Identification of Paraldol-Deoxyguanosine Adducts in DNA Reacted with Crotonaldehyde

Mingyao Wang, Edward J. McIntee, Guang Cheng, Yongli Shi, Peter W. Villalta, and Stephen S. Hecht*

University of Minnesota Cancer Center, Minneapolis, Minnesota 55455

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Crotonaldehyde (**1**) is a mutagen and carcinogen, but its reactions with DNA have been only partially characterized. In a previous study, we found that substantial amounts of 2-(2-hydroxypropyl)-4-hydroxy-6-methyl-1,3-dioxane (paraldol, **7**), the dimer of 3-hydroxybutanal (**8**), were released upon enzymatic or neutral thermal hydrolysis of DNA that had been allowed to react with crotonaldehyde. We have now characterized two paraldol-deoxyguanosine adducts in this DNA: N^2-[2-(2-hydroxypropyl)-6-methyl-1,3-dioxan-4-yl]deoxyguanosine (N^2-paraldol-dG, **13**) and N^2-[2-(2-hydroxypropyl)-6-methyl-1,3-dioxan-4-yl]deoxyguanylyl-(5′−3′)-thymidine [N^2-paraldol-dG-(5′−3′)-thymidine, **14**]. Four diastereomers of N^2-paraldol-dG (**13**) were observed. Their overall structures were determined by ^{1}H NMR, by MS, and by reaction of paraldol with deoxyguanosine and DNA. ^{1}H NMR data showed that two diastereomers had all equatorial substituents in the dioxane ring, while two others had an axial 6-methyl group. Preparation of paraldol with the (*R*)- or (*S*)-configuration at the 6-position of the dioxane ring and the carbinol carbon of the 2-(2-hydroxypropyl) group allowed partial assignment of the absolute configurations of N^2-paraldol-dG (**13**). Four diastereomers of N^2-paraldol-dG-(5′−3′)-thymidine (**14**) were observed. Their overall structure was determined by ^{1}H NMR, MS, and hydrolysis with snake venom or spleen phosphodiesterase. Reactions of nucleosides and nucleotides with paraldol demonstrated that adducts were formed only from deoxyguanosine and its monophosphates. Experiments with DNA that had been reacted with crotonaldehyde indicated that N^2-paraldol-dG-containing adducts in DNA are relatively resistant to enzymatic hydrolysis. The results of this study demonstrate that the reaction of crotonaldehyde with DNA is more complex than previously recognized and that stable N^2-paraldol-dG adducts are among those that should be considered in assessing mechanisms of crotonaldehyde mutagenicity and carcinogenicity.

Introduction

Crotonaldehyde (**1**), or 2-butenal, is mutagenic in *Salmonella typhimurium* and other systems used for detection of genetic damage (*1*). It induces altered liver cell foci, neoplastic nodules, and hepatocellular carcinoma upon oral administration to F344 rats (*1*, *2*). Crotonaldehyde is commonly detected in mobile source emissions, cigarette smoke, and other products of thermal degradation (*1*). It is also a product of lipid peroxidation and a metabolite of the hepatocarcinogen *N*-nitrosopyrrolidine (*3*, *4*).

Consistent with its mutagenic and tumorigenic properties, crotonaldehyde forms adducts with DNA and dG. The exocyclic 1,N^2-propano-dG adducts **2** and **3** (Scheme 1) are the major products characterized to date in reactions of crotonaldehyde with DNA (*5*−*7*). Adducts **4**−**6** have been detected in reactions with dG, but at lower levels than **2** and **3** (*7*−*9*). Reaction with DNA of α-acetoxy-*N*-nitrosopyrrolidine, a precursor to crotonaldehyde, also produces adducts **2**−**5**, with **2** and **3** predominating (*10*). Using ^{32}P-postlabeling, Chung and Nath demonstrated that adducts **2** and **3** are present in DNA of various tissues from untreated laboratory rodents as well as humans, and proposed that these adducts arise from endogenous lipid peroxidation (*3*, *6*, *11*−*13*). They have also detected substantial levels of adducts **2** and **3** in oral tissue DNA, with increased amounts in cigarette smokers (*14*). Thus, crotonaldehyde-derived DNA adducts may have both endogenous and exogenous sources.

Recently, we demonstrated that enzymatic or neutral thermal hydrolysis of DNA that had been reacted with crotonaldehyde or α-acetoxy-*N*-nitrosopyrrolidine produced 2-(2-hydroxypropyl)-4-hydroxy-6-methyl-1,3-dioxane (paraldol,[1] **7**), the dimer of 3-hydroxybutanal (**8**) (*15*) (Scheme 1). Levels of the adducts releasing **7** were higher than those of adducts **2**−**6**, suggesting that they could play a role in the DNA damaging properties of crotonaldehyde. In the study presented here, we investigated the paraldol-releasing adducts in crotonaldehyde-treated DNA and determined the structures of paraldol-dG adducts.

* To whom correspondence should be addressed: University of Minnesota Cancer Center, Box 806 Mayo, 420 Delaware St. SE, Minneapolis, MN 55455. Telephone: (612) 624-7604. Fax: (612) 626-5135. E-mail: hecht002@tc.umn.edu.

[1] Abbreviations: DIBAL, diisobutylaluminum hydride; N^2-paraldol-dG, N^2-[2-(2-hydroxypropyl)-6-methyl-1,3-dioxan-4-yl]deoxyguanosine; N^2-paraldol-dG-(5′−3′)-thymidine, N^2-[2-(2-hydroxypropyl)-6-methyl-1,3-dioxan-4-yl]deoxyguanylyl-(5′−3′)-thymidine; paraldol, 2-(2-hydroxypropyl)-4-hydroxy-6-methyl-1,3-dioxane.

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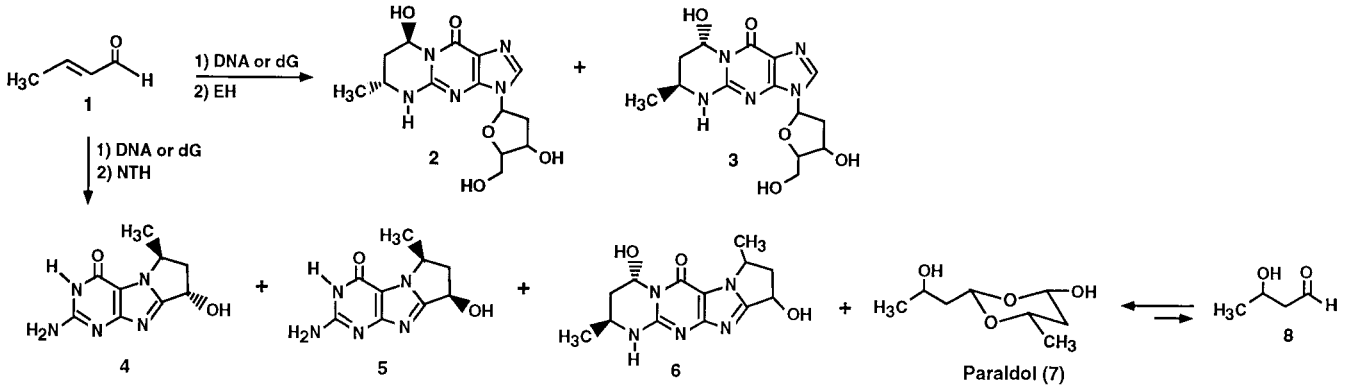
Scheme 1. Products Formed in the Reaction of Crotonaldehyde (1) with DNA or dG[a]



[a] EH, enzymatic hydrolysis; NTH, neutral thermal hydrolysis. Other isomers of **2**, **3**, and **6** have been observed (*5*−*10*, *15*).

Experimental Section

Apparatus. HPLC analyses were carried out with Waters Associates (Waters Division, Millipore, Milford, MA) systems equipped with a model 991 or 996 photodiode array detector, or a RF-10AXL spectrofluorometric detector (Shimadzu Scientific Instruments, Columbia, MD). The HPLC systems also included a HP 1100 series control module with an autosampler (Hewlett-Packard, Wilmington, DE) and a Foxy Jr. fraction collector (Isco Inc., Lincoln, NE). Flow rates were 1 mL/min. The following solvent elution systems were used: (1) two 4.6 mm × 25 cm Supelcosil LC 18-BD columns (Supelco, Bellefonte, PA) with isocratic elution by 5% CH_3CN in 10 mM sodium phosphate buffer (pH 7) for 10 min and then a gradient from 5 to 25% CH_3CN over the course of 60 min and detection by UV (254 nm); (2) the same columns as in system 1, with elution by a gradient of 0 to 30% CH_3CN in 10 mM sodium phosphate buffer (pH 7) over the course of 40 min and detection by UV (254 nm); (3) a 4.6 mm × 25 cm, 5 μm OD5 octadecyl column (Burdick & Jackson, Baxter, McGaw Park, IL) with elution by a gradient from 20 to 80% CH_3CN in H_2O over the course of 40 min with detection by UV (254 nm); (4) the same as system 3 except a gradient from 40 to 60% CH_3CN in H_2O over the course of 40 min with UV detection (365 nm); and (5) two 4.6 mm × 25 cm Partisil-10 SCX strong cation exchange columns (Whatman, Clifton, NJ) with elution by 80 mM ammonium phosphate buffer (pH 2) with detection by fluorescence (excitation at 290 nm and emission at 380 nm).

GC with flame ionization detection was carried out on a HP 6890 series gas chromatograph (Hewlett-Packard) with a 30 m × 0.32 mm i.d., 3.0 μm film thickness DB-1 column (J&W Scientific, Folsom, CA). One microliter of sample was injected in the split mode (1:100). The injector temperature was 200 °C, and He was the carrier gas (32 cm/s at 40 °C). The initial temperature of the oven was 40 °C for 4 min, and then it was programmed at a rate of 10 °C/min to 210 °C. The flame ionization detector was set at 250 °C (H_2, 40 mL/min; air, 400 mL/min; and He, 25 mL/min). The retention times of acetaldehyde, crotonaldehyde, and paraldol were 2.52, 9.10, and 11.97 min, respectively.

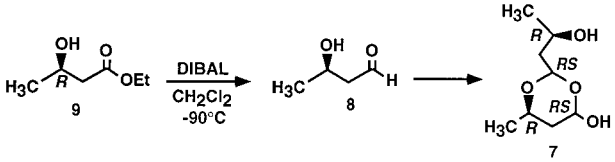
LC−MS and LC−MS/MS analyses were carried out on a SCIEX API-III instrument (Perkin-Elmer Biosystems, Foster, CA) in the positive ion electrospray ionization mode. The mass spectrometer was interfaced with a Waters Alliance HPLC system equipped with an SPD-10A UV−vis detector (Shimadzu). The HPLC system was operated as in system 3 except elution was with 0 to 50% CH_3CN in H_2O over the course of 50 min at a rate of 1 mL/min, with UV detection (254 nm). Some LC−MS analyses (on compound **14** and N^2-paraldol-dG-5′-MP) were performed on a Finnigan MAT LCQ Deca instrument (Thermoquest LC/MS Division, San Jose, CA) interfaced with a Waters 600 HPLC multisolvent delivery system. The API source of the mass spectrometer was set as follows: voltage, 4.5 kV; current, 0.14 μA; and capillary temperature, 350 °C. The analyses were performed in the negative ion electrospray ionization mode using the same column as in HPLC system 1 with elution by a gradient from 0 to 50% 2-propanol in H_2O over the course of 15 min at a flow rate of 1 mL/min with UV detection (254 nm).

NMR data were obtained on a 300 or 800 MHz instrument (Varian, Inc., Palo Alto, CA) using standard 5 mm or Shigemi 3 mm tubes (Shigemi, Inc., Allison Park, PA). Optical rotations were measured on an Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ) at the 589 nm Na D line at 22 °C.

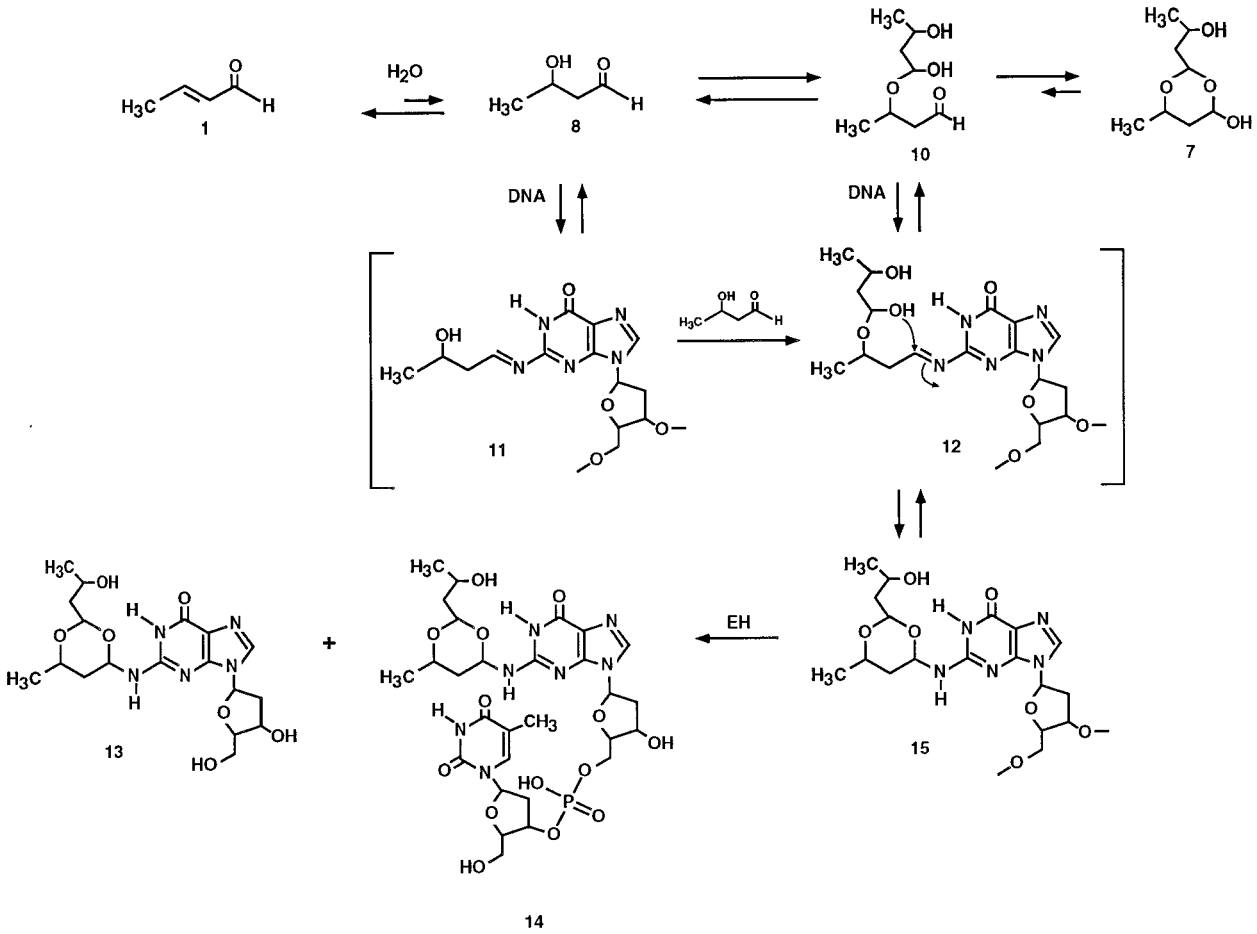
Chemicals and Enzymes. Crotonaldehyde, ethyl (*S*)-(+)- and ethyl (*R*)-(−)-3-hydroxybutyrate, and diisobutylaluminum hydride (DIBAL) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium potassium tartrate was purchased from Fisher Scientific (Springfield, NJ). Calf thymus DNA was obtained from Sigma Chemical Co. (St. Louis, MO). Adducts **2** and **3** and paraldol [mp 93−95 °C, lit. (*16*) 95−98 °C] were prepared as described previously (*5*, *15*, *16*). Alkaline phosphatase was purchased from Boehringer Mannheim Co. (Indianapolis, IN). All other chemicals and enzymes were obtained from Sigma.

2-[2-(*S*)-Hydroxypropyl]-4-hydroxy-6(*S*)-methyl-1,3-dioxane. To a stirred solution of ethyl (*S*)-(+)-3-hydroxybutyrate (1.2 g, 9 mmol) in 25 mL of CH_2Cl_2 at −76 °C was added 7.2 mL of DIBAL (1.5 M in toluene) by syringe over the course of 1 h. After 2 h, the reaction was quenched with 4 mL of methanol and the mixture allowed to warm to room temperature. To the mixture was added 80 mL of a saturated solution of sodium potassium tartrate. The mixture was then stirred overnight. The organic layer was separated, and the aqueous phase was extracted five times with 100 mL portions of ethyl acetate. The combined organic layers were dried (Na_2SO_4) and concentrated to dryness. The resulting oil was subjected to column chromatography on silica gel, with elution by 50% hexane in ethyl acetate and then ethyl acetate. Aliquots of the fractions were treated with 2,4-dinitrophenylhydrazine reagent to identify those containing the product (*15*). The combined fractions (0.26 g, 16% yield) were analyzed by HPLC system 4, and the retention time of the product was identical to that of racemic paraldol (**7**). Its ^{1}H NMR spectrum was also essentially identical to that of **7**. Its optical rotation $[\alpha]_D^{20}$ was −23°. Similarly, 2-[2-(*R*)-hydroxypropyl]-4-hydroxy-6(*R*)-methyl-1,3-dioxane (**7**, Scheme 2), 0.26 g, was prepared from ethyl (*R*)-(−)-3-hydroxybutyrate (**9**, Scheme 2) ($[\alpha]_D^{20}$ = 0.84°).

Scheme 2. Preparation of (*R*)-3-Hydroxybutanal (8) and Its Dimerization to Paraldol (7)



Scheme 3. Proposed Routes of Formation of Adducts 12 and 13 in the Reaction of Crotonaldehyde with DNA[a]



[a] EH, enzymatic hydrolysis.

***N*²-[2-(2-Hydroxypropyl)-6-methyl-1,3-dioxan-4-yl]deoxyguanosine (*N*²-Paraldol-dG, 13, Scheme 3; Peaks 8A,B and 9A,B in Figures 1 and 3).** Peaks 8 and 9 (Figure 1) were collected using HPLC system 1, and further purified with HPLC system 3. Peaks 8A,B (mixture of at least two isomers, 100 μg): ^{1}H NMR (DMSO-d_6) δ 10.6 (bs, 2H, dG-N1-H), 7.94 (s, 1H, dG-C8-H), 7.93 (s, 1H, dG-C8-H), 6.49 (s, 2H, dG-N^2-H), 6.13 (dd, J = 3 and 7 Hz, 2H, 1′-H), 5.37 (m, 2H, dioxane 4-H), 5.26 (d, J = 4 Hz, 1H, 3′-OH), 5.24 (d, J = 4 Hz, 1H, 3′-OH), 4.85 (m, 2H, 5′-OH), 4.79 (m, 2H, dioxane 2-H), 4.44 (d, J = 5 Hz, 1H, 2-hydroxypropyl-OH), 4.38 (d, J = 5 Hz, 1H, 2-hydroxypropyl-OH), 4.35 (m, 2H, 3′-H), 3.79 (m, 4H, 4′-H, dioxane 6-H), 3.70 (m, 2H, 2-hydroxypropyl-2H), 3.56 (m, 2H, 5′-H$_a$), 3.50 (m, 2H, 5′-H$_b$), 2.59 (m, 2H, 2′-H$_a$), 2.17 (m, 2H, 2′-H$_b$), 1.76 (d, J = 11 Hz, 2H, dioxane 5$_{eq}$-H), 1.58 (m, 2H, 2-hydroxypropyl-1H$_a$), 1.51 (m, 2H, 2-hydroxypropyl-1H$_b$), 1.30 (dd, J = 6 and 11 Hz, 2H, dioxane 5$_{ax}$-H), 1.14 (d, J = 6 Hz, 3H, dioxane 6-CH$_3$), 1.13 (d, J = 6 Hz, 3H, dioxane 6-CH$_3$), 1.025 (d, J = 6.4 Hz, 3H, 2-hydroxypropyl-CH$_3$), 1.013 (d, J = 6.1 Hz, 3H, 2-hydroxypropyl-CH$_3$); ESI-MS m/z (relative intensity) 426 (100, M + 1); UV λ_{max} for peak 8A (H$_2$O) 253.2, 270 nm (sh); UV λ_{max} for peak 8B (H$_2$O) 253.2, 270 nm (sh). Peaks 9A,B (mixture of at least two isomers, 100 μg): ^{1}H NMR (DMSO-d_6) δ 10.5 (bs, 2H, dG-N1-H), 7.95 (s, 1H, dG-C8-H), 7.94 (s, 1H, dG-C8-H), 6.49 (s, 2H, dG-N^2-H), 6.13 (dd, J = 3 and 7 Hz, 2H, 1′-H), 5.38 (m, 2H, dioxane 4-H), 5.28 (d, J = 4 Hz, 1H, 3′-OH), 5.24 (d, J = 3 Hz, 3′-OH), 4.85 (dd, J = 5 and 5 Hz, 2H, 5′-OH), 4.80 (m, 2H, dioxane 2-H), 4.44 (d, J = 5 Hz, 1H, 2-hydroxypropyl-OH), 4.35 (d, J = 5 Hz, 1H, 2-hydroxypropyl-OH), 4.34 (m, 2H, 3′-H), 3.82 (m, 2H, dioxane 6-H), 3.76 (m, 2H, 4′-H), 3.72 (m, 2H, 2-hydroxypropyl-2H), 3.53 (m, 2H, 5′-H$_a$), 3.45 (m, 2H, 5′-H$_b$), 2.60 (m, 2H, 2′-H$_a$), 2.18 (m, 2H, 2′-H$_b$), 1.77 (bd, J = 11 Hz, 2H, dioxane 5$_{eq}$-H), 1.6−1.5 (m, 4H, 2-hydroxypropyl-1H$_{a,b}$), 1.29 (dd, J = 11 and 23 Hz, 2H, dioxane 5$_{ax}$-H), 1.14 (d, J = 6 Hz, 3H, dioxane 6-CH$_3$), 1.13 (d, J = 6 Hz, 3H, dioxane 6-CH$_3$), 1.027 (d, J = 6.4 Hz, 3H, 2-hydroxypropyl-CH$_3$), 1.016 (d, J = 6.4 Hz, 3H, 2-hydroxypropyl-CH$_3$); ESI-MS m/z (relative intensity) 426 (100, M + 1); UV λ_{max} for peak 9A (H$_2$O) 253.2, 270 nm (sh); UV λ_{max} for peak 9B (H$_2$O) 253.2, 270 nm (sh).

***N*²-[2-(2-Hydroxypropyl)-6-methyl-1,3-dioxan-4-yl]deoxyguanylyl-(5′−3′)-thymidine [*N*²-Paraldol-dG-(5′−3′)-thymidine, 14, Scheme 3; Peaks 7A−D].** Peak 7 was collected using HPLC system 1 and then further purified in system 3. This gave 25 μg of peaks 7A−D: ^{1}H NMR (DMSO-d_6/D$_2$O) δ 7.93 (s, 1H, dG-C8-H), 7.64 (s, 1H, dThd-C6-H), 6.12 (dd, J = 6 and 9 Hz, 1H, dG 1′-H), 6.09 (dd, J = 6 and 8 Hz, 1H, dThd 1′-H), 5.39 (m, 1H, dioxane 4-H), 4.92 (m, 1H, dioxane 2-H), 4.56 (m, 1H, 3′-H-dThd), 4.48 (m, 1H, 3′-H-dG), 3.95 (m, 1H, dioxane 6-H), 3.86 (m, 3H, 4′-H, 5′-H$_a$-dG), 3.70 (m, 2H, 2-hydroxypropyl-2H, 5′-H$_b$-dG), 3.51 (m, 2H, 5′-H$_{a,b}$-dThd), 2.72 (m, 1H, 2′-H$_a$-dG), 2.19 (m, 1H, 2′-H$_a$-dThd), 2.13 (m, 1H, 2′-H$_b$-dG), 2.03 (m, 1H, 2′-H$_b$-dThd), 1.76 (m, 1H, dioxane 5$_{eq}$-H), 1.73 (s, 3H, dThd-CH$_3$), 1.52 (m, 2H, 2-hydroxypropyl-1H$_{a,b}$), 1.26 (m, 1H, dioxane 5$_{ax}$-H), 1.12 (m, 3H, dioxane 6-CH$_3$), 1.00 (m, 3H, 2-hydroxypropyl-CH$_3$); ESI-MS m/z (relative intensity) 729 (M$^+$, 27), 728 (100), 640 (11); UV λ_{max} for peak 7A (H$_2$O) 258.0, 267 nm (sh); UV λ_{max} for peak 7B (H$_2$O) 258.0, 267 nm (sh); UV λ_{max} for peak 7C (H$_2$O) 256.8 nm; UV λ_{max} for peak 7D (H$_2$O) 256.8 nm.

Reactions. (1) Crotonaldehyde and DNA. For detection of paraldol-releasing adducts and characterization of adducts **13** and **14**, crotonaldehyde (12 mmol) was allowed to react with calf thymus DNA (25 mg) for 96 h, at 37 °C, in 6 mL of 0.1 M phosphate buffer (pH 7). Levels of paraldol-releasing adducts were determined by reaction of each collected peak with 2,4-

dinitrophenylhydrazine reagent as previously described (*15*). For quantitation studies, crotonaldehyde (0.2, 2, 20, and 200 mM) was allowed to react with DNA (20 mg) in 2 mL of 0.1 M phosphate buffer (pH 7) at 37 °C, for different periods of time. The modified DNA was precipitated by addition of ethanol and then washed with 70% ethanol and ethanol until crotonaldehyde could not be detected in the washings, by analysis of its 2,4-dinitrophenylhydrazone. It was subjected to neutral thermal hydrolysis, and the amount of released paraldol was determined (*15*).

(2) Paraldol and Nucleosides, Nucleotides, and DNA. Paraldol (1.2 mmol) was allowed to react with DNA (20 mg) in 1.5 mL of 0.1 M phosphate buffer (pH 7) at 37 °C for 40 h. The DNA was isolated and washed as described above.

Paraldol (0.17 mmol) was allowed to react with dG or dThd (0.034 mmol), or dG and dThd (0.034 mmol of each), in 2.5 mL of 0.1 M phosphate buffer (pH 7) at 37 °C for 96 h. Similarly, paraldol (0.17 mmol) was allowed to react with dG-3′-MP, dG-5′-MP, dT-3′-MP, dT-5′-MP, dAdo, or dCyd (0.034 mmol) in 1 mL of 0.1 M phosphate buffer (pH 7) at 37 °C for 48 h.

Hydrolysis of DNA. Unless specified otherwise, enzymatic hydrolysis was carried out using system 1. The modified DNA (2.5 mg) was dissolved in 1 mL of a 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7). The mixture was incubated at 37 °C for 70 min with DNase I (type II, from bovine pancreas), phosphodiesterase I (type II, from *Crotalus adamanteus* venom), and alkaline phosphatase (from calf intestine) as described previously (*15*). System 2 was the same as system 1, except DNase II (type V, from bovine spleen) and phosphodiesterase II (from calf spleen) were used. In system 3, DNA was initially digested with DNase I as in system 1. Then to the resulting mixture were added 250 μL of a 0.5 M sodium acetate/10 mM $ZnCl_2$ buffer (pH 5.3) and 600 units of nuclease P1 (from *Penicillium citrinum*). After incubation for 2 h, 200 μL of 0.4 M Tris-HCl buffer (pH 8) was added, and then alkaline phosphatase was added as in system 1. The mixture was incubated for an additional 3 h.

Products of enzymatic hydrolysis were analyzed using HPLC system 1. Retention times were as follows: adducts **2** and **3**, 38.4 and 40.4 min; adducts **13**, 46.4, 46.7, 48.3, and 48.7 min; and adducts **14**, 44.1 min. dG was quantified using HPLC system 2.

Adducts **2** and **3**, as the guanine base, were released by acid hydrolysis and analyzed in HPLC system 5.

Hydrolysis of 14. Adduct **14** was dissolved in 300 μL of a 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7). Then 0.075 unit of venom phosphodiesterase I (type II, from *C. adamanteus*) or spleen phosphodiesterase II (from calf spleen) was added, and the mixture was incubated at 37 °C for 1 h. The resulting mixture was analyzed by HPLC system 1.

Results

Freshly opened bottles of crotonaldehyde used in this study were free of paraldol, as determined by GC. There were no detectable impurities. The ratio of *trans*-crotonaldehyde:*cis*-crotonaldehyde was approximately 25:1.

Crotonaldehyde was allowed to react with DNA. The DNA was extensively purified to remove unreacted crotonaldehyde. It was then hydrolyzed enzymatically and analyzed by reversed phase HPLC. This produced the chromatogram illustrated in Figure 1. Peaks 1−11 were not present in hydrolysates of untreated DNA. The indicated peaks are adducts **2** and **3** (Scheme 1) previously identified as products of the reaction of crotonaldehyde with DNA. Our initial goal in this study was to determine which peaks (1−11) would release paraldol upon acid hydrolysis. Paraldol-releasing DNA adducts can be quantified by reaction with 2,4-dinitrophenylhydrazine reagent, which produces a mixture of the 2,4-dinitrophenylhydrazones of 3-hydroxybutanal and crotonaldehyde (*15*). The results of this analysis are summarized in Figure 2. Peaks 6−9 released the greatest amounts of paraldol. As peaks 8 and 9 constituted about 35% of the paraldol-releasing adducts detected in the 20−60 min HPLC eluant, they were the initial focus of this study.

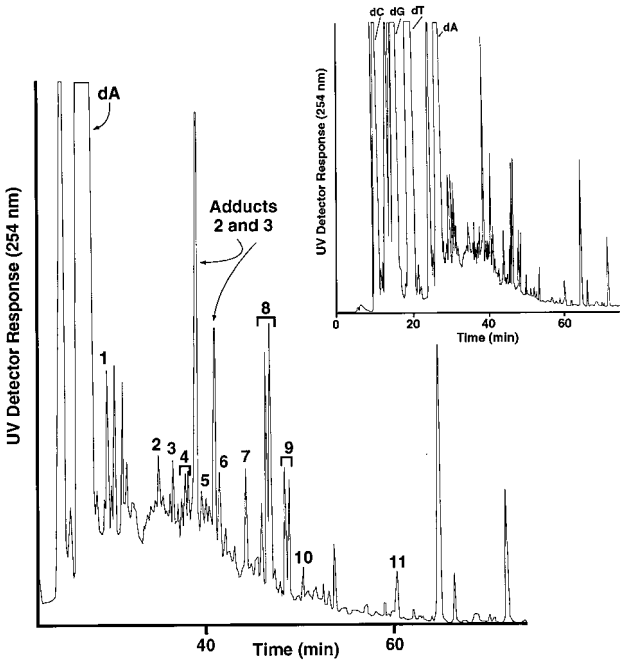


Figure 1. Chromatogram obtained upon HPLC analysis (system 1) of an enzymatic hydrolysate of DNA that had been reacted with crotonaldehyde. Peaks 1−11 were not present in hydrolysates of untreated DNA. Each of these peaks released paraldol (**7**, Scheme 1) upon hydrolysis. Adducts **2** and **3** are the 1,N^2-propano-dG adducts shown in Scheme 1. The inset is the entire chromatogram.

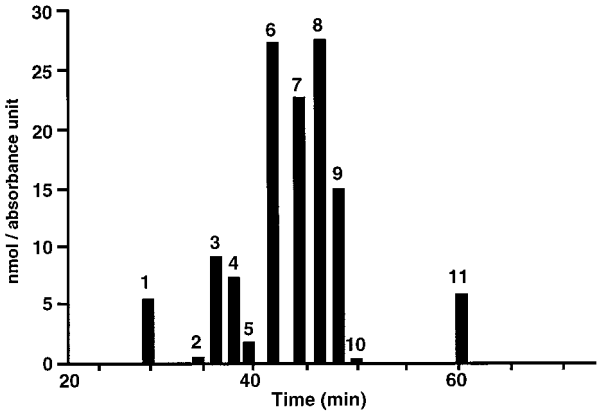


Figure 2. Amounts of paraldol released from each peak (1−11) in Figure 1, upon treatment with 2,4-dinitrophenylhydrazine reagent, which produces the 2,4-dinitrophenylhydrazones of 3-hydroxybutanal and crotonaldehyde (*15*). Time (minutes) refers to the HPLC retention times of the peaks in Figure 1.

Peaks 8 and 9 each consisted of a pair of major HPLC peaks: 8A,B and 9A,B. This is illustrated for the purified peaks in Figure 3. The UV spectra of peaks 8A,B and 9A,B were identical. The spectrum of peak 8A is shown in Figure 4. These UV spectra are very similar to those of N^2-substituted dG adducts which we have previously characterized (*17*).

LC-ESI-MS analysis (positive ion mode) of peaks 8A,B (as a mixture) and peaks 9A,B (as a mixture) gave base peaks of *m*/*z* 426, which is M + 1 of an adduct between

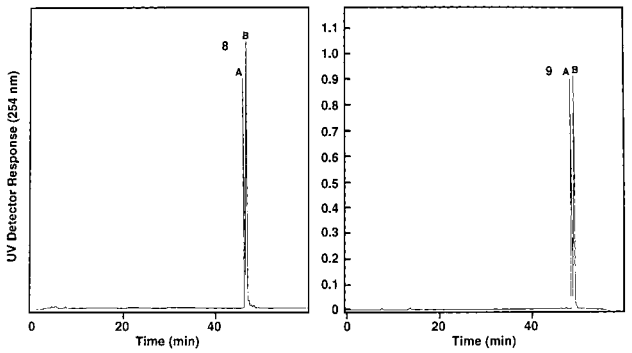


Figure 3. Chromatograms obtained upon HPLC analysis (system 1) of peaks 8 and 9 of Figure 1, after their purification. Each consists of at least two peaks: 8A,B and 9A,B. HPLC system 3 was used.

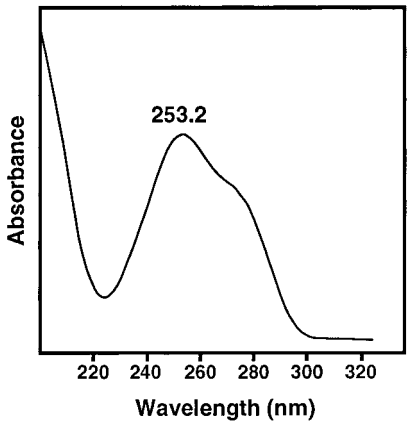


Figure 4. UV spectrum of peak 8A of Figure 3.

paraldol (**7**) and dG. LC-ESI-MS/MS analysis of *m*/*z* 426 of peaks 9A,B gave the spectrum illustrated in Figure 5.

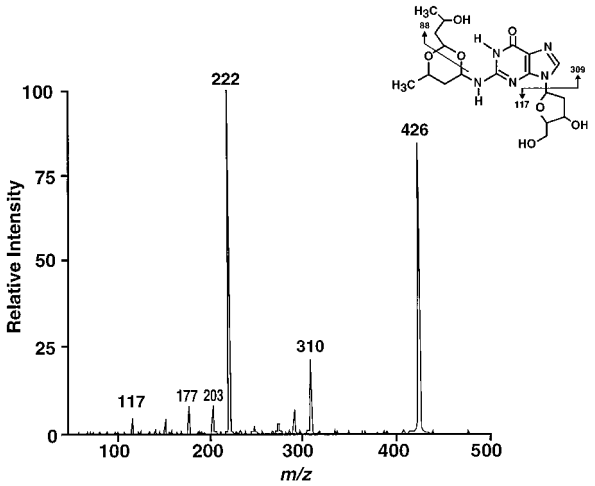
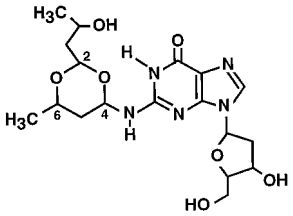


Figure 5. LC−MS/MS analysis (positive ion electrospray mode) of peaks 9A,B (*m*/*z* 426), which is M + 1 of an adduct between paraldol and dG. The peak at *m*/*z* 222 corresponds to the loss of 3-hydroxybutanal (88 mass units) and replacement of the sugar moiety with a proton (net loss of 116 mass units).

Similar data were obtained for peaks 9A,B. The peak at *m*/*z* 310 corresponds to the loss of 116 mass units from *m*/*z* 426 (the sugar unit is replaced with a proton). The peak at *m*/*z* 222 corresponds to the loss of 116 mass units plus 88 mass units (3-hydroxybutanal) and replacement of the sugar unit with a proton (see Figure 5). These data are consistent with an adduct of paraldol and dG.

^{1}H NMR data for peaks 8A,B (as a mixture) are summarized in Table 1. All assignments were confirmed by COSY spectra. The data are consistent with peaks 8A,B being a mixture of at least two major isomers, which are clearly seen in the chromatogram in Figure 3. Peaks for two isomers are observed for the proton at C8 of dG,

Table 1. ^{1}H NMR Data (800 MHz) for Peaks 8A,B and 9A,B[a]



	deoxyguanosyl protons									
	C8-H[b]	N1-H	N^2-H	1′-H	2′-H$_{a,b}$	3′-H	4′-H	5′-H$_{ab}$[b]	3′-OH[b]	5′-OH
peaks 8A,B	7.93(s), 7.94(s)	10.6(bs)	6.49(s)	6.13(dd) (J = 3 and 7 Hz)	2.17(m), 2.59(m)	4.35(m)	3.79(m)	3.50(m), 3.56(m)	5.24(d) (J = 4 Hz), 5.26(d) (J = 4 Hz)	4.85(m)
peaks 9A,B	7.94(s), 7.95(s)	10.5(bs)	6.49(s)	6.13(dd) (J = 3 and 7 Hz)	2.18(m), 2.60(m)	4.34(m)	3.76(m)	3.45(m), 3.53(m)	5.24(d) (J = 3 Hz), 5.28(d) (J = 4 Hz)	4.85(dd) (J = 5 and 5 Hz)

	dioxane protons						2-(2-hydroxypropyl) protons			
	2H	4-H	5-H$_{eq}$	5-H$_{ax}$	6-H	6-CH$_3$[b]	1-H$_{a,b}$	2-H	2-OH[b]	3-CH$_3$[b]
peaks 8A,B	4.79(m)	5.37(m)	1.76(d) (J = 11 Hz)	1.30(m)	3.79(m)	1.13(d) (J = 6 Hz), 1.14(d) (J = 6 Hz)	1.51(m), 1.58(m)	3.70(m)	4.38(d) (J = 5 Hz), 4.44(d) (J = 5 Hz)	1.013(d) (J = 6.1 Hz), 1.025(d) (J = 6.4 Hz)
peaks 9A,B	4.80(m)	5.38(m)	1.77(bd) (J = 11 Hz)	1.29(m)	3.82(m)	1.13(d) (J = 6 Hz), 1.14(d) (J = 6 Hz)	1.5−1.6(m)	3.72(m)	4.35(d) (J = 5 Hz), 4.44(d) (J = 5 Hz)	1.016(d) (J = 6.4 Hz), 1.027(d) (J = 6.4 Hz)

[a] Values are chemical shifts (δ). [b] Separate resonances listed represent at least two isomers.

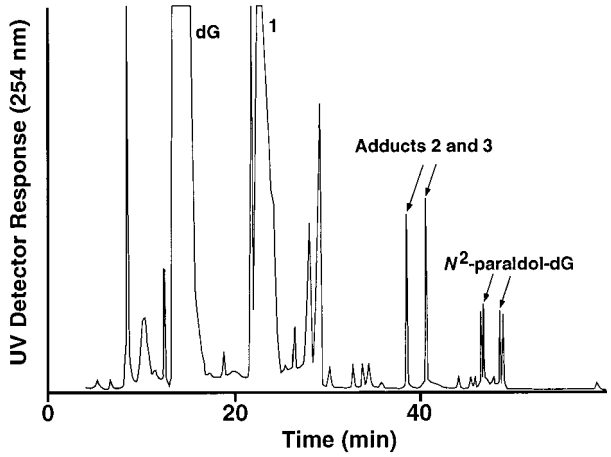


Figure 6. Chromatogram obtained upon HPLC analysis (system 1) of the reaction of paraldol with dG. The peaks marked N^2-paraldol-dG are identical to those obtained upon reaction of crotonaldehyde with DNA (see Figures 1 and 3). Adducts **2** and **3** are the 1,N^2-propano-dG adducts shown in Scheme 1. The peak eluting at 23 min is unreacted crotonaldehyde (**1**).

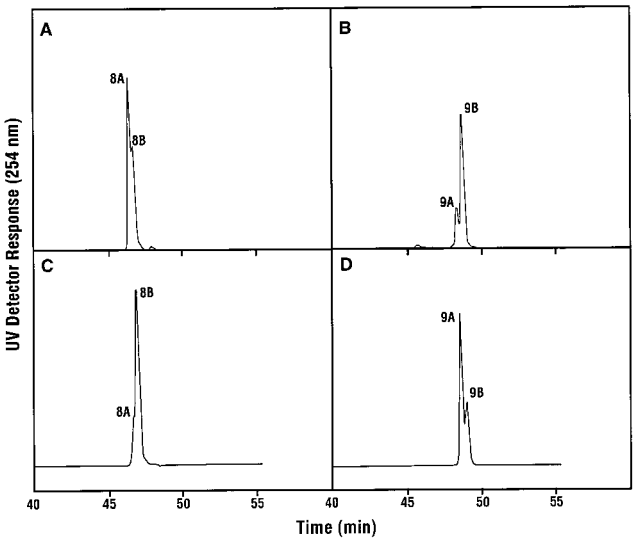


Figure 7. 2-[2(*R*)-Hydroxypropyl]-4-hydroxy-6(*R*)-methyl-1,3-dioxane (**7**, Scheme 2) or the corresponding 2-[2(*S*)-hydroxypropyl]-4-hydroxy-6(*S*)-isomer prepared as shown in Scheme 2 was allowed to react with dG. Peaks 8 and 9 were collected and reanalyzed using HPLC system 1: (A and B) the (*R*)-isomer and (C and D) the (*S*)-isomer.

the 5′- and 3′-OH protons of the deoxyribose ring, the methyl protons at the 6-position of the dioxane ring and on the 2-(2-hydroxypropyl) group, and the 2′-OH protons of the 2-(2-hydroxypropyl) group. Similar doubling of resonances was observed in the spectrum of peaks 9A,B.

The ^{1}H NMR data are completely consistent with the structure illustrated in Table 1, N^2-paraldol-dG (**13**, Scheme 3). There is no resonance corresponding to the exocyclic NH_2 of dG, indicating that substitution has occurred at N^2. This is in agreement with the UV data summarized above. The ^{1}H NMR peaks assigned to N1-H, N^2-H, 3′-OH, 5′-OH, and the OH of the 2-(2-hydroxypropyl) group are all found at positions consistent with these assignments, and all disappear upon addition of D_2O.

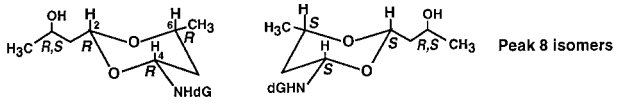
A NOESY spectrum provides evidence that the substituents on the dioxane ring of the peak 8A,B isomers are all equatorial. Strong cross-peaks are observed for the 2−6 and 2−4 axial protons of the dioxane ring, and a weaker cross-peak is observed for the 4−6 axial protons. In contrast, a NOESY spectrum of peaks 9A,B shows a strong cross-peak only for the 2−4 protons, indicating that the methyl group at the 6-position and the protons at the 2- and 4-positions are axial in these isomers. Consistent with this, the COSY spectrum of peaks 8A,B indicates strong coupling of the axial dioxane 5-proton to both the 4- and 6-protons. However, the COSY spectrum of peaks 9A,B indicates strong coupling of the axial dioxane 5-proton to the 4-proton, but weaker coupling to the 6-proton. Collectively, these results are consistent with an all equatorial conformation for the substituents of the dioxane ring in the peak 8A,B isomers and an axial methyl group in the peak 9A,B isomers.

Confirmation of the overall structural assignment was provided by reacting paraldol with dG. This produced the chromatogram illustrated in Figure 6. The indicated peaks coeluted with peaks 8A,B and 9A,B, and had identical UV, MS, and ^{1}H NMR data. The four major N^2-paraldol-dG peaks were also produced in reactions of paraldol with DNA, and of crotonaldehyde with dG.

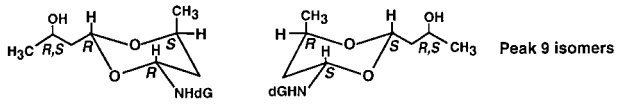
Further information about the stereochemistry of N^2-paraldol-dG was obtained by reduction of commercially available ethyl (*R*)-(−)-3-hydroxybutyrate (**9**) with DIBAL as indicated in Scheme 2. This produces the corresponding enantiomer of 3-hydroxybutanal (**8**) which dimerizes, yielding paraldol (**7**) in which the absolute configurations at the 6-position of the dioxane ring and the carbinol carbon of the 2-(2-hydroxypropyl) group are (*R*). Similarly, ethyl (*S*)-(+)-3-hydroxybutyrate gave the corresponding paraldol with the (*S*)-configuration at these two positions. The paraldol produced in each of these reactions was then allowed to react with dG. Paraldol synthesized from (*R*)-(−)-ethyl 3-hydroxybutyrate produced mainly peaks 8A and 9B, while paraldol synthesized from (*S*)-(+)-ethyl-3-hydroxybutyrate gave predominantly peaks 8B and 9A (Figure 7A−D).

There are 16 stereoisomers of N^2-paraldol-dG. Eight of these are illustrated in Figure 8. When the dioxane ring is in the favored chair conformation, there are four isomers corresponding to peaks 8A,B with all equatorial substituents. Similarly, there are four isomers corresponding to peaks 9A,B with an axial methyl group. Peaks 8A and 9B are therefore enriched in isomers 1 and 7, respectively, while peaks 8B and 9A are enriched in isomers 4 and 6, respectively (Figure 8).

We next investigated the structure of peak 7, which released amounts of paraldol similar to those of peaks 8 and 9. We did not pursue the structure of peak 6 in this study because it was poorly resolved from the second eluting 1,N^2-propano-dG isomer. The UV spectrum of peak 7 is illustrated in Figure 9. It is similar to that of N^2-paraldol-dG (Figure 3). Further analysis of peak 7 in HPLC system 1 demonstrated that it consisted of four peaks, 7A−D, in a 1:1:0.1:0.1 ratio. All had UV spectra like that shown in Figure 9; they are presumed to be isomers. All subsequent data were obtained on peaks 7A−D. Reaction of paraldol with DNA produced peaks 7A−D, as well as the N^2-paraldol-dG adducts discussed above. However, in contrast to the results obtained above, reaction of paraldol with dG did not produce peaks 7A−D. These results suggested that another DNA base was present in the structure. Preliminary ^{1}H NMR data for peaks 7A−D demonstrated the presence of two 1′-protons and a methyl singlet which could correspond to the methyl group of dThd. Thus, it appeared that peaks



isomer	carbinol	dioxane 2	dioxane 4	dioxane 6	HPLC r.t.
1	*R*	*R*	*R*	*R*	peak 8A
2	*S*	*R*	*R*	*R*	?
3	*R*	*S*	*S*	*S*	?
4	*S*	*S*	*S*	*S*	peak 8B



isomer	carbinol	dioxane 2	dioxane 4	dioxane 6	HPLC r.t.
5	*R*	*R*	*R*	*S*	?
6	*S*	*R*	*R*	*S*	peak 9A
7	*R*	*S*	*S*	*R*	peak 9B
8	*S*	*S*	*S*	*R*	?

Figure 8. Isomers of N^2-paraldol-dG in peaks 8 and 9 of Figures 1, 3, and 7. NOESY experiments indicate that peak 8 isomers have all equatorial substituents. The retention times of isomers 1 and 4 are located by the data in Figure 7 as peaks 8A and 8B, respectively; isomers 2 and 3 are most likely formed, but their retention times, e.g., peaks 8A,B, are not known. NOESY experiments indicate that peak 9 isomers have an axial methyl group. Isomers 6 and 7 are located under peaks 9A and 9B, respectively, while the retention times of isomers 5 and 8 are not known.

7A−D were either a cross-linked product or a dinucleoside phosphate. Extensive purification of peaks 7A−D by collection from HPLC systems 1 and 3 ultimately provided a sample that was sufficiently pure for further ^{1}H NMR studies. These data are summarized in Table 2. All assignments were confirmed by COSY spectra. The data are consistent with the structure illustrated in Table 2 and Scheme 3 (compound **14**). The protons of the dioxane ring and the 2-(2-hydroxypropyl) substituent were observed at chemical shifts similar to those seen in the spectra of N^2-paraldol-dG. Moreover, a NOESY spectrum demonstrates connectivity among the 2-, 4-, and 6-protons of the dioxane ring, confirming its presence. Analysis of peaks 7A−D by LC-ESI-MS (negative ion mode) produced a molecular ion at *m/z* 729, corresponding to $C_{28}H_{40}N_7O_{14}P$, and a base peak at *m/z* 728, consistent with the structure illustrated in Table 2. However, these data alone could not completely establish the structure illustrated in Table 2.

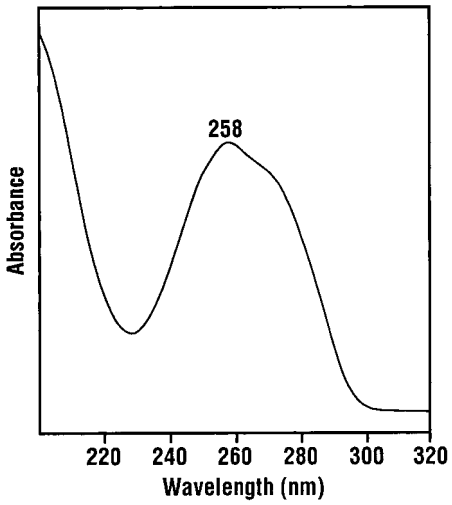
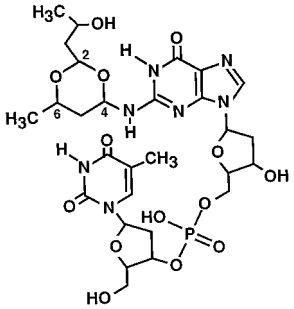


Figure 9. UV spectrum of peak 7 of Figure 1.

Further evidence was obtained by treatment of peaks 7A−D with snake venom phosphodiesterase which liberates 5′-nucleotides from 3′-termini. This produced the chromatogram illustrated in Figure 10. The three peaks were identified as dThd (16.63 min), N^2-paraldol-dG-5′-MP (31.23 min), and unhydrolyzed peak 7 (42.78 min). N^2-Paraldol-dG-5′-MP was prepared by reaction of paraldol with dG-5′-MP. The product was characterized by negative ion ESI-MS which gave a molecular ion at *m/z* 505 and a base peak at *m/z* 504. MS/MS analysis of the

Table 2. ^{1}H NMR Data (800 MHz) for Peak 7[a]



deoxyguanosyl and thymidyl protons							
C8-H(dG)	C6-H(dThd)	1′-H	2′-H	3′-H	4′-H	5′-H$_{a,b}$	CH_3
7.93(s)	7.64(s)	6.12(dd) (J = 6,9 Hz)(dG) 6.09(dd) (J = 6,8 Hz)(dThd)	2.03(m) (dThd) 2.13(m) (dG) 2.19(m) (dThd) 2.72(m) (dG)	4.48(m) (dG) 4.56(m) (dThd)	3.86(m)	3.51(m) (dThd) 3.86(m) (dG) 3.70(m) (dG)	1.73(s)

dioxane protons						2-(2-hydroxypropyl) protons		
2-H	4-H	5-H$_{eq}$	5-H$_{ax}$	6-H	6-CH_3	1-H$_{a,b}$	2-H	3-CH_3
4.92(m)	5.39(m)	1.76(m)	1.26(m)	3.95(m)	1.12(m)	1.52(m)	3.70(m)	1.00(m)

[a] Spectrum obtained in DMSO-d_6 containing one drop of D_2O. Values are chemical shifts (δ).

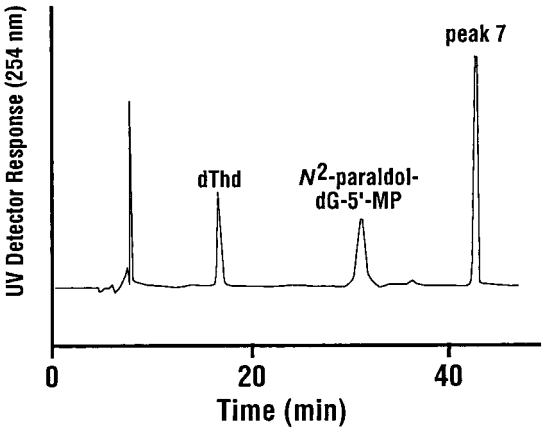


Figure 10. Chromatogram obtained upon HPLC analysis (system 1) of a snake venom phosphodiesterase hydrolysate of peaks 7A−D.

ion at *m*/*z* 505 gave a major fragment at *m*/*z* 416, corresponding to the loss of 3-hydroxybutanal and one hydrogen. The UV spectrum and HPLC retention time of this standard were identical to those of the peak eluting at 31.23 min. Moreover, treatment of the 31.23 min peak with alkaline phosphatase produced N^2-paraldol-dG. These data are completely consistent with the structure shown in Table 2. Additional evidence was obtained by treating peaks 7A−D with spleen phosphodiesterase which liberates 3′-nucleotides from 5′-termini. This produced N^2-paraldol-dG and dT-3′-MP. These results confirm that peaks 7A−D are four isomers of N^2-paraldol-dG-(5′−3′)-thymidine (**14**), with the overall structure shown in Table 2 and Scheme 3. Stereochemical details were not pursued, but similar considerations as discussed for peaks 8 and 9 would apply.

As some of the other peaks in Figure 1 might have resulted from reactions of paraldol with other DNA constituents, we examined the reaction of paraldol with various nucleosides and nucleotides. Products were found only in the reaction of paraldol with dG, dG-3′-MP, and dG-5′-MP. Reactions with dAdo, dCyd, dThd, dT-3′-MP, or dT-5′-MP produced no detectable products.

Various DNA hydrolysis conditions were investigated, as it appeared that other peaks in the chromatogram in Figure 1 might be partially hydrolyzed adducts such as peak 7, and that ultimately these could be completely converted to peaks 8 and 9 by the right combination of enzymes. System 1 was best for the hydrolysis of crotonaldehyde-modified DNA. Hydrolysis of calf thymus DNA proceeds with approximately 90% efficiency using this mixture. Hydrolysis of crotonaldehyde-modified DNA using this mixture gave chromatograms as shown in Figure 1. When the material eluting from 30 to 65 min was collected and rehydrolyzed using a mixture of phosphodiesterases I and II and alkaline phosphatase, the amount of peaks 8 and 9 increased by about 45%, but the other paraldol-releasing adducts, e.g., peaks 1−7, were still incompletely hydrolyzed. We also tried systems 2 and 3, but neither of these worked as well as system 1 with respect to the yield of N^2-paraldol-dG, under either the initial hydrolysis or repeat hydrolysis conditions. In contrast, all enzymatic hydrolysis conditions produced similar amounts of 1,N^2-propano-dG adducts **2** and **3**, and rehydrolysis had no further effect. Collectively, these results suggest that N^2-paraldol-dG-containing adducts in DNA are relatively resistant to enzymatic hydrolysis, in contrast to 1,N^2-propano-dG adducts **2** and **3**.

Table 3. Quantitation of Total Paraldol-Releasing DNA Adducts vs Adducts 2 and 3 Formed in the Reaction of Crotonaldehyde with DNA[a]

crotonaldehyde (mM)	reaction time (h)	total paraldol-releasing adducts (μmol/mol of G)	adducts **2** and **3** (μmol/mol of G)
0.2	4	46	NQ[b]
	20	162	NQ
2.0	4	244	NQ
	20	512	NQ
20.0	4	1040	NQ
	20	2110	28
200	96	11000	850

[a] Reactions were carried out in 2 mL of 0.1 M phosphate buffer (pH 7.0) at 37 °C with 20 mg of DNA. Paraldol-releasing DNA adducts were quantified by neutral thermal hydrolysis of the DNA and formation of 2,4-dinitrophenylhydrazones as described in the Experimental Section. Adducts **2** and **3** were quantified by acid hydrolysis of the DNA and analysis by HPLC. [b] NQ, not quantified.

Table 3 summarizes the levels of total paraldol-releasing adducts in DNA reacted with crotonaldehyde. Depending on time and concentration, levels of these adducts ranged from 46 to 11 000 μmol/mol of guanine. Quantitation of adducts **2** and **3** was only possible at the two highest concentrations and longest reaction times. Their levels were 13−76 times lower than those of total paraldol-releasing adducts. Adducts **13** and **14** were quantified only at a crotonaldehyde concentration of 200 mM and a reaction time of 96 h. Their levels were less than 10% of that of the total paraldol-releasing adducts and similar to those of adducts **2** and **3**. Further analysis of each region of the chromatogram illustrated in Figure 1, obtained by enzymatic hydrolysis of the DNA, demonstrated that approximately 90% of the paraldol released from DNA eluted between 5 and 15 min, the retention time of paraldol itself.

Discussion

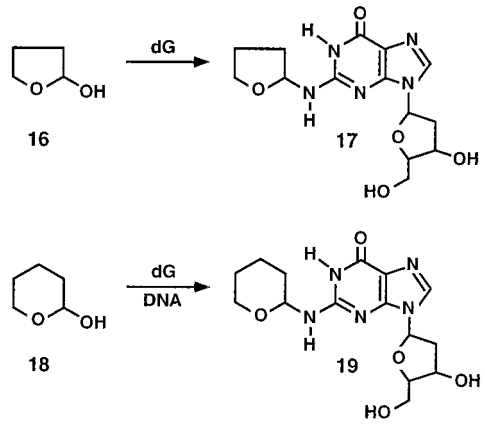
The results of this study demonstrate that, in addition to the previously characterized adducts **2**−**6** of crotonaldehyde resulting from Michael addition (Scheme 1), a second class of DNA adducts is produced by reaction with 3-hydroxybutanal (**8**) or its dimer, paraldol (**7**). The likely origin of these adducts is outlined in Scheme 3. In aqueous solutions, crotonaldehyde is in equilibrium with 3-hydroxybutanal (**8**), otherwise known as aldol, the aldol condensation product of acetaldehyde (*18*). 3-Hydroxybutanal rapidly dimerizes, forming paraldol (**7**) via intermediate **10** (*16*). Either 3-hydroxybutanal or intermediate **10** could form a Schiff base with the exocyclic amino group of dG, giving **11** or **12**. Schiff base **12** could also be formed by further reaction of **11** with 3-hydroxybutanal. Ring closure of **12** produces **15** in DNA. This intramolecular cyclization is analagous to that observed by Sodum and Chung in the reaction of 2,3-epoxy-4-hydroxynonanal with dG (*19*). Enzymatic hydrolysis of this DNA releases the adducts characterized in this study, with overall structures **13** and **14**. Levels of these adducts were comparable to those of the 1,N^2-propano-dG adducts **2** and **3** in enzymatic hydrolysates of crotonaldehyde-treated DNA, and there are clearly other paraldol-releasing adducts in this DNA, as discussed further below. Since, at pH 7.4, the amounts of 3-hydroxybutanal and paraldol in aqueous solutions of crotonaldehyde are

far smaller than that of crotonaldehyde itself, our data imply that the reactions of 3-hydroxybutanal or paraldol with DNA must occur at a rate that is greater than that of crotonaldehyde with DNA. Kinetic studies are required to test this hypothesis.

The relative roles of Michael addition adducts such as **2**−**6** versus N^2-paraldol-dG adducts such as **13** and **14** in crotonaldehyde mutagenesis and tumorigenesis are of course unclear at this point. Site specific mutagenesis studies with 1,N^2-propano-dG, an unsubstituted analogue of adducts **2** and **3**, have been reported (*20*). This adduct is highly mutagenic in *Escherichia coli*, directing the incorporation of dAMP, resulting in dG → dThd transversions. Frameshift mutations have also been observed. However, in simian kidney (COS7) cells, 1,N^2-propano-dG was far less mutagenic, with favored incorporation of dCyd. In a shuttle vector system, crotonaldehyde induced a variety of mutations, including dG → dThd transversions, dG → dAdo transitions, and tandem base substitutions (*21*). Our results clearly demonstrate that, in contrast to adducts **2** and **3**, N^2-paraldol-dG adducts are resistant to enzymatic hydrolysis, indicating that they may cause significant steric perturbations in DNA. We speculate that they may also be resistant to DNA repair. A comparison of the persistence of adducts **2** and **3** versus N^2-paraldol-dG in cells or rodents treated with crotonaldehyde would be of interest.

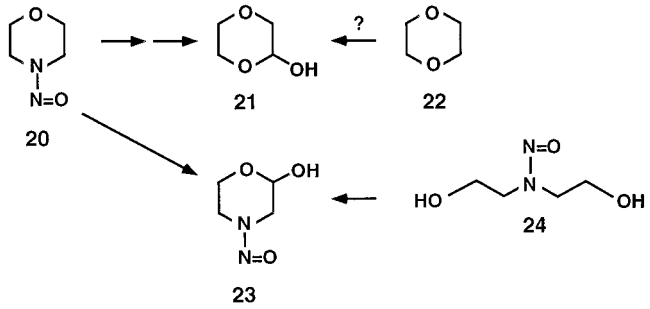
Our results demonstrate that paraldol, a lactol, reacts readily with dG and DNA. The reaction of lactols with DNA may be more common than previously recognized. We showed that both 2-hydroxytetrahydrofuran (**16**) and 2-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran (**18**) react with dG to form N^2-substituted adducts **17** and **19** (Scheme 4) and

Scheme 4. Reactions of Lactols with dG or DNA Resulting in Production of N^2-dG Adducts



that **18** also reacts similarly with DNA (*17*, *22*). We are not aware of any carcinogenicity data on paraldol, **16**, or **18**. However, 5-(3-pyridyl)-2-hydroxytetrahydrofuran, a structurally related lactol, was nontumorigenic in A/J mice (*23*). Acetaldehyde, another precursor to paraldol, is carcinogenic, but we did not observe adduct **13** in reactions of acetaldehyde with DNA (data not shown). 1,4-Dioxane (**22**, Scheme 5) is carcinogenic in rodents and is metabolized to 1,4-dioxane-2-one, most likely via 2-hydroxy-1,4-dioxane (**21**), although **21** has not been isolated in these reactions (*24*, *25*). Compound **21** is also a metabolite of the carcinogen *N*-nitrosomorpholine (**20**) (*26*). Mutagenicity and carcinogenicity data on **21** have not been reported. 2-Hydroxy-*N*-nitrosomorpholine (**23**),

Scheme 5. Metabolic Formation of Lactols from Carcinogens[a]



[a] Lactol formation has been confirmed from **20** and **24**, and is proposed from **22**.

a lactol metabolite of **20** and *N*-nitrosodiethanolamine (**24**), is mutagenic, but lacked tumorigenicity (*27*, *28*). It is plausible that adducts such as those observed here are also formed from these other lactols and may play some role in carcinogenesis.

There are 16 possible stereoisomers of N^2-paraldol-dG. We have consistently observed two pairs of HPLC peaks in our studies of N^2-paraldol-dG, and ^{1}H NMR data are also consistent with the presence of at least four isomers. We have obtained partial information about the presence of four isomers (denoted as isomers 1, 4, 6, and 7 in Figure 8) by reactions with dG of paraldol formed from (*R*)- or (*S*)-3-hydroxybutanal. Thus, peaks 8A,B contain the all (*R*)- and all (*S*)-isomers, respectively, while peaks 9A,B contain the (carbinol-*S*,2*R*,4*R*,6*S*)- and (carbinol-*R*,2*S*,4*S*,6*R*)-isomers, respectively. Inspection of molecular models demonstrates no obvious reason isomers 2, 3, 5, and 8 of Figure 8 should not also be formed in the reaction of paraldol with dG or DNA. Thus, we assume that peaks 8 and 9 (Figures 1 and 3) each contain four isomers of N^2-paraldol-dG, but that they are not separated under our conditions. The formation of the other eight isomers should be less favored on steric grounds. These isomers would have either an axial dG or 2-(2-hydroxypropyl) group if the 1,3-dioxane ring were in the chair conformation, or would have to assume the less favored boat conformation of the 1,3-dioxane ring (*28*).

The adducts identified in this study comprise less than 10% of the total paraldol-releasing adducts in DNA reacted with crotonaldehyde. We focused on these adducts because they were stable under the conditions of enzymatic hydrolysis and could be reproducibly observed upon HPLC analysis of DNA reacted with crotonaldehyde, as illustrated in Figure 1. However, as the study unfolded, it became clear that the major adducts in this DNA are unstable during enzyme hydrolysis, releasing paraldol under these conditions. This release is prevented by treatment of the DNA with $NaBH_3CN$ (data not shown). We hypothesize that the major unstable paraldol-releasing adduct(s) is a Schiff base such as **11** or **12**. Efforts are currently underway to characterize this material.

In summary, we have characterized a new class of dG adducts in DNA reacted with crotonaldehyde. Adducts **13** and **14** are formed from 3-hydroxybutanal and/or its dimer, paraldol. They are stable in DNA and partially resistant to enzymatic hydrolysis. Although they comprise a relatively small proportion of the total paraldol-releasing adducts in this DNA, their stability in DNA and resistance to hydrolysis suggest that they may be

persistent and play an important role in mutagenesis and carcinogenesis by crotonaldehyde.

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