

# Initial Oxidation Products in the Metabolism of Pyrene, Anthracene, Fluorene, and Dibenzothiophene by the White Rot Fungus *Pleurotus ostreatus*

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**The initial metabolites in the degradation of pyrene, anthracene, fluorene, and dibenzothiophene by *Pleurotus ostreatus* were isolated by high-pressure liquid chromatography and characterized by UV-visible, gas-chromatographic, mass-spectrometric, and <sup>1</sup>H nuclear magnetic resonance spectral techniques. The metabolites from pyrene, dibenzothiophene, anthracene, and fluorene amounted to 45, 84, 64, and 96% of the total organic-solvent-extractable metabolites, respectively. Pyrene was metabolized predominantly to pyrene *trans*-4,5-dihydrodiol. Anthracene was metabolized predominantly to anthracene *trans*-1,2-dihydrodiol and 9,10-anthraquinone. In contrast, fluorene and dibenzothiophene were oxidized at the aliphatic bridges instead of the aromatic rings. Fluorene was oxidized to 9-fluorenol and 9-fluorenone; dibenzothiophene was oxidized to the sulfoxide and sulfone. Circular dichroism spectroscopy revealed that the major enantiomer of anthracene *trans*-1,2-dihydrodiol was predominantly in the *S,S* configuration and the major enantiomer of the pyrene *trans*-4,5-dihydrodiol was predominantly *R,R*. These results indicate that the white rot fungus *P. ostreatus* initially metabolizes polycyclic aromatic hydrocarbons by reactions similar to those previously reported for nonligninolytic fungi. However, *P. ostreatus*, in contrast to nonligninolytic fungi, can mineralize these polycyclic aromatic hydrocarbons. The identity of the dihydrodiol metabolites implicates a cytochrome P-450 monooxygenase mechanism.**

There currently is increasing interest in the biodegradation of polycyclic aromatic hydrocarbons (PAHs), since some of these ubiquitous pollutants are suspected to be human carcinogens (10). PAHs are components of coal tar, creosote, and crude oil and are formed by the incomplete combustion of organic material (1). As the molecular weight of PAHs increases, their water solubility decreases, which indicates their potential affinity for bioaccumulation in biological membranes and tissues. This hydrophobicity usually correlates with increasing genotoxicity and decreasing biodegradability (10). PAHs also bind to soils and sediments and persist in the environment (10). Recently, microbial treatment of PAH-contaminated groundwater and soil has proven to be an attractive alternative to traditional cleanup methods (1, 30). Because of the difficulty of isolating microorganisms which can effectively degrade pyrene and other PAHs with four or more fused aromatic rings, recent research efforts have focused on evaluating a wide phylogenetic spectrum of microorganisms for bioremediation of high-molecular-weight-PAH-contaminated wastes (10). A diverse group of ligninolytic and nonligninolytic fungi are able to cometabolically oxidize PAHs to detoxified chemical products (9, 12, 13, 23, 31-33, 39, 47). The ability of white rot fungi to degrade the abundant naturally occurring polymer lignin has made these fungi appropriate candidates for PAH degradation (8, 22, 23, 40). In addition, since white rot fungi produce extracellular enzymes, PAH bioavailability is maximized and toxicity problems for the fungus are avoided.

The white rot fungus *Phanerochaete chrysosporium* has been

used as a model organism, since it is able to oxidize a wide range of PAHs, including the potent carcinogen benzo[*a*]pyrene (2, 5-8, 17, 21, 24, 25, 27, 35-37, 42, 44). Much of the research with *Phanerochaete chrysosporium* has focused on the role of lignin peroxidase in the oxidation of PAHs (23). However, since the rate of PAH degradation often does not correlate with lignin peroxidase activity, several other enzymatic mechanisms are thought to be used by white rot fungi to degrade PAHs (23, 42).

Other white rot fungi, such as *Trametes versicolor*, *Bjerkandera* sp., and *Pleurotus ostreatus*, have also been shown to metabolize PAHs (20, 29, 34, 40, 46). For instance, we have recently reported that *P. ostreatus* degrades phenanthrene, anthracene, pyrene, fluorene, and benzo[*a*]pyrene to CO<sub>2</sub> and water-soluble products (3, 4). Our recent studies and those of other investigators have indicated the potential of *P. ostreatus*, a lignin-degrading fungus, to degrade PAHs. However, virtually no structural elucidation of the PAH metabolites produced by *P. ostreatus* has been reported.

In this study, we report the isolation and identification of metabolites produced by *P. ostreatus* cultures from pyrene, anthracene, fluorene, and a sulfur-containing PAH, dibenzothiophene. We have also determined the absolute configuration of the anthracene *trans*-1,2-dihydrodiol and pyrene *trans*-4,5-dihydrodiol metabolites.

## MATERIALS AND METHODS

**Microorganisms and culture conditions.** Stock cultures of *P. ostreatus* Florida 66 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 (28). Methods for cultivation of *P. ostreatus* and the PAH metabolism experiments were the

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same as those described in the accompanying paper (4). To each flask, 2.5 mg of nonlabeled PAH and 1  $\mu$ Ci of labeled PAH dissolved in 0.3 ml of dimethyl formamide were added. Incubation conditions and control experiments were prepared as described in the accompanying paper (4).

**Extraction and detection of PAH metabolites.** After incubation, the medium 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 residue was redissolved in methanol for thin-layer chromatography and high-pressure liquid chromatography (HPLC) and compared with residues from controls as described in the accompanying paper (4). The UV absorption spectra and radiolabeled metabolites were measured as described in the accompanying paper. The mass balance represents the percent recovery of initially added radioactivity.

**Gas chromatography-mass spectrometry.** Gas chromatography-mass spectrometry analysis of metabolites was performed as described in the accompanying paper (4), except that the samples were injected into the gas chromatograph at 50°C, held isothermally for 2 min, and programmed to rise to 280°C at a rate of 20°C/min, after which the temperature was held isothermally at 280°C for 10 min.

**Nuclear magnetic resonance spectrometry.** Proton 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 acetone- $d_6$ . Chemical shifts are reported on the ppm scale by assigning the residual acetone signal to 2.05 ppm. Spectral parameters are first-order measurements (datum point resolution, 0.22 Hz per point). General procedures for data acquisition and processing have been reported previously (18, 19).

**CD spectroscopy.** Circular dichroism (CD) spectra were obtained in methanol as described in the accompanying paper (4).

**Chemicals.** [9,10- $^{14}$ C]anthracene (58 mCi/mmol) and [4,5,9,10- $^{14}$ C]pyrene (59.5 mCi/mmol) were purchased from Chemsyn Science Laboratories, Lenexa, Kans. [9- $^{14}$ C]fluorene (14.2 mCi/mmol) was purchased from Sigma Chemical Co., St. Louis, Mo. All radiolabeled PAHs had 98% radiochemical purity. Unlabeled anthracene, pyrene, and dibenzothiophene were purchased from Aldrich Chemical Co., Milwaukee, Wis. Fluorene was purchased from Sigma. All metabolite standards were either synthesized in the laboratory or purchased from Aldrich Chemical Co. All solvents were of HPLC grade. All other chemicals were of the highest purity available.

## RESULTS

*P. ostreatus* was incubated with anthracene, pyrene, fluorene, and dibenzothiophene in basidiomycetes rich medium (BRM) for 21 days. HPLC profiles of the ethyl acetate-extractable metabolites are shown in Fig. 1. Since radiolabeled dibenzothiophene was not commercially available, degradation of dibenzothiophene to CO<sub>2</sub> was not determined.

**Pyrene metabolism.** When *P. ostreatus* was grown in the presence of [ $^{14}$ C]pyrene, 91% of the pyrene was metabolized and 0.4% was mineralized to CO<sub>2</sub>. The ethyl acetate extract contained 45% of the pyrene metabolites, mostly as one major metabolite (Fig. 1A). The radioactive product that coeluted with synthetic pyrene *trans*-4,5-dihydrodiol (14.4 min) accounted for 5.7% of the total radioactivity added. The UV-visible (Fig. 1A, inset) and mass-spectral properties, molecular ion [M<sup>+</sup>] at  $m/z$  236, [M<sup>+</sup> - H<sub>2</sub>O] at  $m/z$  218, as well as expected fragment ions at  $m/z$  205, 189, 176, and 95, were consistent with pyrene 4,5-dihydrodiol (26). Since the reversed-phase HPLC system could not separate the *cis* and *trans* isomers, nuclear magnetic resonance spectroscopy analysis was conducted to determine the configuration of this metabolite. Resonance assignments and spectral parameters for the metabolite are as follows: H1/H8, 7.87; H2/H7, 7.64; H3/H6, 7.90; H4/H5, 5.07; and H9/H10, 7.83 ppm; and  $J_{1-2}/J_{7-8}$ , 8.1;  $J_{1-3}/J_{6-8}$ , 1.3; and  $J_{2-3}/J_{6-7}$ , 7.2 Hz. The resonance from the hydroxyl proton was not detected owing to exchange. The chemical shifts and coupling constants of the metabolite were similar to those previously reported for the *trans* isomer (26).

The major enantiomer of pyrene *trans*-4,5-dihydrodiol obtained from incubation of pyrene with liver microsomes of rats treated with 3-methylcholanthrene (MC-microsomes) has been determined previously by Chiu and Yang to possess an *R,R* configuration (15). The pyrene *trans*-4,5-dihydrodiol me-

tabolite obtained from incubation with *P. ostreatus* had a CD spectral pattern similar to that from MC-microsomes, but the Cotton effect was weaker (Fig. 2A). Therefore, the predominant enantiomer of the pyrene *trans*-4,5-dihydrodiol from *P. ostreatus* also had an *R,R* absolute configuration. The pyrene *trans*-4,5-dihydrodiol from MC-microsomes consisted of 89% 4*R*,5*R* enantiomer and 11% 4*S*,5*S* enantiomer (15). Thus, comparison of the relative ellipticity of the CD spectra indicates that the pyrene *trans*-4,5-dihydrodiol from *P. ostreatus* contained 63% 4*R*,5*R* enantiomer and 37% 4*S*,5*S* enantiomer, with an optical purity of 26% (Table 1).

**Anthracene metabolism.** Approximately 74% of the [ $^{14}$ C]anthracene added to *P. ostreatus* was metabolized. Approximately 16.5% of the total radioactivity was recovered in the ethyl acetate extract, and 0.62% was mineralized to CO<sub>2</sub>. In the ethyl acetate extract fraction, 64% of the radioactivity consisted of anthracene metabolites and the rest consisted of non-metabolized anthracene. The HPLC elution profile (Fig. 1B) of the ethyl acetate-extractable metabolites indicated that anthracene was metabolized predominantly to two metabolites, which had retention times and UV-visible spectral properties identical to those of authentic anthracene *trans*-1,2-dihydrodiol (13.3 min;  $\lambda_{\max}$  245, 276, 287, and 298 nm) and 9,10-anthraquinone (26.5 min;  $\lambda_{\max}$  225, 255, 273, and 325 nm) (Fig. 1B, inset) (14, 43). The CD spectrum of the anthracene *trans*-1,2-dihydrodiol obtained from incubation with *P. ostreatus* is shown in Fig. 2B. For comparison, the CD spectra of the anthracene *trans*-1,2-dihydrodiols of known optical purity, obtained from metabolism of anthracene by *Cunninghamella elegans* and by MC-microsomes, are also shown in Fig. 2B. The predominant enantiomers of the anthracene *trans*-1,2-dihydrodiol from *C. elegans* and from MC-microsomes have 1*S*,2*S* and 1*R*,2*R* absolute configurations, respectively (Fig. 2B) (11, 45). The previously published enantiomeric compositions and the optical purity of the metabolites from *C. elegans*, *Rhizoctonia solani*, and MC-microsomes are summarized in Table 1. The anthracene *trans*-1,2-dihydrodiol from *P. ostreatus* had a CD spectrum similar to that from *C. elegans* but a mirror image of that from MC-microsomes (Fig. 2B). Therefore, the major enantiomer of the metabolite from *P. ostreatus* had a 1*S*,2*S* absolute configuration. On the basis of the molar ellipticity of the Cotton effects shown in Fig. 2B and the data in Table 1, we conclude that anthracene *trans*-1,2-dihydrodiol from *P. ostreatus* consists of 58% 1*S*,2*S* enantiomer and 42% 1*R*,2*R* enantiomer, with an optical purity of 16%.

**Fluorene metabolism.** Approximately 96% of the [ $^{14}$ C]fluorene added to cultures was metabolized. Of the total radioactivity, 26% was recovered in the ethyl acetate extraction phase and 0.19% was mineralized to CO<sub>2</sub>. The HPLC elution profile (Fig. 1C) indicates that fluorene was metabolized by *P. ostreatus* to 9-fluorenol and 9-fluorenone. The HPLC retention times were 16.0 and 23.0 min, respectively, and the UV-visible absorption spectra (Fig. 1C, inset) were identical to those of authentic compounds and published spectra (38).

**Dibenzothiophene metabolism.** Approximately 84% of the dibenzothiophene added to cultures was metabolized. HPLC analysis of the products of *P. ostreatus* incubations with dibenzothiophene for 21 days gave a minor peak at 10.3 min and a major peak at 11.0 min, with residual dibenzothiophene eluting at 32.8 min (Fig. 1D). The major metabolite was identified as dibenzothiophene sulfoxide and the minor component was identified as dibenzothiophene sulfone on the basis of comparison of retention times, UV absorbance (Fig. 1D, inset), and gas chromatographic-mass spectral properties. The [M<sup>+</sup>] at  $m/z$  200, [M<sup>+</sup> - O] at  $m/z$  184, [M<sup>+</sup> - CO] at  $m/z$  172, and [M<sup>+</sup> - CHO] at  $m/z$  171 were identical to those of authentic

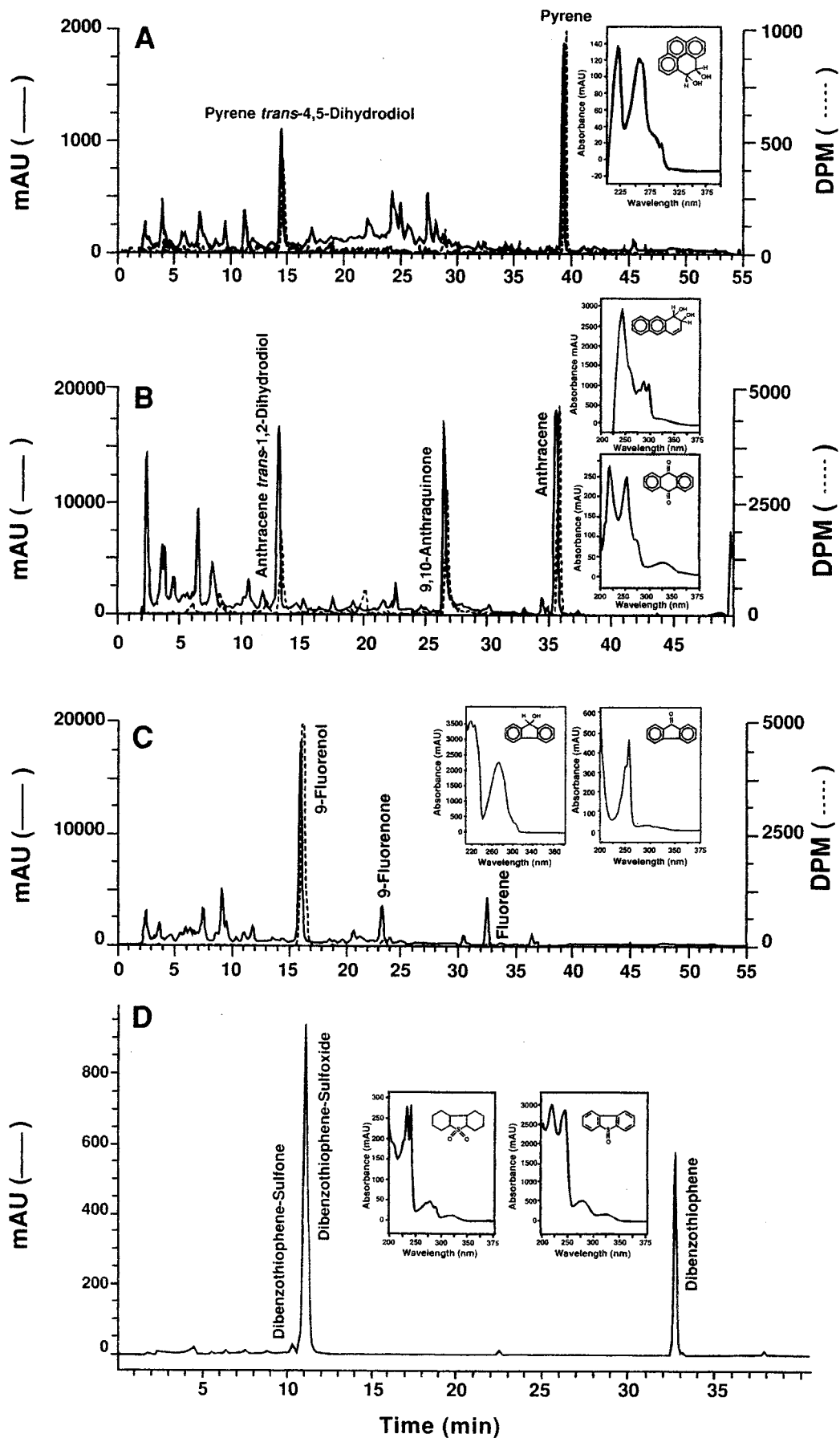


FIG. 1. HPLC elution profiles of metabolites of pyrene (A), anthracene (B), fluorene (C), and dibenzothiophene (D), formed by the incubation of *P. ostreatus* in basidiomycetes rich medium containing PAHs.

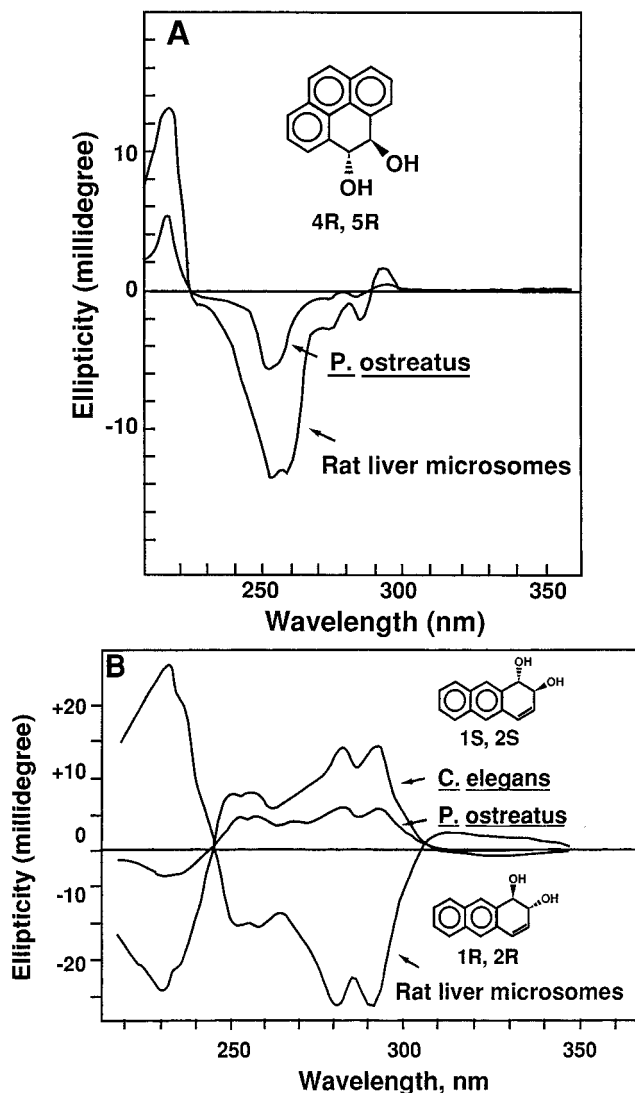


FIG. 2. (A) CD spectra of the pyrene *trans*-4,5-dihydrodiols produced by *P. ostreatus* and MC-microsomes. (B) CD spectra of the anthracene *trans*-1,2-dihydrodiols produced by *P. ostreatus*, *C. elegans*, and MC-microsomes (15, 45).

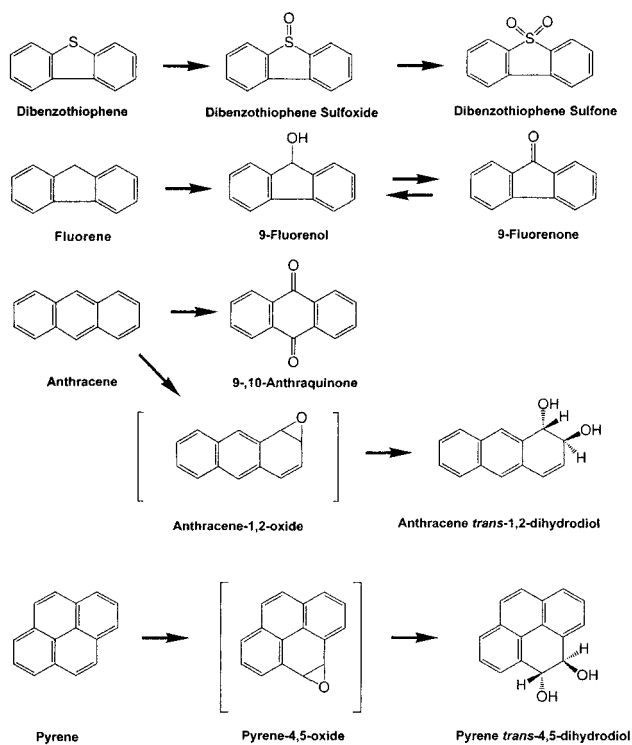


FIG. 3. Proposed pathways for the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene in cultures of *P. ostreatus* grown in basidiomycetes rich medium containing PAHs.

dibenzothiophene sulfoxide; the  $[M^+]$  at  $m/z$  216 and the fragment ion at  $m/z$  187 were identical to those of authentic dibenzothiophene sulfone (16, 41).

## DISCUSSION

The chemical structures of the identified metabolites, as well as proposed pathways for the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by *P. ostreatus*, are shown in Fig. 3. Previously, we showed that *P. ostreatus* had the ability to mineralize these PAHs (3). In this study and the investigation described in the accompanying paper (4), we found that *P. ostreatus* metabolized several PAHs in a highly regio- and stereoselective manner, similar to that reported for nonlignolytic fungi (12, 14, 32, 43).

TABLE 1. Enantiomeric composition of the anthracene *trans*-1,2-dihydrodiols and pyrene *trans*-4,5-dihydrodiols produced by fungi and rat liver microsomes

Metabolite	Organism	% of enantiomer:		Optical purity (%)	Reference
		<i>S,S</i>	<i>R,R</i>		
Anthracene <i>trans</i> -1,2-dihydrodiol	<i>P. ostreatus</i>	58	42	16	This study
	<i>C. elegans</i>	75	25	50	11
	<i>R. solani</i>	60	40	20	43
	Rat liver microsomes				45
	Control	20	80	60	
	MC induced <sup>a</sup>	3	97	94	
PB induced <sup>a</sup>	12	88	76		
Pyrene <i>trans</i> -4,5-dihydrodiol	<i>P. ostreatus</i>	37	63	26	This study
	Rat liver microsomes	11	89	78	15

<sup>a</sup> MC, 3-methylcholanthrene; PB, phenobarbital.

Pyrene was metabolized at the 4,5 bond (K-region) to form an epoxide, which was hydrated to form pyrene *trans*-4,5-dihydrodiol, with the predominant enantiomer in an *R,R* configuration. As in phenanthrene metabolism by *P. ostreatus* (4), the major site of enzymatic attack on pyrene was the K-region to form a dihydrodiol, which was highly stereospecific (4). The formation of *trans* dihydrodiols with the same configuration (*R,R*) raised the possibility that the degradation mechanisms of phenanthrene and pyrene are similar. Cytochrome P-450 monooxygenase and epoxide hydrolase could be responsible for pyrene dihydrodiol formation, as was shown for phenanthrene by incorporation of  $^{18}\text{O}_2$  into phenanthrene dihydrodiol, P-450-specific inhibitor experiments, and measurement of P-450 levels in *P. ostreatus* cultures (4).

Metabolites identified during anthracene degradation by *P. ostreatus* were 9,10-anthraquinone and anthracene *trans*-1,2-dihydrodiol, which had the predominant enantiomer in an *S,S* configuration. The metabolic pathway is shown in Fig. 3. Anthracene *trans*-1,2-dihydrodiol is probably formed via monooxygenation to anthracene 1,2-oxide with enzymatic hydration. The dihydrodiol may be in the pathway of mineralization to  $\text{CO}_2$  via ring cleavage enzymes. The minor and delayed formation of  $^{14}\text{CO}_2$  (3) seems to correlate with the low levels of dihydrodiol formed. The major metabolite was 9,10-anthraquinone, which may be a dead-end or autooxidation product (43). This observation agrees with that of Field et al. (20), who suggested that anthraquinone accumulates as a dead-end metabolite in *Bjerkandera* sp. and *Phanerochaete chrysosporium*. Vyas et al. (46) showed that *P. ostreatus*, *Corioloropsis polyzona*, and *Trametes versicolor* further degrade anthraquinone; *T. versicolor* (20) and *Phanerochaete chrysosporium* (25) mineralize it to  $\text{CO}_2$ . The formation of 9,10-anthraquinone and anthracene *trans*-1,2-dihydrodiol may represent an attack by two different enzymatic systems (laccase and cytochrome P-450, respectively) or by a single enzymatic system that exerts regioselectivity by oxidizing anthracene at different sites. These results and those of previous studies indicate the following major differences between *Phanerochaete chrysosporium* and *P. ostreatus* in anthracene metabolism. (i) In *Phanerochaete chrysosporium*,  $^{14}\text{CO}_2$  is produced from anthracene at the first stage of growth (25), whereas in *P. ostreatus*,  $^{14}\text{CO}_2$  formation is observed only at later stages of growth (3). (ii) Anthracene *trans*-1,2-dihydrodiol was identified only in *P. ostreatus*. (iii) Anthraquinone accumulated in culture media of *P. ostreatus* but is mineralized in *Phanerochaete chrysosporium* (25).

The stereoselectivity of *P. ostreatus* in metabolism of PAHs warrants further discussion. As the results summarized in Table 1 illustrate, although *P. ostreatus* metabolized both pyrene and anthracene to the corresponding pyrene *trans*-4,5-dihydrodiol and anthracene *trans*-1,2-dihydrodiol in a stereoselective manner, its stereoselectivity is much less pronounced than that of *C. elegans* or MC-microsomes. Formation of a *trans* dihydrodiol involves two enzymatic reactions, epoxidation of the PAH substrate (catalyzed by cytochrome P-450), and hydration of the resulting epoxide (catalyzed by epoxide hydrolase). Thus, the low stereoselectivity of *P. ostreatus* during the metabolism of pyrene and anthracene indicates the low stereoselective preference of the cytochrome P-450 isozymes and/or the epoxide hydrolase. It is noteworthy that *P. ostreatus* metabolizes pyrene to produce a *trans*-4,5-dihydrodiol rich in the *R,R* enantiomer but metabolizes anthracene to a *trans*-1,2-dihydrodiol rich in the *S,S* enantiomer.

Dibenzothiophene was oxidized to dibenzothiophene sulfoxide and dibenzothiophene sulfone (Fig. 3), and fluorene was oxidized to 9-fluorenone and 9-fluorenone (Fig. 3). As shown by previous studies on the metabolism of dibenzothiophene and

fluorene by *C. elegans*, these dibenzothiophene and fluorene metabolites may be considered dead-end products mediated by cytochrome P-450 monooxygenases (16, 38, 41).

In conclusion, *P. ostreatus* acts on PAHs like nonligninolytic fungi, such as *C. elegans*; however, in contrast to them, it is able to mineralize PAHs. Since *P. ostreatus* does not contain lignin peroxidase and since PAH metabolism did not correlate with laccase, manganese peroxidase, and manganese-inhibited peroxidase activities (3), it is possible that a cytochrome P-450 monooxygenase is responsible for the initial step in the attack. The ligninolytic mechanism of *P. ostreatus* may be involved in the later steps of metabolism, such as ring cleavage, which leads to  $\text{CO}_2$  evolution.

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