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Metabolism of Phenanthrene by the White Rot Fungus *Pleurotus ostreatus*

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The white rot fungus *Pleurotus ostreatus*, grown for 11 days in basidiomycetes rich medium containing [^{14}C] phenanthrene, metabolized 94% of the phenanthrene added. Of the total radioactivity, 3% was oxidized to CO_2. Approximately 52% of phenanthrene was metabolized to *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene (phenanthrene *trans*-9,10-dihydrodiol) (28%), 2,2′-diphenic acid (17%), and unidentified metabolites (7%). Nonextractable metabolites accounted for 35% of the total radioactivity. The metabolites were extracted with ethyl acetate, separated by reversed-phase high-performance liquid chromatography, and characterized by ^{1}H nuclear magnetic resonance, mass spectrometry, and UV spectroscopy analyses. $^{18}O_2$-labeling experiments indicated that one atom of oxygen was incorporated into the phenanthrene *trans*-9,10-dihydrodiol. Circular dichroism spectra of the phenanthrene *trans*-9,10-dihydrodiol indicated that the absolute configuration of the predominant enantiomer was 9*R*,10*R*, which is different from that of the principal enantiomer produced by *Phanerochaete chrysosporium*. Significantly less phenanthrene *trans*-9,10-dihydrodiol was observed in incubations with the cytochrome P-450 inhibitor SKF 525-A (77% decrease), 1-aminobenzotriazole (83% decrease), or fluoxetine (63% decrease). These experiments with cytochrome P-450 inhibitors and $^{18}O_2$ labeling and the formation of phenanthrene *trans*-9*R*,10*R*-dihydrodiol as the predominant metabolite suggest that *P. ostreatus* initially oxidizes phenanthrene stereoselectively by a cytochrome P-450 monoxygenase and that this is followed by epoxide hydrolase-catalyzed hydration reactions.

Phenanthrene, a polycyclic aromatic hydrocarbon (PAH) containing three fused benzene rings, is produced by combustion of fossil fuels, other industrial processes, and natural occurrences such as forest fires (12). It is not mutagenic or carcinogenic to humans; however, it has been shown to be toxic to aquatic organisms (28, 38, 39, 42). Phenanthrene has been used as a model compound for studying the biodegradation of PAHs since (i) it is found in high concentrations in PAH-contaminated environmental samples; (ii) many PAHs containing a phenanthrene moiety are carcinogenic; and (iii) the regiospecificity and stereoselectivity of oxygenases can be determined in metabolic studies since phenanthrene is the smallest PAH to have both a "bay-region" and a "K-region."

The microbial degradation of phenanthrene has been extensively studied. In general, bacteria that utilize phenanthrene as the sole source of carbon and energy oxidize it via a dioxygenase primarily in the bay-region (the C-3 and C-4 or the C-5 and C-6 positions), to form a phenanthrene *cis*-dihydrodiol. Further metabolism of the *cis*-3,4-dihydrodiol yields 1-hydroxy-2-naphthoic acid and protocatechuic acid or catechol, which ultimately can be mineralized (12). In contrast to these bacteria, *Streptomyces flavovirens* (43) and the marine cyanobacterium *Agmenellum quadruplicatum* (35) metabolize phenanthrene in the K-region, mainly to phenanthrene *trans*-9,10-dihydrodiol with a 9*S*,10*S* absolute configuration similar to that produced by mammalian enzymes (1, 31, 36).

Fungi do not utilize phenanthrene as the sole source of carbon and energy but, instead, cometabolize the PAH to hydroxylated products (13, 40). Many nonligninolytic fungi metabolize phenanthrene in a highly regio- and stereoselective manner, via cytochrome P-450 monoxygenase and epoxide hydrolase, to form phenanthrene *trans*-1,2-, *trans*-3,4-, and *trans*-9,10-dihydrodiol; 1-, 2-, 3-, 4-, and 9-phenanthrols; and sulfate, glucoside, and glucuronide conjugates of the primary metabolites (11, 14, 44). Relatively few nonligninolytic fungi have the ability to degrade PAHs to carbon dioxide (13). In contrast, ligninolytic fungi have the ability to cleave the aromatic rings and mineralize PAHs (2–4, 7–9, 15, 19, 20, 23, 26, 29, 32–34, 41). Since phenanthrene has an ionization potential of 8.19 eV, it is not considered to be a lignin peroxidase substrate (23). However, *Phanerochaete chrysosporium* is able to mineralize phenanthrene, oxidizing it initially to phenanthrene-9,10-quinone and then to the ring cleavage product 2,2′-diphenic acid under ligninolytic conditions (23, 32). Furthermore, it has been shown that lignin peroxidase H8 oxidizes 9-phenanthrol to phenanthrene-9,10-quinone (46). Interestingly, under nonligninolytic conditions, *Phanerochaete chrysosporium* metabolizes phenanthrene to phenanthrene *trans*-3,4- and *trans*-9,10-dihydrodiols; 3-, 4-, and 9-phenanthrols; and a glucoside conjugate of 9-phenanthrol (45). Thus, *Phanerochaete chrysosporium* has multiple enzymatic pathways for the metabolism of phenanthrene (44–46).

We recently reported that the white rot fungus *Pleurotus ostreatus* has the ability to mineralize a wide variety of PAHs, including phenanthrene (3). We also showed that there was no distinct correlation between the activities of laccase, manganese peroxidase, and manganese-inhibited peroxidase and PAH mineralization in *P. ostreatus* cultures. Less is known about the biodegradative pathways, biochemical mechanisms, and enzymes involved in the metabolism of PAHs by *P. ostreatus*.

In this study, we describe the isolation and characterization of metabolites produced from the degradation of phenan-

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threne by *P. ostreatus*. We also provide evidence that a cytochrome P-450 monooxygenase is involved in the degradation mechanism.

MATERIALS AND METHODS

Microorganisms and culture conditions. Stock cultures of *P. ostreatus* Florida f6 were maintained on potato dextrose agar plates (Difco Laboratories, Detroit, Mich.) that had been incubated at 28°C and stored at 4°C until use (21, 27). Mycelium was aseptically transferred to sterile blender cups, each containing 90 ml of basidiomycetes rich medium (BRM), and homogenized for 1 min. BRM contained the same components as basidiomycetes salt medium (BSM) (21) without $MnSO_4$ and with 1% D-glucose and 1% peptone (Difco Laboratories). Aliquots (approximately 10 ml) of the homogenate were used to inoculate 100 ml of BRM in 250-ml Erlenmeyer flasks. To each flask, 2.5 mg of nonlabeled phenanthrene and 1 μCi of [9,10-^{14}C]phenanthrene (19.3 mCi/mmol) dissolved in 0.3 ml of dimethyl formamide were added. Two control flasks were prepared, one with sterile BRM and phenanthrene and the other with mycelium but no phenanthrene. All of the flasks were incubated for 21 days at 28°C in the dark (to prevent photooxidation of the phenanthrene) with shaking at 120 rpm on a rotary shaker.

Extraction and detection of phenanthrene metabolites. After incubation, the medium (pH 7.5) and mycelium were extracted with 3 volumes of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo at 40°C. The aqueous phase was acidified to pH 2.5 with concentrated HCl and extracted in the same manner. The residues were redissolved in methanol for thin-layer chromatography and high-pressure liquid chromatography (HPLC) and compared with residues from controls. Phenanthrene and its metabolites were resolved by reversed-phase HPLC. An Ultrasphere C_{18} octadecylsilane column (25 cm by 4.6 mm [inner diameter]; Altex Scientific, Berkeley, Calif.) with a 40-min linear gradient of water-methanol (50:50 to 5:95, vol/vol) at a flow rate of 1 ml/min was used for separation of neutral metabolites. Acidic metabolites were separated under the same HPLC conditions, except that 1% acetic acid was added to the mobile phase.

The UV absorption spectra of metabolites were obtained with a model 1040A diode array detector (Hewlett-Packard, Palo Alto, Calif.) and analyzed with a Hewlett-Packard model 300 computer. Radiolabeled metabolites eluting from the column were measured with a Radiomatic FLO-ONE model A-525A detector (Packard Instrument Co., Downers Grove, Ill.). Thin-layer chromatography of PAH metabolites was performed on silica gel 60 F_{254} plates (20 by 20 cm; thickness, 0.25 mm; E. Merck, Darmstadt, Germany). The metabolites were separated in benzene-ethanol (9:1, vol/vol). Compounds were observed on the chromatograms with an UV lamp.

GC-MS. Before analysis by gas chromatography-mass spectrometry (GC-MS), the neutral extracts were acetylated with acetic anhydride and pyridine and the acidic extracts were methylated with diazomethane (24). GC-MS analysis of acetylated and methylated metabolites was performed on a model 4000 gas chromatograph mass spectrometer (Finnigan MAT Corp., San Jose, Calif.) equipped with a quadrupole mass filter and a DB5 ms capillary column (0.25 mm [inner diameter] by 0.25 μm [film thickness] by 30 m). The instrument had been upgraded to a model 4500 capability and fitted with a Varian model 3400 gas chromatograph and a Davco temperature-programmable interface. Analyses were performed in the electron ionization mode with an electron energy of 70 eV. Samples were injected into the gas chromatograph at 65°C, held isothermally for 2 min, and programmed to rise to 280°C at 20°C/min, after which the temperature was held isothermally at 280°C for 3 min.

Nuclear magnetic resonance spectrometry. ^{1}H nuclear magnetic resonance spectra were recorded at 500.13 MHz at approximately 29°C on a model AM 500 spectrometer (Bruker Instruments, Billerica, Mass.). The samples were dissolved in 99.95% acetone-d_6. Chemical shifts are reported on the ppm scale; the residual acetone signal was assigned 2.05 ppm. The datum point resolution was 0.215 Hz per point. Analysis was conducted with the aid of spectral simulation by using the Bruker program PANIC. Approximations of four-spin and five-spin systems were made. General procedures for data acquisition and processing have been reported previously (17, 18).

Circular dichroism spectroscopy. Circular dichroism spectra were obtained in methanol with a model 500A spectropolarimeter (Jasco, Tokyo, Japan) and were calibrated with the standard ammonium (1*R*)-(−)-10-camphor sulfonate. The spectra of the metabolites were compared with the circular dichroism spectra published previously (14).

Mineralization experiments. In a biometer flask, 80 ml of culture medium (BRM) was inoculated with 5 ml of blended fungal inoculum from one well-grown plate in 100 ml of physiological saline solution. Phenanthrene (2.0 mg of nonlabeled compound, and 1 μCi of labeled compound) was added to each of the biometer flasks. Ten milliters of 5 M NaOH was used as a $^{14}CO_2$ trap in the biometer flask side arm. Samples (200 μl) of NaOH were taken at each sampling time, and radioactivity was measured in a model TRI-CARB 2000CA liquid scintillation analyzer (Packard Instrument Co.).

$^{18}O_2$ experiments. The incorporation of $^{18}O_2$ into phenanthrene metabolites was determined by incubating *P. ostreatus* in four rubber-stoppered, 125-ml Erlenmeyer flasks containing 50 ml of BRM with 1.3 mg of phenanthrene and 98 atom% $^{18}O_2$ (Cambridge Isotope Laboratories, Andover, Mass.). Before the addition of $^{18}O_2$, each flask was purged with N_2 four times. The isotope composition of the oxygen in the flask, as measured by MS, was 94 to 97 atom% $^{18}O_2$ at the beginning of the experiment. Cultures were incubated for 48 h in the presence of phenanthrene. The phenanthrene *trans*-9,10-dihydrodiol formed was extracted with ethyl acetate and purified by thin-layer chromatography (benzene-ethanol; 9:1). The isotope abundance of the dihydrodiol was calculated from the relative intensities of the molecular ions at *m/z* 212, 214, and 216.

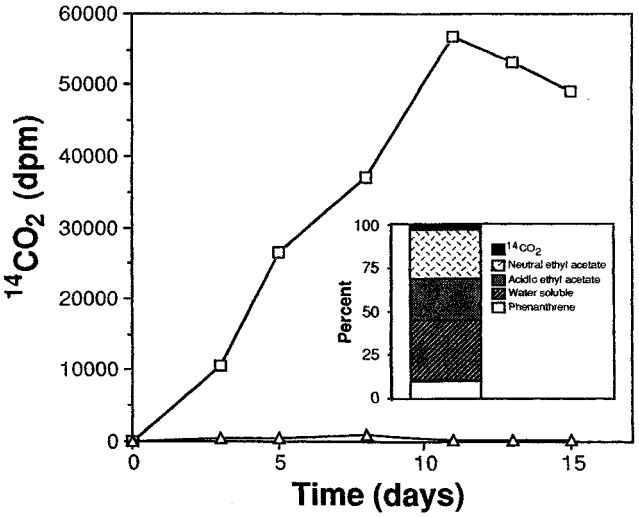

FIG. 1. Mineralization of [9,10-^{14}C]phenanthrene by *P. ostreatus* (□) and control incubation of phenanthrene in BRM without the fungus (△). The insert represents the distribution of phenanthrene metabolism. The mass balance represents the percent recovery of radioactivity initially added.

Cytochrome P-450 determination. A cell-free homogenate was prepared by growing *P. ostreatus* for 48 to 72 h in 2-liter Erlenmeyer flasks containing 700 ml of BSM, which were shaken at 120 rpm and incubated at 27°C in the dark. The mycelia (20 g) were harvested by filtration and resuspended in 20 ml of 50 mM phosphate buffer, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM reduced glutathione (pH 7.4), with 20 g of glass beads (diameter, 0.45 to 0.55 μm) in a glass vessel. The cells were disrupted with a homogenizer (B. Braun, Allentown, Pa.) for a total of 4 min in 20-s bursts followed by 20-s cooling periods. The homogenate was filtered through cotton cloth, and the filtrate was centrifuged twice at 10,000 × *g* for 10 min followed by ultracentrifugation at 105,000 × *g* for 90 min at 4°C. The microsomal pellet and the 105,000 × *g* supernatant were analyzed for cytochrome P-450 by the method of Omura and Sato (37).

Cytochrome P-450 inhibitor experiments. A mycelial suspension of *P. ostreatus* was inoculated into 50 ml of BRM and incubated for 5 days at 28°C and 120 rpm in the dark. To duplicate cultures, the cytochrome P-450 inhibitor 1-aminobenzotriazole (1, 10, and 100 μM [Sigma Chemical Co., St. Louis, Mo.]) SKF 525-A (proadifen, 0.1 mM [Smith Kline Beecham, Philadelphia, Pa.]), or fluoxetine (Prozac; 0.1 and 0.3 M [Eli Lilly, Indianapolis, Ind.]) was added. The cultures with inhibitors were incubated for 20 min, and then phenanthrene (1.25 mg per flask) was added. The cultures were incubated for another 17 h and, then 300 μg of pyrene was added as an internal standard to determine the extraction efficiency. The cultures were extracted with ethyl acetate, and the ethyl acetate extracts were analyzed by HPLC for formation of phenanthrene metabolites.

Chemicals. [9,10-^{14}C]phenanthrene (19.3 mCi/mmol; 95% radiochemical purity) was purchased from Amersham/Searle, Arlington Heights, Ill. Phenanthrene was purchased from Sigma. 2,2′-Diphenic acid was purchased from Fluka AG, Buchs, Switzerland. All solvents were of HPLC grade. All other chemicals were of the highest purity available.

RESULTS

P. ostreatus, grown in BRM with [9,10-^{14}C]phenanthrene for 11 days, was able to mineralize 3% of the added PAH (Fig. 1). In addition, 60.6% of the ^{14}C label initially added was found as ethyl acetate-extractable metabolites and residual phenanthrene; 35% of the radioactivity was found in the nonextractable fraction (Fig. 1, inset).

An analysis of the ethyl acetate-extractable metabolites by HPLC, under neutral and acidic solvent conditions, is shown in Fig. 2. A major peak at 10.6 min (Fig. 2A), which represented

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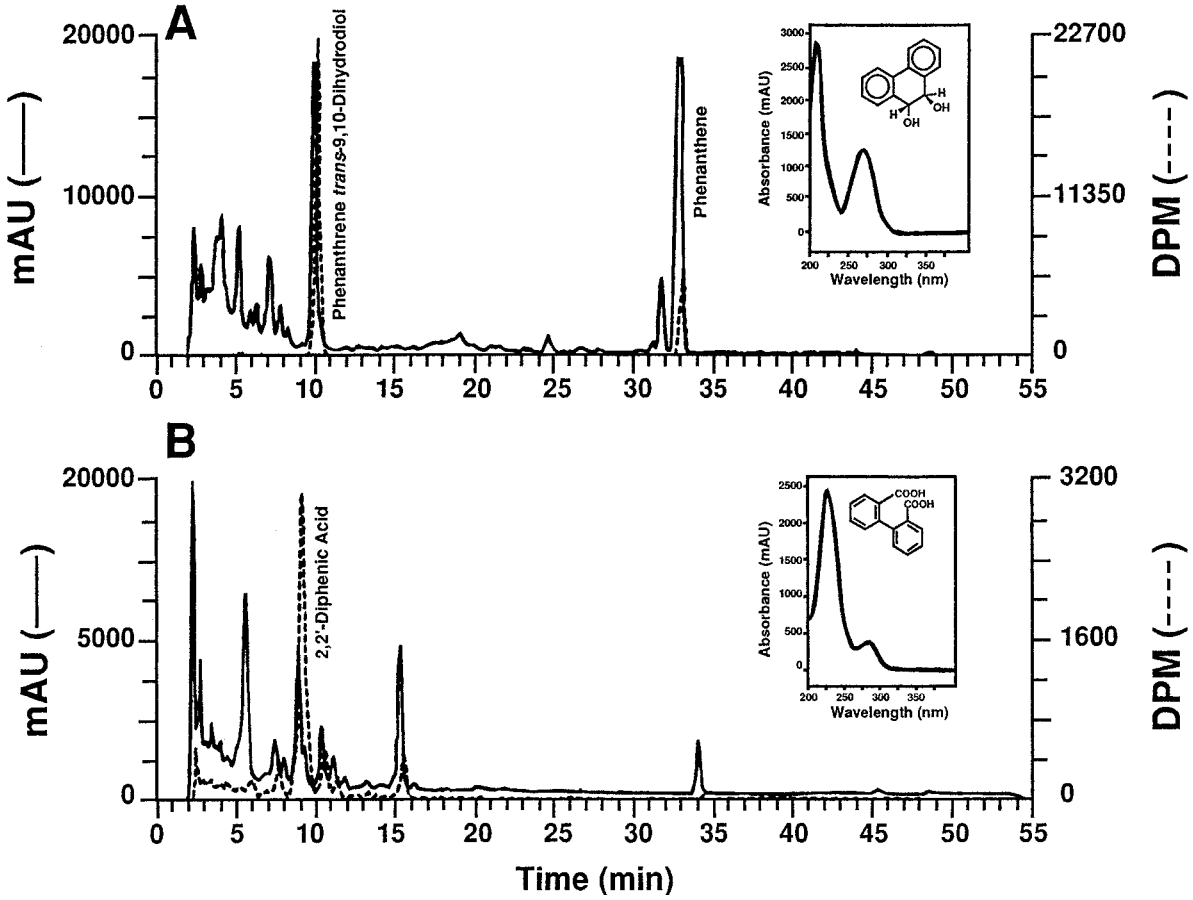

FIG. 2. HPLC elution profile of [9,10-^{14}C]phenanthrene metabolites formed from incubation of *P. ostreatus* with [9,10-^{14}C]phenanthrene for 21 days. (A) HPLC of neutral extract. ——, UV A_{254}; - - - -, radioactivity. Inset, UV spectrum of phenanthrene *trans*-9,10-dihydrodiol produced from phenanthrene by *P. ostreatus*. (B) HPLC of acid extract. ——, UV A_{254}; - - - -, radioactivity. Inset, UV spectrum of 2,2′-diphenic acid produced from phenanthrene by *P. ostreatus*.

all of the neutral metabolites, was identified as phenanthrene *trans*-9,10-dihydrodiol. The UV absorption spectrum (Fig. 2A, inset), λ_{max} (270 nm), GC retention time, GC-MS properties of the diacetylated derivative $M^{+\cdot}$ at *m/z* 296, fragment ions at *m/z* 236 [$M^{+\cdot} - 60$], *m/z* 194 [$M^{+\cdot} - 60 - 42$], and *m/z* 165 [$M^{+\cdot} - 131$], corresponding to the respective sequential losses of acetic acid, ketene, and CHO (Fig. 3A), were identical to those of synthetic phenanthrene *trans*-9,10-dihydrodiol. The ^{1}H nuclear magnetic resonance spectroscopy analysis was conducted to confirm that the phenanthrene 9,10-dihydrodiol was in the *trans* configuration. The resonance and spectral properties are as follows: H1/H8, 7.71; H2/H7, 7.34; H3/H6, 7.36; H4/H5, 7.80; H9/H10, 4.61; OH, 4.71 ppm; and J_{1-2}/J_{7-8}, 7.7; J_{1-3}/J_{6-8}, 1.4; J_{2-3}/J_{6-7}, 7.4; J_{2-4};J_{5-7}, 1.2; and J_{3-4}/J_{5-6}, 7.8 Hz. The nuclear magnetic resonance spectral properties and coupling constants were consistent with those reported previously for phenanthrene *trans*-9,10-dihydrodiol (45), given expected errors of about ± 0.2 Hz. The resonances for H1/H8 and H3/H6 were slightly broadened as a result of unresolved benzylic couplings.

Several additional phenanthrene metabolites were also detected by HPLC analysis of the acid-extractable ethyl acetate-soluble metabolites (Fig. 2B). One major metabolite, which accounted for 84.8% of the total acidic metabolites, had an identical UV spectrum (λ_{max} 234 nm, 285 nm) (Fig. 2B inset) and HPLC retention time (9.2 min) to authentic 2,2′-diphenic acid. The other metabolites were not formed in sufficient quantities for further isolation or structural characterization.

The circular dichroism spectrum (Fig. 4) of the phenanthrene *trans*-9,10-dihydrodiol showed a positive Cotton effect at 228 nm, indicating that the absolute configuration of the predominant enantiomer produced by *P. ostreatus* was 9*R*,10*R*, which is different from that formed by the white rot fungus *Phanerochaete chrysosporium* (44), the cyanobacterium *Agmenellum quadruplicatum* (35), and *Streptomyces flavovirens* (43) but is identical to that formed by the nonligninolytic fungi *Cunninghamella elegans* and *Syncephalastrum racemosum* (Table 1).

MS analysis (Fig. 3B) of the phenanthrene *trans*-9,10-dihydrodiol isolated from the $^{18}O_2$-labeling experiments gave an apparent molecular ion [$M^{+\cdot}$] at *m/z* 214 for the mono-18_2 incorporated metabolite and apparent neutral losses of [$M^{+\cdot} - 18$] (*m/z* 196) and [$M^{+\cdot} - 20$] (*m/z* 194), as well as the expected fragment ions at *m/z* 152, *m/z* 165, and *m/z* 183. Since there was no ion at *m/z* 216 and the molecular ion was at *m/z* 214, the data indicate that only one oxygen atom from $^{18}O_2$ was added to the phenanthrene molecule during the formation of the dihydrodiol.

The formation of phenanthrene *trans*-9,10-dihydrodiol suggested that the initial hydroxylation was mediated by cytochrome P-450 enzymes. To demonstrate the existence of cytochrome P-450 in *P. ostreatus*, a reduced carbon monoxide

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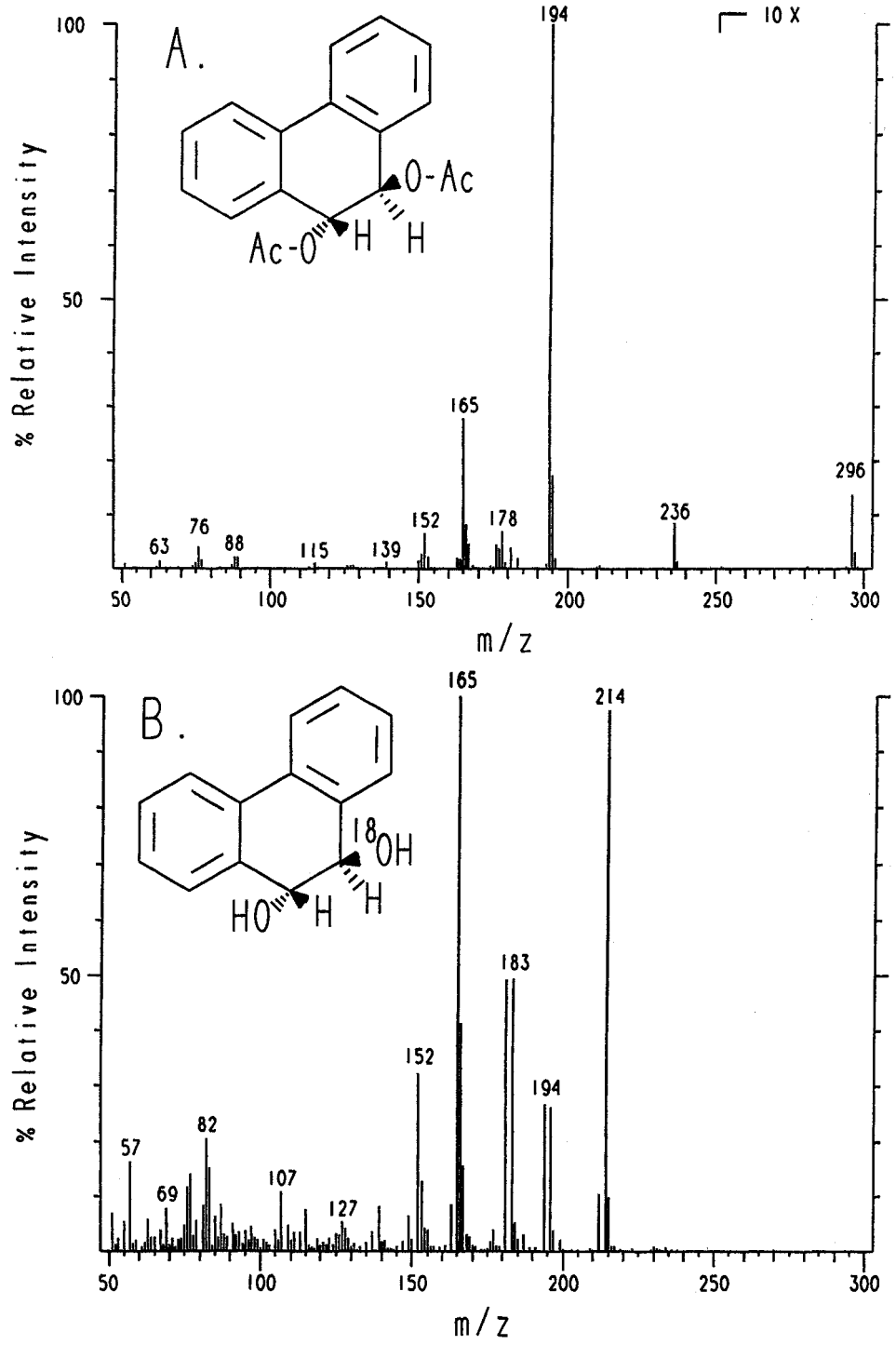

FIG. 3. (A) Mass spectrum of the acetylated derivative of phenanthrene *trans*-9,10-dihydrodiol produced from phenanthrene by *P. ostreatus*. (B) Mass spectrum of phenanthrene *trans*-9,10-dihydrodiol formed in the $^{18}O_2$ incorporation experiment.

difference spectrum was obtained for cytosolic and microsomal fractions. An absorbance peak at 450 nm and a major peak at 420 nm were obtained for both the 100,000 $\times$ *g* supernatant and the pellet (data not shown). In addition, the formation of phenanthrene *trans*-9,10-dihydrodiol in cultures of *P. ostreatus* incubated with the cytochrome P-450 inhibitors proadifen (SKF-525A), 1-aminobenzotriazole, and fluoxetine was reduced by 44-83% (Table 2).

DISCUSSION

Evidence presented in this work demonstrates that *P. ostreatus* is capable of metabolizing phenanthrene to phenanthrene *trans*-9,10-dihydrodiol and 2,2′-diphenic acid as well as mineralizing it to CO_2 (3). A proposed degradation pathway for phenanthrene in *P. ostreatus* is presented in Fig. 5. Previously, we reported that the white rot fungus *P. ostreatus* degraded a

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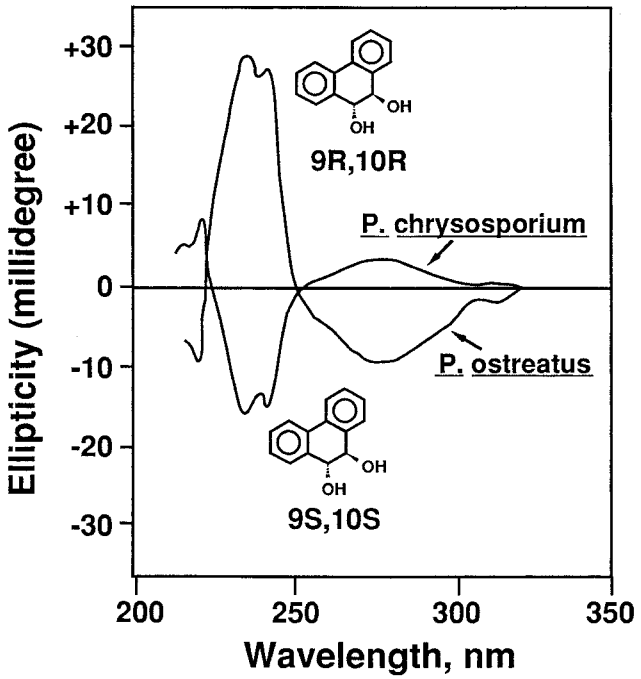

FIG. 4. Circular dichroism spectra of the phenanthrene *trans*-9,10-dihydrodiols produced by *P. ostreatus* and *Phanerochaete chrysosporium*.

wide variety of PAHs, including phenanthrene, with little correlation between PAH degradation and extracellular laccase, manganese peroxidase, or manganese-independent peroxidase activities (3). In this study, the formation of phenanthrene *trans*-9*R*,10*R*-dihydrodiol, in which only one atom of oxygen originated from molecular oxygen, indicates that *P. ostreatus* initially oxidizes phenanthrene stereoselectively, via a cytochrome P-450 monooxygenase and an epoxide hydrolase rather than a dioxygenase, to form the dihydrodiol. The cytochrome P-450 mechanism of oxidation was further supported by the inhibition of phenanthrene *trans*-9,10-dihydrodiol formation by the specific cytochrome P-450 inhibitors SKF 525-A, 1-aminobenzotriazole, and fluoxetine. In addition, we found a minor peak at 450 nm and a major peak at 420 nm in a carbon monoxide difference spectrum of the microsomal fractions. A cytochrome P-450 in *Pleurotus pulmonarius* involved in benzo[*a*]pyrene hydroxylation has recently been reported (30). Although *Phanerochaete chrysosporium*, when grown in high-nitrogen media, exhibits similar regiospecificity to *P. ostreatus* in metabolizing phenanthrene to form the phenanthrene *trans*-9,10-dihydrodiol, there was a difference in stereoselectivity between the species. The dihydrodiol produced by *Phanerochaete chrysosporium* is predominantly the 9*S*,10*S* enantiomer (44), but that produced by *P. ostreatus* was mainly the 9*R*,10*R* enantiomer. The phenanthrene *trans*-9*R*,10*R*-dihydrodiol produced by *P. ostreatus* is similar to the principal enantiomer produced in the nonligninolytic fungi *Cunninghamella elegans* and *Syncephalastrum racemosum* and different from that produced by mammals, cyanobacteria, and streptomycetes (Table 1). Formation of phenanthrene *trans*-9,10-dihydrodiol involves two enzymatic reactions: (i) epoxidation of phenanthrene, catalyzed by cytochrome P-450 isozymes; and (ii) hydration of the resulting K-region 9,10-oxide, catalyzed by epoxide hydrolase. The overall stereoselectivity of these enzymes involved in these two biotransformations results in the formation of either phenanthrene 9*S*,10*S*-dihydrodiol or phenanthrene 9*R*,10*R*-dihydrodiol as the predominant enantiomeric metabolite. The results summarized in Table 1 clearly indicate that the cytochromes P-450 and epoxide hydrolases of *P. ostreatus*, *C. elegans*, and *S. racemosum* favor the stereoselective formation of phenanthrene 9*R*,10*R*-dihydrodiol. On the other hand, metabolisms of phenanthrene by *Phanerochaete chrysosporium*, *A. quadruplicatum* PR-6, *S. flavovirens*, rabbit in vivo, rat in vivo, and rat liver microsomes all produce phenanthrene 9*S*,10*S*-dihydrodiol as the predominant metabolite. Thus, these results provide evidence for different stereoselectivity of the enzymes among these microorganisms and mammals for the metabolism of phenanthrene at the K-region (C-9 and C-10) positions. Our results also provide better a understanding of the regio- and stereoselective biotransformation of PAHs as well as the enzymatic properties of *P. ostreatus*.

TABLE 1. Comparison of the enantiomeric compositions of the phenanthrene *trans*-9,10-dihydrodiols produced by microorganisms and mammals

Microorganism or mammal	Phenanthrene *trans*-9,10-dihydrodiol enantiomer distribution		Reference
	9*S*,10*S*	9*R*,10*R*	
Pleurotus ostreatus	Minor	Major	This study
Cunninghamella elegans	Minor	Major	44
Syncephalastrum racemosum	Minor	Major	44
Phanerochaete chrysosporium	Major	Minor	44
Agmenellum quadruplicatum PR-6	Major	Minor	35
Streptomyces flavovirens	Major	Minor	43
Rabbit in vivo (from urine)	Major	Minor	31
Rat in vivo (from urine)	Major	Minor	31
Rat liver microsomes	Major	Minor	36

We also showed that *P. ostreatus* can cleave the aromatic ring and form 2,2′-diphenic acid and CO_2 from phenanthrene. Hammel et al. (23) reported in 1992 that *Phanerochaete chrysosporium* metabolizes phenanthrene to phenanthrene 9,10-quinone and 2,2′-diphenic acid. Our experiments showed that *P. ostreatus* produced phenanthrene *trans*-9,10-dihydrodiol and 2,2′-diphenic acid. In both cases, ring cleavage reactions may be involved in the enzymatic attack at the C-9 and C-10 positions to form diphenic acid and eventually CO_2; however, the first metabolic step is different in the two fungi.

The role of laccase in PAH degradation is still unclear. Laccase may be able to oxidize nonphenolic compounds under certain conditions. Bourbonnais and Paice (5) and Bourbonnais et al. (6) have shown that the artificial laccase substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) has the capacity to act as a mediator, enabling the oxidation of nonphenolic lignin model compounds that are not laccase substrates on their own.

Laccase may also be involved in further metabolism of PAH metabolites. A laccase from *Coriolus versicolor* (*Trametes ver-*

TABLE 2. Effect of cytochrome P-450 inhibitors on the formation of phenanthrene *trans*-9,10-dihydrodiol in cultures of *P. ostreatus*

Inhibitor	Concn	Inhibition (%)
1-Aminobenzotriazole	1 μM	48
	10 μM	78
	100 μM	83
Proadifen (SKF-525A)	0.1 mM	77
Fluoxetine	0.1 mM	44
	0.3 mM	63

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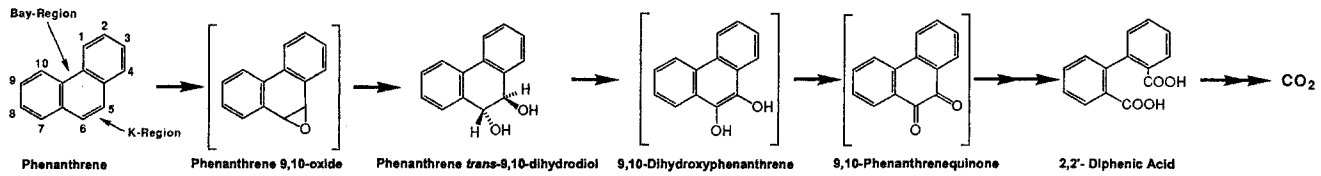

FIG. 5. Proposed pathway for the metabolism of phenanthrene in cultures of *P. ostreatus* grown in BRM.

sicolor) converts 4,6-di-*t*-butyl guaiacol to a ring cleavage product (25). The experiments showed that ^{18}O from $^{18}O_2$ but not from $H_2{}^{18}O_2$ was incorporated into the derivative. The 2,2′-diphenic acid that was formed from phenanthrene in this study, therefore, could also be a product of a reaction catalyzed by *P. ostreatus* laccase. On the other hand, protocatechuate 3,4-dioxygenase, a ring cleavage enzyme previously identified in *P. ostreatus*, could be involved (16). This hypothesis is further supported by the observation that catechol is also mineralized by *P. ostreatus* (3). Since [^{14}C]catechol has only one aromatic ring, the only source of $^{14}CO_2$ has to be from ring cleavage.

Another open question is whether the metabolism of PAHs is related to the degradation of lignin. One may consider that PAHs and their metabolites resemble fragments generated during lignin biodegradation, and therefore it may be hypothesized that a similar enzymatic system is operating at some stages of lignin and PAH degradation. Such enzymes could include monooxygenases, dioxygenases, ligninolytic enzymes, ring cleavage enzymes, dehydrogenases, and decarboxylases. The importance of these enzymes in lignin degradation has previously been proposed (10, 16, 20, 22, 25) and may include the degradation of small lignin fractions resembling PAHs.

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