Reactions of α-Acetoxy-*N*-nitrosopyrrolidine and α-Acetoxy-*N*-nitrosopiperidine with Deoxyguanosine: Formation of N^2-Tetrahydrofuranyl and N^2-Tetrahydropyranyl Adducts

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The goal of this study was to compare the reactions of α-acetoxy-*N*-nitrosopyrrolidine (α-acetoxyNPYR) and α-acetoxy-*N*-nitrosopiperidine (α-acetoxyNPIP) with deoxyguanosine (dG). α-AcetoxyNPYR and α-acetoxyNPIP are stable precursors to the α-hydroxynitrosamines which are formed metabolically from NPYR and NPIP. These α-hydroxynitrosamines are believed to be the proximate carcinogens of NPYR and NPIP. NPYR and NPIP, although structurally similar, have remarkably different carcinogenic properties, and a comparison of the reactions of their metabolically activated forms with dG and ultimately DNA could provide insights on their mechanisms of carcinogenicity. Reactions of α-acetoxyNPYR and α-acetoxyNPIP with dG were carried out at 37 °C and pH 7.0. The products were analyzed by HPLC and characterized by their spectral properties and by comparison to standards. In each reaction, one of the major products was a new type of dG adduct: N^2-(tetrahydrofuran-2-yl)dG (THF-dG) from α-acetoxyNPYR and N^2-(3,4,5,6-tetrahydro-2*H*-pyran-2-yl)dG (THP-dG) from α-acetoxyNPIP. THF-dG was synthesized independently by reaction of either 2-chlorotetrahydrofuran or 2,3-dihydrofuran with dG. Similarly, THP-dG was prepared by reaction of 2-chloro-3,4,5,6-tetrahydro-2*H*-pyran with dG. The structures of THF-dG and THP-dG were established by their UV and ^{1}H-NMR spectra. THF-dG was less stable than THP-dG, but could be readily converted to a stable derivative, N^2-(4-hydroxybutyl)dG, by reaction with $NaBH_4$. THF-dG and THP-dG were converted to dG and 2-hydroxytetrahydrofuran or 2-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran, respectively, upon neutral thermal or acid hydrolysis. This reaction was found to be reversible, with the adducts being produced in substantial amounts by reaction of 2-hydroxytetrahydrofuran or 2-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran with dG. The latter reaction accounts for part of the THF-dG and THP-dG produced from the α-acetoxynitrosamines; stable oxonium ion-derived electrophiles may also be involved in the formation of THF-dG and THP-dG. Comparisons of the yields of various adducts in the reaction of α-acetoxyNPYR and α-acetoxyNPIP with dG showed some major differences. Whereas yields of THF-dG and THP-dG were similar, adducts formed from open chain diazonium ion or related intermediates were formed more extensively from α-acetoxyNPYR than from α-acetoxyNPIP. Adducts formed from enal products of the two nitrosamines were also different. Adduct formation as characterized in this study may account for some of the contrasting carcinogenic properties of NPYR and NPIP.

Introduction

N-Nitrosopyrrolidine (NPYR)[1] and *N*-nitrosopiperidine (NPIP) are structurally related cyclic nitrosamines with remarkably different carcinogenic properties in rats. Their structures are shown in Figures 1 and 2. While NPYR induces mainly liver tumors, NPIP causes tumors of both the esophagus and the liver. Esophageal tumors have not been reported in rats treated with NPYR (*1-3*). These differences in organospecificity may be due to differences in the metabolism of NPYR versus NPIP, to differences in the types of DNA adducts that are formed from these two nitrosamines, to the repair, persistence, or mutagenic properties of the adducts, or perhaps to other factors. Our working hypothesis is that differences in metabolism and DNA adduct formation between NPYR and NPIP are responsible for their contrasting carcinogenic activities.

Human exposure to NPYR and NPIP could occur through the diet or tobacco use, or due to endogenous formation of these nitrosamines. NPYR is one of the most commonly detected dietary nitrosamines and is also a constituent of tobacco and tobacco smoke (*4, 5*). The greatest potential for human exposure to these nitrosamines is however through endogenous formation. The endogenous formation of N-nitrosoproline has been conclusively demonstrated in many situations in which humans are exposed to nitrogen oxides, nitrate, or nitrite (*6*). The generation of nitric oxide from arginine catalyzed by NO synthase is now well recognized under a variety of conditions; this reaction also leads to nitrosa-

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[1] Abbreviations: NPYR, *N*-nitrosopyrrolidine; NPIP, *N*-nitrosopiperidine; α-acetoxyNPYR, α-acetoxy-*N*-nitrosopyrrolidine; α-acetoxyNPIP, α-acetoxy-*N*-nitrosopiperidine; dG, deoxyguanosine; THF-OH, 2-hydroxytetrahydrofuran; THP-OH, 2-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran; THP-Cl, 2-chloro-3,4,5,6-tetrahydro-2*H*-pyran; THF-Cl, 2-chlorotetrahydrofuran; THP-dG, N^2-(3,4,5,6-tetrahydro-2*H*-pyran-2-yl)dG; THF-dG, N^2-(tetrahydrofuran-2-yl)dG; 2,4-DNP reagent, (2,4-dinitrophenyl)hydrazine reagent; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone.

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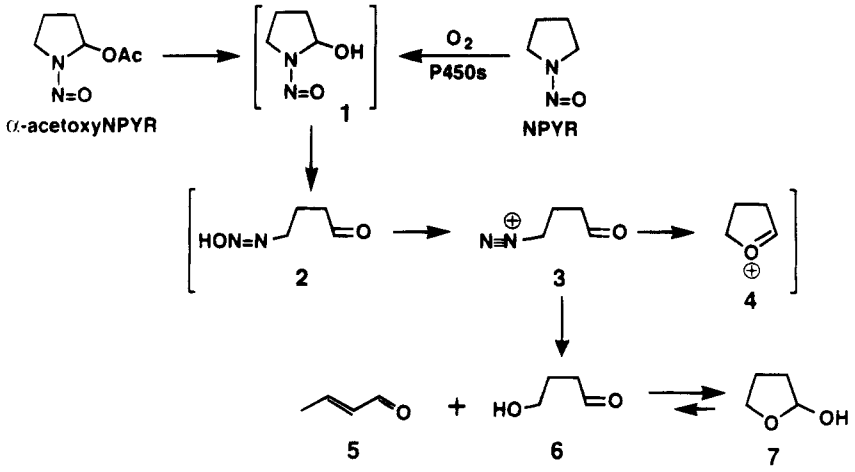


Figure 1. Intermediates and products formed in the solvolysis of α-acetoxyNPYR and upon metabolic α-hydroxylation of NPYR. Compounds **5**–**7** have been identified as metabolites of NPYR.

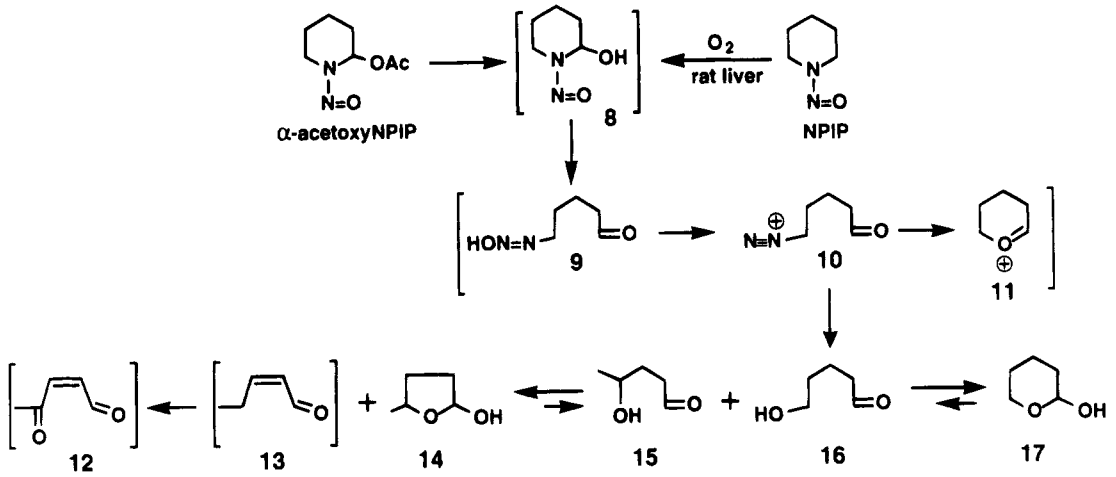


Figure 2. Intermediates and products formed in the solvolysis of α-acetoxyNPIP and upon metabolic α-hydroxylation of NPIP. Compounds **16** and **17** have been identified as metabolites of NPIP.

tion of secondary amines (*7*, *8*). Since pyrrolidine and piperidine occur naturally in humans, being excreted in urine in amounts of 20–30 mg per day, their endogenous nitrosation appears likely (*9*). In fact, the excretion of NPYR and NPIP in human urine has been demonstrated in patients with bladder infections (*9*, *10*). Since NPYR and NPIP are extensively metabolized, DNA adducts or metabolites could perhaps provide dosimeters for their endogenous formation in humans.

Therefore, we and others have studied the metabolism of NPYR and NPIP and have characterized some of the DNA adducts which are formed from these nitrosamines (*11-26*). One of the major pathways of metabolism of NPYR and NPIP is α-hydroxylation, which results in the formation of unstable intermediates- α-hydroxyNPYR or NPIP (**1** and **8**, Figures 1 and 2). Spontaneous decomposition of these intermediates leads to a cascade of reactive intermediates and products, several of which are electrophilic and can potentially react with DNA. In previous studies, we have characterized a number of adducts formed by this process (*15*, *17-19*, *22*, *26*; Figure 3). In this paper and the companion study, we have further examined the reactions with dG and DNA of α-acetoxy-*N*-nitrosopyrrolidine (α-acetoxyNPYR, Figure 1) and α-acetoxy-*N*-nitrosopiperidine (α-acetoxyNPIP, Figure 2), stable precursors to the α-hydroxynitrosamines that are formed metabolically from these two carcinogens.

Experimental Section

Caution. *α-AcetoxyNPYR and α-acetoxyNPIP are mutagens and are chemically activated forms of the carcinogens NPYR and NPIP. They should be handled with extreme care, using appropriate safetywear and ventilation at all times.*

Apparatus. HPLC analyses were carried out with Waters Associates (Millipore, Waters Division, Milford, MA) systems equipped with Models 991 or 994 photodiode array detectors or Perkin-Elmer Models 650-10S or LS-40 fluorescence detectors (Perkin-Elmer Co., Norwalk, CT).

The following HPLC systems were used: (1) Two 3.9 mm × 30 cm μBondapak C-18 columns (Millipore, Waters Division) eluted with 5% CH_3CN in H_2O for 5 min, then a linear gradient to 25% CH_3CN in 30 min at 1 mL/min. (2) A 9.4 mm × 50 cm Partisil 10 ODS-3 Magnum 9 (Whatman, Clifton, NJ) column eluted with a linear 30 min gradient from 5% to 25% CH_3CN in H_2O at 4 mL/min. (3) A 4.6 mm × 25 cm Econosil 5 μm silica column (Alltech, Deerfield, IL) eluted with 7/3 $CHCl_3$/hexane at 1 mL/min. (4) Two 3.9 mm × 30 cm μBondapak C-18 columns eluted with solvent A for 6 min, then with a gradient from 0% to 100% solvent B in 60 min at 1 mL/min, using curve 6. Solvent A was 0.1 M phosphate buffer (pH 5.7), and solvent B was 20% MeOH in H_2O. (5) A 4.6 mm × 25 cm Supelcosil LC 18-DB column (Supelco, Bellefonte, PA) eluted with a gradient from 0% to 30% CH_3CN in 10 mM phosphate buffer (pH 7.0), in 30 min at 1 mL/min, using curve 6. (6) The same as system 5, except H_2O was used instead of phosphate buffer. (7) A 10 mm × 25 cm Supelcosil LC 18-DB column, eluted with the same program as in system 5, except in 40 min at 4 mL/min. (8) Two 3.9 mm × 30 cm μBondapak C-18 columns eluted with 50–100% MeOH in H_2O in 50 min using curve 6 at 1 mL/min. (9) A 4.6

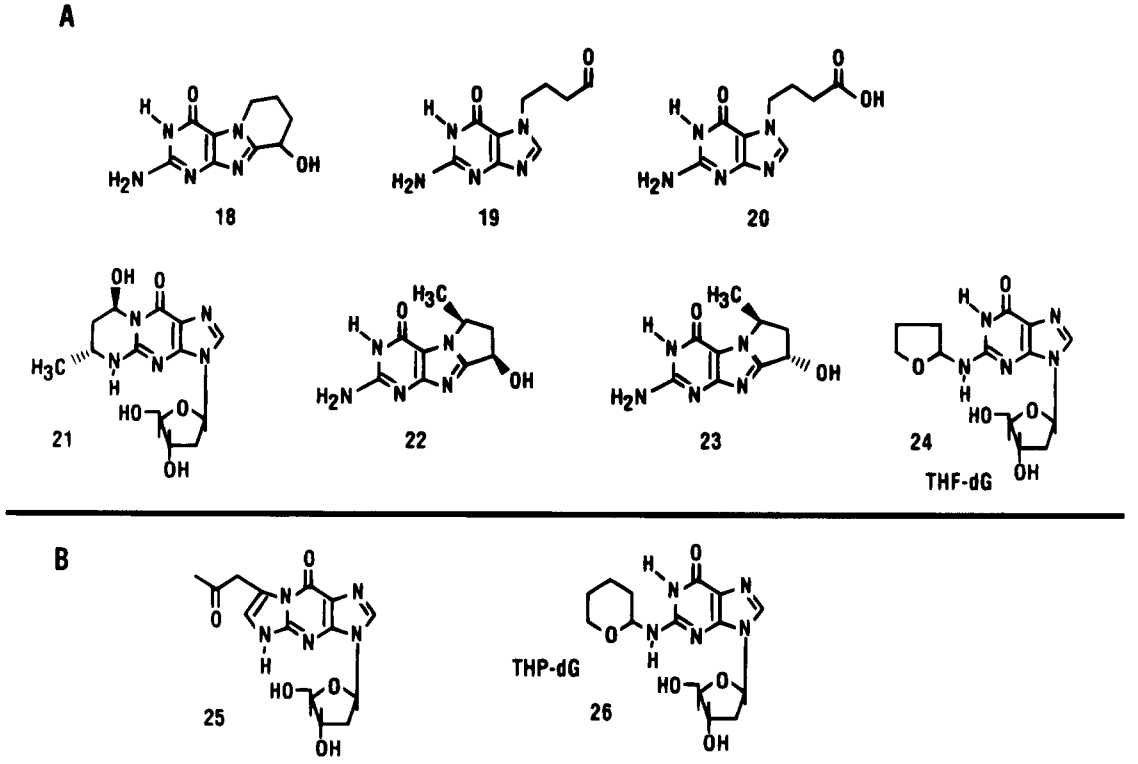


Figure 3. DNA adducts formed upon α-hydroxylation of (A) NPYR and (B) NPIP. Adducts **18**–**23** and **25** have been previously characterized in vitro; **18**, **19**, and **21** have also been detected in vivo. THF-dG (**24**) and THP-dG (**26**) were identified in this study.

mm × 25 cm B&J OD5 octadecyl 5 μm column (Burdick and Jackson, Baxter, McGaw Park, IL) eluted with a gradient from 40% to 70% CH_3CN in H_2O in 60 min at 1 mL/min, using curve 6. (10) Two 4.6 mm × 25 cm Partisil-10 SCX strong cation exchange columns (Whatman) eluted with 0.1 M ammonium phosphate buffer (pH 2.0), at 1 mL/min.

Low resolution and high resolution FAB-MS were run on a VG ZAB-T instrument and a Kratos MS-50 instrument, respectively, at the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry, St. Louis, MO. Electrospray ionization MS was carried out with a VG Quattro instrument. ^{1}H-NMR spectra were determined on a Bruker AM 360 WB spectrometer.

Chemicals and Enzymes. α-AcetoxyNPYR, α-acetoxyNPIP, 4-hydroxybutanal (2,4-dinitrophenyl)hydrazone, 5-hydroxypentanal (2,4-dinitrophenyl)hydrazone, and *cis*-2-pentenal were prepared as described (*11*, *16*, *26-28*). 2-Hydroxytetrahydrofuran (THF-OH), the cyclic and predominant form of 4-hydroxybutanal, and 2-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran (THP-OH), the cyclic form of 5-hydroxypentanal, were prepared from 2,3-dihydrofuran and 3,4-dihydro-2*H*-pyran (Aldrich Chemical Co., Milwaukee, WI), according to literature procedures (*29-31*). Spectral properties of THF-OH (**7**, Figure 1) were as follows: ^{1}H-NMR ($CDCl_3$): δ 5.53 (m, 1H, OC*H*OH), 4.02 (m, 1H, H_α of OCH_2), 3.83 (m, 1H, H_β of OCH_2), 3.48 (bs, 1H, OH), 2.1–1.8 (m, 4H, CH_2CH_2); MS, *m/z* (rel intensity): 88 (M^+, 2), 87 (10), 71 (24), 57 (38), 42 (100). Spectral properties of THP-OH (**17**, Figure 2) were as follows: ^{1}H-NMR ($CDCl_3$): δ 4.82 (m, 1H, OC*H*OH), 4.41 (d, 1H, OH), 3.96 (m, 1H, H_α of OCH_2), 3.50 (m, 1H, H_β of OCH_2), 1.78 (m, 2H, OCHOHC*H* + OCH_2-CH_2C*H*), 1.47 (m, 4H, OCHOHC*H* + OCH_2C*H*_2C*H*); CI-MS, *m/z* (rel intensity): 103 (M + 1, 2), 101 (4), 85 (100).

2-Chloro-3,4,5,6-tetrahydro-2*H*-pyran (THP-Cl) was also prepared essentially as described (*32*): 3,4-Dihydro-2*H*-pyran (12.3 g, 0.15 mol) was stirred in a 50 mL round bottom flask at 0–5 °C, and dry HCl was slowly passed into it until 4.8 g was added. The reaction mixture was stirred for 1 h more and then distilled under vacuum to give 6.7 g (0.06 mol, 40%) of product, the structure of which was confirmed by ^{1}H-NMR (DMSO): δ 6.27 (s, 1H, OCHCl), 4.02 (m, 1H, OCH), 3.75 (m, 1H, OCH), 2.0 (m, 3H), 1.7 (m, 3H). The same procedure was used to prepare 2-chlorotetrahydrofuran (THF-Cl) (*31*) from 2,3-dihydrofuran (14 g, 0.2 mol), yielding 8.0 g, 0.075 mol, 38%. Its structure was confirmed by ^{1}H-NMR (DMSO): δ 6.37 (d, 1H, J = 4.1 Hz, OCHCl), 4.23 (m, 1H, OCH), 4.08 (m, 1H, OCH), 2.4 (m, 1H), 2.3 (m, 2H), 2.0 (m, 1H).

Hog liver esterase and dG were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from Aldrich Chemical Co.

Reactions. (A) Reaction of α-AcetoxyNPIP and dG. This was carried out as described in ref 26. Products were analyzed using HPLC system 1.

(B) Reactions of *cis*- and *trans*-2-Pentenal with dG. Either *cis*-2-pentenal (2 mmol) or *trans*-2-pentenal (1 mmol) was added to a solution of dG (30 mg, 0.1 mmol) in 5 mL of 0.1 M phosphate buffer (pH 7.0). The solutions were incubated in a shaking water bath at 37 °C for 24 h and then extracted twice with CH_2Cl_2. The aqueous portions were analyzed using HPLC system 1.

(C) Reactions of THP-Cl, THF-Cl, and 2,3-Dihydrofuran with dG. Either THP-Cl (2.8 g, 0.023 mol) or THF-Cl (3.8 g, 0.036 mol) was dissolved in 5 mL of 0.1 M phosphate buffer (pH 7.0) at 0–5 °C, and the pH was readjusted to 7.0. The resulting mixture was then added to dG (187 mg, 0.70 mmol) in the same buffer. The mixture was incubated in a shaking water bath at 37 °C for 24 h. Adducts were initially purified using HPLC system 2. Final purification of THP-dG (**26**, Figure 3B) was accomplished using HPLC system 1.

Spectral properties of THP-dG were as follows: UV (H_2O), λ_{max} 255, 272 (sh) at pH 7, 257, 272 (sh) at pH 1, 260 at pH 13; ^{1}H-NMR (DMSO-d_6): δ 10.45 (s, 1H, N1-H), 7.97 (two s, 1H, H-8 of two diastereomers), 7.05 (t, 1H, N^2-H), 6.15 (m, 1H, H-1′), 5.30 (d, 1H, 3′-OH), 5.10 (m, 1H, H-2″), 4.87 (m, 1H, 5′-OH), 4.38 (m, 1H, H-3′), 3.8 (m, 2H, H-6″ + H-4′), 3.6–3.4 (m, 1H, H-6″ + H-5′), 2.63 (1H, m, H-2′), 2.25 (1H, m, H-2′), 1.8–1.3 (m, 6H, H-3″,4″,5″); low resolution FAB-MS (*p*-nitrobenzyl alcohol matrix), *m/z* (rel intensity): 374 (M + Na, 67), 352 (M + H, 83), 236 (M − deoxyribose + 2H, 100), 152 (guanine + H, 80); high resolution FAB-MS: calcd for $C_{15}H_{22}N_5O_5$ 352.16088, found 352.16040.

THF-dG (**24**, Figure 3A) was purified with HPLC systems 5 and 6. THF-dG was also prepared by reaction of 2,3-dihydrofuran (294 mg, 4.2 mmol) with dG (266 mg, 1.0 mmol) in 100 mL of 0.1 M phosphate buffer (pH 7.0), at 37 °C for 20 h. After

extraction with 100 mL of $CHCl_3$, THF-dG was purified by HPLC system 7. The collected solutions were kept in dry ice until analysis. Spectral properties of THF-dG were as follows: UV (H_2O), λ_{max} 255, 272 (sh) at pH 7, 257, 272 (sh) at pH 1, 260 at pH 13; ^{1}H-NMR (DMSO-d_6): δ 7.92 (s, 1H, H-8), 6.16 (m, 1H, H-1′), 5.77 (bs, 1H, H-2″), 4.35 (m, 1H, H-3′), 3.8–3.7 (m, 2H, H-4′ + H-5″), 3.5–3.4 (m, 2H, H-5′ + H-5″), 2.58 (m, 1H, H-2′), 2.18 (m, 1H, H-2′), 2.1–1.7 (m, 4H, H-3″, 4″); high resolution FAB-MS (*p*-nitrobenzyl alcohol matrix): calcd for $C_{14}H_{20}N_5O_5$ 338.14556, found 338.14520.

(D) Neutral Thermal Hydrolysis of THP-dG and THF-dG. THP-dG (0.12 μmol) in 0.2 mL of 0.05 M phosphate buffer (pH 7.0) was heated for 1 h at 100 °C. The reaction mixture was extracted 3 times with $CHCl_3$. The extracts were combined, concentrated, and resuspended in 0.1 mL of H_2O. To this was added 10 μL of 0.13 M (2,4-dinitrophenyl)hydrazine reagent (2,4-DNP reagent) prepared as described (*33*). The sample was extracted twice with $CHCl_3$, and the extracts were concentrated and analyzed for 5-hydroxypentanal (2,4-dinitrophenyl)hydrazone by HPLC system 3. Another aliquot of the neutral thermal hydrolysate was analyzed for THP-dG and dG by HPLC system 1.

THF-dG was isolated by HPLC system 5 from an incubation mixture of α-acetoxyNPYR and dG or from reaction of THF-Cl with dG. It was diluted to 1 mL in 0.1 M phosphate buffer (pH 7.0), and heated at 100 °C for 1 h. To this was added 0.1 mL of 2,4-DNP reagent. The reaction mixture was extracted twice with 15 mL of CH_2Cl_2. The extracts were dried, dissolved in 1.5 mL of MeOH, and analyzed by HPLC system 8 or 9 with UV detection at 365 nm. Another aliquot was analyzed for THF-dG and dG by HPLC system 5.

(E) Conversion of THF-dG to N^2-(4-Hydroxybutyl)-guanine. THF-dG was dissolved in 2 mL of 0.1 M phosphate buffer (pH 7.0), and to this was added 50 μL of an aqueous solution of $NaBH_4$ (92 μg/μL). It was allowed to stand for 24 h and analyzed by HPLC system 5 with UV detection at 254 nm. The product eluted at 20.6 min in this system compared to 21.7 min for THF-dG. Its UV (H_2O) had λ_{max} 254, 278 (sh) at pH 7, 259, 276 (sh) at pH 1, and 259, 278 (sh) at pH 13. The product was collected and concentrated to dryness. The residue was dissolved in 1 mL of 0.1N HCl and heated at 37 °C overnight. The solution was neutralized and N^2-(4-hydroxybutyl)guanine was purified using HPLC systems 5 and 6. Spectral properties were as follows: UV(H_2O) λ_{max} 248, 280 (sh) at pH 7, 252, 280 (sh) at pH 1, 244 (sh), 278 at pH 13; ^{1}H-NMR (DMSO-d_6 + D_2O) δ 7.63 (s, 1H, H-8), 3.40 (t, J = 6.2 Hz, 2H, CH_2OH), 322 (t, J = 6.9 Hz, 2H, CH_2NH), 1.56–1.40 (m, 4H, CH_2CH_2); MS, electrospray ionization positive mode, *m/z* (relative intensity) 246 (M + Na^+, 9), 224 (M + H, 17), 191 [M − (H + CH_2OH), 100].

(F) Reactions of THP-OH and THF-OH with dG. A solution of THP-OH (3.80 mg, 37.3 μmol, 12.3 mM; or 12.9 mg, 126 μmol, 42.1 mM) and dG (8.0 mg, 29.9 μmol, 10.0 mM), in 3 mL of 0.1 M phosphate buffer (pH 7.0), was incubated at 37 °C with shaking. Aliquots were removed at various time intervals, immediately extracted with ice-cold $CHCl_3$/isoamyl alcohol (24/1) to stop the reaction, and stored at 0–5 °C until analysis for THP-dG by HPLC system 1.

THF-OH (14.0 mg, 158 μmol) in 0.5 mL of 0.1 M phosphate buffer (pH 7.0) was mixed with dG (10 mg, 37 μmol) in 3.75 mL of the same buffer and incubated with shaking for 20 h at 37 °C. After extraction with ice-cold $CHCl_3$, the reaction mixture was analyzed for THF-dG by HPLC system 5.

(G) Time-Dependent Formation of dG Adducts from α-AcetoxyNPYR and α-AcetoxyNPIP. A mixture of α-acetoxyNPYR (40.0 mg, 253 μmol, 42.1 mM) or α-acetoxyNPIP (43.6 mg, 253 μmol, 42.1 mM) and dG (15.8 mg, 59.2 μmol) was incubated at 37 °C in 6 mL of 0.1 M phosphate buffer (pH 7.0). Aliquots (0.8 mL) were withdrawn at 5, 10, 20, 40, 120, and 180 min. The aliquots were immediately extracted with 1 mL of ice-cold $CHCl_3$/isoamyl alcohol (24/1) to stop the reaction and were stored at −80 °C. The α-acetoxyNPYR reactions were analyzed as follows: Each aliquot was divided into three 0.1 mL portions. The first portion was analyzed directly for THF-dG using HPLC systems 4 and 5 with UV detection (254 nm).

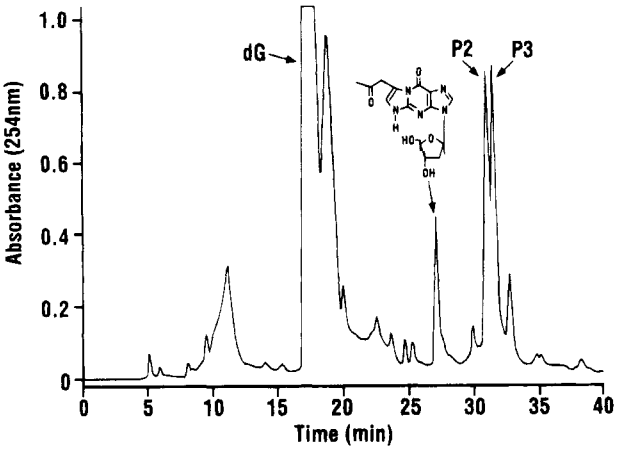


Figure 4. HPLC chromatogram (system 1) obtained upon analysis of an incubation mixture of dG and α-acetoxyNPIP.

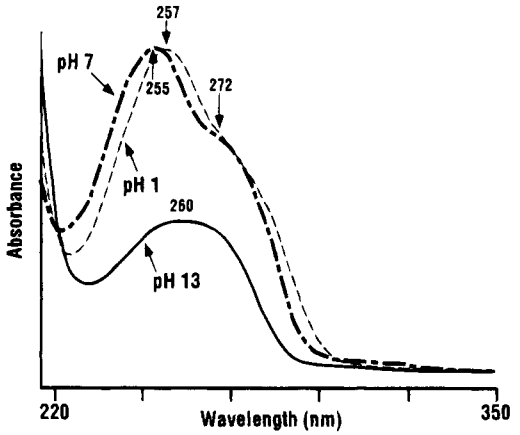


Figure 5. UV spectra of P2 and P3 of Figure 4.

The second was mixed with 0.3 mL of 0.1 M phosphate buffer (pH 7) and then heated at 100 °C for 1 h to release adducts **18**–**20**, **22**, and **23** (Figure 3A). The resulting mixture was analyzed with HPLC system 10 with fluorescence detection (excitation, 290 nm; emission, 380 nm). The third portion was mixed with 0.3 mL of 0.1 N HCl and then heated at 80 °C for 45 min to release adduct **21**, as the corresponding guanine base. This was also analyzed by HPLC system 10 with fluorescence detection. Retention times of the adducts were as follows (min): THF-dG, 76.6 (system 4); **19**, 18.0; **22**, 18.8; **23**, 19.9; **18**, 25.4; **21** (as guanine base), 29.1 (system 10). Adduct yields were quantified using calibration curves generated with standards, except in the case of THF-dG which was quantified using a curve generated from THP-dG because THF-dG was unstable (see Results). The α-acetoxyNPIP reactions were analyzed for THP-dG and adduct **25** (Figure 3B) using HPLC system 1 with UV detection at 254 and 280 nm, respectively. Retention times were as follows (min): THP-dG, 32.3 and 33.0; **25**, 27.5.

Results

Reaction of α-acetoxyNPIP with dG followed by HPLC analysis gave the chromatogram shown in Figure 4. In an earlier study, the peak eluting at 27 min had been identified as 7-(2-oxopropyl)-1,N^2-ethenodG (**25**, Figure 3B) (*26*). The peaks eluting between 30 and 35 min, designated P2 and P3, were the focus of the present study. P2 and P3 had identical UV spectra at pH 7. The effects of pH on the UV spectra of combined P2 and P3 are illustrated in Figure 5. These spectra are similar to those of cyclic 1,N^2-propanodG adducts or N^2-substituted dG adducts, which we have previously characterized (*34*). High resolution MS data demonstrated that the elemental composition of P2 and P3 was $C_{15}H_{22}N_5O_5$, corresponding to the addition of C_5H_9O to dG.

These data suggested that P2 and P3 were diastereomeric 1,N^2-propanodG adducts substituted with an ethyl group, homologous to **21**, Figure 3A. These would be formed by reaction of *cis*- or *trans*-2-pentenal with dG and have recently been characterized by Eder and Hoffman (*35*). This proposal seemed reasonable since, in previous studies of the reaction of α-acetoxyNPYR with dG and DNA, we have identified the 1,N^2-propanodG adducts **21** (*15*). The latter are formed by the reaction of dG with 2-butenal (**8**, Figure 1) which is a solvolysis product of α-acetoxyNPYR (*16*). Although we had not previously detected either *cis*- or *trans*-2-pentenal as solvolysis products of α-acetoxyNPIP (*26*), we investigated their reactions with dG. Reaction of *trans*-2-pentenal with dG gave a pair of adducts with UV spectra similar to those characterized by Eder and Hoffman; they presumably are diastereomeric 1,N^2-propanodG adducts (*35*). They eluted at 33.5 and 34.4 min, different from the retention times of P2 and P3. Reaction of *cis*-2-pentenal with dG also gave a pair of adducts with retention times of 33.5 and 34.4 min, different from those of P2 and P3. Therefore, we concluded that P2 and P3 did not result from reaction of *cis*- or *trans*-2-pentenal with dG. However, the reactions of both *cis*- and *trans*-2-pentenal with dG did produce 7-(2-oxopropyl)-1,N^2-ethenodG (**25**, Figure 3B), presumably via oxidation of the 2-pentenals to 4-oxo-2-pentenal (**12**, Figure 2).

We have previously proposed the intermediacy of oxonium ion **11**, Figure 2, in the solvolysis of α-acetoxyNPIP (*26*). This could react with dG, potentially forming P2 and P3. Therefore, we synthesized THP-Cl and allowed it to react with dG. The reaction mixture was analyzed by HPLC. The major adducts were two peaks having identical retention times and UV spectra to those of P2 and P3. The ^{1}H-NMR spectrum of this material is illustrated in Figure 6. All peaks were assigned based on D_2O exchange and decoupling experiments. Of particular interest was the coupling of H-2″ to N^2-H; irradiation at 5.1 ppm (H-2″) caused collapse of the triplet at 7.05 ppm (N^2-H) to a broad singlet. This experiment demonstrated the coupling of H-2″ with N^2-H and confirmed that substitution had occurred at the exocyclic amino group of dG. The ^{1}H-NMR spectrum is consistent only with the structure shown in Figure 6. This spectrum was essentially identical to that of P2 and P3, formed from the reaction of α-acetoxyNPIP with dG. These data established the structures of P2 and P3 as a pair of diastereomeric N^2-(3,4,5,6-tetrahydro-2*H*-pyran-2-yl)dG adducts (THP-dG, **26**, Figure 3B).

Since the corresponding five-membered oxonium ion **4**, Figure 1, could be formed from α-acetoxyNPYR, we investigated the reaction of THF-Cl with dG in order to prepare a standard. A major product with a UV spectrum similar to that of THP-dG was detected and isolated by HPLC; diastereomers were not separated under our conditions (Figure 7A). The high resolution MS showed an elemental composition of $C_{14}H_{20}N_5O_5$. In contrast to THP-dG, this product was somewhat unstable. Overnight storage at −20 °C resulted in partial decomposition to dG (Figure 7B). The ^{1}H-NMR spectrum is compared to that of THP-dG in Table 1. As in the case of THP-dG, the assignment of H-2″ was based on decoupling experiments which showed that it was coupled with N^2-H. The spectrum was similar to that of THP-dG except that H-2″ was approximately 0.67 ppm further downfield. These data are consistent with the structure shown in Table 1 and Figure 3A, N^2-(tetrahydrofuran-2-yl)dG (THF-dG, **24**).

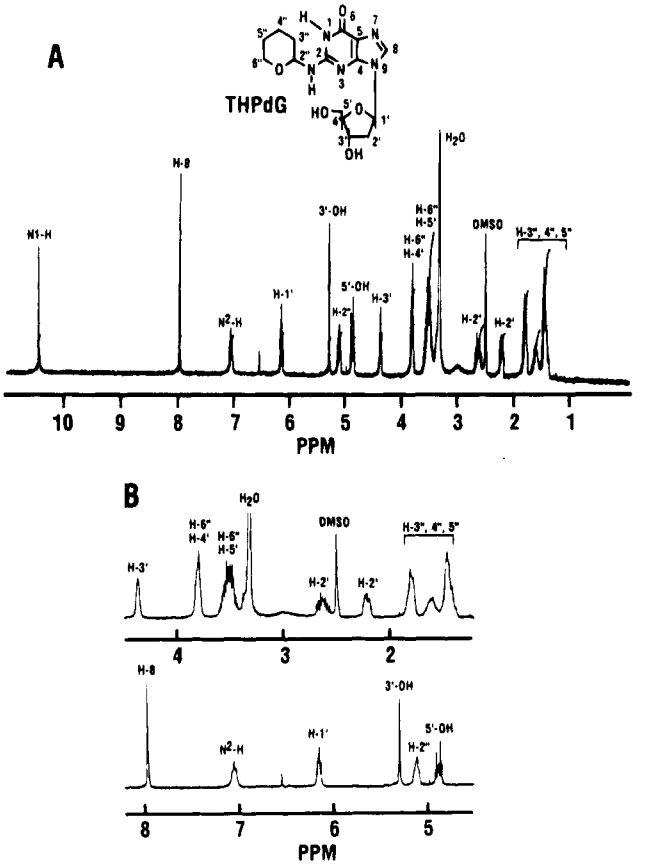


Figure 6. (A) ^{1}H-NMR spectrum (DMSO-d_6) of THP-dG obtained by reacting THP-Cl with dG. (B) Details of spectrum, 1−8 ppm.

We sought methods to produce a stable derivative of THF-dG. Treatment of THF-dG with $NaBH_4$ resulted in near-quantitative conversion to a single product, which, in contrast to THF-dG, was stable. This product had a UV spectrum consistent with an N^2-substituted dG (Figure 7C). Further spectral data were obtained on the corresponding guanine base, prepared by mild acid hydrolysis (Figure 7D). The UV spectrum of this material was similar to that of N^2-(3-hydroxypropyl)guanine (*34*, *36*). The ^{1}H-NMR spectrum of the base is summarized in Table 2. This spectrum was also similar to that of N^2-(3-hydroxypropyl)guanine (*36*). The chemical shifts and splitting patterns of the protons at positions 2′, 3′, and 4′ were similar to those of 4-amino-1-butanol (*37*). The MS was determined using electrospray ionization in the positive ion mode. An M + 1 peak was observed at *m/z* 224, and an M + Na^+ peak was observed at *m/z* 246. The base peak was *m/z* 191 [M − (H + CH_2-OH)]. These data established the structure of the product obtained by $NaBH_4$ reduction followed by hydrolysis as N^2-(4-hydroxybutyl)guanine (**29**, Figure 8). This was formed by hydrolysis of **28**, which in turn was produced by $NaBH_4$ reduction of the open-chain tautomer **27** of THF-dG.

HPLC analysis of reaction mixtures of α-acetoxyNPYR and dG demonstrated the presence of a peak with identical UV spectra and retention time to that of THF-dG (Figure 9). Its presence was further confirmed by $NaBH_4$ reduction to N^2-(4-hydroxybutyl)dG as well as neutral thermal hydrolysis, which caused the peak to disappear with appearance of THF-OH. The latter was identified as its (2,4-dinitrophenyl)hydrazone derivative.

The properties of THP-dG and THF-dG were compared. THP-dG was relatively stable at 37 °C and pH 7. After

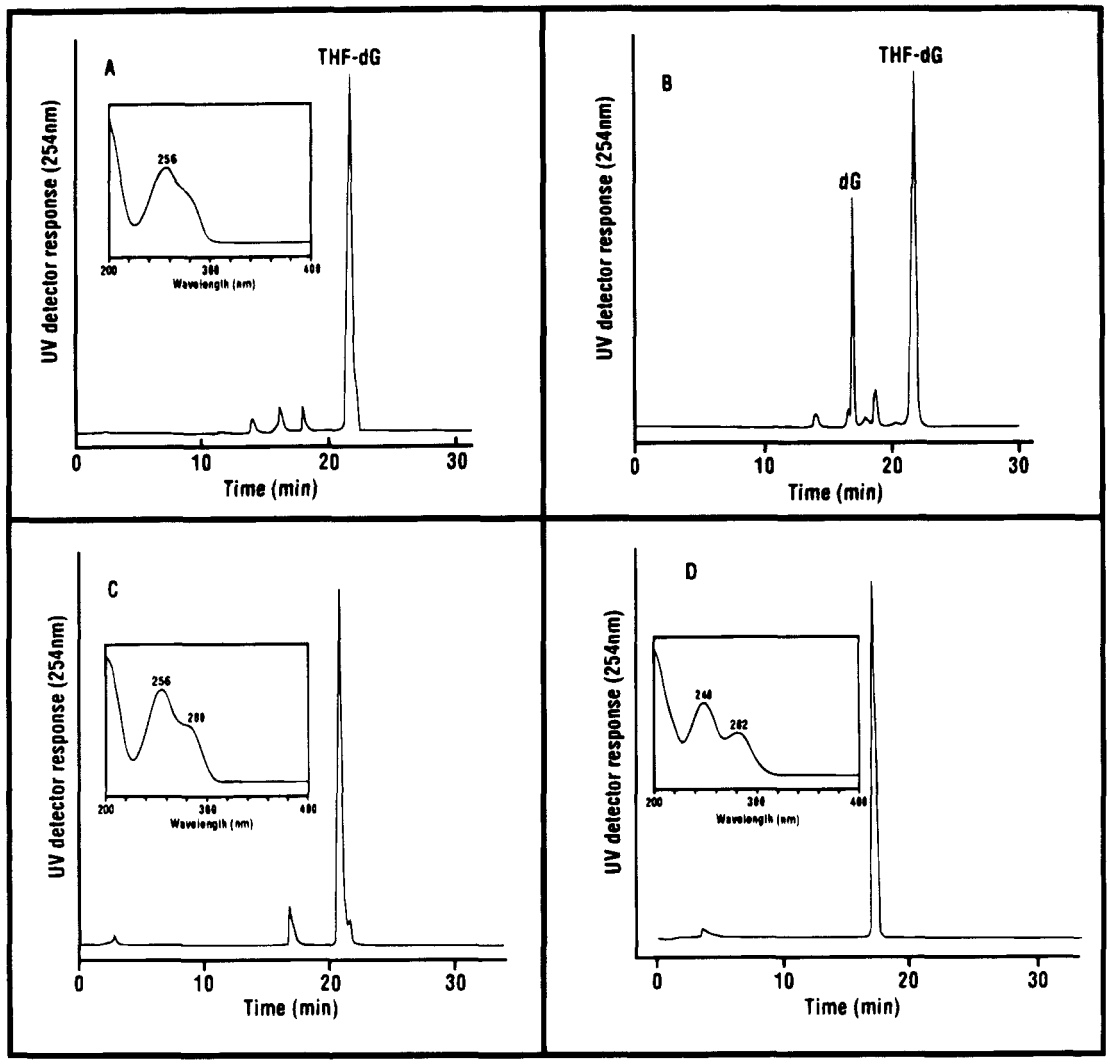
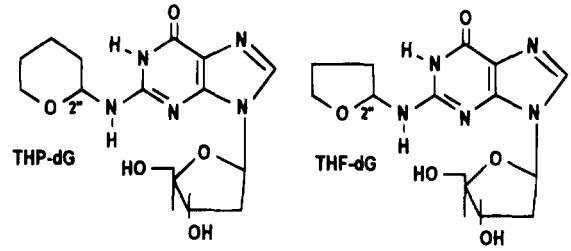


Figure 7. HPLC chromatograms (system 5) and UV spectra (insets) of (A) THF-dG synthesized from THF-Cl; (B) a mixture of THF-dG and dG produced by storing THF-dG in pH 7.0 phosphate buffer at −20 °C overnight; (C) N^2-(4-hydroxybutyl)dG produced by $NaBH_4$ treatment of THF-dG; and (D) N^2-(4-hydroxybutyl)guanine produced by HCl hydrolysis of N^2-(4-hydroxybutyl)dG.

Table 1. ^{1}H-NMR Spectral Data for THP-dG and THF-dG[a]



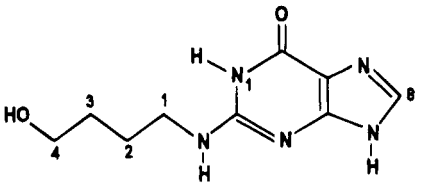
	chemical shifts (ppm)									
	N1-H	H-8	N^2-H	H-2″	H-1′	3′-OH	5′-OH	H-3′	H-4′	H-5′
THP-dG	10.45	7.97	7.05	5.10	6.15	5.30	4.87	4.38	3.8	3.4−3.6
THF-dG	10.60	7.92	7.01	5.77	6.16	5.28	4.96	4.35	3.7−3.8	3.4−3.5

	chemical shifts (ppm)									
	H-6″ or H-5″			H-6″ or H-5″		H-2′		H-3″-H-5″ or H-3″-H-4″		
THP-dG	3.8			3.4−3.6		2.63, 2.25		1.3−1.8		
THF-dG		3.7−3.8			3.4−3.5	2.58, 2.18			1.7−2.1	

[a] In DMSO-d_6.

70 min, 26% had decomposed, with conversion to dG. In contrast, 70% of THF-dG was converted to dG under these conditions. Complete disappearance of THP-dG and THF-dG was noted under neutral thermal hydrolysis conditions, with formation of dG, THP-OH, and THF-OH. The latter were identified as their (2,4-dinitrophenyl)-hydrazones. Reaction of THF-dG with $NaBH_4$ resulted in immediate formation of N^2-(4-hydroxybutyl)dG, while treatment of THP-dG under the same conditions slowly led to a new product (32% conversion after 7.5 h) with a

Table 2. ^{1}H-NMR Spectral Data for N^2-(4-Hydroxybutyl)guanine[a]



position	chemical shifts, ppm (multiplicity)
8	7.63 (s)
N^2-H	6.51 (bs)
4-CH_2	3.40 (t, J = 6.2 Hz)
1-CH_2	3.22 (t, J = 6.9 Hz)
2,3-CH_2	1.40−1.56 (m)

[a] In DMSO-d_6 + D_2O, except for N^2-H which was observed in DMSO only.

characteristic N^2-substituted dG UV spectrum. This is presumed to be N^2-(5-hydroxypentyl)dG.

Based on previous studies (*38*), it seemed likely that THP-dG and THF-dG could be formed by the reaction of THP-OH or THF-OH with dG, followed by ring closure as illustrated for THF-dG in Figure 8. This was tested by reacting THP-OH or THF-OH with dG under the same conditions used for the reactions of the α-acetoxynitrosamines with dG. HPLC analysis of the reaction mixtures demonstrated that both THP-dG and THF-dG were formed in the respective reactions of THP-OH and THF-OH with dG. This is illustrated for THF-dG in Figure 10. The reaction of THF-OH with dG to give THP-dG was complete within 7 h. The yield of THP-dG was 11.7 mmol/mol of dG compared to 12 mmol/mol of dG from α-acetoxyNPIP, when equimolar amounts of THP-OH or α-acetoxyNPIP (42 mM) were reacted with dG. Since the yield of THP-OH in the solvolysis of α-acetoxyNPIP is 29% (*26*), we also investigated the formation of THP-dG starting with 12.3 mM THP-OH; the yield was 3 mmol/mol of dG, about 25% of that formed from 42 mM α-acetoxyNPIP. These results indicate that THP-OH is not the only precursor to THP-dG produced in the reaction of α-acetoxyNPIP with dG. Yields of THF-dG from reactions of equimolar amounts of α-acetoxyNPYR or THF-OH with dG were similar (8.0 vs 10.0 mmol/mol of dG). 2,3-Dihydrofuran also reacted with dG to produce THF-dG.

Previously, we identified several other products in the reaction of α-acetoxyNPYR with dG: three cyclic 7,8-substituted adducts (**18**, **22**, and **23** (Figure 3A)), the 7-substituted aldehyde **19** and acid **20**, and the diastereomeric 1,N^2-propanodG adducts **21** (*15*, *17*, *19*). The formation of these adducts and THF-dG from α-acetoxyNPYR was compared with the formation of THP-dG and 7-(2-oxopropyl)-1,N^2-ethenodG (**25**, Figure 3B) in the reaction of α-acetoxyNPIP with dG. In the first 3 h of reaction, which was sufficient for hydrolysis of most of the α-acetoxynitrosamine in each case (*19*, *26*), the major products from α-acetoxyNPYR were the 7-substituted aldehyde **19** and THF-dG while the major product from α-acetoxyNPIP was THP-dG (Figure 11). At later time points, up to 24 h, the amounts of all the products except for 7-(2-oxopropyl)-1,N^2-ethenodG (**25**) remained essentially constant. However, the amount of this latter adduct increased linearly between 3 and 24 h such that

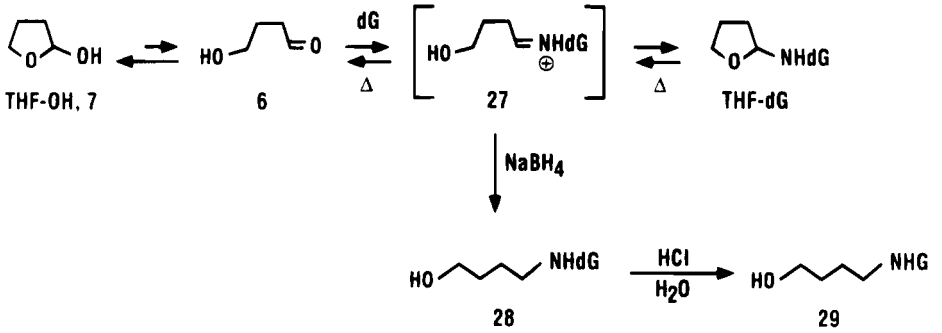


Figure 8. Reversible formation of THF-dG from THF-OH and dG.

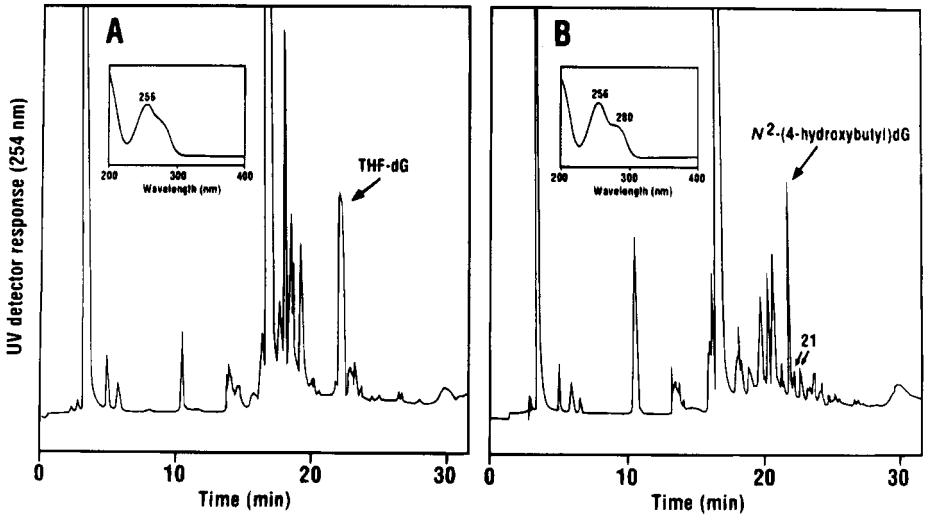


Figure 9. HPLC analysis (system 5) of (A) products from reaction of α-acetoxyNPYR with dG, showing formation of THF-dG and its UV spectrum in the inset, and (B) products formed upon treatment of A with $NaBH_4$. Inset is UV spectrum of the peak marked N^2-(4-hydroxybutyl)dG formed by reduction of THF-dG. Detection of diastereomeric adducts **21** (Figure 3A) is also indicated.

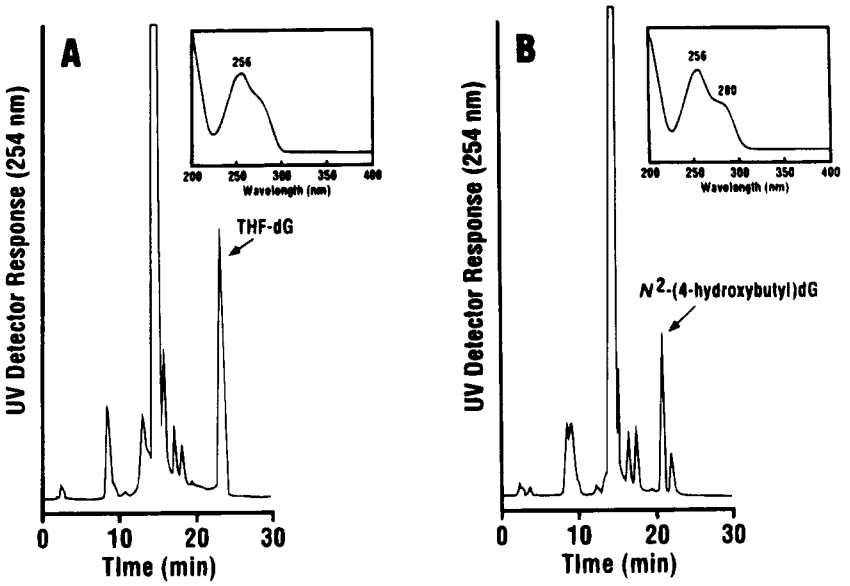


Figure 10. HPLC analysis (system 5) of (A) products from reaction of THF-OH with dG, showing formation of THF-dG and its UV spectrum in the inset, and (B) products formed upon treatment of A with $NaBH_4$. The UV spectrum of N^2-(4-hydroxybutyl)dG, formed by reduction of THF-dG, is shown in the inset.

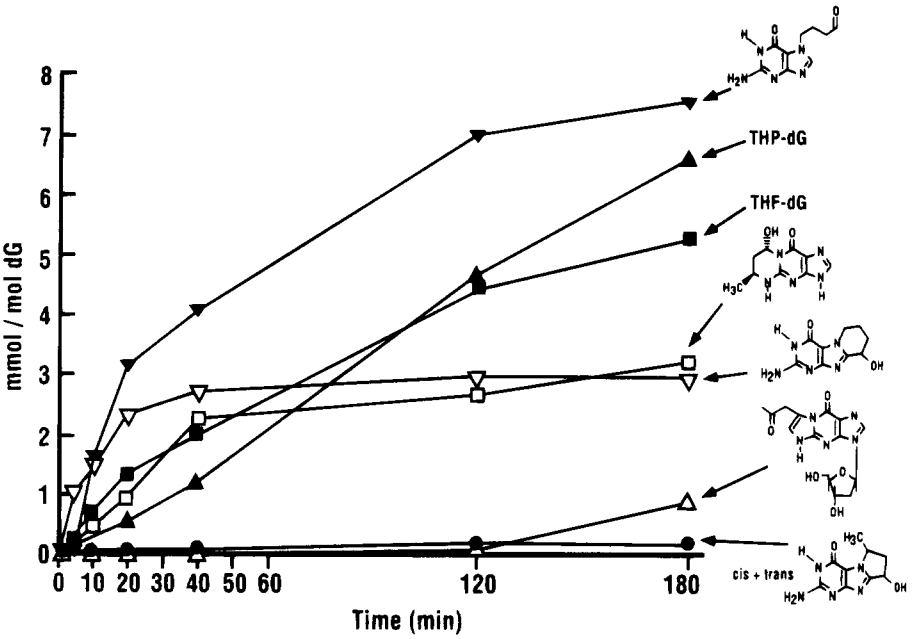


Figure 11. Time-dependent formation of dG adducts of α-acetoxyNPYR (▼, ■, □, ▽, ●, ○) and α-acetoxyNPIP (▲, △). Symbols ● and ○ are superimposed.

its final concentration was more than 3 times as great as that of THP-dG.

Discussion

An important result of this study was the identification of new types of adducts—THF-dG and THP-dG—as products of the reactions of α-acetoxyNPYR and α-acetoxyNPIP with dG. Both are previously unrecognized major adducts formed in these reactions, as illustrated in Figure 11. Their formation in similar yields from the two cyclic nitrosamines indicates some similarities in the chemistry of their reactions with dG, but there are also potentially important differences, as discussed further below.

There are several possible mechanisms that could produce these adducts, as illustrated for THF-dG in Figure 12. First, as we have shown, THF-OH (**7**) and THP-OH (**17**) react with dG to form THF-dG and THP-dG, respectively (path a). Both THF-OH and THP-OH are major products of the solvolysis of the α-acetoxynitrosamines (*11*, *26*). Therefore, path a can account for part of the adduct formation. Our comparative studies of the reactions of THP-OH and α-acetoxyNPIP with dG indicate that approximately 25% of the THP-dG produced from α-acetoxyNPIP arises by path a. The second mechanism is by direct reaction of the cyclic oxonium ion **4** with dG (path b). Previously, we have obtained strong evidence that a cyclic oxonium ion is a product of the α-hydroxylation of the tobacco-specific nitrosamines NNN and NNK (*39*). The formation of cyclic five- and six-membered oxonium ions with structures related to those discussed here has been demonstrated in reactions of unsaturated ketones such as 1-hexen-5-one and 2-hepten-6-one with strong acids (*40*). The third mechanism, path c, would involve reaction of the cyclic oxonium ion with another constituent of the solvolysis mixture to produce

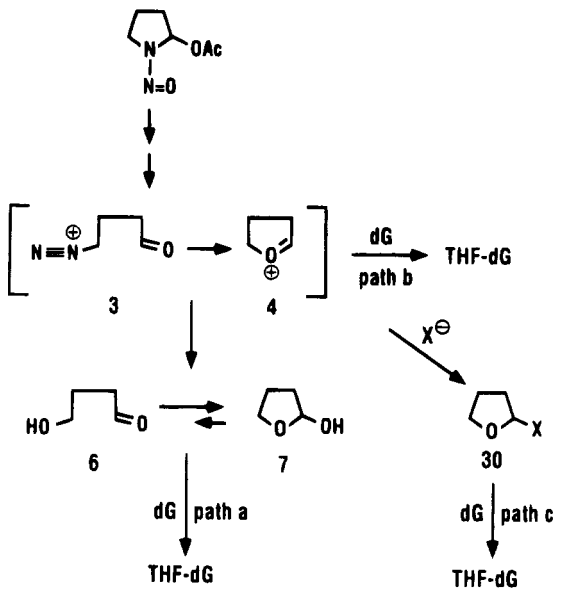


Figure 12. Three paths for formation of THF-dG from α-acetoxyNPYR.

a cyclic oxonium ion-derived electrophile, **30**. For example, tetrahydrofuran-2-yl phosphate could form by reaction of **4** with buffer. This could in turn react with dG, giving THF-dG. Evidence for this type of mechanism was obtained in our studies of the reactions of these cyclic nitrosamines with DNA, as described in the companion paper (*43*). Further studies would be necessary to characterize such cyclic oxonium ion-derived electrophiles.

The open-chain 7-substituted aldehyde adduct **19**, Figure 3A, is a major adduct produced in the reaction of α-acetoxyNPYR with dG, as illustrated in Figure 11. This adduct forms by reaction with dG of open-chain intermediates such as **3**. The cyclic 7,8-substituted adduct **18** is also formed in considerable amounts by concerted reaction of intermediates such as **2** or **3** with the 7- and 8-positions of dG, as indicated in our earlier studies (*17*, *19*). Thus, reactions with dG of open-chain intermediates derived from α-acetoxyNPYR are quantitatively important. In contrast, we have not yet detected the corresponding products which would be produced in the reactions of α-acetoxyNPIP with dG, via intermediates such as **9** or **10** of Figure 2. Neutral thermal hydrolysis of reaction mixtures of α-acetoxyNPIP and dG does lead to small amounts of a fluorescent adduct, insufficient for characterization in our studies to date (data not shown). We are presently carrying out further studies on the possible formation of this kind of adduct from α-acetoxyNPIP.

Enals such as 2-butenal, **5**, produced in the solvolysis of α-acetoxyNPYR or in the metabolic α-hydroxylation of NPYR, also react with dG to give adducts **21**–**23** of Figure 3A (*15*, *17*). Adduct **21** in particular is formed in significant amounts as illustrated in Figure 11. In contrast, we have not observed the corresponding product, 2-pentenal (**13**), in the solvolysis of α-acetoxyNPIP nor have we detected the 1,N^2-propanodG adducts that would be expected products of the reaction of 2-pentenal with dG (*26*). Instead, both *cis*- and *trans*-2-pentenal apparently undergo oxidation yielding 4-oxo-2-pentenal (**12**), which reacts with dG producing 7-(2-oxopropyl)-1,N^2-ethenodG (**25**) (*26*). This may explain why the ethyl-substituted 1,N^2-propanodG adducts are not detected. With time, the 1,N^2-ethenodG adduct **25** becomes the major one formed in the reaction of α-acetoxyNPIP with dG. In contrast, ethenodG adducts of α-acetoxyNPYR have not been observed as major products in the reaction with dG.

Table 3. Adduct Formation from α-AcetoxyNPYR or α-AcetoxyNPIP and dG[a]

	adduct type (mmol/mol of dG)		
	18-20[b] from open-chain electrophiles	THF-dG or THP-dG	**21–23** or **25**[b] from enals
α-acetoxyNPYR	10.6	5.3	3.5[c]
α-acetyoxyNPIP	ND[d]	6.6	0.9[e]

[a] The α-acetoxynitrosamines and dG were incubated at 37 °C for 3 h. [b] Numbers refer to structures in Figure 3. [c] From reaction of 2-butenal with dG. [d] ND = not detected. [e] From reaction of 4-oxo-2-pentenal with dG.

Thus, there are substantial differences in the reactions of α-acetoxyNPYR and α-acetoxyNPIP with dG, at least according to presently available data. The extents of adduct formation after 3 h are summarized in Table 3. Both compounds form similar amounts of THF-dG or THP-dG. However, α-acetoxyNPYR apparently forms larger amounts of open-chain electrophile-derived adducts than does α-acetoxyNPIP while the structures and amounts of the enal-derived adducts formed by the two nitrosamines are markedly different.

THP-dG and THF-dG can be considered as cyclic tautomers of hydroxyalkyl imines such as **27** (Figure 8). Previous studies have demonstrated that the six-membered cyclic tautomers of several hydroxyalkyl imines are more stable than the corresponding five-membered cyclic tautomers, with respect to the ring opened forms (*41*, *42*). Our data are consistent with these results. The six-membered cyclic tautomer THP-dG is more stable at 37 °C (pH 7), than is the five-membered analogue THF-dG. The slower reduction of THP-dG than THF-dG with $NaBH_4$ is also consistent with the greater stability of the six-membered than the five-membered cyclic tautomer. In THF-dG, the hydrogens on carbons 4″ and 5″ of the tetrahydrofuran ring are eclipsed, whereas the corresponding hydrogens on carbons 5″ and 6″ of THP-dG are staggered. The unfavorable steric interactions in THF-dG are likely responsible for its lower stability compared to THP-dG. The open-chain tautomers such as **27** can hydrolyze to dG and the corresponding hydroxyalkanals. This is the reverse of their formation from THF-OH and THP-OH.

In conclusion, this study has demonstrated the formation of substantial amounts of new adducts, THF-dG and THP-dG, in the reaction of α-acetoxyNPYR and α-acetoxyNPIP with dG and has shown some major differences in the products of these reactions. These differences might be related to the differing organospecificities of NPYR and NPIP.

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