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Metabolism of Phenanthrene by *Phanerochaete chrysosporium*

JOHN B. SUTHERLAND, ALLISON L. SELBY, JAMES P. FREEMAN, FREDERICK E. EVANS, AND CARL E. CERNIGLIA*

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

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The white rot fungus *Phanerochaete chrysosporium* metabolized phenanthrene when it was grown for 7 days at 37°C in a medium containing malt extract, D-glucose, D-maltose, yeast extract, and Tween 80. After cultures were grown with [9-^{14}C]phenanthrene, radioactive metabolites were extracted from the medium with ethyl acetate, separated by high-performance liquid chromatography, and detected by liquid scintillation counting. Metabolites from cultures grown with unlabeled phenanthrene were identified as phenanthrene *trans*-9,10-dihydrodiol, phenanthrene *trans*-3,4-dihydrodiol, 9-phenanthrol, 3-phenanthrol, 4-phenanthrol, and the novel conjugate 9-phenanthryl β-D-glucopyranoside. Identification of the compounds was based on their UV absorption, mass, and nuclear magnetic resonance spectra. Since lignin peroxidase was not detected in the culture medium, these results suggest the involvement of monooxygenase and epoxide hydrolase activity in the initial oxidation and hydration of phenanthrene by *P. chrysosporium*.

Polycyclic aromatic hydrocarbons (PAHs), such as phenanthrene, are commonly found as pollutants in soils (21), estuarine waters and sediments (16, 31), and other terrestrial and aquatic sites (10). Phenanthrene has been shown to be toxic to marine diatoms (22), gastropods (26), mussels (27), crustaceans (30), and fish (3).

Although several bacteria (10, 20, 33) and a cyanobacterium (25) are known to metabolize phenanthrene, little is known about the metabolism of phenanthrene by fungi. The zygomycete *Cunninghamella elegans* oxidizes phenanthrene to the *trans*-1,2-dihydrodiol; it also produces smaller amounts of the *trans*-3,4- and *trans*-9,10-dihydrodiols (9, 11) and a glucoside conjugate of 1-phenanthrol (9). The basidiomes of an *Auricularia* sp. have been reported to accumulate 0.2 to 5.1 mg of phenanthrene per kg of dry weight (23).

The white rot fungus *Phanerochaete chrysosporium* has been shown to metabolize a large number of xenobiotic compounds (1, 7, 18). Among these are the PAHs benzo[*a*]pyrene (8, 29), pyrene (19), fluorene (14), and phenanthrene (6, 24). According to Bumpus (6), *P. chrysosporium* was able to convert 7.7% of the labeled carbon atoms in [9-^{14}C]phenanthrene to $^{14}CO_2$ in 27 days at 39°C. After the cultures had been extracted with methylene chloride, radioactive metabolites were detected in the organic, aqueous, and particulate fractions from these cultures but not characterized further (6). Morgan et al. (24) reported that *P. chrysosporium* metabolized 28.0% of the radioactivity in [9-^{14}C]phenanthrene to water-soluble products and 1.3% to $^{14}CO_2$ in 70 days at 30°C. The purified lignin peroxidase H8 from *P. chrysosporium*, which is typically produced in nitrogen-limited media (34), oxidizes benzo[*a*]pyrene to the 1,6-, 3,6-, and 6,12-quinones (17) and pyrene to the 1,6- and 1,8-quinones (19). This enzyme also oxidizes the PAHs benz[*a*]anthracene, anthracene, and perylene but not phenanthrene (19).

The current study represents an effort to characterize the products of phenanthrene oxidation by *P. chrysosporium* cultures in which lignin peroxidase activity was not induced.

* Corresponding author.

MATERIALS AND METHODS

P. chrysosporium ME-446 (ATCC 34541) was maintained on slants of malt extract agar (Difco Laboratories, Detroit, Mich.).

A malt extract-glucose medium (pH 5.5) was used; it contained 20 g of malt extract, 20 g of D-glucose, 2.4 g of D-maltose, 1.8 g of yeast extract, and 50 mg of Tween 80 (polyoxyethylene sorbitan monooleate) per liter of deionized water. The medium was inoculated with the spores of *P. chrysosporium* (ca. 5 μg/liter).

Phenanthrene (Sigma Chemical Co., St. Louis, Mo.) was dissolved in *N*,*N*-dimethylformamide (14 mg in 5.0 ml) and autoclaved separately before it was added aseptically to 1 liter of medium. When radioactive phenanthrene was required, [9-^{14}C]phenanthrene (10.9 mCi/mmol; radiochemical purity, 99%; Sigma) was also added.

Lignin peroxidase activity was assayed by the veratryl alcohol method (34).

For the extraction, isolation, and identification of phenanthrene metabolites, 0.71 mg of unlabeled phenanthrene and 0.37 μCi of [9-^{14}C]phenanthrene were added to 50 ml of malt extract-glucose medium in triplicate 250-ml flasks. The cultures were inoculated with *P. chrysosporium* and incubated at 37°C with shaking at 125 rpm.

After incubation for 7 days, the mycelia were harvested. (The mean dry weight of the mycelia, determined for nonradioactive cultures, was 3.2 ± 0.8 g/liter of medium.) Each of the cultures was extracted with five equal volumes of ethyl acetate. The neutral extracts for each flask were combined and dried over anhydrous Na_2SO_4. The aqueous phase from each flask was acidified to pH 2.5 with concentrated HCl and extracted again with five equal volumes of ethyl acetate. The acid extracts for each flask also were combined and dried over anhydrous Na_2SO_4.

The volumes of the neutral and acid extracts and the aqueous phases were measured. Triplicate 1-ml samples were removed from each extract for liquid scintillation counting in Ultima Gold (Packard Instrument Co., Meriden, Conn.) scintillation fluid (33). When sampling the aqueous phase, care was taken not to remove any ethyl acetate floating on the surface. Samples of the mycelia were combusted; the CO_2 was collected in vials containing 10 ml of

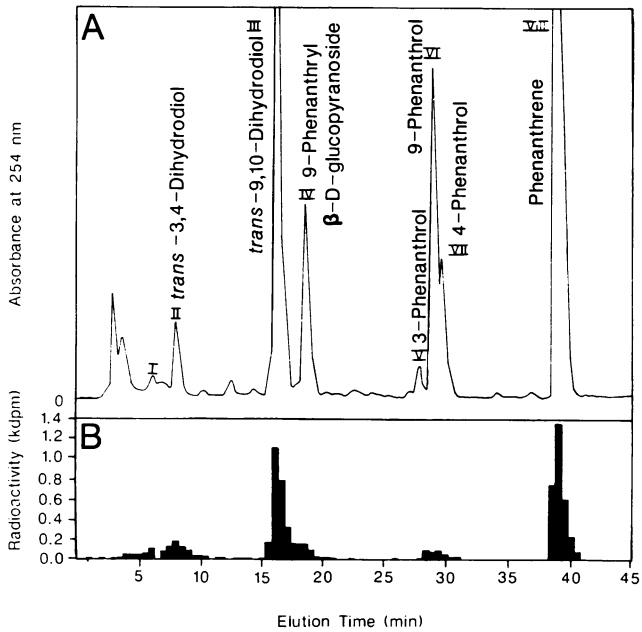

FIG. 1. HPLC elution profile of metabolites produced by *P. chrysosporium* in malt extract-glucose broth with [9-^{14}C]phenanthrene. (A) A_{254}. (B) Radioactivity of 30-s fractions collected during elution.

Permafluor V (Packard) and 7 ml of Carbo-Sorb (Packard) for liquid scintillation counting.

To obtain a sufficient amount of individual phenanthrene metabolites for analysis, multiple 500-ml cultures in 2-liter flasks were inoculated with *P. chrysosporium*. The cultures were incubated at 37°C with shaking at 125 rpm. After 7 days, the cultures were extracted once with ethyl acetate, which was dried over anhydrous Na_2SO_4 and concentrated with a rotary vacuum evaporator (33).

Glass thin-layer chromatography plates coated with silica gel (LK6F Linear-K; 250 μm; Whatman Chemical Separations, Clifton, N.J.) were cleaned with benzene-ethanol (9:1, vol/vol) before they were spotted. The crude ethyl acetate extract was solubilized in acetone and spotted on the thin-layer chromatography plates. The plates were developed in benzene-ethanol (9:1, vol/vol), and the spots were visualized with UV. The spots then were scraped from the plates and eluted from the silica gel with methanol.

Reverse-phase high-performance liquid chromatography (HPLC) analysis of metabolites was performed with a Beckman model 420 liquid chromatograph (Beckman Instruments, Fullerton, Calif.) fitted with a Beckman Ultrasphere C_{18} column (inner dimensions, 25 cm by 4.6 mm; 5-μm particle size). The mobile phase consisted of a 40-min methanol-water gradient (from 50:50 to 95:5, vol/vol) with a flow rate of 1.0 ml/min (33). A Beckman model 160 UV absorbance detector was set at 254 nm to detect metabolites.

In experiments using radioactive phenanthrene, the crude ethyl acetate extract was utilized for HPLC. Fractions (0.5 ml) were collected at 30-s intervals and counted by liquid scintillation methods, using Scintisol (Isolab, Inc., Akron, Ohio) scintillation fluid (33).

UV absorption spectra were determined with a model 1040A diode array spectrophotometer (Hewlett-Packard, Palo Alto, Calif.) and compared to those published previously for phenanthrene metabolites (2, 9, 15, 20, 33).

TABLE 1. Percentage of the radioactivity represented by each individual metabolite among the [9-^{14}C]phenanthrene metabolites produced by *P. chrysosporium*

Metabolite[a]	HPLC retention time (min)	% of radioactivity recovered
I (unknown)	5.6	4.5
II (phenanthrene *trans*-3,4-dihydrodiol)	7.6	11.1
III (phenanthrene *trans*-9,10-dihydrodiol)	15.6	60.8
IV (9-phenanthryl β-D-glucopyranoside)	17.9	14.0
V (3-phenanthrol)	26.6	3.1
VI (9-phenanthrol)	28.2	4.3
VII (4-phenanthrol)	29.1	2.2

[a] Roman numerals refer to the peaks in Fig. 1.

To purify the metabolites obtained from the thin-layer chromatography plates, a semipreparative reverse-phase Beckman Ultrasphere C_{18} column (inner dimensions, 25 cm by 10 mm; 5-μm particle size) was used. Compounds were eluted isocratically with methanol-water (8:2) at a flow rate of 2.0 ml/min and collected in test tubes.

A few of the phenolic metabolites were not separated sufficiently by this method and were purified instead by normal-phase HPLC with a Beckman Ultrasphere Si silica column (inner dimensions, 25 cm by 4.6 mm). The metabolites were eluted isocratically with hexane-ethyl acetate (19:1, vol/vol) at a flow rate of 2.0 ml/min.

After purification, phenanthrene metabolites were analyzed by mass spectrometry with a direct exposure probe (33). Some metabolites were acetylated (9) to obtain additional mass spectral data.

Proton nuclear magnetic resonance (NMR) spectra were obtained at 500 MHz by using a Bruker AM500 spectrometer (Bruker Instruments, Billerica, Mass.). Samples were dissolved in deuterated acetone. The typical data acquisition and processing parameters were the same as those described previously (28). Chemical shifts were reported in parts per million downfield from tetramethylsilane by assigning the acetone peak to 2.05 ppm.

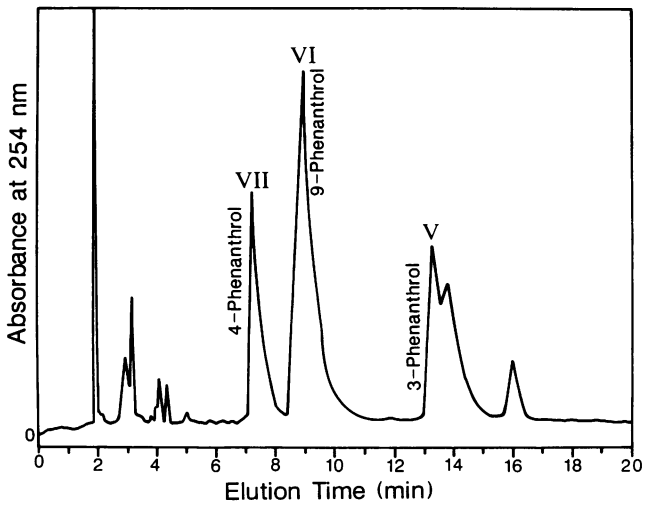

FIG. 2. HPLC elution profile of metabolites V to VII on a normal-phase silica column. Compounds were eluted isocratically with hexane-ethyl acetate (95:5, vol/vol) at a flow rate of 2.0 ml/min.

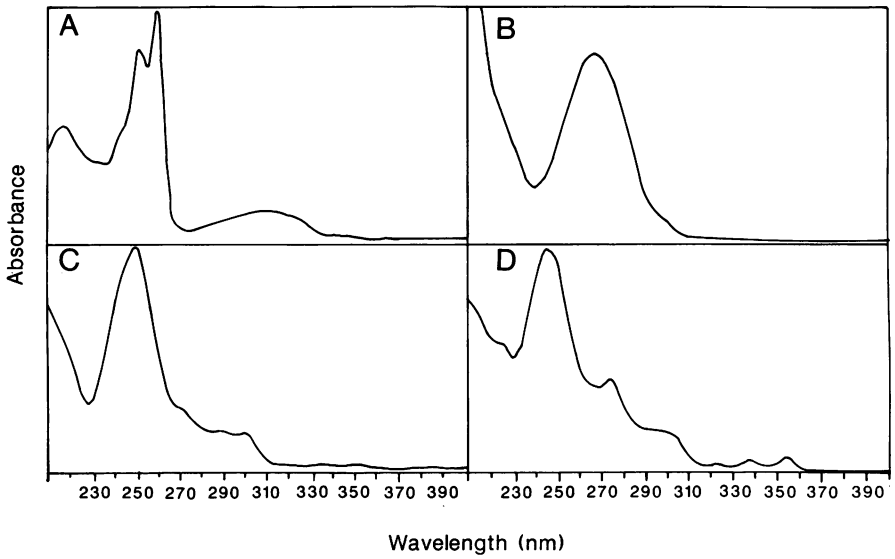

FIG. 3. UV absorption spectra of four of the metabolites produced from phenanthrene by *P. chrysosporium*. (A) Metabolite II, phenanthrene *trans*-3,4-dihydrodiol. (B) Metabolite III, phenanthrene *trans*-9,10-dihydrodiol. (C) Metabolite IV, 9-phenanthryl β-D-glucopyranoside. (D) Metabolite VII, 4-phenanthrol.

RESULTS

Individual phenanthrene metabolites from broth cultures were chromatographed by HPLC and detected by the UV A_{254} (Fig. 1A) and by liquid scintillation counting (Fig. 1B). The metabolites that contained 2% or more of the radioactivity eluted at 5.6, 7.6, 15.6, 17.9, 26.6, 28.2, and 29.1 min and were designated I through VII, respectively (Table 1). The major metabolites were III, II, and IV; each of the other four metabolites represented less than 5% of the radioactivity. Unmetabolized phenanthrene eluted at 39.0 min (Table 1) and represented 41% of the total radioactivity recovered. No lignin peroxidase activity was detected in the *P. chrysosporium* cultures grown in the malt extract-glucose medium.

Three phenanthrene metabolites, V, VI, and VII, were not separated sufficiently by reverse-phase HPLC and thus were purified further by normal-phase HPLC (Fig. 2). The retention times for metabolites V, VI, and VII with the normal-phase column were 13.3, 9.0, and 7.3 min, respectively (Fig. 2).

Metabolite II had a UV absorption spectrum (Fig. 3A) with λ_{max} values at 216, 251, 258, and 308 nm, which is similar to the spectrum reported for synthetic phenanthrene 3,4-dihydrodiol (20). The mass spectrum of metabolite II (Table 2) shows a molecular ion (M^+) at *m/z* 212 and fragment ions at *m/z* 194, 181, 166, 165, and 152, typical of a phenanthrene dihydrodiol (11, 20). The NMR chemical shifts (Table 3) and coupling constants (Table 4) obtained for metabolite II were consistent with the NMR analysis previously reported for phenanthrene *trans*-3,4-dihydrodiol (20).

Metabolite III had a UV absorption spectrum (Fig. 3B) with a λ_{max} at 269 nm, similar to that of phenanthrene 9,10-dihydrodiol (20, 33). The mass spectrum of metabolite III (Table 2) shows a molecular ion (M^+) at *m/z* 212 and fragment ions at *m/z* 194, 181, 166, 165, and 152, similar to the spectrum of phenanthrene 9,10-dihydrodiol (33). The NMR spectral parameters (Tables 3 and 4) for metabolite III were consistent with NMR data previously reported for phenanthrene *trans*-9,10-dihydrodiol (20, 33).

Metabolite IV had a UV absorption spectrum (Fig. 3C) with λ_{max} values at 210, 250, and 300 nm. The mass spectrum of metabolite IV (Table 2) shows a molecular ion (M^+) at *m/z* 356 and fragment ions at *m/z* 194 and 165. To establish the identity of metabolite IV as a glucoside conjugate, it was acetylated. The mass spectrum of the acetylated derivative (Table 2) shows a molecular ion (M^+) at *m/z* 524 and

TABLE 2. Mass spectra of the principal metabolites produced from phenanthrene by *P. chrysosporium*[a]

Metabolite	Characteristic mass ions, *m/z* (with relative abundance)
Phenanthrene *trans*-3,4-dihydrodiol (II)	212 [M^+] (61), 194 (29), 181 (16), 169 (13), 168 (91), 166 (100), 165 (95), 164 (12), 163 (12), 155 (10), 153 (21), 152 (34), 151 (13), 141 (20), 140 (53), 139 (24), 128 (14), 127 (15), 115 (15), 83 (13), 82 (18), 76 (12), 63 (12), 55 (14)
Phenanthrene *trans*-9,10-dihydrodiol (III)	212 [M^+] (72), 194 (35), 181 (79), 166 (53), 165 (100), 164 (11), 153 (15), 152 (26), 82 (24), 77 (17), 76 (11), 51 (10)
9-Phenanthryl β-D-glucopyranoside (IV)	356 [M^+] (0.52), 195 (18), 194 (100), 166 (14), 165 (38)
Acetylated derivative of IV	524 [M^+] (0.35), 331 (30), 169 (100), 165 (21), 127 (19), 109 (66), 79 (26), 60 (14), 52 (18)
3-Phenanthrol (V)	195 (15), 194 [M^+] (100), 165 (27), 97 (11), 82 (15)
9-Phenanthrol (VI)	195 (15), 194 [M^+] (100), 166 (15), 165 (56), 82 (20)
4-Phenanthrol (VII)	195 (15), 194 [M^+] (100), 166 (22), 165 (51), 82 (14)

[a] All mass spectra were obtained by electron impact with a direct exposure probe.

TABLE 3. ^{1}H-NMR chemical shifts of the metabolites formed from phenanthrene by *P. chrysosporium*

Assignment	Chemical shift (ppm) of metabolites					
	Phenanthrene *trans*-3,4-dihydrodiol	Phenanthrene *trans*-9,10-dihydrodiol	9-Phenanthryl β-D-glucopyranoside[a]	3-Phenanthrol	9-Phenanthrol	4-Phenanthrol
1	6.70	7.71	7.84	7.83	7.72	7.48[b]
2	6.23	7.35	7.57	7.23	7.51	7.44
3	4.37	7.37	7.55		7.46	7.22[b]
4	5.37	7.80	8.72	8.13	8.68	
5	8.31	7.80	8.80	8.62	8.78	9.83
6	7.54	7.37	7.72	7.62	7.71	7.62
7	7.45	7.35	7.65	7.59	7.65	7.57
8	7.85	7.71	8.51	7.91	8.38	7.92
9	7.82	4.62		7.61		7.76
10	7.35	4.62	7.50	7.72	7.15	7.75

[a] Chemical shifts of the glucose moiety were as follows: 1', 5.31; 2', 3.73; 3', 3.62; 4', 3.54; 5', 3.68; 6'a, 3.95; 6'b, 3.75.
[b] Assignments may be reversed.

TABLE 4. ^{1}H-NMR coupling constants of the metabolites formed from phenanthrene by *P. chrysosporium*

Proton	Coupling constant (Hz) of metabolites					
	Phenanthrene *trans*-3,4-dihydrodiol	Phenanthrene *trans*-9,10-dihydrodiol	9-Phenanthryl β-D-glucopyranoside[a]	3-Phenanthrol	9-Phenanthrol	4-Phenanthrol
1-2	9.6	7.9	7.9	8.7	8.2	7.5
1-3	ND[b]	1.4	1.6		1.6	1.5
2-3	5.3	7.2	7.0		7.0	7.5
2-4	1.1	1.6	1.2	2.3	1.4	
3-4	1.8	7.5	8.2		8.2	
5-6	8.3	7.5	8.3	8.0	8.2	8.6
5-7	1.3	1.6	1.3	1.4	1.2	1.4
6-7	8.2	7.2	7.0	6.9	6.8	7.0
6-8	1.3	1.4	1.5	1.4	1.4	1.7
7-8	8.2	7.9	8.2	8.4	8.2	7.6
9-10	8.5			9.0		9.0

[a] First-order measurements for the glucose ring (in hertz) were as follows: 1'-2', 7.7; 2'-3', 9.0; 3'-4', 9.2; 4'-5', 9.4; 5'-6'a, 2.7; 5'-6'b, 5.7; 6'a-6'b, −11.9.
[b] Not detected.

fragment ions at *m/z* 331, 169, 165, 127, 109, and 79. The NMR spectrum of metabolite IV (Fig. 4), with the chemical shifts (Table 3) and the coupling constants (Table 4), reveals aliphatic resonances due to a D-glucose residue. The site of substitution was determined to be the 9- position by the pattern of coupling constants (Table 4) and by decoupling experiments.

Metabolite V had a UV absorption spectrum similar to that reported for 3-phenanthrol (15) with an additional λ_{max} at 300 nm (2). The UV spectrum and HPLC retention time of metabolite V were identical to those of 3-phenanthrol prepared by the acid dehydration of phenanthrene *cis*-3,4-dihydrodiol produced by *Beijerinckia* sp. strain B-836 (20). The mass spectrum of metabolite V (Table 2) shows a molecular ion (M^+) at *m/z* 194 and a fragment ion at *m/z* 165, as expected for a phenanthrol (20, 33). The 3-hydroxyl substitution was confirmed by NMR analysis; the expected upfield shift (0.3 to 0.6 ppm) of the protons ortho to the hydroxyl group was noted (Tables 3 and 4).

Metabolite VI had a UV absorption spectrum similar to that reported for 9-phenanthrol (2, 15). The UV spectrum and HPLC retention time of metabolite V were identical to those of 9-phenanthrol prepared by the acid dehydration of phenanthrene *trans*-9,10-dihydrodiol produced by *Streptomyces flavovirens* (33). The mass spectrum of metabolite VI (Table 2) shows a molecular ion (M^+) at *m/z* 194 and a fragment ion at *m/z* 165, as expected for a phenanthrol (20, 33). The 9-hydroxyl substitution was confirmed by NMR analysis (Tables 3 and 4).

Metabolite VII had a UV absorption spectrum (Fig. 3D) with λ_{max} values at 245, 273, 302, 337, and 354 nm, similar to

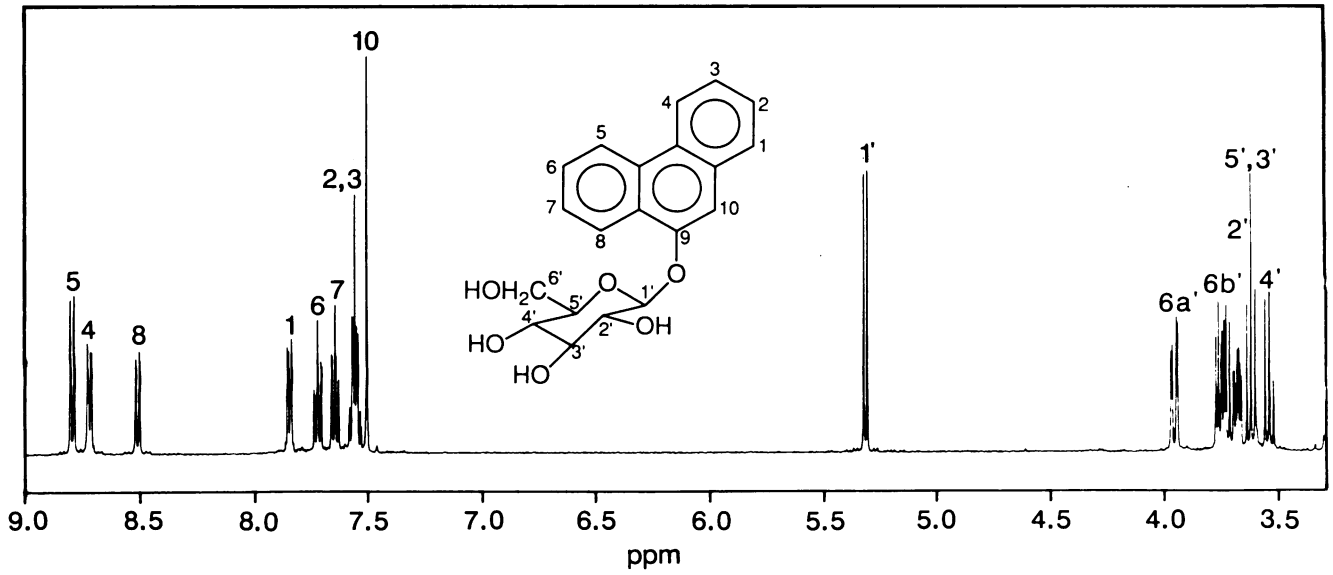

FIG. 4. 500-MHz ^{1}H-NMR spectrum of metabolite IV, 9-phenanthryl β-D-glucopyranoside, produced from phenanthrene by *P. chrysosporium*.

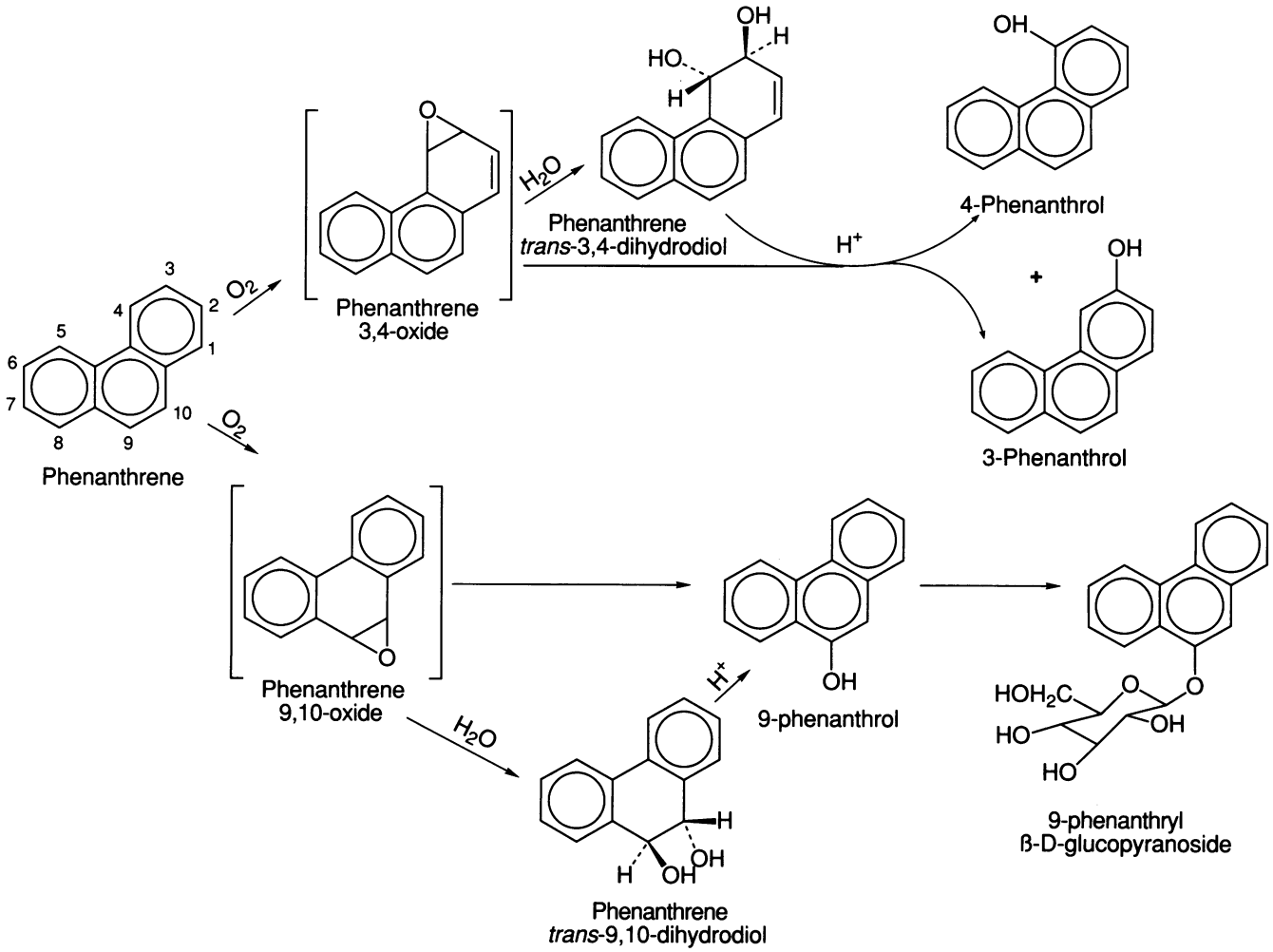

FIG. 5. Proposed pathway for the metabolism of phenanthrene in cultures of *P. chrysosporium* grown in malt extract-glucose broth.

that of 4-phenanthrol (2). The mass spectrum of metabolite VII (Table 2) shows a molecular ion (M^+) at *m/z* 194 and a fragment ion at *m/z* 165, as expected for a phenanthrol (20, 33). Identification of metabolite VII as 4-phenanthrol was confirmed by NMR analysis; the expected upfield shift of the proton ortho to the hydroxyl group and the 1-ppm downfield shift of the proton in a bay position relative to the hydroxyl group were noted (Tables 3 and 4).

Metabolite I, which eluted at 5.6 min (Fig. 1A), was not identified. It had a relatively low UV A_{254}.

In experiments with [9-^{14}C]phenanthrene, the percentage of the radioactivity found in the neutral extract was 46.4 ± 11.2%, that in the acid extract was 2.9 ± 0.8%, that in the aqueous phase after ethyl acetate extraction was 0.5 ± 0.2%, and that in the mycelium was 50.2 ± 18.0%. The relative amount of each metabolite in the neutral extract is shown in Table 1; 79.1% of the radioactivity was found in metabolites oxidized at the 9,10- positions and 16.4% was found in metabolites oxidized at the 3,4- positions.

DISCUSSION

Previous workers (6, 24) have reported that *Phanerochaete chrysosporium* metabolizes PAHs; however, no products other than CO_2 have been identified. According to Sanglard et al. (29), *P. chrysosporium* metabolizes [^{14}C]benzo[*a*]pyrene to several unidentified water-soluble and ethyl acetate-soluble compounds. Whereas purified lignin peroxidase oxidizes benzo[*a*]pyrene to compounds that may be phenols, epoxides, or quinones, intact mycelia metabolize benzo[*a*]pyrene to compounds that may also include dihydrodiol epoxides (29). Bumpus (6) reported that *P. chrysosporium* metabolizes [9-^{14}C]phenanthrene to radioactive compounds that can be separated into water-soluble, methylene chloride-soluble, and insoluble fractions.

In this report, the initial compounds produced in the metabolism of phenanthrene by *P. chrysosporium* have been identified. A proposed pathway that includes the ethyl acetate-extractable metabolites produced from phenanthrene by cultures of *P. chrysosporium* is shown in Fig. 5.

Phenanthrene *trans*-9,10- and *trans*-3,4-dihydrodiols, which would be expected to be formed by the successive activities of monooxygenases and epoxide hydrolases (10), were detected in the medium. However, the phenanthrene 9,10- and 3,4-oxides that are likely to be transient intermediates in this process (4, 20) were not found. PAH arene oxides are generally unstable in aqueous solution and either isomerize to phenols (4, 10) or are hydrated by epoxide hydrolase to the corresponding *trans*-dihydrodiols (10, 12).

Phenanthrene *trans*-9,10-dihydrodiol is the predominant isomer produced from phenanthrene by lobsters and sharks (32) and by most mammalian hepatic microsomes (12). This isomer is also produced by the cyanobacterium *Synechococcus* sp. (*Agmenellum quadruplicatum*) (25) and by the actinomycete *S. flavovirens* (33). The regiospecificity of *P. chrysosporium* differs from that of *C. elegans*, which produces principally the *trans*-1,2-dihydrodiol with smaller amounts of the *trans*-3,4- and *trans*-9,10-dihydrodiols (9, 11).

The three phenanthrols that we found were most likely produced either by dehydration of the *trans*-dihydrodiols (20) or by rearrangement of the postulated arene oxides (4). All three of these phenanthrols are also produced by mammalian hepatic microsomes (12). The sequence of elution of the phenanthrols from the Ultrasphere C_{18} reverse-phase column, 3-, 9-, and 4-, was the same as that found by Bao and Yang (2) under similar chromatographic conditions.

The novel 9-phenanthryl β-D-glucopyranoside produced by *P. chrysosporium* differed from the 1-phenanthryl β-D-glucopyranoside produced by *C. elegans* (9). Since *P. chrysosporium* produced mainly the *trans*-9,10-dihydrodiol and *C. elegans* produced mainly the *trans*-1,2-dihydrodiol (11), the regiospecificity of glucoside formation by these two fungi corresponds with that of dihydrodiol formation.

Thus far, no conclusive evidence for the involvement of the lignin peroxidase system of *P. chrysosporium* in phenanthrene metabolism has been obtained. Significantly, Hammel et al. (19) found that isozyme H8, the most abundant form of lignin peroxidase, does not oxidize phenanthrene. Phenanthrene 9,10-quinone, which would be a potential product of peroxidase activity, was sought but not detected in our study. It is conceivable that phenanthrene, which induces cytochrome P-450 monooxygenase activity in *Cunninghamella bainieri* (13), may also induce monooxygenase activity in *P. chrysosporium*. If such a monooxygenase exists, it could be involved in the formation of arene oxides from phenanthrene so that an epoxide hydrolase could produce the *trans*-dihydrodiols (20).

Because of its ability to produce extracellular lignin peroxidases, *P. chrysosporium* has been proposed for use in the bioremediation of xenobiotic environmental pollutants (1, 7, 18). The present study shows that this fungus can also transform a PAH that is not a substrate for lignin peroxidase (19). Although the K-region metabolites, phenanthrene 9,10-oxide and 9-phenanthrol, are weakly mutagenic (5), other metabolites, including the conjugate 9-phenanthryl β-D-glucopyranoside, may be considered detoxification products of phenanthrene (9, 10).

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