Products of the Direct Reaction of the Diazonium Ion of a Metabolite of the Carcinogen *N*-Nitrosomorpholine with Purines of Nucleosides and DNA

Charles N. Zink, Nicolas Soissons, and James C. Fishbein*

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, Maryland 21228

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A number of putative purine nucleoside and nucleobase adducts of the diazonium ion derived from 3-hydroxy-*N*-nitrosomorpholine have been synthesized as dimethylacetals. These are converted, in most cases nearly quantitatively, to the aldehydes, or in two cases to their derivatives, on treatment with mild acid to yield standards for a quantitative investigation of alkylation of purine nucleosides and DNA by the above metabolite of the powerful carcinogen *N*-nitrosomorpholine. The stability of the resulting nucleobase ethoxyacetaldehyde (EA) adducts has been characterized under a number of conditions with respect to their propensity to decompose. The stabilities, compared to that of the previously characterized adduct of the model benzimidazole, are generally unexceptional. Deposition of adducts on purine nucleosides and DNA were quantified in reactions in which 3-hydroperoxy-*N*-nitrosomorpholine was reduced to the hydroxy metabolite by a water-soluble phosphine at 21 ± 2 °C. The adduct profile is highly similar to that observed from simpler α-hydroxy metabolites of acyclic dialkylnitrosamines, with the three most abundant ethoxyacetaldehyde (EA) adducts in reactions of duplex DNA being N7-EA-Gua ∼ O^6-EA-Gua > N3-EA-Ade. The initial rate kinetics of formation of hydroxyethyl (HE) lesions from the initially formed EA lesions have been determined in the case of the major products in the cases of both the nucleoside and DNA adducts. The rates of formation of HE adducts are accelerated in DNA, relative to the nucleosides in the cases of the N7-EA-Ade, N7-EA-Gua, and O^6-EA-Gua adducts by factors of 7, 14, and 54, respectively. The initial rates of depurination of the N3-EA-Ade, N7-EA-Gua, and N7-EA-Gua adducts have also been quantified, and they are unexceptional in comparison with what has been previously reported for simple alkyl adducts. The adduct profiles reported here stand in significant contrast to what has been reported previously for structurally closely related α-substituted cyclic nitrosamines. In part or whole, this may be due to methodological differences in the conduct of the present and previous reports.

1. Introduction

N-Nitrosomorpholine (NMOR), **1** Scheme 1, is a powerful liver carcinogen in rats and mice and a respiratory tract carcinogen in hamsters (*1*−*3*). NMOR is found in groundwater, wastewater effluents, foods, cosmetics and toiletries (*4*−*6*), petroleum based fluids, and some industrial workplace airspaces, particularly in the rubber industry (*7*−*10*). NMOR is one of seven nitrosamine carcinogens for which NIOSH has specifically developed a valid analytical detection methodology in the form of Protocol 2522 for workspace monitoring (*11*). NMOR appears to be formed endogenously. NMOR has been detected in human urine (*12, 13*) and human gastric juices (*14*), and levels are elevated in individuals who simultaneously consume nitrate-rich and protein-rich foods (*13*).

Though NMOR is widely used as model for the induction of liver cancer in animal models, little is known of the mutation or DNA damage spectrum from this compound. It has been demonstrated that mutations in Hras are largely transversions at both GC and AT sites, quite different from the textbook GC → AT transitions that typify the simple nitrosamines (*15*). The DNA damage profile for NMOR is marginally characterized. Isolation of *N*7-hydroxyethyl-Gua (N7-HE-Gua) from rats treated with NMOR was reported 30 years ago (*16*). A dGua adduct derived from crotonaldehyde formed in the decay of the diazoic in Scheme 1 in the presence of dGua has been isolated (*17*).

There is good evidence from studies of the metabolism of the radiolabeled compound that a significant part of the metabolism involves simple α-hydroxylation, path a in Scheme 1, as indicated by the isolation of the metabolite, bottom of Scheme 1 (*18, 19*). Largely through the efforts of the Loeppky group, a second pathway involving β hydroxylation has been elaborated (path b, Scheme 1) (*20*−*26*). This pathway gives rise to an overlapping set of DNA damaging agents some of which have been characterized. While it was reported earlier that α-hydroxyNMOR, **2**, yields "almost entirely" acetaldehyde (*27, 28*), this was later demonstrated to be incorrect (*29*). About one-quarter of the diazonium ion derived from α-hydroxyNMOR does undergo rearrangement (k_r, Scheme 1) generating acetaldehyde and glycoaldehyde. Predominantly though, α-hydroxyNMOR forms 2-hydroxyethoxyacetaldehyde or its cyclic form by either the k_{H2O} and k_{IM} pathways, respectively, in Scheme 1.

Anticipation that *direct* reaction of the diazonium ion in Scheme 1 might give rise to DNA adducts, classical nitrosamine

* To whom correspondence should be addressed. Tel: 410-455-2190. E-mail: jfishbei@umbc.edu.

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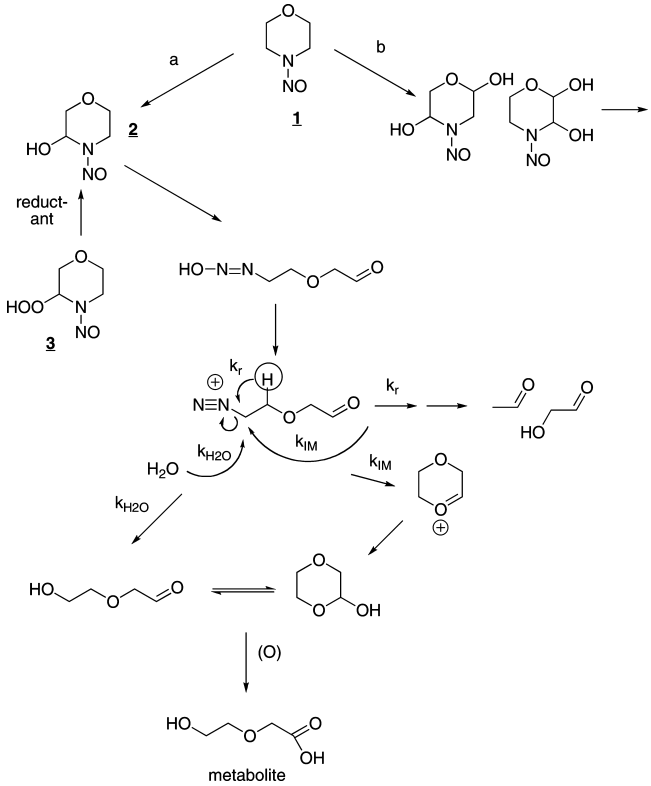
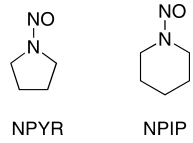
Scheme 1. Metabolic Fate of *N*-Nitrosomorphloline

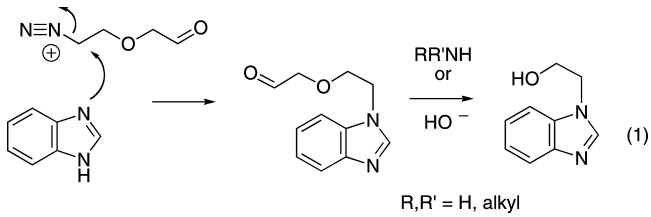


damage, is tempered by the extensive studies of Hecht on the DNA adducts from *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosopiperidine (NPIP) (*30*−*40*).



Of the nine nucleoside adducts so far derived from reactions of α-hydroxyNPYR with nucleosides or DNA, only two of these, one being the most abundant among all the known adducts, are claimed to be derived from *direct* reaction of the analogous diazonium ion. To date, not a single detected nucleoside adduct derived from reactions of α-hydroxyNPIP with nucleosides or DNA involves *direct* reaction of the diazonium ion. In the case of both NPYR and NPIP, the adducts not derived from *direct* reaction with diazonium ions are due to electrophilic products of diazonium ion decay.

Notwithstanding, it was recently established that the diazonium ion derived from α-hydroxyNMOR deposits an ethoxyacetaldehyde (EA) on the "model nucleobase" benzimidazole presaging actual EA-nucleobase adducts, eq 1 (*41*).



It was additionally demonstrated that the EA-benzimidazole adduct slowly decays to a hydroxyethyl (HE) adduct and that this decay is accelerated by primary and secondary amines or base (eq 1). This latter observation rationalizes the known chemistry of the diazonium ion, which does not appear to produce hydroxyethylating equivalents, with the observation 30 years ago, above, of the product 7-hydroxyethylGua (7-HEGua) isolated from rats administered NMOR (*16*).

In the present article, a number of the putative purine-EA nucleobase adducts have been synthesized as their stable dimethyl acetals. Treatment of dilute solutions of these with acid nearly quantitatively generates, in most cases, the aldehydes, chiefly in the hydrate form. These solutions are then employed as standards for the analysis and quantitation of the products of reactions of α-hydroxyNMOR, derived from hydroperoxide **3** (Scheme 1) with purine nucleosides and DNA, subsequent to acid catalyzed depurination. The stability of the EA moiety and/or its propensity toward conversion to HE adducts on the purine nucleobases, alkylated nucleosides, and in calf thymus DNA has been studied. Surprisingly, on the basis of the dearth of diazonium ion adducts from other cyclic nitrosamines, the spectrum of adducts and relative quantities reflect what is observed for simple diazonium ions derived from the α-hydroxy metabolites of acyclic dialkylnitrosamines. In addition, it is observed that nucleobase-EA adducts are not of exceptional reactivity, but in the cases of some adducts, duplex DNA significantly accelerates the conversion to HE adducts.

2. Experimental Section

2.1. Chemicals. Except as noted, chemicals, solvents, and isotopically enriched reagents employed in synthetic and analytical procedures were obtained from readily available commercial sources. Deionized water was obtained in house and filtered for HPLC and LC/MS experiments. Solvents such as methylene chloride, acetonitrile, ethyl acetate, and hexanes were dried and distilled from calcium hydride. Chloroform was distilled from sodium sulfate. Methanol and ethanol were distilled from magnesium turnings. Ether and tetrahydrofuran were dried and distilled from sodium. Methanol and acetonitrile used in HPLC and LC/MS experiments were purchased as HPLC grade solvents and were not further purified.

2.2. Analytical Determinations. Measurements of pH were performed on an Orion pH meter, model SA720, with a combination electrode. Two point calibrations were done before pH values were recorded. Calibrations were performed using commercially available standards. Elemental analyses were performed by Atlantic Microlabs, Inc., Norcross, GA. High resolution mass spectra were obtained at the UMBC Center for Biological Mass Spectrometry. Low resolution mass spectra and nucleobase adduct quantification were performed on a Waters 2695 liquid chromatograph attached to a Waters Micromass ZQ Mass Spectrometer.

2.3. Synthesis. The compounds ($^{15}N^2$)-2′-deoxyguanosine (*42*), ($^{15}N^6$)-2′-deoxyadenosine (*42*−*44*), α-hydroperoxy-*N*-nitrosomorpholine (*29, 41*), 2-(6-amino-7*H*-purin-7-yl)ethanol (*45*), 2-(9*H*-purin-6-ylamino)ethanol (*46*), 2-(6-amino-3*H*-purin-3-yl)ethanol (*47*), 2-(2-amino-9*H*-purin-6-yloxy)ethanol (*48*), 2-amino-7-(2-hydroxyethyl)-1*H*-purin-6(7*H*)-one (*49*), 9-((2*R*,4*R*,5*R*)-4-hydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-2-(2-hydroxyethylamino)-1*H*-purin-6(9*H*)-one (*50*), and 2-amino-9-((2*S*,4*S*,5*S*)-4-hydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-1-(2-hydroxyethyl)-1*H*-purin-6(9*H*)-one (*51, 52*) were synthesized as described in the references cited and exhibited ^{1}H NMR and ^{13}C NMR spectra consistent with either what was previously reported or, in the absence of such, the structure indicated. Hydroxyethylated nucleobases from the last two compounds were liberated by acid hydrolysis and purified by neutralization and recrystallization in water.

2.4. 2-(2,2-Dimethoxyethoxy)ethyl methanesulfonate (4). A solution of 2-(2,2-dimethoxyethoxy)ethanol (*53*) (20.1 g, 0.134 mol) in dry pyridine (40 mL) and dry CH_2Cl_2 (80 mL) was cooled to 0 °C under a nitrogen atmosphere. Methanesulfonyl chloride (15.5 mL, 0.201 mol) was added dropwise to the stirring solution. The

solution was stirred for 1 h at 0 °C and then 2 h at room temperature. The resulting suspension was then cooled to 0 °C and 150 mL of H_2O was added slowly. The solution was then extracted with CH_2Cl_2 (3 × 200 mL). The organic extracts were combined, washed sequentially with 1 M HCl (1× 100 mL), saturated $NaHCO_3$ (1× 100 mL), and brine (1× 100 mL), and were then dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure to yield a pale yellow liquid (90% yield) that was used without further purification. ^{1}H NMR ($CDCl_3$) δ 3.04 (3H, s), 3.37 (6H, s), 3.52 (2H, d, J = 5.0 Hz), 3.74−3.76 (2H, m), 4.34−4.36 (2H, m), 4.47 (1H, t, J = 5.0 Hz). ^{13}C NMR ($CDCl_3$) δ 102.8, 71.1, 69.5, 69.2, 54.2, 37.9.

2.5. 7-(2-(2,2-Dimethoxyethoxy)ethyl)-7*H*-purin-6-amine (5). To a solution of *N*-(4,6-diaminopyrimidin-5-yl)formamide (*47*) (500 mg, 3.3 mmol) in dry DMF (10 mL) was added sodium hydride (130 mg, 3.3 mmol, 60% in mineral oil). The solution was heated at 50 °C. After 30 min, a solution of **4** (782 mg, 3.3 mmol) in DMF (2 mL) was added dropwise to the solution. The solution was heated at 50 °C for 9 h and then cooled to room temperature and concentrated. To the residue was added H_2O (10 mL), and the solution was neutralized with acetic acid. The solution was concentrated and purified by silica gel column chromatography (90:10:0.5 $CHCl_3$/MeOH/NH_4OH) (R_f 0.25). The collected product (605 mg) was suspended in acetic anhydride (20 mL) and heated at 150 °C. After 1 h, the solution was concentrated to dryness and to the residue was added 20 mL of ammonium hydroxide (28% ammonia). The solution was transferred to a sealed vessel and heated at 60 °C for 1 h. The solution was concentrated to dryness and purified by silica gel column chromatography (90:10:0.5 $CHCl_3$/MeOH/NH_4OH) (R_f 0.15) to yield a white solid (50% yield). ^{1}H NMR (DMSO) δ 3.17 (6H, s), 3.36 (2H, d, J = 5.0 Hz), 3.72 (2H, t, J = 5.0 Hz), 4.31 (1H, t, J = 5.5 Hz), 4.54 (2H, t, J = 4.6 Hz), 6.87 (2H, s), 8.18 (2H, s).^{13}C NMR (DMSO) δ 159.8, 152.0, 151.6, 146.4, 111.1, 102.1, 70.2, 69.99, 53.5, 46.3. λ_{max} = 270 nm (*47, 54*). Anal. Calcd for $C_{11}H_{17}N_5O_3$: C, 49.43; H, 6.41; N, 26.20. Found: C, 49.40; H, 6.48; N, 26.10.

2.6. *N*-(2-(2,2-Dimethoxyethoxy)ethyl)-9*H*-purin-6-amine (6). 6-Chloropurine (200 mg, 1.3 mmol) was dissolved in 2-(2,2-dimethoxyethoxy)ethanamine (*55*) (∼1 mL, 5.2 mmol). The solution was stirred at 70 °C for 2 h. The solution was cooled to room temperature and concentrated to dryness. The resulting solid was purified by silica gel column chromatography (85:15:0.5 $CHCl_3$/MeOH/TEA) (R_f 0.20) to yield a white solid that was recrystallized from ethanol (95% yield).^{1}H NMR (D_2O) δ 3.40 (6H, s), 3.62 (2H, d, J = 5.5 Hz), 3.79−3.85 (4H, m), 4.59 (1H, t, J = 5.0 Hz), 8.12 (1H, s), 8.20 (1H, s).^{13}C NMR (D_2O) δ 153.1, 151.7, 148.6, 139.9, 116.4, 102.7, 69.62, 69.48, 54.6, 40.2. Anal. Calcd for $C_{11}H_{17}N_5O_3$: C, 49.43; H, 6.41; N, 26.20. Found: C, 49.44; H, 6.42; N, 26.06.

2.7. 3-(2-(2,2-Dimethoxyethoxy)ethyl)-3*H*-purin-6-amine (7). To a solution of *N*-(4,6-diaminopyrimidin-5-yl)formamide (*47*) (1.0 g, 6.5 mmol) in dry DMF (75 mL) under N_2 was added 4 Å molecular sieves (4.8 g). The suspension was stirred and heated at 90 °C until the amide was dissolved. To this solution was added **4** (2.23 g, 9.75 mmol), and this solution was heated at 90 °C for 11 days. The resulting suspension was cooled to room temperature and filtered. The filtrate was concentrated under vacuum to yield an oil that was purified by silica gel column chromatography (90:10:0.5 $CHCl_3$/MeOH/NH_4OH) (R_f 0.29) yielding a white solid (7.5%).^{1}H NMR ($CDCl_3$) δ 3.28 (6H, s), 3.41 (2H, d, J = 5.5 Hz), 3.94 (2H, t, J = 5.0 Hz), 4.34 (1H, t, J = 5.0 Hz), 4.54 (2H, t, J = 4.6 Hz), 8.02 (1H, s), 8.1 (1H, s).^{13}C NMR ($CDCl_3$) δ 155.2, 153.7, 150.3, 143.6, 121.1, 102.6, 70.9, 68.5, 54.2, 50.2. λ_{max} = 272 nm (*47, 54*). Mass Calcd for $C_{11}H_{18}N_5O_3$: 268.1406. Mass Obsd: 268.1404.

2.8. 9-((2*R*,4*R*,5*R*)-4-(*tert*-Butyldimethylsilyloxy)-5-((*tert*-butyldimethylsilyloxy)methyl)-tetrahydrofuran-2-yl)-6-(2-(2,2-dimethoxyethoxy)ethoxy)-9*H*-purin-2-amine (8). The compound 2-amino-9-((2*R*,4*R*,5*R*)-4-(*tert*-butyldimethylsilyloxy)-5-((*tert*-butyldimethylsilyloxy)methyl)-tetrahydrofuran-2-yl)-1*H*-purin-6(9*H*)-one (diTBDMS-dG) (*56*) (1.0 g, 2 mmol), triphenylphosphine (680 mg, 2.6 mmol), and 2-(2,2-dimethoxyethoxy)ethanol (400 mg, 2.6 mmol) were suspended in dry THF (40 mL) and stirred at 0 °C under a N_2 atmosphere. To the suspension was added diisopropyl azodicarboxylate (0.75 mL, 2.6 mmol). The solution was stirred at 0 °C for 10 min and then at room temperature for 18 h. The solution was concentrated and purified by silica gel column chromatography (100% diethyl ether) (R_f 0.27) to yield a clear liquid that crystallized upon standing (65% yield). ^{1}H NMR ($CDCl_3$) δ 0.06 (6H, s), 0.08 (6H, s), 0.89 (18H, s), 2.33 (1H, m), 2.55 (1H, m), 3.37 (6H, s), 3.58 (2H, d, J = 5.0 Hz), 3.70−3.80 (2H, m), 3.90 (2H, t, J = 5.5 Hz), 3.95 (1H, m), 4.49 (2H, m), 4.56 (1H, m), 4.63 (2H, t, J = 5.0 Hz), 4.80 (2H, s), 6.30 (1H, t, J = 6.9 Hz), 7.88 (1H, s).

2.9. (2*R*,3*R*,5*R*)-5-(2-Amino-6-(2-(2,2-dimethoxyethoxy)ethoxy)-9*H*-purin-9-yl)-2-(hydroxymethyl)-tetrahydrofuran-3-ol (9). Compound **8** (250 mg, 0.4 mmol), potassium fluoride (196 mg, 3.4 mmol), and 18-crown-6 (105 mg, 0.4 mmol) were dissolved in DMF (5 mL) and stirred at 60 °C for 2 h. The solution was cooled to room temperature and concentrated under reduced pressure. The residue was dissolved in water (10 mL) and washed with CH_2Cl_2 (3 × 15 mL). The aqueous extract was collected, concentrated, and purified by silica gel column chromatography (90:5:5 CH_3CN/H_2O/NH_4OH) (R_f 0.35) to yield a clear solid (90% yield).^{1}H NMR (DMSO) δ 2.21 (1H, m), 2.53 (1H, m), 3.26 (6H, s), 3.47 (2H, d, J = 5.0 Hz), 3.48−3.60 (2H, m), 3.78−3.82 (3H, m), 4.35 (1H, m), 4.46−4.52 (3H, m), 4.99 (1H, t, J = 5.5 Hz), 5.27 (1H, d, J = 4.1 Hz), 6.20 (1H, t, J = 6.0 Hz), 6.44 (2H, s), 8.09 (1H, s).^{13}C NMR (DMSO) δ 160.2, 159.7, 154.0, 137.8, 113.9, 102.3, 87.6, 82.8, 70.8, 70.2, 68.9, 64.9, 61.7, 53.4, 39.4. Mass Calcd for $C_{16}H_{26}N_5O_7$: 400.1833. Mass Obsd: 400.1827.

2.10. 6-Chloro-7-(2-(2,2-dimethoxyethoxy)ethyl)-7*H*-purin-2-amine (10). 2-Amino-6-chloropurine (1.0 g, 5.9 mmol) was suspended in DMF (15 mL) under a N_2 atmosphere. 1,8-Diazabicyclo[5.4.0]undec-7-ene (1.08 mL, 7.25 mmol) and **4** (1.34 g, 5.89 mmol) were added, and the solution was stirred at 80 °C for 18 h. The solution was cooled to room temperature and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (100 mL) and H_2O (100 mL). The organic extract was collected, and the aqueous extract was washed with ethyl acetate (2× 100 mL). The organic extracts were combined, dried over Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography (95:5 $CHCl_3$/EtOH) (R_f 0.15) to yield a white solid (15.6% yield). ^{1}H NMR ($CDCl_3$) δ 3.30 (6H, s), 3.42 (2H, d, J = 5.0 Hz), 3.82 (2H, t, J = 5.0 Hz), 4.35 (1H, t. J = 5.0 Hz), 4.49 (2H, t, J = 5.0), 5.06 (2H, s), 8.04 (1H, s).

2.11. 2-Amino-7-(2-(2,2-dimethoxyethoxy)ethyl)-1*H*-purin-6(7*H*)-one (11). The compound **10** (100 mg, 0.33 mmol), K_2CO_3 (46 mg, 0.33 mmol), and 1,4-diazabicyclo[2.2.2]octane (46 mg, 0.41 mmol) were suspended in water (7 mL) and heated in a sealed reaction vial at 110 °C for 3 h. The solution was cooled to room temperature, and the pH of the solution was adjusted to 7 using solid KH_2PO_4. The precipitate that formed was filtered and dried under vacuum to yield a white solid (95% yield). ^{1}H NMR (DMSO) δ 3.21 (6H, s), 3.37 (2H, d, J = 5.5 Hz), 3.76 (2H, t, J = 5.0 Hz), 4.30−4.36 (3H, m), 6.10 (2H, s), 7.85 (1H, s), 10.8 (1H, bs).^{13}C NMR (DMSO) δ 160.0, 154.9, 152.9, 143.6, 107.8, 102.2, 69.85, 69.45, 53.4, 45.8. HRMS Calcd for $C_{11}H_{17}N_5O_4$: 284.1366. Obsd: 284.1353.

2.12. 2-(2-(2,2-Dimethoxyethoxy)ethylamino)-9-((2*R*,4*R*,5*R*)-4-hydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-1*H*-purin-6(9*H*)-one (12). The compound 9-((2*R*,4*R*,5*R*)-4-(*tert*-butyldimethylsilyloxy)-5-((*tert*-butyldimethylsilyloxy)methyl)-tetrahydrofuran-2-yl)-2-fluoro-6-(4-nitrophenethoxy)-9*H*-purine (*57*) (380 mg, 0.59 mmol) was dissolved in 6 mL of methanolic 2-(2,2-dimethoxyethoxy)ethanamine (1 M, 6.45 mmol) and stirred at room temperature for 18 h. The solution was concentrated under reduced pressure. The residue was dissolved in DMF (10 mL), and potassium fluoride (290 mg, 5.02 mmol) and 18-crown-6 (156 mg, 0.59 mmol) were added. The suspension was heated at 60 °C for 18 h. The suspension was cooled to room temperature and filtered. The filtrate was concentrated under reduced pressure and purified as a single peak by HPLC using a CH_3CN/H_2O gradient on a 5 μm C18 250 mm × 21.2 mm semipreparative column (30% yield). Major form. (89%) ^{1}H NMR (DMSO) δ 2.20 (1H, m), 2.60 (1H, m), 3.27 (6H, s), 3.43−3.60 (8H, m), 3.80 (1H, m), 4.35 (1H, m), 4.46 (1H, t, J = 5.0), 4.88

(1H, t, $J = 5.5$), 5.28 (1H, d, $J = 3.7$), 6.15 (1H, t, $J = 6.0$), 6.46 (1H, bs), 7.91 (1H, s), 10.6 (1H, s).^{13}C NMR (DMSO) δ 156.7, 152.6, 150.40, 135.84, 116.93, 102.2, 87.6, 82.8, 70.89, 70.0, 69.1, 61.83, 53.4, 39.2. Minor form. (11%) ^{1}H NMR (DMSO) δ 2.23−2.28 (1H, m), 2.69 (1H, m), 3.27 (6H, s), 3.43−3.60 (8H, m), 4.08 (1H, m), 4.28 (1H, m), 4.46 (1H, t, $J = 5.0$ Hz), 4.81 (1H, t, $J = 5.5$ Hz), 5.49 (1H, d, $J = 4.1$ Hz), 6.15 (1H, t, $J = 6.0$ Hz), 6.46 (1H, bs), 8.01 (1H, s), 10.6 (1H, s).^{13}C NMR (DMSO) δ 156.7, 152.6, 150.30, 136.3, 116.63, 102.2, 88.0, 82.8, 70.53, 70.0, 69.1, 61.55, 53.4, 39.2. HRMS Calcd for $C_{16}H_{26}N_5O_7$: 400.1836. Obsd: 400.1827.

2.13. 2-Amino-9-((2*S*,3*R*,4*S*,5*S*)-3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-1-(2-(2,2-dimethoxyethoxy)ethyl)-1*H*-purin-6(9*H*)-one (13). Guanosine hydrate (1.0 g, 3.53 mmol) and powdered K_2CO_3 (0.73 g, 5.30 mmol) were suspended in DMF (25 mL). The suspension was heated at 90 °C to solublize the guanosine. To the solution was added 2-(2-bromoethoxy)-1,1-dimethoxyethane (*58*) (1.13 g, 5.30 mmol), and the solution was heated at 90 °C for 24 h. The solution was cooled to room temperature and filtered through Celite. The filtrate was concentrated under reduced pressure and purified by silica gel column chromatography (90:5:5 $CH_3CN/H_2O/NH_4OH$) (R_f 0.20) to yield a white solid (80% yield). ^{1}H NMR (DMSO) δ 3.24 (6H, s), 3.43 (2H, d, $J = 5.0$ Hz), 3.50−3.65 (4H, m), 3.86 (1H, q), 4.09 (1H, t, $J = 4.5$ Hz), 4.14 (2H, t, $J = 5.5$ Hz Hz), 4.41 (2H, t, $J = 5.5$), 5.02 (1H, bs), 5.13 (1H, bs), 5.40 (1H, bs), 5.70 (1H, d, $J = 6.0$ Hz), 6.93 (2H, s), 7.95 (1H, s).^{13}C NMR (DMSO) δ 156.5, 154.3, 149.5, 135.9, 116.0, 102.2, 86.0, 85.2, 73.6, 70.4, 70.1, 68.2, 61.4, 53.5, 40.8. HRMS Calcd for $C_{16}H_{26}N_5O_8$: 416.1780. Obsd: 416.1776.

2.14. 2-(2,3-Dihydro-1,4-oxazin-4-yl)-1-*H*-purin-6(9*H*)-one (14). To 25 mg of **12** was added 5 mL of a solution of D_2O containing 0.1 M DCl, and this was stirred at 80 °C for 30 min. Analysis by HPLC, employing a 10 mM Ammonium Acetate pH 6.9/CH_3CN gradient at a flow rate of 1 mL/min and a C18(2) 250 × 4.6 mm column, indicated a minor component of fused peaks at 38−39 min followed by the major peak at 49 min, the balance of which was isolated by use of a semipreparative column. The eluate was concentrated in vaccuo, but not to dryness, and to this concentrated fraction was added 5 volume equivalents of D_2O. This procedure was repeated 5 times subsequently, the last two times employing 99.996 atom % D, D_2O, after which the product was analyzed spectroscopically. ^{1}H NMR (DMSO) δ 3.85 (2H, t, $J = 4.6$ Hz),4.11 (2H, t, $J = 4.9$ Hz), 6.17 (1H, d, $J = 5.0$ Hz), 6.59 (1H, d, $J = 5.0$ Hz), 8.27 (1H, s), 11.45 (1H, bs).^{13}C NMR (DMSO) δ 155.8, 151.8, 148.2, 137.8, 130.1, 118.1, 112.1, 105.5, 64.3, 39.9. [MH+] = 220.2.

2.15. Generation and Characterization of EA-Nucleobase Standards. The dimethoxy-protected nucleobases or nucleosides **5**, **6**, **7**, **9**, **11**, **12**, and **13** were subjected to mild acid hydrolysis to liberate their corresponding aldehydes. They were subsequently employed as standards in later experiments carried out to characterize nucleoside and DNA alkylation by the diazonium ion derived from α-hydroxynitrosomorpholine. Deprotection was carried out by preparing a solution of the nucleobase/nucleoside in D_2O (99.996% D) and adding DCl to yield a final concentration of ∼7 mM nucleobase/nucleoside and 0.1 M DCl. The reaction solution was maintained at 25 °C (nucleobase) or 80 °C (nucleoside). The reactions were monitored by ^{1}H NMR following the decay of the dimethoxy methine signal (∼4.50 ppm) and the decay of the H1′ signal from the ribose sugar (∼6.40 ppm) (nucleoside products). With two exceptions (see Results), the resulting aldehyde is seen predominantly in its hydrate form with the new methine signal forming around 5.05 ppm. Dimethylformamide was used as an internal standard to quantify the product yields. After completion of the reaction and characterization, the resulting solutions were stored at −20 °C and were used in experiments as stock solutions for authentic standards. Periodic checks indicated that the products were stable to the storage conditions.

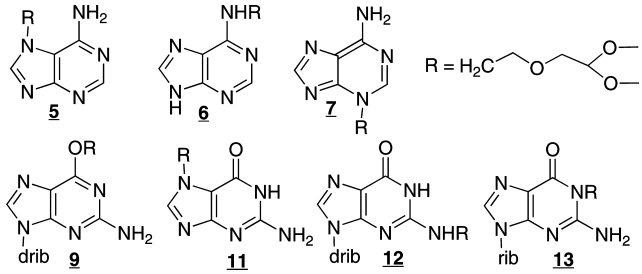
2.16. Products of Alkylation by α-Hydroxynitrosomorpholine. 2.16.1. Alkylation of Nucleosides. These experiments were initiated when a solution of **3** (Scheme 1) in CH_3CN was added to a buffered aqueous solution (0.05 M 70% anion cacodylic acid buffer, pH 6.9) containing tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and ($^{15}N^6$)-2′-deoxyadenosine or ($^{15}N^2$)-2′-deoxyguanosine. The final concentrations were 10 mM in **3**, 10 mM in TCEP, and 0.5 mM in ($^{15}N^6$)-2′-deoxyadenosine or ($^{15}N^2$)-2′-deoxyguanosine. The reaction was stirred at room temperature for 10 s with bubbling occurring. The solution was acidified to 0.1 M HCl and subjected to mild acid hydrolysis (30 min, 80 °C). The solution was cooled briefly, neutralized with NaOH (pH 7), and diluted with synthetic (^{14}N) EA standards. The products were chromatographically separated and analyzed by LC/MS employing single ion monitoring that monitored masses of 222.22 [M(^{14}N)H$^+$] and 223.22 [M(^{15}N)H$^+$] for (2-ethoxyacetaldehyde)-adenine (EA-Ade), 180.08 [M(^{14}N)H$^+$] and 181.08 [M(^{15}N)H$^+$] for (2-hydroxyethoxy)-adenine (HE-Ade), 238.09 [M(^{14}N)H$^+$] and 239.09 [M(^{15}N)H$^+$] for (2-ethoxyacetaldehyde)-guanine (EA-Gua), and 196.08 [M(^{14}N)H$^+$] and 197.08 [M(^{15}N)H$^+$] for (2-hydroxyethyl)-guanine (HE-Gua). The identity and the yields of products from the reactions were determined from the peaks of mass [M(^{15}N)H$^+$], which coeluted with the peaks of the standards [M(^{14}N)H$^+$]. Product yields were calculated from the peak areas corrected for the contribution of the M + 1 signal of the [M(^{14}N)H$^+$] standard. The yields, standard deviations, and percent error were determined from the averages of three experiments with the value from each experiment being an average of at least three injections. Control experiments involved the incubation of the purine nucleosides in the decomposition products of **3** in aqueous buffer, followed by acid hydrolysis, neutralization, separation, and analysis by LC/MS.

2.16.2. Alkylation of DNA. These experiments were initiated when a solution of **3** (Scheme 1) in acetonitrile was added to a buffered aqueous solution (0.05 M 70% anion cacodylic acid buffer, pH 6.9) containing tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and calf thymus DNA. The final concentrations were 10 mM in **3**, 10 mM TCEP, and ∼0.5 mg/mL calf thymus DNA. The reaction was stirred at room temperature for 15 s with bubbling occurring. The solution was acidified to 0.1 M HCl and subjected to mild acid hydrolysis (30 min, 80 °C). The solution was cooled briefly, neutralized with NaOH (pH 7), and separated into three aliquots. Two of the aliquots were chromatographically separated and analyzed by LC/MS employing single ion monitoring for the analysis of EA-Ade (MH+ = 222.22), HE-Ade (MH+ = 180.08), EA-Gua (MH+ = 238.09), and HE-Gua (MH+ = 196.08) products. The third aliquot was chromatographically separated and analyzed by HPLC employing a UV/vis PDA detector to determine the concentrations of Ade and Gua in the calf thymus DNA.

The products of the reaction were identified using authentic standards with the yields being calculated from standard curves. Product yields, standard deviations, and percent error were determined from the averages of three experiments with the value from each experiment being an average of at least three injections. Control experiments involved the incubation of **2**, generated from the reduction of **3**, in aqueous buffer at 23 °C for 15 s. Calf thymus DNA was then incubated in the reaction solution containing the decomposition products of **2** for 10 s, followed by acid hydrolysis, neutralization, separation, and analysis by LC/MS. No EA-nucleobase products were detected in these control experiments.

2.17. Kinetics. 2.17.1. General. All rate constants were determined under initial rate conditions monitoring less than 10% of conversion. Kinetics of decay of the ethoxyacetaldehyde (EA) nucleobase adducts were determined by monitoring the disappearance of the starting material monitored with a photodiode array detector and by comparison with the internal standard 5-benzimidazolecarboxylic acid. Rates of formation of hydroxyethyl (HE) adducts from EA adducts of nucleoside or DNA that were formed in the reactions of α-hydroxynitrosomorpholine were determined by monitoring the appearance of the HE adducts with time by LC/MS. Rates of depurination of EA adducts from DNA were determined by monitoring the appearance and EA-nucleobase adduct as a function of time by LC/MS using the internal standard 5-benzimidazolecarboxylic acid.

Scheme 2. Precusors Synthesized for the Detection of Some Prospective Purine Adducts of the Diazonium Ion from *N*-Nitroso-3-hydroxy-morpholine



2.17.2. Examination of the Aqueous Stability of 2-(2-Ethoxyacetaldehyde)-Nucleobase Standards. The decay of the (2-ethoxyacetaldehyde)-nucleobase (EA-nucleobase) standards was studied in aqueous buffered media at 25 °C using a Waters 717 HPLC with an autosampler, a temperature controlled carousel, and a Waters 996 photodiode array detector monitoring at 270 nm. Separations were performed on a Restek Pinnacle II reverse phase C18 5 μm, 250 × 4.6 mm column and effected by means of a H_2O/CH_3CN gradient. The buffers used in these experiments were carbonate, phosphate, and ethanolamine. Reaction solutions were maintained at ionic strength $\mu = 1.0$ M, using KCl. Experiments were initiated when a solution containing the EA-nucleobase dissolved in water was injected into the reaction vial and then injected onto the HPLC. The final concentration of the EA-nucleobase was $(1-2) \times 10^{-4}$ M.

2.17.3. Formation of Hydroxyethylated Nucleobases. Experiments monitoring the rates of formation of HE adducts from nucleosides or DNA reacted with α-hydroxynitrosomorpholine were initiated when a solution of **3** in acetonitrile was added to a buffered aqueous solution (0.05 M 70% anion cacodylic acid buffer, pH 6.9) containing tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and nucleoside (0.5 mM) or calf thymus DNA (0.5 mg/mL). The final concentrations of **3** and TCEP were 10 mM. The reaction was stirred at room temperature for 15 s, with evolution of gas noted during this period. Aliquots of the reaction were removed periodically and subjected to acid hydrolysis by neutralization of the buffer base and addition of HCl from a concentrated stock to give a concentration equal to 0.1M, after which the sample was hydrolyzed at 80 °C for 30 min. The products were separated and analyzed by LC/MS. Single ion monitoring was employed to analyze for masses 180.08 (HE-Ade) and 196.08 (HE-Gua) over the course of the reaction.

2.17.4. Rates of Depurination of Duplex DNA Subsequent to Reaction with α-Hydroxynitrosomorpholine. Experiments monitoring the rates of depurination of DNA reacted with α-hydroxynitrosomorpholine were initiated when a solution of **3** in acetonitrile was added to a buffered aqueous solution (0.05 M 70% anion cacodylic acid buffer, pH 6.9) containing TCEP and calf thymus DNA. The final concentrations were 10 mM in **3**, 10 mM TCEP, and ~0.5 mg/mL calf thymus DNA. The reaction was stirred at room temperature for 15 s, with evolution of gas noted during this period. Aliquots of the reaction were removed periodically, and products were chromatographically separated and analyzed by LC/MS without acid induced hydrolysis. Single ion monitoring was employed to analyze for masses 222.22 (EA-Ade) and 238.09 (EA-Gua) over the course of the reaction.

3. Results

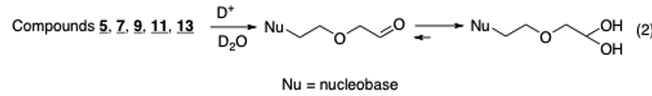
3.1. Generation and Characterization of EA-Nucleobase Standards from Dimethoxyprotected Nucleobases and Nucleosides. Structures of the relevant compounds are summarized in Scheme 2.

3.1.1. Hydrolysis of 5, 7, 9, 11, and 13. The dimethoxy-protected compounds were subjected to mild acid hydrolysis using 0.1 M DCl in D_2O at 20 ± 2 °C (nucleobases) or 80 °C (nucleosides) to liberate the corresponding aldehydes and to generate the free nucleobases, respectively, as in eq 2.

Table 1. Removal of the Dimethoxy Protecting Group and the Ribose Sugar from Nucleobase and Nucleoside Standards Using 0.1 M DCl in D_2O and 7.5 mM DMF (Internal Standard)

nucleoside[a]	temperature	dimethoxy removal	sugar cleavage
5	R.T.	92%/48 h	
6	R.T.	98%/24 h[b]	
7	R.T.	98%/48 h	
9	R.T.	95%/48 h	95%/15 min
	80 °C	95%/30 min	100%/15 min
11	R.T.	91%/44 h	
	80 °C	96%/30 min	
12	R.T.	100%/33 h[c]	95%/19 h
	80 °C	100%/20 min[d]	100%/20 min
13	80 °C	100%/30 min	90%/5 h

[a] Concentration is 6−7 mM. [b] 77% diol, 22% carbinolamine, and 1% aldehyde forms. [c] 33% diol, 45% carbinolamine, and 22% alkene forms. [d] 12% diol, 16% carbinolamine, and 72% alkene forms.



The reactions were monitored by following the decay of the dimethoxy methine signal (~4.50 ppm) and the decay, where applicable, of the H1′ signal from the ribose sugar (~6.40 ppm). The corresponding aldehyde under these conditions is in its hydrate form (>98%) with the new methine hydrogen signal appearing around 5.05 ppm. The conversion times and percent yields, based on the comparison with an internal standard DMF, for these reactions are illustrated in Table 1. Injection, after neutralization of a portion of the reaction solution, on HPLC with UV/vis detection resulted in a single UV absorbing peak with an absorption spectrum consistent with what has been reported for simple alkyl group substitution at the site expected. Use of ESI/MS detection again yielded a peak with a spectrum consistent with the hydrate form. In some cases, hydrolysis also resulted in conversion to the hydroxyethylated nucleobase, to the extent of 1−3%.

3.1.2. Hydrolysis of 6. Monitoring of the ^{1}HNMR spectrum of the decay of **6** in 0.1 M DCl in D_2O at room temperature for 24 h gave evidence of the complete conversion, on the basis of the internal standard DMF, of the methinyl hydrogen to three different forms consistent with chemical shifts expected of the aldehyde, 9.65 ppm (~1%), the aldehyde hydrate, 5.05 ppm (77%), and a carbinolamine, 6.56 ppm (22%). Analysis by HPLC with diode array detection exhibited a single UV-absorbing peak, while ESI/MS detection similarly indicates a single peak containing signals with MH+ consistent with all three structures. Neutralization to pD = 5.1 and reanalysis by ^{1}HNMR indicates a shift in the equilibrium of the three species with the carbinolamine form becoming dominant at 83%, the aldehyde hydrate form being 16%, and the aldehyde remaining at ~1%. Analysis of the ^{1}H−^{13}C coupling of this sample by HMBC (Figure 1) indicates a structure consistent with **15** on the basis of coupling between H3′ (6.56 ppm) and both C5′ (40.1 ppm) and C6 (153.1 ppm).

3.1.3. Hydrolysis of 12. Monitoring of the ^{1}HNMR spectrum of the decay of **12** in 0.1 M DCl in D_2O at room temperature 20 ± 2 or 80 °C for 30 min indicates the complete disappearance of the methinyl H of **12** to yield three different forms consistent with chemical shifts expected of the aldehyde, 9.65 ppm, the aldehyde hydrate, 5.05 ppm, and a carbinolamine,

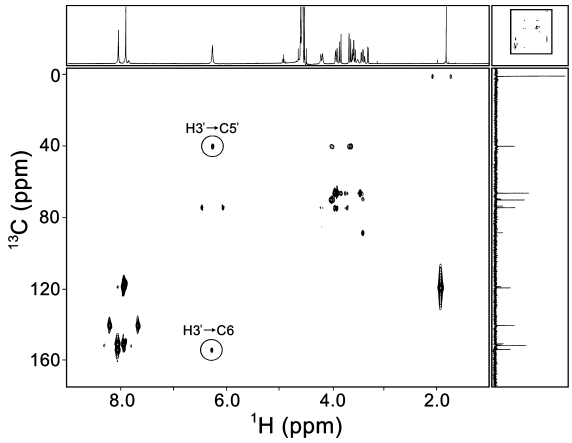
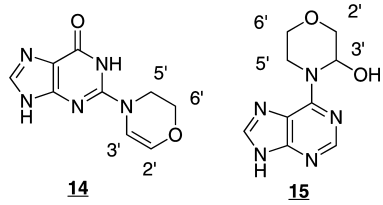


Figure 1. HMBC spectrum of the acid hydrolysis product **15** of compound **6**. Key long-range couplings are circled.



14 **15**

6.56 ppm. The aldehyde is in trace quantities, <1%, under both hydrolysis conditions. In addition, under both conditions, two doublets in the alkene region, 6−7 ppm region, appear. Isolation and characterization of the compound responsible for these resonances was effected by chromatography. The HMBC spectrum (Figure 2) indicates the H3′ → C5′, H3′ → C2, and H5′ → C2 couplings consistent with the structure of compound **14**. The alkene predominates, at the expense of the aldehyde, aldehyde hydrate, and carbinolamine in the higher temperature hydrolysis. For hydrolysis at room temperature, the ratio of hydrated aldehyde/carbinolamine/alkene, calculated from ^{1}H NMR, is 33/45/22, but changes to 12/16/72 when the temperature of hydrolysis is raised to 80 °C.

The hydrolysis time required and the conversion yields for the deprotection and or deglycosylation to yield the EA-nucleobase standards are summarized in Table 1.

3.1.4. Stability of Nucleobase Standards in Neutral and Basic Media: Decay of EA Adducts. The rate of decomposition of nucleobase standards was monitored under various conditions including phosphate and carbonate buffered media, in some cases containing ethanolamine. Typical data for the initial rates of decay of Gua adducts in carbonate buffer, pH 10.4, monitored by UV detection and employing the internal standard 5-benzimidazole carboxylic acid, are shown in Figure 3, panel A. It was not possible to accurately measure rate constants for decay at neutral pH buffered with phosphate without the addition of ethanolamine, which considerably elevated the rate of decay. Calculated rate constants for the disappearance of the EA adducts under different conditions are summarized in Table 2. Values of k_{obsd} for the EA adduct attached to the exocyclic amino group of Gua, N^2-EA-Gua, could not be measured due to irregularities in peak shape possibly due to the equilibrium between the hydrated aldehyde/aldehyde/alkene forms that could not be separated. Finally, for comparison, the rate constants were also measured for the EA adduct of benzimidazole, whose chemistry has been previously characterized, and these values are also included in Table 2 (*41*).

3.2. Alkylation of Purine Nucleosides and Purines in DNA by α-Hydroxynitrosomorpholine. α-Hydroperoxynitrosomorpholine, **3**, was decomposed by the addition of TCEP to solutions of ^{15}N^6-dAde, ^{15}N^2-dGua, or DNA, and the EA-containing nucleobases, after acid hydrolysis, were separated, detected, and quantified by HPLC-MS facilitated by selected ion monitoring. In the case of the nucleoside reactions, identification and quantitation were effected by the addition of natural abundance isotope-containing adduct standards after deprotection (as described above). In the case of DNA, identification was confirmed by coelution under two different chromatography conditions and spiking with authentic standards, and quantitation was effected by interpolation from three point standard curves. Typical chromatograms, for the alkylation of dAde and DNA, are presented in Figures 4 and 5, respectively. In the reactions with DNA, three adducts, **15**, N1-EA-Gua, and N^2-Gua adducts in the form of either N^2-EA-Gua (hydrate) or compound **14**, were undetected using a normal 10 μL sample injection. A 10-fold larger injection volume revealed a quantifiable amount of the last of these: N^2-EA-Gua (hydrate). A summary of the percentage yield of each of the adducts detected is include in Table 3.

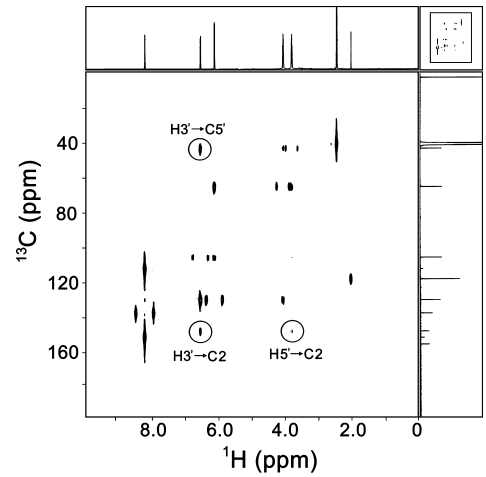


Figure 2. HMBC spectrum of the acid hydrolysis product **14** of compound **12**. Key long-range couplings are circled.

3.3. Transformation of EA Adducts of Nucleosides and DNA Reacted with α-Hydroxynitrosomorpholine. 3.3.1. Formation of HE Purine Adducts. The conversion of the EA-Ade and EA-Gua products, which were generated in the reactions of **2**, from **3**, in the presence of nucleosides or DNA, to HE adducts was examined by monitoring the formation of HE adducts as a function of time. In these reactions, **3** was reduced with TCEP in a buffered solution containing dAde, dGua, or DNA. The solution was stirred at room temperature, and aliquots were removed periodically. The aliquots were hydrolyzed in acid and neutralized, and the products were chromatographically separated and analyzed by LC/MS. Increases in the formation of HE adducts as a function of time were plotted as illustrated in Figure 3 for the N7-HE-Gua and O^6-HE-Gua adducts of dGua (panel B) and all four of the major adducts observed in DNA (panel C). Slopes of such plots allowed the calculation of the values of k_{obsd}, which are summarized in Table 4.

3.3.2. Spontaneous Depurination of EA Adducts from DNA Reacted with α-Hydroxynitrosomorpholine. α-Hydroperoxy-*N*-nitrosomorpholine, **3**, was reduced by TCEP in the presence of calf thymus DNA. As a function of time, aliquots of the reaction solution were removed and subjected to LC separation, without prior acid hydrolysis, and free nucleobase EA adducts were detected by MS. Typical plots of the increases in liberated nucleobase EA adducts as a function of time are indicated for the adenine adducts in Figure 3, panel D. Rate

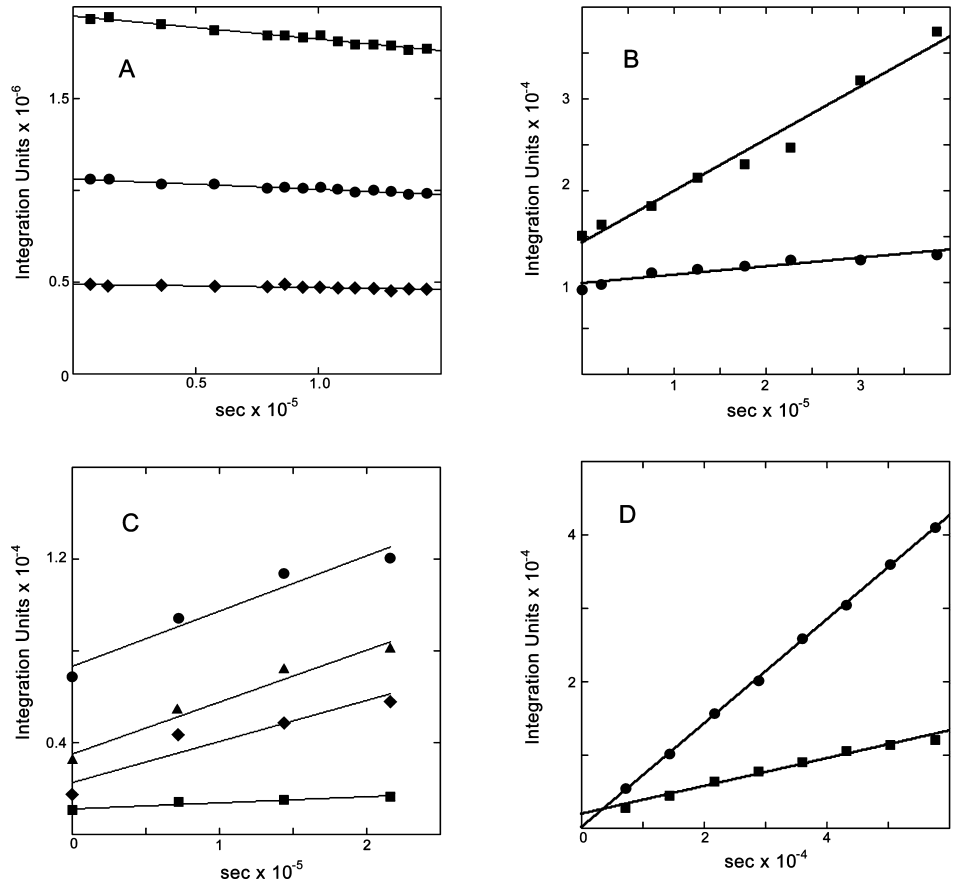


Figure 3. Kinetics of decay or formation of adducts on nucleobases or DNA. (A) Decay of EA-Gua adducts, N1-EA-Gua (■), N7-EA-Gua (●), and O6-EA-Gua (♦) as a function of time at 25 °C, pH 10.4, and 0.01 M carbonate buffer. (B) Formation of N7-HE-Gua (■) and O6-HE-Gua (●) adducts as a function of time at 25 °C, 0.05 M 70% anion cacodylic acid buffer, and pH 6.8. (C) Formation of O6-HE-Gua (♦), N7-HE-Gua (■), N3-HE-Ade (▲), and N7-HE-Ade (●) adducts from the respective EA-DNA adducts as a function of time at 25 °C, 0.05 M 70% anion cacodylic acid buffer, and pH 6.9. (D) Spontaneous depurination to form EA-Ade nucleobase adducts N3-EA-Ade (■) and N7-EA-Ade (●) from reactions with DNA at 25 °C, 0.05 M 70% anion cacodylic acid buffer, and pH 6.9.

Table 2. Rate Constants for the Decay of EA-Nucleobase Standards and EA-Benzimidazole (bzi) in Aqueous Buffered Solutions (0.1 M) at 25 °C and 1 M Ionic Strength (KCl)

	$10^7 \times k_{obsd}$ (s^{-1})			
	carbonate pH 10.4	carbonate pH 9.6	phosphate pH 7.4	phosphate pH 7.4 0.1 M ethanolamine
bzi	17.6	4.91	<0.02	63.6
N3-Ade	15.5	8.18	<1.59	37.3
N^6-Ade	11.2	6.74	<0.07	<1.1
N7-Ade	11.8	3.75	<0.17	36.0
N1-Gua	6.52	2.71	<0.17	18.9
O^6-Gua	4.39	<1.24	<0.30	48.8
N7-Gua	5.32	2.54	<0.38	51.5

constants based on the initial rates of the reaction were calculated assuming an end point of complete depurination, on the basis of the initial yield of the EA adduct calculated from acid hydrolysis of an aliquot after the initial alkylation reaction. The rate constants and calculated half-times for the relevant modified nucleobases are summarized in Table 5.

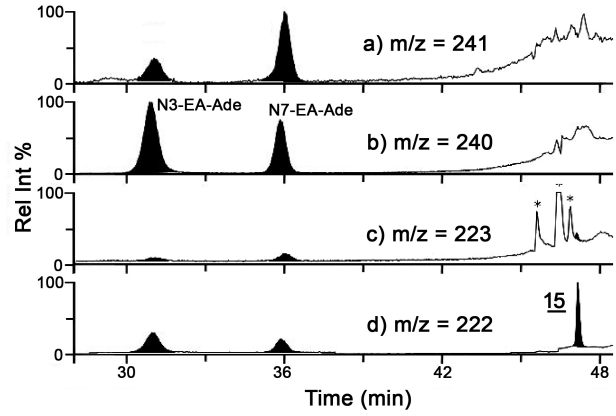


Figure 4. Typical LC/MS chromatogram with single ion monitoring for the reaction of **3**, reduced with TCEP, with ($^{15}N^6$)-2′-deoxyadenosine, 25 °C, 0.05 M 70% anion cacodylic acid buffer, and pH 6.9 and analysis for EA-Ade adducts. (a) m/z = 241 for N3 and N7 ($^{15}N^6$)-EA-Ade (hydrate forms). (b) m/z = 240 for N3 and N7 ($^{14}N^6$)-EA-Ade standards (hydrate forms). (c) m/z = 223 for N3 and N7 ($^{15}N^6$)-EA-Ade (aldehyde form) and ($^{15}N^6$)-**15**. (d) m/z = 222 for N3 and N7 ($^{14}N^6$)-EA-Ade (aldehyde form) and ($^{14}N^6$)-**15**. The peaks marked with an asterisk were identified in the control experiments.

4. Discussion

4.1. Dimethylacetal Precursors to Ethoxyacetaldehyde Adducts of Purine Nucleobases. The use of dimethylacetals to generate dilute solutions of nucleobases containing the ethoxyacetaldehyde moiety was adopted on the basis of preliminary work in which attempts to make pure nucleobase adducts containing the pendant aldehyde met with failure due to oligomer- and polymerization and other instabilities manifest by the aldehyde functionality. Generally, under the influence of acid and in some cases heat for deglycosylation, the dilute solutions of acetals were converted to predominantly the aldehyde hydrate form in nearly quantitative yield (Table 1). Two exceptions were the EA adducts attached to the exocyclic amino groups of the purines. Cleavage of the dilute acetals **6**

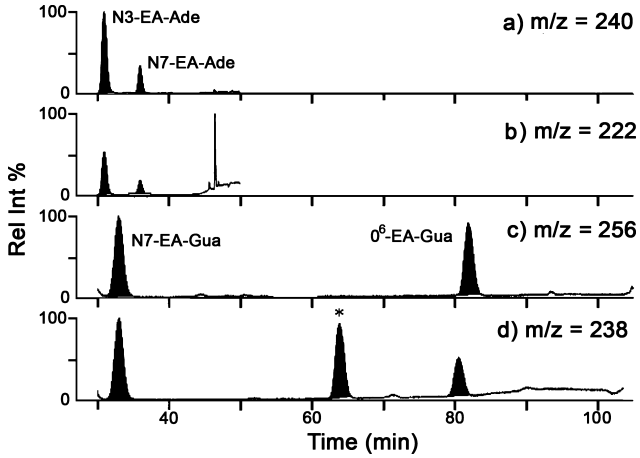


Figure 5. Typical LC/MS chromatogram with single ion monitoring for the reaction of **3**, reduced with TCEP, with calf thymus DNA, 25 °C, 0.05 M 70% anion cacodylic acid buffer, and pH 6.9, and analyzing for EA-Ade and EA-Gua. (a) m/z = 240 for N3 and N7-EA-Ade (hydrate forms). (b) m/z = 222 for N3 and N7-EA-Ade (aldehyde forms) and **15**. (c) m/z = 256 for N7- and O^6-EA-Gua (hydrate form). (d) m/z = 238 for N7-, O^6-, and N^2-EA-Gua (aldehyde form). N^2-EA-Gua is not observed in this chromatogram (see Results). The peak marked with an asterisk (*) was identified in the control experiments.

Table 3. Percent Yields of EA Products in Reactions of Purine Nucleosides and DNA with HOONMOR in Aqueous Media at 23 °C and pH 6.8

	nucleoside reactions		DNA reactions	
nucleobase adduct	10^2 × average % yield	% error	10^2 × average % yield	% error
N3-EA-Ade	0.06	3.2	6.14	4.7
N^6-EA-Ade	0.04	10.6	<0.035	
N7-EA-Ade	0.37	3.0	1.52	2.4
N1-EA-Gua	0.34	4.7	<0.005	
N^2-EA-Gua	0.11	9.7	0.15	2.8
O^6-EA-Gua	4.92	1.4	38.3	0.8
N7-EA-Gua	8.65	1.6	41.1	2.5

Table 4. Rate Constants of HE-Ade and HE-Gua Formation in Aqueous Buffered Media at 23 °C

	$10^6 \times k_{obsd}$ (s^{-1})		
	nucleosides		DNA
nucleobase adduct	0.1 M phosphate (pH 7.4)	0.1 M cacodylate (pH 6.8)	0.1 M cacodylate (pH 6.8)
N3-HE-Ade	0.10	1.20	1.16
N7-HE-Ade	0.04	0.62	4.51
N7-HE-Gua	0.01	0.06	0.81
O^6-HE-Gua	0.06	0.02	1.08

Table 5. Rates of Depurination from the DNA of EA Adducts at pH 6.8, 23 °C and of Methylated (Me) Adducts at pH 7.2, 37 °C

nucleobase adduct	$10^6 \times k_{obsd}$ (s^{-1})	$t_{1/2}$ (h)	nucleobase adduct refs *61 and 62*	$10^6 \times k_{obsd}$ (s^{-1})	$t_{1/2}$ (h)
N3-EA-Ade	5.94	32.4	N3-Me-Ade	6.42	30
N7-EA-Ade	47.9	4.0	N7-Me-Ade	68.7	2.8
N7-EA-Gua	1.29	150	N7-Me-Gua	1.34	144

and **12**, gave rise to cyclic structures **15** and **14**, respectively. The alkene-containing **14** predominated when the cleavage was accelerated by heat and was used for quantifying adduct levels (vide infra). Heating of **15** at 80 °C initiated the formation of some ^{1}H NMR resonances in the alkene region, but this was not investigated further. The formation at room temperature of **15** and the hydrate and aldehyde forms that are in equilibrium with it were essentially quantitative and gave a single well-defined peak in HPLC/MS that could be used in further adduct quantitation and analysis.

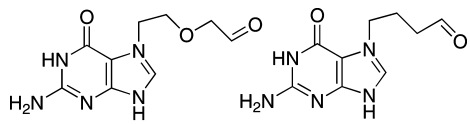
The EA-nucleobase adduct liberated by acid hydrolysis from the acetals was not markedly destabilized by attachment to the various heteroatoms of the purines. While decomposition to the HE-nucleobase was detected by mass spectrometry to the extent of 1−3% during acid hydrolysis of the dimethylacetals, the kinetics of decay studied at neutral and basic pH indicated that the stability of the EA moiety attached to the purines is comparable to that when attached to benzimidazole. In an earlier study, it was noted that the EA-fragment attached to benzimidazole was quite stable at neutral pH, but its decay was accelerated in basic media and in the presence of primary and secondary amines (*41*). Thus, the possibility that the pendant amines of the purines might destabilize the EA fragment was anticipated. To the contrary, examination of the data in Table 2 indicates, in general, a very similar stability, with rate constants for decay generally within a factor of 3 of that observed with the benzimidazole adduct. In no case was it possible to reliably determine the rate constants for decay in nearly neutral phosphate buffer (Table 2) due to the sluggishness of the reaction. As previously noted with the benzimidazole adduct, ethanolamine accelerates the decay, except in the case of the N^6-EA-Ade adduct. The reason for enhanced stability of the N^6-EA-Ade adduct is unclear; it may have to do with adoption of the carbinolamine form, **15**, though this does not appear to imbue stability in basic pH, to the extent that it exists as such under those conditions. Stability of the N^2-EA-Gua adduct could not be reliably monitored by initial rate methods due to the poor chromatographic resolution that resulted in excessive scatter in the initial rate plots of concentration against time.

4.2. Deposition of EA Adducts in the Decay of α-Hydroxynitrosomorpholine in the Presence of Nucleosides and Duplex DNA. 4.2.1. Nucleosides. Consistent with what is expected on the basis of alkylation by simple primary diazonium ions, all sites investigated are targeted by the diazonium ion intermediate and, similarly, with a distinct preference for the N7 and O^6 atoms of dGua. The 1-propyldiazonium ion similarly targets, for *n*-propylation, all heteroatoms on the purine nucleosides, with the O^6 and N7 of dGua being an average of 7-fold more abundant than the third-most abundant adduct studied here, N7 of dAde (*59*). Interestingly, the range of yields is an order of magnitude larger in the case of the diazonium ion derived from α-hydroxynitrosomorpholine compared to what is observed in *n*-propylation by the 1-propyldiazonium ion. The reason for this is presently unclear; possibly, it is due to methodological differences. In any case, there is no reason to expect quantitative uniformity given that the diazonium ion in the present case is sterically larger and can also undergo intramolecular capture, both of which factors can impact product yields differently depending upon the structure of the encounter complex with the different atom sites on the nucleosides.

4.2.2. Duplex DNA. The O^6 and N7 atoms of Gua are the predominant sites of deposition in duplex DNA with N3 of Ade being the third most targeted. This is qualitatively what is observed with the ethyldiazonium ion and *n*-propylation by 1-propyldiazonium ions (*59*). Remarkably, EA deposition at the N3 atom of Ade in the duplex increases 100-fold over what is observed in the nucleoside, substantially larger than the 8-fold increase in *n*-propylation at N3 observed in the case of the 1-propyldiazonium ion. The disappearance of adducts at N1 of Gua and N^6 of Ade in going from the nucleoside to the DNA duplex was also noted in the earlier propylation study. Similarly, the N^2-EA-Gua remains a minor product in reactions of duplex

DNA. Though minor, its biological significance is unknown. Certainly, considering the importance of lesion persistence, it is the only one of the adducts presently observed that will not either spontaneously depurinate or for which there is no specific repair mechanism such as the alkyl guanyl transferases in the case of O^6-Gua adducts. The activity of this repair protein is presently undocumented for the lesions derived from α-hydroxynitrosomorpholine, but the activity is known to be inhibited and accelerated, relative to the removal of a simple methyl group, by factors that similarly affect simple S_N2 reactions. Increased steric bulk and electron withdrawal decelerate rates of repair. Conjugation adjacent to the reactive center accelerates repair. The predominant form of the EA adduct, the hydrate, contains both an electron withdrawing group and increased steric bulk relative to a simple methyl group, suggesting the activity of alkylguanyl transferase might be anticipated to be relatively reduced compared to that of methyl. However, it is notable that the protein retains substantial activity against the pyridyloxobutyl lesion (*60*).

4.2.3. Comparison with Other Cyclic α-Hydroxynitrosamines. Although a precise quantitative comparison is not possible, the extant literature indicates that the diazonium ion derived from α-hydroxynitrosomorpholine is of efficiency with respect to alkylation of the N7 atom of Gua in the nucleoside and DNA that is comparable to that of α-hydroxynitrosopyrrolidine. The diazonium ions from α-hydroxyNMOR and α-hydroxynitrosopyrrolidine generated the adducts **16** and **17**, respectively. The alcohol derived from borohydride reduction of **17** was the species actually detected. The N7 adduct, **17**, is the major product of alkylation of dGua by the precursor α-acetoxynitrosopyrrolidine, with a yield of ∼8 mmol of adduct per mol of Gua (*31*).



16 **17**

Upon normalizing for the differences in the amounts of alkylating agents to which the DNA was subjected in that and the present study, the diazonium ion from α-hydroxynitrosomorpholine is 3-fold *less* effective. With DNA as a target, **17** is the third most abundant product, but the most abundant *diazonium ion* adduct with a yield of 6.6 mmol of adduct per mol of Gua (*32*). Again correcting for the differences in amounts of alkylating agent in the two studies, the diazonium ion derived from α-hydroxynitrosomorpholine is 6-fold *more* efficient than that derived from α-hydroxynitrosopyrrolidine. Methodological differences between the present study and those of the Hecht group are such that there is doubt about the precision of these comparisons. For now, it is sufficient to suggest a similar efficiency in the DNA alkylation by these diazonium ions until such time as a direct comparison can be made using identical precursors and reaction conditions.

The present results appear to contrast markedly with the damage profile so far elucidated for the congener of α-hydroxynitrosomorpholine, α-hydroxynitrosopiperidine. While the alkylation profile by the former is typical of simpler diazonium ions, not a single diazonium ion derived product has been reported for the latter (*30*−*32, 39*). The major product from a reaction of α-acetoxynitrosopiperidine involves the reaction of the exocyclic amino group of dGua with the aldehyde equivalent derived from hydrolysis of the diazonium ion. An etheno adduct has also been reported. In some part, the difference may lie in the differences in approach taken here and previously. The present study has focused on diazonium ion adducts, using synthetic standards and chromatographic methods to quantify the product yields. By their nature, the diazonium ions are relatively unselective and short-lived. In the present study for the reactions with DNA, only ∼1% of the diazonium ions are trapped by DNA. The overwhelming portion reacts with water or by internal capture by the carbonyl of the diazonium ion and yields much more stable aldehyde equivalents, with some smaller fraction undergoing diazonium rearrangement and carbocation hydration, yielding other, again, more stable carbonyl electrophiles. The earlier studies, with α-acetoxynitrosopiperidine, are carried out with much longer exposure times in which these more stable carbonyl electrophiles can modify DNA, possibly substantially transforming the initial product profile so that any initially deposited diazonium ion derived products become relatively minor. The present study, using α-hydroperoxynitrosomorpholine, generates the α-hydroxynitrosomorpholine in seconds, thereby generating the diazonium ion in seconds (*29*), and after a total reaction time of only 15 s, the products are subjected to acid hydrolysis, chromatographic resolution, and detection. We stress that it is currently unknown which adducts from which electrophiles engender the potent mutagenic effects of any of the cyclic nitrosamines.

4.3. Formation of HE Adducts on Nucleosides and DNA. Rate constants for the formation of HE adducts are in some cases markedly accelerated in duplex DNA. Rate constants for HE formation were determined for a limited number of EA adducts, those that predominated in deposition on DNA. Decomposition of α-hydroxynitrosomorpholine in the presence of either nucleosides or DNA yielded the EA adducts, and the subsequent formation of HE adducts was monitored as a function of time. Comparison of the two rightmost columns in Table 4 indicate that the incorporation of the EA adduct into duplex DNA accelerates the formation of the HE adduct in the cases of the N7-EA-Ade, N7-EA-Gua, and O^6-EA-Gua adducts by factors of 7, 14, and 54, respectively. The basis for these rate accelerations and their differences is currently uncertain. There is no detectable acceleration of the formation of the HE adduct upon incorporation into the DNA duplex in the case of the N3-EA-Ade adduct. Of the four adducts studied, this is the only one that lies in the minor groove, although at this point, it is unknown if this is any more than coincidence.

4.4. Kinetics of Depurination of EA Adducts. Rate constants derived from initial rate measurements of DNA treated with α-hydroxynitrosomorpholine presented in the left half of Table 5 suggest that these adducts are unremarkable in their reactivity. The order of reactivity, N7-EA-Ade > N3-EA-Ade > N7-EA-Gua, is identical with what has been observed for the analogously methylated bases in DNA, as indicated by the data in the right half of Table 5 (*61, 62*). These data are for the reaction of the methylated bases at 37 °C, while the data for the EA adducts are at 22 °C. A literature compilation of values for N7-Me-Gua depurination suggests one might anticipate an increase in rate constant by about a factor of ∼2 at 37 °C (*61, 62*). This would make the purine EA adducts somewhat more reactive than the methylpurines, but well within the range of values observed for the depurination of N7-alkyl-Gua residues containing an electron withdrawing group a few atoms proximal to the N7 atom.

4.5. Summary of Most Significant Findings. The first purine adduct profile of the diazonium ion derived from α-hydroxynitrosomorpholine demonstrates remarkable similarity to those

observed for the diazonium ions derived from simple acyclic α-hydroxynitrosamines. It contrasts for the most part with the damage profiles reported to date for structurally related α-substituted cyclic nitrosamines. The ethoxyacetaldehyde moiety initially deposited is relatively stable but slowly decays to a hydroxyethyl fragment, and this decay is accelerated in duplex DNA compared to that in nucleosides.

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