Trapping of a Cross-Link Formed by a Major Purine Adduct of a Metabolite of the Carcinogen N-Nitrosomorpholine by Inorganic and Biological Reductants

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · APRIL 2013

Impact Factor: 3.53 · DOI: 10.1021/tx3005289 · Source: PubMed

2 AUTHORS, INCLUDING:

Niangoran Koissi

Morgan State University

14 PUBLICATIONS **32** CITATIONS

SEE PROFILE

Chemical Research in Toxicology

Article

pubs.acs.org/crt

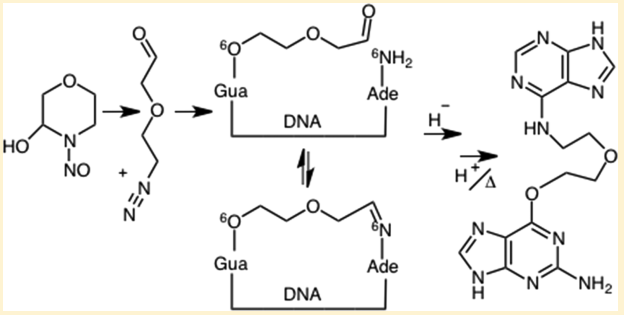
Trapping of a Cross-Link Formed by a Major Purine Adduct of a Metabolite of the Carcinogen *N*-Nitrosomorpholine by Inorganic and Biological Reductants

Niangoran Koissi and James C. Fishbein*

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland 21250, United States

Supporting Information

ABSTRACT: 3-Hydroperoxy-*N*-nitrosomorpholine in buffered aqueous media in the presence of calf thymus DNA was treated with a phosphine reductant to generate the transient α-hydroxynitrosamine and subsequent diazonium ion that alkylated the DNA, as previously reported. Subsequent addition of hydride donors, for 30 min, followed by acid hydrolysis of the mixture allowed detection and quantification of 6-(2-{2-[(9*H*-purin-6-yl)amino]ethoxy}ethoxy)-9*H*-purin-2-amine, the reduced cross-link formed from deposition, via the diazonium ion, of a 3-oxapentanal fragment on O^6-Gua, and condensation with N^6-Ade, presumably in the vicinity. Decreasing the temperature of the reaction mixtures and decreasing the pH modestly increased the yields of the trapped cross-link. Among three borohydride reductants, $NaNCBH_3$ is superior, being ∼4 times more effective on a molar basis, as opposed to a hydride equivalent basis, than $NaBH_4$ or $Na(AcO)_3BH$. For trapping with $NaNCBH_3$, it is deduced that the reaction likely occurs with the iminium ion that is in protonic equilibrium with its conjugate base imine. In an experiment in which the hydroperoxide was decomposed and $NaNCBH_3$ was introduced after various periods of time, the amount of cross-link was observed to increase, nearly linearly, by ∼4-fold over 1 week. These data indicate that there are a minimum of two populations of cross-links, one that forms rapidly, in minutes, and another that grows in with time, over days. Reduced nicotinamide cofactors and ascorbate are observed to effect reduction (over 3 days) of the cross-links, confirming the possibility that otherwise reversible cross-links might be immortalized under biological conditions.



INTRODUCTION

N-Nitrosomorpholine (**1**) is a potent liver and respiratory tract carcinogen in rodents.[1−3] There are numerous avenues of exposure to *N*-nitrosomorpholine in the human environment, and it is formed in humans endogenously.[4−13] A major pathway of metabolism in the rat is hydroxylation α to the *N*-nitroso functionality, likely mediated by P450 enzymes, as indicated in Scheme 1.[14,15] The α-hydroxy-*N*-nitrosomorpholine (**2**) metabolite rapidly decomposes to a diazonium ion capable of alkylating DNA.[16] Purine alkylation by the diazonium ion in Scheme 1 has been reported, and the pattern of adduct distribution is qualitatively similar to that observed for the simpler methyl- and ethyldiazonium ions.[17] A major adduct observed in reactions with DNA is that from capture of the diazonium ion by the O^6 atom of Gua. Unlike the adducts of simple alkyldiazonium ions, those derived from direct reaction of the diazonium ion of *N*-nitrosomorpholine, and indeed the diazonium ions of all cyclic nitrosamines, contain a pendant aldehyde moiety. The promiscuity of this functional group is such that it was anticipated that it might participate in DNA cross-linking, possibly transiently, even involving, as it were, more than one "player".

In this work, we sought to obtain evidence of such liaisons by attempting to trap cross-links using reductants, as indicated for the specific case involving the N^6-Ade in Scheme 1. Indeed, evidence of cross-linking reactions has been obtained, and various conditions that affect trapping yields of cross-links using borohydride reductants have been examined. Observations of the kinetics of trapped cross-link evolution with time indicate that there are at least two populations of cross-links: those that are rapidly formed, in minutes, and a larger pool of cross-links that form over days. Finally, it is reported that there are biological reagents that are capable of "immortalizing" otherwise reversible cross-links.

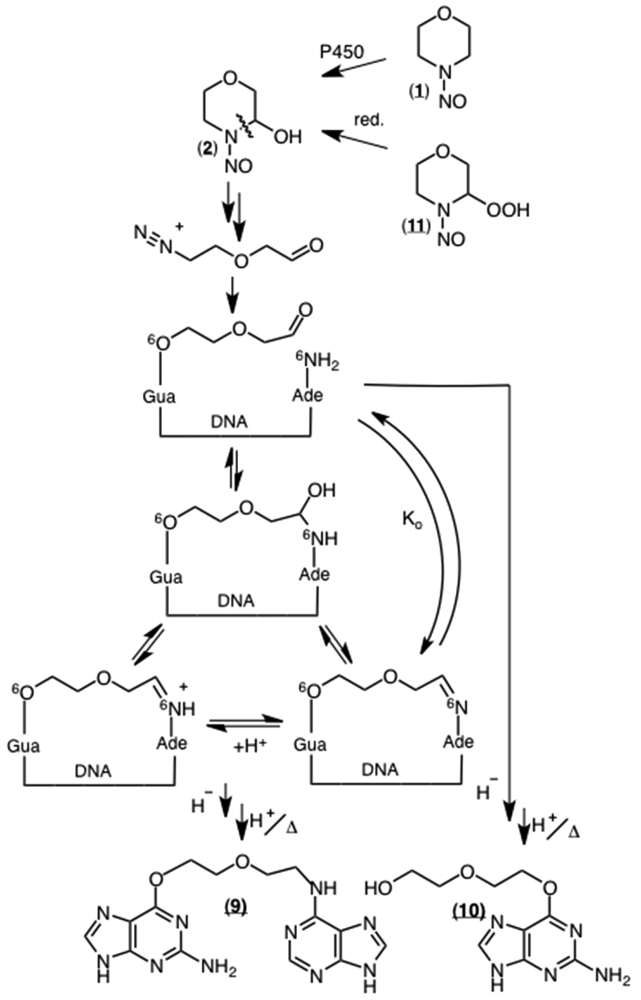
EXPERIMENTAL PROCEDURES

Materials. Except as noted, chemicals, reagents, and solvents obtained from commercial sources were of analytical or synthetic grade and were used as received. 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. Methanol was 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

Received: December 31, 2012

   dx.doi.org/10.1021/tx3005289 | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

Scheme 1. Formation and Distribution of Cross-Links Derived from Activation of *N*-Nitrosomorpholine (1), or Reduction of 11, to Metabolite 2 in the Presence of DNA Followed by Reduction (H^-) and Acid Hydrolysis To Yield Cross-Linked Nucleobases 9 and Un-Cross-Linked 10



were not further purified. Calf thymus DNA was purchased from commercial sources and purified after precipitation with ethanol by three successive washings and precipitations, at 0 °C, with a 70% ethanol/30% water mixture (v/v).

Analytical Determinations. ^{1}H NMR and ^{13}C NMR spectra were acquired using a JEOL AS 400 (400 MHz) instrument. Measurements of pH were performed using an Orion pH meter (model SA720), with a combination electrode. Two point calibrations, using commercially available standards, were conducted prior to recording pH values. High-resolution mass spectra were obtained at the University of Maryland, Baltimore County Center for Biological Mass Spectrometry.

HPLC. Some purification steps employed a Waters 2695 liquid chromatograph with a diode array UV detector monitoring at 260 nm using a Phenomenex Luna column (250 mm × 4.6 mm, 1 mL/min) and the following gradient elution method. The two solvents were 10 mM aqueous ammonium acetate (pH 6.9) and acetonitrile. The gradient entailed initially 5% acetonitrile for 5 min and then a 20 min linear gradient to 40% acetonitrile followed by a 20 min linear gradient to 60% acetonitrile, which was kept isocratic for 5 min followed by a 5 min linear gradient to the initial condition. A period of 15 min of equilibration time was allowed between injections.

LC/MS/MS analyses of cross-links were conducted using a Water Micromass ZQ triple quadrupole mass spectrometer interfaced with a Waters 2695 HPLC system equipped with a BDS Hypersil C18 analytical column (150 mm × 2.1 mm, 0.2 mL/min). The gradient used for this analysis entailed initially 0% acetonitrile for 5 min followed by a 10 min linear gradient to 10% acetonitrile and then a 10 min linear gradient to 30% acetonitrile, which was kept isocratic for 5 min followed by a 5 min linear gradient to the initial condition. A period of 15 min of equilibration time was allowed between injections.

Synthesis of the 6-(2-{2-[(9*H*-Purin-6-yl)amino]ethoxy}-ethoxy)-9*H*-purin-2-amine Reduced Cross-Link Standard. The series of reactions indicated in Scheme 2 were used for the synthesis of an authentic standard of a prospective cross-linked base pair that could be stabilized in DNA by reduction and isolated after acid hydrolysis, as described in further detail, below.

2-[2-(2-Hydroxyethoxy)ethyl]isoindoline-1,3-dione (3a). To a solution of 2-(2-chloroethoxy)ethanol (5 mL, 47.4 mmol) in *N*,*N*-dimethylformamide (35 mL) was added potassium phthalimide (8.8 g, 47.51 mmol), and the reaction mixture was stirred for 20 h at 120 °C. The reaction mixture was left to cool, concentrated, and then diluted with 300 mL of dichloromethane. This solution was washed four times with 20 mL each time of water and once with 20 mL of saturated brine and then dried over anhydrous magnesium sulfate. The solvent was removed, and the residue was purified by flash column chromatography on silica gel (3:2 hexane/ethyl acetate mixture) to give the desired compound as a white solid (8.91 g, 76.13%) having an R_f of 0.10 (solvent, 3:2 hexane/ethyl acetate mixture). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.85 (dd, 2H, J = 5.96, 3.24 Hz), 7.72 (dd, 2H, J = 5.96, 3.20 Hz), 3.92 (t, 2H, J = 5.48 Hz), 3.76 (t, 2H, J = 5.48 Hz), 3.72–3.66 (2H, m), 3.61 (t, 2H, J = 5.52 Hz), 2.54 (t, 1H, J = 5.50 Hz). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 168.60, 134.11, 132.10, 123.39, 72.25, 68.47, 61.81, 37.60.

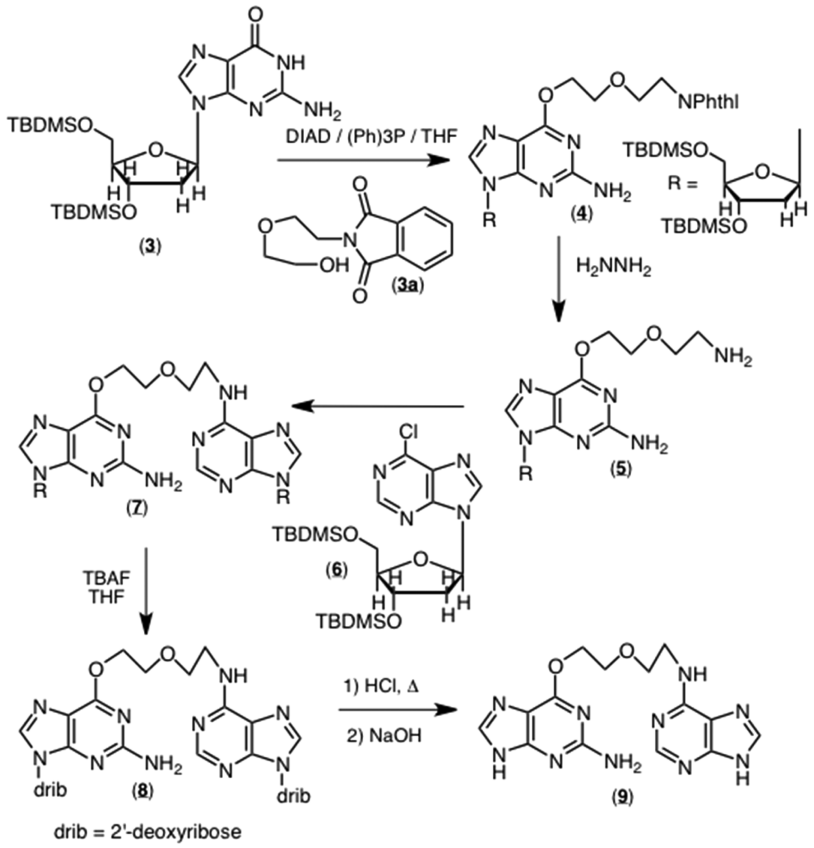
2-[2-(2-{[2-Amino-9-((2*R*,4*S*,5*R*)-4-[(*tert*-butyldimethylsilyl)-oxy]-5-{[(*tert*-butyldimethylsilyl)oxy]methyl}tetrahydrofuran-2-yl)-9*H*-purin-6-yl]oxy}ethoxy)ethyl]isoindoline-1,3-dione (4). The TBDMS-protected guanine nucleoside (**3**) (443 mg, 0.89 mmol) was stirred at 0 °C with triphenylphosphine (2 equiv, 467 mg) and 2-[2-(2-hydroxyethoxy)ethyl]isoindoline-1,3-dione (2 equiv, 420 mg) in 45 mL of dry THF. To this was added, dropwise, diisopropyl azodicarboxylate (DIAD, 2 equiv, 0.36 mL). The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was evaporated to dryness. The mixture was dissolved in ether, and the precipitate was filtered off. The residue was dried again, redissolved in CH_2Cl_2, washed with water (10 mL) and saturated $NaHCO_3$ (10 mL), dried over Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The crude product was purified on a silica gel column with a hexane/ethyl acetate mixture (3:2) as the eluant to give 370 mg (58% yield) of compound **4** as a light brownish oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84 (s, 1H, H-8), 7.78–7.65 (m, 4H, phthal), 6.29 (t, 1H, H-1′, J = 9.19 Hz), 4.87 (br, 2H, NH_2), 4.56 (q, 3H, H-10, H-3′), 3.95 (q, 1H, H-4′), 3.87 (q, 4H, H-11, H-13), 3.81–3.73 (m, 4H, H-14, H-5′, H-5″), 2.55–2.50 (m, 1H, H, H-2′), 2.35–2.29 (m, 1H, H-2″), 0.89 [s, 18H, Si-C$(CH_3)_3$], 0.08 (s, 6H, Si-CH_3), 0.06 (s, 6H, Si-CH_3).

6-[2-(2-Aminoethoxy)ethoxy]-9-((2*R*,4*S*,5*R*)-4-[(*tert*-butyldimethylsilyl)oxy]-5-{[(*tert*-butyldimethylsilyl)oxy]-methyl}tetrahydrofuran-2-yl)-9*H*-purin-2-amine (5). To a solution of **4** (300 mg, 0.768 mmol) in THF (10 mL) was added hydrazine hydrate (0.1 mL, 2 mmol), and the solution was stirred at room temperature overnight. The resulting red solution contained a precipitate that was filtered over Celite and discarded. The filtrate was dissolved in 20 mL of CH_2Cl_2, washed with water (10 mL) and saturated $NaHCO_3$ (10 mL), dried over Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The crude product was purified by silica gel chromatography with 2% MeOH in CH_2Cl_2 to provide 110 mg (45% yield) of compound **5** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (s, 1H, H-8), 6.30 (t, 1H, H-1′, J = 9.19 Hz), 4.88 (br, 2H, NH_2), 4.63 (t, 2H, H-10, J = 4.60 Hz), 4.56 (q, 1H, H-3′), 3.97 (q, 1H, H-4′), 3.86 (t, 2H, H-11), 3.81–3.68 (m, 4H, H-14, H-5′, H-5″), 3.57 (t, 1H, H-13), 2.86 (t, 2H, CH_2-NH_2), 2.55 (m, 1H, H-2′), 2.33 (m, 1H, H-2″), 0.89 [ds, 18H, Si-C$(CH_3)_3$], 0.09 (s, 6H, Si-CH_3), 0.06 (ds, 6H, Si-CH_3).

9-((2*R*,5*R*)-4-[(*tert*-Butyldimethylsilyl)oxy]-5-{[(*tert*-butyldimethylsilyl)oxy]methyl}tetrahydrofuran-2-yl)-6-[2-(2-

dx.doi.org/10.1021/tx3005289 | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

Scheme 2. Synthetic Scheme for Formation of Reduced Cross-Linked Nucleobases Gua and Ade



{[9-((2*R*,4*S*,5*R*)-4-[(*tert*-butyldimethylsilyl)oxy]-5-{[(*tert*-butyldimethylsilyl)oxy]methyl}tetrahydrofuran-2-yl)-9*H*-purin-6-yl]amino}ethoxy)ethoxy]-9*H*-purin-2-amine (7). A solution of **5** (330 mg, 0.567 mmol) in ethanol (10 mL) was added to a solution of 5′,3′-di-*O*-TBDMS-6-chlorodeoxyadenosine (**6**) (0.85 g, 1.7 mmol) in 40 mL of ethanol. The mixture was heated slowly to 78 °C and stirred overnight under reflux. The solvent was removed *in vacuo* and the residue dissolved in 20 mL of CH_2Cl_2, washed with water (20 mL) and saturated $NaHCO_3$ (20 mL), dried over Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The mixture was purified by silica gel chromatography with a CH_2Cl_2/MeOH mixture (9:1) to give 220 g (37% yield) of compound **7** as a light red oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.33 (s, 1H, NH), 8.03 (s, 1H, H-2), 7.88 (s, 1H, H-8), 6.42 (t, 1H, H-1′), 6.30 (t, 1H, H-1′), 4.97 (br, 2H, NH_2), 4.65 (t, 2H, H-10), 4.56 (m. 2H, 2 × H-3′), 4.00−3.94 (dq, 2H, 2 × H-4′), 3.89−3.68 (m, 10H, H-11, H-13, H-14, 2 × [H-5′ and H-5″]), 2.64−2.55 (m, 2H, 2 × H-2′), 2.43−2.30 (m, 2H, 2 × H-2″), 0.89 [ds, 36H, 4 × Si-C$(CH_3)_3$], 0.09 (s, 12H, 4 × Si-CH_3), 0.06 (ds, 12H, 4 × Si-CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ 160.98, 159.34, 154.80, 153.67, 153.04, 138.03, 137.53, 132.23, 123.11, 128.62, 128.43, 120.26, 115.60, 87.98, 87.70, 84.29, 83.73, 71.97, 70.02, 69.18, 65.64, 62.85, 41.14, 40.82, 26.08, 25.86, 18.38, 18.05, −4.60, −4.77, −5.34, −5.43.

(2*R*,5*R*)-5-(2-Amino-6-{2-[2-({9-[(2*R*,4*S*,5*R*)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-9*H*-purin-6-yl}amino)-ethoxy]ethoxy}-9*H*-purin-9-yl)-2-(hydroxymethyl)-tetrahydrofuran-3-ol (8). Compound 7 (50 mg, 0.048 mmol) was dissolved in THF (2 mL), and to this mixture was added 0.5 mL of a 1 M solution of tetrabutylammonium fluoride (TBAF) in THF (0.5 mmol); this mixture was stirred at room temperature for 1 h. The colorless solution became purple in this time. The solvents were removed under reduced pressure, and the residue was dissolved in water (10 mL) and washed twice with CH_2Cl_2 (10 mL). The crude compound was extracted into the aqueous layer. The compound was purified by HPLC using a buffered aqueous acetonitrile gradient (see HPLC) to produce **8** as a faintly red solid in 90% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.30 (s, 1H, H-8), 8.18 (s, 1H, H-2), 8.05 (s, 1H, H-8), 7.77 (br, 1H, NH), 6.40 (br, 2H, NH_2), 6.31 (t, 1H, H-1′), 6.17 (t, 1H, H-1′), 5.29 (d, 1H, OH), 5.26 (d, 1H, OH), 5.16 (t, 1H, OH), 4.98 (t, 1H, OH), 4.49 (t, 2H, H-10), 4.39 (q, 1H, H-3′), 4.34 (q, 1H, H-3′), 3.85 (q, 1H, H-4′), 3.80−3.75 (m, 3H, H-4′, H-11), 3.63−3.45 (m, 8H, H-13, H-14, 2 × [H-5′ and H-5″]), 2.67 (m, 1H, H-2′), 2.54 (m, 1H, H-2′), 2.26−2.17 (m, 2H, 2 × H-2″). HRMS (FAB+): *m*/*z* calcd for $C_{24}H_{32}N_{10}O_8$ $[M + H]^+$ 589.2484, found 589.2473

6-(2-{2-[(9*H*-Purin-6-yl)amino]ethoxy}ethoxy)-9*H*-purin-2-amine (9). Compound **8** (20 mg, 0.034 mmol) was dissolved in HCl (1 mL, 5 M) and 5 mL of water. The mixture was stirred at 80 °C for 30 min and then cooled and neutralized with NaOH (1 mL, 5 M). The mixture was purified by HPLC using the buffered aqueous acetonitrile gradient (see HPLC) to yield 10.52 mg (87%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.36 (s, 1H, N**H**), 8.16 (s, 1H, H-8), 8.03 (s, 1H, H-8), 7.75 (s, 1H, H-2), 6.20 (br, 2H, N**H**$_2$), 4.50 (t, 2H, C**H**$_2$), 3.78 (t, 2H, C**H**$_2$), 3.64 (m, 4H, 2 × C**H**$_2$). λ_{max} = 241 and 282 nm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 160.56, 160.13, 153.60, 152.81, 149.98, 144.42, 138.70, 140.35, 120.52, 108.69, 69.45, 68.89, 65.29. HRMS (FAB+): *m*/*z* calcd for $C_{14}H_{16}N_{10}O_2$ $[M + H]^+$ 357.1537, found 357.1527.

2-{2-[(2-Amino-9*H*-purin-6-yl)oxy]ethoxy}ethanol (10). To 0.176 mmol of the precursor aldehyde[17] in 1 mL of water was added $NaBH_4$ (10 mg). Stirring was continued for 10 min. The reaction mixture was neutralized and purified by HPLC as a single peak (90:10 H_2O/MeCN mixture) on a 5 μm, C18, 250 mm × 21.2 mm semipreparative column to yield the product as a white solid (70%). ^{1}H NMR (D_2O): δ 8.38 (1H, s), 4.77 (2H, m), 3.99 (2H, m), 3.73 (4H, m). ^{13}C NMR (D_2O): δ 160.0, 156.2, 150.6, 142.3, 106.6, 71.8, 68.3, 67.4, 60.3. HRMS: *m*/*z* calcd for $[M + H]^+$ $C_9H_{14}N_5O_3$ 240.1091, found 240.1090.

Trapping Cross-Links. *Standard Protocol.* A solution of 3-hydroperoxy-*N*-nitrosomorpholine [HOONOMO, **11** (Scheme 1)][16] in acetonitrile was added to a buffered aqueous solution [0.05 M 70% anion cacodylic acid buffer (pH 6.9)] containing tris(2-carboxyethyl)-

dx.doi.org/10.1021/tx3005289 | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

phosphine hydrochloride (TCEP) from a freshly neutralized stock and calf thymus DNA. The final concentrations were 10 mM α-HOONOMO, 10 mM TCEP, and ~0.5 mg/mL calf thymus DNA. The reaction mixture was stirred at room temperature for 30 s, during which the evolution of gas was observed. A solution of freshly prepared hydride donor (for details, *vide infra*) was added. The time of exposure to the reductant varied (for details, *vide infra*). In most experiments that used boron hydride reductants, after being exposed for 30 min, the solution was acidified to 0.1 M HCl and subjected to mild acid hydrolysis (30 min at 80 °C). The solution was cooled briefly, neutralized with NaOH (pH 7), and separated into three aliquots. One aliquot was used for LC/MS analysis of total O^6-Gua alkylation that generated un-cross-linked **10** (Scheme 1) by monitoring for the MH^+ ion at m/z 240.1. A second aliquot was used for LC/MS analysis of the reduced cross-link [**9** (Scheme 1)] with monitoring for the MH^+ ion at m/z 357.17, isolation, and quantification by analysis of a daughter fragment as described in Results. The third aliquot was used for determination of the total amount of Gua by LC with UV detection. The products of the reaction were identified by co-elution with authentic standards under two different elution protocols and verified occasionally by spiking a replicate injection with the standard. Product yields were calculated on the basis of interpolation from standard curves.

Effect of Varying the Reducing Agent on Cross-Link Trapping. Calf thymus DNA was incubated as previously mentioned with 10 μM HOONOMO and TCEP at 21 °C. Cross-link trapping was conducted via addition of 10 mM $NaBH_4$, $NaCNBH_3$, or $NaBH(OAc)_3$ and various concentrations of other organic and natural reductants, including dithiothreitol, NADH, NADPH, ascorbate, and GSH.

Effect of the Variation of Temperature on Cross-Link Trapping. Calf thymus DNA was incubated as previously mentioned with 10 μM HOONOMO in 250 μL of cacodylic buffer (pH 6.9) at 21, 37, and 50 °C, and trapping at the same temperature employed treatment with $NaBH_4$ or $NaNCBH_3$ for 30 min.

Effect of pH on Cross-Link Trapping. The reaction was conducted generally according to the standard protocol with the exception that the values of the solution pH were 5.0, 6.8, and 8.5 and were maintained by 0.055 M cacodylic acid buffers. Reduction of the cross-links was effected by treatment with 10 mM $NaCNBH_3$ for 30 min.

Time Course of A–G Cross-Link Formation. Cross-link formation was monitored over 7 days. A batch reaction mixture was prepared and the reaction carried out as described in Standard Protocol using a final concentration of hydride donor $NaCNBH_3$ of 10 mM. Aliquots were removed at various times and subjected to reduction (10 mM $NaCNBH_3$ for 30 min) and hydrolysis as described in Standard Protocol.

RESULTS

The yield of the un-cross-linked O^6-Gua adduct, detected after reduction and depurination as **10** (Scheme 1), was 0.31% (±0.03), on the basis of four determinations.

Evidence of cross-links formed by the aldehyde function of the O^6-Gua adduct formed in the capture of the diazonium ion derived from α-hydroxy-*N*-nitrosomorpholine was sought in reactions with calf thymus DNA that were similarly subjected to reduction, depurination, and LC/MS analysis. This was guided by authentic standard **9** (Scheme 1) that was synthetically generated. A typical LC/MS profile is indicated in Figure 1A, where the upper chromatogram, in which monitoring at [M + H] m/z 357 was initiated at 22.5 min, is the experimental while the lower chromatogram is that of the synthetic standard. Collision-induced dissociation of the $[M + H]^+$ m/z 357 parent ion of the standard in a triple quadrapole instrument gave rise to two major fragment ions with nominal masses of $[M + H]^+$ m/z 162 and 206 (Figure 1B). These masses are consistent with structures depicted Figure 1B, presumably because of the cleavages indicated by the hash marks in Scheme 3. The most

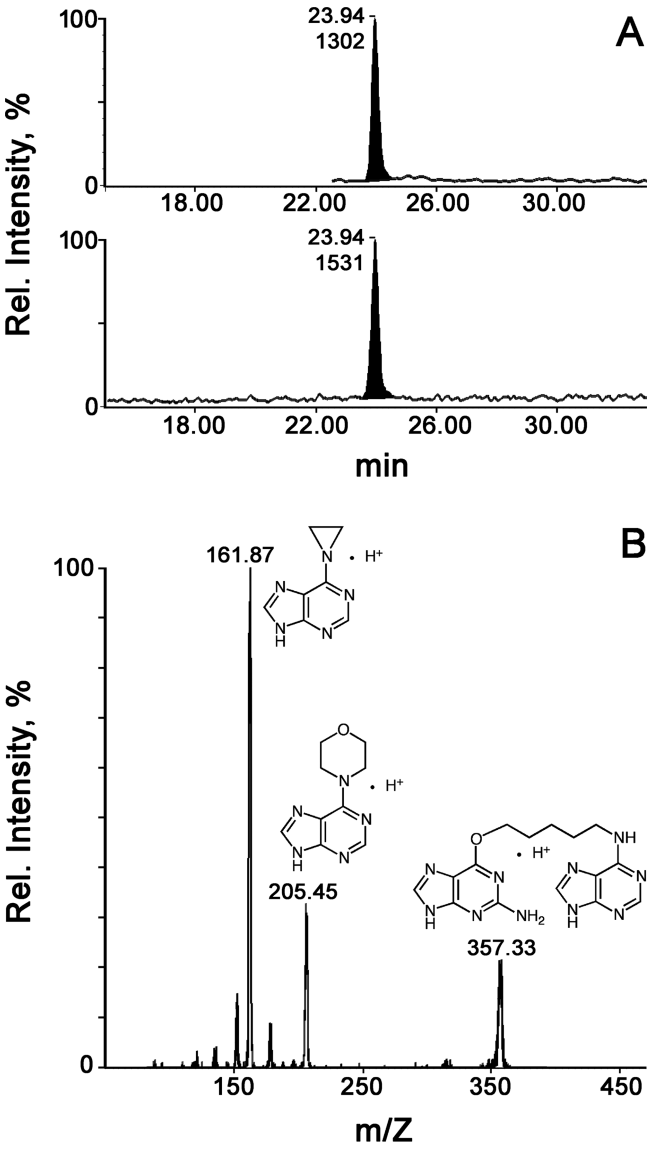


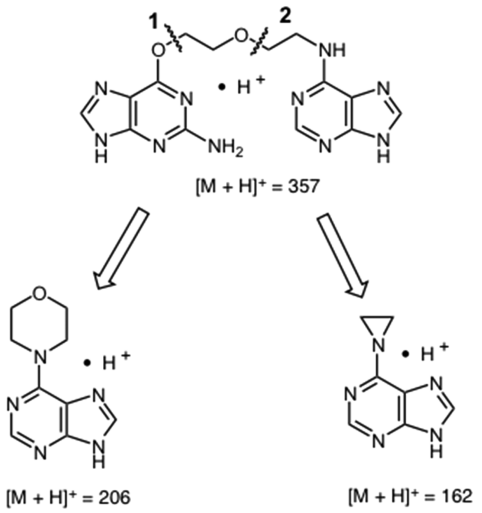
Figure 1. LC/MS chromatogram and fragmentation pattern of the reduced cross-link, **9** (Scheme 1). (A) The bottom chromatogram is the signal monitored at m/z 357.3 of the synthetic standard [**9** (Scheme 1)]. The top chromatogram is the signal monitored at m/z 357.3, starting at 22.5 min, of the experimental sample derived from the treatment of calf thymus DNA, as detailed in Experimental Procedures. (B) Fragmentation of the m/z 357.3 species at 23.9 min. Proposed structures are assumed on the basis of the nominal masses.

intense ion, at m/z 162, was used for quantification. For reduction by borohydride reagents, the reduction was typically conducted for 30 min on the basis of the following observation. Comparison, at 21 and 37 °C, of reduction by 25 mM $NaBH_4$ for 30 and 120 min generated yields of the reduced cross-link that decreased <2% (21 °C) or <4% (37 °C) at the longer reduction time. These differences are likely within the precision of the determinations.

Figure 2 summarizes experimental results of cross-links trapped by borohydride reductants (30 min reduction time). Borohydride reductants were introduced from freshly prepared stock aqueous solutions. Panel A indicates the results for yields of reduced cross-links as a function of reductant, in this case $NaBH_4$, concentration, and temperature. Panel B indicates the effect of temperature on the yields of $NaNCBH_3$ (10 mM)

dx.doi.org/10.1021/tx3005289 | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

Scheme 3. Proposed Structures for Collision-Induced Dissociation Detected via Triple Quadrupole MS of the Reduced Cross-Link Standard



trapping. A comparison of yields with different borohydride reductants is indicated in panel C. In the first experiment using $NaBH(OAc)_3$ (data indicated by the third bar from the left in Figure 2C), the preparation of the stock solution of the reductant in water was accompanied by fuming and a significant decrease in the pH of the stock that was attributed to hydrolysis of the borohydride ester. In a second experiment with this reductant (data indicated by the right-most bar in Figure 2C), the reductant stock was prepared with addition, prior to dissolution of the borohydride, of 2 equiv of Na_2HPO_4 for each -OAc function. The stock solution exhibited an approximately neutral pH (by pH paper) upon mixing with a minimum of gas evolution. The data presented in panel D indicate the effect of pH on the yield of the reduced cross-link with 25 mM $NaNCBH_3$ as the reductant.

An experiment was conducted to monitor the change, over 7 days, of trappable cross-link [**9** (Scheme 1)] as a function of time prior to addition of the reductant. The results are indicated by the circles in Figure 3. The earliest time points, two indistinguishably overlapping circles, are for reduction 30 s after decomposition of the hydroperoxide. In a second experiment, the amount of trappable cross-link was measured over 3 days, indicated by squares in Figure 3. The earliest time point was, again, 30 s after decomposition of the hydroperoxide. In this second experiment, the total amount of 2-{2-[(2-amino-9*H*-purin-6-yl)oxy]ethoxy}ethanol [**10** (Scheme 1)] formed from reduction of the initially deposited O^6-Gua aldehyde adduct decreased by 14% after 1 day and by 31% after 3 days. In both data sets, error bars for the data are within the dimensions of the symbols.

In the experiments summarized in Figure 4, the reduced cross-link was quantified after exposure to the three biological reducing agents indicated for a period of 3 days. At concentrations of 5−50 mM, neither GSH nor dithiothreitol, after incubation of these reducing agents for 3 days, produced detectable amounts of the reduced cross-link.

DISCUSSION

Analytical Method. The major purine adducts derived from the decay of α-hydroxynitrosomorpholine in the presence of DNA have been previously reported.[17] The O^6-Gua adduct

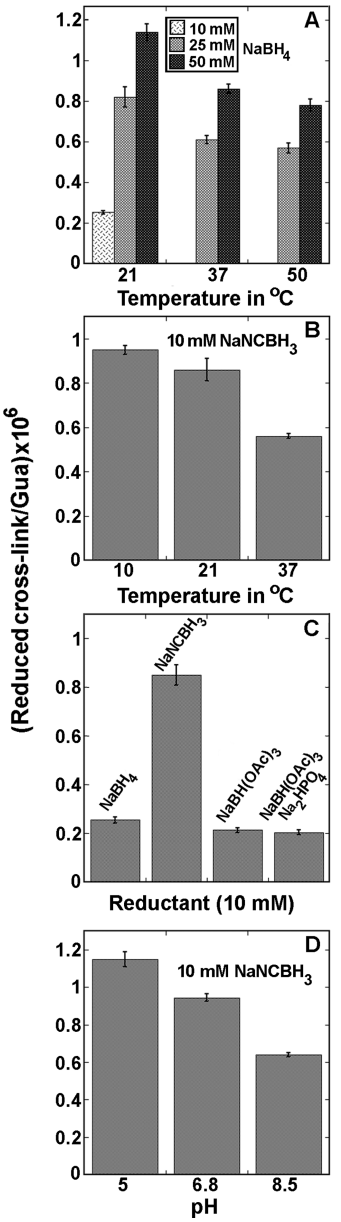


Figure 2. Yields of reduced cross-links from borohydride reductants as a function of varying conditions, for formation for 30 s and reduction for 30 min. (A) Reduction by $NaBH_4$ as a function of reductant concentration and temperature (of cross-link formation and reduction). (B) Reduction by $NaNCBH_3$ as a function of temperature (of cross-link formation and reduction). (C) Comparison of different borohydride reducing agents (see the text). (D) Yields for $NaNCBH_3$ reduction as a function of pH (of cross-link formation and reduction).

was a major product with a yield of 0.38%. That method entailed simple depurination and quantitation, in most cases, of the aldehyde or its hydrate form. In the method reported here, the yield of the un-cross-linked O^6-Gua adduct detected subsequent to reduction and then depurination (**10** in Scheme 1) amounted to 0.31%, in reasonable agreement with the result of the alternative method of the previous report.

Overview of Cross-Links. The appropriately oxidized metabolites of cyclic nitrosamines decompose to ambident electrophiles with a diazonium ion distal to an aldehyde functionality as in the example for the metabolite of NMOR (**2** in Scheme 1). To the best of our knowledge, the Results

dx.doi.org/10.1021/tx3005289 | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

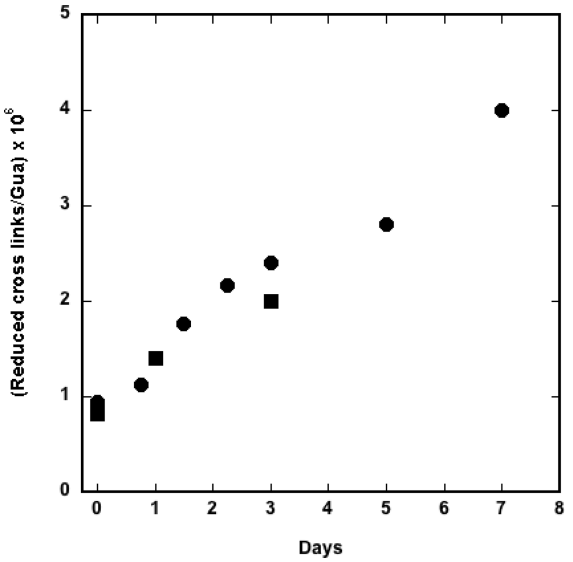


Figure 3. Yields of reduced cross-links [**9** (Scheme 1)] upon a 30 min incubation with 10 mM $NaCNBH_3$, after decomposition of **11** (Scheme 1) and incubation (25 °C) for the period of time indicated on the *X* axis. Circles depict data from an experiment conducted for 7 days and squares data for a similar experiment conducted for 3 days in which the total amount of reduced O^6-Gua adduct [**10** (Scheme 1)] was also determined (see the text).

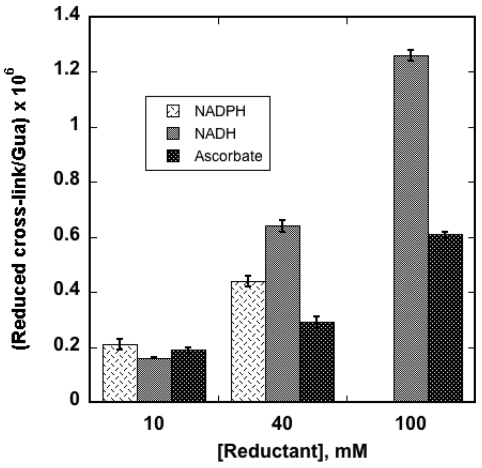


Figure 4. Yields of reduced cross-links formed from reduction by biological reducing agents after 3 days in the presence of each reducing agent at the given concentration. An experiment employing 100 mM NADPH was not conducted.

present the first evidence of trappable cross-links involving the pendent aldehyde moiety from a major diazonium ion-derived adduct from a cyclic α-hydroxynitrosamine. In all the experiments reported here, the amount of cross-link trapped [**9** (Scheme 1)] is a small portion of the total amount of O^6-Gua adduct deposited [**9** + **10** (Scheme 1)] between 0.1 and 1%. It is emphasized that this is the amount of trappable cross-links and does not exclude the possibility of a much larger pool of cross-links that are unreactive to reductants, as might be expected for some of the species in Scheme 1. This is discussed further below. Formation of cross-links by pendant aldehyde functions of other DNA adducts, from electrophiles both endogenously and exogenously derived, has precedent.[18,19]

Effects on Yields of Reduced Cross-Links. Most experiments were conducted by exposing calf thymus DNA to TCEP and 3-hydroperoxy-*N*-nitrosomorpholine (which is instantaneously decomposed by TCEP) for 30 s, more than sufficient time for decomposition of the α-hydroxynitrosamine and adduct deposition.[16] Introduction of a cross-link reducing reagent was typically followed by a 30 min incubation prior to acidification and depurination.

Both the reductant concentration and the temperature affect the yields of the detected trappable cross-link. In most experiments, borohydride-based reductants were employed. The yield is a function, albeit a nonlinear one, of hydride donor concentration as indicated by comparison of any set of bars in Figure 2A at a given temperature when $NaBH_4$ was employed. The same panel further indicates a diminishing yield with an increasing temperature (at the time of both adduct deposition and cross-link reduction). A similar effect is seen (Figure 2B) with $NaNCBH_3$. With both reductants, an approximately 30 °C increase in temperature results in a 25−30% decrease in the level of trappable cross-link.

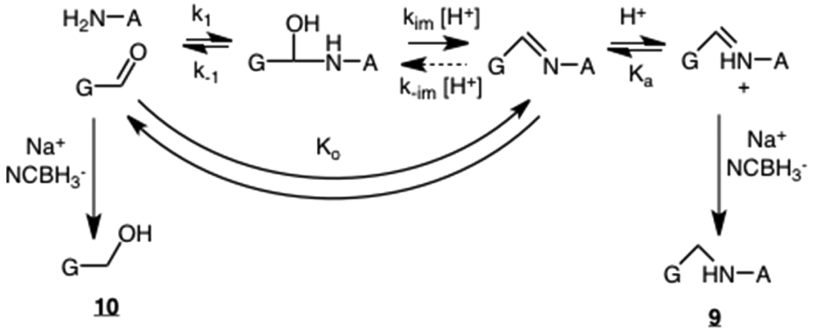
A direct comparison of different borohydride-based reductants is presented Figure 2C, and $NaNCBH_3$ is clearly superior. The data as presented are not normalized for total hydride equivalents, the molar concentration of total reagent being 10 mM in each case. $NaNCBH_3$ is more effective than $NaBH_4$ by a factor of almost 4-fold, though it contains only three-fourths of the total reducing equivalents. It is similarly superior to either of the experimental conditions in which $NaBH(OAc)_3$ was employed (*vide infra*), though normalizing for the total number of reducing equivalents would make $NaNCBH_3$ only approximately half again as effective as $NaBH(OAc)_3$, under either condition. In any case, it is unclear if normalization in conducting comparisons is valid on the basis of the nonlinear effects of trapping upon $NaBH_4$ concentration observed in Figure 2A. It is emphasized that in either approach, normalized or not, the data in Figure 2C indicate that $NaNCBH_3$ is the generally superior agent.

The exact nature of the reductant in experiments employing $NaBH(OAc)_3$ is uncertain. In an experiment employing a freshly prepared aqueous stock solution of the reductant (third column of Figure 2C), the initial preparation of the stock solution was accompanied by some fuming and a decrease in the pH of the stock. This was attributed potentially to hydrolysis of the triester, presumably to a mixture of mono- and diesters and $HB(OH)_3^-$. It is also possible that acidification resulted in a loss of some reducing equivalents. In an effort to minimize this loss, an experiment was conducted in which the $NaHB(OAc)_3$, in preparation of the stock, was dissolved in an aqueous solution containing sufficient Na_2HPO_4 to buffer against complete borate triester hydrolysis. These data appear in the rightmost column in Figure 2C. Comparison of these data with those in the adjacent column in Figure 2C indicates that the reducing equivalents were not significantly compromised by the observed acidification of the stock solution of reductant, and in both experiments, the trapping effectiveness is essentially equal to that of $NaBH_4$. Nonetheless, the identity of the reducing specie(s) is unknown.

Steric inhibition or accessibility does not appear to be dominant in determining the effectiveness of borohydride-based reducing agents. In Figure 2C, BH_4^- is the smallest such reagent whereas $BH(OR)_3^-$ (*vide supra*) is the largest, yet in each experiment, they are essentially equally effective trapping agents. The most effective trap, $NCBH_3^-$, is intermediate in size.

dx.doi.org/10.1021/tx3005289 | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

Scheme 4. Detailed Reaction Scheme for Trapping of Cross-Linked Imine and Iminium Ion by $NaNCBH_3$



Target(s) of Borohydride Reductants and Implications. The yield of the reduced cross-link and the absence of a significant effect of pH on the yield of the reduced cross-link are consistent with borohydride trapping of an iminium ion and its conjugate base imine, with which it is likely in protonic equilibrium. Reference to Scheme 4 is useful at this point. The pK_a of the iminium ion is anticipated to be <0 (Supporting Information). Two observations militate against the possibility that only the iminum ion, and none of the conjugate base imine, is accessible to trapping upon addition of $NaNCBH_3$. First, on the basis of the low pK_a of the iminium ion, an increase in the yield of the reduced cross-link of $>10^3$ is predicted for a decrease in pH from 8.5 to 5, in contrast with what is observed in Figure 2D. This assumes little difference in adduct deposition in this pH range, which is reasonable given the negligible change in ionization states of the nucleobases in the same pH range.[20] Second, the observed yield of the reduced cross-link at pH 6.8 is 10^3 times greater than predicted on the basis of the maximal theoretical yield of imine formed from the observed 0.0031 mol of O^6-Gua adduct [$K_o \sim \infty$ (Scheme 4)], and pK_a < 0 for the iminium ion. Even with a low pK_a value, it can be computed that the imine is likely in protonic equilibrium with the iminium ion (Supporting Information) and thus is trapped as the reduced cross-link [**9** (Schemes 1 and 4)]. The possibility that the neutral imine is trapped directly, possibly in association with Lewis acids or metals or H-bond donors, cannot be ruled out.[21−24]

The carbinolamine is likely in rapid equilibrium with the aldehyde and is thus not trapped as the imine or iminium ion on the basis of the chemistry of imines reported in the literature. The rate-limiting step in imine formation for an aldehyde and an amine of low conjugate acid pK_a is anticipated to be the acid-catalyzed reaction k_{im} in Scheme 4, so that $k_{-1} \gg k_{im}[H^+]$.[25,26] Because of this, upon introduction of $NaNCBH_3$, the carbinolamine reverts quantitatively to aldehyde that is immediately trapped as alcohol **10** (Scheme 1).

Thus, the amount of trapped reduced cross-link, overwhelmingly in the imine form at pH 6.8, reflects the equilibrium constant K_o (Scheme 4). An estimate for the K_o of ~0.03 M^{-1} can be deduced on the basis of the assumption that each O^6-Gua adduct is in the suitable proximity of two adenines (Supporting Information). This is similar to the K_o value of ~0.1 M^{-1} that can be estimated from literature for the formation of imines from amines and substituted benzaldehydes.[27,28] Finally, it is emphasized that the equilibrium constant for the total amount of cross-link formation, a number that would include the untrapped carbinolamine (Scheme 4), could be substantially greater on the basis of what has been reported in the literature.[18,19,25]

Implications of the Kinetics. The kinetics of formation of trappable cross-links are presented in Figure 3 and require that there are a minimum of two sets of trappable cross-links, a rapidly formed set, trapped on day 0, and a more slowly formed set that emerges over, and predominates after, days. In these experiments, TCEP-mediated reduction of 3-hydroperoxy-*N*-nitrosomorpholine in the presence of DNA is followed at various times (indicated on the abscissa of Figure 3) by a 30 min exposure to 10 mM $NaNCBH_3$, after which the purines are acid hydrolyzed and the cross-link yields are quantified. On the day 0 time points, $NaNCBH_3$ treatment occurs for 30 min after a 30 s exposure of DNA to the *α*-hydroxy-*N*-nitrosomorpholine (=TCEP + 3-hydroperoxy-*N*-nitrosomorpholine). The quantities of the reduced cross-links on "day 0" are nearly one-quarter of those detected on day 7, when the $NaNCBH_3$ treatment (30 min) occurs 7 days after decomposition of *α*-hydroxy-*N*-nitrosomorpholine in the presence of DNA. The accumulation of trappable cross-links is not due to accumulation of new O^6-3-oxapentanal-containing lesions over days. The diazonium ion giving rise to such lesions (Scheme 1) is destroyed in, likely,[29] milliseconds, and there is evidence against the formation of additional lesions from a long-lived phantom electrophile as has been proposed in other cases.[30] Indeed, in the experiment in Figure 3 indicated by squares, the total amount of O^6-3-oxapentanal-containing lesions was also monitored, by reduction [to **10** (Scheme 1)] and hydrolysis, and found to decrease over 3 days (see Results). This is likely a result of hydrolysis to hydroxyethyl lesions, as has been reported by us previously.[17] These data indicate that while a significant proportion of trappable cross-links form on the minute time scale or sooner, a larger proportion form, or can be trapped, much more slowly, presumably after some reconfiguration of local structure. This bifurcated behavior contrasts sharply with what has been observed in the case of acrolein-derived cross-links that, though similarly increasing over days, are effectively undetectable on day 0.[31]

Biological Reductants. The data in Figure 4 establish that cross-links can be covalently immortalized by biological reductants, the nicotinamide cofactors NADPH and NADH as well as ascorbate. While the concentrations of NADPH and NADH employed here are well above those observed *in vivo*,[32−35] as are the higher concentrations of ascorbate, ascorbate reaches millimolar concentrations in certain cells and is imported against its concentration gradient from the plasma where its concentration is tens of micromolar.[36−39] The experiments depicted involved exposure of the DNA to the biological reductants for 3 days. This is a relatively brief exposure given that hepatocyte lifetimes have been estimated to be 6−18 months.[40−42] The N^2-ethyl-dG adduct has been

dx.doi.org/10.1021/tx3005289 | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

attributed to biological reduction of a form of the imine derived from acetaldehyde, and indeed, reduction of such a species by biological reducing agents has been observed.[43−45] The potential generality of this phenomenon has been suggested for pendant aldehyde-containing lesions[18] and is confirmed here.

The impact of such reactions on mutagenesis by NMOR is presently unknown, but the mutation spectrum of 3-hydroxy-*N*-nitrosomorpholine will be reported in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional observations and references. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (410) 455-2190. Fax: (410) 455-2608. E-mail: jfishbei@umbc.edu.

Funding

This work was supported by National Institutes of Health Grant NCI R01-CA52881.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DIAD, diisopropyl azodicarboxylate; HOONOMO, 3-hydroperoxy-*N*-nitrosomorpholine; Pthal, phthalyl; TBAF, tetrabutylammonium fluoride; TBDMS, *tert*-butyldimethylsilane; TCEP, tris(2-carboxyethyl)phosphine hydrochloride

■ REFERENCES

(1) Druckrey, H., Preussmann, R., Ivankovic, S., and Schmahl, D. (1967) [Organotropic carcinogenic effects of 65 various N-nitroso-compounds on BD rats]. *Z. Krebsforsch. 69*, 103−201.

(2) Lijinsky, W. (1992) *Chemistry and biology of N-nitroso compounds*, Cambridge University Press, Cambridge, U.K.

(3) Mohr, U. (1979) Carcinogenesis of N-nitroso-morpholine and derivatives in Syrian golden hamsters. *Prog. Exp. Tumor Res. 24*, 235−244.

(4) Charrois, J. W., Arend, M. W., Froese, K. L., and Hrudey, S. E. (2004) Detecting N-nitrosamines in drinking water at nanogram per liter levels using ammonia positive chemical ionization. *Environ. Sci. Technol. 38*, 4835−4841.

(5) Sanches-Filho, P. J., Rios, A., Valcarcel, M., Zanin, K. D., and Caramao, E. B. (2003) Determination of nitrosamines in preserved sausages by solid-phase extraction-micellar electrokinetic chromatography. *J. Chromatogr., A 985*, 503−512.

(6) Spiegelhalder, B., and Preussmann, R. (1984) Contamination of toiletries and cosmetic products with volatile and nonvolatile N-nitroso carcinogens. *J. Cancer Res. Clin. Oncol. 108*, 160−163.

(7) Fajen, J. M., Carson, G. A., Rounbehler, D. P., Fan, T. Y., and Vita, R. (1979) N-Nitrosamines in the Rubber and Tire Industry. *Science 205*, 1262−1264.

(8) Monarca, S., Feretti, D., Zanardini, A., Moretti, M., Villarini, M., Spiegelhalder, B., Zerbini, I., Gelatti, U., and Lebbolo, E. (2001) Monitoring airborne genotoxicants in the rubber industry using genotoxicity tests and chemical analyses. *Mutat. Res. 490*, 159−169.

(9) Oury, B., Limasset, J. C., and Protois, J. C. (1997) Assessment of exposure to carcinogenic N-nitrosamines in the rubber industry. *Int. Arch. Occup. Environ. Health 70*, 261−271.

(10) Reh, B. D., and Fajen, J. M. (1996) Worker exposures to nitrosamines in a rubber vehicle sealing plant. *Am. Ind. Hyg. Assoc. J. (1958−1999) 57*, 918−923.

(11) Dallinga, J. W., Pachen, D. M., Lousberg, A. H., van Geel, J. A., Houben, G. M., Stockbrugger, R. W., van Maanen, J. M., and Kleinjans, J. C. (1998) Volatile N-nitrosamines in gastric juice of patients with various conditions of the gastrointestinal tract determined by gas chromatography-mass spectrometry and related to intragastric pH and nitrate and nitrite levels. *Cancer Lett. 124*, 119−125.

(12) Kakizoe, T., Wang, T. T., Eng, V. W., Furrer, R., Dion, P., and Bruce, W. R. (1979) Volatile N-nitrosamines in the urine of normal donors and of bladder cancer patients. *Cancer Res. 39*, 829−832.

(13) van Maanen, J. M., Pachen, D. M., Dallinga, J. W., and Kleinjans, J. C. (1998) Formation of nitrosamines during consumption of nitrate- and amine-rich foods, and the influence of the use of mouthwashes. *Cancer Detect. Prev. 22*, 204−212.

(14) Brunnemann, K. D., Hecht, S. S., and Hoffmann, D. (1982) N-Nitrosamines: Environmental occurrence, in vivo formation and metabolism. *J. Toxicol. Clin. Toxicol. 19*, 661−688.

(15) Hecht, S. S., and Young, R. (1981) Metabolic α-hydroxylation of N-nitrosomorpholine and 3,3,5,5-tetradeutero-N-nitrosomorpholine in the F344 rat. *Cancer Res. 41*, 5039−5043.

(16) Kim, H. J., and Fishbein, J. C. (2003) Reexamination of the aqueous chemistry of N-nitroso-3-hydroxymorpholine, a metabolite of the carcinogen N-nitrosomorpholine. *Chem. Res. Toxicol. 16*, 715−720.

(17) Zink, C. N., Soissons, N., and Fishbein, J. C. (2010) Products of the direct reaction of the diazonium ion of a metabolite of the carcinogen N-nitrosomorpholine with purines of nucleosides and DNA. *Chem. Res. Toxicol. 23*, 1223−1233.

(18) Minko, I. G., Kozekov, I. D., Harris, T. M., Rizzo, C. J., Lloyd, R. S., and Stone, M. P. (2009) Chemistry and biology of DNA containing 1,N(2)-deoxyguanosine adducts of the α,β-unsaturated aldehydes acrolein, crotonaldehyde, and 4-hydroxynonenal. *Chem. Res. Toxicol. 22*, 759−778.

(19) Stone, M. P., Cho, Y. J., Huang, H., Kim, H. Y., Kozekov, I. D., Kozekova, A., Wang, H., Minko, I. G., Lloyd, R. S., Harris, T. M., and Rizzo, C. J. (2008) Interstrand DNA cross-links induced by α,β-unsaturated aldehydes derived from lipid peroxidation and environmental sources. *Acc. Chem. Res. 41*, 793−804.

(20) Blackburn, G. M. (1996) *Nucleic Acids in Chemistry and Biology*, 2nd ed., Oxford University Press, New York.

(21) Barrero, A. F., Alvarez-Manzaneda, E. J., Chahboun, R., and Arteaga, A. F. (2004) Degradation of the side chain of (−)-scaleol: A very short synthesis of nor-ambreinolide and ambrox. *Synth. Commun. 34*, 3631−3643.

(22) Bartoli, G., Cupone, G., Dalpozzo, R., De Nino, A., Maiuolo, L., Procopio, A., and Tagarelli, A. (2002) Stereoselective reduction of enaminones to syn γ-aminoalcohols. *Tetrahedron Lett. 43*, 7441−7444.

(23) Corey, E. J., and Helal, C. J. (1998) Reduction of carbonyl compounds with chiral oxazaborolidine catalysts: A new paradigm for enantioselective catalysis and a powerful new synthetic method. *Angew. Chem., Int. Ed. 37*, 1986−2012.

(24) Mohanta, P. K., Davis, T. A., Gooch, J. R., and Flowers, R. A. (2005) Chelation-controlled diastereoselective reduction of α-fluoroketones. *J. Am. Chem. Soc. 127*, 11896−11897.

(25) Jencks, W. P. (1964) Mechanism and catalysis of simple carbonyl group reactions. *Prog. Phys. Org. Chem. 2*, 63−128.

(26) Cordes, E. H., and Jencks, W. P. (1962) On the mechanism of Schiff base formation and hydrolysis. *J. Am. Chem. Soc. 84*, 832−837.

(27) Cordes, E. H., and Jencks, W. P. (1962) Nucleophilic catalysis of semicarbazone formation by anilines. *J. Am. Chem. Soc. 84*, 826−831.

(28) Zuman, P. (1951) The reaction of the carbonyl group with primary amines. *Collect. Czech. Chem. Commun. 15*, 839−873.

(29) Agnew, T. E., Kim, H.-J., and Fishbein, J. C. (2005) Diazonium ion chemistry: Replacement of H by alkyl at the central carbon accelerates an SN2 substitution reaction. *J. Phys. Org. Chem. 17*, 483−488.

(30) Wang, M., Young-Sciame, R., Chung, F., and Hecht, S. S. (1995) Formation of N^2-Tetrahydropyranyl Adducts in the Reactions of α-Acetoxy-N-nitrosopyrrolidine and α-Acetoxy-N-nitrosopiperidine with DNA. *Chem. Res. Toxicol. 8*, 617.

(31) Sanchez, A. M., Kozekov, I. D., Harris, T. M., and Lloyd, R. S. (2005) Formation of inter- and intrastrand imine type DNA-DNA cross-links through secondary reactions of aldehydic adducts. *Chem. Res. Toxicol. 18*, 1683−1690.
(32) Polakis, E. S., and Bartley, W. (1966) Changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides during the aerobic growth cycle of yeast on different carbon sources. *Biochem. J. 99*, 521−533.
(33) Klaidman, L. K., Leung, A. C., and Adams, J. D., Jr. (1995) High-performance liquid chromatography analysis of oxidized and reduced pyridine dinucleotides in specific brain regions. *Anal. Biochem. 228*, 312−317.
(34) Wosikowski, K., Mattern, K., Schemainda, I., Hasmann, M., Rattel, B., and Loser, R. (2002) WK175, a novel antitumor agent, decreases the intracellular nicotinamide adenine dinucleotide concentration and induces the apoptotic cascade in human leukemia cells. *Cancer Res. 62*, 1057−1062.
(35) Yu, Q., and Heikal, A. A. (2009) Two-photon autofluorescence dynamics imaging reveals sensitivity of intracellular NADH concentration and conformation to cell physiology at the single-cell level. *J. Photochem. Photobiol., B 95*, 46−57.
(36) Hornig, D. (1975) Distribution of ascorbic acid, metabolites and analogues in man and animals. *Ann. N.Y. Acad. Sci. 258*, 103−118.
(37) Franceschi, R. T., Wilson, J. X., and Dixon, S. J. (1995) Requirement for Na^+-dependent ascorbic acid transport in osteoblast functio. *Am. J. Physiol. 268*, C1430−C1439.
(38) May, J. M., Li, L., Hayslett, K., and Qu, Z. C. (2006) Ascorbate transport and recycling by SH-SY5Y neuroblastoma cells: Response to glutamate toxicity. *Neurochem. Res. 31*, 785−794.
(39) Montecinos, V., Guzman, P., Barra, V., Villagran, M., Munoz-Montesino, C., Sotomayor, K., Escobar, E., Godoy, A., Mardones, L., Sotomayor, P., Guzman, C., Vasquez, O., Gallardo, V., van Zundert, B., Bono, M. R., Onate, S. A., Bustamante, M., Carcamo, J. G., Rivas, C. I., and Vera, J. C. (2007) Vitamin C is an essential antioxidant that enhances survival of oxidatively stressed human vascular endothelial cells in the presence of a vast molar excess of glutathione. *J. Biol. Chem. 282*, 15506−15515.
(40) Macdonald, R. A. (1961) "Lifespan" of liver cells. Autoradiographic study using tritiated thymidine in normal, cirrhotic, and partially hepatectomized rats. *Arch. Intern. Med. 107*, 335−343.
(41) Grisham, J. W. (1962) A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H3. *Cancer Res. 22*, 842−849.
(42) Mason, W. S., Jilbert, A. R., and Summers, J. (2005) Clonal expansion of hepatocytes during chronic woodchuck hepatitis virus infection. *Proc. Natl. Acad. Sci. U.S.A. 102*, 1139−1144.
(43) Fang, J. L., and Vaca, C. E. (1995) Development of a ^{32}P-postlabelling Method for the Analysis of Adducts Arising throught the Reaction of Acetaldehyde with 2′-Deoxyguanosine-3′-monophosphate and DNA. *Carcinogenesis 16*, 2177.
(44) Fang, J. L., and Vaca, C. E. (1997) Detection of DNA Adducts of Acetaldehyde in Peripheral White Blood Cells of Alcohol Abusers. *Carcinogenesis 18*, 627.
(45) Vaca, C. E., Fang, J. L., and Schweda, E. K. (1995) Studies of the reaction of acetaldehyde with deoxynucleosides. *Chem.-Biol. Interact. 98*, 51−67.