Misincorporation of Nucleotides opposite Five-Membered Exocyclic Ring Guanine Derivatives by *Escherichia coli* Polymerases in Vitro and in Vivo: 1,N^2-Ethenoguanine, 5,6,7,9-Tetrahydro-9-oxoimidazo[1,2-*a*]purine, and 5,6,7,9-Tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*]purine[†]

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ABSTRACT: A variety of exocyclic modified bases have been shown to be formed in DNA from various procarcinogens (e.g., acrolein, malonaldehyde, vinyl chloride, urethan) and are also found in untreated animals and humans, presumably arising as a result of lipid peroxidation. 1,N^2-Ethenoguanine (1,N^2-ϵ-Gua), a product known to be formed from several 2-carbon electrophiles, was placed in a known site (6256) in bacteriophage M13MB19 and mutations were analyzed in *Escherichia coli,* with 2.05% G→A, 0.74% G→T, and 0.09% G→C changes found in uvrA$^-$ bacteria. 5,6,7,9-Tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*]purine (HO-ethanoGua), formally the hydrated derivative of 1,N^2-ϵ-Gua, is a stable DNA product also derived from vinyl halides. When this base was placed in the same context, the mutation rate was 0.007−0.19% for G→A, C, or T changes. The saturated etheno ring derivative of 1,N^2-ϵ-Gua, 5,6,7,9-tetrahydro-9-oxoimidazo[1,2-*a*]purine (ethanoGua) produced G→A and G→T mutations (0.71% each). All mutants were SOS-dependent and were attenuated by uvrA activity in *E. coli*. In vitro studies with four polymerases showed strong blocks to addition beyond the adduct site in the order ethanoGua > HO-ethanoGua > 1,N^2-ϵ-Gua. Both *E. coli* polymerases (pol) I exo$^-$ and II exo$^-$ and bacteriophage pol T7 exo$^-$ showed extensive misincorporation opposite ethanoGua in vitro, with pol I exo$^-$ incorporating G and T, pol II exo$^-$ incorporating A, and pol T7 exo$^-$ incorporating A and G. All modified bases reduced the use of the minus strand bearing the modified guanine in *E. coli* cells. It is of interest that even though the normal base pairing site of guanine is completely blocked, all of the five-membered ring derivatives incorporate the normal base (C) in >80% of the replication events in *E. coli*. Major differences in blockage and misincorporation are seen due to what might appear to be relatively modest structural differences, and polymerases can differ dramatically in their selectivities.

Modification of nucleic acids with bifunctional electrophiles results in the formation of a number of different base derivatives that contain an exocyclic five- or six-membered ring (*1*−*4*). Some similar compounds have been identified in tRNAs (*5*). In DNA, most of the chemicals that form such derivatives are known mutagens and carcinogens, and there is considerable interest in the hypothesis that the exocyclic bases are major contributors to the mutagenicity of these chemicals (*3*). In support of this view, the five-membered exocyclic derivatives N^2,3-ϵ-Gua,[1] 1,N^6-ϵ-Ade, and 3,N^4-ϵ-Cyd (Scheme 1) have all been shown to produce some mutations when examined in various systems, although direct comparisons have not been made (*6*−*10*). The six-

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[1] Abbreviations: ϵ, etheno; Gua or G, guanine; 1,N^2-ϵ-Gua, 1,N^2-ethenoguanine (or 9-oxoimidazo[1,2-*a*]purine); ethanoGua, 1,N^2-ethanoguanine (5,6,7,9-tetrahydro-9-oxoimidazo[1,2-*a*]purine); HO-ethanoGua, 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazopurine; dGuo, deoxyguanosine; ethanodGuo, 5,6,7,9-tetrahydro-9-oxoimidazo[1,2-*a*]-purine 2-deoxyribosyl nucleoside; M_1Gua, pyrimido[1,2-*a*]purin-10-(3*H*)one; propanoGua, 1,N^2-propanoguanine; propanodGuo, 1,N^2-propano-2′-deoxyguanosine; HO-propanoGua, 8-hydroxypropanoGua; CGE, capillary gel electrophoresis; MOPS, 3-(*N*-morphilino)propanesulfonic acid; SSC, saline sodium citrate buffer (15 mM sodium citrate buffer (pH 7.0) containing 150 mM NaCl); SSPE, 10 sodium phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 150 mM NaCl; Kf$^-$, *E. coli* polymerase I (Klenow fragment) exo$^-$; pol II$^-$, *E. coli* polymerase II exo$^-$; T7$^-$, bacteriophage T7 polymerase exo$^-$/thioredoxin complex; HIV RT, human immunodeficiency virus-1 reverse transcriptase; ES MS, electrospray mass spectrometry; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)acetic acid.

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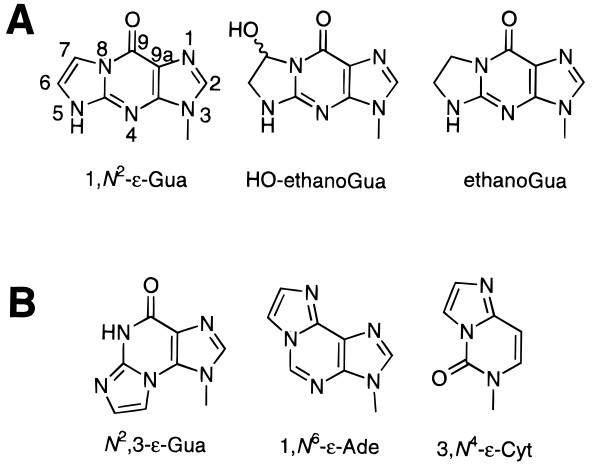
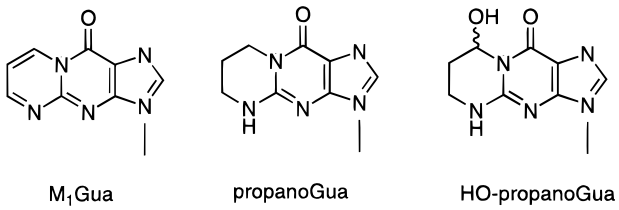
Scheme 1: Structures of (A) Five-Membered Ring Exocyclic Gua DNA Adducts Considered in This Work and (B) Other Adducts



Scheme 2: Structures of Six-Membered Ring Exocyclic Gua DNA Adducts

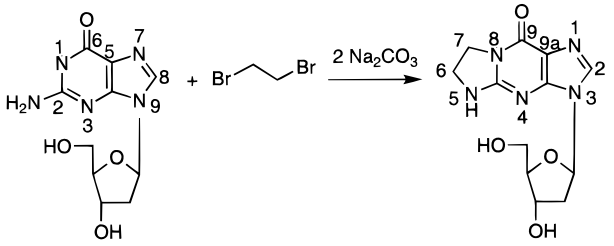


membered ring derivatives M_1Gua and propanoGua (Scheme 2) have been previously considered for mutagenicity in one of our laboratories and elsewhere and shown to be miscoding in vitro and in vivo (*11*, *12*). It is of particular interest that some of these adducts have been found not only in DNA of animals treated with chemicals but also in DNA of animals and humans not knowingly exposed to known precursors (*13*), except perhaps certain chlorinated compounds found in disinfected drinking water (*14*). N^2,3-ϵ-Gua, 1,N^6-ϵ-Ade, 3,N^4-ϵ-Cyt, M_1Gua, and HO-propanoGua have all been found at levels of 1 per 10^6-10^8 bases in liver DNA (*13, 15−18*). The contribution of such bases to diseases such as cancer and to aging is a matter of interest (*19, 20*).

Recently we have been interested in questions related to mechanisms of misincorporation opposite the five-membered exocyclic Gua adducts containing the ring structure shown in Scheme 1A [the so-called "linear" (as opposed to "angular") adducts (*21*)]. We previously quantified 1,N^2-ϵ-Gua and HO-ethanoGua in DNA treated with 2-chlorooxirane (vinyl chloride epoxide) (*22*). Methods were developed for the synthesis of oligonucleotides containing each of these two bases at a defined position, and in vitro misincorporation studies were done with several purified polymerases (*23*). The results collectively showed that both bases tend to strongly block replication at and beyond the site of substitution and to favor the misincorporation of dATP and dGTP across from both adducts. The selectivity of the polymerases varied. Analysis of steady-state kinetic results suggested that both *Escherichia coli* pol I (Kf^-) and pol II^- misincorporated >35% of the time, and frameshifts were also seen.

In this work we examined miscoding opposite these two adducts, 1,N^2-ϵ-Gua and HO-ethano Gua, in an *E. coli*/M13MB19-based system that has also been used to study

Scheme 3: Synthesis of EthanoGua



the closely related six-membered ring homologues M_1Gua and propanoGua (*11, 12*). The work was also extended to comparisons with the saturated model ethanoGua (Scheme 3). This adduct, which has not been identified in DNA, was used to provide in vivo and in vitro comparisons with the other two five-membered ring exocyclic derivatives.

EXPERIMENTAL PROCEDURES

Reagents. Reagents for oligonucleotide synthesis were purchased from PerSeptive Biosystems (Framingham, MA). Polymerases (all exonuclease$^-$) were purified by L. L. Furge (Department of Biochemistry) from recombinant expression vectors described elsewhere (*23−25*), and the extinction coefficients cited were used to determine concentrations of diluted enzymes.

Instrumental Analysis. UV spectra were recorded using a modified Cary 14/OLIS instrument operating at ambient temperature (On-Line Instrument Systems, Bogart, GA). Positive ion electrospray mass spectra were obtained using direct loop injection into a Finnigan TSQ 7000 instrument (Finnigan, Sunnyvale, CA). NMR spectra were recorded in the Vanderbilt facility using a Bruker AM-300 instrument (Bruker, Billerica, MA). CGE was done with a Beckman P/ACE 2000 or P/ACE 5000 instrument (Beckman, Fullerton, CA) using a ssDNA 100 gel capillary. Samples were applied at −5 kV and run at −10 kV and 30 °C.

Synthesis of EthanoGua-Modified Oligonucleotides

EthanodGuo. The approach (Scheme 3) was patterned after that used by Marinelli et al. (*26*) for propanodGuo. dGuo (270 mg, 1.0 mmol) and Na_2CO_3 (212 mg, 2.0 mmol, finely ground with a pestle) were stirred in 4.0 mL of $(CH_3)_2$-SO under dry argon. 1,2-Dibromoethane (86 mL, 190 mg, 1.0 mmol) was added in a single portion, and the mixture was stirred at room temperature overnight. The reaction was diluted with 2 vol of H_2O, and the pH was adjusted to ∼5 with CH_3CO_2H. HPLC analysis was done with a 10 × 250 mm Beckman Ultrasphere octadecylsilane column (5 μm; Beckman, San Ramon, CA) and mixtures of two solvents, (A) 50 mM $NH_4CH_3CO_2$, pH 5.5, and (B) 90% CH_3OH/10% H_2O, v/v, with a flow rate of 4.0 mL min^{-1} and UV detection at both 254 and 290 nm. The system was programmed from a solvent mixture of 95% A/5% B (t = 0) to 7% A/30% B (at 25 min) and then to 60% A/40% B (at 30 min) (all v/v). Residual dGuo eluted at t_R 12.5 min (14% of the total A_{254} area), followed by two major peaks (t_R 17.2 and 30.7 min) and three minor ones. The t_R 17.2 min peak accounted for ∼50% of the total A_{254} area and was subsequently shown to be ethanodGuo. Further reaction (to 40 h) did not appreciably change the HPLC profile. The

gradient was modified slightly to improve the preparative separation of the peak of interest (85% A/15% B at $t = 0$, 78% A/22%B at 15 min, 60% A/40% B at 20 min, 85% A/15% B at 25 min; elution of ethanodGuo at t_R 9.4 min). CH_3OH was removed in vacuo, and $NH_4CH_3CO_2$ and H_2O were removed by repeated lyophilization to yield 71 mg of ethanodGuo, characterized by the following properties. UV (in HPLC solvent) λ_{max}, nm: 253, 285. Positive ion electrospray mass spectrum, *m*/*z* 294.4 (rel abundance 100, MH^+), 178.8 (24, M-116, loss of deoxyribose). ^{1}H NMR (300 MHz, $(C^2H_3)_2SO$) δ 2.22 (m, 1H, H-2″), 2.48 (m, 1H, H-2′), 3.53 (m, 1H, H-5″ or -5′), 3.51 (m, 1H, H-5″ or -5″), 3.54 (app t, 2H, $-CH_2-$ of ethano ring), 3.78 (m, 1H, H-4′), 4.04 (app t, 2H, $-CH_2-$ of ethano ring), 4.30 (m, 1H, H-3′), 4.95 (bs, 1H, 5′-OH), 5.27 (bs, 1H, 3′-OH), 6.09 (app t, 1H, H-1′), 7.81 (s, 1H, NH), 7.91 (s, 1H, H-2). ^{13}C NMR (75 MHz, $(C^2H_3)_2SO$) δ 42.2 (C-3′), 87.6 (C-1′), 70.7 (C-10), 135.4 (C-2), 150.4 (C-3a), 155.4 and 155.5 (C-6 and C-9) (the two ethano $-CH_2-$ peaks were not observed but were presumed to be obscured by the solvent, 38−40 ppm).

5′-O-(4,4′-Dimethoxytrityl)ethanodGuo. The ethanodGuo (57 mg, 0.19 mmol) from above was dried under vacuum in a P_2O_5 desiccator and then reacted with 4,4′-dimethoxytrityl chloride and *N*,*N*-diisopropylethylamine in pyridine (6 mL) under argon for 15 h at 4 °C using the general procedure described elsewhere (*27*). A 2-fold excess of 4,4′-dimethoxytrityl chloride and *N*,*N*-diisopropylethylamine was then added, and the reaction was allowed to proceed for another 4 h, after which the reaction was quenched with 26 mL of CH_3OH (with stirring for 10 min) and concentrated to dryness in vacuo. The residue was dissolved in CH_2Cl_2 and washed with 10% aq K_2CO_3 (w/v), and the product was purified by flash chromatography on silica gel, eluting with a mixture of $CH_2Cl_2/CH_3OH/(C_2H_5)_3N$ (95:5:0.3, v/v/v). The fractions containing the product, as judged by TLC, were combined and concentrated in vacuo to yield 51 mg of 5′-*O*-(4,4′-dimethoxytrityl)ethanodGuo (48% yield). ^{1}H NMR (C^2HCl_3) δ 2.44 (m, 1H, H-2″), 2.69 (m, 1H, H-2′), 3.32 (m, 1H, H-5′ or -5″), 3.67 (m, 1H, H-5′ or -5″), 3.73 (app t, 2H, $-CH_2-$ of ethano ring), 4.12 (m, 1H, H-4′), 4.23 (t, 2H, $-CH_2-$ of ethano ring), 4.60 (m, 1H, H-3′), 5.15 (s, 1H, 3′-OH), 6.19 (s, 1H, 1′-OH), 6.80 and 6.82 (s, 3H, $-OCH_3$ groups), 7.22−7.42 (aromatic region), 7.59 (s, 1H H-2).

3′-O-[(N,N-Diisopropylamino)-(2-cyanoethyl)phosphoinyl]-5′-O-4,4′-dimethoxytrityl)ethanodGuo. The 5′-*O*-(4,4′-dimethoxytrityl)ethanodGuo from above (16.2 mg, 26 μmol) was dried by using repeated evaporation from anhydrous pyridine and reacted with 1*H*-tetrazole (6.9 mg, 31 μmol, 1.2 equiv) and 2-cyano- *N*,*N*,*N*′,*N*′-tetraisopropylphosporamidite (12 μL, 37 mg, 38 μmol, 1.5 equiv) in 2 mL of CH_2Cl_2 for 2 h (*27*), and the progress of the reaction was monitored by TLC. The product was purified by extraction (with 10% aq $NaHCO_3$, w/v, from CH_2Cl_2) and flash chromatography ($CH_2Cl_2/CH_3OH/(C_2H_5)_3N$, 95:5:0.5, v/v/v) to yield 29 mg of the phosphoramidite. ^{31}P NMR (C^2H_3CN) δ 27.83.

The product was dissolved in CH_3CN and used in the synthesis of the 8-mer d(5′-GGTG*TCCG-3′) and the 19-mer d(5′-CAGTGGGTG*TCCGAATTGA-3′) for these studies (G* = ethanoGua). The coupling efficiency was ∼50% at the site of addition in both cases. The oligonucleotides (with 4-*tert*-butylphenoxyacetyl protecting groups) were treated with 0.10 N NaOH at room temperature overnight to deprotect.

Oligonucleotide Purification. The different oligonucleotides were purified using a 10 × 250 mm octadecylsilane reversed-phase HPLC column (YMC-Pack ODS-AQ, 5 μm, YMC, Wilmington, NC), first with a linear gradient of 0−30% CH_3CN (v/v) in 50 mM $NH_4CH_3CO_2$ buffer, pH 5.0, over 50 min. Solvent was removed in vacuo and by lyophilization, and the oligonucleotides were further purified using a second linear gradient of 20−26% CH_3CN (v/v) in the same buffer over 50 min. The oligonucleotides were then purified on 20% polyacrylamide (w/v) gels buffered with sodium MOPS (pH 7.0). Purity of all oligonucleotides was checked by CGE, and purity of >99% was achieved before use (Figure 1, vide infra).

Characterization of Modified Oligonucleotides. The 10-mer "primer" oligonucleotide d(5′-TCAATTCGGA-3′) was prepared by automated synthesis and purified as described previously (*23*). The 8-mer d(5′-GGTGTCCG-3′) was prepared in the same manner: ES MS *m*/*z* 2441.0 (MH^-), theor 2441.6. The 1,N^2-ϵ-Gua and HO-ethanoGua monomers were synthesized as described and used to prepare the same 8-mer with the most central Gua substituted. 1,N^2-ϵ-Gua 8-mer, ES MS *m*/*z* 2465.0 (MH^-), theor 2465.0. HO-ethanoGua 8-mer, ES MS *m*/*z* 2483.2 (MH^-), theor 2483.0. The 19-mer "template" oligomers, d(5′-CAGTGGGTG*TCCGAATTGA-3′) (G* = modified G), were prepared as described previously (*23*).

The 8-mer and 19-mer containing ethanoGua were characterized after digestion with 8 μg of nuclease P_1 (Sigma Chemical Co., St. Louis, MO) at 37 °C for 3 h followed by digestion with 9 μg of snake venom phosphodiesterase (Sigma) and 6 μg of alkaline phosphatase (Sigma) at 37 °C for another 3 h. Digested products were then analyzed by HPLC using a 10 × 250 mm YMC octadecylsilane column (vide supra) and a gradient formed with 50 mM $NH_4CH_3CO_2$, pH 4.5, (A) and CH_3CN (B): 0 min (100% A, 0% B), 25 min (90% A, 10% B), 35 min (80% A, 20% B), 45 min (50% A, 50% B), 55 min (50% A, 50% B), and 60 min (100% A, 0% B) (flow 1.0 mL min^{-1}). Under these conditions ethanodGuo was eluted at t_R 38 min (Figure 2).

For all oligonucleotides, extinction coefficients were estimated using the method of Borer (*28*).

General Methods for Site-Specific Mutagenesis

Bacterial Strains. Three *E. coli* strains were used in this study: LM102 (AB1157 F′ [*traD36 proAB lacIQlacZ*Δ*M15*]), LM103 (AB1157 uvrA6 F′ [*traD36 proAB lacIQlacZ*Δ*M15*]), and JM105 (*supE thi rspL endA sbcB15 hsd R4* Δ*(lac-proAB) F′ [traD36 proAB lacIQZ*Δ*M15])*).

M13MB102 Preparation. The general approach described by Benamira and Marnett (*29*, *30*) was used. Briefly, bacteriophage M13MB102 corresponds to a M13mp19 genome in which 27 bp of the *his*D3052 sequence of *Salmonella typhimurium* has been incorporated at position 6256 (*30*). Single-stranded M13MB102 was grown in the presence of JM105, and the Qiagen (Chatsworth, CA) Maxi-prep procedure was followed to extract double-stranded DNA, while single-stranded DNA was recovered by precipitation with 2.5% poly(ethylene glycol) 8000 (w/v).

DNA Construction, Transfection, and Mutagenesis. Gapped duplex DNA was obtained by dialysis of DNA linearized

by *Bss*HII and *Ksp*I in the presence of a 12-fold excess of single-stranded DNA, from 95% (v/v) to 0% formamide (*30*). Phosphorylated 8-mer oligonucleotides (containing or not containing the adduct) were ligated into the gapped duplex for 4 h at 16 °C with 400 units of DNA ligase (Boehringer-Mannheim, Indianapolis, IN) in 50 mM sodium MOPS buffer (pH 7.0). Ligated products were purified by centrifugation through Ultrafree Probind filters (0.45 μm, Millipore, Bedford, MA), recovered from a 0.8% (w/v) low-melting-point agarose gel with 5 units of β-agarase (New England Biolabs, Beverly, MA), and concentrated with a Microcon 3 device (Amicon, Beverly, MA) following the manufacturer's instructions. *E. coli* cells were grown to mid-log phase, isolated, resuspended, and irradiated with a hand-held UV light (254 nm) to induce the SOS system, as previously described (*33*). The dose of UV was determined in separate experiments (varying time from 0 to 3 min) to reduce bactrial survival to 1−10%. Transfection of DNA into *E. coli* was performed by electroporation using a Gibco BRL Cell-porator *E. coli* system (Gaithersburg, MD). Bacteria were plated on LB plates in the presence of isopropyl β-D-thiogalactoside (*31*) and grown overnight. The phage population was recovered by shaking the plates in 10 mM Tris buffer containing 1.0 mM EDTA (pH 7) for 3 h. *E. coli* JM105 was then infected with the phage DNA population and plated in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma Chemical Co., St. Louis, MO) and isopropyl β-D-thiogalactoside to obtain 300−400 blue plaques per plate.

Hybridization. Single-stranded DNA (+ strand) was lifted onto nitrocellulose membranes (Schleicher and Schull, Keene, NH) after chilling for 2 h and then baked under vacuum at 80 °C for 2 h. Hybridizations were performed at 32 °C with the following hybridization media: 2× SSPE, 20% formamide (v/v), 10% SDS (w/v), 2.5 mg/mL calf thymus DNA, and 2.5% Denhart's solution (*32*). Labeled 13-mer oligonucleotides bearing the four different nucleotides opposite position 6256 were used as specific probes and designated as follows: probe A (for G→A mutations), d(5′-GCCGCGGT*A*TCCG-3′); probe G (wild type), d(5′-GCCGCGGT*G*TCCG-3′); probe C (G→C), d(5′-GCCGCGGT*C*TCCG-3′); and probe T (G→T), d(5′-GCCGCGGT*T*TCCG-3′). Finally, washes were performed in 2× SSC twice for 20 min at the hybridization temperature, and the membranes were exposed to X-ray film for 6−24 h at −80 °C.

T Construct. To study the strand utilization and estimate the blockage of replication that occurred in the presence of the adducts, another vector, termed the "T construct" in this study, was used. This construct differed by the presence of a T in the plus strand at position 6256, corresponding to the adduct site (*12*, *33*). The same procedure was used with this construct as with M13MB102, except that only hybridizations with the A probe were performed.

In Vitro Experiments. The general experimental procedures used were as described previously for the related ϵ adducts (*23*). Briefly, a 10-mer primer (2 μM) was 5′-labeled with T4 kinase (Boehringer-Mannheim), annealed at 90 °C for 10 min, and slowly cooled to room temperature with a 19-mer template containing Gua or ethanoGua at position 11. Either a mixture of of the four dNTPs (100 μM) or a single dNTP (100 μM) was incubated in 50 mM sodium MOPS buffer (pH 7.0) containing 2 μg/mL bovine serum albumin, 4 mM dithiothreitol, and 8 mM $MgCl_2$ in the presence of one of the polymerases.

Table 1: Percentages of Base-Pair Substitutions opposite Gua and Gua Derivatives in Pre-Irradiated *E. coli*[a]

base	uvrA phenotype	G→A, A probe (%)	G→T, T probe (%)	G→C, C probe (%)
Gua	+	<0.09	<0.07	<0.10
1,N^2-ϵ-Gua	+	0.34 ± 0.16	0.25 ± 0.12	<0.08
Gua	−	<0.10	<0.05	<0.11
HO-ethanoGua	−	2.05 ± 0.20	0.74 ± 0.25	0.09 ± 0.02
Gua	+	<0.09	<0.09	<0.11
HO-ethanoGua	+	0.14 ± 0.09	0.09 ± 0.03	0.12 ± 0.04
Gua	−	<0.07	<0.07	<0.07
HO-ethanoGua	−	0.11 ± 0.04	0.19 ± 0.09	0.07 ± 0.04
Gua	+	<0.08	<0.18	<0.09
ethanoGua	+	0.17 ± 0.14	0.11 ± 0.05	<0.08
Gua	−	<0.07	<0.07	<0.06
ethanoGua	−	0.71 ± 0.18	0.71 ± 0.23	<0.19

[a] Results are expressed as percent mutation frequencies ± SD and were obtained by screening ~1000 plaques per experiment for each probe. Values are the average of 2−9 independent experiments. An experiment with Gua was done at the same time as each experiment with a Gua derivative. The "<" designations indicate that no mutations were observed at the indicated limit of detection. Limits of detection differ because of differences in the number of plaques among experiments. Values for G probes approach 100% but are not shown. Values for nonirradiated cells were all in the range of <0.06 to <0.18 in all cases but are not shown.

The concentration of a single dNTP was varied to determine k_{cat} and K_m, which were estimated from measurements of the percent extension of the primer with a nonlinear fitting program (k•cat, BioMetallics, Princeton, NJ), using an approach patterned after that of Boosalis et al. (*34*). Concentrations of polymerases and reaction times were varied to keep the extent of primer extension less than ~20%. The products were analyzed on 20% (w/v) denaturing polyacrylamide−urea gels and exposed using a Molecular Dynamics model 400E Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA). Representative details of concentrations and time are presented in the appropriate tables and figures.

Sequence analyses were performed by following the procedure described by Maxam and Gilbert (*35*, *36*) except for the T-specific reaction, where the modification described by Friedman and Brown (*37*) was used.

RESULTS

Mutagenicity of 1,N^2-ϵ-Gua and HO-EthanoGua in E. coli. Oligonucleotides containing 1,N^2-ϵ-Gua and HO-ethanoGua were synthesized and characterized as described previously (*23*), ligated into an M13MB102 gap duplex genome, transfected into LM102 (a wild-type *E. coli* strain), and compared to a construct containing Gua at the same position (6256). When 1,N^2-ϵ-Gua was substituted for G, the frequency of mutation to A was 0.34%, the frequency of mutation to T was 0.25%, and mutation to C was undetectable (Table 1). Lower mutation frequencies were observed when HO-ethanoGua was incorporated into the same G site of M13MB102 phage (0.14 ± 0.09% A, 0.09 ± 0.034% T, and 0.12 ± 0.04% C). These changes were all highly dependent upon induction of the SOS response by UV irradiation. Without induction, no mutants were observed

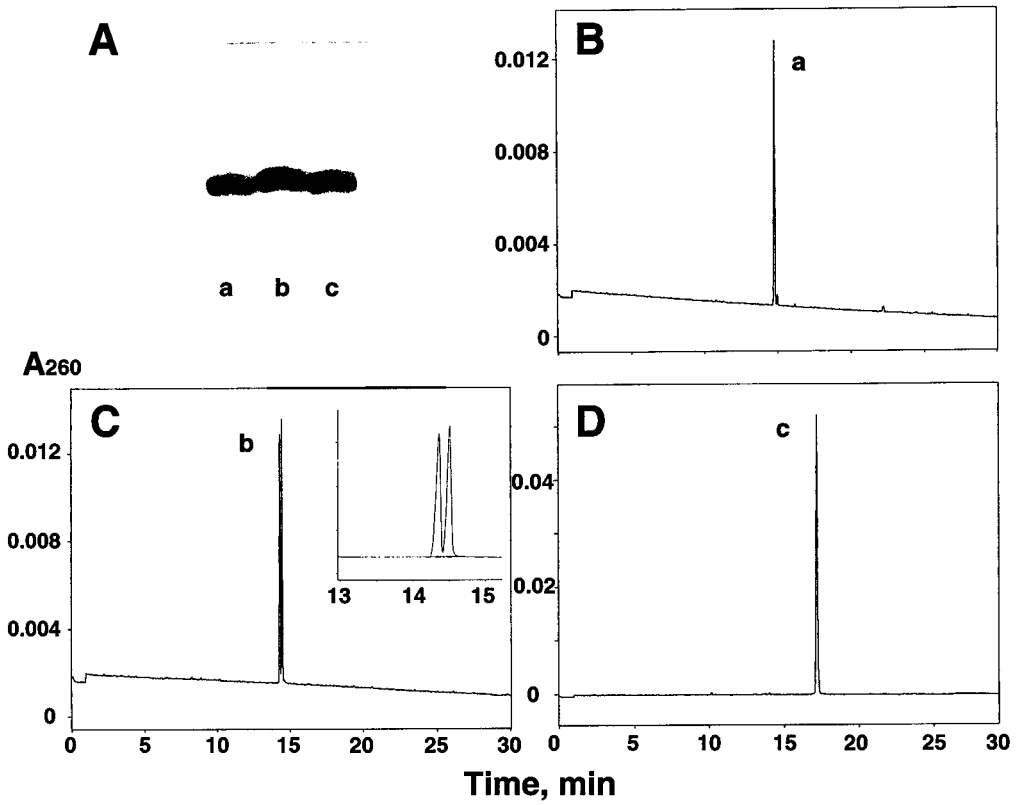


FIGURE 1: CGE profiles of oligonucleotides containing ethanoGua. In panel A, three preparations of an 8-mer containing ethanoGua were end-labeled with ^{32}P and subjected to electrophoresis in a polyacrylamide-urea gel (20%, w/v), and the results were visualized by phosphorimaging. The three preparations are labeled a, b, and c and were analyzed by CGE in panels B, C, and D, respectively. The conditions were different in the three CGE runs, which were done at different times, so the migration times are not directly comparable. Only preparation c was used for mutagenesis studies.

after hybridization for any of the three misincorporation products opposite the adduct.

The uvrA protein plays an essential role in the nucleotide excision repair system in wild-type *E. coli*, and comparisons were made with a deficient strain, LM103. No mutants were detectable without induction (of the SOS system) (Table 1). With induction, 1,N^2-ϵ-Gua increased the G→A mutation frequency to 2.05%, the G→T frequency was 0.74%, and the G→C frequency was still very low (0.09 ± 0.02%). Interestingly, no significant increase of the overall mutation frequency due to HO-ethanoGua was detectable in the uvrA-deficient strain.

Synthesis of EthanoGua, the Saturated Homologue of 1,N^2-ϵ-Gua. Different mutation results were observed with 1,N^2-ϵ-Gua and HO-ethanoGua, which formally differ only by a molecule of H_2O (Table 1). A third exocyclic Gua adduct, ethanoGua, the saturated homologue of 1,N^2-ϵ-Gua (Scheme 1), was examined. dGuo was modified to form ethanodGuo (Scheme 3), which was incorporated into oligonucleotides after protection of the hydroxyls with dimethoxytrityl and phosphoramidite groups. After deprotection, HPLC, and electrophoretic purification, purity was evaluated using two different techniques: (i) ^{32}P-labeling of oligonucleotides with T4 polynucleotide kinase and analysis on 20% acrylamide gels and (ii) CGE. Three preparations of the 8-mer oligonucleotide containing ethanoGua are shown (Figure 1). Electrophoretic analysis showed a single band in all three cases. In contrast, CGE analysis demonstrated that preparation a contained an impurity and preparation b was a mixture of two major peaks. Preparation c was of high purity. The

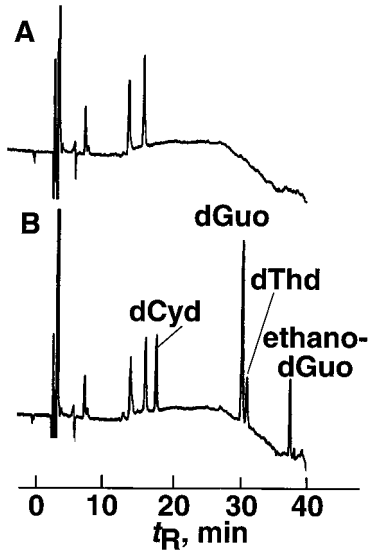


FIGURE 2: Characterization of the 8-mer d(5′-GGTGTCCG-3′) containing ethanoGua. The 8-mer was digested enzymatically as described in Experimental Procedures. (A) HPLC with no materials injected. (B) HPLC of a digest of the 8-mer. Standard deoxyribonucleosides were used to identify the peaks in a separate HPLC separation (not shown).

8-mer and 19-mer oligonucleotides containing ethanoGua were digested with nucleases and phosphatases, and the products were analyzed by HPLC (Figure 2). The impurities in preparations a and b were not identified. Preparation c was ligated into the vector for mutational analysis. These and other observations with oligonucleotides indicate that caution needs to be taken regarding purity of oligonucleotides

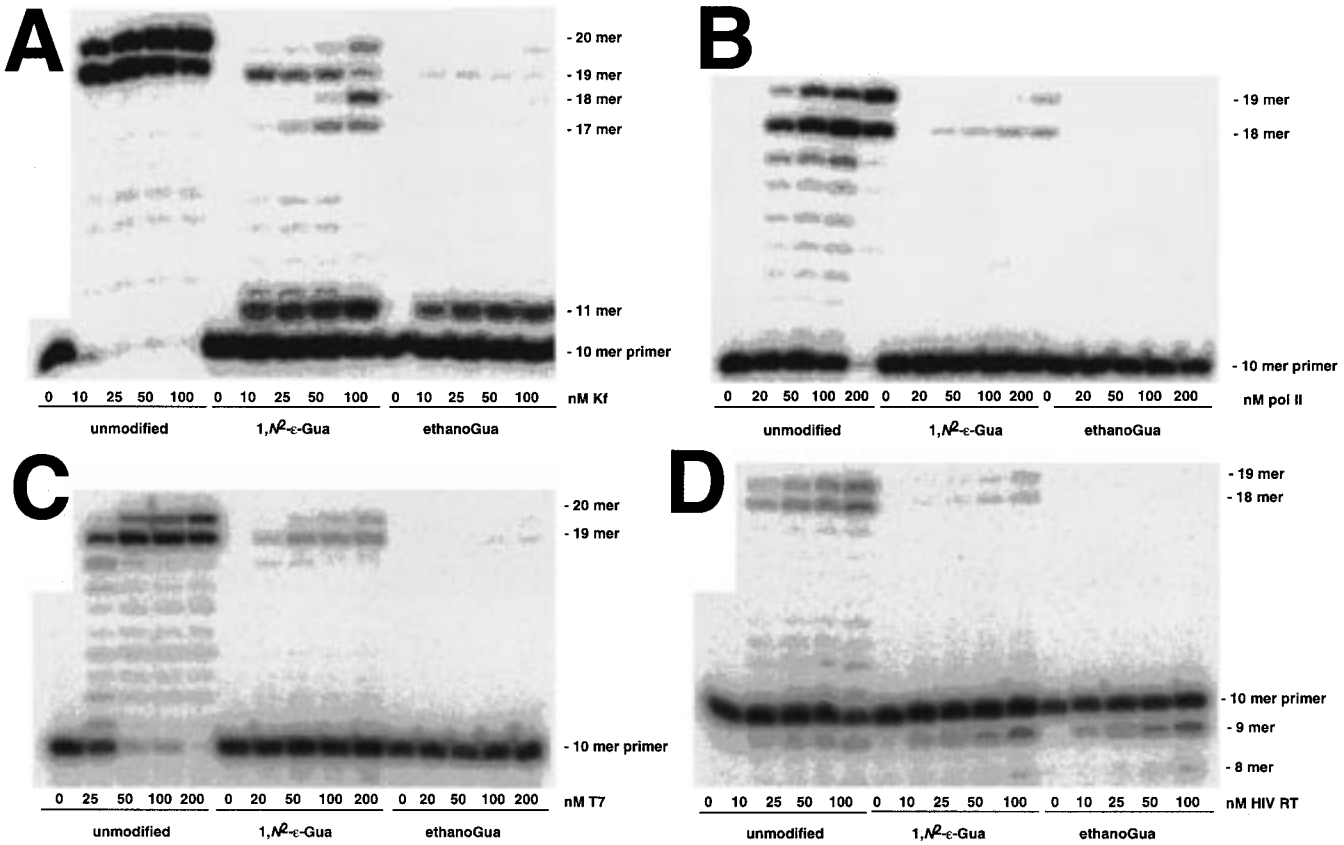
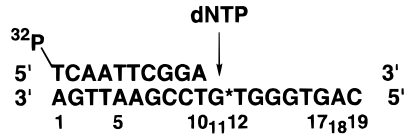


FIGURE 3: Extension of 10-mer primers by polymerases in the presence of all four dNTPs and various 19-mers containing Gua, 1,N^2-ϵ-Gua, or ethanoGua at position 11. Results are shown for the unmodified 19-mer and the 19-mer templates containing 1,N^2-ϵ-Gua and ethanoGua, with varying concentrations of (A) Kf$^-$, (B) pol II$^-$, (C) T7$^-$, and (D) HIV RT. The reaction time was 30 min in all cases, and 100 μM each of dATP, dCTP, dGTP, and dTTP was present.

Scheme 4: Oligonucleotides Used in EthanoGua Misincorporation Assays



used in such studies, particularly in vivo work, and underline the necessity of using high-resolution, independent techniques such as CGE to verify the purity of oligonucleotides following separations.

Misincorporation of dNTPs opposite EthanoGua in the Presence of Purified Polymerases. Extension of a 10-mer primer annealed with a 19-mer template containing ethanoGua was compared with an unmodified template (Scheme 4) using each of four polymerases, Kf$^-$, pol II$^-$, T7$^-$, and HIV RT, as in a previous study (*23*). Full-length extension (in the presence of the four dNTPs) was highly retarded with all four polymerases (Figure 3). Blockage of extension by ethanoGua was more extensive than in the case of 1,N^2-ϵ-Gua. Some 11-mer product was observed with Kf$^-$ and, to a lesser extent, with pol II$^-$, corresponding to blockage after incorporation of one base opposite the adduct (Scheme 4).[2]

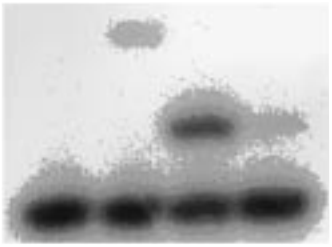


FIGURE 4: Extension of 10-mer primer with Kf$^-$ in the presence of single dNTPs.

Full-length extension products obtained by primer extension in the presence of all four dNTPs were sequenced (*35*, *36*). Analysis clearly showed that the 19-mer product obtained by extension in the presence of Kf$^-$ corresponded to an incorporation of G opposite the adduct, while A was incorporated at this site in the presence of pol II$^-$ or T7$^-$ (data not shown), as reported earlier for 1,N^2-ϵ-Gua and HO-ethanoGua (*23*) and also seen with HIV RT and *S*-glutathionylethyl-substituted Gua derivatives.[3]

Primer extension assays were done in the presence of individual dNTPs (Figure 4), and G was mainly incorporated in the presence of Kf$^-$; C and T were also incorporated. The incorporation of more than one base with dCTP, yielding a 13-mer product, can be explained by a slippage to the three following G's present (two nucleotides after the adduct)

[2] Some degradation of the primer was observed with HIV RT, when the template contained either 1,N^2-ϵ-Gua or ethanoGua (Figure 3D). The basis of this is unknown, but similar results have been seen with other blocking lesions [*23*; M.-S. Kim and F. P. Guengerich (*Chem. Res. Toxicol.*, in press)] and with several commercial preparations of HIV RT (with M_1Gua).

[3] M. S. Kim and F. P. Guengerich (*Chem. Res. Toxicol.*, in press).

Table 2: Products of Polymerase Extension Assays of 10-mer Primer in the Presence of All Four dNTPs (Extension Assays) or a Single dNTP (1-Base Incorporation) in the Presence of 19-mers

		products	
	polymerase	base = Gua	base = ethanoGua
extension assays	Kf$^-$	19 + 20 mer	11 + 17 + 18 + 19 + 20 mer
	pol II$^-$	18 + 19 mer	18 + 19 mer
	T7$^-$	19 + 20 mer	19 + 20 mer
	HIV RT	18 + 19 mer	9 + 8 + 7 mer
1-base incorporation	Kf$^-$	C > A, G, T	G > T, C
	pol II$^-$	C > A, G, T	A, C
	T7$^-$	C	A, G
	HIV RT	−	−

(Scheme 4). Similar experiments were performed in the presence of pol II$^-$ and T7$^-$ (Table 2); A was mainly incorporated, as well as some C in the presence of pol II$^-$ and some G in the case of T7$^-$.

Steady-state kinetic assays of single dNTP incorporation were used to characterize incorporation parameters more quantitatively (Table 3; Figure 5). In the presence of Kf$^-$, a significant decrease of K_m was observed when dGTP or dTTP was incorporated, compared to the normal base dCTP. In contrast, k_{cat} values with Kf$^-$ were similar for dCTP and dTTP incorporation and increased slightly for dGTP. With pol II$^-$, the k_{cat} value for dATP incorporation opposite the adduct was increased compared to dCTP, and the K_m values were similar for the two nucleotides. When comparisons are made between rates of incorporation of dNTPs opposite ethanoGua with pol II$^-$, the k_{cat} values are similar to that seen for misincorporation of dATP opposite Gua. With Kf$^-$, the k_{cat} values were all lower for all incorporations opposite ethanoGua than for any dNTPs opposite Gua. K_m values varied considerably. The misincorporation ratios [defined as $(k_{cat}/K_m)_{dNTP}/(k_{cat}/K_m)_{dCTP}$, where dNTP ≠ dCTP (*37*)] were all very high compared to the values obtained for 1,N^2-ϵ-Gua and HO-ethanoGua under the same conditions [0.15−1.2 (*23*)] and indicate a strong preference against incorporation of dCTP.

EthanoGua-Induced Mutations in E. coli. Both LM102, the wild-type strain for DNA repair, and LM103, the uvrA-deficient strain, were transformed with phage DNA containing ethanoGua or Gua at position 6256 of the minus strand. No mutations were detectable without SOS induction (by UV light) in either the LM102 or the LM103 *E. coli* strain (Table 1). After induction, G→A (0.17%) and G→T mutations (0.11%) were detected with the ethanoGua derivative in *E. coli* LM102. Significant increases of mutation to T and A at the adduct site were observed in the nucleotide excision repair deficient background (Table 1). The mutation frequencies reached equivalent values for G→A and G→T mutation frequencies (0.71% each). In contrast, the G→C mutation frequency (corresponding to G incorporation opposite the adduct) remained below the limit of detection.

Effect of Five-Membered Exocyclic Ring Gua Derivatives on Template Strand Utilization. To evaluate the utilization of the plus versus minus strand in the presence of each of the three exocyclic Gua adducts, we performed experiments using M13MB102 containing a T positioned opposite the adduct and probed with an oligonucleotide containing A at the position equivalent to 6256. Determination of the percentage of plaques that hybridize with A permitted estimation of the frequency of utilization of the plus strand in vivo (*12*). The plus strand was used as a template 30% of the time in both LM102 and LM103 strains when no adducts were present in the plus strand (Table 4). However, in the presence of 1,N^2-ϵ-Gua, the minus strand was used 78% and 82% of the time in LM102 and LM103, respectively. The difference reflects blockage of replication by polymerases in the presence of the adduct or replication following repair of the adduct. Taking these results into account, a corrected value of 16% total mutation frequencies in the uvrA-deficient strain can be calculated with 1,N^2-ϵ-Gua (Table 4). The presence of ethanoGua in the vector led to 51% plus strand use in LM102 and 33% in LM103. Such observations allow correction of the total mutation frequency to 2.2% for ethanoGua in LM103. HO-ethanoGua also increased the use of the plus strand (to 72% in LM102 and 56% in LM103), yielding a corrected total mutation frequency of ∼0.8% in the uvrA$^-$ strain.

DISCUSSION

A key result of this study is that 1,N^2-ϵ-Gua and ethanoGua were both mutagenic in *E. coli*, yielding both G→A transitions and G→T tranversions, while HO-ethanoGua was much less mutagenic. The work clearly establishes that 1,N^2-ϵ-Gua, long recognized as a reaction product of Gua nucleosides or DNA treated with several bifunctional alkylating agents (*38*), is mutagenic in vivo. All mutations seen with 1,N^2-ϵ-Gua, ethanoGua, and HO-ethanoGua were dependent on SOS induction by UV light, suggesting that polymerase III is involved in adduct bypass and introduction of mutations. The mutations seen with 1,N^2-ϵ-Gua and ethanoGua were attenuated by the presence of a functional uvrA gene, indicating the involvement of nucleotide excision repair in removal (Table 1). The levels of mutations seen with HO-ethanoGua were too low to readily determine whether nucleotide excision repair has a role or not. 1,N^2-ϵ-Gua, ethanoGua (Figure 3), and HO-ethanoGua (*23*) pose strong blocks to replication by several polymerases. A partial block also appears to occur in vivo in *E. coli*, as shown by the increased replication from the opposite plus strand (Table 4). These results can be used to normalize mutation frequencies to reflect the percent of misincorporation events occurring as polymerases insert nucleotides opposite the lesion (in uvrA$^-$ *E. coli*) (*39*). The result is a trend of miscoding in the order 1,N^2-ϵ-Gua (16%) > ethanoGua (2.2%) > HO-ethanoGua (0.8%) (Table 4). Thus, even though the entire base-pairing region of Gua is blocked, polymerases still appear to insert C more than 80% of the time, assuming that the mutation frequencies are not further influenced by other DNA repair processes. The incorporation of any nucleotide, particulary dATP, should not preserve the overall geometry of a canonical purine:pyrimidine pair.

The misincorporation patterns seen here with these five-membered exocyclic Gua derivatives can be compared with those observed with the six-membered homologues (Scheme 3) (*11, 12*). In in vitro studies with Kf$^-$, propanoGua led to the misincorporation of mainly G and A (*40*), similar to the results seen with both 1,N^2-ϵ-Gua and HO-ethanoGua with Kf$^-$ (pol II$^-$, T7$^-$, and HIV RT tended to insert only A) (*23*). With ethanoGua, the preference for incorporation by Kf$^-$ was G > T ≈ C ≫ A (Figure 3; Table 3). In *E. coli*, both propanoGua and M_1Gua produced G→A and G→T

Table 3: Steady-State Kinetic Parameters for dNTP Incorporation opposite EthanoGua

polymerase	template base	substrate	k_{cat} (min^{-1})	K_m (μM)	misincorporation ratio[a]
Kf^-	ethanoGua	dCTP	$9.9(\pm0.9) \times 10^{-4}$	950 ± 190	
	ethanoGua	dGTP	$24.7(\pm1.7) \times 10^{-4}$	45 ± 9	53
	ethanoGua	dTTP	$4.5(\pm0.2) \times 10^{-4}$	38 ± 7	11
	Gua	dCTP	16 ± 5	0.044 ± 0.024	
	Gua	dGTP	$3.8(\pm0.4) \times 10^{-1}$	39 ± 7	2.7×10^{-5}
	Gua	dTTP	$1.8(\pm0.3) \times 10^{-1}$	28 ± 11	1.8×10^{-5}
pol II^-	ethanoGua	dCTP	$1.9(\pm0.2) \times 10^{-4}$	26 ± 10	
	ethanoGua	dATP	$5.1(\pm0.5) \times 10^{-4}$	11 ± 4	6
	Gua	dCTP	15 ± 2	3 ± 1	
	Gua	dATP	$3.5(\pm1.2) \times 10^{-3}$	287 ± 111	2.5×10^{-6}

[a] Misincorporation ratio = $(k_{cat}/K_m)_{dNTP}/(k_{cat}/K_m)_{dCTP}$, where dNTP ≠ dCTP (*34*) (also termed the misincorporation frequency in this and other references). Results indicate SE within experiments as calculated by the analysis program. The values for dCTP incorporation include incorporation of 2- and 3-base addition products that occur due to slipped intermediates (*23*), and values for dATP include the 2-base extension product that results because of the rapid addition of an additional dATP opposite the T 3′ of the ethanoGua adduct. See Figure 5 for plots of results obtained with Kf^- and ethanoGua. In experiments done with pol II^- and ethanoGua, the concentration of pol II^- was 5 nM, the concentration of primer−template complex was 100 nM, and the dNTP concentrations were 1, 5, 10.25, 50, 75, and 100 μM; the reaction time was 30 min. In experiments with Kf^- and Gua-containing template (100 nM), the concentration of Kf^- was 1 nM in the case of dCTP (reaction time 5 min); with dGTP and dTTP the concentration of Kf^- was 10 nM, and the reaction time was 30 min. With pol II^- and the Gua-containing template (100 nM), the concentration of pol II^- was 0.1 nM with dCTP (reaction time 5 min) and 100 nM with dATP (reaction time 30 min).

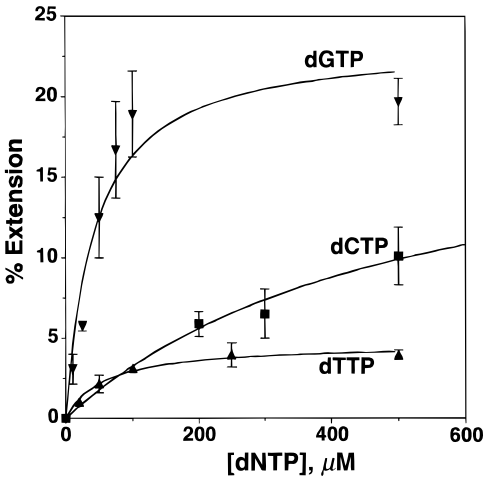


FIGURE 5: Incorporation of dNTPs opposite ethanoGua by Kf^- as a function of dNTP concentration. Incubations were done with 80 nM primer−template concentration for 30 min with the indicated concentration of each dNTP. The concentration of Kf^- was 25 nM in the experiments with dGTP and dTTP and 75 nM in the experiments with dCTP. The percent primer extension, determined by phosphorimager analysis, is shown as the mean ± SD of three determinations at each dNTP concentration, without correction for the enzyme concentration. Calculated kinetic parameters: dCTP, k_{cat} $9.9(\pm0.9) \times 10^{-4}$ min^{-1}, K_m $950(\pm190)$ μM; dGTP, k_{cat} $25(\pm2) \times 10^{-4}$ min^{-1}, K_m $45(\pm9)$ μM; dTTP, k_{cat} $4.5(\pm0.2) \times 10^{-4}$ min^{-1}, K_m $38(\pm7)$ μM (Table 3).

mutations at equal frequencies (*11*, *12*), and a similar result was observed with ethanoGua (Table 1). With 1,N^2-ϵ-Gua, there was a consistently greater fraction of G→A than G→T mutations, most readily seen in the absence of uvrA (Table 2). PropanoGua was more mutagenic than M_1Gua in previous studies (*11*, *12*), with the total mutation frequency rising to ∼39% when corrected for strand use. With the five-membered exocyclic rings, the unsaturated adduct (1,N^2-ϵ-Gua) was more mutagenic than the saturated analogue (ethanoGua) (Table 1).

Differences were seen in the manner in which polymerases process the five-membered exocyclic adducts. Some variations were seen when the purified polymerases were used with the ethanoGua-containing template (Table 3), as with 1,N^2-ϵ-Gua and HO-ethanoGua (*23*), although all were strongly blocked (Figure 3). The work presented in Table 3 also reinforces the observation of blockage by the ethanoGua lesion, although it must be emphasized that the rate-limiting step in these reactions is unknown (under the conditions used in the assays). The misincorporation patterns seen in *E. coli*, where pol III is probably involved, are quite different from those observed with pol I (Kf^-) and pol II^-. Pol III requires the SOS response for all activities observed here (Table 1). Other DNA adducts have been shown to yield varying misincorporation results with different polymerases (*23*−*25*).[3] These results argue that mutagenesis should be considered a kinetic process, as opposed to a thermodynamic process, and that details of mutagenesis cannot be understood only through structural studies, at least in the absence of polymerases.

Table 4: Strand Usage opposite 1,N^2-ϵ-Gua, HO-ethanoGua, and EthanoGua in T Constructs

(+) ——T——

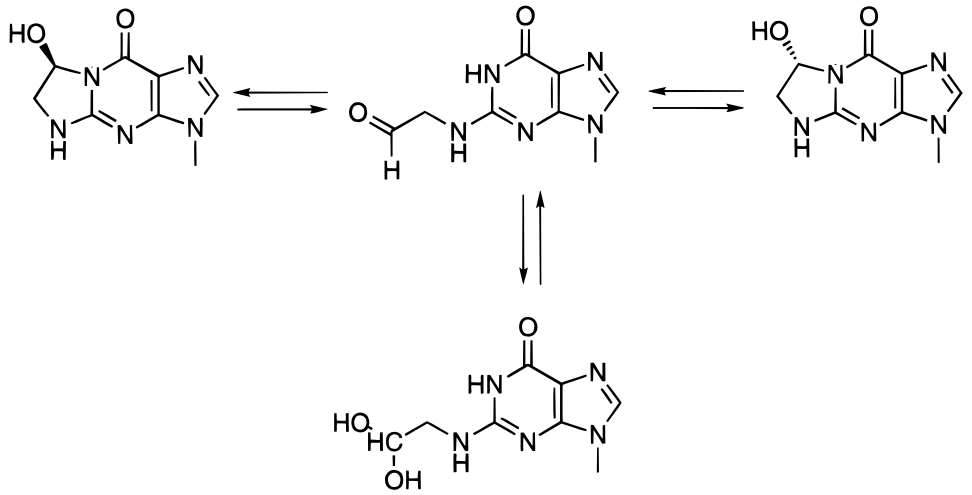
(−) ——G*——

		% use of plus strand,[a] base = G*		
E. coli strain	Gua	1,N^2-ϵ-Gua	ethanoGua	HO-ethanoGua
LM102	29 ± 6	78 ± 7	51 ± 13	72 ± 8
LM103 ($uvrA^-$)	28 ± 2	82 ± 5	33 ± 1	56 ± 2
		corrected total mutation frequency (%)[b]		
LM103 ($uvrA^-$)		16	2.2	0.8

[a] Results are presented as means ± SD of three experiments, with the percentage being determined by dividing the number of plaques with DNA hybridized to a ^{32}P——**A**—— probe divided by the total number of plaques (*12*). [b] Results from Table 1 are divided by the fraction of use of the minus strand containing the Gua derivative.

The three five-membered exocyclic rings considered here appear similar at a casual glance (Scheme 1A). 1,N^2-ϵ-Gua was more mutagenic in *E. coli* and also showed a shift to G→A transitions, in the absence of uvrA (Table 1). This might possibly be explained by the increased tendency of the saturated ethanoGua to pucker. It is possible that this minor structural variation is responsible for the consistently greater blocking of purified polymerases by ethanoGua > 1,N^2-ϵ-Gua (Figure 3). Indeed, in most cases there is very

Scheme 5: Equilibration of Forms of OH-EthanoGua



limited information available regarding the mechanisms underlying blockage of polymerases by adducts. HO-ethanoGua has biological properties quite different from those of 1,N^2-ϵ-Gua or ethanoGua. The saturation of the ring (sp^3 binding) cannot be responsible for the differences in considering ethanoGua. The low mutation frequency seen with HO-ethanoGua is not the result of a strong in vivo block to replication, as demonstrated in the results of strand use (Table 4). One possibility that cannot be excluded is that other repair systems, for example, glycosylases, are highly active and have not been accounted for in this study. Bacterial glycosylases have been shown to have activity toward some of the ϵ adducts (*41*). Another explanation for the low mutagenicity of HO-ethanoGua is that the structure exists in multiple forms, as shown previously (Scheme 5) (*23*). The two epimers at the carbinol exist, as shown by CD measurements with the nucleoside (*23*). However, if one epimeric form were misincorporating and the other were not, the mutation frequency would presumably only be decreased 2-fold. However, the ring-opened form, N^2-(2-oxoethyl)Gua, might be more prominent at the replication fork complex and be less mutagenic. A suitable model for this might be N^2-(2-acetamido)Gua, but to our knowledge, no studies have been done with this adduct.

Other stable Gua hemiaminal products include those derived from glyoxal (*42*), acrolein (*43*), and crotonaldehyde and other bifunctional alkylating agents (*44, 45*). The latter, HO-propanoGua, is the six-membered ring homologue of HO-ethanoGua (Scheme 3) and has been identified in liver samples of humans who have not been knowingly exposed to potential mutagens (*16*). A possible explanation for its formation has been proposed, with the adduct arising from reaction of an epoxide of the lipid peroxidation product 4-hydroxynonenal (*46, 47*). The suitability of HO-ethanoGua as a model for these adducts has not been investigated. However, the synthetic approaches we have utilized here and previously (*23*) should be applicable, at least for HO-propanoGua.

The relevance of these studies to the entire issue of mutagenesis and carcinogenesis caused by vinyl halides and other compounds is not yet clear. The ϵ adducts 1,N^2-ϵ-Ade, 3,N^4-ϵ-Cyt, N^2,3-ϵ-Gua, and 1,N^2-ϵ-Gua have now all been shown to be mutagenic in various bacterial systems, and HO-ethanoGua is marginally mutagenic. However, treatment of isolated DNA with 2-chlorooxirane yields adducts in the order 1,N^6-ϵ-Ade > HO-ethanoGua > N^2,3-ϵ-Gua > 3,N^4-ϵ-Cyt > 1,N^2-ϵ-Gua (*22*). Definitive studies of the rates of removal of all of these adducts from mammalian systems have not been completed, and few other direct comparisons of mutagenicity have been made in bacterial or mammalian systems.

In summary, a total of five structurally related DNA adducts have now been compared for site-specific mutagenesis at one site in a single vector system, in this and previous work (*12, 39*). The significance of this direct comparison is emphasized, because assessments of mutagenicity are often influenced by sequence contexts and vectors (*8*). A general similarity was seen in that the saturated and unsaturated five- and six-membered exocyclic ring derivatives are similar, in that all are mutagenic and produce G→A transitions and G→T transversions in vivo (Tables 1 and 4) (*12, 39*). All mutations were strongly SOS-dependent, and nucleotide excision repair was implicated. The presence of a (hemiaminal) hydroxyl group in the saturated (five-membered) ring attenuated the mutagenic response in this system, and if the analogy holds, then HO-propanoGua (Scheme 2) might be expected to show low mutagenic acitivity as well.

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