Published in final edited form as:

Chem Res Toxicol. 2013 May 20; 26(5): 732–740. doi:10.1021/tx3005289.

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 USA 21250

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-((9H-purin-6-yl)amino)ethoxy)ethoxy)-9H-purin-2-amine, the reduced cross-link formed from deposition, via the diazonium ion, of a 3-oxa-pentanal fragment on O⁶-Gua, and condensation with N⁶-Ade, presumably in the vicinity. Decreasing temperature of the reactions and decreasing pH modestly increased the yields of trapped crosslink. Among three borohydride reductants, NaNCBH₃ is superior, being ~4 times more effective on a molar basis, as opposed to a hydride equivalent basis, than NaBH₄ or Na(AcO)₃BH. For trapping with NaNCBH₃, 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 NaNCBH₃ was introduced after various times, the amount of cross-link was observed to increase, nearly linearly, by about four-fold over one week. These data indicate that there are a minimum of 2 populations of cross-links, one that forms rapidly, in minutes, and another that grows in with time, over days. Reduced nicotinamide co-factors 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 ethyl-diazonium ions.¹⁷ A major adduct observed in reactions with DNA is that from capture of the diazonium ion by the O⁶ atom of Gua. Unlike the adducts of simple alkyl diazonium 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 “players”.

*Fishbein: Tel. 410-455-2190; Fax, 410-455-2608; jfishbei@umbc.edu

In the present report it was sought to obtain evidence of such liaisons by attempting to trap cross-links using reductants, as indicated for the specific case involving the N⁶-Ade in Scheme 1. Indeed evidence is obtained for cross-linking reactions and various conditions that affect trapping yields of cross-links using borohydride reductants have been examined. Observations on 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 crosslinks.

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 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 70% ethanol/30% water (v/v).

Analytical determinations

¹H NMR and ¹³C 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 carried out prior to recording values of pH. High resolution mass spectra were obtained at the UMBC 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 used 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 held 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 carried out using a Water Micromass ZQ Triple Quadrupole Mass Spectrometer interfaced to 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 then a 10 min linear gradient to 30% acetonitrile and was held 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-((9H-purin-6-yl)amino)ethoxy)ethoxy)-9H-purin-2-amine reduced cross-link standard

The series of reactions indicated in Scheme 2 were used for 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-dimethyl-formamide (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 4 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) to give the desired compound as a white solid (8.91 g, 76.13 %) having an $R_f = 0.10$ (solvent 3:2, hexane:ethyl acetate). ^1H NMR (CDCl_3 , 400MHz,) 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-((2R,4S,5R)-4-((tert-butylidimethylsilyl)oxy)-5-(((tert-butylidimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-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 eq, 467 mg) and 2-(2-(2-hydroxyethoxy)ethyl)isoindoline-1,3-dione (2 eq, 420 mg) in 45 ml of dry THF. To this was added, dropwise, diisopropyl azodicarboxylate (DIAD, 2 eq, 0.36 ml). The reaction was allowed to warm at room temperature and was 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, re-dissolved in CH_2Cl_2 and washed with water (10 ml), saturated NaHCO_3 (10ml), dried over Na_2SO_4 , filtered and evaporated to dryness under reduced pressure. The crude product was purified on a silica gel column with hexane-ethyl acetate (3:2) as eluant to give 370 mg (yield 58%) of the compound **4** as a light brownish oil. ^1H 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' & 5''), 2.55-2.50 (m, 1H, H, H-2'), 2.35-2.29 (m, 1H, H-2''), 0.89 (s, 18H, Si- $\text{C}(\text{CH}_3)_3$), 0.08 (s, 6H, Si- CH_3), 0.06 (s, 6H, Si- CH_3).

6-(2-(2-Aminoethoxy)ethoxy)-9-((2R,4S,5R)-4-((tert-butylidimethylsilyl)oxy)-5-(((tert-butylidimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-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 CH_2Cl_2 and washed with water (10 ml), saturated NaHCO_3 (10ml), 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 (yield 45 %) of the compound **5** as a colorless oil. ^1H 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' & 5''), 3.57 (t, 1H, H-13), 2.86 (t, 2H, $\text{CH}_2\text{-NH}_2$), 2.55 (m, 1H, H-2'), 2.33 (m, 1H, H-2''), 0.89 (ds, 18H, Si- $\text{C}(\text{CH}_3)_3$), 0.09 (s, 6H, Si- CH_3), 0.06 (ds, 6H, Si- CH_3).

9-((2R,5R)-4-((tert-Butylidimethylsilyl)oxy)-5-(((tert-butylidimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-6-(2-(2-((9-((2R,4S,5R)-4-((tert-

butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)ethoxy)ethoxy)-9H-purin-2-amine (7)

A solution of **5** (330 mg, 0.567 mmol) in ethanol (10ml) was added to a solution of 5',3'-di-*O*-TBDMS-6-chloro-deoxyadenosine (**6**) (0.85 g, 1.7 mmol) in 40 ml 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 CH₂Cl₂ and washed with water (20 ml), sat NaHCO₃ (20 ml), dried over Na₂SO₄, filtered and evaporated to dryness under reduced pressure. The mixture was purified by silica gel chromatography with CH₂Cl₂-MeOH (9:1) to give 220g (yield 37 %) of the compound **7** as light red oil. ¹H NMR (400 MHz, CDCl₃): 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, NH₂), 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' & 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(CH₃)₃), 0.09 (s, 12H, 4×Si-CH₃), 0.06 (ds, 12H, 4×Si-CH₃). ¹³C NMR (400 MHz, CDCl₃): 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.

(2R,5R)-5-(2-Amino-6-(2-(2-((9-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)ethoxy)ethoxy)-9H-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-ol (8)

The compound **7** (50 mg, 0.048 mmol) was dissolved in THF (2 ml), and to this was added 0.5 mL of a 1 M solution of tetrabutylammonium fluoride (TBAF) in THF (0.5 mmol) and the mixture was stirred at room temperature for 1 hr. 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 CH₂Cl₂ (10 ml). The crude compound was extracted into the aqueous layer. The compound was purified by HPLC using buffered aqueous-acetonitrile gradient (see **HPLC**) to obtain **8** as a faintly red solid in a 90% yield. ¹H NMR (400 MHz, DMSO-*d*₆): 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, NH₂), 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' & 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 C₂₄H₃₂N₁₀O₈ [M + H]⁺ 589.2484 found 589.2473

6-(2-(2-((9H-Purin-6-yl)amino)ethoxy)ethoxy)-9H-purin-2-amine (9)

The compound **8** (20 mg, 0.034 mmol) was dissolved in HCl (1 ml, 5M) and 5ml of water. The mixture was stirred at 80 °C for 30 min and then cooled and neutralized with NaOH (1ml, 5M). The mixture was purified by HPLC using the buffered aqueous-acetonitrile gradient (see **HPLC**) to yield 10.52 mg (87%). ¹H NMR (400 MHz, DMSO-*d*₆): 12.36 (s, 1H, NH), 8.16 (s, 1H, H-8), 8.03 (s, 1H, H-8), 7.75 (s, 1H, H-2), 6.20 (br, 2H, NH₂), 4.50 (t, 2H, CH₂), 3.78 (t, 2H, CH₂), 3.64 (m, 4H, 2×CH₂). λ_{max} = 241, 282 nm. ¹³C 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 C₁₄H₁₆N₁₀O₂ [M + H]⁺ 357.1537 found 357.1527.

2-(2-((2-amino-9H-purin-6-yl)oxy)ethoxy)ethanol (10)

To 0.176 mmol of the precursor aldehyde ¹⁷ in 1 mL of water was added NaBH₄ (10 mg). Stirring was continued for 10 min. The reaction mixture was neutralized and purified by HPLC as a single peak (90:10 H₂O/MeCN) on a 5 μm C18 250 mm × 21.2 mm

semipreparative column to yield product as white solid (70%). ^1H 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 Calcd for $[\text{M}+\text{H}]^+$ $\text{C}_9\text{H}_{14}\text{N}_5\text{O}_3$: 240.1091 Obsd: 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)phosphine hydrochloride (TCEP) from a freshly neutralized stock and calf thymus DNA. The final concentrations were 10 mM in α -HOONOMO, 10 mM TCEP, and ~ 0.5 mg/mL calf thymus DNA. The reaction was stirred at room temperature for 30 seconds 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 30 min of exposure, 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. One aliquot was used for LC/MS analysis of total O⁶Gua alkylation that generated uncross-linked **10**, Scheme 1, by monitoring for the MH⁺ ion with $m/z = 240.1$. A second aliquot was used for LC/MS analysis of the reduced cross-link (**9**, Scheme 1) with monitoring for the MH⁺ ion with $m/z = 357.17$, isolation and quantification by analysis of a daughter fragment as described in **Results**. The third aliquot was used for determination of total 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 standard. Product yields were calculated on the basis of interpolation from standard curves.

Effect of varying reducing agent on the cross-link trapping—Calf thymus DNA was incubated as previously mentioned with 10 μM HOONOMO and TCEP at 21 °C. The cross-link trapping was carried out by addition of 10 mM of NaBH_4 , NaCNBH_3 , or $\text{NaBH}(\text{OAc})_3$, and various concentrations of other organic and natural reductants including dithiothreitol, NADH, NADPH, ascorbate, and GSH.

Effect of variation of temperature on the 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 NaCNBH_3 for 30 min.

Effect of pH on cross-link trapping—The reaction was carried out generally according to the standard protocol with the exception that the values of solution pH were 5.0, 6.8 and 8.5 and they 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 was prepared and carried out as in the standard protocol using a final concentration of the hydride donor $\text{NaCNBH}_3 = 10$ mM. Aliquots were removed at various times and subjected to reduction (10 mM NaCNBH_3 , 30 min) and hydrolysis as in the standard protocol.

Results

The yield of uncross-linked O⁶-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 O⁶-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 the 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 $[M + H] = 357$ was initiated at 22.5 min, is the experimental while the lower chromatogram is that of the synthetic standard. Collision-induced dissociation of the $[M + H]^+ = 357$ parent ion of the standard in a triple quadrupole instrument gave rise to two major fragment ions with nominal masses $[M + H]^+ = 162$ and 206, Figure 1B. These masses are consistent with structures depicted Figure 1B, presumably due to cleavages indicated by the hash marks in Scheme 3. The most intense ion, 162, was used for quantification. For reduction by borohydride reagents, the reduction was typically carried out for 30 min on the basis of the following observation. Comparison, at 21 °C and 37 °C, of reduction by 25 mM NaBH₄ for 30 or 120 min generated yields of the reduced cross-link that decreased <2% (21 °C) and <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 NaBH₄, concentration and temperature. Panel B indicates the effect of temperature on the yields of NaNCBH₃ (10 mM) 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 third bar from the left in panel 2C), the preparation of the stock solution of reductant in water was accompanied by fuming and a significant decrease in pH of the stock that were attributed to hydrolysis of the borohydride ester. In a second experiment with this reductant (data indicated by the right-most bar in panel 2C) the reductant stock was prepared with addition, prior to dissolution of the borohydride, of 2 equivalents of Na₂HPO₄ for each -OAc function. The stock solution exhibited 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 reduced cross-link with 25 mM NaNCBH₃ as the reductant.

An experiment was carried out to monitor the change, over 7 days, of trappable cross-link (**9**, Scheme 1) as a function of time prior to addition of reductant. The results are indicated by the circles in Figure 3. The earliest time points, two indistinguishably overlapping circles, are for reduction 30 seconds after decomposition of the hydroperoxide. In a second experiment, the amount of trappable cross-link was measured over three days, indicated by squares, Fig. 3. The earliest time point was, again, 30 sec after decomposition of the hydroperoxide. In this second experiment, the total amount of 2-(2-((2-amino-9H-purin-6-yl)oxy)ethoxy)ethanol (**10**, Scheme 1) formed from reduction of the initially deposited O⁶-Gua aldehyde adduct decreased by 14% after one day and by 31% after three days. In both data sets, error bars for the data are with the dimensions of the symbols.

In the experiments summarized in Figure 4, 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 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 O⁶-Gua adduct 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 presently reported, the yield of uncross-linked O⁶-Gua adduct detected subsequent to *reduction* and then depurination, **10** in Scheme 1, amounted to 0.31%, in reasonable agreement with the alternative method of the previous report.

Overview on 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 our knowledge the **Results** 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 O⁶-Gua adduct deposited (**9** + **10**, Scheme 1), between 0.1 – 1%. It is emphasized that this is the amount of *trappable* crosslinks 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, is well precedented.^{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 sec, more than sufficient time for decomposition of the α -hydroxynitrosamine and adduct deposition.¹⁶ Introduction of cross-link reducing reagent was typically followed by a 30 minute incubation prior to acidification and depurination.

Both reductant concentration and temperature affect the yields of trappable cross-link detected. In most experiments borohydride based reductants were employed. The yield is a function, albeit a non-linear one, of hydride donor concentration as indicated by comparison of any set of bars in Fig 2A at a given temperature when NaBH₄ was employed. The same panel further indicates a diminishing yield with increasing temperature (at the time of both adduct deposition and cross-link reduction). A similar effect is seen, Fig 2B, with NaNCBH₃. With both reductants an approximately 30 °C increase in temperature results in a 25-30% decrease in trappable cross-link.

A direct comparison of different borohydride based reductants is presented Fig 2C and NaNCBH₃ 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. NaNCBH₃ is more effective than NaBH₄ by a factor of almost 4-fold, though it contains only ¾ 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 NaNCBH₃ only ~1/2 again as effective as NaBH(OAc)₃, under either condition. In any case, it is unclear if normalization in carrying out comparisons is valid on the basis of the non-linear effects of trapping upon NaBH₄ concentration observed in Fig 2A. It is emphasized that in either approach, normalized or not, the data in Fig 2C indicate that NaNCBH₃ is the generally superior agent.

The exact nature of the reductant in experiments employing $\text{NaBH}(\text{OAc})_3$ is uncertain. In an experiment employing a freshly prepared aqueous stock solution of the reductant (third column, Fig 2C), initial preparation of the stock solution was accompanied by some fuming and a decrease in pH of the stock. This was attributed potentially to hydrolysis of the tri-ester, presumably to a mixture of mono- and di- esters and $\text{HB}(\text{OH})_3^-$. It is also possible that acidification resulted in a loss of some reducing equivalents. In an effort to minimize this loss, an experiment was carried out in which the $\text{NaBH}(\text{OAc})_3$, in preparation of the stock, was dissolved in an aqueous solution containing sufficient Na_2HPO_4 to buffer against complete borate tri-ester hydrolysis. This data is the rightmost column in Fig. 2C. Comparison of this data with that in the adjacent column in Fig. 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 Fig. 2C, BH_4^- is the smallest such reagent whereas $\text{BH}(\text{OR})_3^-$ (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.

Target(s) of borohydride reductants and implications

The yield of reduced cross-link and the absence of a significant effect of pH on the yield of 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 iminium ion, and none of the conjugate base imine, is accessible to trapping upon addition of NaNCBH_3 . First, based on the low pK_a of the iminium ion, an increase in yield of 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 Fig 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 range of pH.²⁰ Second, the observed yield of reduced cross-link at $\text{pH} = 6.8$ is 10^3 greater than predicted on the basis of the maximum theoretical yield of imine formed from the observed 0.0031 moles of O^6 -Gua adduct ($K_o \sim \infty$, Scheme 4) and $\text{pK}_a < 0$ for the iminiumion. 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**, Scheme 1 and Scheme 4). It cannot be ruled out that the neutral imine is trapped directly, possibly in association with Lewis acids – metals or H-bond donors.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 $k_{-1} \gg k_{\text{im}}[\text{H}^+]$.^{25, 26} Because of this, upon introduction of the NaNCBH_3 , the carbinolamine reverts quantitatively to aldehyde that is immediately trapped as alcohol, **10**, Scheme 1.

Thus, the amount of trapped reduced crosslink, overwhelmingly in the imine form at $\text{pH} 6.8$, reflects the equilibrium constant K_o , Scheme 4. An estimate of $K_o \sim 0.03 \text{ M}^{-1}$ can be deduced based on the assumption that each O^6 -Gua adduct is in suitable proximity to two adenines (Supporting Information). This is similar the value of $K_o \sim 0.1 \text{ M}^{-1}$ that can be estimated from literature on 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 Fig 3 and requires that there are a minimum of two sets of trappable cross-links, a rapidly formed set, trapped on day zero, 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 in the abscissa of Fig 3) by a 30 min exposure to 10 mM NaNCBH₃ after which the purines are acid hydrolysed and the cross-link yields are quantified. On the day 0 time points, NaNCBH₃ treatment occurs for 30 min after 30 sec exposure of DNA to the α -hydroxy-N-nitrosomorpholine (= TCEP + 3-hydroperoxy-N-nitrosomorpholine). The quantity of the reduced cross-links on “day 0” are nearly one quarter of those detected on day 7, when the NaNCBH₃ 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 O⁶-3-oxa-pentanal-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 Fig. 3 indicated by squares, the total amount of O⁶-3-oxa-pentanal-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 This data indicates that while a significant proportion of trappable cross-links form on the minutes time scale or sooner, a larger proportion form, or are trappable, 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 which, though similarly increasing over days, are effectively undetectable on day zero.³¹

Biological reductants

The data in Fig 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 it is tens of micromolar.³⁶⁻³⁹ The experiments depicted involved exposure of the DNA to the biological reductants for three days. This is a relatively brief exposure given that hepatocyte lifetimes have been estimated to be 6 – 18 months.⁴⁰⁻⁴² The N²-ethyl-dG adduct has been 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 lesions¹⁸ and is confirmed here.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Information: This work was supported by funds from the National Institutes of Health, NCI R01-CA52881.

References

1. Druckrey H, Preussmann R, Ivankovic S, Schmahl D. Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats. *Z Krebsforsch.* 1967; 69:103–201. [PubMed: 4230610]
2. Lijinsky, W. Chemistry and biology of N-nitroso compounds. Cambridge University Press; Cambridge, UK: 1992.
3. Mohr U. Carcinogenesis of N-nitroso-morpholine and derivatives in Syrian golden hamsters. *Prog Exp Tumor Res.* 1979; 24:235–244. [PubMed: 538246]
4. Charrois JW, Arend MW, Froese KL, Hrukey SE. Detecting N-nitrosamines in drinking water at nanogram per liter levels using ammonia positive chemical ionization. *Environ Sci Technol.* 2004; 38:4835–4841. [PubMed: 15487793]
5. Sanches-Filho PJ, Rios A, Valcarcel M, Zanin KD, Caramao EB. Determination of nitrosamines in preserved sausages by solid-phase extraction-micellar electrokinetic chromatography. *J Chromatogr A.* 2003; 985:503–512. [PubMed: 12580519]
6. Spiegelhalder B, Preussmann R. Contamination of toiletries and cosmetic products with volatile and nonvolatile N-nitroso carcinogens. *J Cancer Res Clin Oncol.* 1984; 108:160–163. [PubMed: 6746708]
7. Fajen JM, Carson GA, Rounbehler DP, Fan TY, Vita R. N-Nitrosamines in the Rubber and Tire Industry. *Science.* 1979; 205:1262–1264. [PubMed: 472741]
8. Monarca S, Feretti D, Zanardini A, Moretti M, Villarini M, Spiegelhalder B, Zerbini I, Gelatti U, Lebbolo E. Monitoring airborne genotoxicants in the rubber industry using genotoxicity tests and chemical analyses. *Mutat Res.* 2001; 490:159–169. [PubMed: 11342241]
9. Oury B, Limasset JC, Protois JC. Assessment of exposure to carcinogenic N-nitrosamines in the rubber industry. *Int Arch Occup Environ Health.* 1997; 70:261–271. [PubMed: 9342627]
10. Reh BD, Fajen JM. Worker exposures to nitrosamines in a rubber vehicle sealing plant. *Am Ind Hyg Assoc J.* 1996; 57:918–923. [PubMed: 8865601]
11. Dallinga JW, Pachen DM, Lousberg AH, van Geel JA, Houben GM, Stockbrugger RW, van Maanen JM, Kleinjans JC. 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.* 1998; 124:119–125. [PubMed: 9500200]
12. Kakizoe T, Wang TT, Eng VW, Furrer R, Dion P, Bruce WR. Volatile N-nitrosamines in the urine of normal donors and of bladder cancer patients. *Cancer Res.* 1979; 39:829–832. [PubMed: 427771]
13. van Maanen JM, Pachen DM, Dallinga JW, Kleinjans JC. Formation of nitrosamines during consumption of nitrate- and amine-rich foods, and the influence of the use of mouthwashes. *Cancer Detect Prev.* 1998; 22:204–212. [PubMed: 9618041]
14. Brunnemann KD, Hecht SS, Hoffmann D. N-nitrosamines: environmental occurrence, in vivo formation and metabolism. *J Toxicol Clin Toxicol.* 1982; 19:661–688. [PubMed: 6761448]
15. Hecht SS, Young R. Metabolic alpha-hydroxylation of N-nitrosomorpholine and 3,3,5,5-tetradeutero-N-nitrosomorpholine in the F344 rat. *Cancer Res.* 1981; 41:5039–5043. [PubMed: 7307006]
16. Kim HJ, Fishbein JC. Reexamination of the aqueous chemistry of N-Nitroso-3-hydroxymorpholine, a metabolite of the carcinogen N-nitrosomorpholine. *Chem Res Toxicol.* 2003; 16:715–720. [PubMed: 12807354]
17. Zink CN, Soissons N, Fishbein JC. Products of the direct reaction of the diazonium ion of a metabolite of the carcinogen N-nitrosomorpholine with purines of nucleosides and DNA. *Chemical research in toxicology.* 2010; 23:1223–1233. [PubMed: 20443589]

18. Minko IG, Kozekov ID, Harris TM, Rizzo CJ, Lloyd RS, Stone MP. Chemistry and biology of DNA containing 1,N(2)-deoxyguanosine adducts of the alpha,beta-unsaturated aldehydes acrolein, crotonaldehyde, and 4-hydroxynonenal. *Chem Res Toxicol*. 2009; 22:759–778. [PubMed: 19397281]
19. Stone MP, Cho YJ, Huang H, Kim HY, Kozekov ID, Kozekova A, Wang H, Minko IG, Lloyd RS, Harris TM, Rizzo CJ. Interstrand DNA cross-links induced by alpha,beta-unsaturated aldehydes derived from lipid peroxidation and environmental sources. *Accounts of chemical research*. 2008; 41:793–804. [PubMed: 18500830]
20. Blackburn, GM. *Nucleic Acids in Chemistry and Biology*. 2nd. Oxford University Press; 1996.
21. Barrero AF, Alvarez-Manzaneda EJ, Chahboun R, Arteaga AF. Degradation of the side chain of (-)-scaleol: a very short synthesis of nor-ambreinolide and ambrox. *Synthetic Communications*. 2004; 34:3631–3643.
22. Bartoli G, Cupone G, Dalpozzo R, De Nino A, Maiuolo L, Procopio A, Tagarelli A. Stereoselective reduction of enamines to syn gamma-aminoalcohols. *Tetrahedron Letters*. 2002; 43:7441–7444.
23. Corey EJ, Helal CJ. Reduction of carbonyl compounds with chiral oxazaborolidine catalysts: a new paradigm for enantioselective catalysis and a powerful new synthetic method. *Angew Chem Int Ed*. 1998; 37:1986–2012.
24. Mohanta PK, Davis TA, Gooch JR, Flowers RA. Chelation-controlled diastereoselective reduction of a-fluoroketones. *J Am Chem Soc*. 2005; 127:11896–11897. [PubMed: 16117501]
25. Jencks WP. Mechanism and catalysis of simple carbonyl group reactions. *Prog Phys Org Chem*. 1964; 2:63–128.
26. Cordes EH, Jencks WP. On the mechanism of Schiff base formation and hydrolysis. *J Am Chem Soc*. 1962; 84:832–837.
27. Cordes EH, Jencks WP. Nucleophilic catalysis of semicarbazone formation by anilines. *J Am Chem Soc*. 1962; 84:826–831.
28. Zuman P. The reaction of the carbonyl group with primary amines. *Coll Czech Chem Comm*. 1951; 15:839–873.
29. Agnew TE, Kim HJ, Fishbein JC. Diazonium ion chemistry: Replacement of H by alkyl at the central carbon accelerates an SN2 substitution reaction. *Journal of Physical Organic Chemistry*. 2005; 17:483–488.
30. Wang M, Young-Sciame R, Chung F, Hecht SS. Formation of N²-Tetrahydropyranyl Adducts in the Reactions of a-Acetoxymethyl-N-nitrosopyrrolidine and a-Acetoxymethyl-N-nitrosopiperidine with DNA. *Chemical research in toxicology*. 1995; 8:617. [PubMed: 7548743]
31. Sanchez AM, Kozekov ID, Harris TM, Lloyd RS. Formation of inter- and intrastrand imine type DNA-DNA cross-links through secondary reactions of aldehydic adducts. *Chem Res Toxicol*. 2005; 18:1683–1690. [PubMed: 16300377]
32. Polakis ES, Bartley W. Changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides during the aerobic growth cycle of yeast on different carbon sources. *The Biochemical journal*. 1966; 99:521–533. [PubMed: 4381585]
33. Klaidman LK, Leung AC, Adams JD Jr. High-performance liquid chromatography analysis of oxidized and reduced pyridine dinucleotides in specific brain regions. *Analytical biochemistry*. 1995; 228:312–317. [PubMed: 8572312]
34. Wosikowski K, Mattern K, Schemainda I, Hasmann M, Rattel B, Loser R. WK175, a novel antitumor agent, decreases the intracellular nicotinamide adenine dinucleotide concentration and induces the apoptotic cascade in human leukemia cells. *Cancer Res*. 2002; 62:1057–1062. [PubMed: 11861382]
35. Yu Q, Heikal AA. Two-photon autofluorescence dynamics imaging reveals sensitivity of intracellular NADH concentration and conformation to cell physiology at the single-cell level. *Journal of photochemistry and photobiology B, Biology*. 2009; 95:46–57.
36. Hornig D. Distribution of ascorbic acid, metabolites and analogues in man and animals. *Annals of the New York Academy of Sciences*. 1975; 258:103–118. [PubMed: 1106295]

37. Franceschi RT, Wilson JX, Dixon SJ. Requirement for Na(+)-dependent ascorbic acid transport in osteoblast function. *The American journal of physiology*. 1995; 268:C1430–1439. [PubMed: 7611363]
38. May JM, Li L, Hayslett K, Qu ZC. Ascorbate transport and recycling by SH-SY5Y neuroblastoma cells: response to glutamate toxicity. *Neurochemical research*. 2006; 31:785–794. [PubMed: 16791474]
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 MR, Onate SA, Bustamante M, Carcamo JG, Rivas CI, Vera JC. 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*. 2007; 282:15506–15515. [PubMed: 17403685]
40. Macdonald RA. “Lifespan” of liver cells. Autoradio-graphic study using tritiated thymidine in normal, cirrhotic, and partially hepatectomized rats. *Archives of internal medicine*. 1961; 107:335–343. [PubMed: 13764742]
41. Grisham JW. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H3. *Cancer Res*. 1962; 22:842–849. [PubMed: 13902009]
42. Mason WS, Jilbert AR, Summers J. Clonal expansion of hepatocytes during chronic woodchuck hepatitis virus infection. *Proc Natl Acad Sci U S A*. 2005; 102:1139–1144. [PubMed: 15657132]
43. Fang JL, V CE. Development of a 32P-postlabelling Method for the Analysis of Adducts Arising through the Reaction of Acetaldehyde with 2'-Deoxyguanosine-3'-monophosphate and DNA. *Carcinogenesis*. 1995; 16:2177. [PubMed: 7554072]
44. Fang JL, V CE. Detection of DNA Adducts of Acetaldehyde in Peripheral White Blood Cells of Alcohol Abusers. *Carcinogenesis*. 1997; 18:627. [PubMed: 9111191]
45. Vaca CE, Fang JL, Schweda EK. Studies of the reaction of acetaldehyde with deoxynucleosides. *Chem Biol Interact*. 1995; 98:51–67. [PubMed: 7586051]

Abbreviations

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

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

Figure 2.

Yields of reduced cross-links from borohydride reductants as a function of varying conditions, for 30 sec of formation and 30 min of reduction. A. Reduction by NaBH₄ as function of reductant concentration and temperature (of cross-link formation and reduction). B. Reduction by NaNCBH₃ as a function of temperature (of cross-link formation and reduction). C. Comparison of different borohydride reducing agents, see text. D. Yields for NaNCBH₃ reduction as a function of pH (of cross-link formation and reduction).

Figure 3. Yields of reduced cross-links (**9**, Scheme 1) upon 30 min incubation with 10 mM NaCNBH₃, after decomposition of **11**, Scheme 1, and incubation (25 °C) for the period of time indicated in the X axis. Circles for an experiment carried out for seven days, squares for a similar experiment carried out for 3 days in which the total amount of reduced O⁶-Gua adduct (**10**, Scheme 1) was also determined (see 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 executed.

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 uncross-linked (10).

Scheme 2.

Synthetic scheme for formation of reduced cross-linked nucleobases Gua and Ade.

Scheme 3.

Proposed structures for collision - induced dissociation detected in triple quad MS of the reduced cross-link standard.

Scheme 4.

Detailed reaction scheme for trapping of cross-linked imine and iminium ion by $NaNCBH_3$.