Synthesis, Spectral Analysis, and Mutagenicity of 1-, 3-, and 6-Nitrobenzo[a]pyrene

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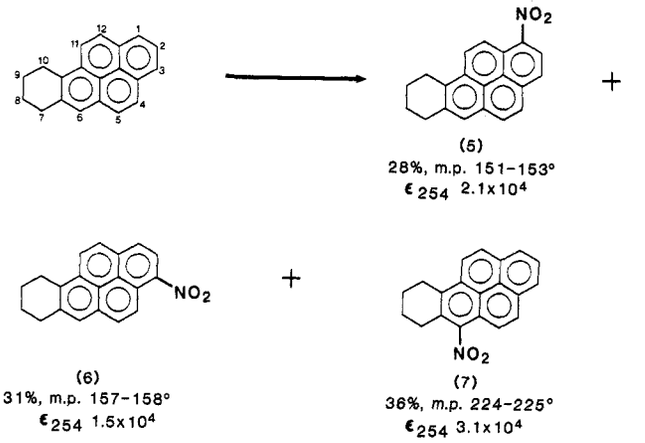
The mutagenic environmental pollutants 1-, 3-, and 6-nitrobenzo[a]pyrene were synthesized. Nitration of 7,8,9,10-tetrahydrobenzo[a]pyrene with sodium nitrate in trifluoroacetic acid and acetic anhydride at ambient temperature gave a mixture of 1-, 3-, and 6-nitro-7,8,9,10-tetrahydrobenzo[a]pyrene, which was separated by chromatography. Dehydrogenation of the isolated nitrotetrahydrobenzo[a]pyrenes with 2,3-dichloro-4,5-dicyano-1,6-benzoquinone produced 1-, 3-, and 6-nitrobenzo[a]pyrene in high yield. Comparison of the spectral data of these compounds with those obtained from direct nitration of benzo[a]pyrene confirmed that 1- and 3-nitrobenzo[a]pyrenes are indeed the minor products of the latter reaction. This confirmation also verifies that 1- and 3-nitrobenzo[a]pyrene were the minor nitrated products of benzo[a]pyrene formed in model air atmospheres. The 1-, 3-, and 6-nitrobenzo[a]pyrene were mutagenic in *Salmonella typhimurium* tester strains TA98 and TA100 in the presence of a mammalian microsomal (S9) activating system. Both 1- and 3-nitrobenzo[a]pyrene, but not 6-nitrobenzo[a]pyrene, were also direct-acting mutagens in these strains. However, only 6-nitrobenzo[a]pyrene exhibited weak mutagenic activity when tested in Chinese hamster ovary cells, while only 3-nitrobenzo[a]pyrene produced a concentration-dependent decrease in cellular survival.

Nitro polycyclic aromatic hydrocarbons (nitro-PAHs) have been found as mutagenic components in fly ash, diesel emissions, photocopier toners, cigarette smoke, and other environmental samples.[1-8] Recently, several nitro-PAHs have also been shown to be carcinogenic in experimental animals.[8-12] A major concern now is the possible hazard these compounds pose for human health.

The nitro-PAHs, 1-nitrobenzo[a]pyrene (1-nitro-BaP; 1), 3-nitrobenzo[a]pyrene (3-nitro-BaP; 2), and 6-nitrobenzo[a]pyrene (6-nitro-BaP; 3) were found in model atmospheres containing trace quantities of benzo[a]pyrene (BaP), nitrogen oxide, and nitric acid.[13] 6-Nitro-BaP has also been detected as an air pollutant[14] and both 3- and 6-nitro-BaP have been isolated from diesel exhaust emissions.[7] Although 6-nitro-BaP has been unequivocally identified, the characterization of 1- and 3-nitro-BaP was only tentative.[13] Direct nitration of BaP with fuming nitric acid in acetic anhydride has been reported to yield 6-nitro-BaP, a predominant product that has been well characterized, and a mixture of mononitro-BaP in small quantity.[15] On the basis of molecular orbital calculations, 1- and 3-nitro-BaP were predicted to be the minor products, but no identification has been achieved.[15] Since characterization of 1-, 3-, and 6-nitro-BaP in the model atmosphere experiment was based on the comparison of the TLC R_f values and GC retention times of the products with those of the nitro-BaP from the direct nitration reaction described above,[13] the characterization of 1- and 3-nitro-BaP requires confirmation. Because nitration of BaP yielded 1- and 3-nitro-BaP at levels less than 5% and because separation of 1- and 3-nitro-BaP is very difficult, it was desirable to develop a synthetic model that could provide both compounds pure and in high yield. In this paper, we report (1) a novel synthesis of 1-, 3-, and 6-nitro-BaP, (2) the full characterization and structural analysis of the compounds from their UV–visible absorption, mass and high-resolution 500-MHz proton NMR spectra data, (3) the confirmation of the formation of 1- and 3-nitro-BaP obtained from direct nitration of BaP, and (4) the mutagenicity of 1-, 3-, and 6-nitro-BaP in *Salmonella typhimurium* tester strains TA98 and TA100, with and without mammalian (S9) activation, and in the Chinese hamster ovary cell system.

Scheme I



Results

Synthetic Chemistry. Synthesis starts from a commercially available chemical, 9,10-dihydrobenzo[a]pyren-7(8*H*)-one, which was reduced to 7,8,9,10-tetrahydro-BaP (4) via the Wolff–Kishner reduction[16,17] in near-quantitative yield. Direct nitration of 4 was accomplished by several different nitration reagents, including fuming nitric acid, silver nitrate, and sodium nitrate, to yield the desired compounds 1-nitro-7,8,9,10-tetrahydro-BaP (5), 3-nitro-7,8,9,10-tetrahydro-BaP (6), and 6-nitro-7,8,9,10-tetra-

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Table I. Proton NMR (500-MHz) Spectral Data for 1-, 3-, and 6-Nitrobenzo[a]pyrene[a]

Compound	Chemical Shift, (δ)											
	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	H_9	H_{10}	H_{11}	H_{12}
BaP	8.33	8.03	8.18	8.02	8.09	8.65	8.37	7.88	7.82	9.18	9.21	8.43
1-NO_2-BaP	—	8.78	8.36	8.19	8.39	8.94	8.49	7.94	8.02	9.32	9.57	9.12
3-NO_2-BaP	8.50	8.63	—	8.64	8.47	8.93	8.49	7.94	8.01	9.30	9.48	8.59
6-NO_2-BaP	8.56	8.21	8.42	8.35	7.95	—	8.17	8.06	8.06	9.39	9.36	8.67

Compound	Coupling Constant (Hz)						
	$J_{1,2}$	$J_{2,3}$	$J_{4,5}$	$J_{7,8}$	$J_{8,9}$	$J_{9,10}$	$J_{11,12}$
BaP	7.7	7.5	9.0	8.2	6.8	8.2	7.0
1-NO_2-BaP	—	8.3	9.2	8.1	6.9	8.6	9.6
3-NO_2-BaP	8.6	—	9.6	8.1	6.8	8.5	9.1
6-NO_2-BaP	7.9	7.6	9.2	8.5	7.0	8.7	9.2

[a] Samples were dissolved in acetone-d_6. Chemical shifts are reported in ppm downfield from internal tetramethylsilane. Coupling constants were first-order measurements.

hydro-BaP (7) (Scheme I). When fuming nitric acid in ice-cold acetic anhydride was employed for reaction with 4 overnight, considerable amounts of dinitro-7,8,9,10-tetrahydro-BaP, BaP-1,6-, -3,6-, and -6,12-quinone, and unidentified materials were formed as byproducts. Although the yields of the undesired products were reduced when shorter reaction times (2–6 h) were used, the conversion of 4 into the desired mononitro-7,8,9,10-tetrahydro-BaP was also decreased. Nitration of 4 employing sodium nitrate in trifluoroacetic acid and acetic anhydride at ambient temperature under argon provided much cleaner results. This reaction yielded compounds **5**, **6**, and **7** in yields of 28%, 31%, and 36%, respectively, together with 5% of **4**. Unlike the results reported by Spitzer and Stewart[18] that nitration of benzene and toluene can be achieved by sodium nitrate in neat trifluoroacetic acid, we found that nitration of 4 by sodium nitrate in neat trifluoroacetic acid proceeded extremely slowly (less than 5% conversion after 15 h). However, when acetic anhydride was used as cosolvent, nitration was nearly complete in 6 h at room temperature.

Separation of the reaction mixture was accomplished first by column chromatography with a Florisil column. Compound **7** and the recovered **4** were each easily separated, but compounds **5** and **6** were eluted as a mixture, which were then separated by normal-phase HPLC employing either an analytical or a preparative column.

Aromatization of **5–7** was achieved by heating under reflux with 2,3-dichloro-4,5-dicyano-1,6-benzoquinone (DDQ) in benzene. The yields were all nearly quantitative when reactions were carried out in a dilute solution. Presumably due to formation of the charge-transfer complex between DDQ and the products (nitro-BaP), the yields were much lower (down to 50%) when reaction was performed at higher concentration.

To obtain the suspected 1- and 3-nitro-BaP described by Dewar et al.[15] for spectral characterization, direct nitration of BaP by fuming nitric acid in ice-cold acetic anhydride was also carried out. Spectral analysis of the products confirmed that both 1- and 3-nitro-BaP were indeed minor products from direct nitration of BaP. Direct nitration of BaP by sodium nitrate in trifluoroacetic acid and acetic anhydride was also performed and was found to result in higher yields with much lower amounts of byproducts (BaP-1,6- and -3,6-quinones, and dinitro-BaP) formed.

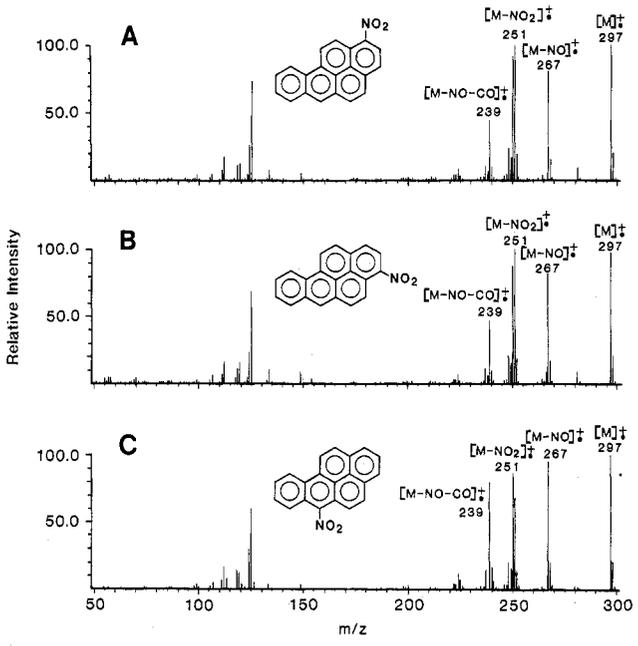

Figure 1. Mass spectra of (A) 1-nitro-BaP, (B) 3-nitro-BaP, and (C) 6-nitro-BaP.

Spectral Identification and Conformation of Products. The products 1-, 3-, and 6-nitrobenzo[a]pyrene were unequivocally characterized by analysis of their UV–visible absorption and mass and high-resolution 500-MHz proton NMR spectra. The mass spectra (Figure 1) all exhibited similar patterns with molecular ions (M^+) at m/z 297. The fragment ions at m/z 267 (loss of NO), m/z 251 (loss of NO_2), and m/z 239 (loss of NO and CO) are typical of nitro-PAHs. The structures of these three nitro-PAHs were further confirmed by high-resolution 500-MHz proton NMR spectroscopy. NMR spectra are shown in Figure 2 and the resonance assignments and coupling constants of nitro-BaP and BaP are given in Table I. The proton NMR resonance assignments were determined by extensive homonuclear decoupling experiments, including de-

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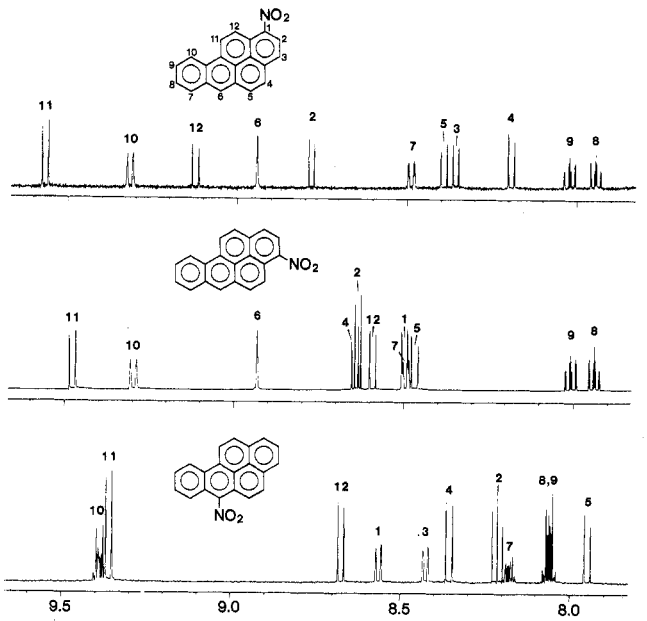

Figure 2. Proton NMR (500 MHz) spectra of 1-nitro-BaP, 3-nitro-BaP, and 6-nitro-BaP dissolved in acetone-d_6. Chemical shifts are in ppm downfield from tetramethylsilane.

coupling of the long-range peri $^4J_{1,12}$, $^4J_{5,6}$ and bay $^5J_{10,11}$ couplings, as well as by comparison to assignments of BaP.

The NMR spectrum of 6-nitro-BaP, which lacks a singlet resonance peak, clearly indicates the nitro substituent located at the C-6 position of BaP. The chemical shift of the peri protons H5 and H7 of 6-nitro-BaP are upfield (0.14 and 0.21 ppm, respectively) as compared with those of BaP (Table I). These data can confirm the structural assignment and also can be used to determine the conformation of the compound. The magnitude and direction of the chemical shift changes of the peri protons should be dependent on the orientation about the C–N bond. This is because of the anisotropy of the nitro group.[19] The shielding effect of the nitro substituent on H5 and H7 suggests that the plane of the nitro group be either perpendicular or near perpendicular to the aromatic moiety of 6-nitro-BaP.[19,20] Such an orientation may result from steric hindrance between the nitro substituent and the H5 and H7 protons. This orientation would result in nonconjugation or little conjugation between the π-electrons of the nitro group and the π-electrons of the aromatic ring. This conclusion was consistent with the result that the UV–visible spectrum of 6-nitro-BaP was similar to that of BaP (see Figure 3). This conclusion was also consistent with the observation by Rodenburg et al.[20] that the orientation of the nitro groups of 2-*tert*-1-nitropyrene and 2,4-di-*tert*-butyl-1-nitropyrene prevents this group from conjugation with the π-system, leading to an unperturbed pyrene electronic spectrum.

Distinction between the structures of 1-nitro-BaP and 3-nitro-BaP was made by analysis of their high-resolution proton NMR spectra. The most prominent effects of the nitro group were large downfield shifts of the protons ortho and peri to the substituent. The downfield shifts of H2 is 0.6 ppm for both compounds. The downfield shift of H12 (0.7 ppm) of 1-nitro-BaP and the downfield shift of H4 (0.6 ppm) of 3-nitro-BaP provide a convenient method for distinguishing between these two mononitro-BaP isomers. The downfield shifts of H12 of 1-nitro-BaP and H4 of 3-nitro-BaP suggest that the oxygen atom of the nitro substituent is toward the peri protons (H12 and H4, respectively). Such an orientation would result in partial conjugation between the nitro group and the aromatic ring. As expected, the UV–visible absorption spectra of 1- and 3-nitro-BaP are different from that of BaP and exhibit extended π–π conjugation (Figure 3). Thus, the results of NMR and UV–visible spectra of 1-, 3-, and 6-nitro-BaP can be employed for studying the conformations of these compounds.

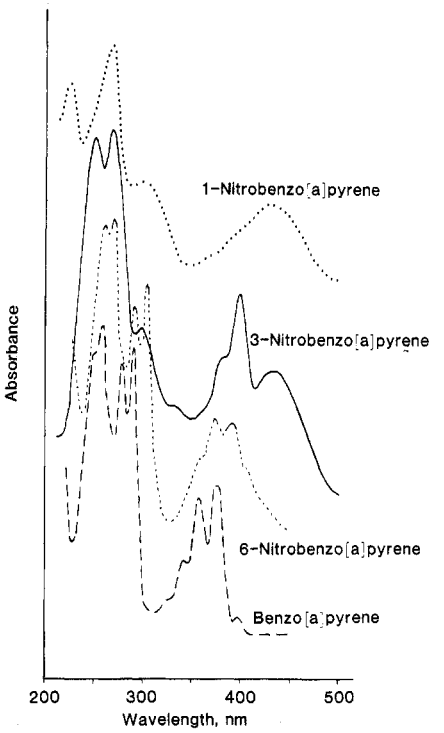

Figure 3. Ultraviolet–visible absorption spectra of 1-nitro-BaP, 3-nitro-BaP, 6-nitro-BaP, and BaP measured in methanol.

Mutagenicity. The mutations induced by 1-nitro-BaP, 3-nitro-BaP, 6-nitro-BaP, and BaP were compared in *Salmonella typhimurium* tester strains TA98 and TA100 (Figure 4). In the presence of S9 activation, all of the four compounds produced a strong mutagenic response in both strains. In general, the number of revertants produced by the nitro-BaP in strain TA98 was much higher than that observed in strain TA100. The numbers of revertants produced by BaP in the two tester strains were similar. In strain TA98, 1-nitro-BaP and 3-nitro-BaP were ca. 4-fold more mutagenic than BaP, while 6-nitro-BaP was ca. 2-fold more mutagenic than BaP. These results were consistent with those obtained by Pitts et al. although that report did not specify the compound concentrations for assay and did not distinguish between 1- and 3-nitro-BaP.[21] In strain TA100 with S9 activation, BaP and 3-nitro-BaP were the most mutagenic followed by 6-nitro-BaP and 1-nitro-BaP. In the presence of S9, all four compounds reverted the nitroreductase-deficient strains TA98NR and TA100NR in a manner similar to that found for the respective parent strains (data not presented).

Both 1- and 3-nitro-BaP were also mutagenic in TA98 and TA100 without S9. Concentration-dependent increases in the numbers of revertants produced by 1- and 3-nitro-BaP were found with use of less than 5 nmol/plate

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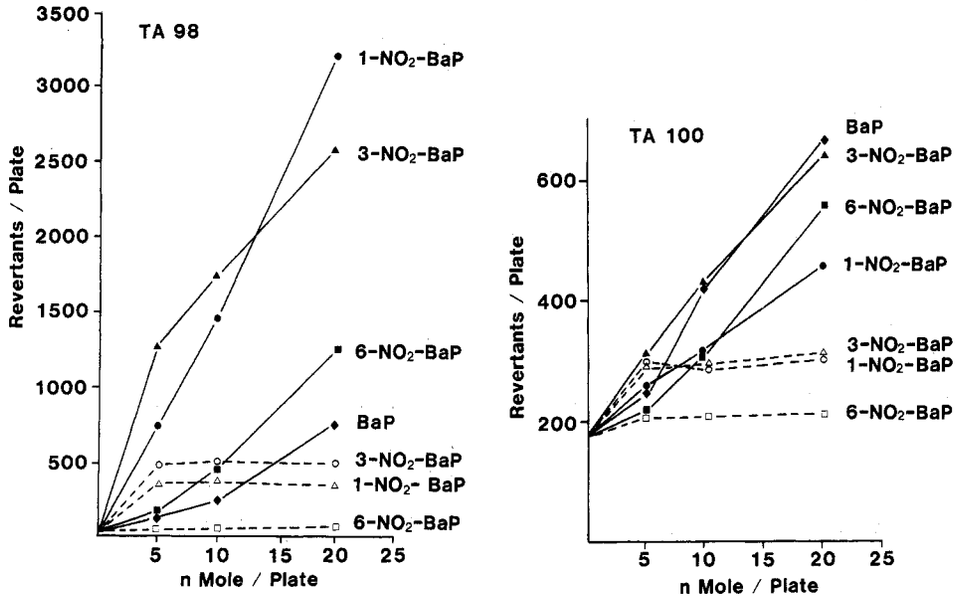

Figure 4. Reversions induced by BaP (♦), 1-nitro-BaP (●), 3-nitro-BaP (▲), and 6-nitro-BaP (■) in *Salmonella typhimurium* tester strains TA98 and TA100 with (closed symbols) and without (open symbols) S9 activation. Each point represents the averaged results of assays performed in triplicate.

Table II. Mutagenic Activity of 6-Nitrobenzo[a]pyrene in the CHO/HGPRT Assay

	Mutants/10^6 Survivors[a]		
Dose (μg/ml)	Exp I	Exp II	Exp III
0	2.0	2.0	3.0
1.0	----	31.0	----
3.0	27.0	----	69.0
6.0	33.0	----	109.0

[a] Mutation frequency as defined in Experimental Section.

of these compounds. Both were less mutagenic in the nitroreductase-deficient strains than the parent tester strains (data not shown). Without S9 activation, 6-nitro-BaP did not induce reversions in any of the tester strains.

When the three isomeric nitro-BaP were evaluated in a mammalian mutation system, the CHO/HGPRT assay, the results were quite different. In the absence of metabolic activation, 6-nitro-BaP consistently displayed a concentration-dependent increase in 6-thioguanine-resistant mutants. The increase ranged from 13-fold to 36-fold, depending on the concentration (Table II). Benzo[a]pyrene, under the same conditions of assay, displayed less than a 2-fold increase relative to the background level (data not shown). This latter response is considered negative. Under similar conditions, 1-NO_2-BaP and 3-NO_2-BaP were also negative, although 3-NO_2-BaP consistently induced a concentration-dependent cytotoxic response (reduction in cloning ability). This effect ranged from a 10% decrease in survival at 1 μg/mL to a 50% decrease at 10 μg/mL (data not shown). Genotoxic effects of these compounds are now being studied in this system in the presence of various exogenous metabolic activation systems.

Discussion

In this paper we have reported the novel synthesis, spectral characterization, and mutagenicity of the environmental contaminants, 1-, 3-, and 6-nitro-BaP. Spectral analysis of these compounds demonstrated that 1- and 3-nitro-BaP are indeed the minor products obtained from direct nitration of BaP by fuming nitric acid.[15] These results also verify that the prediction by Dewar et al.[15] based on molecular orbital calculations and confirm the conclusion of Pitts et al. that 1- and 3-nitro-BaP were the minor products obtained from model atmospheres containing BaP and nitric acid.[13]

We have also demonstrated that the synthesized nitro-BaP are potent bacterial mutagens. Both 1- and 3-nitro-BaP are direct-acting mutagens in the *Salmonella* reversion assay. This direct mutagenic activity, which is characteristic of many nitro-substituted PAHs, was apparently mediated by bacterial nitroreduction since both compounds were less mutagenic in tester strains deficient in nitroreductase activity. Consistent with previous findings, 6-nitro-BaP was not a "direct-acting" mutagen in the *Salmonella* assay.[22] In Chinese hamster ovary cells, the direct mutagenic activity of these compounds was the reverse of that seen with bacteria. Only 6-nitro-BaP was mutagenic, while both 1- and 3-nitro-BaP were inactive, although 3-nitro-BaP was cytotoxic. The mechanism underlying the differential mutagenic responses displayed by these compounds in the bacterial and mammalian cell assays is presently under investigation.

In strain TA98 with S9, all of the nitro-BaP isomers were more mutagenic than the parent BaP. The higher mutagenic potential of the nitro-BaPs in TA98 relative to TA100 suggests that these compounds induced reversions by a frame-shift mechanism. With S9, BaP was approximately equally mutagenic in strains TA98 and TA100 and displayed mutagenic activity equal to or greater than the nitro-BaP isomers in TA100. These observations suggest that nitro substitution influences the nature of the mutagenic DNA damage produced in the *Salmonella* by BaP. Since the nitro-BaPs were equally mutagenic in both nitroreductase-proficient and -deficient tester strains, ring oxidation, not bacterial nitroreduction, may be involved in the S9-mediated mutagenicity of these compounds. Indeed, although nitrosubstitution at the 6-position of BaP inhibited dihydrodiol formation,[23] ring hydroxylation was involved in the metabolic activation of this compound.[22]

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In other studies, both the *trans*-7,8-dihydrodiol and the *trans*-9,10-dihydrodiol were found as the predominant metabolites of 1- and 3-nitro-BaP incubated with rat-liver microsomes.[22-25] These metabolites may be responsible for the S9-mediated mutagenicity of 1- and 3-nitro-BaP.

All the gathered data have indicated that 1-, 3-, and 6-nitro-BaP are mutagenic environmental compounds and therefore may be hazardous to human health. The synthesis described in this paper, providing each mononitro-BaP without contamination by other isomer(s), facilitates further biological studies of these compounds. Because of the availability of these compounds, we have been studying the metabolic activation pathways of 6-nitro-BaP and metabolic fates of 1- and 3-nitro-BaP.[22-26] The spectral data presented in this paper would be useful as standard references for identification of 1-, 3-, and 6-nitro-BaP from other environmental sources. The distinct differences in NMR shifts of the protons peri to the nitro substituent and in the UV–visible absorption spectra have been shown to be applicable for the conformational analysis of nitro-PAHs.

Experimental Section

General Procedures. All melting points were taken on a Thomas-Hoover capillary melting point apparatus and were uncorrected. Ultraviolet–visible absorption spectra were obtained by using a Beckman Model 25 spectrophotometer. Mass spectral analyses were performed on a Finnigan 4000 GC/MS with a solid probe by electron impact at 70 eV at an ionizer temperature at 250 °C. Fourier-transform proton NMR spectra were obtained with a Bruker WM 500 spectrometer. HPLC was performed with a Waters Associates system consisting of two 6000A pumps, a 660 solvent programmer, a U6K injector, and a 440 UV detector (254 nm).

Nitration of 7,8,9,10-Tetrahydrobenzo[*a*]pyrene (4). Compound 4 (1.28 g, 5 mmol) in acetic anhydride (300 mL) was added to a solution of sodium nitrate (425 mg, 5 mmol) in trifluoroacetic acid (200 mL). The resulting solution was stirred at ambient temperature under argon for 15 h. The reaction products were partitioned between ethyl acetate and water containing 5 mL of concentrated sulfuric acid. The organic layer was collected, washed with water (2 × 300 mL), and dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The yellowish residue was column chromatographed over Florisil (2 × 20 cm). Elution with hexane gave the unreacted 4 (65 mg). Elution with benzene–hexane (4:1, v/v) gave 6-nitro-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7) (630 mg, 36% yield): mp 224–225 °C (benzene); mass spectrum (70 eV), *m/z* 301; UV (methanol) ϵ_{254} 3.1×10^4. Further elution with benzene–hexane (4:1, v/v) and benzene gave a mixture of 1-nitro-7,8,9,10-tetrahydro-BaP (5) and 3-nitro-7,8,9,10-tetrahydro-BaP (6) in various ratios. The mixture of 5 and 6 was separated by normal-phase HPLC employing a DuPont Zorbax SIL semipreparative column (9.4 × 250 mm) and eluting isocratically with 2% tetrahydrofuran in hexane at a flow rate of 4 mL/min. Compound 5 was eluted at 18.2 min: mass spectrum (70 eV), *m/z* 301; mp 151–153 °C (benzene); UV (methanol) ϵ_{254} 2.1×10^4. Compound 6 was eluted at 19.2 min: mass spectrum (70 eV), *m/z* 301; mp 157–158 °C (benzene); UV (methanol) ϵ_{254} 1.5×10^4. Analysis of the mixture indicated that 5 and 6 were produced in the yield of 28% and 31%, respectively.

The mixture of 5 and 6 could also be separated in a larger scale by employing a preparative column (21.2 × 250 mm) using a Beckman HPLC system and eluting isocratically with 2% tetrahydrofuran in hexanne at a flow rate of 11.2 mL/min. The retention times of 5 and 6 were 21.5 and 22.7 min, respectively.

Nitration of 4 employing fuming nitric acid in ice-cold acetic anhydride gave 5–7 each in 20–25% yields together with the byproduct dinitro-4, the recovered 4, and unidentified materials.

1-Nitrobenzo[*a*]pyrene (1). A solution of 5 (100 mg, 0.33 mmol) and DDQ (230 mg, 1 mmol) in benzene (500 mL) was heated for reflux under argon for 8 h. The reaction mixture was washed with water (5 × 300 mL). The organic layer was separated and dried with anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The resulting residue was applied onto a Florisil column (2 × 20 cm). Elution with ethyl acetate–benzene (1:9, v/v) gave product 1 as an orange solid in 97 mg (99% yield): mp 250–250.5 °C; mass spectrum (70 eV), *m/z* 297 (M^+), 267, 251, 239; UV spectrum, Figure 3; NMR spectrum, Figure 2 and Table I. Anal. ($C_{20}H_{11}NO_2$) C, H, N.

When a similar reaction was performed with use of only 100 mL of benzene, compound 1 was obtained only in 55% yield.

3-Nitrobenzo[*a*]pyrene (2). Dehydrogenation of 6 (52 mg, 0.17 mmol) by DDQ, performed as described for 1, yielded 2 in 50 mg (99% yield) as an orange solid: mp 211–212 °C; mass spectrum, Figure 1; UV spectrum, Figure 3; NMR spectrum, Figure 2 and Table I. Anal. ($C_{20}H_{11}NO_2$) C, H, N.

6-Nitrobenzo[*a*]pyrene (3). Dehydrogenation of 7 (100 mg, 0.33 mmol) by DDQ (230 mg, 1 mmol) in refluxing benzene (500 mL) for 15 h gave 3 in 84 mg (85% yield) as an orange solid: mp 255–256 °C (lit.[15] mp 252–253 °C); mass spectrum, Figure 1; UV spectrum, Figure 3; NMR spectrum, Figure 2 and Table I.

Nitration of Benzo[*a*]pyrene. Nitration of benzo[*a*]pyrene by fuming nitric acid in ice-cooled acetic anhydride was carried out according to the previously published procedures of Dewar et al.[15] The products were analyzed by HPLC and GC,[20] confirming that 1- and 3-nitrobenzo[*a*]pyrene were minor products in a total yield of less than 5%.

Bacterial Mutagenicity Analysis. Reversion to prototrophy using *Salmonella typhimurium* histidine auxotrophic strains TA98, TA98NR, TA100, and TA100NR was measured essentially as described by Ames et al.[27] The mutagen, dissolved in 0.1 mL of glass-distilled dimethyl sulfoxide, was added to a tube containing 2.5 mL of molten top agar (0.6% agar, 0.6% NaCl, 0.05 mM L-histidine, 0.05 mM biotin), 0.1 mL of the bacterial tester strain, and 0.5 mL of S9 mix. The S9 mix consisted of 6 parts of Aroclor 1254-induced rat-liver homogenate and 14 parts 33 mM KCl, 5 mM glucose 6-phosphate, 4 mM NADP, and 8 mM $MgCl_2$ in 100 mM sodium phosphate buffer, pH 7.4. The contents of the tube were mixed and poured into 100-mm petri dishes containing Vogel's minimal salts agar with glucose. After the agar had solidified, the plates were inverted and incubated at 37 °C for 48 h in the dark. Colonies were counted manually.

Bacterial mutagenicity assays were performed in triplicate, using 0, 5, 10, and 20 nmol of compound/plate both with and without the addition of S9. The mean background (uninduced) reversion frequencies were as follows: TA98, 32.5; TA98 (+S9), 50.5; TA100, 131.5; TA100 (+S9), 162.9; TA98NR, 30.5; TA98NR (+S9), 46.8. The variation in revertants/plate between replicate assays was generally less than ±10%.

Mammalian Cell Mutagenicity. Chinese hamster ovary cells (CHO-K1-BH4) were used as the indicator cell to detect mutation induction at the hypoxanthine–guanine phosphoribosyltransferase (HGPRT) locus by assessing resistance to 6-thioguanine. The nitro-BaP compounds were dissolved in Me_2SO and appropriate amounts were added to treatment medium to achieve the concentrations shown in Table II. The solvent concentration never exceeded 1%. Cells were treated for 18 h. The procedure used to isolate induced mutants was essentially the same as that recommended by Hsie et al.[28] Mutations were calculated as 6-thioguanine-resistant cells per 10^6 surviving cells.

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Ames, Berkeley, CA, while strains TA98NR and TA100NR were obtained from H. S. Rosenkranz, Cleveland, OH. CHO-K_1-BH_4 cells were provided by A. W. Hsie, Oak Ridge, TN.

Appendix

Elemental Analytical Data. 1-Nitrobenzo[*a*]pyrene (1). Anal. Calcd for $C_{20}H_{11}NO_2$: C, 80.80; H, 3.73; N, 4.71. Found: C, 80.69; H, 3.80; N, 4.80. **3-Nitrobenzo[*a*]pyrene (2).** Anal. Calcd for $C_{20}H_{11}NO_2$: C, 80.80; H, 3.73; N, 4.71. Found: C, 80.59; H, 3.60; N, 4.63.

Registry No. 1, 70021-99-7; 2, 70021-98-6; 3, 63041-90-7; 4, 17750-93-5; 5, 88598-56-5; 6, 88598-57-6; 7, 88598-58-7; benzo[*a*]pyrene, 50-32-8.

Potential Antitumor Agents: Synthesis and Biological Properties of Aliphatic Amino Acid 9-Hydroxyellipticinium Derivatives

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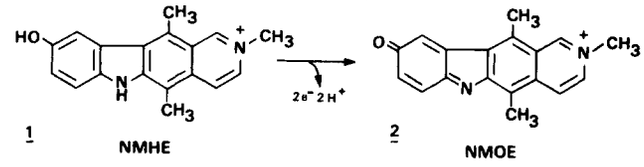
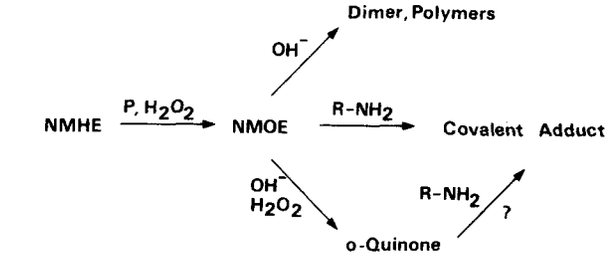
Aliphatic amino acids glycine, alanine, valine, and leucine were conjugated to the antitumor drug N^2-methyl-9-hydroxyellipticinium (NMHE) through a peroxidase-catalyzed oxidation reaction. NMR studies of the adducts so obtained have indicated (i) that the amino acids were linked to NMHE between the nitrogen of their primary amine and the C-10 position of the ellipticine ring and (ii) that a double bond was present between the nitrogen and the α-carbon of the amino acid moiety. All amino acid–NMHE adducts exhibit a higher *lipophilic* property than the parent compound (NMHE) directly correlated with the length of the aliphatic chain of the amino acids. The adducts interact with DNA through an intercalating process with apparent binding constant ranging from 2 $\times 10^5$ to 5 $\times 10^5$ M^{-1} at pH 7.40. The presence of the amino acid moiety linked to NMHE results (i) in a slight decrease of the cytotoxicity on L1210 cells in vitro (ID_{50} ranged from 0.20 to 0.50 μM) as compared to NMHE (ID_{50} = 0.05 μM), (ii) in a decrease of the antitumor efficiency in vivo against L1210 leukemia for leucine–NMHE and valine–NMHE (ILS at $LD_0/2$ = 35% and 31%, respectively), (iii) in a suppression of the antitumor activity for alanine–NMHE and glycine–NMHE (ILS < 25%), (iv) in a strong increase in the bacteriostatic activity on the quaternary ammonium sensitive *Escherichia coli* BL101 strain and on *Salmonella typhimurium* TA98 strain. The bacteriostatic effect is directly correlated with the lipophilic property of the drugs. These finding are discussed in terms of a structure–activity relationship.

N^2-Methyl-9-hydroxyellipticinium (NMHE) (see Scheme I) exhibits a high cytotoxic activity against various experimental tumor cells[1] and is actually used in the treatment of osteolytic breast cancer metastases.[2] In the series of ellipticines, previous works have suggested that the limiting factor in the antitumor efficiency of these drugs was the transport through tumor cell membranes.[3,4] In connection with this finding, it must be noted that the clinically active drug NMHE is a charged, large, and hydrophilic compound exhibiting consequently the most unfavorable structure in terms of membrane transport.[5] A possible strategy suitable to increase the transport of such a molecule across cell membranes is the conjugation with lipophilic compounds of biological interest. Promising results have been obtained in this way with daunorubicine bound to leucine and leucine-containing dipeptides.[6,7] Taking advantage of the ability of the oxidized form of NMHE to readily undergo a nucleophilic addition with N donors,[8] we have prepared the homologous series of aliphatic amino acid–NMHE adducts, namely, glycine–, alanine–, valine–, and leucine–NMHE. The present article describes the preparation and the study of some physicochemical and pharmacological properties of these compounds.

Scheme I



Scheme II



Results

Reaction between N^2-Methyl-9-oxoellipticinium and Amino Acids. In aqueous medium, NMHE can be readily oxidized by an enzymatic system such as peroxidase–hydrogen peroxide (P–H_2O_2) to the corresponding quinone imine[9] N^2-methyl-9-oxoellipticinium (NMOE, compound **2** in Scheme I) as indicated in eq 1.

$$\text{NMHE} + H_2O_2 \xrightarrow{P} \text{NMOE} + 2H_2O \qquad (1)$$

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