, 1996; Furge & Guengerich, 1997), although insertion of G and T has not been reported. Very recently, Shibutani et al. (1996) reported the misincorporation of different bases at another ϵ-modified lesion, 3,N^4-ϵ-cytosine, by individual polymerases.

In summary, both 1,N^2-ϵ-Gua and HO-ethanoGua have been found to have miscoding potential and should be included in considerations of the genotoxicity of the set of DNA adducts derived from vinyl halides and other carcinogens that are activated to similar epoxides or equivalent alkylating species.

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SUPPORTING INFORMATION AVAILABLE

CD spectra of HO-ethanoGua derivatives, a polyacrylamide gel electrophoretogram of the HO-ethanoGua-containing 19-mer, and ES mass spectra of oligonucleotides (5 pages). Ordering information is given on any current masthead page.

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