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An Evaluation of the Mutagenicity, Metabolism, and DNA Adduct Formation of 5-Nitrobenzo[*b*]naphtho[2,1-*d*]thiophene

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Thioarenes, sulfur-containing polycyclic aromatic compounds, are environmental contaminants suspected of posing human health risks. In this study, 5-nitrobenzo[*b*]naphtho[2,1-*d*]thiophene (5-nitro-BNT), a nitrated-thioarene, was examined for its mutagenicity, metabolism and subsequent formation of DNA adducts. 5-Nitro-BNT was weakly mutagenic in *Salmonella typhimurium* strains TA98 and TA100 without Aroclor-1254-induced rat liver S9 (S9), and its activity was increased in the presence of S9. Anaerobic metabolism of 5-nitro-BNT by S9 or xanthine oxidase (XO) produced one major metabolite, identified as 5-amino-BNT by NMR, MS, and UV spectroscopy and by comparison with an authentic standard. Aerobic S9 metabolism of 5-nitro-BNT produced a major metabolite, identified as *trans*-9,10-dihydroxy-9,10-dihydro-5-nitro-BNT (5-nitro-BNT-9,10-diol). Also present was a minor amount of 5-amino-BNT and *trans*-9,10-dihydroxy-9,10-dihydro-5-amino-BNT (5-amino-BNT-9,10-diol). DNA adduct analyses were performed using the ³²P-postlabeling assay and reversed-phase HPLC. Three major XO-derived calf thymus DNA adducts were detected. On the basis of their chromatographic mobilities, two adducts were identified as reaction products of 5-nitro-BNT with 2'-deoxyguanosine and one adduct with 2'-deoxyadenosine. Incorporation of allopurinol (a specific XO inhibitor) in the incubation mixture resulted in loss of all three adducts, confirming enzymatic mediation by XO. Aerobic S9 activation of 5-nitro-BNT with calf thymus DNA produced three adducts. On the basis of their chromatographic mobilities, two were identified as reaction products of 5-nitro-BNT with 2'-deoxyguanosine and one with 2'-deoxyadenosine. Incorporation of 1-aminobenzotriazole (a P450 inhibitor) in the incubation mixture resulted in a loss of these adducts, confirming enzymatic mediation by P450. Aerobic S9-catalyzed metabolism of 5-nitro-BNT-9,10-diol produced the same DNA adducts as observed with 5-nitro-BNT. Aerobic S9-catalyzed metabolism of 5-amino-BNT-9,10-diol produced the same deoxyadenosine-derived DNA adducts as observed with 5-nitro-BNT and 5-nitro-BNT-9,10-diol. These results provide additional information that both ring oxidation and nitroreduction are involved in the metabolism, DNA adduct formation and mutagenicity of 5-nitro-BNT.

Introduction

Thioarenes, sulfur-containing polycyclic aromatic hydrocarbons (PAHs),¹ have been detected in the combustion emissions of brown-coal (1), hard-coal (2), lubricating oil (3, 4), and diesel exhaust (5). Thioarenes account for more than 50% of the polycyclic aromatic compounds in some crude oil samples (6). Additionally, it has been

determined that 25% of the polycyclic aromatic compounds emitted from coal-fired residential furnaces are thioarenes (5). Benzo[*b*]naphtho[2,1-*d*]thiophene (BNT), a representative member of this class of chemicals has also been detected in extracts of diesel particles (7) and in rural air in Denmark (8). The carcinogenic activity of BNT was similar to chrysene when implanted in the lungs of rats (9). Jacob and co-workers studied the metabolism of BNT by rat liver microsomes and found only products that represented oxidation of the sulfur atom (sulfone and sulfoxide) (10). When BNT metabolism

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was studied using Aroclor-induced rat liver S9, the metabolites identified included both the sulfone and sulfoxide of BNT as well as products of ring oxidation, with 1,2- and 3,4-dihydrodiols of BNT formed in significant quantities (11). BNT was mutagenic in *Salmonella typhimurium* TA100 and TA98 (12, 13) and is known to induce mixed-function monooxygenase activity in rat liver (14, 15). BNT and BNT-3,4-diol have been shown to have comparable mutagenicity while BNT-1,2-diol was less mutagenic, suggesting that BNT-3,4-diol is a proximate mutagen (16).

For several years, our focus has been on the investigation of the genotoxicity of nitrated polycyclic aromatic hydrocarbons (NO_2 -PAHs), which constitute a large class of environmental contaminants. Some of these are known to be tumorigenic and others have been shown to contribute substantially to the mutagenicity of diesel exhaust in the *Salmonella* assay, indicating a potential risk to human health. The relationship between exposure and cancer risk of NO_2 -PAHs is an important unresolved question and has led to studies focusing on their environmental occurrence, DNA adduct forming potential, and carcinogenicity. Despite the current knowledge on thioarenes, very little information exists on the toxicology of nitrated-thioarenes. Pitts et al. (17) and Jager (18) were the first to show that PAHs react with oxides of nitrogen (e.g., nitric acid) to form nitroaromatics under conditions that are expected in polluted air and in combustion processes. Electrophilic nitration of BNT by nitric and sulfuric acid yielding predominantly 5-nitro-BNT has been demonstrated synthetically (19). These results suggest that electrophilic nitration of BNT to 5-nitro-BNT may occur in the atmosphere with high levels of photochemical oxidants and nitrogen dioxide. In this study, we have (i) examined the mutagenicity of 5-nitro-BNT in *Salmonella* TA100 and TA98, (ii) investigated its metabolism under both aerobic and anaerobic conditions, and (iii) analyzed the metabolic activation of 5-nitro-BNT to DNA adducts under both aerobic and anaerobic conditions.

Experimental Section

Chemicals. **Caution!** 5-Nitro-BNT and the derivatives described in this paper may be carcinogenic to laboratory animals. Therefore, protective clothing should be worn and appropriate safety procedures should be followed when working with these compounds. Calf thymus DNA, nuclease P1 (NP1), and calf spleen phosphodiesterase (SPDE) were purchased from CalBiochem Corporation (LaJolla, CA). Polyethyleneimine-cellulose (PEI-cellulose) TLC plates were purchased from Alltech (Avondale, PA). Aroclor-1254-induced rat liver S9 was purchased from MOLTOX, Boone, NC. 2'-Deoxyguanosine-3'-phosphate (3'

dGMP) and 2'-deoxyadenosine-3'-monophosphate (3'-dAMP) were purchased from Pharmacia (Piscataway, NJ). Micrococcal nuclease (MN) and allopurinol were purchased from Sigma Chemical Co., (St. Louis, MO). 1-Aminobenzotriazole (1-ABT) was a gift from Dr. Michael J. Devito (U.S. Environmental Protection Agency). T₄ polynucleotide kinase (3'-phosphatase free) was obtained from Boehringer Mannheim (Indianapolis, IN). [$\gamma^{32}\text{P}$]ATP (>3000 Ci/mmol) in an aqueous solution containing 5 mM 2-mercaptoethanol was obtained from Amersham (Arlington Heights, IL). Flo-Scint II was obtained from Packard Instruments Company, Inc. (Downers Grove, IL). The internal UV standard, *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene, was a gift from Dr. David H. Phillips, Haddow Laboratories, The Institute of Cancer Research (Sutton, Surrey U.K.). All other chemicals were reagent grades and obtained from commercial sources.

Instrumentation. UV spectra were determined on a Beckman model DU-70. Proton NMR spectra were recorded on a Bruker AVANCE-300 instrument at 300.13 MHz. Chemical shifts are reported as parts per million referenced to tetramethylsilane. 2D-COSY, homonuclear decoupling, and 1D-NOE difference spectra were recorded according to established manufacturer's protocols. Electron impact/mass spectrometry (EI/MS) was obtained on a Hewlett-Packard model 5998A mass spectrometer and high-pressure liquid chromatography/electrospray/mass spectrometry (HPLC/ES/MS) on a HP-1100 MSD instrument. Positive chemical ionization/gas chromatography/mass spectrometry (PCI/GC/MS) was performed using an Extrel ELQ-400-3 triple quadrupole mass spectrometer coupled to a Varian model 3400 gas chromatograph and model 1094 Septum Programmable Injector using methane as the reagent gas. Positive chemical ionization/liquid chromatography/mass spectrometry (PCI/LC/MS) was performed on an Extrel ELQ-400-3 triple quadrupole mass spectrometer coupled to an Eldex 9600 HPLC via Extrel's Thermabeam particle beam interface using methane as the reagent gas. Exact mass determinations using EI were recorded on a VG70-250SEQ hybrid mass spectrometer at 10 000 resolution, using a solid probe. Analytical and preparative TLC were carried out on fluorescent silica gel plates or C18 reversed-phase plates. Bands were visualized with short- and long-wavelength UV lamps.

Separation of 5-nitro-BNT metabolites was performed on a Hewlett-Packard 1100 HPLC System equipped with a model G1311A quaternary solvent delivery system, a model HP G1322A-degasser, a model HP 1315A diode-array detector and a model HP G1313A autosampler.

Separation of the ^{32}P -postlabeled 3',5'-bisphosphate adducts was carried out on a Waters Associates HPLC System (Millipore; Waters Division, Milford, MA) equipped with a model 600 solvent delivery system, a model U6K septumLess injector, a model 490E UV-vis wavelength detector, a model 600E automated gradient controller and a model 717 autosampler.

Mutagenicity Assays. The *Salmonella* plate incorporation assay was performed as described by Ames et al. (20) with modifications by Claxton et al. (21). *Salmonella* strains TA98 and TA100 were kindly provided by Dr. Bruce N. Ames (University of California, Berkeley, CA). 5-Nitro-BNT was dissolved in DMSO and assayed in both strains, in triplicate, on the same day, without and with Aroclor-induced rat liver S9, a dose range of 0.36, 1.08, 1.79, 3.60, and 10.80 nmol/plate. All concentrations were tested in triplicate.

Synthesis of 5-Nitro-BNT. 5-Nitro-BNT was synthesized using the general procedure of Alunni-Bistocchi et al. (19). A mixture of nitric acid (0.4 mL) and sulfuric acid (0.4 mL) in acetic acid (2 mL) was added dropwise to a stirring solution of BNT (468 mg, 0.2 mmol) in acetic acid (20 mL) at 0 °C. Within a few minutes, a yellow precipitate was formed that was filtered, washed with water, and dried. The crude product was purified by silica gel column chromatography with elution by hexane: ethyl acetate (9:1) to yield 370 mg (67%) of 5-nitro-BNT; mp 206–208 °C (lit. 202 °C (18)). EI/MS (m/z , relative intensity): 279 [M]⁺ (100); 231 [M – 58 (NO, CO)]⁺ (39). ¹H NMR (CD₂Cl₂,

¹ Abbreviations: BNT, benzo[b]naphtho[2,1-d]thiophene; 5-nitro-BNT, 5-nitrobenzo[b]naphtho[2,1-d]thiophene; 5-nitro-BNT-9,10-diol, *trans*-9,10-dihydroxy-9,10-dihydro-5-nitrobenzo[b]naphtho[2,1-d]thiophene; 5-amino-BNT-9,10-diol, *trans*-9,10-dihydroxy-9,10-dihydro-5-aminobenzo[b]naphtho[2,1-d]thiophene; 1-ABT, 1-aminobenzotriazole; 5-amino-BNT, 5-aminobenzo[b]naphtho[2,1-d]thiophene; 3'-dAMP, 2'-deoxyadenosine-3'-monophosphate; 3'-dGMP, 2'-deoxyguanosine-3'-phosphate; MN, micrococcal nuclease; EI/MS, electron impact/mass spectrometry; HPLC/ES/NCI/MS, high-pressure liquid chromatography/electrospray/negative chemical ionization/mass spectrometry; PCI/GC/MS, positive chemical ionization/gas chromatography/mass spectrometry; PCI/LC/MS, positive chemical ionization/liquid chromatography/mass spectrometry; NO_2 -PAHs, nitrated polycyclic aromatic hydrocarbons; NP1, nuclease P1; PAHs, polycyclic aromatic hydrocarbons; PEI-cellulose, polyethyleneimine-cellulose; POM, polycyclic organic matter; RRT, relative retention time; SPDE, calf spleen phosphodiesterase; XO, xanthine oxidase; S9, Aroclor-1254 induced rat liver S9.

ppm): 7.56–7.63 (m, 2H, H₈ and H₉); 7.72–7.82 (m, 2H, H₂, and H₃); 7.98–8.03 (m, 1H, H₁₀); 8.23–8.30 (m, 2H, H₇ and H₁); 8.68 (m, 1H, H₄); 8.93 (s, 1H, H₆).

Synthesis of 5-Amino-BNT. The method of Alunni-Bistocchi et al. (19) was used with the substitution of zinc dust for nickel as the catalyst. In a flame dried flask, 5-nitro-BNT (10 mg, 35.8 mmol) was dissolved in 2 mL of ethanol and hydrazine hydrate (30 mg, 75 mmol) was added followed by zinc dust. The reaction mixture was refluxed for 4 h, and was monitored by TLC on silica gel (hexane and CH₂Cl₂, 1/1) for the appearance of a fluorescent product and until the starting material was consumed. The reaction mixture was cooled to room temperature and ice–water (15 mL) was added. The resulting pale yellow precipitate was filtered and washed with water. The crude product was purified by silica gel column chromatography with hexane and then increasing concentrations of methylene chloride. This gave 8.2 mg (93%) of 5-amino-BNT as colorless needles. PCI/GC/MS *m/z* (relative intensity): 278 [M + 29 (C₂H₅)⁺] (10.7); 264 [M + 15 (CH₅)⁺] (3.8); 250 [M + 1 (H)⁺] (100). ¹H NMR (acetone-*d*₆, ppm) 7.45–7.52 (m, 2H, H₂, H₃); 7.54–7.68 (m, 2H, H₈, H₉); 7.63 (s, 1H, H₆); 7.98–8.07 (m, 2H, H₇, H₁₀); 8.14–8.2 (m, 1H, H₄); 8.24–8.28 (m, 1H, H₁).

Aerobic S9-Mediated Metabolism of 5-Nitro-BNT. To evaluate the aerobic metabolism of 5-nitro-BNT with corresponding identification of metabolites, two large-scale incubations of 5-nitro-BNT with S9 were carried out at 37 °C with shaking as described by Murphy et al. (11). Briefly, the total volume of each incubation mixture was 220 mL containing S9 (0.9 mg/mL), 50 mM Tris-HCl buffer (pH 7.4), 0.11 M KCl, 8 mM MgCl₂, 1 mM of NADPH, and 40 μM 5-nitro-BNT (previously dissolved in 4 mL of DMSO). After 1 h, the reactions were quenched by the addition of an equal volume of ice-cold acetone. The separate aqueous layers of the two metabolite mixtures were extracted twice with 440 mL of ethyl acetate and the extracts combined and evaporated to dryness in vacuo. Control incubations using heat deactivated S9 were performed and extracted as described above.

Anaerobic S9-Mediated Metabolism of 5-Nitro-BNT. To evaluate the anaerobic S9 metabolism of 5-nitro-BNT with corresponding identification of its metabolites, two large-scale incubations were performed as described above with the following modifications. Briefly, 170 mL of each incubation mixture containing, 50 mM Tris-HCl buffer (pH 7.4), 0.11 M KCl, and 8 mM MgCl₂ were purged of oxygen by allowing the solutions to equilibrate overnight in an anaerobic chamber (Coy Laboratory Product, Ann Arbor, MI) containing 10% H₂, 85% N₂, and 5% CO₂, National Specialty Gases, Raleigh, NC). 5-Nitro-BNT (40 μM, previously dissolved in 4 mL of DMSO), NADPH (1 mM), and S9, (39 mg/mL) were placed in the anaerobic chamber the following morning and purged of oxygen for 2 h. The 5-nitro-BNT and the S9 solutions were added to the Tris-HCl buffer and the reaction initiated by adding NADPH. After 1 h, the reaction mixtures were capped, removed from the anaerobic chamber and the aqueous layers of the two metabolite mixtures were extracted as described. Control incubations using heat deactivated S9 were performed and extracted as described above.

Anaerobic XO-Mediated Metabolism of 5-Nitro-BNT. To evaluate the anaerobic XO-catalyzed metabolism of 5-nitro-BNT with corresponding identification of metabolites, two large-scale incubations of 5-nitro-BNT with XO were carried out at 37 °C with shaking as described (22–24). Briefly, 196 mL of 50 mM potassium phosphate buffer (pH 5.8) containing 3.7 mM hypoxanthine was purged of oxygen by allowing the solutions to equilibrate overnight in an anaerobic chamber as described above. XO and 5-nitro-BNT were placed in the chamber the following morning and purged of oxygen for 2 h. XO (100 units) and 5-nitro-BNT (40 μM) (previously dissolved in DMSO) were added to the described solution (total volume 200 mL) and incubated anaerobically for 4 h at 37 °C. The reaction mixtures were capped, removed from the anaerobic chamber and the aqueous layers of the metabolite mixtures were extracted as

described. Control incubations using heat deactivated XO were performed and extracted as described above.

HPLC Analysis of 5-Nitro-BNT Metabolites. The extracted metabolite residues were resuspended in 4 mL of methanol, filtered, and multiple aliquots (200 μL) were applied to a semipreparative 5 μm, 9.5 mm × 250 mm Zorbax ODS column (MAC-MOD Analytical, Inc., Chadds Ford, PA) and eluted with a linear gradient using the following solvent system: solvent A, 40% water; solvent B, 60% methanol. The linear gradient was 0 to 26 min, 60 to 100% B; 26 to 30 min, 100% B; 30 to 35 min, 85% B; 35 to 60 min, 60% B. The flow rate was 2.5 mL/min and the column was allowed to equilibrate at the initial solvent ratio (40% A:60% B) for 15 min before subsequent analysis. Metabolite fractions containing UV-absorbing peaks were collected and evaporated to dryness in vacuo. The metabolite residues were resuspended in 2 mL of methanol, filtered and multiple aliquots (100 μL) were applied to a 5 μm, 4.6 mm × 250 mm Zorbax ODS column (MAC-MOD Analytical, Inc., Chadds Ford, PA) and eluted with the same linear gradient as described for the semipreparative column, using a flow rate of 1.0 mL/min. Metabolite fractions containing UV-absorbing peaks were collected and evaporated to dryness in vacuo. The major isolated metabolites were resuspended in 500 μL of methanol and 100 μL was used to obtain UV spectra. The remaining metabolite suspensions were evaporated to dryness in vacuo and stored at –80 °C for metabolite identification.

Reduction of Metabolic 5-Nitro-BNT-9,10-diol. An analytical amount of the metabolically formed *trans*-9,10-dihydroxy-9,10-dihydro-5-nitroBNT (5-nitro-BNT-9,10-diol) was reduced to *trans*-9,10-dihydroxy-9,10-dihydro-5-amino-BNT (5-amino-BNT-9,10-diol) using the method of Alunni-Bistocchi et al. (19) as modified above. The product was identified from its mass spectra and NMR spectra. HPLC/ES/NCI/MS *m/z* (relative intensity): 282 [M – 1][–] (100); 264 [M – 19 (H, H₂O)][–] (40). ¹H NMR (acetone-*d*₆, ppm) 4.64 (m, H₉); 5.04 (m, H₁₀); 6.0 (m, H₈); 6.66 (m, H₇); 7.16 (s, H₆); 7.49–7.58 (m, H₂, H₃); 8.02 (m, H₄); 8.19 (m, H₁).

XO-Mediated in Vitro Modification of Calf Thymus DNA with 5-Nitro-BNT. The method used for the generation of calf thymus DNA adducts by the XO-catalyzed nitroreduction of 5-nitro-BNT was essentially as described in the anaerobic XO-mediated metabolism of 5-nitro-BNT section. Briefly, XO (0.5 units/mL) and 5-nitro-BNT (40 μM) in the presence of calf thymus DNA (1 mg/mL) (total volume 2 mL) were incubated anaerobically for 4 h at 37 °C. Samples were removed from the anaerobic chamber and the reaction was stopped by the addition of 10 mL ethyl acetate. Inhibition experiments were conducted similarly, but in the presence of allopurinol (100 μM) as described by Howard et al. (23).

Aerobic and Anaerobic S9-Mediated in Vitro Modification of Calf Thymus DNA. Aerobic incubation of 5-nitro-BNT (40 μM), or anaerobic incubation of 5-amino-BNT (40 μM), and 5-nitro-BNT-9,10-diol with calf thymus DNA (1.0 mg/mL) in the presence of S9 (1.6 mg/mL) for 4 h at 37 °C was performed as previously described (24, 25). Inhibition experiments under aerobic conditions were conducted using the same conditions as described above except that 1-ABT (10 mM) was included in the S9 incubation as described by Ortiz de Montellano et al. (26). The reaction mixtures were terminated by the addition of 10 mL of ethyl acetate. The incubations were extracted three times with 10 mL of ethyl acetate. The modified calf thymus DNA adducts were isolated as described by Amin et al. (27). The aqueous portion was cooled to 0 °C, and 30 mL of cold ethanol was added slowly. The DNA was precipitated, spooled out with a glass rod, washed with 10 mL of ethanol, 10 mL of acetone, and 10 mL of ether, and dried under a stream of nitrogen at 37 °C. The modified DNA sample residues were resuspended in 1.2 mL of HPLC-grade water and stored at –80 °C until time of adduct analyses.

In Vitro Preparation of 5-Nitro-BNT-Modified Nucleotide Adduct Standards. The generation of 5-nitro-BNT-

Table 1. Mutagenicity of 5-Nitro-BNT in *Salmonella typhimurium* TA98 and TA100 in the Presence of Rat Liver S9

sample	nmol	mean revertants/plate ^a TA98				mean revertants/plate ^a TA100			
		-S9	±SD	+S9	±SD	-S9	±SD	+S9	±SD
5-nitro-BNT	0.00	22	1.3	34	2.1	73	1.0	108	7.6
	0.36	54	3.2	51 ^b	1.4	154 ^b	1.1	139 ^b	1.0
	1.08	69 ^b	4.0	82 ^b	1.7	167 ^b	3.2	278 ^b	8.7
	1.79	70 ^b	5.3	200 ^b	3.7	176 ^b	2.3	385 ^b	10.0
	3.60	92 ^b	9.3	932 ^b	0.8	183 ^b	3.0	709 ^b	12.3
	10.80	137 ^b	4.2	2023 ^b	12.7	258 ^b	3.4	1459 ^b	7.8

^a Mean revertants/plate was calculated from triplicate plates at each dose. ^b Significantly different at $P < 0.05$ using a pairwise multiple comparison procedure (Dunnett's Method).

modified 3'-dGMP and 3'-dAMP adduct standards by XO-catalyzed nitroreduction of 5-nitro-BNT or by aerobic S9 activation was performed as described above for calf thymus DNA. Solutions of 3'-dGMP (10 mg/mL) and 3'-dAMP (10 mg/mL) were treated as described. The reaction mixture was extracted three times with 1 vol of ethyl acetate:diethyl ether (1:1). Removal of the unreacted nucleotides was achieved by loading the reaction mixture on primed Sep-Pak cartridges and washing with 10 mL of HPLC-grade water and 10 mL of methanol to elute the modified nucleotides. The methanol was evaporated to dryness in vacuo and the sample resuspended in 1.2 mL of HPLC water and stored at -80 °C until time of adduct analysis.

³²P-Postlabeling Analysis. The calf thymus DNA (50 µg) of each modified DNA sample was digested to mononucleotides at 37 °C for 3.5 h with MN and SPDE as described (24). 5-Nitro-BNT-modified calf thymus DNA adducts and 5-nitro-BNT-modified nucleotide standards (3'-dGMP and 3'-dAMP reaction products) were subjected to both adduct enrichment procedures (butanol extraction method and NP1 treatment) (24). The samples were incubated with 50 µCi [γ ³²P]ATP and 3.5 units T₄ polynucleotide kinase for 30 min at 37 °C, and the total incubates spotted onto PEI-cellulose TLC plates.

TLC Separation and Preparation of Samples for HPLC Analysis. The labeled digests of the 5-nitro-BNT-modified calf thymus DNA adduct samples (both XO and S9-mediated) and the 5-nitro-BNT-3'-dGMP and 3'-dAMP reaction products (both XO and S9-mediated) were applied to 10 cm × 10 cm PEI-cellulose sheets and separated using a TLC system (D1 direction as described) (24, 28). Radioactivity of spots was located by autoradiography and the samples were prepared for HPLC analyses as previously described (24).

HPLC Analysis of ³²P-Postlabeled Adducts. The extracts of the excised adducts (100 µL) were applied to a 5 µm, 4.6 mm × 250 mm Zorbax Phenyl modified column and eluted with a linear gradient using the following solvent system: solvent A, 0.3 M NaH₂PO₄ buffer (pH 2.0); solvent B, 90% methanol and 10% solvent A. The gradient was 0 to 12.5 min, 10 to 43% B; 12.5 to 45 min, 43 to 95% B; 45 to 50 min, 95% B; 50 to 65 min, 95 to 10% B. The flow rate was 1 mL/min and the column was allowed to equilibrate at the initial solvent ratio (90% A:10% B) for 15 min before subsequent analysis. Radiolabeled nucleotides were detected by an in-line flow-through scintillation counter (model A-500 Flow-One β Radiomatic Instruments & Chemical Co., Inc., Tampa FL). The retention times of ³²P-postlabeled DNA adducts are expressed as a relative retention time (RRT), which was calculated by dividing the retention time of the ³²P-postlabeled DNA adducts by the retention time of the internal standard (*cis*-9,10-dihydroxy-9,10-dihydrophenanthrene). The reproducibility of the RRT was ±0.03.

Results

Mutagenicity. The mutagenicity of 5-nitro-BNT was examined in *Salmonella* TA98 and TA100 and the results are shown in Table 1. Toxicity was observed in both strains at doses above 11 nmol/plate, hence the mutagenic activity was determined at a dose range of 0.36,

1.08, 1.79, 3.60, and 10.80 nmol/plate. 5-Nitro-BNT displayed weak direct-acting mutagenicity in the absence of S9 in TA98 and TA100, respectively. In the presence of S9, 5-nitro-BNT was more mutagenic in TA98 and TA100, respectively.

Aerobic S9-Mediated Metabolism of 5-Nitro-BNT. The metabolism of 5-nitro-BNT was studied under aerobic conditions using S9. Following two sequential separations using reverse-phase semipreparative HPLC to separate metabolites from 5-nitro-BNT, analytical reversed-phase HPLC was used in the final separation of the ethyl acetate-extractable metabolites as shown in Figure 1a. Four metabolites were detected. The peak eluting at 37.8 min contained 5-nitro-BNT. The structure of the major aerobic metabolite (metabolite 3) was deduced from NMR and MS and found to be *trans*-9,10-dihydroxy-9,10-dihydro-5-nitro-BNT (5-nitro-BNT-9,10-diol) based on the following analyses (Scheme 1). Low resolution PCI/LC/MS *m/z* (relative intensity): 314 [M + 1(H)]⁺ (72.9); 296 [M + 1-18 (H₂O)]⁺ (37.3); 280 [M + 1-34 (H₂O,O)]⁺ (22.9); 296 [M + 1-48 (H₂O, NO)]⁺ (100); 250 [M + 1-64 (H₂O, NO₂)]⁺ (27.1) suggested a dihydrodiol structure. High-resolution EI/MS confirmed a precise mass of 313.0409 for the empirical formula C₁₆H₁₁O₄NS (313.0389).

The NMR analyses of metabolite 3 revealed a singlet at 8.72 (H₆), two downfield aromatic proton multiplets, 8.5 (H₄) and 8.33 (H₁), and an aromatic multiplet at 7.8–7.85 (H₂,H₃) (Figure 2). The *peri* proton, H₄, was down-shifted due to the anisotropic effect of the nitro group (29). Also observed were: two coupled olefinic protons, a doublet of doublets at 7.02 (H₇, *J*_{7,8} = 9.7 Hz, *J*_{7,9} = 2.6 Hz); a doublet of doublets at 6.15 (H₈, *J*_{8,7} = 9.7 Hz, *J*_{8,9} = 2.6 Hz); and two carbinol protons, a doublet at 5.17 (H₁₀, *J*_{10,9} = 11.2 Hz) and a triplet of doublets at 4.73 (H₉, *J*_{9,10} = 11.2 Hz, *J*_{9,8} = *J*_{9,7} = 2.6 Hz). These couplings were confirmed by COSY spectra (data not shown). The large coupling constant between dihydrodiol protons, H₉ and H₁₀, suggested that they were in a quasi-diequatorial conformation. 1D-NOE spectra conclusively corroborated the assignment of metabolite 3 as 5-nitro-BNT-9,10-diol. Irradiation of proton H₆ produced an NOE effect (5.12%) on olefinic proton H₇ (7.02) (Figure 2). Concomitantly, irradiation of proton H₇ produced NOE effects in protons H₆ (8.72) and H₈ (6.15) (data not shown). For comparison, irradiation of 5-nitro-BNT at proton H₆ gave an NOE effect at proton H₇ (5.08%). The observed NOE effects were consistent with the calculated interatomic distances (Å) between H₆ and H₇: 5-nitro-BNT, 2.362, 5-nitro-BNT-9,10-diol, 2.308. The UV spectra of metabolite 3 is shown in Figure 1d. In summary, the experimental evidence strongly support the identification of metabolite 3 as 5-nitro-BNT-9,10-diol. Metabolite 2 remains unidentified.

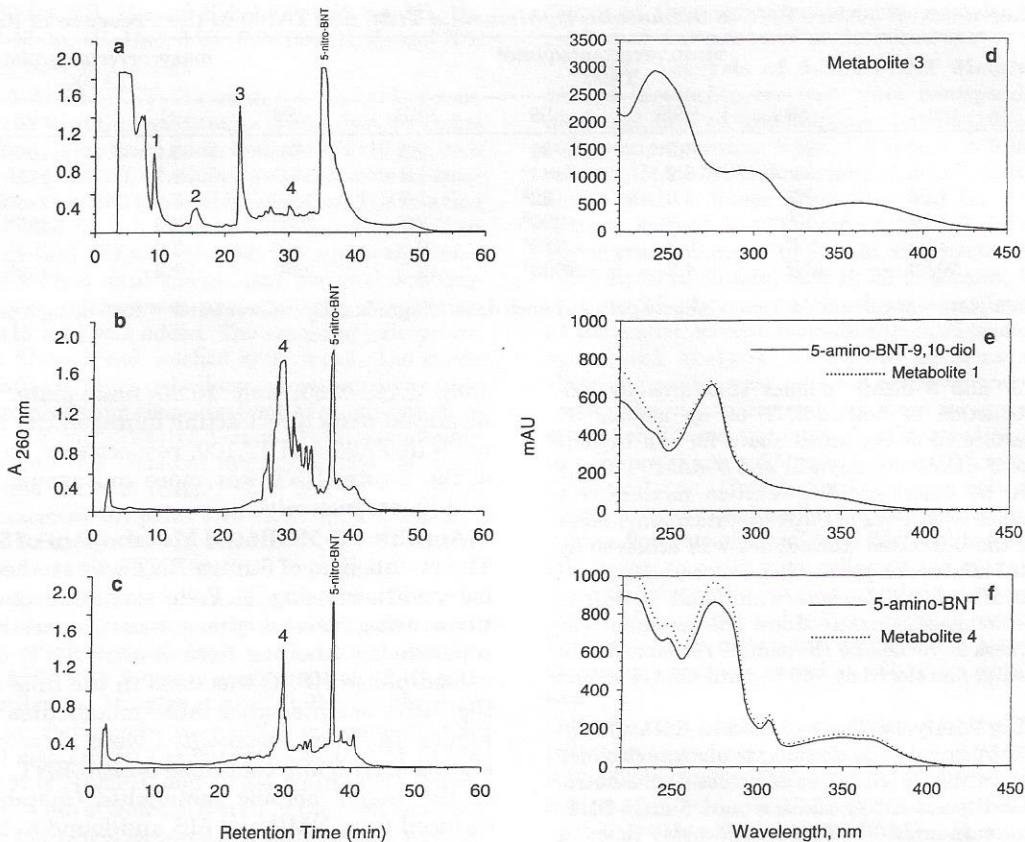
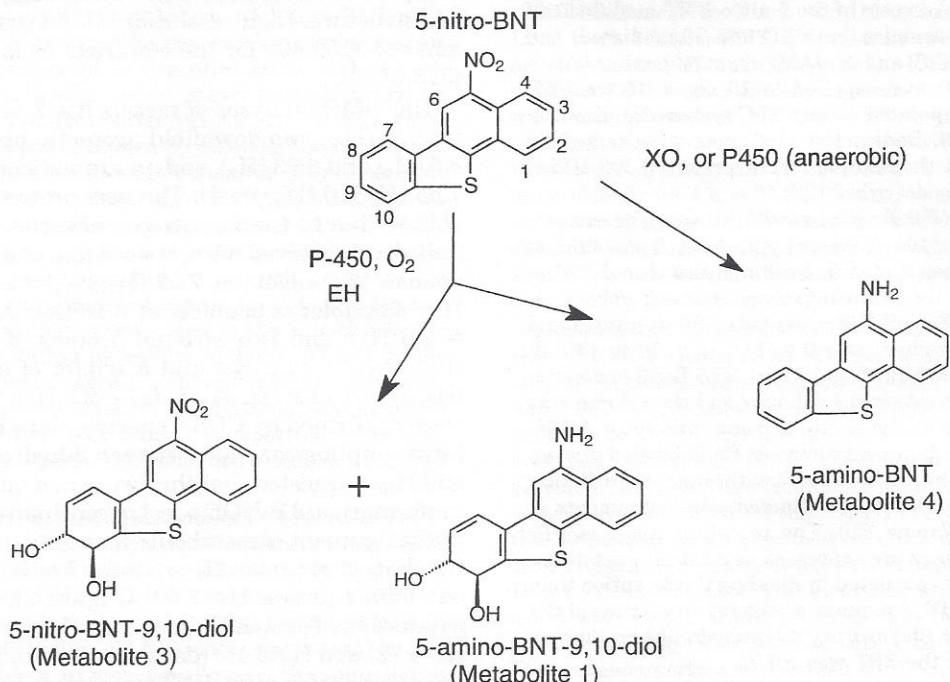


Figure 1. Reversed-phase HPLC profiles of ethyl acetate-extractable metabolites obtained from (a) aerobic incubation of 5-nitro-BNT with S9, (b) anaerobic incubation of 5-nitro-BNT with XO and (c) anaerobic incubation of 5-nitro-BNT with S9. 5-Nitro-BNT eluted at 37.8 min. UV spectra of (d) metabolite 3, (f) 5-amino-BNT and metabolite 4, (e) 5-amino-BNT-9,10-diol and metabolite 1. The UV spectra were obtained in methanol.

Scheme 1. Proposed Pathways for the Formation of 5-Nitro-BNT Metabolites^a



^a The (*R,R*) configuration of the dihydrodiols is illustrated; however, the absolute configurations have not been determined.

Metabolic 5-nitro-BNT-9,10-diol (metabolite 3) was chemically reduced to its amino analogue, 5-amino-BNT-9,10-diol, using a modification of the method of Alunni-Bistocchi et al. (19). The mass spectra and fragmentation analyses were consistent with the proposed structure.

The NMR spectra revealed the expected upfield shifts of protons H₆ and H₁ with the remaining protons having chemical shifts similar to those found in 5-nitro-BNT-9,10-diol. The HPLC chromatogram of the aerobic S9 metabolism of 5-nitro-BNT revealed the amino-diol (me-

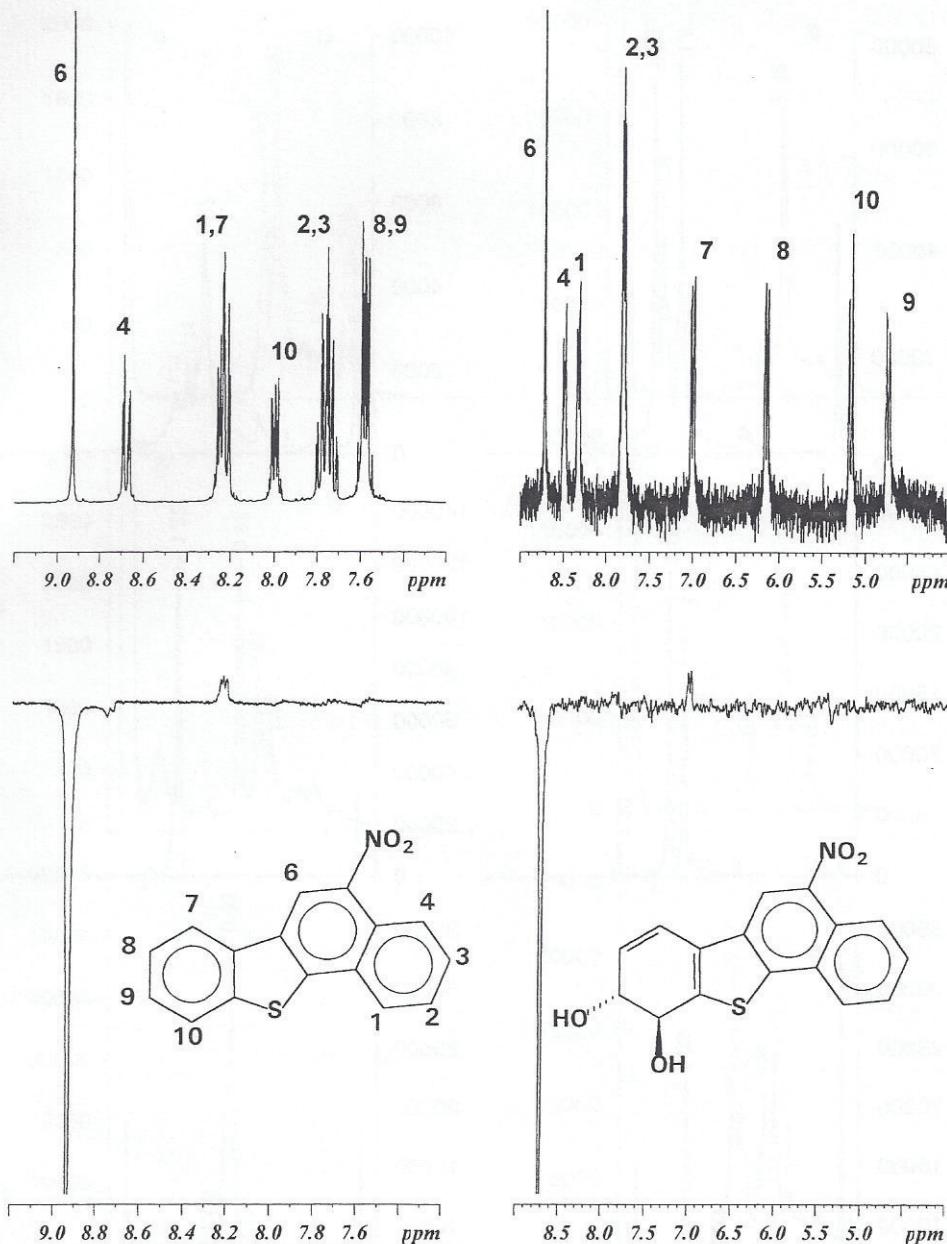


Figure 2. Proton 300 MHz NMR spectra of 5-nitro-BNT (left panels) and metabolite 3 [5-nitro-BNT-9,10-diol] (right panels). (Upper panels) 1D NOE; (lower panels) 1-D NOE NMR difference spectra. The negative band represents the point of NOE irradiation. NMR spectrum of 5-nitro-BNT was taken in CD_2Cl_2 and metabolite 3 [5-nitro-BNT-9,10-diol] in acetone- d_6 .

tabolite **1**, (Figure 2a). The UV spectra of 5-amino-BNT-9,10-diol and metabolite **1** were identical (Figure 1e, Scheme 1).

Also detected in the aerobic S9 incubation mixture of 5-nitro-BNT was a trace amount of 5-amino-BNT (metabolite **4**) (Figure 1a, Scheme 1). This was confirmed by co-chromatography of the aerobic S9-derived 5-nitro-BNT metabolites with the synthetic standard, 5-amino-BNT (data not shown). No metabolites were detected in control incubations (data not shown).

Anaerobic XO and S9-Mediated Metabolism of 5-Nitro-BNT. Metabolism of 5-nitro-BNT under anaerobic conditions with XO resulted in the production of one major metabolite (Figure 1b). The peak eluting at 37.8 min contained the parent compound 5-nitro-BNT. The structure of the major anaerobic metabolite (metabolite **4**) was identified by comparison of its NMR, MS, and UV spectra with that of an authentic standard of 5-amino-BNT. The UV spectra (Figure 1f), MS, and NMR spectra (data not shown) of the metabolite and standard were identical. In addition, synthetic 5-amino-BNT co-chromatographed with the anaerobic metabolic product by HPLC (data not shown). No metabolites were detected in control incubations (data not shown).

(data not shown) of the metabolite and standard were identical. In addition, synthetic 5-amino-BNT co-chromatographed with the anaerobic metabolic product by HPLC (data not shown).

Anaerobic metabolism of 5-nitro-BNT in the presence of S9 resulted in the production of one major and several minor metabolites (Figure 1c). The peak elution at 37.8 min contained the parent compound 5-nitro-BNT. The structure of the major anaerobic metabolite (metabolite **4**) was identified by comparison of its NMR, MS, and UV spectra with that of an authentic standard of 5-amino-BNT. The UV spectra (Figure 1f), MS, and NMR spectra (data not shown) of the metabolite and standard were identical. In addition, synthetic 5-amino-BNT co-chromatographed with the anaerobic metabolic product by HPLC (data not shown). No metabolites were detected in control incubations (data not shown).

In summary, as described in Scheme 1, aerobic S9 incubation of 5-nitro-BNT produced predominantly 5-ni-

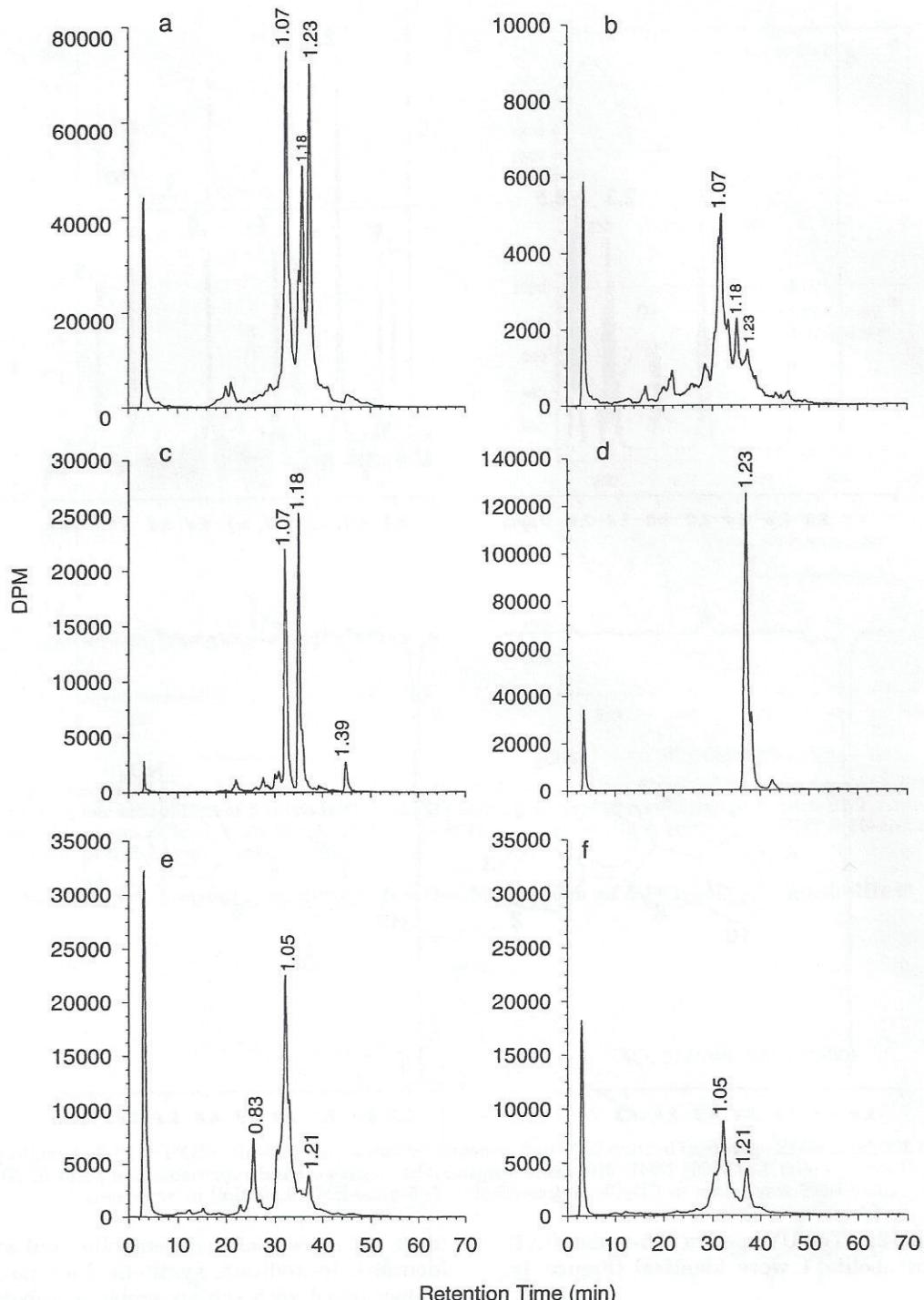


Figure 3. HPLC profiles of the ^{32}P -postlabeled adducts isolated from calf thymus DNA or 3'-mononucleotide phosphates incubated anaerobically with 5-nitro-BNT DNA and XO or aerobically with S9 as described in the Experimental Section. (a) 5-nitro-BNT, calf thymus DNA and XO with butanol extraction; (b) 5-nitro-BNT, calf thymus DNA, XO and allopurinol with butanol extraction; (c) 5-nitro-BNT, 3'-dGMP and XO with butanol extraction; (d) 5-nitro-BNT, 3'-dAMP and XO with butanol extraction; (e) 5-nitro-BNT, calf thymus DNA and S9 with NP1 treatment; (f) 5-nitro-BNT, calf thymus DNA, S9, and 1-aminobenzotriazole with NP1 treatment.

tro-BNT-9,10-diol, a significant amount of 5-amino-BNT-9,10-diol and a trace amount of 5-amino-BNT. Anaerobic XO or S9 incubation of 5-nitro-BNT produced predominantly 5-amino-BNT.

Analysis of XO-Derived Calf Thymus DNA Adducts of 5-Nitro-BNT. Replicate samples of 5-nitro-BNT-calf thymus DNA adducts were prepared. One was subjected to butanol extraction and the other subjected to NP1 to screen for the presence of arylamine-derived DNA adducts using ^{32}P -postlabeling/HPLC analysis. Butanol extraction of the first sample resulted in the

separation of three major adducts (RRT 1.07, 1.18, and 1.23) when examined by HPLC (Figure 3a). When the duplicate sample was subjected instead to NP1 treatment, there was a complete loss of the three major adducts (data not shown). Duplicate incubations were also prepared with allopurinol, a specific inhibitor of XO. When the first sample was subjected to butanol extraction, the major adducts (RRT 1.07, 1.18, and 1.23) were reduced by 15, 38, and 76%, respectively (Figure 3b). When the second sample was subjected to NP1 treatment, no adducts were observed (data not shown).

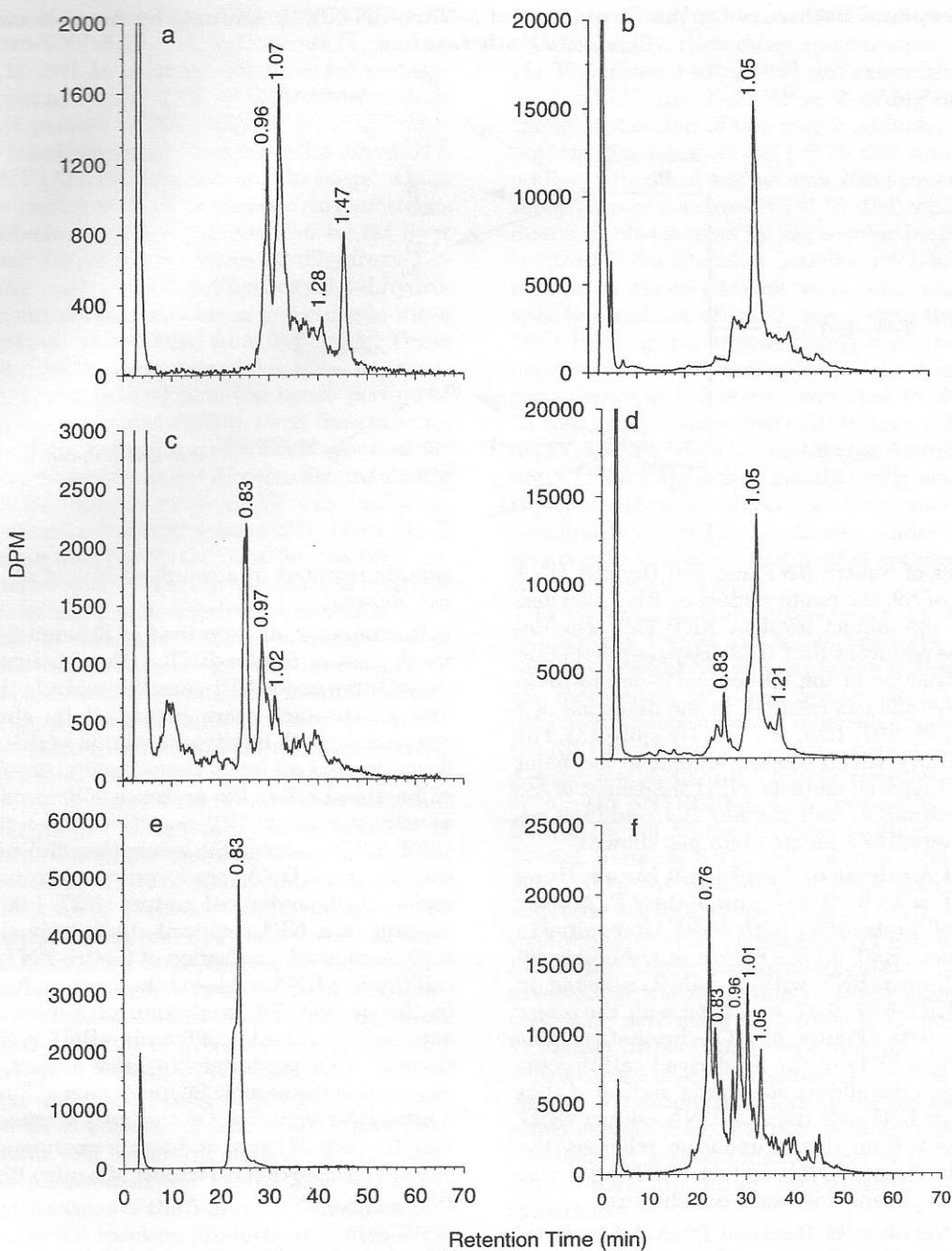
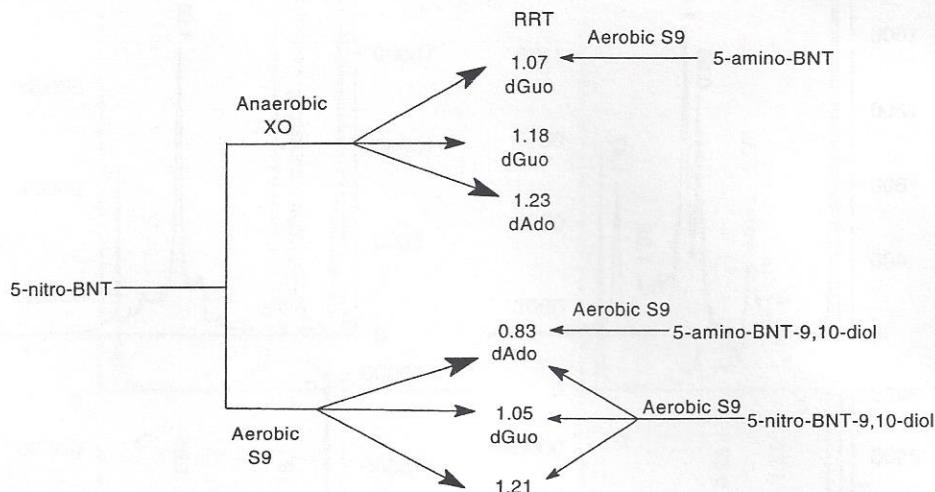


Figure 4. HPLC profiles of the ^{32}P -postlabeled adducts isolated from calf thymus DNA incubated aerobically with 5-amino-BNT and S9, 5-nitro-BNT, 3'-mononucleotide phosphates and S9, with 5-nitro-BNT-9,10-diol, 5-amino-BNT-9,10-diol, and S9 or metabolized anaerobically with 5-nitro-BNT-9,10-diol, XO and calf thymus DNA as described in the Experimental Section. (a) 5-amino-BNT and S9 with butanol extraction; (b) 5-nitro-BNT, 3'-dGMP and S9 with NP1 treatment; (c) 5-nitro-BNT, 3'-dAMP and S9 with NP1 treatment; (d) 5-nitro-BNT-9,10-diol and S9 with NP1 treatment; (e) 5-amino-BNT-9,10-diol, calf thymus DNA and S9 with NP1 treatment; (f) 5-nitro-BNT-9,10-diol, calf thymus DNA and XO with butanol extraction.

DNA Adduct Analysis of XO Incubations of 5-Nitro-BNT with 3'-dGMP and 3'-dAMP. Anaerobic incubation of 5-nitro-BNT with XO, 3'-dGMP, and 3'-dAMP provided adduct standards that were used in the identification of XO-derived 5-nitro-BNT-calf thymus DNA adducts. HPLC analysis of the butanol-extracted reaction products of 5-nitro-BNT with 3'-dGMP resulted in separation of two major adducts occurring at a RRT of 1.07 and 1.18, respectively (Figure 3c). HPLC analysis of the butanol-extracted reaction products of 5-nitro-BNT with 3'-dAMP resulted in observation of one major adduct (RRT 1.23) (Figure 3d). Chromatographic comparisons of the RRTs and co-chromatography of the XO-derived calf thymus DNA (data not shown) with the adduct standards

provided evidence that the major 5-nitro-BNT-calf thymus DNA adducts with RRT of 1.07 and 1.18 were derived from deoxyguanosine, whereas the major 5-nitro-BNT-calf thymus DNA adduct (RRT 1.23) was derived from deoxyadenosine (3'-dAMP).

Analysis of Aerobic S9-Derived DNA Adducts of 5-Nitro-BNT and 5-Amino-BNT. The HPLC profiles of the ^{32}P -postlabeled aerobic S9-derived 5-nitro-BNT-calf thymus DNA and 5-amino-BNT adducts are shown in Figure 3, panels e and f, and Figure 4, panel a-c. NP1 treatment of the S9-derived 5-nitro-BNT-calf thymus DNA adducts resulted in the detection of three adducts (RRT 0.83, 1.05, and 1.21), with the major adduct at RRT 1.05 (Figure 3e). When 1-ABT was included in the

Scheme 2. Proposed Pathways for the Formation of 5-Nitro-BNT DNA Adducts by Aerobic and Anaerobic Pathways

reaction mixture of 5-nitro-BNT and calf thymus DNA in the presence of S9, the major adduct at RRT 1.05 was reduced 3-fold, the adduct level at RRT 1.21 was unchanged, and the adduct at RRT 0.83 disappeared (Figure 3f). Butanol extraction of the S9-derived 5-amino-BNT-calf thymus DNA adducts resulted in the detection of 4 adducts (RRT 0.96, 1.07, 1.28, and 1.47) (Figure 4a). The predominant adduct (RRT 1.07) was similar to the major adduct of the XO-derived adducts. NP-1 treatment of the S9-derived 5-amino-BNT-calf thymus DNA adducts resulted in no observable adducts (data not shown).

DNA Adduct Analysis of Aerobic S9 Incubations of 5-Nitro-BNT with 3'-dGMP and 3'-dAMP. Aerobic S9 incubations of 5-nitro-BNT with 3'-dGMP resulted in one major adduct (RRT 1.05) (Figure 4b). Aerobic S9 incubations of 5-nitro-BNT with 3'-dAMP, resulted in three adducts (RRT 0.83, 0.97, and 1.02) with the major adduct at RRT 0.83 (Figure 4c). Co-chromatographic comparisons of the RRTs of the S9-derived calf thymus DNA adducts with the adduct standards, indicated that the major 5-nitro-BNT-calf thymus DNA adduct (RRT 1.05) was derived from deoxyguanosine whereas the 5-nitro-BNT-calf thymus DNA adduct (RRT 0.83) was derived from deoxyadenosine (data not shown).

Analysis of Aerobic S9-Derived DNA Adducts of 5-Nitro-BNT-9,10-diol, 5-Amino-BNT-9,10-diol, and Anaerobic XO-Derived DNA Adducts of 5-Nitro-BNT-9,10-diol. The aerobic S9 activation of the major aerobic metabolite **3** (5-nitro-BNT-9,10-diol) with calf thymus DNA was examined. HPLC analysis of the NP1 treatment of the 5-nitro-BNT-9,10-diol adducts resulted in the detection of three adducts (RRT 0.83, 1.05, and 1.21) with the major adduct at RRT 1.05 (Figure 4d). This adduct pattern was similar to the adduct pattern observed in the aerobic S9-derived DNA adducts of 5-nitro-BNT (Figure 3e). The HPLC analysis of the NP1 resistant calf thymus DNA adducts from the aerobic S9 activation of 5-amino-BNT-9,10-diol, revealed one DNA adduct, RRT 0.83 (Figure 4e). The anaerobic XO metabolic activation of 5-nitro-BNT-9,10-diol with calf thymus DNA was examined. HPLC analysis of the butanol-extracted XO-activated 5-nitro-BNT-9,10-diol-calf thymus DNA adducts resulted in the separation of one major polar adduct (RRT 0.76) and four minor adducts (0.83, 0.96, 1.01, and 1.05) (Figure 4f). NP1 treatment of a duplicate

sample resulted in a complete loss of all adducts (data not shown).

In summary, as described in Scheme 2, anaerobic XO incubation of 5-nitro-BNT with calf thymus DNA produced three major NP1-sensitive adducts (RRT 1.07, 1.18, and 1.23) which were identified by chromatographic comparison with in vitro standards as dGuo-, dGuo-, and dAdo-derived adducts, respectively. Aerobic incubation of 5-amino-BNT in the presence of S9 produced predominantly a similar NP1-sensitive dGuo-derived adduct (RRT 1.07). Aerobic S9 incubation of 5-nitro-BNT with calf thymus DNA produced predominantly a NP1-resistant dGuo-derived adduct (RRT 1.05) and a minor amount of a NP1-resistant dAdo-derived adduct, RRT 0.83. Aerobic S9 incubation of 5-nitro-BNT-9,10-diol with calf thymus DNA produced the same adducts as observed in the aerobic S9 incubation of 5-nitro-BNT. Finally, aerobic S9 incubation of 5-amino-BNT-9,10-diol with calf thymus DNA produced the same adduct, RRT 0.83, as observed in the aerobic S9 incubation of 5-nitro-BNT, and 5-nitro-BNT-9,10-diol. On the basis of these experimental results, each of these adducts is presumed to be a 9,10-diol-7,8-epoxide-derived adduct of 5-nitro-BNT or 5-amino-BNT.

Discussion

In this study, we have shown that 5-nitro-BNT is weakly mutagenic in *Salmonella* strains TA98 and TA100 without S9, and that this activity is increased in the presence of S9. The mutagenic response of these strains in the absence of S9 suggests the production of the proximate mutagen via nitroreduction. Nitroreduction has been demonstrated to be carried out by a family of nitroreductases present in *Salmonella* (30). In contrast, the mutagenic response in strains TA98 and TA100 in the presence of S9 provides evidence for the formation of proximate mutagens by oxidation. Thus, the results of the present study indicate that the biological effects of nitro-PAHs are mediated by their metabolic activation to reactive electrophiles by nitroreduction and/or oxidation (31, 32).

Additional evidence supporting this conclusion is provided by the results of the metabolism of 5-nitro-BNT under both aerobic and anaerobic conditions (Schemes 1

and 2). The aerobic metabolism of 5-nitro-BNT by S9 produces 5-nitro-BNT-9,10-diol, 5-amino-BNT, and 5-amino-BNT-9,10-diol. In contrast, S9-mediated metabolism of the parent molecule, BNT, produced dihydrodiols in the naphthal portion of the molecule yielding *trans*-1,2-dihydroxy-1,2-dihydro-BNT and *trans*-3,4-dihydroxy-3,4-dihydro-BNT (11). By comparison, chrysene, which has a structure similar to BNT (a benzene ring substituting for a thiophene ring) was metabolized by rat liver enzymes to two dihydrodiols, *trans*-1,2-dihydroxy-1,2-dihydrochrysene, and *trans*-3,4-dihydroxy-3,4-dihydrochrysene, both metabolites similar in structure to those metabolic dihydrodiols obtained from BNT (33). These results suggest that the presence of the NO₂-naphthal group in BNT directs oxidation to the benzo portion of the molecule. This shift in metabolism away from the ring containing the nitro moiety has also been reported for 6-nitrochrysene, where the major dihydrodiol metabolite from Aroclor-1254 induced rat-liver S9 was *trans*-1,2-dihydroxy-1,2-dihydro-6-nitrochrysene (34). The 1,2-diol of 6-nitrochrysene and the 9,10-diol of 5-nitro-BNT are structurally similar with respect to the spatial relationships between the nitro and dihydrodiol moieties.

The detection of 5-amino-BNT as a metabolite of 5-nitro-BNT provides evidence for the occurrence of nitroreduction under aerobic conditions. Similar results, indicating the production of amino metabolites under aerobic conditions, have been reported in studies examining the metabolism of 1-nitropyrene by respiratory tract tissues and cells (35, 36), and 6-nitrochrysene by preweanling mouse liver-S9 (37). The anaerobic metabolism of 5-nitro-BNT using S9 or XO produced 5-amino-BNT as the predominant metabolite. Similarly, nitroreduction of other NO₂-PAHs such as 1-nitropyrene, 5-nitroacenaphthene, 1-nitronaphthalene, and 6-nitrochrysene has been observed when Aroclor-induced rat liver S9 incubations were conducted in an atmosphere of 4% O₂ and N₂ (34). Additionally, under anaerobic conditions, XO has been used to catalyze the binding to DNA of 1-nitropyrene and dihydrodiol derivatives of nitro-PAHs (23, 38).

The ³²P-postlabeling assay coupled to HPLC was used to separate the DNA adducts of 5-nitro-BNT produced under both aerobic and anaerobic conditions. The diagnostic selective metabolism (S9 versus XO) and chromatographic comparisons of the 5-nitro-BNT-modified-calf thymus DNA with the reaction products of 5-nitro-BNT and 3'-dGMP or 3'-dAMP were used to identify the DNA adducts formed. Chromatographic comparisons of the XO-derived 5-nitro-BNT adducts with reaction products of 5-nitro-BNT and 3'-dGMP or 3'-dAMP identified these calf thymus DNA adducts as dGuo or dAdo adducts. When allopurinol, a specific inhibitor of XO was included in the 5-nitro-BNT incubation mixtures, DNA adduct levels were decreased, confirming that nitroreduction and subsequent DNA binding was enzymatically mediated by XO (23, 38). Similar results were reported in the identification of both dGuo and dAdo adducts of 6-nitrochrysene in the lungs of CD-1 mice following *in vivo* exposures (39). Aerobic metabolism of the isolated 5-amino-BNT metabolite with S9 and calf thymus DNA produced a major 3'-dGMP adduct which is similar to the major calf thymus DNA adduct detected following anaerobic incubations of 5-nitro-BNT with XO. These results implicate the production of an N-hydroxy derivative of 5-nitro-BNT in the formation of DNA adducts. Similar results were reported on the N-hydroxy derived DNA adduct following

exposure of rat hepatocytes to 6-aminochrysene (40).

Additionally, chromatographic comparisons of the aerobic S9-derived 5-nitro-BNT adducts with the products of 5-nitro-BNT and 3'-dGMP or 3'-dAMP of S9 resulted in the identification of two major adducts: a dGuo adduct derived from 5-nitro-BNT-9,10-diol and a single dAdo adduct. The dGuo adduct was also formed by the aerobic incubation of 5-nitro-BNT-9,10-diol, while the dAdo adduct was also formed by the aerobic incubation of either 5-nitro-BNT-9,10-diol or 5-amino-BNT-9,10-diol. The formation of these adducts were inhibited by 1-ABT, a specific inhibitor of P450, suggesting that the observed DNA binding was enzymatically mediated by P450. The observation of a 5-amino-BNT-9,10-diol-Guo adduct is reminiscent of the results reported for 6-nitrochrysene. In that study, one major calf thymus DNA adduct with dGuo resulted when the metabolite 6-aminochrysene-1,2-dihydrodiol was further metabolically activated by 3-methylcholanthrene-induced rat liver microsomes (41). A 6-aminochrysene-1,2-dihydrodiol adduct has also been observed *in vivo* after treatment of preweanling mice with 6-nitrochrysene (42).

In summary, the mutagenicity of 5-nitro-BNT in *Salmonella* TA100 and TA98 suggests involvement of both nitroreductive and oxidative pathways. To clarify the mechanism by which 5-nitro-BNT is metabolically activated, the aerobic and anaerobic metabolism of 5-nitro-BNT by S9 and XO *in vitro* were studied (Scheme 1). Aerobic metabolism of 5-NBNT resulted in the formation of 5-nitro-BNT-9,10-diol, 5-amino-BNT and 5-amino-BNT-9,10-diol. Under aerobic conditions 5-nitro-BNT-9,10-diol, 5-amino-BNT, and 5-amino-BNT-9,10-diol were involved in the formation of DNA adducts (three, one, and 1one adducts, respectively). Under anaerobic conditions using S9 or XO, 5-amino-BNT was the predominant metabolite. Under anaerobic conditions, 5-nitro-BNT was involved in the formation of three adducts. Thus, the metabolic activation of 5-nitro-BNT and DNA adduct formation involves nitroreduction and ring oxidation.

Knowledge of specific adduct structures obtained under various activation conditions in the present study should prove useful for establishing a linkage between specific metabolites, activation pathways and carcinogenicity of 5-nitro-BNT and other subsequent identified nitrated thioarenes.

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