Identification of a Novel, N7-Deoxyguanosine Adduct as the Major DNA Adduct Formed by a Non-Bay-Region Diol Epoxide of Benzo[*a*]pyrene with Low Mutagenic Potential†

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ABSTRACT: A metabolite of benzo[*a*]pyrene, 9-*r*,10-*t*-dihydroxy-7,8-*c*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-III), that is not thought to be involved in carcinogenesis has nevertheless been shown to bind extensively to DNA in vitro. The adducts formed by this non-bay-region diol epoxide in Chinese hamster ovary cells are much less mutagenic than those formed by an isomeric diol epoxide that is carcinogenic. We have isolated and characterized three major adducts formed by in vitro reaction of BPDE-III with DNA. The major adduct, accounting for over half of the total is formed by reaction of BPDE-III with the N7 position of dGuo and is recovered after enzymatic digestion as an N7-Gua adduct. A second major adduct involves the N^2 position of dGuo, while the third adduct is tentatively identified as a C8-substituted dGuo. Little or no reaction with deoxyadenosine residues is detected. The N7 adduct is unstable in DNA at 37 °C and is released as the modified base with a half-life of about 24 h. This adduct lability apparently leads to single-strand breaks and alkali-sensitive sites in the DNA and may account in part for some of the biological properties of BPDE-III adducts. This represents the first description of an N7-dGuo adduct that is formed in DNA as the major adduct by a diol epoxide derived from a carcinogenic polycyclic aromatic hydrocarbon.

Polycyclic aromatic hydrocarbons, an often-studied group of chemical carcinogens, produce many of their biological effects by way of electrophilic metabolites that attack cellular macromolecules (Miller & Miller, 1981; Pelkonen & Nebert, 1982; Weinstein, 1981; Harvey, 1991), in particular DNA. Attempts to correlate DNA binding with carcinogenic potential have been generally successful (Brookes & Lawley, 1964; Meehan & Straub, 1979; Phillips et al., 1979). However, metabolites exist that bind covalently to DNA in vitro (Meehan & Straub, 1979; MacLeod et al., 1982) and in vivo (Baird & Diamond, 1977; Ivanovic et al., 1978; MacLeod et al., 1980; MacLeod et al., 1991a; MacLeod et al., 1991b; Pruess-Schwartz & Baird, 1986) but do not appear to be carcinogenic. Although quantitative differences in the in vivo formation of such adducts play a major role in differences in carcinogenicity, in several cases such quantitative differences are insufficient to explain the large differences in carcinogenicity between isomeric and enantiomeric metabolites (Buening et al., 1978; Slaga et al., 1977; Slaga et al., 1979). Therefore, comparisons of the formation, stability, repair, and biological effect of adducts derived from such metabolites with the same properties of more carcinogenic adducts represent a potentially fruitful approach to understanding the basic mechanisms of carcinogenesis.

One such interesting comparison is provided by the isomeric diol epoxides of benzo[*a*]pyrene. The carcinogenicity of benzo[*a*]pyrene is generally attributed to the (+) enantiomer of 7-*r*,8-*t*-dihydroxy-9,10-*t*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-I[1]), while the (–) enantiomer and both enantiomers of 7-*r*,8-*t*-dihydroxy-9,10-*c*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene are inactive (Buening et al., 1978; Slaga et al., 1979). In addition, the positional isomer 9-*r*,10-*t*-dihydroxy-7,8-*c*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-III) is generally considered noncarcinogenic (Slaga et al., 1977). However, all of these diol epoxides bind to DNA in vitro (Meehan & Straub, 1979; MacLeod et al., 1982), albeit to different extents, and several of them have been shown to have measurable mutagenic activities (Huberman et al., 1976; Wood et al., 1977; Brookes & Osborne, 1982; MacLeod et al., 1991a).

In particular, adducts derived from BPDE-III appear to be very different from BPDE-I adducts conformationally (MacLeod et al., 1982) and are substantially less toxic in bacterial cells (Tang et al., 1992) and less mutagenic in mammalian cells (MacLeod et al., 1991a) than equal numbers of BPDE-I adducts. In order to attempt to relate confor-

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[1] Abbreviations: BPDE-I, 7-*r*,8-*t*-dihydroxy-9,10-*t*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (sometimes referred to as *anti*-BPDE); BPDE-III, 9-*r*,10-*t*-dihydroxy-7,8-*c*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; FAPY, formamidopyrimidine; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; BPTT, 7,8,9,10-tetrahydro-8,9,10-trihydroxybenzo[*a*]pyrene; NOE, nuclear Overhauser effect; LD, linear dichroism.

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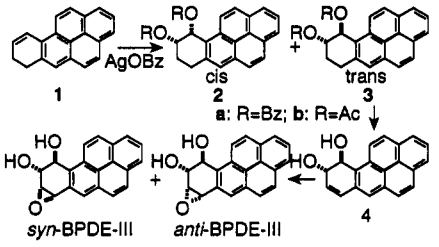
Scheme 1



mational differences at the adduct level to biological differences, one must know the chemical structure of the adducts. In the present work three major BPDE-III adducts have been prepared from treated DNA and identified by chemical and spectroscopic means. Interestingly, the major adduct formed by BPDE-III involves the N7 position of dGuo, a position not found to be the primary target for other polycyclic aromatic hydrocarbon diol epoxides.

MATERIALS AND METHODS

Chemicals. Since BPDE-III was not available from the National Cancer Institute's Chemical Carcinogen Repository in sufficient quantities for the preparation and identification of DNA adducts, synthesis of the racemic compound from 7,8-dihydrobenzo[*a*]pyrene (**1**) was undertaken via the sequence in Scheme 1. Compound **1** was prepared from 7,8,9,-10-tetrahydrobenzo[*a*]pyrene (50 mmol) by stirring its solution in dry benzene in the dark with an equimolar amount of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) for 2.5 h at room temperature under nitrogen. Filtration of the mixture followed by chromatography on a short column of Florisil eluted with benzene gave a mixture of **1** (65%), unreacted starting compound (15%), and benzo[*a*]pyrene (20%). The identity of **1** was confirmed by its characteristic NMR spectrum: δ ($CDCl_3$) 2.50 (m, 2, H_8), 3.22 (t, 2, H_7), 5.87 (m, 1, H_9), 7.25 (d, 1, H_{10}); $J_{9,10}$ = 8.14 Hz), 7.9–8.6 (m, 8, Ar) ppm. Due to the difficulty of separation and the facility of decomposition of **1**, this mixture was employed directly in the next step.

A suspension of silver benzoate (13.2 g, 58 mmol) and I_2 (6.40 g, 25 mmol) in 200 mL of dry benzene was heated at reflux for 30 min under a nitrogen atmosphere; then a solution of the above mixture (containing 5.85 g, 23 mmol of **1**) in 150 mL of dry benzene was added, and the resulting suspension was heated at reflux for 2 days. The mixture was filtered through Celite; then the filtrate was concentrated to less than 100 mL and passed through a column of Florisil eluted with a solvent gradient of hexanes to CH_2Cl_2 to yield 10.91 g (68%) of a mixture of the *cis* and *trans* dibenzoates (**2a** and **3a**) as a tan solid, mp 171–175 °C: NMR ($CDCl_3$) δ 2.53 (m, 2, H_8), 3.22 (t, 2, H_7), 7.25–8.43 (m, 19, Ar + H_{10}) ppm. Anal. Calcd for $C_{34}H_{24}O_4$: C, 82.24; H, 4.87. Found: C, 82.35; H, 4.96.

To a solution of the mixture of **2a** and **3a** (10.0 g, 20 mmol) in 250 mL of THF was added a solution of NaOMe (2.7 g, 5 mmol) in 300 mL of MeOH. The resulting brown solution was stirred at 60 °C for 45 min and then evaporated to dryness. The residue was triturated with 50 mL of cold ether; then 100 mL of water was added, and the solid product was filtered, washed with water and small portions of cold ether, and then dried in air. The solid (5.0 g) was dissolved in 50 mL of dry pyridine, 50 mL of Ac_2O was added, and the mixture was stirred under a nitrogen atmosphere for 4 h. The solution was poured onto ice water and stirred for 30 min; then the precipitate was filtered and washed with water. Purification through a short column of Florisil eluted with CH_2Cl_2 gave a 1:3 mixture of the *cis* and *trans* diacetates (**2b** and **3b**) (5.84 g, 78%). Crystallization from acetone at 5 °C furnished **2b** (1.2 g, 16%), mp 192.5–194 °C, as bright-yellow crystals: NMR ($CDCl_3$) δ 2.10 (s, 3, CH_3), 2.16 (s, 4, CH_3 + H_8), 2.45 (m, 1, H_8), 3.49 (m, 2, H_7), 5.47 (dt, 1, H_9, J = 12.9 Hz), 7.26 (H_{10} overlapping with $CHCl_3$), 7.96–8.22 (m, 8, ArH) ppm. Anal. Calcd for $C_{24}H_{20}O_4$: C, 77.40; H, 5.41. Found: C, 77.35; H, 5.28. Concentration of the mother liquor and precipitation with hexanes provided **3b** as a pale-yellow powder (3.9 g, 52%), mp 175–177 °C (lit. mp 181–183 °C [McAustland et al., 1976]): NMR ($CDCl_3$) δ 2.02 (s, 3, CH_3), 2.10 (s, 3, CH_3), 2.35 (m, 2, H_8), 3.32 (m, 2, H_7), 5.47 (apparent q, 1, H_9, J = 3.4 Hz), 6.86 (d, 1, H_{10}, J = 3.2 Hz), 7.97–8.23 (m, 8, ArH) ppm. Attempted preparation of **2b** and **3b** by direct reaction of **1** with silver acetate by a previously reported method (Thakker et al., 1978), instead of with silver benzoate, gave a very low yield.

To a refluxing solution of **3b** (840 mg, 2.3 mmol) in 300 mL of CCl_4 was added *N*-bromosuccinimide (488 mg, 2.8 mmol) and benzoyl peroxide (two batches of 60 mg each added at 30-min intervals), and reflux was continued for an additional 30 min. Then the solvent was evaporated to dryness, the residue was dissolved in 300 mL of ether, and the solution was washed with water (4 × 250 mL), dried over $MgSO_4$, and evaporated to dryness, and the residue was triturated with cold hexanes. The crude product was dissolved in THF (90 mL), K_2CO_3 (5 g) and DBN (1.4 mL) were added to the solution, and it was heated at 65 °C for 10 min under N_2. The solution was allowed to cool; then 10 mL of MeOH was added, and N_2 was bubbled through the solution for 15 min. A solution of NaOMe (550 mg, 10.2 mmol) in 18 mL of MeOH was added, and the heating at 65 °C was continued for an additional 10 min. The solution was concentrated to half volume by evaporation of solvent under vacuum; then water was added. The precipitate was filtered, washed with water, dried, and chromatographed on a Florisil column eluted with a solvent gradient of CH_2Cl_2 to 20% EtOAc in CH_2Cl_2. The combined fractions were concentrated to ~20 mL, and hexanes was added to give the pure *trans* dihydrodiol **4** (250 mg, 39%) as a cream-colored precipitate, mp 207–209 °C (lit.[1] mp 209–210 °C): NMR ($CDCl_3$) δ 4.33 (m, 1, H_9), 5.06 (d, 1, OH), 5.34 (d, 1, OH), 5.48 (d, 1, H_{10}), 6.32 (dd, 1, H_8, J = 9.5, J = 5.4 Hz), 6.98 (d, 1, H_7, J = 9.6 Hz), 8.26 (m, 8, ArH) ppm.

A solution of **4** (305 mg, 1.1 mmol) and *m*-chloroperbenzoic acid (3.8 g, 22 mmol) in 100 mL of dry THF was stirred at ambient temperature under nitrogen for 2 h. TLC showed complete conversion to two products (R_f 0.47 and 0.32 in THF–hexane, 2:1). The solution was cooled, diluted with EtOAc, washed with cold 5% aqueous NaOH and cold water (2×), and dried over Na_2SO_4. *Heating was avoided in the workup due to the thermal instability of the diol epoxide isomers.* Evaporation of the solvent in vacuo gave a mixture of *anti*- and *syn*-BPDE-III (2.3:1 by NMR) as a beige solid (260 mg, 65%). This solid was triturated with 5 mL of dry THF and then diluted with 5 mL of dry ether. The insoluble solid phase was filtered, and the filtrate was concentrated to give *syn*-BPDE-III (73 mg) as a tan solid (TLC: one spot R_f 0.47), mp 179.0–180.5 °C: the NMR spectrum was in agreement with that reported for the *syn*-isomer (Thakker et al., 1978). NMR (DMSO-d_6 + D_2O) δ 3.99 (m, 1, H_8), 4.50 (d, 1, H_7) $J_{7,8}$ = 3.8 Hz), 4.60 (m, 1, H_9), 5.49 (m, 1, H_{10}), 8.10–8.54 (m, 8, ArH) ppm. Trituration of the insoluble beige solid with 3 mL of THF and 3 mL of ether afforded *anti-*

BPDE-III (100 mg) as a beige solid (TLC: one spot R_f 0.32), mp 197.5–198.5 °C: the NMR spectrum was in agreement with that reported for the *anti*-isomer (Thakker et al., 1978). NMR (DMSO-d_6) δ 3.93 (dd, 1, H_8), 4.14 (d, 1, H_9), 4.48 (d, 1, H_7), 515 (d, 1, H_{10}), 5.70 (d, 1, OH), 5.86 (d, 1, OH), 8.09–8.33 (m, 8, ArH) ppm; $J_{7,8}$ = 4.5 Hz; $J_{8,9}$ = 1.6 Hz; $J_{9,10}$ = 7.4 Hz; the OH protons exchanged with D_2O.

Salmon sperm DNA (Sigma Chemicals, St. Louis, MO) was purified and quantitated as previously described (MacLeod & Zachary, 1985). Enzymes were obtained from Sigma Chemicals (St. Louis, MO); poly(dGuo-dCyt) was from Pharmacia Biotechnology (Piscataway, NJ), and poly(dAdo-dThy) was from Boehringer Mannheim (Indianapolis, IN). All other chemicals used were analytical reagent grade or better.

Preparation of Adducts. Salmon sperm DNA was dissolved in 10 mM Tris, pH 7.4, at 100 μg/mL. BPDE-III was dissolved in dry tetrahydrofuran; concentrations were determined spectrophotometrically (MacLeod & Selkirk, 1982). Small-scale reactions were initiated by adding 1/20 volume of an appropriate BPDE-III stock (generally 1.5–2.5 mM) to DNA at room temperature under subdued lighting. For large-scale preparation of adducts, 1/10 volume of BPDE-III was routinely used. A total of 74 mg of DNA was treated with 55.5 μmol of BPDE-III to prepare adducts for NMR analysis. After 6 or 24 h, NaCl was added to 0.1 M, hydrolysis products and unreacted diol epoxide were removed by extraction with ethyl acetate and ether, and the DNA was precipitated by the addition of 2.5 volumes of ethanol at –20 °C. To remove the last straces of noncovalently bound hydrolysis products, the DNA precipitate was rinsed twice with ether before dissolving in 10 mM Tris, pH 7.4. Adduct levels in the repurified DNA were estimated from the absorbance at 352 nm (MacLeod et al., 1991a).

Previously developed enzymatic methods for obtaining the adducts as deoxyribonucleosides (Baird & Brookes, 1973; MacLeod et al., 1982; MacLeod et al., 1991a) were modified to limit the quantities of nucleases needed and to shorten the length of time that the adducts were subjected to elevated temperature (37 °C). Modified DNA (240 μg/mL) was first digested 3 h with DNase I (45 units/mL) at 37 °C. The pH was raised by the addition of 1/20 volume of 1.0 M Tris, pH 9.0, and digestion continued for 4 h with venom phosphodiesterase (0.33 μg/mL) and then overnight with bacterial alkaline phosphatase (11.7 μg/mL). The digests were adjusted to 20% methanol, and the adducts were adsorbed to SepPak C_{18} cartridges and eluted in 100% methanol (MacLeod et al., 1982). Control experiments indicated quantitative recovery of the adducts by this method (MacLeod et al., 1991a).

HPLC analysis and adduct preparation were carried out on an Aquapore RP-300 column as described for BPDE-I adducts (MacLeod et al., 1988).

NMR and Mass Spectral Analysis. Samples (approximately 60–110 μg) were dissolved in approximately 0.6 mL of Me_2SO-d_6 or methanol-d_4 (99.96 atom % ^2H) and placed in a 5-mm sample tube. ^1H NMR spectra were recorded at 500.13 MHz using a Bruker AM500 spectrometer. Chemical shifts are reported on the δ scale by assigning the residual proton signal of the Me_2SO peak to 2.49 ppm or the methanol peak to 3.30 ppm. Typical data acquisition conditions for 1-D ^1H NMR spectra were as follows: sweep width, 7042 Hz; pulse width, 80°; data size, 32K; relaxation delay, 0; and acquisition time, 2.33 s. In some cases, the residual solvent resonance was presaturated, and in other cases selective saturation or decoupling was performed. The number of scans typically ranged from 800 to 1400. Measurements were made at ambient temperature unless indicated otherwise. Data were generally processed with resolution enhancement using a Gaussian window function (LB = –0.5; GB = 0.17). For coupling constant measurements, data were zero-filled to 64K before processing. Coupling constants are first-order measurements, except for the case of adduct **1**, where spectral simulation using Bruker software was used to aid in key coupling constant measurements. COSY 2-D NMR spectra were also obtained in selected cases.

Fast atom bombardment (FAB) mass spectra were acquired using a Finnigan MAT TSQ-70 equipped with an Ion Tech atom gun. FAB ionization was induced by Xe atoms accelerated to energies of 8–10 keV. FAB full-scan mass spectra were acquired by scanning the third quadrupole from m/z 200 to m/z 750. Normally, 25 scans were acquired per probe loading.

Nitrous Acid Deamination. HPLC-purified adducts were dissolved in 3.6 M sodium acetate buffer, pH 3.5, and treated with sodium nitrite as described (Shapiro & Pohl, 1968). Adducts were repurified from the reaction mixtures on Sep Pak cartridges and analyzed by HPLC. Spectrophotometric determinations of the deamination of deoxyguanosine (Shapiro & Pohl, 1968) under these conditions gave rate constants similar to literature values.

Analysis of DNA Damage. Preparations of supercoiled plasmid DNA were modified with BPDE-III at a final concentration of 20 μM as described above for 6 h at room temperature. The repurified DNA was dissolved in 10 mM Tris, pH 7.4, and aliquots were incubated at 37 °C for varying periods of time. The control and incubated DNAs were analyzed by electrophoresis on 0.8% agarose gels. Supercoiled and nicked DNA bands were quantitated by densitometry of the negatives obtained by photographing the ethidium bromide stained gels. A standard factor of 1.4 was applied to the density of the supercoiled band to correct for differential staining of supercoiled and nicked DNA (Lloyd et al., 1978).

Plasmid DNA was either mock-modified or modified with 10 or 20 μM BPDE-III or with 10 μM BPDE-I. A restriction fragment containing the 5′ end of the hamster APRT gene was prepared and end-labeled with ^{32}P by standard methods. The labeled fragment was denatured in formamide (95 °C, 2 min), with or without prior treatment with 1.0 M piperidine for 30 min at 95 °C, and immediately analyzed by electrophoresis on an 8% polyacrylamide/8 M urea gel. Gels were dried and exposed to X-ray film to visualize the labeled bands at single-nucleotide resolution. Alternatively, the dried gels were exposed to PhosphorImager screens, and radioactivity in individual bands was quantitated after scanning on a PhosphorImager 400A (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Preliminary Studies of BPDE-III Adducts. Numerous spectroscopic studies and theoretical computations based on NMR-derived distance constraints support a model for the conformation of the major DNA adduct derived from BPDE-I in which the pyrene moiety lies in the minor groove of the DNA (Cosman et al., 1992) at an angle of about 45° with respect to the long axis of the double helix. Previous spectroscopic studies of BPDE-III-modified DNA indicated a different conformation for these adducts (MacLeod et al., 1982). To clarify this difference, BPDE-III-modified salmon sperm DNA (absorbance spectrum shown in Figure 1A) was analyzed by linear dichroism (LD), using the hydrodynamic

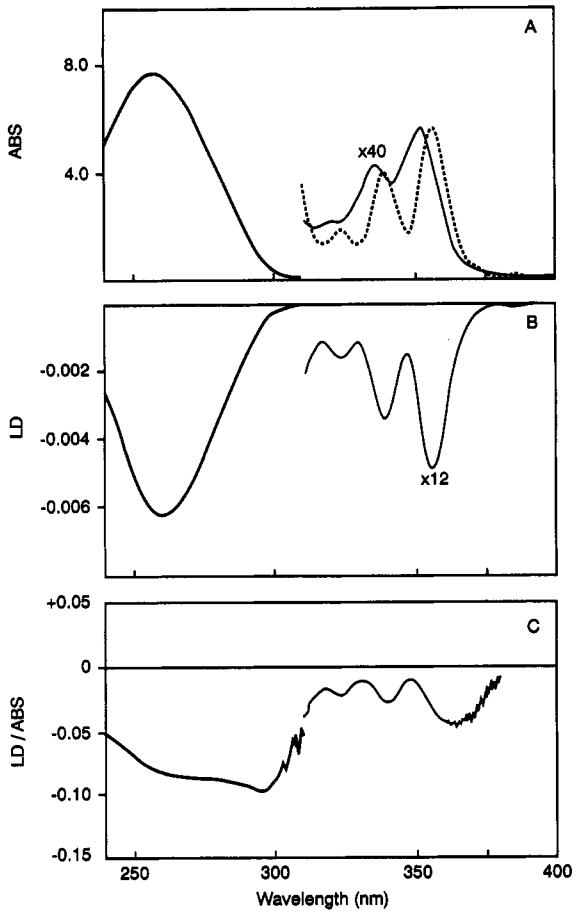


FIGURE 1: Absorption and linear dichroism of BPDE-III-modified DNA. Salmon sperm DNA was modified with BPDE-III and repurified as described in Materials and Methods. This treatment removes essentially all noncovalently bound hydrolysis products, leaving only covalent adducts. (A) The absorbance spectrum (ABS) was measured in a 1.0-cm cell (solid line). The dashed line represents the inverted LD spectrum from panel B, with the inverted LD maximum at 356 nm normalized to the height of the absorption maximum at 352 nm. (B) The linear dichroism signal of the same sample was measured with a 0.1-cm optical path length; the vertical scale is in absolute LD units. Because of limitations in the dynamic range of the LD apparatus, the data below 310 nm was determined with a solution diluted by a factor of 10. (C) The reduced linear dichroism (LD/ABS) was calculated from the data in panels A and B.

flow gradient of a Couette cell to orient the long axis of the DNA molecules as previously described (Geacintov et al., 1987; Geacintov, 1988). The LD signal (defined as the absorbance of light polarized parallel to the direction of flow minus the absorbance of light polarized perpendicular to the flow) was negative in the region of the DNA absorption band (Figure 1B) and resembled an inverted DNA absorption spectrum as expected, since the bases tend to be oriented with their planes perpendicular to the flow lines (Geacintov, 1988). The LD signal at wavelengths above 310 nm, where the pyrenyl chromophore absorbs, was also negative in sign (Figure 1B), with rather sharp minima at 324, 339, and 356 nm. The negative LD spectrum indicated that the planar pyrenyl residues tend to be roughly parallel to the planes of the DNA bases, suggesting a conformation that may be close to but not necessarily identical to that of a classical intercalation complex.

If there were only one type of adduct with a defined orientation, the negative LD spectrum would have been expected to resemble the absorption spectrum of the pyrenyl chromophore; the latter, however, exhibited broader absorption bands with maxima at 335 and 352 nm (Figure 1A). The

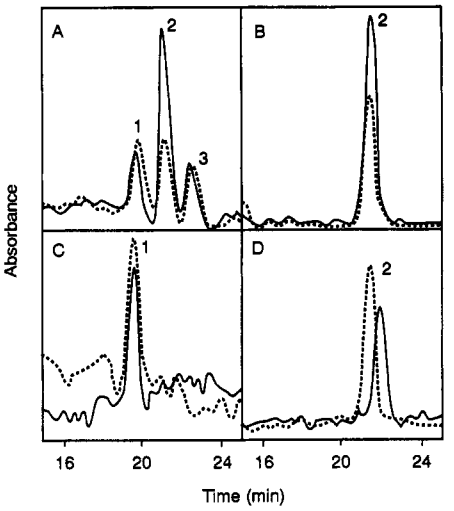


FIGURE 2: HPLC analysis of BPDE-III–DNA adducts. Adducts were prepared by enzymatic digestion of DNA treated with BPDE-III for 6 h at room temperature as described in Materials and Methods and analyzed by HPLC using an Aquapore RP-300 column operated at 35 °C. (A) The purified DNA was subjected to enzymatic digestion either immediately (solid line) or after incubation at 37 °C for 24 h (dashed line). (B) Material released from the DNA following incubation at 37 °C for 24 h (dashed line) or 72 h (solid line) and soluble in ethyl acetate was also analyzed. (c) Adduct peak 1 was collected and treated with nitrous acid in sodium acetate buffer (dashed line) or with buffer only (solid line) before rechromatography. (D) Adduct peak 2 was collected and treated with nitrous acid in sodium acetate buffer (dashed line) or with buffer only (solid line) before rechromatography.

spectral differences are particularly evident when the LD spectrum is inverted and normalized (Figure 1A, dotted line) for direct comparison. These characteristics point to a heterogeneous distribution of adduct conformations, such as has previously been observed with BPDE-I–DNA (Geacintov et al., 1984) and BPDE-I–polynucleotide adducts (Roche et al., 1991). The reduced LD signal above 310 nm (LD/absorbance, Figure 1C) confirmed that the adduct conformations are heterogeneous; if a single, homogeneously oriented adduct were present, the reduced LD signal would be constant as a function of wavelength (Geacintov et al., 1987). Instead, the reduced LD signal exhibits negative extrema at 323, 340, and 360 nm. These LD data indicate that there are at least two different adduct conformations in BPDE-III-modified DNA and raise the possibility that several chemically heterogeneous adducts may be formed from BPDE-III.

To test this possibility, BPDE-III was reacted with DNA for 6 h at room temperature and adducts were prepared by enzymatic digestion to the deoxyribonucleoside level. Three major adducts were separable by reverse-phase HPLC (Figure 2A). Analytical HPLC runs of several digests derived from DNA reacted with BPDE-III for 6 h gave 20.2 ± 1.9% adduct **1**, 56.5 ± 2.8% adduct **2**, and 23.4 ± 3.2% adduct **3**. The retention times of these adducts were similar to those of BPDE-I–dGuo adducts in the same system and dissimilar to the retention times of dAdo adducts (MacLeod et al., 1988). Furthermore, HPLC analysis using fluorescence detection also gave no evidence for late-eluting, dAdo adducts (data not shown). We have recently shown that fluorescence detection dramatically increases the sensitivity for BPDE-I–dAdo adducts relative to BPDE-I–dGuo adducts (Chen et al., 1993). This suggested that the major BPDE-III adducts might be modified dGuo residues. Spectrophotometric determinations of covalent binding to nucleic acids of varying base compositions were consistent with this suggestion. Absorbance spectra of BPDE-III covalently bound to salmon sperm DNA

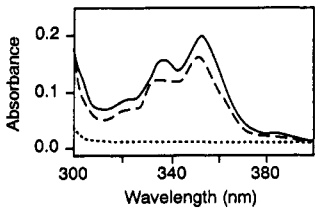


FIGURE 3: Covalent binding of BPDE-III to nucleic acids. Solutions of salmon sperm DNA (solid line), poly(dGuo-dCyt) (dashed line), or poly(dAdo-dThy) (dotted line), each at a concentration of 100 μg/mL in 10 mM Tris, pH 7.4, were allowed to react with BPDE-III (75 μM) for 24 h at room temperature. The nucleic acids were repurified as described in Materials and Methods and dissolved in 10 mM Tris, pH 7.4, and absorbance spectra were recorded from 300 to 400 nm. Assuming an extinction coefficient of 29 500 for the covalent adduct (MacLeod et al., 1991a), the fraction of added BPDE-III that was recovered as adduct was calculated (see text). Expansion of the absorbance scale for the modified poly(dAdo-dThy) preparation did not reveal any adduct-specific absorbance above background noise.

and to the alternating copolymers poly(dGuo-dCyt) and poly-(dAdo-dThy) under identical reaction conditions are shown in Figure 3. Levels of covalent binding of BPDE-III to poly-(dGuo-dCyt) and to salmon sperm DNA were similar (3.4 and 4.1% of added BPDE-III bound to the nucleic acid, respectively) while binding to poly(dAdo-dThy) was not detected (<0.1% of added BPDE-III bound).

When BPDE-III-treated DNA was held at 37 °C for increasing periods of time and then extracted with ethyl acetate prior to enzymatic digestion, the relative amount of adduct peak 2 that was recovered from the digest progressively decreased (Figure 2A); the apparent half-life for this change was about 24 h. When the ethyl acetate extract of the BPDE-III-treated, 37 °C incubated DNA was analyzed, a single peak coincident with adduct peak 2 was obtained (Figure 2B). The amount of peak 2 recovered in the ethyl acetate extract increased with increasing incubation time at 37 °C. This lability to mild heating suggested the possibility that peak 2, which represented over 50% of the adducts in the unheated sample, might represent an adduct formed at the N7 position of dGuo.

To test this, the three adduct peaks were isolated by HPLC and subjected to deamination with nitrous acid in sodium acetate buffer (Shapiro & Pohl, 1968). The HPLC retention times of the nitrous acid treated adducts were compared with the retention times of adducts treated with buffer only. The rationale for this was the fact that nitrous acid deamination only occurs if the N^2 of dGuo is unsubstituted (Koreeda et al., 1976). The adduct obtained from peak 1 did not demonstrate an altered retention time after treatment with nitrous acid (Figure 2C), suggesting that the adduct was formed at the exocyclic amino group. Adduct peak 2 (Figure 2D) exhibited a significant change in its retention time after nitrous acid treatment, consistent with the possibility that it was not derived from addition to the exocyclic amino group, but from adduction at some other position, possibly N7. Adduct peak 3 exhibited multiple peaks with altered retention times after nitrous acid treatment (data not shown), again suggesting that it was not an N^2 adduct.

Identification of Three Major Adducts. To obtain direct evidence for the nature of these adducts, large-scale reactions were carried out and enzymatically digested to prepare sufficient quantities of adducts for NMR and mass spectral analysis. The overall yields (as percent BPDE-III added) after HPLC purification were about 0.2% for adducts **1** and **3** and 0.4% for adduct **2**. ^{1}H NMR spectra of the adducts were obtained at 500.13 MHz in both Me_2SO-d_6 and methanol-d_4. The Me_2SO spectrum of adduct **1** is shown in Figure 4, and spectral parameters are listed in Table 1. All 12 nonexchangeable resonances from the 7,8,9,10-tetrahydro-8,9,10-trihydroxybenzo[a]pyrene moiety (BPTT) were detected, as well as four exchangeable protons that were vicinal to H7, H8, 89, and H10 (Figure 4). The assignments were based primarily on homonuclear decoupling experiments involving vicinal and long-range coupling constants, NOEs, and the disappearance of the exchangeable protons following addition of a trace of D_2O. A description of the general strategy used for making resonance assignments has previously been given for the case of mercaptopurine adducts derived from BPDE-I (MacLeod et al., 1991c). Several additional approaches were used here. A routine COSY spectrum aided in resonance assignments and enabled measurement of chemical shifts in several congested regions (e.g. H8, H9, H3′). The distinction between the assignment of H1 and H3 of the BPTT moiety is based on an NOE experiment involving selective saturation of H4.

The most important assignments were those of H7 and H8, as well as the protons to which they were coupled, because C7 and C8 are the candidate sites for the attachment to the nucleic acid base. The data clearly show that both H7 and H8 exhibit vicinal couplings to an exchangeable proton (Table 1). For H7, the exchangeable proton is at 6.80 ppm (J = 9.2 Hz),

Table 1: ^{1}H NMR Spectral Parameters for C7-Substituted BPDE-III 2′-Deoxyguanosine and Guanine Adducts[a]

	chemical shift (ppm) (coupling constant (Hz))		
assignment	**1** (N^2-dGuo)	**2** (N7-G)[b]	**3** (C8-dG)[c]
1	8.29 (7.7)	8.31 (7.7)	8.27 (8.1)
2	8.04	8.05	8.01
3	8.24 (7.7)	8.25 (7.5)	8.21 (7.7)
4	8.11 (9.0)	8.06 (9.0)	8.02 (9.2)
5	8.07	7.94	7.91
6	8.17	7.49	7.49
7	5.72 (9.1)[d]	5.82 (9.2)[d]	4.97 (10.3)[d]
8	4.23 (2.2)[d]	4.90 (2.2)[d]	
9	4.24 (3.5)[d]	4.33 (3.9)[d]	4.22 (3.4)[d]
10	5.47	5.58	5.49
11	8.50 (9.5)	8.53 (9.5)	8.51 (9.3)
12	8.26	8.30	8.25
OH-8	5.14 (5.4)[e]	5.30 (6.2)	
OH-9	5.31 (3.6)	5.39 (3.9)[e]	
OH-10	5.77 (6.2)	5.18 (10.1)	
1′	6.18 (6.2; 7.7)		
2′	2.59		
2″	2.18		
3′	4.25		
4′	3.74		
5′[h]	3.48		
5″[h]	3.50		
G8[f]	7.98	8.48	*g*
OH-3′	5.19 (4.1)		
OH-5′	4.83 (5.4)		
NH/NH_2-2	6.80 (9.2)[e]	6.14	
NH-1	10.59	10.45	10.60

[a] Measurements were made at approximately 29 °C with Me_2SO-d_6 as solvent, except as indicated below. Vicinal coupling constants in Hz are shown in parentheses, as available. Resonance overlap prevented first-order measurements in several cases. Only one vicinal coupling constant for each coupled pair of protons is listed. [b] Assignments for major subspectrum. [c] Structure identification tentative. Listed spectral parameters represent partial analysis. [d] The coupling constants shown were obtained with methanol-d_4 as solvent because the parameters were less difficult to measure than in Me_2SO-d_6. For adduct **1**, the coupling constants were determined by spectral simulation using final values of 4.465 and 4.472 ppm for the chemical shifts of H8 and H9, respectively. [e] Estimated value due to resonance overlap or line broadening. [f] C8 proton of guanine ring. [g] Extensive attempts to locate a possible C8 resonance of the guanine ring using methanol-d_4 as well as Me_2SO-d_6 as solvents failed to show evidence of this proton. [h] Assignments may be reversed.

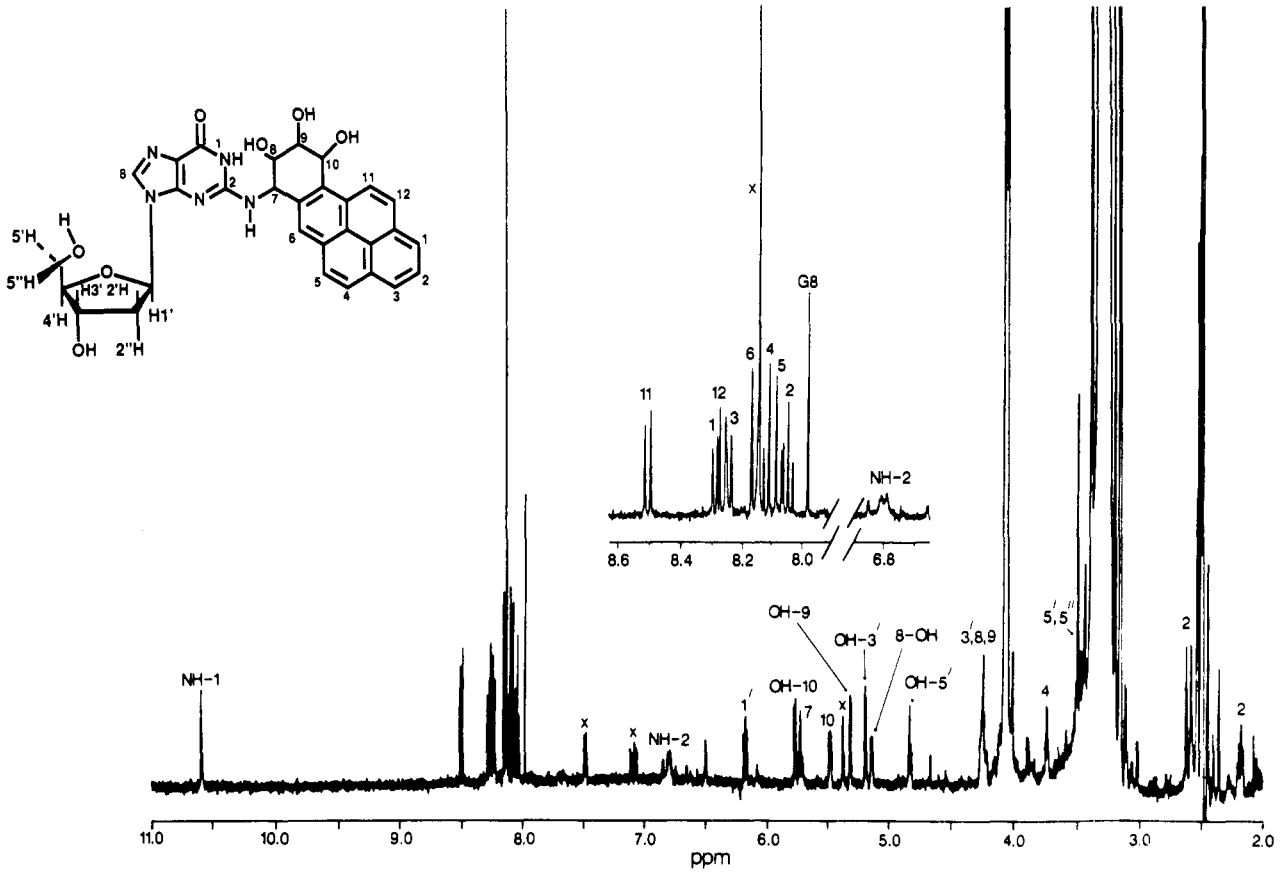


FIGURE 4: ^{1}H NMR spectrum of adduct **1**. The 500-MHz spectrum of adduct **1** in Me_2SO-d_6 at 29 °C is shown with resonance assignments. The inset is an expansion of the most congested region. The large singlet at about 8.17 ppm was determined to be an impurity on the basis of integration in a spectrum recorded under quantitative conditions and on the basis of its appearance in the other samples.

characteristic of an NH group (Jeffrey et al., 1976b; Roy et al., 1991), while for H8 it is at 5.14 ppm (J = 5.4 Hz), characteristic of an OH group (MacLeod et al., 1991c; MacLeod et al., 1993). Thus, the data indicate attachment of an NH group at C7 and the presence of an OH group at C8 of BPTT. The magnitude of J_{7-8} (9.1 Hz) shows that H7 and H8 (and, therefore, the OH and nucleic acid base substituents at the same positions) are *trans* (Table 1). This coupling constant, as well as J_{8-9} and J_{9-10}, are similar in magnitude to those previously reported for BPDE-I adducts substituted at C10 (MacLeod et al., 1991c; MacLeod et al., 1993; Cheng et al., 1989), suggesting the same type of *trans–cis–trans* configuration for the tetrahydro ring. In addition, it indicates that the time-averaged conformation of the tetrahydro ring is similar to that in BPDE-I adducts, even though the nucleic acid base is substituted at the bay region in the BPDE-I adducts.

Other spectral parameters indicate that the NH substituent at C7 of BPTT arises from the N^2 position of 2′-deoxyguanosine. A nonexchangeable singlet at 7.98 ppm and an exchangeable singlet at 10.59 ppm are characteristic of 2′-deoxyguanosine-derived C8 and NH-1 resonances (Jeffrey et al., 1976b; Roy et al., 1991). A total of seven additional aliphatic protons and two exchangeable protons with spectral parameters characteristic of a 2′-deoxyribose ring (Evans et al., 1980) indicate that the sugar is present and that substitution must be through the base. The appearance of the resonance for an NH group rather than an NH_2 group clearly shows that the substitution is through N^2 of the guanine ring. As noted above, this exchangeable proton is coupled to H7 of the BPTT moiety. We conclude that adduct **1** is *trans*-7-(deoxyguanosin-N^2-yl)-8,9,10-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Figure 4). The results of mass spectral analysis were consistent with this interpretation. A cationized molecular ion was obtained at m/z 592; this would be expected for an N^2-substituted adduct + Na^+.

If adduct peak 1 was composed of two adducts derived from the reaction of both the (+) and (−) enantiomers of BPDE-III, it would of course be diastereomeric due to the 2′-deoxyribose. Although the spectral differences between such diastereomers are subtle (Jeffrey et al., 1976b; Cheng et al., 1989; Chadha et al., 1989; Roy et al., 1991; Sayer et al., 1991), it is likely that they would also be detectable for BPDE-III adducts by high-field ^{1}H NMR. However, careful examination of each of the resonances of adduct **1** in expanded form in resolution-enhanced spectra gave no indication of such extra resonances (data not shown). Considering the purity of the sample and the signal to noise ratio of the spectrum, it is estimated that a second *trans* diastereomer would have been detected if present at a level in excess of 15%. Furthermore, there was no chromatographic evidence for heterogeneity of this adduct. The chromatographic system used routinely allows separation of diastereomeric, N^2-dGuo adducts derived from BPDE-I (MacLeod et al., 1988; MacLeod et al., 1989). These data strongly suggest that the reaction of BPDE-III with N^2-dGuo in double-stranded DNA is enantiomerically selective, producing predominantly a single diastereomer.

The ^{1}H NMR spectrum of adduct **2** in Me_2SO was conspicuously different from that of adduct **1**, in part because of the lack of resonances attributable to a 2′-deoxyribose ring (Figure 5). This suggested the possibility that the BPTT might be attached only to a nucleic acid base. Analysis of the spectrum, according to procedures similar to those described

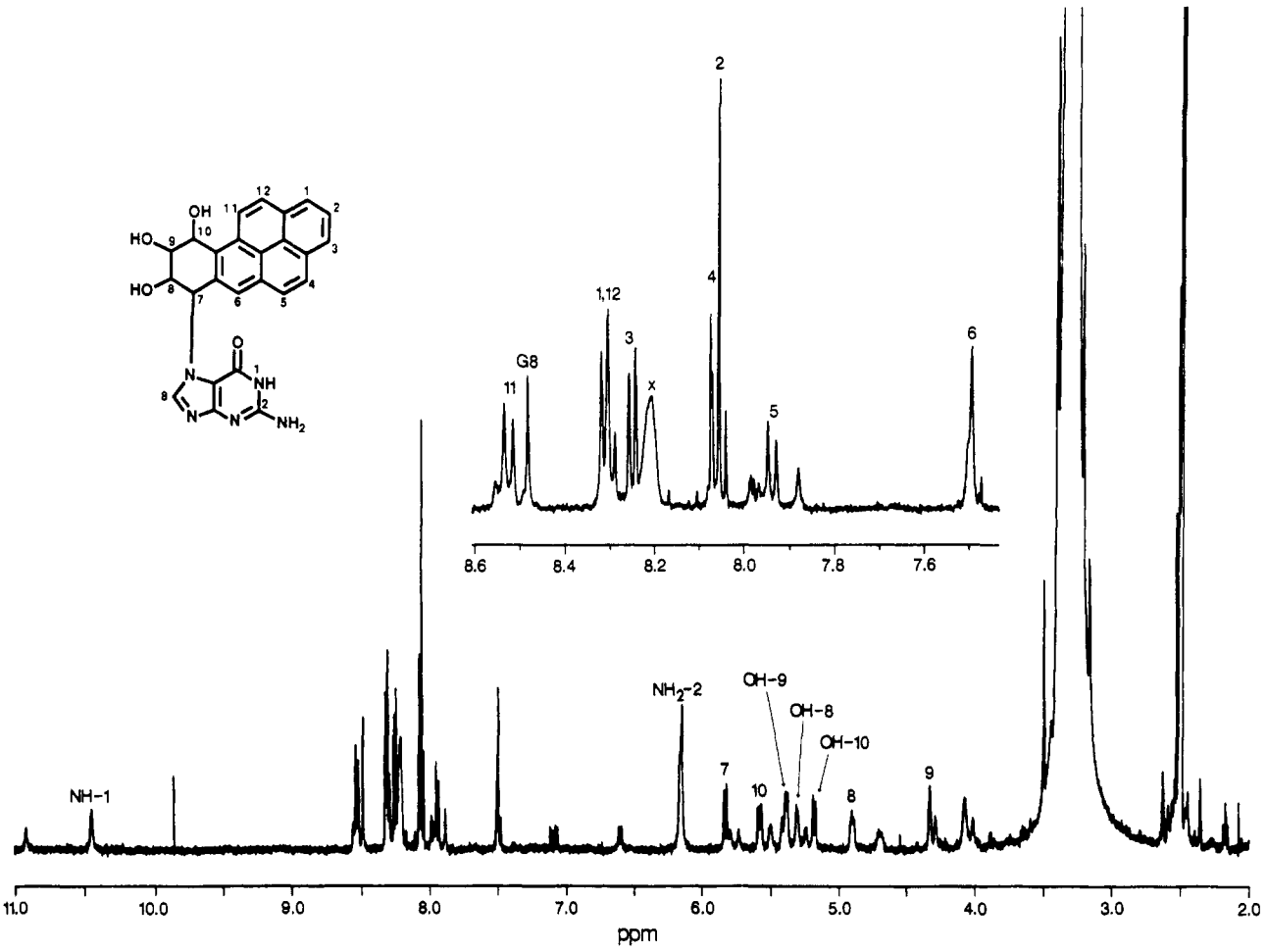


FIGURE 5: ^{1}H NMR spectrum of adduct **2**. The 500-MHz spectrum of adduct 2 in Me_2SO-d_6 at 29 °C is shown with resonance assignments. The inset is an expansion of the most congested region.

above, revealed the presence of all 12 nonexchangeable protons of BPTT. However, unlike adduct **1**, there were only three resonances from exchangeable protons of BPTT and no exchangeable proton was coupled to H7. This showed that substitution had again occurred at C7 but that there was no exchangeable proton vicinal to H7. The coupling constants of the tetrahydro ring indicated the same *trans–cis–trans* configuration and a similar conformation as that found in adduct **1** (Table 1). Three additional resonances could be attributed to the nucleic acid base. Exchangeable singlets at 10.45 and 6.14 ppm were assigned to NH-1 and NH_2-2 of the guanine ring, respectively. The singlet at 8.48 ppm, which was nonexchangeable, was assigned to the guanine C8 proton. The presence of these protons indicates that the guanine ring cannot be substituted at C8, N1, N^2, or O^6 and strongly suggests that adduct **2** is 7-(guanin-7-yl)-8,9,10-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-III–N7-Gua; Figure 5).

There are several characteristics of the ^{1}H NMR spectrum of adduct **2** that deserve special comment. The spectrum contains several relatively small resonances. Although some of these are impurities, others are actually subspectra. The equilibrium between two distinct forms is demonstrated by negative cross peaks between corresponding resonances in a phase-sensitive NOESY spectrum recorded at 29 °C with Me_2SO as solvent (mixing time = 300 ms, data now shown). The assignments that are reported are those of the major subspectrum (Table 1). It is likely that there is restricted rotation at the site of attachment of the carcinogen to the nucleic acid base. Minimum-energy conformations would be expected to occur with the BPTT rotated away from the guanine ring in order to reduce unfavorable steric interactions between the guanine ring and the BPTT. One characteristic of such a conformation is that the H6 proton of BPTT would experience an upfield shift due to the ring current field of the guanine ring. An unusual upfield shift of H6 was noted. The assignment of H6 has been confirmed by NOE experiments (data not shown). A more detailed study of the conformation and dynamics is in progress.

Alternatively, it is conceivable that adduct **2** has been isolated as a ring-opened derivative of BPDE-III–N7-Gua. By analogy to the well-studied aflatoxin B1–dGuo adducts, attack of hydroxyl ion at the C8 position might lead to cleavage of the C8–N9 bond, resulting in a formamidopyrimidine (FAPY) derivative. The subspectra seen in the NMR analysis would in that case result from the partial double-bond character of the C8–N7 bond. Three pieces of evidence argue against this possibility. First, the postulated ring opening would lead to a second NH_2 group at the N9 position; there was no evidence for this in the NMR spectra. Second, FAB mass spectra of adduct **2** gave prominent molecular ions at m/z 454 and 476, corresponding to $(M + H)^+$ and $(M + Na)^+$, respectively. The postulated ring-opened products would be 18 mass units heavier than this due to the addition of H_2O, and corresponding ions were not detected above background. Finally, when BPDE-III-modified DNA was treated at 37 °C for 60 min in sodium carbonate buffer, pH 9.5, conditions which quantitatively cause ring opening of an aflatoxin B1–N7-dGuo adduct (Hertzog et al., 1987), subsequent release of adduct **2** by mild heating (48 h at 37 °C, neutral pH) was

inhibited by 50–60%. Furthermore, when adducts were prepared from this base-treated DNA by enzymatic digestion, adduct **2** was found in greatly reduced amounts, but several peaks with novel retention times were seen (data not shown). These data are inconsistent with the suggestion that adduct **2** was isolated as a ring-opened form.

Preliminary NMR experiments have been conducted on adduct 3. It was possible to locate all 12 nonexchangeable protons of the BPTT moiety (Table 1). In addition, the coupling constants for the tetrahydro ring indicated a *trans–cis–trans* addition and a similar conformation as for the other two adducts. These data strongly suggested another C7-substituted adduct. The ^{1}H NMR spectrum was complex due to chemical exchange (data not shown), and the sample was relatively unstable in Me_2SO, limiting the analysis. As a result many of the peaks are unassigned. An extensive search for a possible singlet attributable to the C8 proton of the Gua ring was conducted using both methanol-d_4 and Me_2SO-d_6 as solvents and, in the case of methanol, by conducting measurements as a function of temperature. There was no evidence of a C8 proton in any NMR spectrum, and we tentatively assign this adduct as a C8-substituted dGuo adduct, with the substitution being directly at C7 of BPTT. The unusual upfield shift of H7 compared to the cases of the other two adducts supports this proposal (Table 1). This upfield shift can be explained, on the basis of comparison with model compounds (Chamberlain, 1974), as a substituent effect from attachment to a carbon (C8) instead of a nitrogen (N7 or N^2). Had the adduct been an N7-substituted guanosine nucleoside, a downfield shift of the H7 resonance from the BPTT moiety might have been expected due to the positive charge in the adjacent guanine ring. In fact, the H7 resonance of adduct **2** was observed to shift downfield slightly upon addition of acid (data not shown). The chemical exchange may be a consequence of restricted rotation at the site of attachment of the carcinogen to the nucleic acid base, like that of the N7-substituted adduct. The sugar resonances for adduct **3** were not readily observable at ambient temperature due to the chemical exchange. A similar phenomenon has been described in an N^2-substituted guanosine adduct of a pyrene derivative (Evans et al., 1993). The lack of a singlet referred to above is also inconsistent with peak 3 being due to a FAPY derivative. If this adduct were ring-opened, the resonance from the formyl proton (or resonances due to *cis–trans* isomerism about the amide bond) should have been found (Beranek et al., 1983; Tomasz et al., 1987; Humphreys and Guengerich, 1991). In no case was there rapid deuterium exchange of the formyl proton. Furthermore, FAPY adducts still containing a sugar moiety have exhibited additional resonances for each proton (Tomasz et al., 1987), which is also inconsistent with our NMR spectra.

Analysis of Single-Strand Breaks and Alkali-Sensitive Sites. Previous studies have indicated that loss of labile N7-dGuo adducts in DNA generates apurinic sites which can be cleaved by hot alkali (Haseltine et al., 1980; Osborne et al., 1981). Furthermore, studies of supercoiled plasmid DNA treated with BPDE-III indicated the presence of single-strand breaks in the absence of heat or alkali treatments (Tang et al., 1992); these were not seen with other diol epoxides that react primarily with the exocyclic amino group. Both single-strand breaks and alkali-sensitive sites could derive from the breakdown of adduct peak 2 in BPDE-III-treated DNA. Single-strand breaks in BPDE-III-treated, supercoiled plasmid were analyzed by agarose gel electrophoresis with photographic quantitation of the relative amounts of supercoiled and relaxed

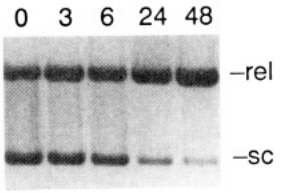


FIGURE 6: Analysis of single-strand breaks in BPDE-III-modified plasmid DNA. DNA from a plasmid (pGAZ-22) containing a portion of the hamster APRT gene was modified with BPDE-III to a level of 13 adducts per plasmid as determined by absorbance measurements. This modified DNA was held at 37 °C in 10 mM Tris, pH 7.4, for increasing times before aliquots were analyzed by electrophoresis in 0.8% agarose gels. The ethidium bromide-stained gel was photographed, and the negative was scanned with a digital camera (Milligen BioImage, Ann Arbor, MI). The relative amounts of DNA in the supercoiled (sc) and relaxed (rel) bands were quantitated with BioImage whole band software.

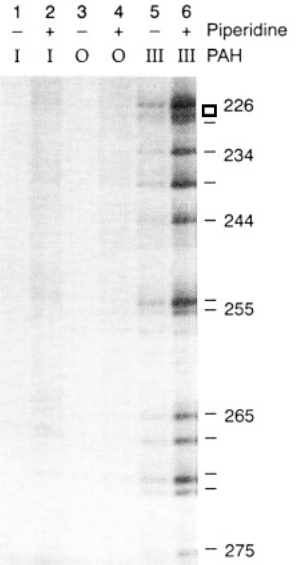


FIGURE 7: Analysis of DNA damage in BPDE-III-treated DNA. A fragment of the hamster APRT gene was prepared from a restriction digest of plasmid DNA modified with 20 μM BPDE-I (lanes 1 and 2), or with 10 μM BPDE-III (lanes 5 and 6) or mock-modified with solvent only (lanes 3 and 4). The gel-purified fragment was end-labeled with ^{32}P in the coding strand. Samples were either treated with piperidine (lanes 2, 4, and 6) or left untreated (lanes 1, 3, and 5) before being analyzed by gel electrophoresis. The region of the gel containing the promoter is shown with horizontal tic marks at each dGuo residue in the sequence.

DNA. When DNA initially containing an average of 13 BPDE-III adducts/molecule was analyzed (Figure 6, lane 1), about half of the molecules were supercoiled; mock-modified DNA was about 80% supercoiled (data now shown). This relaxation of a portion of the BPDE-III-treated molecules has been seen in previous studies and is not seen with other diol epoxides of benzo[*a*]pyrene (Tang et al., 1992). When the BPDE-III-modified DNA was incubated at 37 °C prior to electrophoresis, the apparent fraction of nicked molecules increased (Figure 6, lanes 2–5). The time course for the conversion of supercoiled to relaxed molecules was similar to that of release of adduct peak 2 from the DNA at the same temperature.

To determine the distribution of single-strand breaks and alkali-sensitive sites, ^{32}P-end-labeled recombinant DNA was treated with BPDE-III and analyzed on sequencing gels; as controls, the same DNA was either treated with BPDE-I or left untreated. As seen in Figure 7, lane 5, without further treatment the BPDE-III-treated DNA exhibited a series of bands at every dGuo residue which were not seen in the

untreated (lane 3) or BPDE-I-treated (lane 1) DNA. Since this DNA was not exposed to alkali, but simply denatured with formamide, these evidently represent single-strand breaks present in the treated DNA molecules. The intensity of all of the dGuo-specific bands increased when the samples were treated with piperidine to hydrolyze N7-dGuo adducts and cleave apurinic sites (lane 6). Again, this effect was specific to BPDE-III-treated DNA and did not occur to an appreciable extent in the controls (lanes 2 and 4). For a series of 15 dGuo-specific bands between positions 202 and 275 after piperidine treatment, the fraction of total radioactivity in individual bands varied by about a factor of 3 from the most intensely labeled to the least intensely labeled band. Similar sequence-dependent differences between adduction levels were seen previously using a DNA repair enzyme to detect the adducts (Tang et al., 1992). The overall increase in intensity of these dGuo-specific bands upon piperidine treatment was about fourfold.

DISCUSSION

The major DNA adducts derived from diol epoxides of several polycyclic aromatic hydrocarbons have been identified and in virtually all cases involve the exocyclic amino groups of either dGuo or dAdo or both (Jeffrey et al., 1976a; Koreeda et al., 1976; Jeffrey et al., 1979; Melikian et al., 1984; Osborne et al., 1986; Reardon et al., 1987; Pruess-Schwartz et al., 1987; Agarwal et al., 1987; Chadha et al., 1989; Cheng et al., 1989; Sayer et al., 1991; Jerina et al., 1991; Cheh et al., 1993). An N7-Gua adduct has been found to constitute about 15% of the DNA adducts derived from a bay-region diol epoxide of benz[a]anthracene (Cheh et al., 1993). Although a labile N7-dGuo adduct derived from BPDE-I has been described (Osborne et al., 1981; RamaKrishna et al., 1992), it is generally considered a minor adduct in DNA treated with BPDE-I. The relative occurrence of single-strand breaks and alkali-sensitive sites in BPDE-I- and BPDE-III-treated DNA seen in previous studies (Tang et al., 1992) and analyzed in more detail in the present study (Figure 6) clearly indicate that labile N7-dGuo adducts are at best a minor feature of BPDE-I-treated DNA but are major components of BPDE-III-treated DNA, amounting to 56.5% of the damage present after 6 h of reaction. This represents a slight underestimate of the relative amount of the N7 adduct formed, due to the lability of this adduct during the reaction. However, direct measurements of the lability at room temperature (data not shown) suggest that the correction for this is no more than about 5%.

Our studies of adduct stability (Figure 2) and stability of DNA damage sites (Figures 6 and 7) indicate that the major adduct, BPDE-III–N7-dGuo, breaks down in vitro primarily to give apurinic sites, but that formation of single-strand breaks also occurs. Although the rates of these conversions observed in vitro are fairly slow, it is possible that they are accelerated in vivo, either due to the chemical composition of the intracellular milieu or enzymatically by way of glycosylase activity. In vivo conversion of BPDE-III–N7-dGuo adducts to apurinic sites could in part explain some of the relative differences in biological effects between BPDE-I and BPDE-III previously described. For example, in bacterial cells BPDE-III adducts are much less toxic than BPDE-I adducts (Tang et al., 1992). Most apurinic sites are repaired in an error-free manner by the action of apurinic endonucleases and resynthesis (Lindahl, 1982). Furthermore, mutation spectra obtained in SOS-induced *Escherichia coli* with DNA containing apurinic sites indicate that the bacterial DNA polymerase is able to replicate past an apurinic site by insertion of dATP (Kunkel, 1984; Lawrence et al., 1990). This is in agreement with in vitro studies of the ability of bacterial polymerases to insert dATP opposite an apurinic site (Sagher & Strauss, 1983; Hevroni & Livneh, 1988). Thus, although apurinic sites derived from labile BPDE-III–N7-dGuo adducts might be expected to be mutagenic, they might have reduced cytotoxicity, in agreement with our experimental results (Tang et al., 1992). In contrast, DNA polymerases appear to have very little ability to replicate past a BPDE-I adduct in DNA (Moore & Strauss, 1979; Hruszkewycz et al., 1992; Shibutani et al., 1993), and in the absence of repair such adducts may be lethal. Indeed, in excision-repair deficient bacteria, a single BPDE-I adduct is sufficient to inactivate a duplex ΦX 174 DNA molecule (Tang et al., 1992). Alternatively, it may be that BPDE-III adducts do not represent a substantial block to bacterial DNA polymerases, a possibility pointed out previously (Tang et al., 1992).

In mammalian cells BPDE-III adducts appear to be more cytotoxic than BPDE-I adducts, although they are less mutagenic (MacLeod et al., 1991a). A recent mutation spectrum obtained in human lymphoblastoid cells with a plasmid containing apurinic sites indicates that, in contrast to the bacterial results, apurinic sites tend to induce short deletions (100–150 bp), whereas BPDE-I adducts tend to induce base substitutions (Klinedinst & Drinkwater, 1992). If BPDE-III–N7-dGuo adducts give rise to deletions with high frequency in mammalian cells by way of apurinic sites, an increased cytotoxicity might be expected to result.

Previous studies of the quenching of BPDE-III–DNA fluorescence by acrylamide (MacLeod et al., 1982) suggested a high degree of interaction between the pyrene moiety and the DNA. In addition, the absorbance spectrum of BPDE-III–DNA is similar to spectra of noncovalently bound pyrene-containing compounds and different from the spectrum of the major *trans*-N^2-dGuo adduct of (+)-BPDE-I (MacLeod et al., 1982). The solution structure of this adduct in DNA was recently shown to position the pyrene moiety in the minor groove of the DNA with little disruption to the helix (Cosman et al., 1992). The linear dichroism spectrum of BPDE-III–DNA is also dissimilar to that of BPDE-I–DNA and indicates the presence of at least a portion of the adducts in a quasi-intercalated conformation. Several bulky carcinogens that form N7- or C8-dGuo adducts, including aflatoxin B1 (Gopalakrishnan et al., 1990), sterigmatocystin (Gopalakrishnan et al., 1992), and *N*-acetylaminofluorene (AAF) (Shapiro et al., 1989; O'Handley et al., 1993), have spectroscopic properties analogous to those of the BPDE-III adducts and have been suggested to exist in quasi-intercalated conformations. In the case of aflatoxin B1 and sterigmatocystin, the presence of two saturated rings in the molecule imparts a flexibility that allows the adduct to intercalate 5′ to the base to which it is linked. However, in the case of the AAF adduct an *anti* to *syn* rotation of the glycosidic bond of the affected dGuo residue has been postulated to bring the fluorene rings into the helix, parallel to the planes of the base pairs (Shapiro et al., 1989). The fact that the majority of the BPDE-III adducts are at either C8 or N7 raises the intriguing possibility that a similar *anti* to *syn* rotation could bring the pyrene moiety into a conformation parallel to the base planes. This could explain the spectroscopic properties referred to above. The postulated rotation about the glycosyl bond might result from unfavorable steric interactions between the bulky carcinogen and the adjacent 3′-nucleotide (Evans et al., 1984). More detailed studies of the conformation of BPDE-III adducts will be necessary to determine whether either of these general

mechanisms is applicable.

As pointed out above, the major DNA adducts derived from the bay-region diol epoxides of a variety of polycyclic aromatic hydrocarbons almost without exception involve the exocyclic amino group of either dGuo or dAdo. In contrast to this, exocyclic amino moieties are relatively poor targets for most simple alkylating agents, which react preferentially at the N7 position of dGuo. Analogous N7-dGuo adducts also appear to be major derivatives of certain nitrogen mustards (Brookes and Lawley, 1961), platinum chemotherapeutic agents (Sherman et al., 1985), and aflatoxin B1 (Essigman et al., 1977). Examination of space-filling, CPK models of an isolated BPDE-III–N7-Gua indicated restricted rotation about the C7–N7 bond, due primarily to interactions between O^6 of the base and the H6 and OH7 groups of BPTT. However, when the analogous model was attempted with BPDE-I, severe overlap of the base O^6 with the H12 proton or the OH9 appeared to be inescapable. Such steric interference may be a general reason for the apparent low reactivity of bay-region diol epoxides of polycyclic aromatic hydrocarbons with N7-dGuo. It will be of interest to determine whether non-bay-region diol epoxides other than BPDE-III target the N7 position of dGuo.

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