Nucleoside and DNA Adducts from *N*-Nitrosotolazoline

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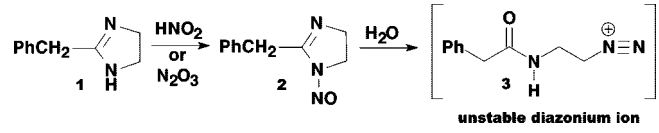
The reaction of *N*-nitrosotolazoline, the nitrosation product of a representative imidazoline receptor drug tolazoline, with DNA, deoxyguanosine (dG), or deoxyadenosine (dA) produces adducts containing the 2-phenylacetamidoethyl group. The synthesis and characterization of 2-phenylacetamidoethyl-guanine derivatives (O^6-dG, O^6-Gua, N^2-Gua, and 7-Gua) and 2-phenylacetamidoethyladenine derivatives (1-Ade, 3-Ade, 7-Ade, and N^6-Ade) are described. In addition to the use of an established UV spectral method for confirming the structure of the alkyl adenines, a new ^{13}C NMR method for determining the *N*-alkylation site is presented. In combination with the synthesized standards, HPLC MS/MS methods were used to determine the nature and the quantity of adducts produced. *N*-Nitrosotolazoline reacted with dG to give 7-(2-phenylacetamidoethyl)deoxyguanosine (major), O^6-(2-phenylacetamidoethyl)deoxyguanosine, and 5′-O-phenyacetyldeoxyguanosine. The reaction of *N*-nitrosotolazoline with dA produced the 1-, 3-, 7-, N^6, and 5-O′-2-phenylacetamidoethyl adenine and dA derivatives as well as several phenylacetyl adducts. Reaction of *N*-nitrosotolazoline with DNA in vitro resulted in the detection of 2-phenylacetamidoethyl adducts (adduct, relative %): 7-Gua, 60%; 3-Ade, 30%; O^6-Gua, 8%; and 7-Ade, 2%. Comparison of these data with appropriate literature data, as well as our work on the mechanism of *N*-nitrosotolazoline hydrolytic decomposition, is consistent with the adducts being produced from a 2-phenylacetamidoethyldiazonium intermediate. The results show that *N*-nitrosotolazoline, and presumably other *N*-nitrosoimidazolines, if produced by endogenous nitrosation pathways, are capable of alkylating DNA without additional metabolic transformation and are probable carcinogens.

Introduction

Tolazoline **1**, an over-the-counter drug, has a structure exemplary of many imidazoline receptor drugs, which are 2-substituted imidazolines (*1*). The endogenous nitrosation of drugs or other substances containing the imidazoline moiety could result in carcinogenic or other deleterious toxicological consequences (*1, 2*). An essential chemical feature of demonstrating that this may be so involves showing that the compounds themselves or intermediates derived from them, in the case of mutagenesis and carcinogenesis, readily form DNA[1] base adducts. We have shown that the nitrosation of tolazoline results in the production of a cascade of reactive diazonium ions and other reactive electrophiles (*1, 2*). *N*-Nitrosotolazoline **2**, the principal initial product of the nitrosation reaction, nitrosates more rapidly than the substrate, and the generation of the cascade of electrophiles follows this transformation. When limited nitrosating agent concentrations are employed, *N*-nitrosotolazoline can be detected but so can nitrosation and decomposition products derived from it (*1, 2*). We have synthesized *N*-nitrosotolazoline **2** and examined its decomposition as a function of pH (*2*). The structures of its decomposition products are consistent with the formation of the 2-phenylacetamidoethyldiazonium ion **3** during its hydrolytic decomposition (Scheme 1). This diazonium ion should readily alkylate DNA to give, among others, the guanine and adenine adducts **4**–**10** shown in the top half of Figure 1. Here, we report our studies on the reaction of **2** with guanine and adenine deoxynucleosides, DNA, as well as the synthesis and the characterization of adducts that are, indeed, derived from **3**. In the case of deoxyribonucleoside reactions with **2**, we also obtained MS evidence for the formation of sugar adducts **12**–**17**.

Scheme 1



Experimental Procedures

Caution: *Most nitrosamines are potent carcinogens. Considerable care should be taken in their use so as to avert exposure to humans and to avoid environmental contamination. We routinely perform all operations with these substances, except for dilute solutions thereof, in well-ventilated fume hoods. We rinse all nitrosamine-contaminated glassware with a solution of concentrated HBr in glacial acetic acid (1:1), which is effective in cleaving the NO group from the amine nitrogen atom. The action of this agent in aprotic solutions is also effective in nitrosamine destruction. Aqueous solutions are treated with either Ni(R) or Al in concentrated sodium hydroxide. This process may produce hydrazines.*

General. Melting points were determined on a Thomas-Hoover capillary tube apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on Bruker ARX 250 (250 MHz for ^1H and 62.5 MHz for ^{13}C), DRX 300 (300 MHz for ^1H and 75 MHz for ^{13}C), or DRX 500 (500 MHz for 1H and 125 MHz for ^{13}C) in $CDCl_3$ unless otherwise stated. HPLC was performed with a Waters system equipped with model 510 solvent delivery pumps, Waters model 490 programmable multiwavelength detector, Waters model 712 WISP autosampler, FC 203 Gilson fraction collector, and

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[1] Abbreviations: CNL, constant neutral loss; CT-DNA, calf thymus DNA; dA, deoxyadenosine; dG, deoxyguanosine; DMF, *N*,*N*-dimethylformamide; ENU, ethylnitrosourea; HMBC, heteronuclear multibond correlation; MRM, multireaction monitoring; SIM, selected ion monitoring.

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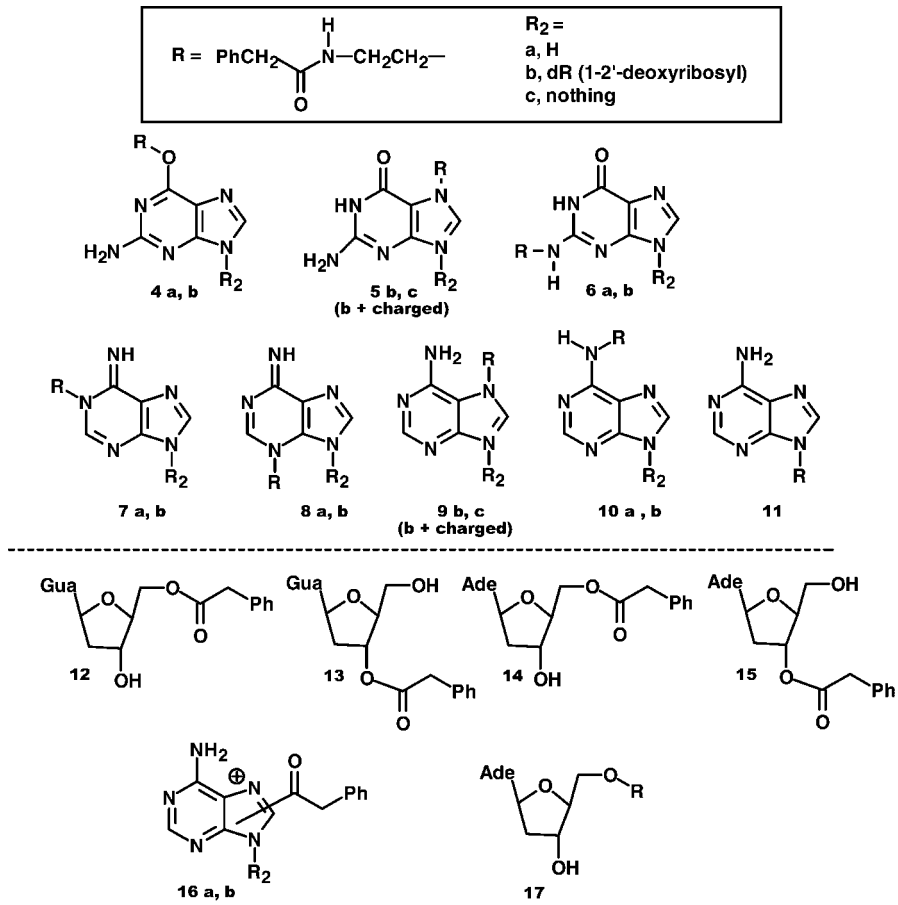


Figure 1. Guanine and adenine adducts (except for **11**) formed from the direct reaction of *N*-nitrosotolazoline **2** with deoxyribonucleosides and/or DNA. Adducts 4, 5, and 7–10 were formed in vitro in CT-DNA.

Millennium control software (Version 2.15). Two types of HPLC column were used, a 4.6 mm × 25 cm Zorbax SB-C8 analytical column and a 10 mm × 25 cm Zorbax SB-C8 semipreparative column, both from Agilent, Inc. Thin-layer chromatography was performed on EM Science aluminum plates precoated with 0.20 mm silica gel. Merck Kieselgel 230–400 mesh silica gel was used for flash column chromatography. The UV–vis spectra were taken on a Hewlett-Packard 8453 UV–visible spectrophotometer. LC/MS was performed on a Finnigan triple-stage quadrapole TSQ 7000 mass spectrometer equipped with the following components: mobile phase pumps (TSP 4000, Quaternary pump), autosampler (SpectraSYSTEM AS 3000), UV detector (TSP UV 6000LP), and MS detector (Finnigan TSQ MS). The LC/MS data were acquired and processed with Xcalibur software.

Materials. Solvents used for moisture-sensitive reactions were dried and distilled by common procedures. All other chemicals were either synthesized as described below or were purchased in reagent grade from commercial sources and used without further purification. HPLC solvents were degassed and filtered prior to use.

***N*-(2-Aminoethyl)phenylacetamide 33 (*3*).** This compound was prepared by the reaction of phenylacetyl chloride with ethylene diamine (*3*). ^{1}H NMR ($CDCl_3$): δ 7.25–7.33 (m, 5 H), 6.14 (br, s, 1 H), 3.56 (s, 2 H), 3.25 (t, 2 H, $J = 5.9$ Hz), 2.75 (t, 2 H, $J = 6.0$ Hz), 1.26 (br, s, 2 H). ^{13}C NMR ($CDCl_3$): δ 171.40, 135.00, 129.26, 128.85, 127.17, 43.70, 42.08, 41.15.

***N*-(2-Bromoethyl)phenylacetamide 35 (*4*).** *N*-(2-Bromoethyl)phenylacetamide was prepared from 2-bromoethylamine hydrobromide and phenylacetyl chloride; mp 84–85 °C. ^{1}H NMR ($CDCl_3$): 7.37 (m, 5 H), 5.80 (b, 1 H), 3.62 (t, $J = 5.7$ Hz, 2 H), 3.59 (s, 2 H), 3.42 (t, $J = 5.7$ Hz, 2 H). ^{13}C NMR ($CDCl_3$): 171.1, 134.5, 129.4, 129.1, 127.5, 43.7, 41.1, 32.3.

6-Hydroxy-2-mercaptopurine. The literature procedure (*5*) for the synthesis of 6-hydroxy-2-mercaptopurine was modified slightly; mp > 300 °C (dec.). ^{1}H NMR (DMSO-d_6): δ 13.52 (br s, 1 H), 13.30 (br s, 1 H), 12.17 (s, 1 H), 8.05 (s, 1 H). ^{13}C NMR (DMSO-d_6): δ 173.4, 153.4, 149.0, 141.5, 110.5.

2-Bromo-6-hydroxypurine 32. The method of Beaman, Gerster, and Robins (*6*) was modified to prepare 2-bromo-6-hydroxypurine. 6-Hydroxy-2-mercaptopurine (3.4 g, 20.4 mmol) was added to a mixed solution of methanol (20 mL) and HBr (40 mL, 48%). The suspension was then cooled to 8 °C, and the addition of bromine (8 mL) was started. After 1.5 h, the bromine addition was completed, and the suspension was stirred for another 4 h at 0 °C. The mixture was then filtered, and the solid was then immediately washed with acetone (20 mL). This dry solid was stirred with cold water (30 mL), filtered, and washed with acetone and cold water. The crude product was dissolved in a 0.1 M NaOH and precipitated with dilute HCl. Recrystallization from methanol yielded 2.4 g of the 2-bromo-6-hydroxypurine (53.5%); mp > 300 °C. ^{1}H NMR (DMSO-d_6): δ 13.04 (br s, 2 H), 8.13 (s, 1 H). ^{13}C NMR (DMSO-d_6): δ 155.5, 140.9, 135.5, 132.1, 123.0.

***N*^{2}-(2-Phenylacetamido)ethylguanine 6a.** A procedure similar to that of Wright and Dudycz was followed (*7*). A 100 mL round-bottom flask equipped with a condenser was charged with water (10 mL), 2-methoxyethanol (30 mL), 2-bromo-6-hydroxypurine **32** (1.6 g, 7.4 mmol), and *N*-(2-aminoethyl)phenylacetamide **33** (1.78 g, 10 mmol). The mixture was stirred at reflux temperature (110 °C) for 4 h. Cooling gave a yellow suspension, and all solvent was removed in vacuo. The solid residue was washed with 2 mL of water and then dissolved in hot methanol (30 mL). Recrystallization from methanol afforded 1.3 g of N^2-(2-phenylacetamido)ethylguanine **6a** as a light yellow solid (56% yield); mp > 300 °C. ^{1}H NMR (DMSO-d_6): δ 10.00 (br, 1 H), 8.40 (b, 1 H), 8.13 (s, 1 H), 7.80 (b, 1H), 0.7.28 (m, 5 H), 7.20 (s, 1H), 3.43 (s, 2H), 3.35 (t, $J =$ 5.7 Hz, 2 H), 3.22 (t, $J = 5.7$ Hz, 2 H). ^{13}C NMR (DMSO-d_6): δ 171.0, 154.3, 153.2, 144.3, 142.9, 136.3, 129.0, 128.0, 126.1, 112.3, 42.2, 39.9, 38.0. HRMS (FAB): *m/z* calcd for $C_{15}H_{16}N_6O_2 + H$, 313.1413; found, 313.1412.

5′-*O*-Phenylacetyl-2-deoxyguanosine 12. To a 4 mL *N*,*N*-dimethylformamide (DMF) solution of 2-deoxyguanosine (100 mg, 0.375 mmol), which had been dried by evaporation twice from anhydrous pyridine, 115 mg of pyridine (1.49 mmol) and phenylacetyl chloride (144 mg, 0.938 mmol) was added. The reaction mixture was stirred at room temperature for 6 h. The product was separated by HPLC on a C-18 semiprep column (methanol and 0.025 M formic acid). In the crude reaction mixture, the product yield was 56%; mp > 250 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.63 (s, 1 H), 7.82 (s, 1 H), 7.22–7.33 (m, 5 H), 6.46 (s, 2 H), 6.13 (t, J = 6.6 Hz, 1 H), 5.42 (d, J = 3.0 Hz, 1 H), 4.37 (m, 1 H), 4.22 (m, 2 H), 3.98 (m, 1 H), 3.66 (s, 2 H), 2.58 (m, 1 H), 2.23 (m, 1 H). ^{13}C NMR (DMSO-d_6): δ 171.00, 156.68, 153.63, 150.92, 135.12, 134.17, 129.30, 128.29, 126.79, 116.73, 83.91, 82.33, 70.51, 64.36, 40.05, 38.68. MS/MS (ESI): 385.2, 152.3, 151.3. HRMS (FAB): *m*/*z* calcd for $C_{18}H_{19}N_5O_5$ + H, 386.1464; found, 386.1455.

2-Amino-6-mesitylsulfonoxy-9-[3′,5′-bis(*t*-butyldimethylsilyl)-2′-deoxyribosyl]purine 29. To a stirred solution of 3′,5′-bis(*t*-butyldimethylsilyl)-2′-deoxyguanosine (*8*) (107 mg, 0.216 mmol) in a mixture of hexamethylphosphoramide (HMPA, 300 μL) and dichloromethane (1.5 mL) were added triethylamine (120 μL, 80.71 mg, 0.86 mmol), 4-dimethylaminopyridine (DMAP, 5.8 mg, 0.04 mmol), and 2-mesitylenesulfonyl chloride (110 mg, 0.502 mmol). After it was stirred at room temperature overnight, the resulting solution was washed with saturated solution of sodium bicarbonate (2 mL × 2) and brine (2 mL × 2). The solution was dried with anhydrous sodium sulfate. After the solvent was removed in vacuo, the residue was purified by flash chromatography (ethyl acetate/hexane, 30/70, v/v) to obtain 128 mg of the 6-mesitylsulfonoxy nucleoside **29** as a white solid (yield: 90%). ^{1}H NMR ($CDCl_3$): 7.95 (s, 1 H), 6.26 (t, J = 6.5 Hz, 1 H), 4.82 (b, s, 2 H), 4.55 (m, 1 H), 3.95 (m, 1 H), 3.74 (m, 2 H), 2.73 (s, 6 H), 2.53 (m, 1 H), 2.36 (m, 1 H,), 2.31 (s, 3 H), 0.89 (s, 18 H), 0.08 (s, 6 H), 0.04 (s, 6 H).

3′,5′-Bis(*t*-butyldimethylsilyl)-*O*^{6}-(2-phenylacetylamino)ethyl-2′-deoxyguanosine 31. Using an adaptation of a literature procedure (*9*), 1,4-diazabicyclo[2.2.2]octane (DABCO, 2 molar equiv), 50 mg of 4 Å molecular sieves, and 5 molar equiv of 2-phenylacetamidoethanol **27** were added to a solution of **29** (96 mg, 0.14 mmol) in 1.4 mL of 1,2-dimethoxyethane. After the mixture was allowed to stir at room temperature for 30 min, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU, 1.5 molar equiv) was added. The mixture was allowed to stir at room temperature for 24 h and then diluted with EtOAc. The organic layer was dried over Na_2SO_4 and evaporated. The crude products were chromatographed on silica gel using hexane and EtOAc (1:1, v/v). The yield of **31** (white solid) was 67%. ^{1}H NMR ($CDCl_3$): 7.92 (s, 1 H), 7.26 (m, 5 H), 6.34 (t, J = 5.5 Hz, 1 H), 6.20 (b, 1 H), 4.84 (s, 2 H), 4.57 (m, 3 H), 4.00 (m, 1 H), 3.83 (m, 2 H), 3.72 (m, 2H), 3.55 (s, 2 H), 2.58 (m, 1H), 2.39 (m, 1H), 0.93 (s, 18 H), 0.12 (s, 6 H), 0.10 (s, 6 H).

***O*^{6}-(2 Phenylacetamidoethyl)deoxyguanosine 4b.** To 20 mg of **31** in 0.5 mL of THF (0.031 mmol) was added 1.0 M TBNF in THF solution (0.15 mL, 0.15 mmol). After 30 min of stirring at room temperature, TLC showed that the starting material had disappeared. Purification on a flash column (methanol and CH_2Cl_2, 1:9 v/v) provided 14 mg of **4b** as a white solid in 96% yield; mp 151–153 °C. ^{1}H NMR (DMSO-d_6): 8.30 (t, J = 5.5 Hz, 1 H), 8.09 (s, 1 H), 7.23 (m, 5 H), 6.40 (s, 2H), 6.22 (t, J = 6.1 Hz, 1 H), 5.24 (b, 1 H), 4.99 (b, 1H), 4.43 (t, J = 5.5 Hz, 2 H), 4.35 (m, 1H), 3.83 (m, 1 H), 3.48 (t, J = 5.4 Hz, 2 H), 3.42 (s, 2 H), 2.55 (m, 1 H), 2.22 (m, 1 H). ^{13}C NMR (DMSO-d_6): 170.46, 160.22, 159.63, 153.93, 137.72, 136.31, 128.92, 128.10, 136.23, 113.90, 87.60, 82.81, 70.77, 64.33, 61.73, 42.19, 39.50, 38.42. HRMS (FAB): *m*/*z* calcd for $C_{20}H_{24}N_6O_5$ + Li, 435.1968; found, 435.1965.

7-(2-Phenylacetamido)ethylguanine 5c. To a solution of 2′-deoxyguanosine (100 mg, 0.37 mmol) in 5 mL of DMF, *N*-(2-bromoethyl)phenylacetamide **35** (200 mg, 0.83 mmol) was added. The reaction mixture was heated at 80 °C for 6 h and then cooled to room temperature. Because 7-alkylguanines are sparingly soluble in any organic solvent, the reaction mixture was diluted with 0.1 N HCl aqueous solution and purified on a preparative HPLC column with methanol and formic acid (0.02 M in water) as the mobile phase. The methanol in the collected eluent was evaporated in vacuo. The aqueous solution was neutralized with concentrated ammonia hydroxide to pH 6.5. Upon neutralization, a white powder [7-(2-phenylacetamido)ethylguanine **5c**] precipitated. The powder was filtered and washed with cold water three times and ethyl ether three times. A trace amount of water was removed in vacuo at 80 °C for 5 h; mp > 300 °C. ^{1}H NMR (DMSO-d_6): δ 10.75 (s, 1 H), 8.11 (t, 1 H, J = 5.2 Hz), 7.69 (s, 1 H), 7.16–7.29 (m, 5 H), 6.07 (s, 2 H), 4.20 (t, 2 H, J = 5.3 Hz), 3.45 (t, 2 H, J = 5.3 Hz), 3.34 (s, 2 H). ^{13}C NMR (DMSO-d_6): δ 170.14, 152.34, 151.16, 142.99, 135.90, 132.86, 128.65, 127.84, 125.99, 108.04, 45.36, 42.05, 39.43. HRMS (FAB): *m*/*z* calcd for $C_{15}H_{17}N_6O_2$ + H, 313.1413; found, 313.1412.

***O*^{6}-(2-Phenylacetamidoethyl)guanine 4a.** To a solution of *N*-2-hydroxyethylphenylacetamide (**27**, 400 mg, 2.23 mmol) in 5 mL of anhydrous DMF was added 48 mg of NaH (2 mmol) in one portion. The reaction mixture was stirred at room temperature for 30 min followed by the addition of 2-amino-6-chloropurine (70 mg, 0.37 mmol). Semipreparative HPLC was used for the purification of the product, *O*^{6}-(2-phenylacetamidoethyl)guanine; mp 165–166 °C. ^{1}H NMR (DMSO-d_6): δ 12.39 (s, 1 H), 8.34 (b, 1 H), 7.81 (s, 1 H), 7.23 (m, 5 H), 6.21 (s, 2 H), 4.39 (t, J = 5.6 Hz, 2 H), 3.47 (t, J = 5.6 Hz, 2 H), 3.34 (s, 2 H). ^{13}C NMR (DMSO-d_6): δ 170.5, 159.8, 159.7, 155.6, 138.0, 136.4, 129.0, 128.2, 126.3, 112.7, 64.2, 42.2, 38.8. HRMS (FAB): *m*/*z* calcd for $C_{15}H_{17}N_6O_2$ + H, 313.1413; found, 313.1418.

1-(2-Phenylacetamidoethyl)adenine 7a. Using the literature as a guide (*10, 11*), to a solution of 100 mg of adenosine (0.38 mmol) in 2 mL of anhydrous DMF, 267 mg of *N*-(2-bromoethyl)phenylacetamide **35** (1.1 mmol) and 80 mg of $CaCO_3$ (0.8 mmol) were added. The solution was stirred for 8 h at 80 °C under anhydrous conditions. The solvent was evaporated to dryness, and the residue was hydrolyzed in 2 mL of acetic acid at 90 °C for 6 h. Purification on semipreparative HPLC gave **7a** (40.5 mg, 36%) as a white solid; mp 250 °C (dec.). ^{1}H NMR (DMSO-d_6): δ 8.29 (br t, J = 5.7 Hz, 1 H), 7.86 (s, 1 H), 7.83 (s, 1 H), 7.17–7.28 (m, 7 H), 4.19 (t, J = 5.6 Hz, 2 H), 3.40 (t, J = 5.6 Hz, 2 H), 3.38 (s, 2 H). ^{13}C NMR (DMSO-d_6): 173.3, 165.9, 148.1, 142.1, 141.0, 135.9, 129.0, 128.2, 126.4, 119.4, 52.0, 42.2, 37.1. HRMS (FAB): *m*/*z* calcd for $C_{15}H_{17}N_6O$ + H, 297.1464; found, 297.1453.

3-(2-Phenylaceamidoethyl)adenine 8a and 9-(2-Phenylaceamidoethyl)adenine 11. Using the literature as a guide (*12*), a mixture of adenine (135 mg, 1 mmol) with *N*-(2-bromoethyl)phenylacetamide (**35**, 362 mg, 1.5 mmol) in 1 mL of DMF was heated at 80 °C while stirring for 4 h. After the solvent was evaporated in vacuo, the residue was purified on aluminum oxide (activated, basic) with CH_2Cl_2 and MeOH (9:1, v/v) as an eluting solvent. The collected fraction contained two products, **8a** and **11**, which were then separated on silica gel with CH_2Cl_2 and MeOH (5:1, v/v). Purification on silica gel alone could not separate **8a** from adenine. For 3-(2-phenylaceamido)ethyladenine **8a**: mp 231–234 °C. ^{1}H NMR (DMSO-d_6): δ 8.21 (br, t, J = 5.5 Hz, 1 H), 8.10 (s, 1 H), 7.95 (b, 1 H), 7.90 (b, 1 H), 7.77 (s, 1 H), 7.15–7.29 (m, 5 H), 4.35 (t, J = 5.6 Hz, 2 H), 3.40 (t d, J = 5.6, 5.5 Hz, 2 H), 3.35 (s, 2 H). ^{13}C NMR (DMSO-d_6): 171.2, 154.5, 149.0 (2C), 145.6, 136.1, 129.0, 128.2, 126.4, 116.4, 49.0, 42.2, 37.6. HRMS (FAB): *m*/*z* calcd for $C_{15}H_{17}N_6O$ + H, 297.1464; found, 297.1470. For 9-(2-phenylaceamido)ethyladenine **11**: mp 212–214 °C. ^{1}H NMR (DMSO-d_6): δ 8.15 (br, 1 H), 8.13 (s, 1 H), 7.95 (s, 1 H), 7.13–7.26 (m, 7 H), 4.19 (t, J = 5.2 Hz, 2 H), 3.40 (t, J = 5.2 Hz, 2 H), 3.34 (s, 2 H). ^{13}C NMR (DMSO-d_6): 170.6, 156.0, 152.4, 149.6, 141.0, 136.3, 129.0, 128.2, 126.3, 118.7, 55.1, 42.5, 42.3. HRMS (FAB): *m*/*z* calcd for $C_{15}H_{17}N_6O$ + H, 297.1464; found, 297.1460.

7-(2-Phenylacetylaminoethyl)adenine 9c. To a solution of 135 mg of adenine (1 mmol) in 2 mL of anhydrous DMF was added 324 mg of 1-phenylacetylaziridine **40** (2 mmol) (*13*). The mixture was stirred for 4 h at 70 °C, and the solvent was evaporated in vacuo. The residue, containing a mixture of all of the ring *N*-alkylated isomers (**7a**, **8a**, **9c**, and **11**) was chromatographed over silica gel with CH_2Cl_2 and MeOH as eluting solvents (from 9:1,

v/v to pure MeOH). The 7-isomer **9c** was collected as a mixture with **8a**. The mixture was then separated on preparative HPLC with MeOH and 0.1% formic acid as the mobile phase. After concentration, 7-(2-phenylacetylaminoethyl)adenine **9c** was obtained as white solid in 17% yield from adenine. For **9c**: mp 223–225 °C. ^{1}H NMR (DMSO-d_6): δ 8.35 (br, 1 H), 8.17 (s, 1 H), 8.08 (s, 1 H), 7.19–7.31 (m, 5 H), 7.07 (b, 2 H), 4.38 (t, J = 6.1 Hz, 2 H), 3.41 (s, 2 H), 3.34 (t, J = 6.1 Hz, 2 H). ^{13}C NMR (DMSO-d_6): 171.4, 160.0, 152.2, 151.3, 145.9, 135.9, 129.1, 128.2, 126.4, 110.7, 44.8, 42.0, 40.5. MS/MS (ESI) of MH^+: 162.3, 161.4, 136.1, 135.2. HRMS (FAB): m/z calcd for $C_{15}H_{17}N_6O$ + H, 297.1464; found, 297.1473.

***N*^{6}-(2-Phenylacetylamino)ethyladenine (10a).** 6-Chloropurine **39** (77.3 mg, 0.5 mmol), *N*-(2-aminoethyl)phenylacetamide **33** (53.4 mg, 0.3 mmol), and Et_3N (0.5 mL) in 4 mL of *n*-butanol were refluxed for 3 h. The reaction mixture was concentrated in vacuo. Flash chromatography ($CHCl_3$−MeOH, 9:1 v/v) on silica gel gave pure **10a** (76.4 mg, 86%) as a white solid; mp 212–214 °C. ^{1}H NMR (DMSO-d_6): δ 12.93 (br, 1 H), 8.18 (s, 2 H), 8.09 (s, 1 H), 7.65 (b, 1 H), 7.17–7.26 (m, 5 H), 3.56 (t, J = 5.9 Hz, 2 H), 3.39 (s, 2 H), 3.31 (t, J = 5.9 Hz, 2 H). ^{13}C NMR (DMSO-d_6): 170.3, 154.5, 152.4, 149.8, 138.8, 136.4, 129.0, 128.2, 126.3, 119.0, 42.4, 39.6, 38.7. HRMS (FAB): m/z calcd for $C_{15}H_{17}N_6O$ + H, 297.1464; found, 297.1466.

Reaction of *N*-Nitrosotolazoline 2 with 2′-Deoxyguanosine. To 4.0 mg of dG (0.014 mmol) dissolved in 1 mL of phosphate buffer (pH 5.0), **2** (15 mg, 0.0794 mmol) was added in one portion. The mixture was sealed in a plastic vial and shaken in an incubator at 37 °C for 24 h. The resulting solution was filtered and then subjected to HPLC/MS analysis.

Detection of dG Adducts by LC/MS. The mobile phase used for eluting the analytes from LC columns consisted of MeOH (A) and 0.1% aqueous formic acid (B), at a flow rate of 1.0 mL/min. A gradient elution was performed as follows: from 10 to 60% A in 25 min and then isocratic at 80% A for 5 min. Nitrogen served both as sheath and auxiliary gases, with argon as the collision gas (1.8 mTorr) in the ESI-MS. The spray capillary voltage was 4.0 kV, and the temperature of the heated inlet capillary was 350 °C. The mass spectra were recorded in the positive mode. For selective detection of dG adducts, constant neutral loss mode (CNL, positive ions) was used, in which first quadrapole scanned from 200 to 700 m/z with a total scan duration of 1 s for a single spectrum while the third quadrapole simultaneously scanned with an offset of 116 m/z to the first quadruple. The collision cell (second quadrapole) was applied with 20 eV energy for the fragmentation of parent ions. To improve sensitivity of adduct detection, single ion monitoring (SIM) was also used following CNL MS^n. Chromatograms were obtained by scanning as follows with a width of 0.6: m/z 429, 7-R-dG (**5b**), O^6-R-dG (**4b**); m/z 386, O'^5-$PhCH_2CO$-dG (**12**); m/z 313, 7-R-Gua (**5c**), O^6-R-Gua (**4a**), and N^2-R-Gua (**6a**).

Effect of Nitrosamine Equivalents and pH on the Formation of Adducts. To four vials of 1 mL of phosphate buffer (0.5 M, pH 5.0, each containing 3.12 mg of 2′-deoxyguanosine) was added 7.8, 14.5, 19.8, or 54.9 mg of *N*-nitrosotolazoline **2**, respectively. The mixtures were incubated at 37 °C for 24 h and then subjected to HPLC quantitative analysis. For studying the effect of buffer pH on the formation of dG adducts, dG was dissolved in phosphate buffers (0.5 M, pH 3.49, 4.01, 4.53, 5.02, and 5.53, respectively) to an initial concentration of 4.0 mg/mL along with 6.1 equiv of **2**. The mixtures were incubated at 37 °C for 24 h and then subjected to HPLC quantitative analysis.

Reaction of 2′-Deoxyadenosine (dA) with *N*-Nitrosotolazoline 2; Detection of the Modified Adenine Bases by HPLC/ESI/MS^n. To a 1 mL phosphate buffer solution of dA (4.0 mg, 0.0159 mmol, pH 5.0), **2** (15 mg, 0.0794 mmol) was added in one portion. The mixture was sealed in a vial and shaken in an incubator at 37 °C for 24 h. The resulting solution was acidified with 90 μL of concentrated HCl followed by heating at 60 °C for 1 h. After it was cooled, the hydrolyzed solution was neutralized with 17 M NH_4OH, filtered, and then subjected to HPLC/MS analysis. The methodology was similar to that described for the reaction of **2** with dG. SIM mode was used for the detection of adducts from the reaction of **2** with dA with the following scan events at a scan width of 0.6: m/z 297, **7a**, **8a**, **9c**, and **10a**; m/z 370, **14**–**16**; and m/z 413, **7b**, **8b**, **9b**, and **10b**.

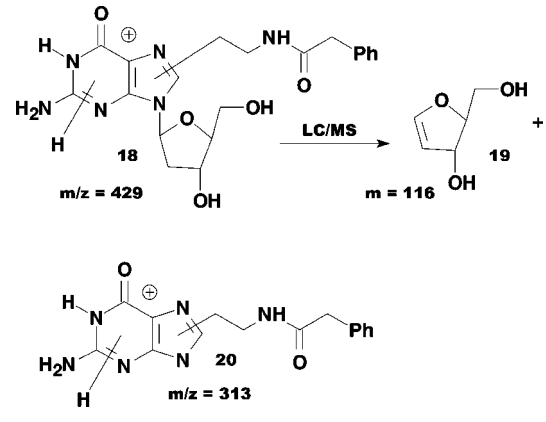
Reaction of *N*-Nitrosotolazoline 2 with DNA. A mixture of 1 mL of calf thymus DNA (CT-DNA) solution (in pH 6.0 phosphate buffer, 1.01 mg/mL determined by UV) and 20 mg of *N*-nitrosotolazoline **2** was shaken at 37 °C for 24 h. The resulting DNA solution was extracted with three equal volumes of CH_2Cl_2 to remove non-DNA-related decomposition products. The aqueous DNA solution was acidified by adding 100 μL of concentrated HCl and then heated at 70 °C for 1 h to release all of the modified and normal bases. The hydrolyzed solution was neutralized with 17 M NH_4OH, and adjusted to a final volume of 1.5 mL. The resulting solution was loaded in a Microcon YM-3 centrifugal filter and centrifuged at 1.2×10^3 rpm for 30 min. The solution in the lower level of the Microcon filter was subject to the HPLC/MS analysis.

Quantitative HPLC/ESI/MS″ Analysis of Hydrolyzed DNA Samples. The hydrolyzed DNA solution was injected to HPLC/MS for the quantitative analysis of modified bases. MRM mode was used to improve the selectivity. MS/MS experiments were performed with a collision energy of 20 eV, with a total scan duration of 1 s. The instrument was tuned by optimizing the response of m/z 313.0 while infusing 10 ppm of **5c** at 10 μL/min into a mobile phase of 0.1% formic in water/MeOH (1:1) at 0.2 mL/min. The transition monitored for the target analytes were as follows: modified adenine (**7a**, **8a**, **9c**, and **10a**), m/z 297.0→136.0 and m/z 297.0→162.0; modified guanine (for **4a** and **5c**), m/z 313.0→152.0 and 313.0→162.0; internal standard, m/z 196.0→152.0; spray voltage, 4.0 kV; and capillary temperature, 350 °C. Another sample of the hydrolyzed DNA was injected into Supelco Discovery BIO Wide Bore C18 Column via an autosampler. Using the general method described by Choi et al. (*14*), an internal standard, O^6-hydroxyethylguanine (1.0 ppm), was postcolumn infused to the eluent at a rate of 10 μL/min rate via Harvard AH 55-1199 syringe pump. HPLC binary eluent mobile phase (0.2 mL/min): (A) methanol; (B) 0.1% aqueous formic acid. Gradient (min, % A) 0, 10%; 20, 50%; and 24, 90%.

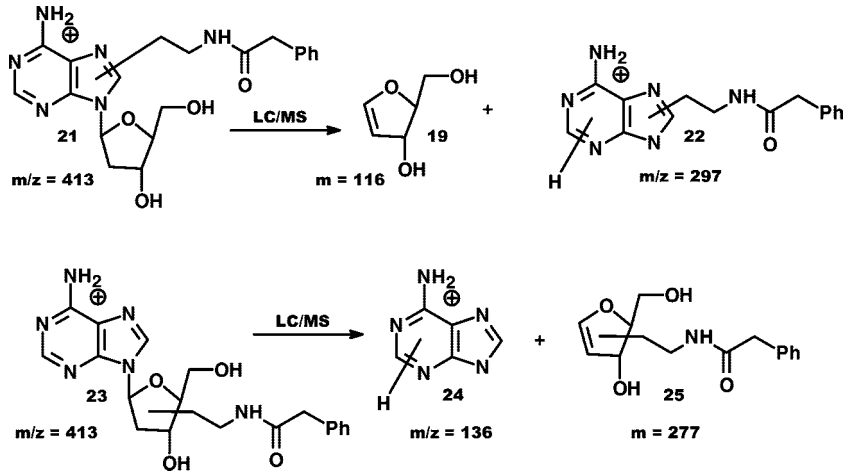
Results and Discussion

Deoxyguanosine Alkylation. Preliminary experiments of the reaction of a 7-fold excess of *N*-nitrosotolazoline **2** at pH 5.5 and 37 °C with either dG (deoxyguanosine) or dA led to a complex mixture as assayed by HPLC. Comparison of the chromatograms with those derived from the hydrolytic decomposition of **2** alone showed that many of the peaks resulted from the decomposition of the substrate. To simplify our analysis (see Scheme 2), we utilized HPLC-MS/MS and constructed a chromatogram based on ions arising from the loss of the neutral dehydroribose fragment **19** (M = 116, CNL). Examination of the dG reaction mixture using this CNL MS method showed five peaks that were not in the control. MS/MS analysis revealed

Scheme 2

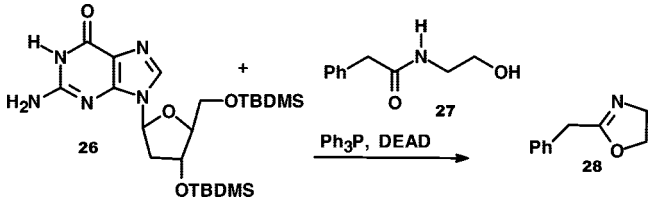


Scheme 3



that three of the parents of these peaks, 10.7, 18.7, and 22.8 min, had an $m/z = 429$, the mass of dG plus the $PhCH_2CONHCH_2CH_2$ fragment (**18**). The peak at 22.8 min also had an ion with a mass increase of 23 (from 428), which was judged to be the Na^+ adduct. Operation of the MS in the SIM mode set to detect ions at 429 showed two prominent peaks (10.7 and 22.8 min) and one very minor one (18.8 min) in the reaction mixture. Because we anticipated alkylation at *N*-7, a process that can lead to the facile cleavage of the deoxyribosyl-base linkage (depurination), we also scanned for peaks with a $m/z = 313$, which is the mass of guanine bound to the same fragment noted above, and found a prominent peak at 13.4 min. Acidic hydrolysis of the adduct mixture, followed again by SIM scanning at $m/z = 313$, resulted in enhancement of the peak at 13.4 min but also gave a new peak at 16.7 min. These peaks were later proven to be 7-(2-phenacetamidoethyl)guanine **5c** and O^6-(2-phenacetamidoethyl)guanine **4a**, respectively (see below).

Scheme 4



Deoxyadenosine Alkylation. Similar methods were used to examine the nature of the reaction mixture produced from the reaction of **2** with dA (Scheme 3). Utilization of SIM scanning at $m/z = 413$ (dA plus the $PhCH_2CONHCH_2CH_2$ fragment, **21**) gave a chromatogram consisting of one major and four minor peaks at 11.7 (major), 21.0, 22.3, 22.9, and 24.2 min, peaks 1–5, respectively (identified as described below as **8b**, **17**, **7b**, **9b**, and **10b**, respectively). MS/MS analysis of each peak showed that peaks 1 and 3–5 all gave a fragment with a m/z of 297 **22**, which is consistent with their being isomeric adenine alkylated adducts. The substance giving rise to peak 2 fragmented to produce adenine (**24**, $m/z = 136$), indicating a product of dA alkylation on a deoxyribosyl OH **25**. The reaction mixture was also scanned using SIM at m/z 297, the mass of the adenine alkylation product that results from depurination. Two peaks, 9.9 (**8a**) and 14.7 (**9c**) min, were observed. Acid hydrolysis of the reaction mixture enhanced these peaks and added others, but authentic synthesis and characterization of both the dG and the dA adducts was required in order for definitive structural characterization prior to quantitation and examination of the products arising from the reaction of **2** with DNA.

Synthesis of Guanine Derivatives (Adducts). In prior work (*15*), we were able to prepare O^6-hydroxyethyldeaoxyguanonsine by using the Mitsunobu reaction to couple 2-acetoxyethanol and an appropriately protected derivative of dG **26**. However, a similar coupling reaction failed, as is shown in Scheme 4 due to the cyclization of 2-phenacetamidoethanol **27** to 2-benzyl-1,3-oxazoline **28**.

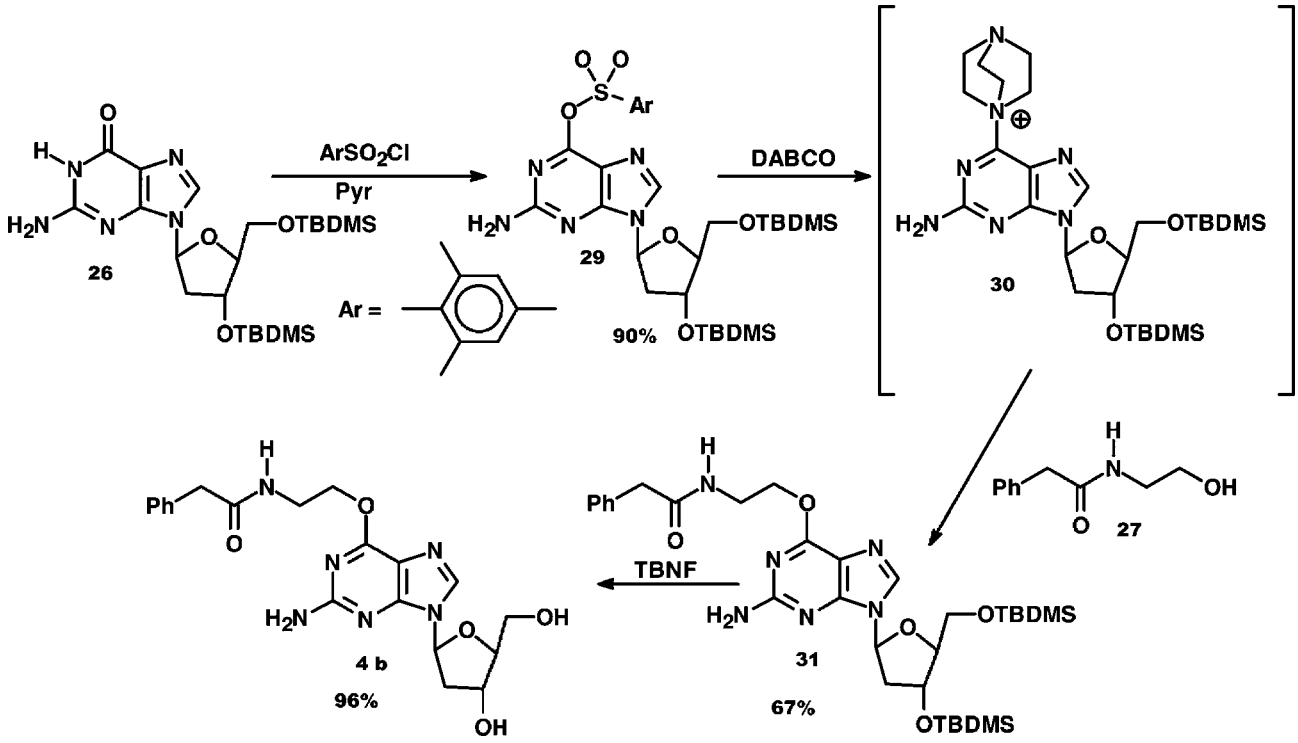
We were able to synthesize the desired O^6 adduct **4b** utilizing a modification of a procedure reported by Laksham et al. (*9*) as shown in Scheme 5. We did not isolate the ammonium salt **30** but reacted it directly with the amidoalcohol **27** to give **31**. The desired adduct **4b** was obtained from dG in four steps in 57% overall yield. The spectral data for **4b** are completely consistent with its assigned structure. HPLC-MS showed that it was the substance giving rise to the peak at 22.8 min (see above). We were able to make the corresponding guanine derivative **4a** by the reaction of 6-chloro-2-aminopurine with **27**.

The *N*-2-Gua **6a** adduct was also synthesized for reference in a straightforward manner from 2-bromoguanine **32** as shown in Scheme 6. Again, the spectral characteristics of this compound were consistent with its structure.

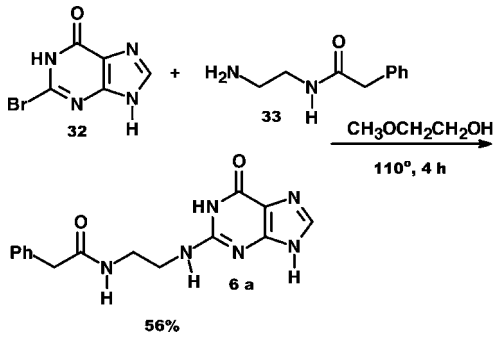
Several methods were tried to generate the 7-dG adduct **5b** by direct alkylation. These included the attempted alkylation of dG with the tosylate of **27** and the alkylation of dG with the tosylate of *N*-2-hydroxyethylsuccinimide. No alkylation was observed without decomposition of the reactants in DMF at 110 or 130 °C in the first case. We did obtain the alkylation product in the second case, but we could not open the succinimide with hydrazine to generate the desired amine. dG was successfully alkylated with the bromide **35**, as shown in Scheme 7, to give **5c**. Even though it is well-known that the direct alkylation of dG or Guo with mild alkylating agents gives rise to reaction at *N*-7, this process constitutes an ambiguous synthesis. To make sure that we had the correct isomer, we utilized NMR ^1H NOESY. The CH_2 at δ 4.20 showed a strong cross-peak with the C-8 H at δ 7.69, confirming alkylation at *N*-7 and the assigned structure (see Supporting Information). This substance had the same HPLC retention time, 13.4 min, as the depurination product described above.

Synthesis of Adenine Derivatives (Adducts). Except for the synthesis of 6-alkylamino derivatives, the synthesis of the other *N*-alkyl adenine isomers is often ambiguous, unless laborious ring closure reactions are used. Because we converted the various dA nucleosides to their free bases by hydrolysis for further analysis, we undertook the synthesis of the needed *N*-alkyl isomers using existing synthetic art. This was followed by rigorous characterization of each isomer, as we describe below.

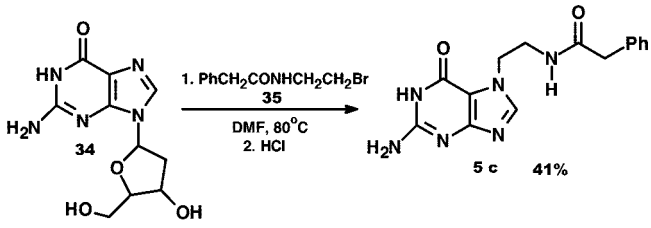
Scheme 5



Scheme 6



Scheme 7



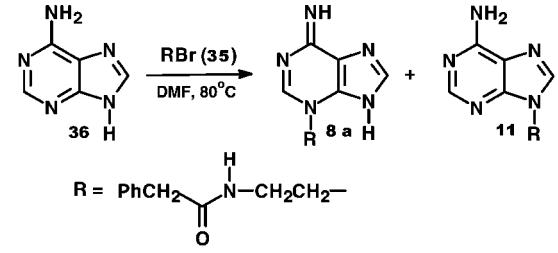
The direct alkylation of adenine **36** with the bromide **35** led to a mixture of two isomeric products (Scheme 8), which were separated by flash SiO_2 chromatography. The major (45%), and more polar substance, was judged to be the 3-substituted product **8a** on the basis of its UV spectrum, its NMR shifts, and literature precedent (*12*). Its complete characterization is described below. The minor product (12%) was assigned the structure of the 9-isomer **11**.

The synthesis of the 1-alkyl isomer **7a** from adenosine **37** is shown in Scheme 9 and is an adaptation of literature methodology (*10*). The ribosyl group was cleaved from **38** with acetic acid. HCl catalysis led to the hydrolytic opening pyrimidine ring. As with the other isomers, structural confirmation is described below.

The N^6-alkyl derivative **10a** was prepared in an unambiguous manner from 6-chloropurine as shown in Scheme 10.

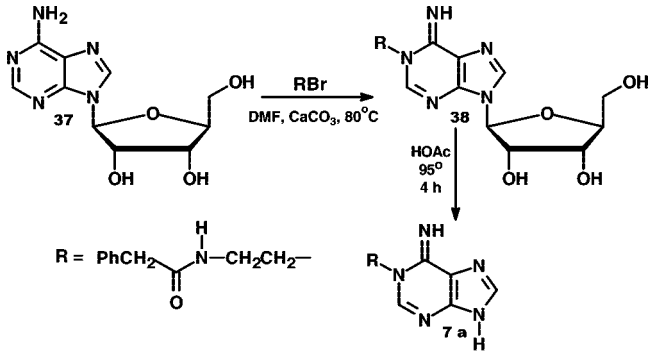
The synthesis of the 7-alkyladenine derivative **9c** was more problematic. Normally, 7-substituted adenines are made either by imidazole ring closure of the appropriate pyrimidine or by the direct alkylation of 3-benzyl adenine at 130 °C followed by hyrogenolytic removal of the benzyl group (*16*). The latter process was attempted with the bromide **35** but led to its decomposition through intramolecular cyclization. Because we had all of the other adenine derivatives of interest, we chose to alkylate adenine with a compound that would not readily cyclize to the oxazoline **28**. This requirement was met by 1-phenyacetylaziridine **40** as shown in Scheme 11 (*2*). Reaction of adenine with **40** gave four products. These were separated by a combination of SiO_2 flash and semipreparative HPLC reversed phase chromatographies to give the desired 7-alkyisomer **7a** in 17% yield. Even though this is not a particularly satisfying direct synthesis, it is economical in effort. The structural characterization of **7a** is described below.

Scheme 8

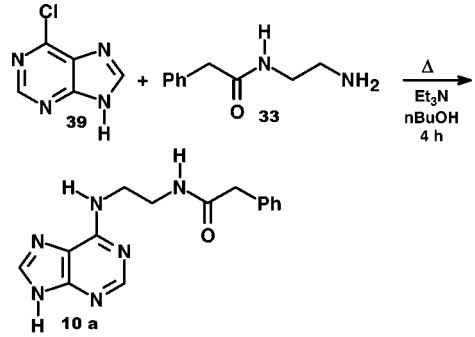


Structural Characterization of 2-Phenacetamidoethyladenine Isomers. Simple ^1H and ^{13}C NMR spectral data are insufficient indicators of the site of adenine alkylation. Even more sophisticated two-dimensional NMR methods are often problematic because of uncertainties in chemical shifts of the ring CH groups and the frequently observed long-range coupling of the alkyl substituent to more than one ring proton (hetero-

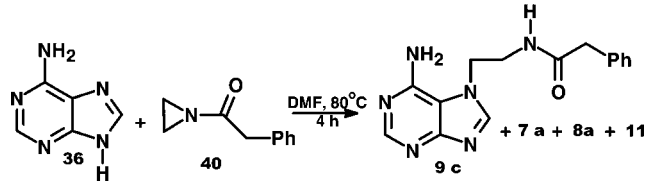
Scheme 9



Scheme 10



Scheme 11



nuclear mulitbond correlation, HMBC). Surprisingly, a UV method developed many years ago remains one of the most reliable methods of making structural assignments (*17–19*). This method involves the recording of the adenine derivative's spectrum in acid, base, and neutral solution. A distinctive and reproducible pattern of spectral shifts occurs as the pH is changed. These shifts are reliably correlated with the alkylation position regardless of the nature of the alkyl group. The UV spectra for the five isomeric 2-phenacetamidoethyl-adenines are given in Figure 2. The pH-induced spectral shifts for each isomer are consistent with the structural assignments and literature data for isomeric *N*-alkyl adenines.

We present here another method for determining the adenine alkylation site through the use of ^{13}C NMR data. We obtained the ^{13}C chemical shift data for adenine and all of the *N*-methyladenines from the literature (*19*). The chemical shift value of a given carbon in adenine was then subtracted from that for the corresponding carbon in a specific *N*-methyladenine. These ^{13}C Δ shift data are plotted for each isomer in Figure 3 (solid lines). Upfield shifts are negative, and downfield shifts are positive. The ^{13}C Δ shifts are smallest for the N^6- and 9-methyl isomers and are plotted on a smaller scale to clearly show the differences. The ^{13}C shift data for the isomeric *N*-2-phenylacetamidoethyladenines were subjected to the same treatment as the methyladenines and are given in Figure 3 as the dashed lines. In several cases, we had too little sample to obtain shifts for the quaternary carbons. In these cases, there are no lines connecting the points. It is readily apparent that there is a strong correlation that allows confirmation and determination of the location of the *N*-alkyl substituent. Given the distinctly different nature of the substituents being compared, CH_3 vs 2-phenylacetamidoethyl, the correlation is excellent and suggests the value of this methodology for *N*-alkyladenine structural assignment. Several effects of the substituents on the ^{13}C shifts are evident. Generally, as has been well-documented in the ^{13}C NMR literature, substituents near a carbon give rise to an upfield shift, which is sometimes called a steric compression shift or a γ-effect. This effect is greater for the larger substituent. Compare, for example, the relative shifts for C-6 for the N^6-alkyl substituents. It is interesting that with the exception of the N^6-alkyl isomers, all shifts at C-8 are positive. The magnitudes, however, are least for the 7- and 9-alkyl isomers where the substituent is closest to this carbon. We have used this type of methodology extensively in our research. We have made no corrections for solvent effects here but expect them to be small as compared to isomer to isomer structural effects on the respective ^{13}C chemical shifts. This type of correlation is most safely utilized when the spectra of different isomers are available, as they are here.

2-Phenylacetyl Adducts. The hydrolytic decomposition of *N*-nitrosotolazoline **2** occurs by attack of H_2O at C-2 of the imidazoline ring (*2*). We also observed the formation of *S*-2-phenylacety-*N*-acetylcysteine when **2** was reacted with *N*-acetylcysteine and proposed that this substance arose from the competitive attack of the thiol on C-2 of *N*-nitrosotolazoline (*2*). Anticipating the existence of similar transformations with dA or dG, we scanned the reactions mixtures of each nucleoside for the respective adducts **12–16** using the MS methods described above. In the case of dG, no adducts of MWt 385 (dG + $PhCH_2C{=}O$) were found in the CNL scan for loss of the ribosyl fragment **19** (m = 116). However, SIM scans at 386 showed two compounds (21.7 and 26.1 min). Each of these compounds gave rise to an unaltered guanine group (m/z 152) when subjected to MS/MS. Thus, the acylation must have occurred on the deoxyribosyl OH groups to give **12** and **13**. Confirmation was achieved by synthesizing 5′-*O*-phenylacetyldeoxyguanosine **12** by the reaction of dG with phenylacetyl chloride. This compound had an HPLC retention time of 21.7 min. We did not make the other isomer but presume it to be the substance with the 26.1 min rt.

The dA *N*-nitrosotolazoline reaction mixture was analyzed in a similar manner. The SIM scan at 370 indicated the presence of four possible isomers [17.0, 22.4, 24.8 (major), and 28.8 min]. MS/MS data revealed that two of these were acylated on the ribosyl group, **14** and **15**, since an unaltered adenine ion (m/z 136) was produced from each. Analysis of the other two peaks indicated probable *N*-acylation (**16a** and **16b**), but the yields were very low, and we did not pursue further separation or characterization.

Yields of dG Adducts. The yields of adducts produced from the reaction of dG with **2** were explored as a function of two variables, reactant ratio and pH. The data are presented in Figure 4. The 7-(2-phenylacetamidoethyl)dG adduct **5b** is formed in greatest yield (detected for quantitation purposes as **5c**), followed by the corresponding O^6-adduct **4b**. (A chromatogram is shown in Figure 5.) This pattern is typical of that produced by diazonium ion-derived alkylation (*20–23*). As expected, adduct yields increase as the molar ratio **2**/dG increases. pH has little effect on *N*-alkylation, but the yield of the acyl adduct **12** does appear to be subject to base catalysis. This adduct presumably forms by attack of the deoxyribosyl 5′-OH at the 2-carbon of **2**, a process that is likely subject to base catalysis (*2*). The N^2-adduct **6** was synthesized as the base **6a**, not the nucleoside

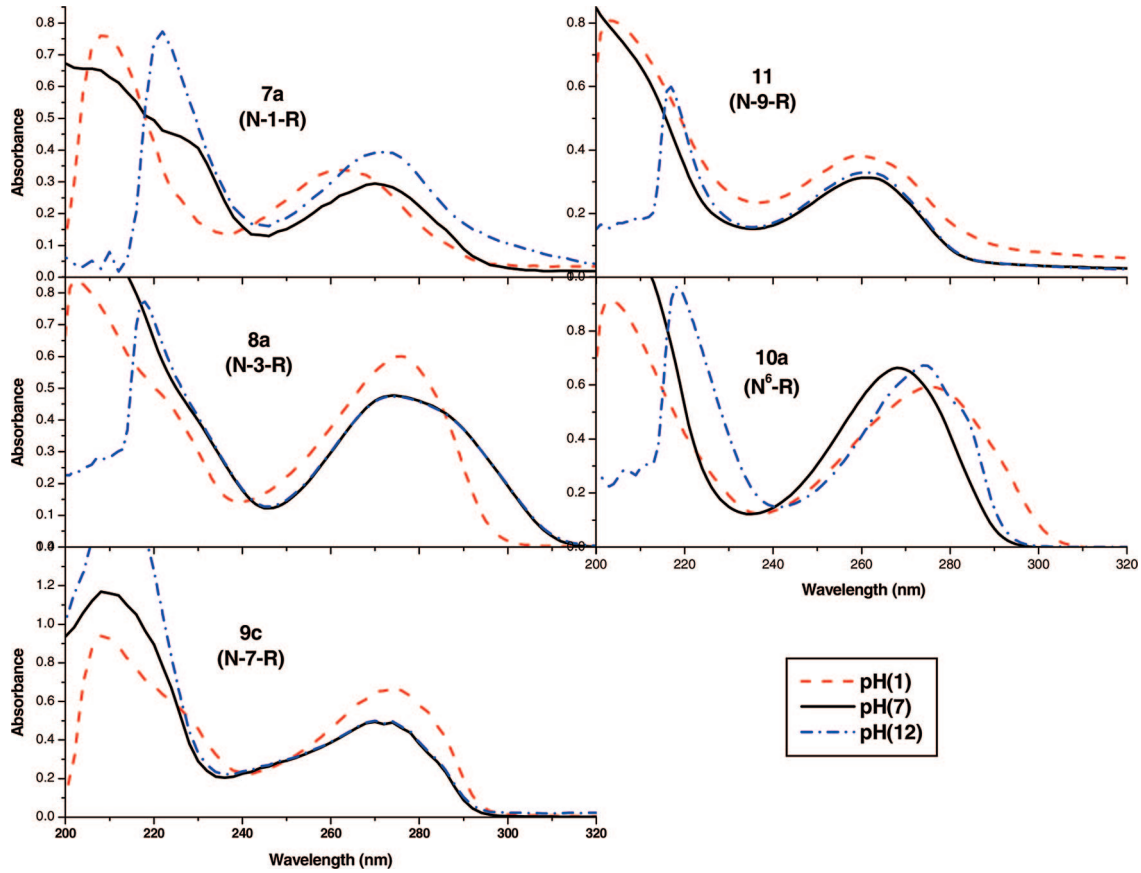


Figure 2. UV spectra in acid, base, and neutral pH solutions for each of the isomeric *N*-2-phenylacetamidoethyl adenine adducts (R = 2-phenylacetamidoethyl). The spectral shifts with pH are characteristic of the position of substitution.

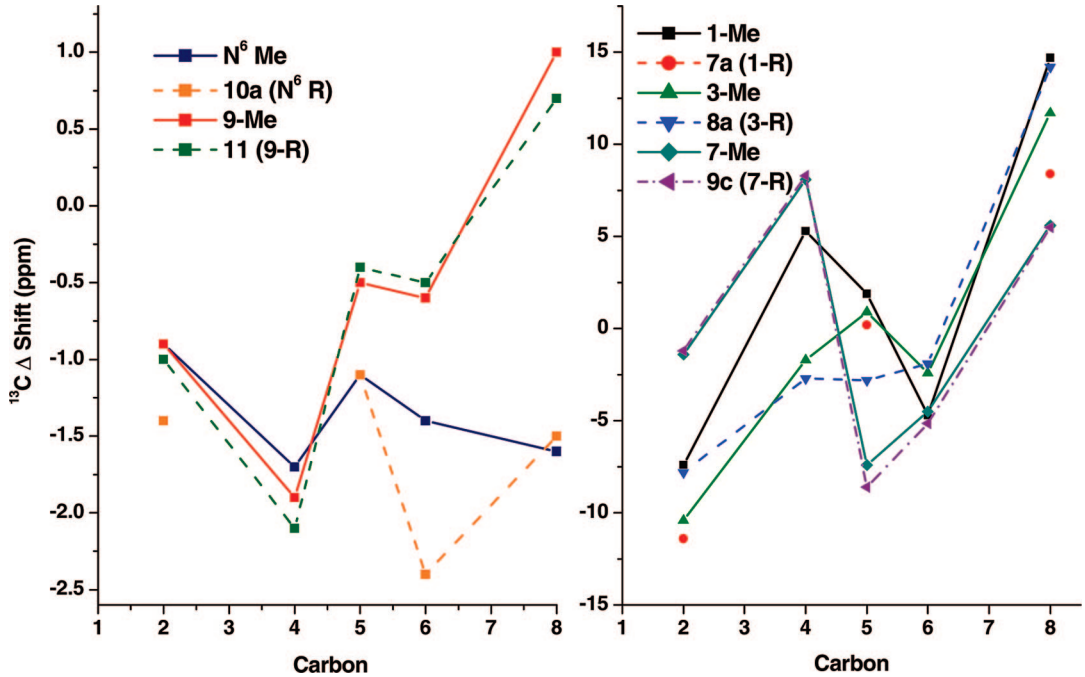


Figure 3. ^{13}C Δ chemical shifts obtained by subtracting the corresponding δ value of the adenine carbon from either that carbon in *N*-methyladenine (solid lines) or the *N*-2-phenylacetamidoadenine (dashed lines). The correlation of the patterns permits assignment of the *N*-alkylation position.

6b. Hydrolysis of the dG alkylation mixture to remove the sugar did not reveal the presence of **6a**.

The dA adducts were not quantified at this stage. A representative HPLC-MS-SIM chromatogram is given in Figure 5. This was obtained after hydrolysis with HCl to cleave the glycosidic linkage.

DNA/*N*-Nitrosotolazoline Aadducts. The nature and quantity adducts produced from the in vitro reaction of **2** with CT-DNA were determined by the reaction of these substances at 37 °C, pH 6 (phosphate buffer), for 24 h. The DNA was hydrolyzed with 1 M HCl at 70 °C for 1 h to liberate the bases. After neutralization of this mixture, the filtrate was analyzed by HPLC/

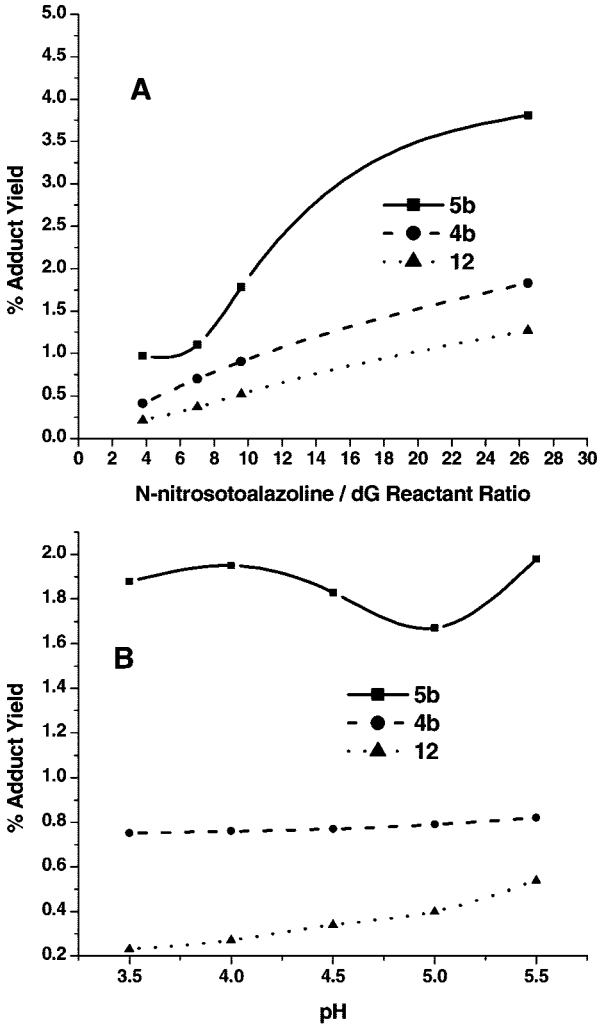


Figure 4. 2-Phenylacetamidoethyl adducts of dG. (A) The yield of each adduct increases as the ratio of **2** to dG increases. (B) Except for the 5′-O-phenacetyl adduct **12**, pH has little effect on adduct yield.

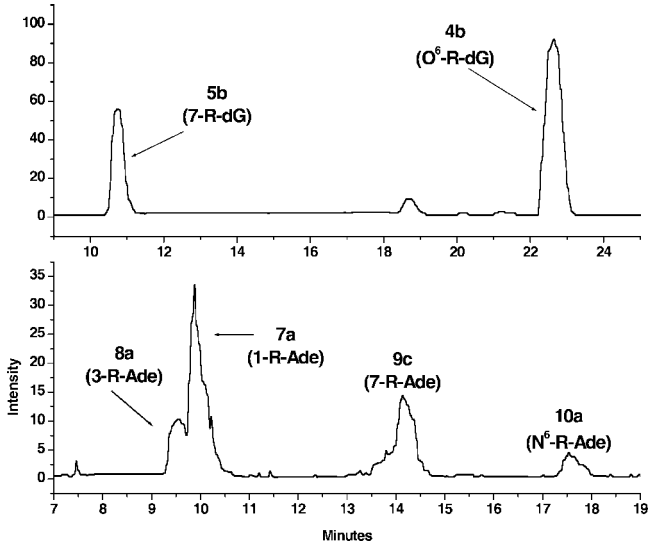
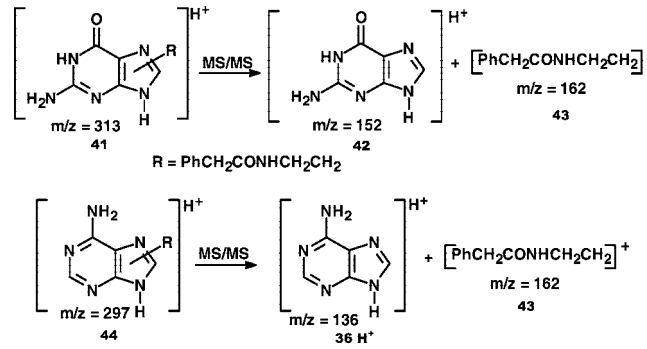


Figure 5. Top panel: The HPLC MS chromatogram resulting from a SIM scan of the alkylation reaction mixture of **2** and dG at *m*/*z* 429. Bottom panel: A HPLC MS chromatogram (*m*/*z* 297) showing the adenine adducts generated after the alkylation of dA with **2**, followed by acidic hydrolysis.

MS/MRM (multi reaction monitoring). The analysis required the development of a new method. While there may be other

Scheme 12



adducts, we only attempted to detect those, which we had synthesized, where the base had been modified by the incorporation of the 2-phenylacetamidoethyl fragment from **2**. The method has two important components: (i) The use of MS^n to monitor ions produced from a given parent ion and (ii) the constant addition of an internal standard by syringe pump following the column separation for the purposes of quantitation. In this method, interference is minimized and selectivity is maximized by meeting the following criteria: (i) the same *m*/*z* of selected parent ion, (ii) the same *m*/*z* of the selected daughter ion, and (iii) the same retention time by HPLC as the analyte standard to which the MS was tuned. The MS/MS fragmentation is shown in Scheme 12 and is based on our work with the standards. The daughter fragment ions (**42**, **43**, and **36**-H^+) shown are the major ones produced from each of the alkylated standards. Because the intensity of the daughter ions varies somewhat from adduct to adduct, we monitored two transformations for each parent ion, 313 → 152 and 313 → 162 and 297 → 136 and 297 → 162, to detect the adduct guanines and adenines, respectively. This approach was particularly important for the 1-alkyl adenine **7a**, which gave a relatively weak *m*/*z* 136 fragment ion intensity.

Unquestionably, the best method for quantifying adducts in MS determinations is through the use of isotopically labeled standards of the molecules being determined. However, because seven or more adducts could be produced from the reaction of **2** with DNA, we sought a method that did not involve all of the synthesis and still promised good, yet possibly less precise, quantitation. Our method involves the postcolumn infusion of the internal standard, O^6-hydroxyethylguanine **45**. Calibration curves (see Supporting Information) were constructed using the synthesized standards of the adducts. The MS/MS transition monitored for the internal standard was *m*/*z* 196 → 152. The calibration curves exhibited high linearity ($r^2 > 0.99$) over the concentration range of interest. All bases could be detected at the 100 pg level with a 20 μL injection. Other parameters, particularly the chromatographic ones, which need adjustment to achieve maximum sensitivity, are given in the Experimental Procedures.

The results of our analysis are given in the chromatograms of Figure 6 and in Table 1. We were only able to detect the 7- and O^6-adducted guanines **5a** and **4a** and the 3- and 7-alkyl adenines **8a** and **9c**. Our analysis assumes complete hydrolytic release of the adducted bases from DNA without further transformation, which may not be true. As seen in Table 1, the 7-Guo **5a** is the major adduct followed by the 3-Ade adduct **8a**. We have compared the relative percentages of the four adducts with ethyl adducts derived from the reaction of CT-DNA with *N*-ethyl-*N*-nitrosourea (ENU) [work of Beranek et al. (*20*)] and also with more recent research on adducts derived from the 1-propyldiazonium ion (*24*). Even though ENU alkylation involves the ethyl diazonium ion and our DNA

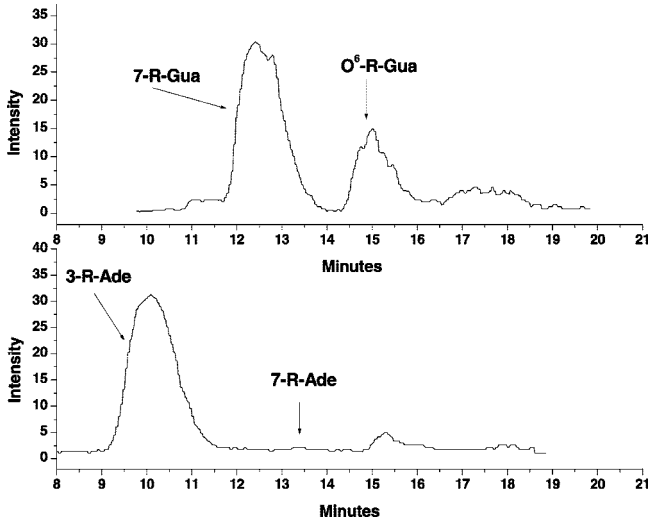


Figure 6. Chromatograms for the quantitative determination of 2-phenylacetamidoethyl adducts in CT-DNA arising from **2** are given. The upper panel gives the scan at for 313 → 152 transformation, and the lower one monitors 297 → 136.

Table 1. Levels of 2-Phenylacetamidoethyl (R) Adducts Produced in DNA by Reaction with 2

adduct	7-Guo (**5c**)	O^6-Guo (**4a**)	3-Ade (**8a**)	7-Ade (**9c**)
concentration (ppm)	1.54	0.22	0.78	0.046
adducts/10^4 bases	62.7	8.9	31.8	1.9
relative % R	60	9	30	1.8
relative % ethyl[a]	46	40	11	1.6

[a] Data taken from Beranek et al. (*20*).

hydrolysis conditions were the same as those used by these researchers, the comparison may not be completely apt because the DNA could affect catalytic decomposition of either of these precursors to the respective diazonium ions. Differences could affect the site of base alkylation. The largest differences are in the relative amounts of 3-Ade and O^6-Guo alkylated bases. The amount of 3-R-Ade **8a** is large in comparison with other data for diazonium ion alkylation. The amount of O^6-ethylation produced by ENU is likewise abnormal. The relative amount of O^6-R **4** produced from **2** is rather typical of what has been observed for other diazonium alkylators, as are the relative amounts of the other adducts derived from **2** (*20–24*).

Concluding Discussion

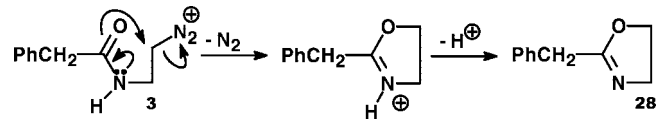
The authors of a study of the decomposition kinetics and products of a group of *N*-nitroso-2-phenylimidazolines akin to **2** suggested that these compounds had little alkylating potential because of intramolecular trapping of the diazonium ion (*25*). This process is illustrated for the diazonium ion **3**, produced from **2**, in Scheme 13. Assisted carbonyl displacement of the nitrogen would give **28**, 2-benzyloxazoline. While this process may occur to some extent, the data presented here clearly and unequivocally demonstrate that *N*-nitrosotolazoline **2** is a potent DNA alkylator. This process does not require metabolic activation, as is necessary for nitrosamines. The alkyl fragment **43**, expected from the diazonium ion **3**, is found in DNA and the alkylation pattern is that expected from a diazonium ion. Key here is the formation of the O^6-2-phenylacetamidoethyl-adducted guanine **4a**. O^6-Alkylated guanines are known to possess considerable carcinogenic potential (*18, 26–28*). Adenines and guanines alkylated at the 3- and 7-positions can also give rise to possibly mutagenic transformation through the generation of apurinic sites. Thus, the alkylating potential of **2**, to which other *N*-nitrosoimidazolines derived from drugs and other commercial chemicals are likely analogous, must be considered seriously. The results delineated in this paper as well as our examination of the properties of **2** (*2*) strongly suggest that it and analogous *N*-nitrosoimidazolines are direct-acting mutagens and carcinogens.

Other DNA adducts could arise from the decomposition of **2**, but we think that we have identified the major purine adducts. We did not look for the N^6-phenylacetyladenine adduct in DNA. Other adducts may arise from attack of nucleophilic centers in DNA at the 2-position of **2**. This process can be conceived to generate a diazonium ion (not shown). Because **2** is nitrosated much faster than its precursor **1** and gives rise to a cascade of diazonium ions (*1, 2*), other adducts could arise from endogenous nitrosation. The nature of these adducts is predictable from our studies of this chemistry.

The key question arising from this research (*1, 2*) is do imidazoline-based drugs and related substances pose a toxicological or carcinogenic threat through the process of endogenous nitrosation? Either gastric nitrosation or NOx nitrosation at sites of chronic inflammation are possible. Our work clearly demonstrates that *N*-nitrosotolazoline, and likely similar compounds, have toxic potential. The critical question then reduces to whether endogenous nitrosation occurs. The instability of **2** may present limits to its detection. However, our chemistry and DNA adduct studies provide a basis for addressing this question.

Another fruitful attribute to this research arises from our discovery that ^{13}C NMR can be used to determine the alkylation site of adenine and its nucleosides. Although the data set is small, we are confident that the method will be of utility.

Scheme 13



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Supporting Information Available: ^1H and ^{13}C spectra, NOESY for **5c**, calibration curves, HPLC MS chromatograms, and an apparatus diagram. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Loeppky, R. N., Shi, J., Barnes, C., and Geddam, S. (2008) A diazonium cascade from the nitrosation of tolazoline, an imidazoline containing drug. *Chem. Res. Toxicol. 21*, 295–307.

(2) Loeppky, R. N., and Shi, J. (2008) *N*-Nitrosotolazoline: Decomposition studies of a typical *N*-nitrosoimidazoline. *Chem. Res. Toxicol. 21*, 0000.

(3) Choi, S.-W., Elmaleh, D. R., Hanson, R. N., and Fischman, A. J. (1999) Design, synthesis, and biological evaluation of novel non-piperazine analogues of 1-[2-(diphenylmethoxy)ethyl]- and 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazines as dopamine transporter inhibitors. *J. Med. Chem. 42*, 3647–3656.

(4) Casadei, M. A., Moracci, F. M., Zappia, G., Inesi, A., and Rossi, L. (1997) Electrogenerated superoxide-activated carbon dioxide. A new mild and safe approach to organic carbamates. *J. Org. Chem. 62*, 6754–6759.

(5) Elion, G. B., Lange, W. H., and Hitchings, G. H. (1956) Condensed pyrimidine systems. XIII. Some amino-substituted derivatives of guanine and 6-thioguanine. *J. Am. Chem. Soc. 78*, 217–220.

(6) Beaman, A. G., Gerster, J. F., and Robins, R. K. (1962) Potential purine antagonists. XXVIII. Preparation of bromopurines. *J. Org. Chem. 27*, 986–990.

(7) Wright, G. E., and Dudycz, L. W. (1984) Synthesis and characterization of N2-(p-n-butylphenyl)-2′-deoxyguanosine and its 5′-triphosphate and their inhibition of HeLa DNA polymerase α. *J. Med. Chem. 27*, 175–181.
(8) Ogilvie, K. K. (1973) The tert-butyldimethylsilyl group as a protecting group in deoxynucleosides. *Can. J. Chem. 51*, 3799–3807.
(9) Lakshman, M. K., Ngassa, F. N., Keeler, J. C., Dinh, Y. Q. V., Hilmer, J. H., and Russon, L. M. (2000) Facile synthesis of O6-alkyl-, O6-aryl-, and diaminopurine nucleosides from 2′-deoxyguanosine. *Org. Lett. 2*, 927–930.
(10) Itaya, T., Tanaka, F., Fujii, T., and Leonard, N. J. (1977) An improved procedure for the synthesis of 1-alkyladenines: Removal of the ribofuranosyl group from 1-benzyl-, 1-(3-methyl-2-butenyl)-, and 1-allyladenosine hydrobromide in acetic acid. *Chem. Pharm. Bull. 25*, 1449–1452.
(11) Robins, M. J., and Trip, E. M. (1973) Sugar-modified N6-(3-methyl-2-butenyl)adenosine derivatives, N 6 -benzyl analogs, and cytokinin-related nucleosides containing sulfur or formycin. *Biochemistry 12*, 2179–2187.
(12) Fujii, T., Walker, G. C., and Leonard, N. J. (1979) 3-Substituted adenines. In vitro enzyme inhibition and antiviral activity. *J. Med. Chem. 22*, 125–129.
(13) Boggs, G. R., and Gerig, J. T. (1969) Nitrogen inversion in *N*-benzoylaziridines. *J. Org. Chem. 34*, 1484–1486.
(14) Choi, B. K., Gusev, A. I., and Hercules, D. M. (1999) Postcolumn introduction of an internal standard for quantitative LC-MS analysis. *Anal. Chem. 71*, 4107–4110.
(15) Gu, F. (1997) The Chemistry of Reactive Nitrosamines and Related DNA Adducts, Ph.D. dissertation, p 253, University of Missouri, Columbia, MO.
(16) Leonard, N. J., and Fujii, T. (1963) Synthesis of 7-substituted adenines through the use of a blocking group at the 3-position. *J. Am. Chem. Soc. 85*, 3719.
(17) Townsend, L. B., Robins, R. K., Loeppky, R. N., and Leonard, N. J. (1964) Purine nucleosides, (VIII). Reinvestigation of the position of glycosidation in certain synthetic ′7′-substituted-6-dimethylaminopurine nucleosides related to puromycin. *J. Am. Chem. Soc. 86*, 5320–5325.
(18) Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.
(19) Fujii, T., and Itaya, T. (1999) Systematic tables of mono- and poly-*N*-methylated adenines: Acid dissociation constants and UV and NMR spectral data. *Heterocycles 51*, 2255–2277.
(20) Beranek, D. T., Weis, C. C., and Swenson, D. H. (1980) A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. *Carcinogenesis 1*, 595–606.
(21) Lawley, P. D. (1984) Carcinogenesis by alkylating agents. In *Chemical Carcinogens* (Searle, C. E., Ed.) pp 325–484, American Chemical Society, Washington, DC.
(22) Lawley, P. D., and Shah, S. A. (1972) Methylation of ribonucleic acid by the carcinogens dimethyl sulfate, *N*-methyl-*N*-nitrosourea, and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. Comparisons of chemical analysis at the nucleoside and base levels. *Biochem. J. 128*, 117–132.
(23) Blans, P., and Fishbein, J. C. (2004) Determinants of selectivity in alkylation of nucleosides and DNA by secondary diazonium ions: Evidence for, and consequences of, a preassociation mechanism. *Chem. Res. Toxicol. 17*, 1531–1539.
(24) Lu, X., Heilman, J. M., Blans, P., and Fishbein, J. (2005) The structure of DNA dictates purine atom site selectivity in alkylation by primary diazonium ions. *Chem. Res. Toxicol. 18*, 1462–1470.
(25) Iley, J., Norberto, F., and Rosa, E. (1989) *N*-Nitroso compounds. Part 1. Structure and decomposition of *N*-nitroso-2 arylimidazolines in aqueous acidic media. *J. Chem. Soc., Perkin Trans. 2* 1471–1475.
(26) Singer, B. (1976) All oxygens in nucleic acids react with carcinogenic ethylating agents. *Nature 264*, 333–339.
(27) Snow, E. T., Foote, R. S., and Mitra, S. (1984) Base-pairing properties of O6-methylguanine in template DNA during in vitro DNA replication. *J. Biol. Chem. 259*, 8095–8100.
(28) Swann, P. F. (1990) Why do O6-alkylguanine and O4-alkylthymine miscode? The relationship between the structure of DNA containing O6-alkylguanine and O4-alkylthymine and the mutagenic properties of these bases. *Mutat. Res. 233*, 81–94.

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