Synthesis, Chemical Properties and Mutagenicity of 1,6- and 3,6-Dinitrobenzo[*a*]pyrenes

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Nitration of benzo[*a*]pyrene (BaP) with HNO_3 ($d=1.38$) produced a mixture of dinitroBaPs (1,6- and 3,6-isomers) and mononitroBaPs (1-, 3- and 6-isomers). Pure 1,6-dinitroBaP and 3,6-dinitroBaP were obtained by the reduction of the dinitroBaPs mixture with NaSH to yield the separable products 1-amino-6-nitroBaP and 3-amino-6-nitroBaP, followed by conversion to dinitroBaPs *via* the the diazonium salts. The half-wave potentials ($E_{1/2}$) corresponding to the one-electron reduction of dinitroBaPs were measured and the relationship of these values to the mutagenicity is discussed.

Keywords dinitrobenzo[*a*]pyrene; nitroarene; benzo[*a*]pyrene; nitration; reduction; cyclic voltammetry; mutagenicity

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are formed in various combustion processes and as a result are widely distributed in the environment, for example, in fly ash, diesel emissions, photocopier toners and cigarette smoke.[1] Recently, several nitro-PAHs have been shown to be carcinogenic in experimental animals.[2] Polynitro-PAHs such as dinitropyrenes have also been found to be among the most potent bacterial mutagens.[3] These findings suggest that nitro-PAHs may be a serious health hazard to humans. Many chemical studies on the mutagenicity and carcinogenicity of nitro-PAHs have been done.[4–8]

Nitration of benzo[*a*]pyrene (BaP, **1**) in the presence of N_2O_4 gives mainly 6-nitroBaP (**2**) together with 1- and 3-nitroBaPs (**3** and **4**) as minor products.[9] The characterization and structural analysis of mononitroBaPs have already been studied.[9,10] While **2** is not mutagenic in *Salmonella typhimurium* histidine auxotrophic strain TA98 without addition of a metabolic activation system (S9 mix), **3** and **4** are more mutagenic than the parent BaP in strain TA98 with or without S9 mix.[10] Since polynitropyrenes or polynitrofluorenones generally have strong mutagenic activities, polynitroBaPs may also be viewed as model compounds in this class of environmental pollutants. This paper reports the first synthesis of 1,6- and 3,6-dinitroBaPs (**5** and **6**). Their chemical properties and mutagenic activities are also discussed.

Results and Discussion

Nitration of BaP (**1**) with 1.5 eq of HNO_3 ($d=1.38$) in

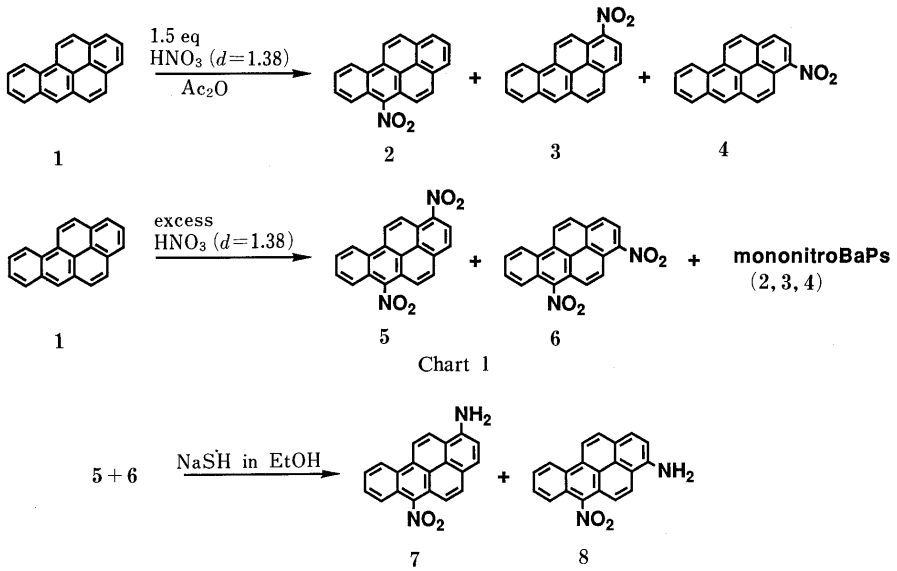

Chart 1

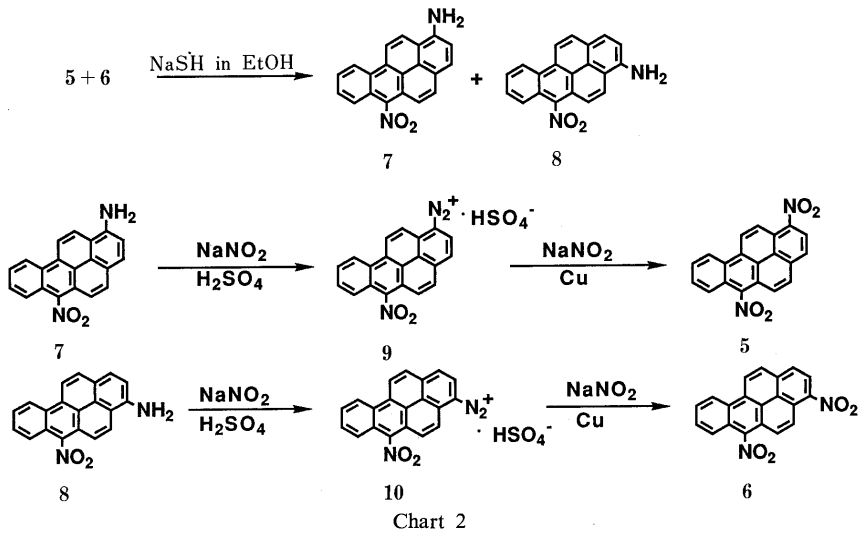

Chart 2

TABLE I. Proton NMR (400 MHz) Spectral Data of MononitroBaPs and DinitroBaPs Measured in DMSO-d_6

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}
1,6-DinitroBaP	—	8.90	8.60	8.48	8.15	—	8.18	8.14	8.14	9.49	9.68	9.10
3,6-DinitroBaP	8.73	8.77	—	8.75	8.23	—	8.19	8.14	8.14	9.50	9.61	8.80
1-NitroBaP	—	8.81	8.38	8.22	8.41	8.99	8.49	7.95	8.02	9.31	9.59	9.04
3-NitroBaP	8.53	8.66	—	8.58	8.49	8.98	8.50	7.95	8.01	9.33	9.52	8.62
6-NitroBaP	8.57	8.21	8.43	8.36	7.91	—	8.12	8.05	8.05	9.41	9.38	8.68

acetic anhydride (Ac_2O) afforded 1-, 3- and 6-nitroBaPs (**3**, **4** and **2**; 8% , 4% and 44% yields). When BaP was nitrated with excess HNO_3 (d=1.38), 1,6-dinitroBaP (**5**), 3,6-dinitroBaP (**6**) and a mixture of mononitroBaPs were formed in 28%, 13% and 41% yields as determined by high-performance liquid chromatographic (HPLC) analysis (Chart 1). The purification of dinitroBaPs from the reaction mixture at this stage was difficult. Therefore, the reaction mixture was reduced with NaSH to give aminonitroBaPs (1-amino-6-nitroBaP (**7**) and 3-amino-6-nitroBaP (**8**)), which were easily separated by silica gel column chromatography. Subsequently, each aminonitroBaP (**7**, **8**) was converted to its corresponding dinitroBaP (**5**, **6**) *via* the diazonium salt (**9**, **10**) (Chart 2).

The nuclear magnetic resonance (NMR) and ultraviolet (UV)-visible spectra of dinitroBaPs reflected the different chemical properties of the two nitro substituents at the 1- (or 3-) and 6-positions. The NMR spectra of dinitroBaPs (Table I), which lacked a singlet resonance peak, clearly indicate the presence of the nitro substituent located at the C-6 position of BaP. The protons *peri* to the 6-nitro substituent (H_5 and H_7) of dinitroBaPs were shifted upfield compared with those of 1- and 3-nitroBaPs. The chemical shifts of H_{12} (1,6-dinitroBaP) and H_4 (3,6-dinitroBaP) were 9.10 and 8.75 ppm, being downfield from those of the H_{12} and H_4 protons of 6-nitroBaP. The protons (H_2) *ortho* to the nitro substituents of dinitroBaPs were also shifted downfield, the signals appearing at 8.90 ppm (1,6-dinitroBaP) and 8.77 ppm (3,6-dinitroBaP). These data show that the nitro substituent at the 1-position of 1,6-dinitroBaP and 3-position of 3,6-dinitroBaP produces a different shielding effect on the *ortho* and *peri* protons compared to the 6-nitro substituent. The UV-visible spectra of 1,6- and 3,6-dinitroBaPs were similar to those of 1-nitroBaP and 3-nitroBaP, respectively (Fig. 1); similarly, it was reported that the UV-visible spectrum of 6-nitroBaP was similar to that of BaP.[10b] It is suggested that the plane of the nitro group is either perpendicular or close to perpendicular to the aromatic moiety of BaP and the 1- or 3-nitro group is conjugated with the aromatic ring of BaP.

The mutagenic activities of 1,6- and 3,6-dinitroBaPs in strain TA98 and the nitroreductase-deficient strain TA98NR without S9 mix are shown in Table II. In the absence of S9 mix, 1,6- and 3,6-dinitroBaPs produced a strong mutagenic response in both strains. 3,6-DinitroBaP, in particular, showed strong mutagenic activity in both strains, nearly equal to that of 1,6- and 1,8-dinitropyrenes. DinitroBaPs showed strong mutagenicity even when tested in TA98NR, while the mutagenicity of 1- and 3-nitroBaPs was greatly decreased in TA98NR as compared to TA98. These results suggest that the mechanism of expression of

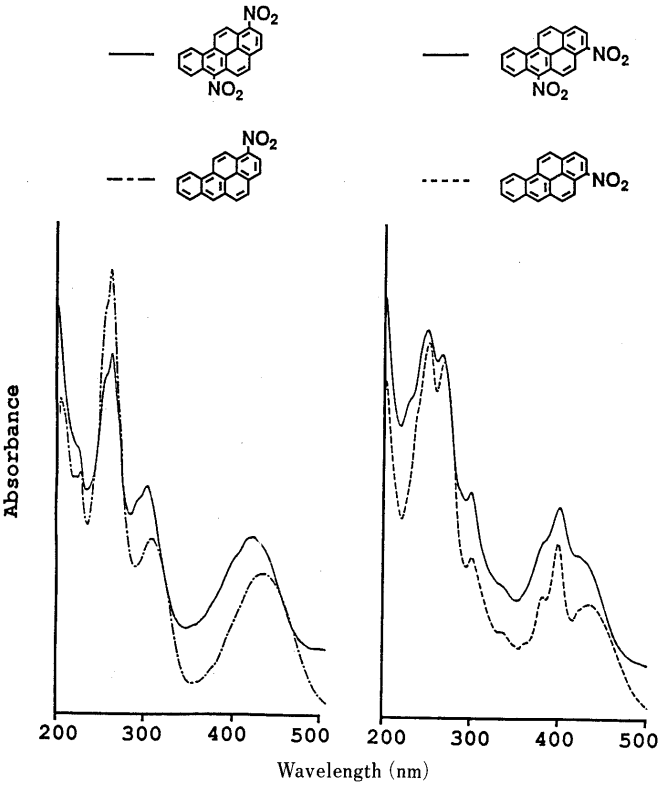

Fig. 1. UV Absorption Spectra of MononitroBaPs and DinitroBaPs Measured in Methanol

TABLE II. Mutagenic Activities of MononitroBaPs and DinitroBaPs in *Salmonella typhimurium* Tester Strains TA98 and TA98NR

Compound	Specific activity (rev./nmol/plate)	
	TA98	TA98NR
1,6-DinitroBaP	6300	3400
3,6-DinitroBaP	150000	56000
1-NitroBaP	830	90
3-NitroBaP	2400	90
6-NitroBaP	0[a)]	0[a)]
1,6-Dinitropyrene	60000	63000
1,8-Dinitropyrene	140000	190000

a) As determined by Pitts *et al.*[9)]

the mutagenicity of 1,6- and 3,6-dinitroBaPs is similar to that of 1,6- and 1,8-dinitropyrenes.

Studies with nitro-PAHs[5,8)] indicate that reduction of the nitro function is required for the expression of mutagenicity in bacteria. Klopman *et al.* have demonstrated that the mutagenicity of a series of nitro-PAHs in *Salmonella* is correlated with the case of their nitroreduction, as measur-

TABLE III. Cyclic Voltammetric Data for MononitroBaPs, DinitroBaPs and Dinitropyrenes

Compound	$-E_{1/2}$ *vs.* S.C.E.	Number of electrons
1,6-DinitroBaP	0.65	2
3,6-DinitroBaP	0.67	2
1-NitroBaP	0.89	1
	1.41	1
3-NitroBaP	0.90	1
	1.42	1
1,6-Dinitropyrene	0.67	2
1,8-Dinitropyrene	0.69	2

S.C.E., standard calomel electrode.

ed from their half-wave potentials.[5a] To gain a better understanding of the relationship of the chemical properties to the diverse mutagenic potencies exhibited by dinitroBaPs, the electrochemical properties of dinitroBaPs and other nitroarenes were examined and their half-wave potentials were compared to ascertain the ease of reduction of the nitro function.

The electrochemical reduction potentials of dinitroBaPs, mononitroBaPs and dinitropyrenes were measured by cyclic voltammetry (Table III). Within the range of 0 to -1.5 V, all nitro arenes showed reversible waves. Each mononitroBaP had two electrochemical reducing steps each involving the transfer of one electron. Their $E_{1/2}$ values (V) were -0.89 and -1.41 (1-nitroBaP), -0.90 and -1.42 (3-nitroBaP) and -0.96 and -1.31 (6-nitroBaP). DinitroBaPs had only one reducing potential, which involved the transfer of two electrons. The $E_{1/2}$ values (V) were -0.65 (1,6-dinitroBaP) and -0.67 (3,6-dinitroBaP). The corresponding $E_{1/2}$ values (V) for dinitropyrenes were -0.67 (1,6-dinitropyrene) and -0.69 (1,8-dinitropyrene). This indicates that dinitroBaPs are more easily reduced than mononitroBaPs. The reduction of dinitroBaPs proceeded in one step, whereas in the case of mononitroBaPs the reduction was a two-step process. This ease of electrochemical reduction of dinitroBaPs is due to the fact that the 1-nitro function of 1,6-dinitroBaP and the 3-nitro function of 3,6-dinitroBaP are made more susceptible to reduction by the effect of the 6-nitro function. DinitroBaPs were chemically reduced with NaSH to give 1-amino-6-nitroBaP and 3-amino-6-nitroBaP. A preliminary experiment indicated that the same reduction occurred on incubation of dinitroBaPs with TA98. It seems that dinitroBaPs induce their mutagenic activation by the reduction of the 1- or 3-nitro function of dinitroBaPs and that the 6-nitro function of each dinitroBaP does not directly influence the activation, since it is not reduced under chemical or biological conditions. It is suggested that the first reduction potentials of these nitrated BaPs are correlated with mutagenicity, and that the mutagenicity of dinitroBaPs can be predicted from their redox potentials.

Experimental

All melting points were determined with a Yanagimoto MP-S3 apparatus. UV spectra were determined with a Shimadzu UV-240 instrument. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded using a Varian VXR-400S spectrometer. The proton chemical shifts were measured relative to tetramethylsilane and are expressed in ppm. Mass spectra (MS) were taken with a JEOL DX-300 mass spectrometer. The cyclic voltammetric measurements were performed with a Yanagimoto P-1000 apparatus, using a platinum working electrode and a saturated calomel electrode as a reference. HPLC was performed on an LC-6A HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a Nucleosil 50-5 column (5 μm, 4.3 mm i.d. × 150 mm). Eluates were monitored with a Shimadzu SPD-6A spectrophotometric detector set at 254 nm.

Nitration of BaP (1) Method A: A solution of HNO_3 (d=1.38, 0.018 ml) in Ac_2O (0.5 ml) was added to a solution of BaP (**1**, 100 mg, 0.4 mmol) in Ac_2O (5 ml) at room temperature. The reaction mixture was stirred for 24 h, then water was added, and stirring was continued for an additional 3 h. The resulting mixture was extracted with CH_2Cl_2. The extracts were washed with water, dried (Na_2SO_4) and concentrated to dryness. The products (105 mg) were analyzed by HPLC (10% CH_2Cl_2–*n*-hexane), and shown to contain 1-nitroBaP (**3**, 8%), 3-nitroBaP (**4**, 4%), 6-nitroBaP (**2**, 44%) and BaP (**1**, 44%).

Method B: To 100 mg (0.4 mmol) of BaP (**1**), 1.0 ml (13 mmol) of HNO_3 (d=1.38) was added dropwise with stirring at room temperature. Stirring was continued for an additional 30 min. The reaction mixture was treated with 50 ml of water and filtered. The dark brown precipitates were washed with water and dried under reduced pressure. The reaction mixture (120 mg) was analyzed by HPLC (10% CH_2Cl_2–10% EtOAc–*n*-hexane), and shown to contain mononitroBaPs (**2**, **3** and **4**, 41%), 1,6-dinitroBaP (**5**, 28%) and 3,6-dinitroBaP (**6**, 13%).

1-Amino-6-nitroBaP (7) and 3-Amino-6-nitroBaP (8) The mixture of the dinitroBaPs (method B, 100 mg) was dissolved in 100 ml of ethanol, and 5 ml of 10% NaSH solution was added at room temperature. The reaction was followed by thin layer chromatography (TLC). Two new spots appeared and their amounts were maximum after 15—20 min. The reaction mixture was evaporated under reduced pressure at 40 °C and the residue was dissolved in CH_2Cl_2. The solution was washed with water, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified on a silica gel column with 30% EtOAc–*n*-hexane. The fractions were analyzed by TLC (EtOAc) and the appropriate fractions were combined to give 1-amino-6-nitroBaP (Rf=0.85) and a mixture of 3-amino-6-nitroBaP (Rf=0.88) and by-products. Finally, 14.0 mg (11.2% yield) of 1-amino-6-nitroBaP (**7**) was obtained, mp 255—256 °C. ^{1}H-NMR (Me_2SO-d_6): 9.29 (d, H_{10}, J=8.4 Hz), 9.12 and 8.80 (d, H_{11} and H_{12}, J=9.4 Hz), 8.10 (d, H_3, J=8.3 Hz), 8.07 (d, H_4, J=9.2 Hz), 7.99 (d, H_7, J=8.4 Hz), 7.94 and 7.88 (dd, H_8 and H_9), 7.45 (d, H_5, J=9.2 Hz), 7.38 (d, H_2, J=8.3 Hz). 3-Amino-6-nitroBaP (**8**) was purified from the mixture of 3-amino-6-nitroBaP (**8**) and by-products on a silica gel column with CH_2Cl_2, 9.7 mg (7.8% yield), mp 202—203 °C. ^{1}H-NMR (Me_2SO-d_6): 9.18 (d, H_{10}, J=8.3 Hz), 8.88 (d, H_{11}, J=9.0 Hz), 8.47 (d, H_4, J=9.7 Hz), 8.35 (d, H_{12}, J=9.0 Hz), 8.22 (d, H_1, J=8.6 Hz), 7.99 (d, H_7, J=8.4 Hz), 7.91 and 7.85 (d, H_8 and H_9), 7.60 (d, H_5, J=9.7 Hz), 7.49 (d, H_2, J=8.6 Hz).

1,6-DinitroBaP (5) $NaNO_2$ (60 mg) was dissolved in 0.4 ml of concentrated H_2SO_4 (d=1.84) at 0 °C. 1-Amino-6-nitroBaP (10 mg, 0.032 mmol) was dissolved in 0.6 ml of AcOH. The resulting thin slurry was dropped slowly into the cold solution of nitrosylsulfuric acid with stirring. Throughout the addition, and for 30 min thereafter, the temperature was kept below 20 °C. Dry ether (2 ml) was added slowly with stirring, and the temperature of the mixture was kept at 0 °C for 30 min. At the end of this period, the precipitate (**9**) was collected, washed with ether and dissolved in 0.5 ml of iced water. An aqueous solution containing 0.3 g (1.9 mmol) of $CuSO_4$ was treated with an aqueous solution of 0.3 g (2.4 mmol) of Na_2SO_3. The resulting greenish brown precipitates were collected, washed with water, and then stirred into a solution of 0.6 g (8.7 mmol) of $NaNO_2$ in 1 ml of water. The cold aqueous solution of the diazonium salt (**9**) was then added slowly to the above solution and the mixture was stirred for 1 h. The reaction mixture was extracted with CH_2Cl_2 (3 times), washed with water, dried (Na_2SO_4) and evaporated below 20 °C under shielding from light. The residue was purified on a silica gel column with 40% CH_2Cl_2–*n*-hexane to give the pure material (**5**) as an orange solid (light-sesitive in organic solvents), 6.8 mg (0.020 mmol, 62.0%), mp 283—285 °C (dec.). MS m/z [rel. intensity]: 342 [M^+, 100], 312 [26], 296 [14], 282 [40], 266 [30], 250 [69], 238 [39], 226 [20]. ^{1}H-NMR (Me_2SO-d_6): 9.68 (d, H_{11}, J=9.7 Hz), 9.49 (d, H_{10}, J=7.3 Hz), 9.10 (d, H_{12}, J=9.7 Hz), 8.90 (d, H_2, J=8.3 Hz), 8.60 (d, H_3, J=8.3 Hz), 8.48 (d, H_4, J=9.4 Hz), 8.18 (m, H_7), 8.15 (d, H_5, J=9.4 Hz), 8.14 (m, H_8 and H_9).

3,6-DinitroBaP (6) 3,6-DinitroBaP (**6**) was synthesized from 3-amino-6-nitroBaP (**8**) (10 mg, 0.032 mmol) by a similar procedure to that described for 1,6-dinitroBaP (**5**). Yellow solid (light-sensitive in organic solvents), 9.0 mg (0.026 mmol, 82.1%), mp 286—288 °C (dec.). MS m/z [rel.

intensity]: 342 [M^+ 100], 312 [29], 296 [13], 282 [32], 266 [35], 250 [48], 238 [48], 226 [17]. ^{1}H-NMR (Me_2SO-d_6): 9.61 (d, H_{11}, J=9.3 Hz), 9.50 (d, H_{10}, J=8.3 Hz), 8.80 (d, H_{12}, J=9.3 Hz), 8.77 (d, H_2, J=8.3 Hz), 8.75 (d, H_4, J=9.8 Hz), 8.73 (d, H_1, J=8.3 Hz), 8.23 (d, H_5, J=9.8 Hz), 8.19 (m, H_7), 8.14 (m, H_8 and H_9).

Mutagenicity Test *Salmonella typhimurium* strains TA98 and TA98NR were used for the mutation assays with and without metabolic activation by rat S9 mix. Each test sample was dissolved in dimethyl sulfoxide and the mutagenic activity was measured by means of the Ames test with preincubation at 37 °C for 20 min. Positive controls included 1,6- and 1,8-dinitropyrenes, and their specific activities (revertants per nmol per plate) toward TA98 and TA98NR were 60000 and 63000 (1,6-dinitropyrene) and 140000 and 190000 (1,8-dinitropyrene), respectively.

Electrochemical Reduction by Cyclic Voltammetry Dimethylformamide containing 0.1 M tetraethylammonium perchlorate was used as the solvent. After transfer of the solution containing the test chemical to the cell, it was purged of oxygen by bubbling with N_2 for 10 min. The cyclic voltammograms were recorded at a scan rate of 100 mV/s while maintaining the test solution under a steady stream of N_2.

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