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Fungal Metabolism of Acenaphthene by *Cunninghamella elegans*

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The filamentous fungus *Cunninghamella elegans* ATCC 36112 metabolized within 72 h of incubation approximately 64% of the [1,8-^{14}C]acenaphthene added. The radioactive metabolites were extracted with ethyl acetate and separated by thin-layer chromatography and reversed-phase high-performance liquid chromatography. Seven metabolites were identified by ^{1}H nuclear magnetic resonance, UV, and mass spectral techniques as 6-hydroxyacenaphthenone (24.8%), 1,2-acenaphthenedione (19.9%), *trans*-1,2-dihydroxyacenaphthene (10.3%), 1,5-dihydroxyacenaphthene (2.7%), 1-acenaphthenol (2.4%), 1-acenaphthenone (2.1%), and *cis*-1,2-dihydroxyacenaphthene (1.8%). Parallel experiments with rat liver microsomes indicated that the major metabolite formed from acenaphthene by rat liver microsomes was 1-acenaphthenone. The fungal metabolism of acenaphthene was similar to bacterial and mammalian metabolism, since the primary site of enzymatic attack was on the two carbons of the five-member ring.

Polycyclic aromatic hydrocarbons (PAHs) are classified as priority toxic pollutants by the U.S. Environmental Protection Agency (18). Many PAHs are toxic and/or carcinogenic and are considered to be potential health risks to humans (18). Acenaphthene, a PAH containing three fused rings, is produced by combustion or pyrolysis of fossil fuels (17) and is also a constituent of coal tar (13), tobacco smoke (23), automobile exhaust (14), and urban air (10). Acenaphthene is used in the synthesis of plastics, dyes, and pesticides (30) and has been detected in effluents from petrochemical, pesticide, and wood-preservative industries (29). Groundwater contamination by acenaphthene has also been previously reported (19).

Although acenaphthene is considered nonmutagenic, it has been shown to induce nuclear and cytological changes in plants and microorganisms (31). In fish, a concentration of 413 μg of acenaphthene per liter of water caused lethargic behavior among juvenile fathead minnows (*Pimephales promelas*) (1). Cairns and Nebeker (1) considered this behavior to be a sensitive indicator of toxicity.

Information on the microbial degradation of acenaphthene is limited (4). Chapman (9) studied the metabolism of acenaphthene with *Pseudomonas* sp. and reported that oxidation in the C-1 and C-2 bridge forms 1-acenaphthenol and 1-acenaphthenone. A *Beijerinckia* sp. was shown to cooxidize acenaphthene to 1-acenaphthenone, 1-acenaphthenol, acenaphthene-*cis*-1,2-diol, and acenaphthenequinone (26).

Under anaerobic conditions, no degradation of acenaphthene in sediment samples was observed (21). However, Mihelcic and Luthy (21) observed microbial degradation of acenaphthene under denitrification conditions after an acclimation period of several weeks prior to the onset of microbial degradation.

The fungus *Cunninghamella elegans*, studied extensively in our laboratory, has shown the ability to metabolize low as well as high-molecular-weight PAHs to compounds which are usually less mutagenic than the parent compounds (4, 7, 25). The metabolic pathways have shown a greater tendency toward detoxification than the bioactivation pathways found more commonly in mammals (5–8, 20, 22, 25). Since the use of specific microorganisms for biodegradation of PAHs and detoxification of PAH-polluted sites appears to be a promising means of bioremediation of PAHs in the environment (7), we have continued to investigate the metabolism of PAHs by *C. elegans*.

We now report the isolation and identification of the acenaphthene metabolites formed by *C. elegans*. We also compared the metabolism of acenaphthene by *C. elegans* to metabolites formed from hepatic microsomes of 3-methylcholanthrene-treated rats incubated with acenaphthene.

MATERIALS AND METHODS

Chemicals. Acenaphthene (99.0% purity) and 1-acenaphthenol (99.0% purity) were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). [1,8-^{14}C]acenaphthene (specific activity, 15.7 mCi/mmol; radiochemical purity, >98%; Dynapol, Palo Alto, Calif.) was a gift from James Mueller, SBP Technologies, U.S. Environmental Protection Agency, Gulf Breeze, Fla. High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Chemical Co., Pittsburgh, Pa. All other chemicals were of reagent grade and of the highest purity available.

Microorganism and culture conditions. Stock cultures of *C. elegans* ATCC 36112 were maintained on Sabouraud dextrose agar plates (Difco Laboratories, Detroit, Mich.) and stored at 4°C. Spores and mycelia from several plates were used to inoculate flasks containing 30 ml of Sabouraud dextrose broth. After 48 h of incubation, 20 mg (130 μmol) of acenaphthene dissolved in 0.5 ml of dimethyl sulfoxide was added to cultures containing *C. elegans* (approximately 3 g [dry weight]) growing in Sabouraud dextrose broth. All flasks were incubated for an additional 72 h. After 72 h, the contents of the flasks were pooled and filtered aseptically to separate the broth from the mycelia. The mycelia were then extracted with three equal volumes of ethyl acetate. Preparation of sterile control flasks, incubation conditions, and ethyl acetate extraction procedures were identical to those

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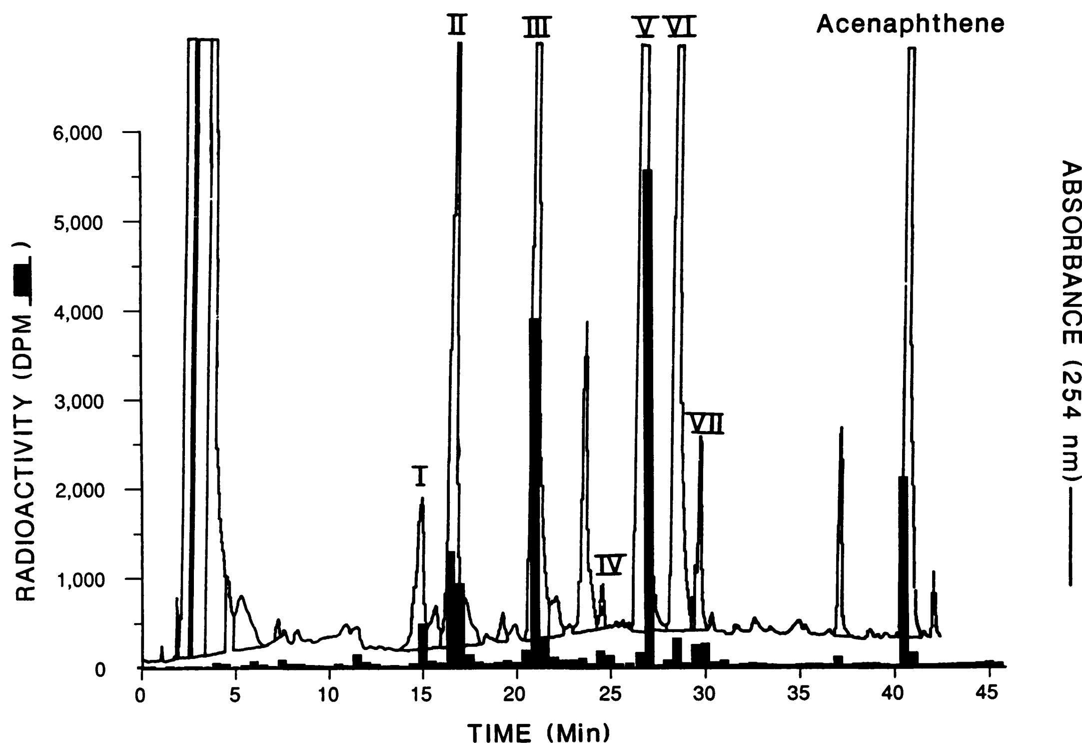

FIG. 1. HPLC elution profile and radioactivity of the ethyl acetate-soluble metabolites formed from [1,8-^{14}C]acenaphthene by *C. elegans*. Fractions eluting from the column were collected at 0.5-min intervals, and radioactivity was measured by liquid scintillation counting.

described previously (24). The ethyl acetate-extractable metabolites were concentrated, and the residue was dissolved in methanol and analyzed by HPLC and thin-layer chromatography.

Experiments with radiolabeled acenaphthene were conducted as described above with [1,8-^{14}C]acenaphthene (1.06 μCi) added to duplicate flasks. As usual, 20 mg (130 μmol) of unlabeled acenaphthene dissolved in 0.5 ml of dimethyl sulfoxide was also added to each flask. After incubation for 72 h, the entire flask contents were extracted and analyzed for metabolites. No metabolites were detected in the control flasks.

Rat liver microsomal metabolism of acenaphthene. Metabolites of acenaphthene from rat liver were obtained by in vitro incubation (8 μmol of acenaphthene dissolved in 1 ml of dimethyl sulfoxide) in the dark at 37°C for 60 min in a 100-ml reaction mixture containing 5 mmol of Tris-HCl (pH 7.5), 0.3 mmol of magnesium chloride, 10 U of glucose 6-phosphate dehydrogenase (type XII; Sigma Chemical Co., St. Louis, Mo.), 10 mg of $NADP^+$, 65 mg of glucose 6-phosphate, and 100 mg of liver microsomal protein from 3-methylcholanthrene-treated male Sprague-Dawley rats. After 60 min, the reaction was terminated by the addition of 100 ml of acetone. Acenaphthene and its metabolites were extracted with ethyl acetate (200 ml). The ethyl acetate extract was dried with anhydrous magnesium sulfate and evaporated to dryness under reduced pressure. The residue was redissolved in methanol and analyzed by HPLC.

Physical and chemical analyses. Fungal and rat liver microsomal metabolites of acenaphthene were separated with a Perkin-Elmer series 10 HPLC equipped with an LC-95 UV-visible absorbance detector (Perkin-Elmer Cetus, Inc., Norwalk, Conn.) operated at 254 nm and HPLC fitted with a Zorbax octyldecyl silane column (inside diameter, 25 cm by 4.6 mm; DuPont Co., Wilmington, Del.). The mobile phase was a 40-min linear gradient of methanol-water (from 30:70 to 95:5 [vol/vol]) at a flow rate of 1.0 ml/min. Compounds were collected from repeated injections of the culture extracts. Fractions with similar compositions and HPLC retention times were pooled and concentrated in a Speed Vac concentrator (Savant Instruments, Hicksville, N.Y.).

In experiments with [1,8-^{14}C]acenaphthene, 0.5-ml fractions were collected every 0.5 min and added to scintillation vials containing 7.0 ml of scintillation fluid (Ultima Gold; Packard Instrument Co., Inc., Meriden, Conn.). The amount of radioactivity was determined with a Packard 2000CA Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

UV-visible absorption spectra of isolated metabolites were determined in methanol with a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, Calif.).

Silica gel F_{500} glass plates (E. Merck, Darmstadt, Germany) were used for thin-layer chromatography to further purify isolated metabolites. The solvent used for thin-layer chromatography was benzene-ethanol (9:1 [vol/vol]).

Mass spectral analyses of acenaphthene metabolites were done by direct-exposure probe, electron impact (EI) mass spectrometry as described previously (24), except that the quadrupole was scanned from 50 to 650 Da in 1 s; samples were dissolved in methanol, and 2 μl of solution was applied to the direct-exposure probe wire and then allowed to evaporate prior to measurements.

The ^{1}H nuclear magnetic resonance (NMR) measurements were carried out at 500 MHz and 29°C on a Bruker AM500 spectrometer (Bruker Instruments, Billerica, Mass.). Metabolites were dissolved in 0.6 ml of deuterated acetone. In

some cases, 10 μl of D_2O was added to remove exchangeable protons. Chemical shifts are reported in parts per million by assigning the acetone resonance to 2.05 ppm. Spectral acquisition conditions were identical to those described previously (24), except that the number of scans for the metabolites varied from 40 to 240. For phenols scans varied from 650 to 1,700 because of their lower concentrations.

RESULTS

Accumulation of acenaphthene metabolites. Figure 1 shows the reversed-phase HPLC elution profile of the ethyl acetate-extractable metabolites formed by incubation of [1,8-^{14}C]acenaphthene with *C. elegans* for 72 h. Acenaphthene was metabolized to seven compounds, which were seen to elute at 15.0, 16.8, 21.4, 24.5, 26.9, 28.4, and 29.8 min. These are referred to as compounds I through VII, respectively. Acenaphthene was seen to elute at 40.8 min (Fig. 1).

At time zero extraction, approximately 75% of the recovered radioactivity accounted for acenaphthene. However, the amount of acenaphthene decreased to less than 10% within 72 h while metabolites I to VII accounted for approximately 64% of the total recovered radioactivity. The major metabolite V (Fig. 1) accounted for 24.8% of the total metabolism at 72 h of incubation. Metabolites I, II, III, IV, VI, and VII accumulated to 2.7, 10.3, 19.9, 1.8, 2.1, and 2.4%, respectively, of the total radioactivity recovered as ethyl acetate-extractable metabolites.

Identification of acenaphthene metabolites. The EI mass spectrum for compound I contained the molecular ion (*m/z* 186) [M^+] and characteristic fragment ions at *m/z* 168 [$M^+ - H_2O$] and at *m/z* 139 [$M^+ - H_2O - HCO$]. The loss of H_2O indicates the probability that at least one hydroxy group was located on the fused five-member saturated ring. On the basis of the UV-visible absorption data and mass spectral data (Table 1), compound I was proposed to be a dihydroxylated derivative of acenaphthene.

The structural identification of compound I was confirmed by NMR analysis. The ^{1}H NMR spectrum of compound I (Fig. 2A) shows that the nonexchangeable proton H_{1a} has a chemical shift characteristic of hydroxy substitution (12). The C-1 hydroxy group causes the adjacent protons H_{2a} and H_{2b} to become nonequivalent and exhibit large couplings to each other and to H_{1a}; this also is characteristic of C-1-substituted acenaphthene (12). It is apparent from the number of resonances that compound I (Fig. 2A) also contains one aromatic ring substituent. In addition, the ion fragmentation pattern seen in the mass spectrum of compound I is consistent with hydroxylation on both the aromatic ring and the fused five-member saturated ring (Table 1). The upfield shift of H_4 and the downfield shift of H_6, because of *ortho* and *peri* effects, respectively, show that the phenol substitution is at C-5. The phenolic proton and the hydroxy proton (C-1-OH) were not detected because of rapid exchange. The hydroxy proton of metabolite I was detected indirectly by the elimination of a coupling constant at H_{1a} after the addition of a trace of D_2O (data not shown). On the basis of these spectral parameters, compound I was identified as 1,5-dihydroxyacenaphthene.

The mass spectra of compounds II and IV appeared similar to those of compound I, except that the *m/z* 157 fragment ion was not present. The presence of more intense ions at *m/z* 168 [$M^+ - H_2O$] and *m/z* 140 [$M^+ - H_2O - CO$] indicates that both hydroxy groups were on the saturated ring (positions C-1 and C-2) (Table 1). The comparison of UV-visible absorption spectral data with those from published reports (26) and mass spectral data suggested that compounds II and IV were dihydroxylated intermediates of acenaphthene.

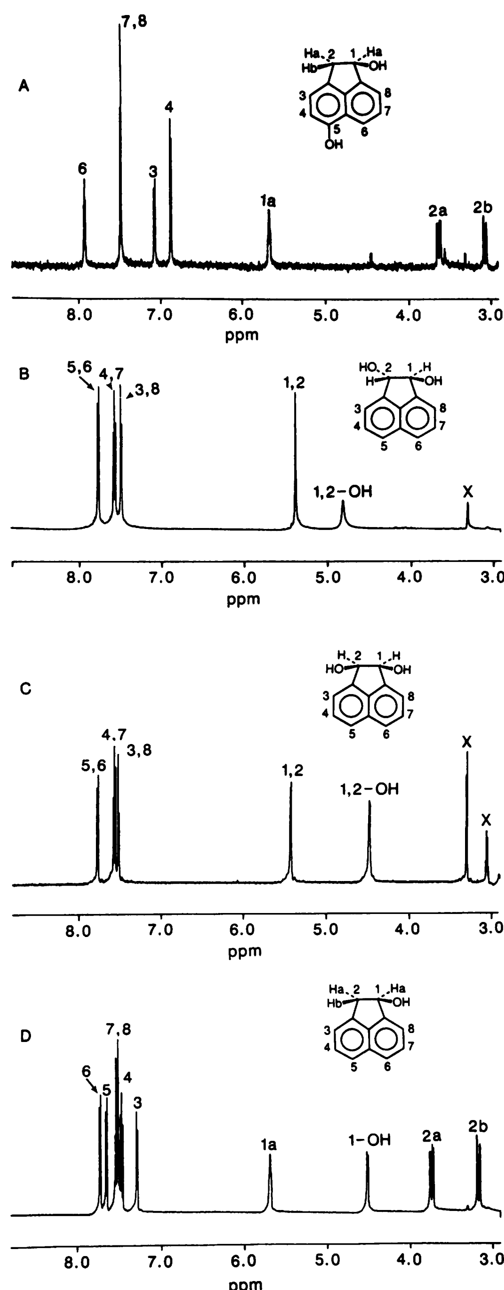

FIG. 2. 500-MHz ^{1}H NMR spectra of 1,5-dihydroxyacenaphthene (metabolite I) (A), *trans*-1,2-dihydroxyacenaphthene (metabolite II) (B), *cis*-1,2-dihydroxyacenaphthene (metabolite IV) (C), and 1-acenaphthenol (metabolite VII) (D) shown with structure and resonance assignments.

The structural identification of compounds II and IV was confirmed by NMR analyses. The ^{1}H NMR spectra of metabolites II and IV are shown in Fig. 2B and C. The NMR spectral parameters were similar to those described for compound I for the hydroxy groups on the C-1 and C-2 positions. However, the reduction in the number of resonances for metabolites II and IV is a consequence of

molecular symmetry and is consistent with the metabolites being *cis* and *trans* isomers. Also, the hydroxy protons were observable for both compounds II and IV. The compounds II and IV were identified as *trans*-1,2-dihydroxyacenaphthene and *cis*-1,2-dihydroxyacenaphthene, respectively. The NMR methods developed to assign the *cis* and *trans* configuration will be discussed elsewhere.

Direct-probe mass spectrometry analysis of metabolite VII showed a molecular ion [M^+] at *m/z* 170, with fragment ions at *m/z* 152 [$M^+ - H_2O$] and at *m/z* 141 [$M^+ - HCO$], suggesting hydroxy substitution on the fused five-member saturated ring (Table 1). On the basis of the NMR spectral parameters described for compounds I, II, and IV and the observable hydroxy proton (Fig. 2D), compound VII was identified as 1-acenaphthenol.

The EI mass spectrum of compound V indicates a compound with two oxygens, with the molecular ion [M^+] at *m/z* 184 and fragment ions at *m/z* 182, *m/z* 154, and *m/z* 126 (Table 1). Therefore, a keto-phenol was tentatively proposed for compound V. The keto substitution at C-1 for metabolite V was confirmed by its ^1H NMR spectrum by the absence of a proton attributable to H_1 and by the equivalence of the H_2 protons (Fig. 3A). Metabolite V also lacked one aromatic proton because of the hydroxy substitution at C-6. This substitution caused an upfield shift of H_7 and a downfield shift of H_5 because of *ortho* and *peri* effects, respectively. The phenolic proton was not detected because of rapid exchange. Thus, compound V was found to be 6-hydroxyacenaphthenone.

Direct-probe mass spectrometry analysis of compound VI showed a molecular ion at *m/z* 168 and a strong fragment ion at *m/z* 140 [$M^+ - CO$], indicating that the compound is a ketone. On the basis of NMR spectroscopy results similar to those described for compound V, compound VI was identified as 1-acenaphthenone (Fig. 3B).

The EI mass spectrum of compound III showed a molecular ion at *m/z* 182 and fragment ions at *m/z* 154 [$M^+ - CO$] and *m/z* 126 [$M^+ - CO - CO$]. These strong fragment ions indicate a dione. Further structural analysis of metabolite III was conducted by NMR spectroscopy to determine the identity of this compound. Because of symmetry, compound III (Fig. 3C) exhibited only three aromatic resonances. Thus, compound III was found to be acenaphthenedione.

To compare the fungal metabolism of acenaphthene with that of the mammalian metabolism of acenaphthene, rat liver microsomes from a 3-methylcholanthrene-treated rat were incubated with acenaphthene. The HPLC elution profile of metabolites formed from acenaphthene by rat liver microsomes showed that the major metabolite formed was 1-acenaphthenone. Identification of this metabolite was based on the fact that the retention time, UV-visible absorption spectrum, and EI mass spectrum were identical to those of the fungal metabolite VI (Table 1).

TABLE 1. HPLC retention times and spectrophotometric and mass spectral data of metabolites formed from acenaphthene by *C. elegans*

Compound	Assignment	HPLC retention time (min)	Mass spectral properties (*m/z*)[a]	UV-visible absorption (nm)[b]
I	1,5-Dihydroxyacenaphthene	15.0	187 (13), 186 (100) [M^+], 185 (45), 169 (43), 168 (42), 157 (21), 141 (10), 140 (11), 139 (21), 129 (12), 128 (28), 127 (14), 70 (11), 63 (11)	**214**, 237, 305, 330
II	*trans*-1,2-Dihydroxyacenaphthene	16.8	187 (10), 186 (75) [M^+], 169 (18), 168 (100), 155 (20), 154 (24), 141 (22), 140 (97), 139 (53), 129 (31), 128 (39), 127 (32), 70 (28)	**224**, 285, 314, 348
III	1,2-Acenaphthenedione	21.4	182 (56) [M^+], 155 (13), 154 (100), 127 (11), 126 (99), 76 (11), 74 (15), 63 (17)	218, **224**, 302, 312
IV	*cis*-1,2-Dihydroxyacenaphthene	24.5	187 (10), 186 (76) [M^+], 169 (16), 168 (100), 155 (20), 141 (21), 140 (98), 139 (55), 129 (29), 128 (38), 127 (32), 126 (11), 69 (15)	**224**, 274, 284, 314
V	6-Hydroxyacenaphthenone	26.9	184 (12) [M^+], 183 (10), 182 (54), 155 (14), 154 (100), 127 (14), 126 (99), 76 (11), 75 (10), 74 (13), 63 (19)	**206**, 210, 224, 284
VI	1-Acenaphthenone	28.4	169 (15), 168 (100) [M^+], 141 (11), 140 (86), 139 (66), 69 (17)	**216**, 314, 337, 376
VII	1-Acenaphthenol	29.8	171 (11), 170 (96) [M^+], 169 (100), 153 (30), 152 (34), 151 (11), 141 (36), 139 (21), 115 (24)	**224**, 276, 287, 314
Parent	Acenaphthene	40.8	155 (12), 154 (100) [M^+], 153 (94), 152 (42), 151 (18), 77 (12), 76 (21)	**226**, 279, 288, 299

[a] Relative intensities (in percentages) are in parentheses; molecular ions are in brackets.
[b] Maxima are indicated by boldface numbers.

DISCUSSION

The fungus *C. elegans* metabolized acenaphthene via hydroxylation of both the aliphatic and aromatic rings to hydroxylated and keto derivatives of acenaphthene. We propose that the primary site of enzymatic attack by *C. elegans* is on the C-1 and C-2 bridge, which resulted in the formation of the secondary alcohol 1-acenaphthenol. This oxidative pathway is likely, since previous studies on the transformation of acenaphthene by bacteria (26) have postulated formation of alcohol as an initial oxidative step. Sub-

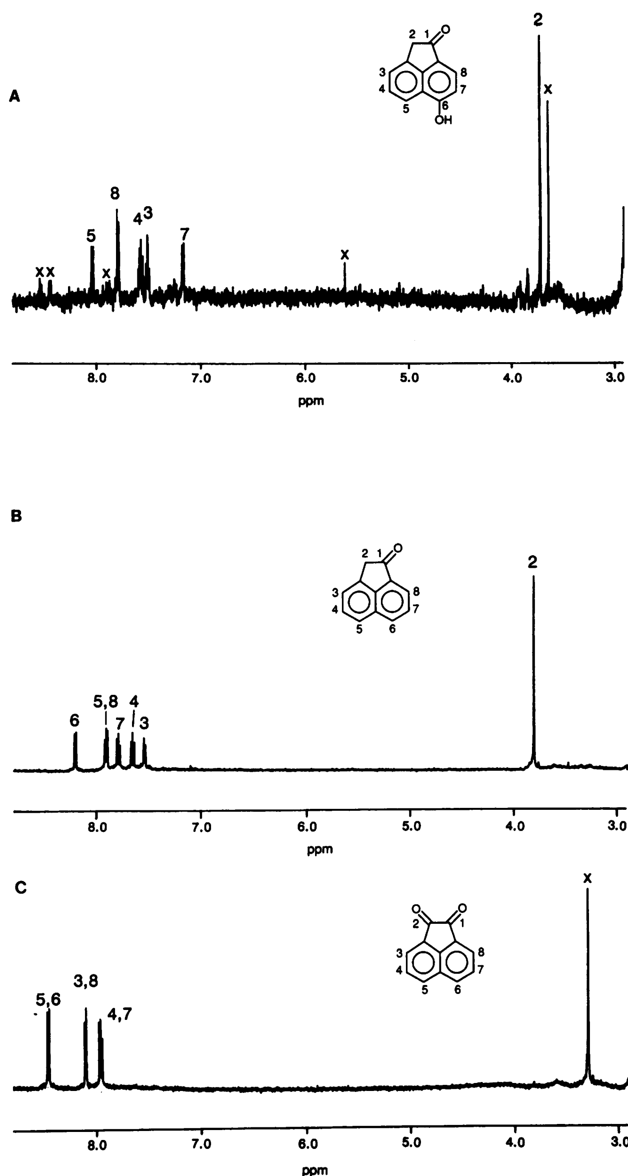

FIG. 3. 500-MHz ^{1}H NMR spectra of 6-hydroxyacenaphthenone (metabolite V) (A), 1-acenaphthenone (metabolite VI) (B), and 1,2-acenaphthenedione (metabolite III) (C) shown with structure and resonance assignments.

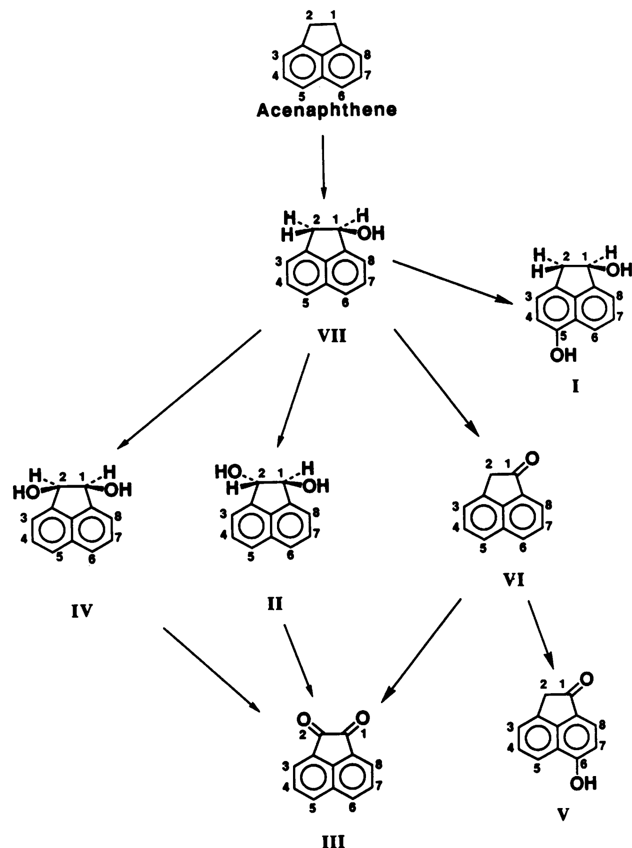

FIG. 4. Proposed pathways for the metabolism of acenaphthene by *C. elegans*. Designations: I, 1,5-dihydroxyacenaphthene; II, *trans*-1,2-dihydroxyacenaphthene; III, 1,2-acenaphthenedione; IV, *cis*-1,2-dihydroxyacenaphthene; V, 6-hydroxyacenaphthenone; VI, 1-acenaphthenone; VII, 1-acenaphthenol.

sequently, 1-acenaphthenol appears to have been oxidized to *cis*- and *trans*-1,2-dihydroxyacenaphthene.

The existing literature on bacterial and mammalian metabolism of acenaphthene indicates the formation of metabolites 1-acenaphthenol, 1-acenaphthenone, acenaphthene-*cis*-1,2-diol, 2-hydroxyacenaphthenone, and acenaphthenequinone (9, 11, 15, 16, 26, 27). The oxidation of acenaphthene by *Beijerinckia* sp. was shown to occur via two successive monooxygenation steps and two subsequent dehydrogenation steps (26). On the basis of these reports and the metabolites observed in our study, the pathway illustrated in Fig. 4 is proposed. The initial oxidative attack at the C-1 and C-2 positions of acenaphthene, with subsequent hydroxylation to form *cis*- and *trans*-dihydroxyacenaphthene, was expected, on the basis of the chemical reactivity and the ease of benzylic hydroxylation compared with aromatic ring hydroxylation.

Another proposed pathway (Fig. 4) includes the hydroxylation at both the C-1 and C-2 bridge and the aromatic ring of acenaphthene to form 1,5-dihydroxyacenaphthene. Additionally, formation of 1-acenaphthenone with subsequent hydroxylation of the aromatic ring to form 6-hydroxyacenaphthenone is proposed. Similar studies (2) show that naphthalene is oxidized by *C. elegans* via a cytochrome P-450 monooxygenase to form naphthalene 1,2-oxide. Naphthalene 1,2-oxide isomerizes spontaneously to 1-naphthol via an NIH shift mechanism, indicating that naphthalene 1,2-oxide is a metabolic intermediate (2). In the present study, oxidation at the C-5 and C-6 positions to form 1,5-dihydroxyacenaphthene and 6-hydroxyacenaphthenone is analogous to the rearrangement of naphthalene 1,2-oxide to form 1-naphthol (2).

Previous studies have shown that *C. elegans* metabolizes 3-methylcholanthrene, a potent carcinogenic PAH, via monohydroxylation of the methylene bridge to form 1-hydroxy- and 2-hydroxy-3-methylcholanthrene. The 1- and 2-keto derivatives of 3-methylcholanthrene and 1-hydroxy-3-methylcholanthrene *trans*-9,10-dihydrodiol were also previously reported (3). The dihydrodiol has been implicated as a proximate carcinogen in mammalian systems. Similarly to the oxidation of the methylene bridge of 3-methylcholan-

threne, the initial oxidation of acenaphthene by *C. elegans* is on the C-1 and C-2 bridge to form 1-acenaphthenol.

Since phenols, quinones, *trans*-dihydrodiols, and epoxides have been identified as detoxification products of PAHs produced by *C. elegans* (7, 28), the metabolic pathways reported in the present study may be helpful in determining the utility of fungi in the detoxification of this environmental pollutant.

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