Identification of Mutagenic Metabolites Formed by C-Hydroxylation and Nitroreduction of 5-Nitroacenaphthene in Rat Liver

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ABSTRACT

The metabolism of the mutagen and carcinogen, 5-nitroacenaphthene, by the 9000 × g supernatant from the livers of Aroclor-pretreated rats was studied. The major primary metabolites were 1-hydroxy-5-nitroacenaphthene and 2-hydroxy-5-nitroacenaphthene. These metabolites were oxidized to 1-oxo-5-nitroacenaphthene and 2-oxo-5-nitroacenaphthene, hydroxylated to cis-1,2-dihydroxy-5-nitroacenaphthene and trans-1,2-dihydroxy-5-nitroacenaphthene, and reduced to 1-hydroxy-5-aminacenaphthene and 2-hydroxy-5-aminacenaphthene. Reduction of 1- and 2-oxo-5-nitroacenaphthene to 1-oxo- and 2-oxo-5-aminacenaphthene was also observed. When incubations were carried out in a N2-enriched atmosphere (10% O2 in N2), the major metabolites were 1-hydroxy- and 2-hydroxy-5-nitroacenaphthene and 2-oxo-5-aminacenaphthene. Selected metabolites were tested for mutagenicity toward Salmonella typhimurium TA 98. The most mutagenic of the metabolites tested, in the presence or absence of rat liver 9000 × g supernatant, were 1-hydroxy-5-nitroacenaphthene and 1-oxo-5-nitroacenaphthene. These results indicate that the 9000 × g supernatant from the livers of Aroclor-pretreated rats is capable of catalyzing both the oxidation and reduction of 5-nitroacenaphthene and that the reduced derivatives of 1-hydroxy- or 2-hydroxy- or 1-oxo- or 2-oxo-5-nitroacenaphthene are proximate mutagens.

INTRODUCTION

5-Nitroacenaphthene (Compound 1; see Chart 1) is in the class of nitropolynuclear aromatic hydrocarbons which are important because of their mutagenic and carcinogenic activities and their possible presence in the environment (8, 13, 16, 17, 21). It is mutagenic toward Salmonella typhimurium, with and without activation, and induces tumors in rats, mice, and hamsters (9, 12, 15, 20, 22). However, no studies have been reported on the metabolism of 5-nitroacenaphthene and only limited data are available on the metabolic activation of nitropolynuclear aromatic hydrocarbons.

Our interest in 5-nitroacenaphthene was stimulated by the results of comparative mutagenicity and carcinogenicity assays of 5-nitroacenaphthene and 1-nitronaphthalene (12, 14, 15). The latter is less mutagenic than the former and appears to be noncarcinogenic (14). Thus, the presence of a 2-carbon bridge (C-1 and C-2 of 5-nitroacenaphthene) seems to be a key structural factor in its biological activity.

In the present study, we have determined the major metabolic pathways of 5-nitroacenaphthene in vitro, under conditions similar to those used in mutagenicity assays. The results indicate that upon incubation with cofactors and liver 9000 × g supernatant from Aroclor-pretreated rats 5-nitroacenaphthene is both oxidized and reduced and that both pathways are involved in its activation.

MATERIALS AND METHODS

Instrumentation. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. 1H NMR spectra were recorded on a Hitachi Perkin-Elmer R-24 high-resolution NMR spectrometer and on a Varian XL-100 spectrometer equipped with a Nicolet Technology pulsed Fourier transform accessory. The proton resonance shifts were measured relative to tetramethylsilane and are expressed in ppm on the δ scale. Mass spectrometry was performed on a Hewlett-Packard Model 5892A instrument. UV spectra were determined with a Cary Model 118 instrument. HPLC was performed with a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model U6K septumless injector, a Model 440 UV/visible detector, and a 6-mm x 30-cm C18Bondapak column (Waters Associates, Inc., Milford, Mass.). For the analyses of head space gases (O2 and N2), we used a Hewlett-Packard Model 7820A gas chromatograph equipped with a thermal conductivity detector and a 5-ft x 1/4-inch MS 5A column. The conditions were: column temperature, ambient; detector temperature, 210°C; and flow rate (helium), 30 ml/min. Elemental analysis was performed by Galbraith Laboratories, Inc., Knoxville, Tenn. High-resolution MS analyses were performed by Shrader Analytical and Consulting Laboratories, Detroit, Mich.

Chemicals. Glucose-6-phosphate, NADP+, and potassium phosphate buffer (pH 7.4) were obtained from Sigma Chemical Co. (St. Louis, Mo.). 5-Bromoacenaphthene, 1-hydroxyacenaphthene, m-chloroperbenzoic acid, CDC13, and pyridinium chlorochromate were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Silicar CC-7 was procured from Mallinckrodt, Inc., St. Louis, Mo. Thin-layer chromatography was performed on Silica Gel 60 F-254 glass plates (EM Laboratories, Inc., Elmsford, N. Y.). Copper wire and cuprous chloride were obtained from ThioKol/Ventron Division, Danvers, Mass.

5-Nitroacenaphthene (Compound 1). In a steel bomb were placed 46.6 g (0.2 mol) of 5-bromoacenaphthene, 2.7 g of copper wire, 108 g (1.8 mol) of 28% aqueous ammonia, and 2.4 g of Cu2Cl2. The reaction vessel was cooled to −78°C, evacuated, allowed to stand until it reached room temperature, and then heated at 190°C for 36 hr. After cooling, the solid material was filtered and washed with EtOAc and the filtrate was extracted with ETOAc. The combined ETOAc solutions were...
dried and concentrated, and the residue was purified by chromatography on Silica CC-7 using 5% EtOAc-hexane as eluent to give 7.0 g (0.04 mol; 20% yield) of pure 5-aminoacenaphthene, m.p. 103–105° from benzene-hexane [literature (10), 106°; literature (3), 104°]. 5-Aminoacenaphthene (0.5 g; 0.003 mol) in CH₂Cl₂ (20 ml) was added dropwise to m-chloroperbenzoic acid (15 g; 0.009 mol) in 100 ml CH₂Cl₂ at room temperature with continuous stirring. After 45 min, the reaction was worked up as described for 3,2-dimethyl-4-nitrotoluene (7). The pure 5-nitroacenaphthene was obtained in 55% yield (0.33 g; 0.002 mol; m.p. 101–103° [literature (10), 101.5–102°]; MS, m/e 199 (M⁺, 82), 169 (100), and 152 (36); NMR (CDCl₃) δ 3.35 (s, 4H, CH₂, H-1), 7.40 (m, 3H, aromatic), and 8.40 (2 d’s, J = 6 Hz, 2H, H-4 and H-6).

1-Acenaphthenone. It was obtained by oxidation of recrystallized 1-acenaphthenol with pyridinium chlorochromate in 43% yield; m.p. 124–126° [literature (11), 121°].

1-Oxo-5-nitroacenaphthene (Compound 2). It was obtained by nitration of 1-acenaphthenone as described previously; m.p. 215–216° [literature (18), 218°]; MS, m/e 213 (M⁺, 100), 183 (39), 167 (23), and 139 (97).

1-Oxo-5-aminoacenaphthene (Compound 12). Compound 2 was reduced with excess zinc and NH₄Cl in ethanol (95%) to give Compound 12 in 60% yield; m.p. 101–103° [literature (18) 100–101°]; MS, m/e 183 (M⁺, 98), 155 (45), and 154 (100).

1-Hydroxy-5-nitroacenaphthene (Compound 4). To a solution of Compound 2 (107 mg; 0.5 mmol) in methanol (150 ml), 95 mg (2.5 mmol) of NaBH₄ were added in small portions with stirring. After 30 min, 25 ml of H₂O were added and the methanol was evaporated under reduced pressure. The remaining aqueous solution was extracted with EtOAc, dried, concentrated, and purified by preparative thin-layer chromatography with elution by 10% EtOAc in benzene to give 7.4 mg of Compound 4 (0.4 mmol; 80% yield); m.p. 109–111° (from EtOAc-hexane); MS, m/e 215 (M⁺, 77), 198 (75), and 168 (100); NMR (CDCl₃), δ 2.22 (bs, OH), 3.28 (dd, J = 20, 2 Hz, 1H-H-2), 3.88 (dd, J = 20, 6 Hz, 1H-H-2), 5.85 (bs, 1H, H-1), 7.42 (m, 1H, aromatic), 8.20 (m, 2H, aromatic), 8.60 (d, J = 8 Hz, 1H, H-6), and 8.69 (d, J = 8 Hz, 1H-H-3).

C₂₃H₂₃NO₃
Calculated: C 66.97, H 4.22, N 6.51
Found: C 67.08, H 4.23, N 6.54

1-Hydroxy-5-aminoacenaphthene (Compound 14). To a suspension of 45 mg (1.1 mmol) of LiAlH₄ in 15 ml of dry tetrahydrofuran was added a solution of 50 mg (0.23 mmol) of Compound 2 with stirring at room temperature. Stirring was continued overnight, after which the reaction mixture was added slowly to ice-cold H₂O and then extracted with EtOAc. The EtOAc layer was dried and evaporated, and the crude residue was purified by column chromatography (Silica CC-7, 50% EtOAc-hexane; m.p. 137–139° (EtOAc-benzene); MS, m/e 185 (M⁺, 100), 167 (72), and 156 (62); NMR (CDCl₃) δ 2.89 (d, J = 20 Hz, 1H-H-2), 3.45 (dd, J = 20, 6 Hz, 1H-H-2), 5.45 (bs, 4H, NH₂, OH, H-1), 6.61 (d, J = 8 Hz, 1H, H-3), 7.00 (d, J = 8 Hz, 1H, H-6), 7.40 (poorly resolved d, 2H), and 7.83 (t, J = 4.5 Hz, 1H); high-resolution MS calculated for C₁₂H₁₀NO₂, 185.0840; found, 185.0842.

5-Nitroacenaphthene. Compound 4 (0.32 g; 1.54 mmol) was heated under reflux for 30 min in dry benzene (100 ml) containing p-toluene sulfonic acid (5 mg) as a catalyst. The reaction mixture was filtered and the benzene was evaporated. The residue was triturated with hexane, and the hexane was left to evaporate to give 5-nitroacenaphthene (0.205 g; 1.1 mmol; 73% yield); m.p. 99–100° [literature (6), 113–114.5°]; MS, m/e 197 (M⁺, 60), 167 (18), 151 (100), and 150 (87); NMR (CDCl₃) δ 7.00 (dd, J = 15, 6 Hz, 2H, H-1, H-2), 7.55 (m, 3H, aromatic), and 8.40 (2 overlapping d’s, J = 6 Hz, 2H, H-4 and H-6).

cis-1,2-Dihydroxy-5-nitroacenaphthene (Compound 7). To a suspension of 70 mg of OsO₄ in dry benzene (20 ml), 5-nitroacenaphthene (50 mg; 0.25 mmol) in dry benzene (30 ml) was added dropwise. Dry pyridine (1.5 ml) was added to the resulting mixture. After 15 min, the color changed from yellow to red and finally to deep brown. After an additional 15 min, crystals separated and stirring was continued for an additional 10 min. The solvent was evaporated, the brown residue was treated with 20 ml of a saturated solution of NaHSO₃ and 10 ml of pyridine, and the mixture was stirred for 15 min. The reaction mixture was extracted with CH₂Cl₂ and concentrated. The residue was crystallized from CH₃OH-tetrahydrofuran to give 22 mg (0.1 mmol; 36% yield) of the desired product as a colorless solid.

Metabolism in Vitro. Incubations were carried out in 50-ml Erlenmeyer flasks containing 20 ml of 5-9 mix and 2.0 ml of substrate which had been dissolved in 200 µl DMSO. The ratio of S-9 mix to substrate was the same as that which gave maximum response in the mutagenicity assays, i.e., 200 µl S-9 mix per plate per 20 µg 5-nitroacenaphthene (see Chart 7). The S-9 mix contained, per ml, 100 µmol of potassium phosphate buffer (pH 7.4), 8 µmol of MgCl₂, 1.65 µmol of KCl, 5 µmol of glucose-6-phosphate, 4 µmol of NADP⁺, and 0.5 ml of 9000 × g supernatant (33 mg protein per ml) from the livers of male F344 rats (250 to 350 g) which had been given i.p. injections of 500 mg Aroclor 1254 per kg in corn oil 5 days prior to sacrifice. The preparation of the 9000 × g supernatant has been described previously (5). The flasks were shaken at 37° for the required time. Reactions were quenched by addition of 20 ml of ice-cold absolute ethanol. The protein was filtered and the ethanol solution was diluted with 50 ml distilled H₂O and extracted 3 times with 50 ml EtOAc. The extracts were dried (MgSO₄) and evaporated under reduced pressure at 40°. The residue was dissolved in 200 µl of tetrahydrofuran, and 10 µl were analyzed by HPLC using a linear gradient from 25% methanol-water to 80% methanol-water in 1 hr with a flow rate of 2 ml/min. Control experiments were performed as described above except that heat-denatured S-9 mix was used. For incubations in an N₂-enriched atmosphere, S-9 mix was added to a “Reactiflask” (Pierce Chemical Co., Rockford, Ill.) and flushed with N₂ prior to addition of substrate. The amounts of O₂ and N₂ in the head space were determined by gas chromatography.

Mutagenicity Assays. S. typhimurium strains TA 98 and TA 100 were kindly provided by Dr. Bruce Ames of the University of California, Berkeley. The procedure of Ames et al. (1) was used in performing these assays. The experimental details have been described previously (5). The purity of all chemicals tested was more than 99% based on compatibility with pyridinium chlorochromate.

Chart 1. The metabolic pathways for 5-nitroacenaphthene (Compound 1) in vitro.

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Results

Nitration of acenaphthene gives a mixture of 3-nitroacenaphthene and 5-nitroacenaphthene (10). To avoid possible complications arising from the presence of 3-nitroacenaphthene, we used 5-nitroacenaphthene which was synthesized from 5-bromoacenaphthene. The metabolic studies were carried out with the same rat liver 9000 x g supernatant as used in the mutagenicity assays. Chart 2 shows a HPLC trace of the EtOAc-soluble metabolites of 5-nitroacenaphthene formed in a 60-min incubation. Ten peaks, A to J, were observed in addition to unchanged 5-nitroacenaphthene. Peaks A to J were not present in control incubations.

The major metabolites were Peaks G and H. The HPLC retention volume, UV spectrum, and MS (see Chart 3) of Peak G were identical to those of synthetic 1-hydroxy-5-nitroacenaphthene (Compound 4). The MS of Peak H (Chart 3) was similar to that of 1-hydroxy-5-nitroacenaphthene, suggesting that it was 2-hydroxy-5-nitroacenaphthene (Compound 5). Its UV spectrum was also similar to that of 1-hydroxy-5-nitroacenaphthene, and neither spectrum changed markedly upon addition of aqueous NaOH. Oxidation of metabolite Peaks G and H with pyridinium chlorochromate resulted in conversion to Peaks I and J. Peak I had MS and HPLC retention volume identical to those of synthetic 1-oxo-5-nitroacenaphthene. The MS of Peak J was similar to that of Peak I. These data confirmed the identities of Peaks G, H, I, and J as 1-hydroxy-5-nitroacenaphthene (Compound 4), 2-hydroxy-5-nitroacenaphthene (Compound 5), 1-oxo-5-nitroacenaphthene (Compound 2), and 2-oxo-5-nitroacenaphthene (Compound 3), respectively.

The MS of Peaks D and F and that of cis-1,2-dihydroxy-5-nitroacenaphthene (Compound 7) are shown in Chart 4. Peak F, which coeluted with the reference standard on HPLC, was thus identified as Compound 7. Peak D was the major metabolite of 5-nitroacenaphthylene (1,2-dehydro-5-nitroacenaphthene) under the same conditions used for metabolism of 5-nitroacenaphthene. These data indicate that Peak D is trans-1,2-dihydroxy-5-nitroacenaphthene, Compound 6.

Peaks A, B, C, and E were extracted from the mixture of metabolites by dilute acid, indicating that they were amines. This was confirmed by comparison of their MS and HPLC retention volumes to those of 1-hydroxy-5-aminoacenaphthene and 1-oxo-5-aminoacenaphthene. Peaks A and B were thus assigned as 1-hydroxy-5-aminoacenaphthene (Compound 14), 1-oxo-5-aminoacenaphthene (Compound 15), and 1-oxo-5-nitroacenaphthene (Compound 16), respectively.

Chart 2. HPLC trace of metabolites formed from incubation of 5-nitroacenaphthene (Compound 1) with rat liver 9000 x g supernatant for 60 min.

Chart 3. MS of synthetic 1-hydroxy-5-nitroacenaphthene (Compound 4, A) and isolated metabolites, Peak G (B) and Peak H (C).

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Chart 4. MS of synthetic cis-1,2-dihydroxy-5-nitroacenaphthene (Compound 7, A) and isolated metabolites, Peak F (B) and Peak D (C).

and 2-hydroxy-5-aminoacenaphthene (Compound 15) and peaks C and E as 1-oxo-5-aminoacenaphthene (Compound 12) and 2-oxo-5-aminoacenaphthene (Compound 13).

The initially formed metabolites of 5-nitroacenaphthene were 1-hydroxy-5-nitroacenaphthene (Compound 4) and 2-hydroxy-5-nitroacenaphthene (Compound 5). Only these metabolites were observed when 5-nitroacenaphthene was incubated with rat liver 9000 × g supernatant for 5 min. Further metabolism of Compounds 4 and 5 gave Compounds 2, 3, 6, 7, and 12 to 15. The formation of Compounds 3, 6, 7, 14, and 15 from Compounds 4 and 5 as a function of time is illustrated in Chart 5.

When incubations of 5-nitroacenaphthene were carried out in an atmosphere of 10% O₂ in N₂, 3 major metabolite peaks were observed, as shown in Chart 6. These were identified by their MS and HPLC retention volumes as 2-oxo-5-aminoace-

naphthene (Compound 13) and 1- and 2-hydroxy-5-nitroacenaphthene (Compounds 4 and 5).

The mutagenic activities toward S. typhimurium TA 98 of 5-nitroacenaphthene and of representative metabolites were determined with and without activation, as shown in Chart 7. In the experiments conducted with activation, 5-nitroacenaphthene and 1-oxo-5-nitroacenaphthene (Compound 2) were the most mutagenic at the lower doses; at higher doses, 1-hydroxy-5-nitroacenaphthene (Compound 1) was also a potent muta-
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The primary metabolic process was still bridge oxidation. When incubations were carried out in an atmosphere of 10% O2 in N2, the relative amount of a reduced metabolite, Compound 73, increased, but the absence of rat liver 9000 x g supernatant, only 1-hydroxy-5-nitroacenaphthene, 1-oxo-5-nitroacenaphthene, and 5-nitroacenaphthene showed significant mutagenic activity, and all 3 compounds were less mutagenic than in the presence of rat liver S-9 fraction (a) and in the absence of rat liver S-9 fraction (b). Compounds 7, 12, and 14 were not mutagenic in the absence of S-9 fractions. REV, revertants.

DISCUSSION

The results of the study demonstrate that the rat liver 9000 x g supernatant, isolated from animals that had been treated with Aroclor, is capable of catalyzing both the oxidation and reduction of 5-nitroacenaphthene. Oxidation to give 1-hydroxy-5-nitroacenaphthene (Compounds 4) and 2-hydroxy-5-nitroacenaphthene (Compound 5) is clearly the predominant metabolic process. All of the metabolites which we detected were derived from Compounds 4 and 5. Facile benzylic hydroxylation has also been observed in the metabolism of 2,4-dinitrotoluene by primary rat hepatocytes (2). When incubations were carried out in an atmosphere of 10% O2 in N2, the relative amount of a reduced metabolite, Compound 13, increased, but the primary metabolic process was still bridge oxidation.

The results of the mutagenicity assays indicate that 1-hydroxy-5-nitroacenaphthene and 1-oxo-5-nitroacenaphthene are proximate mutagens of 5-nitroacenaphthene. Their mutagenic activities are probably expressed through the corresponding hydroxylamines or nitroso compounds since both compounds were also mutagenic in the absence of rat liver 9000 x g supernatant and S. typhimurium is known to possess a family of nitroreductase systems (19). We did not test 2-hydroxy-5-nitroacenaphthene or 2-oxo-5-nitroacenaphthene, but their mutagenic activities would presumably be similar to those of the 1-substituted compounds. The higher mutagenic activities, without activation of 1-hydroxy-5-nitroacenaphthene and 1-oxo-5-nitroacenaphthene compared to 5-nitroacenaphthene suggest that the hydroxylamines (Compounds 8 and 10) or nitroso derivatives have relatively high intrinsic mutagenic activities. However, we were unsuccessful in preparing these compounds with purities suitable for mutagenicity assays.

We also considered the possibility that a stable benzylic carbonium ion might be formed from 1-hydroxy-5-nitroacenaphthene and that this might contribute to its mutagenicity. However, we found that 1-hydroxyacenaphthene was inactive toward S. typhimurium TA 98 (data not shown) and consequently such a mechanism seems unlikely.

cis-1,2-Dihydroxy-5-nitroacenaphthene, which is the major metabolite of 1-hydroxy-5-nitroacenaphthene, can be regarded as a detoxification product since it was less mutagenic than 5-nitroacenaphthene, with activation, and was inactive in the absence of rat liver 9000 x g supernatant. The final products of nitro reduction, 1-oxo-5-aminoacenaphthene and 1-hydroxy-5-aminoacenaphthene, were also less mutagenic with activation than 5-nitroacenaphthene and were not mutagenic without activation. The lack of mutagenic activity of both 1-oxo-5-aminoacenaphthene and 1-hydroxy-5-aminoacenaphthene in the absence of rat liver 9000 x g supernatant is typical for aromatic amines.

It can be concluded from this study that nitro reduction is an important pathway in the metabolic activation of 5-nitroacenaphthene to a mutagen. In the absence of 9000 x g supernatant, the proximate mutagen is probably 5-hydroxylaminoacenaphthene or 5-nitrosoacenaphthene. In the presence of 9000 x g supernatant, bridge hydroxylation is the primary metabolic pathway and the mutagenic intermediates are the corresponding bridge-oxidized hydroxylamines or nitroso derivatives. Since 5-nitroacenaphthene is also carcinogenic, it will be important to determine if similar mechanisms of metabolic activation occur in vivo and if these mechanisms account for its greater carcinogenicity than 1-nitronaphthalene. These studies are currently in progress.

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