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Microbial metabolism and detoxification of 7,12-dimethylbenz[*a*]anthracene

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SUMMARY

Six strains of fungi grown on Sabouraud dextrose broth in the presence of 7,12-dimethylbenz[*a*]anthracene (DMBA) were surveyed for their ability to metabolize DMBA. Experiments with [^{14}C]DMBA indicated that the extent of formation of organic-soluble metabolites ranged from 6 to 28% after 5 days of incubation, depending on the organism tested. The yields of water-soluble metabolites also varied, and ranged from 1 to 33% after 5 days. *Cunninghamella elegans* ATCC 36112 and *Syncephalastrum racemosum* UT-70 exhibited the highest DMBA-metabolizing activity among the organisms surveyed. *S. racemosum* metabolized DMBA primarily to 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-OHM-12-MBA) and 7,12-dihydroxymethylbenz[*a*]anthracene (7,12-diOHMBA). Minor metabolites included 7-OHM-12-MBA-*trans*-5,6-, 8,9- and 10,11-dihydrodiols, and glucuronide and sulfate conjugates of phenolic derivatives of DMBA. In contrast, the major DMBA metabolites produced by *C. elegans* were water-soluble. The predominant organic-soluble metabolites produced by *C. elegans* included 7-OHM-12-MBA-*trans*-5,6-, 8,9- and 10,11-dihydrodiols. DMBA-*trans*-3,4-dihydrodiol was also detected. Circular dichroism spectral analysis revealed that the major enantiomer of the 7-OHM-12-MBA-*trans*-8,9-dihydrodiol formed by each organism had an *S,S* absolute configuration, while the major enantiomers of the 5,6-, 10,11- and 3,4-dihydrodiols had an *R,R* configuration. The mutagenic activity of extracts from *S. racemosum* exposed to DMBA were determined in *Salmonella typhimurium* TA98. The mutagenicity of DMBA decreased by 36% over a period of 5 days as 33% of the compound was metabolized. Comparison of these results with previously reported results in mammalian systems suggests that there are similarities and differences between the fungal and mammalian oxidation of DMBA and that the overall balance of fungal metabolism is towards a detoxification rather than a bioactivation pathway.

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INTRODUCTION

Possible human exposure to carcinogenic methylbenz[*a*]anthracenes (MBAs) from environmental sources has prompted considerable research on the bioaccumulation, metabolism, and disposition of these compounds in aquatic and terrestrial ecosystems. Although the accumulation of MBAs in the environment can only be inferred at this time, evidence exists which suggests that alkylated derivatives of polycyclic aromatic hydrocarbons (PAHs) are being produced in sediments by thermal processes at environmental temperatures [1]. The environmental loss of MBAs can occur by a variety of mechanisms including bio-, photo- and chemical degradation. Microbial degradation may be the most important factor affecting the ultimate fate of these pollutants because many hydrophobic compounds in aquatic ecosystems occur primarily in sediments which are below the photolytic zone [7].

The carcinogenicity of MBAs in mammals has been known for more than two decades [13,25,26]. In general, MBAs and many other carcinogenic PAHs must be metabolized by cytochrome *P*-450 and epoxide hydrolase in a highly stereoselective manner to bay-region *trans*-dihydrodiol-epoxides in order to exert their carcinogenic effects [12,17,18,21]. For example, an evaluation of the mutagenic and tumorigenic activity of benz[*a*]anthracene, benzo[*a*]pyrene and chrysene has indicated that biological activity is dependent on absolute configuration [2,24,28]. A comparison of the metabolic profiles from mammalian and fungal metabolism of certain PAHs has indicated that fungi also oxidize these compounds to *trans*-dihydrodiols via cytochrome *P*-450 and epoxide hydrolase enzyme systems [3]. However, an analysis of the fungal metabolites of 7-MBA and 4-MBA has demonstrated that there are similarities and differences in the regio- and stereoselectivity in MBA metabolism when compared to that of rat liver microsomes [5,6,14].

7,12-Dimethylbenz[*a*]anthracene (DMBA) is a potent mammalian carcinogen that has been used as a model compound to investigate mechanisms of PAH-induced carcinogenicity. Mammalian metabolism studies combined with DNA binding and mutagenicity assays using synthetic derivatives of DMBA support the contention that the 3,4-dihydrodiol-1,2-epoxide metabolite is the ultimate carcinogenic form of DMBA (Fig. 1) [10,19,26]. However, evidence also exists which suggests that sulfate esterification of 7-hydroxymethyl-12-methylbenz[*a*]anthracene may be another potential mechanism of bioactivation [22,23].

In contrast to the large number of studies concerning the mammalian bioactivation of DMBA, relatively little information exists about the microbial metabolism of this carcinogen or the biological activity of microbial DMBA metabolites. Wu and Wong [29] reported that cultures of *Pseudomonas aeruginosa* and *Penicillium notatum* were able to oxidize the methyl groups of DMBA to form hydroxymethyl metabolites. Recently, Wong and coworkers [27] reported that the filamentous fungus *Cunninghamella elegans* ATCC 9245 metabolized DMBA primarily to *trans*-3,4- and 8,9-dihydrodiols. These investigators also reported the isolation of the respective 7- and 12-hydroxymethyl-*trans*-dihydrodiol metabolites noted above, and suggested that *C. elegans* may play a role in the environmental carcinogenesis of PAHs. However, rigorous structural elucidation of the position of oxidative attack on the methyl groups was not carried out and the relative stereochemistry of the dihydrodiols was not determined. Recent work from our laboratory suggests that metabolism of PAHs by *C. elegans* ATCC 36112 is a detoxification process [8]. This observation is important because interest in the use of microorganisms to detoxify PAHs in the environment has become an active area of research.

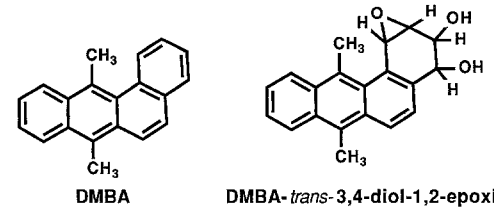

Fig. 1. Structure of 7,12-DMBA and its probable ultimate carcinogenic metabolite, DMBA-3,4-dihydrodiol-1,2-epoxide.

Thus, the objectives for this study were to screen fungi for their ability to metabolize DMBA and to determine the structure and absolute stereochemistry of the metabolites formed from those organisms with the highest DMBA-metabolizing activity. The relationship between the extent of DMBA metabolism by *Syncephalastrum racemosum* and the resultant mutagenic activity of the extracts in the *Salmonella typhimurium* reversion assay was also examined, to determine whether fungi other than *C. elegans* have the ability to detoxify carcinogenic PAHs.

MATERIALS AND METHODS

Microorganisms and culture conditions. Stock cultures of the filamentous fungi *C. elegans* ATCC 36112, *C. elegans* ATCC 9245, *Mucor* sp., *Penicillium chrysogenum, S. racemosum* strain UT-70, and the yeast *Candida lipolytica* strain 37-1 were maintained at 4°C on Sabouraud dextrose slants (Difco Laboratories, Detroit, MI). Microorganisms were inoculated into 125-ml Erlenmeyer flasks containing 30 ml of Sabouraud dextrose broth and incubated at 25°C on a rotary shaker operated at 150 rpm. Fungi were grown for 48 h to achieve sufficient cell density (7 g, dry weight). The cells were removed by aseptic filtration or centrifugation and transferred to sterile 125-ml Erlenmeyer flasks containing 30 ml of fresh media. A dose of 1 mg of DMBA dissolved in 0.10 ml dimethylformamide and 0.5 μCi of [^{14}C]DMBA dissolved in 0.05 ml of benzene were added simultaneously to each flask. All flasks were incubated on a rotary shaker in the dark at 25°C for 5 days. Experiments performed in the absence of microorganisms showed no degradation of the substrate. Batch culture experiments with *S. racemosum* and *C. elegans* ATCC 36112 were performed to obtain a sufficient quantity of metabolites for identification and analysis. Ten flasks of each organism were inoculated and dosed as above with the exception that no radiolabeled DMBA was used.

Isolation and detection of metabolites. The contents of each flask were filtered and extracted with 6 volumes of ethyl acetate. Organic fractions were combined and dried over sodium sulfate, and the solvent was removed under reduced pressure. Each residue was dissolved in methanol and analyzed by a reversed-phase high performance liquid chromatography (HPLC) system which consisted of two Beckman model 100A pumps (Beckman Instruments, Inc., Irvine, CA) and a model 155-10 variable-wavelength absorbance detector adjusted to 254 nm. A 5 μm C_{18} ultrasphere ODS column (4.6 mm × 25 cm; Altex Scientific, Berkley, CA) was used to separate DMBA and DMBA metabolites. Separation was achieved with a programmed 30-min linear gradient of methanol/water (1:1 to 19:1, v/v) at a solvent flow rate of 1.0 ml/min. Fractions (0.5-ml) were collected in scintillation vials at 0.5-min intervals and 7 ml of Scintisol (Isolab, Inc., Akron, OH) were added to each vial. Organic-soluble metabolism was determined by the radioactivity in each fraction measured in a Beckman LS-500C liquid scintillation counter. Water-soluble metabolism was quantified by determining the radioactivity which remained in the aqueous phase after rigorous ethyl acetate extraction. Metabolites from the batch cultures were separated as described above and collected after repeated injections into the HPLC system. Metabolites were pooled and concentrated in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, NY), and identified by their UV-visible, mass and NMR spectra.

Physical and chemical analyses. The UV-visible absorbance spectra of the metabolites were determined in methanol on a Beckman model 25 recording spectrophotometer. Circular dichroism (CD) spectra were determined in methanol at room temperature in a glass cuvette of 1-cm path length on a Jasco 500A spectropolarimeter (Japan Spectroscopic Co., Ltd, Tokyo, Japan). CD spectra are expressed as ellipticity ($\theta\ \lambda$ in millidegrees) for methanol solutions that read 1.0 absorbance in a UV-visible spectrophotometer at the wavelength of maximum absorbance.

Mass spectral analyses of the metabolites were performed with a Finnigan model 4023 mass spectrometer (Finnigan-MAT, San Jose, CA) operated at 70 eV. Samples were dissolved in methanol and

placed in glass sample cups. Spectra were recorded as the probe temperature was increased ballistically from 30 to 300°C. The ion source temperature was 270°C. The mass spectrometer scan range was m/z 50–750 with a 2-s scan time. NMR spectra were obtained on a Bruker WM 500 spectrometer (Bruker Instruments, Inc., Billerica, MA) in acetone-d_6. The data were acquired under the following conditions: data size, 32000; sweep width, 7042 Hz; temperature, 305 K; flip angle, 68°. The proton shifts are reported in parts per million (ppm) downfield from the internal standard tetramethylsilane. Proton assignments were determined by homonuclear decoupling, nuclear Overhauser enhancement (NOE) difference spectra, and consideration of substituent effects.

Direct resolution of the optically active dihydrodiol metabolites was achieved with a Pirkle I-A chiral stationary-phase HPLC column (4.6 × 250 mm) (Regis Chemical Co., Morton Grove, IL) packed with (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine ionically or covalently bonded to spherical particles (5 μm diameter) of γ-aminopropylsilanized silica. The HPLC system consisted of a Waters Associates (Millipore Corp., Milford, MA) model 600A liquid chromatograph, and a Tracor (Tracor Instruments, Austin, TX) 970A detector adjusted to 254 nm. Metabolites were eluted isocratically with a mixture of 15% ethanol/acetonitrile (2:1, v/v) in hexane at a flow rate of 2 ml/min. Resolved enantiomers were characterized by their UV-visible absorbance spectra on a Hewlett-Packard (Hewlett-Packard, Sunnyvale, CA) 1040A spectrophotometric detector, and the percentage of the *S,S* and *R,R* enantiomers was determined by measurement of peak height.

Deconjugation experiments. After the flask contents from the *S. racemosum* incubation had been extracted with 6 volumes of ethyl acetate, the aqueous phase was divided into three 7-ml portions, and diluted with 0.2 M sodium acetate buffer (1:1, v/v; pH 4.5). Deconjugation enzymes β-glucuronidase (type H-1, Sigma Chemical Co., St. Louis, MO) and arylsulfatase (type V, Sigma) were used to determine the presence of glucuronide or sulfate conjugates of DMBA as previously described [4]. After 24 h of incubation, samples were extracted with 4 volumes of ethyl acetate, dried under reduced pressure, and analyzed by HPLC as described above for the separation and fraction collection of radiolabeled compounds.

Kinetics of DMBA metabolism and detoxification. DMBA (1 mg) and 0.5 μCi [^{14}C]DMBA were administered to flasks containing cells from *S. racemosum* and incubated as described above. At the designated time intervals, the contents of one flask were extracted and analyzed by HPLC to quantify metabolism as described above. The mutagenic activity of each extract (50 μg) was determined according to the revised method of Maron and Ames [20] using *Salmonella typhimurium* tester strain TA98, both in the presence and absence of hepatic post-mitochondrial supernatant (S9) prepared from Aroclor 1254-pretreated male Sprague-Dawley rats. All assays were performed in triplicate, and the mean number of His$^+$ revertants was determined for each time point.

Chemicals. DMBA was purchased from Fluka Chemical Corp., Haupauge, NY. Radiolabeled [^{14}C]DMBA (97.4 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Both compounds were determined to be >99% pure by HPLC and MS. 7-Hydroxymethyl-12-methylbenz-[*a*]anthracene (7-OHM-12-MBA), 12-OHM-7-MBA, and 7,12-diOHMBA standards were synthesized according to previously published methods [16]. HPLC solvents were purchased from Fisher Chemical Co., Pittsburgh, PA, and all other chemicals used were of reagent grade or the highest available purity.

RESULTS

Identification of DMBA metabolites

Fig. 2 shows the reversed-phase HPLC elution profiles from ethyl acetate-soluble extracts of spent medium from *S. racemosum* and *C. elegans* cultures grown in the presence of DMBA for 5 days. The major metabolites produced by *S. racemosum* (Fig. 2A) had HPLC retention times of 29.0 and 18.5 min, which were identical to those of 7-OHM-12-

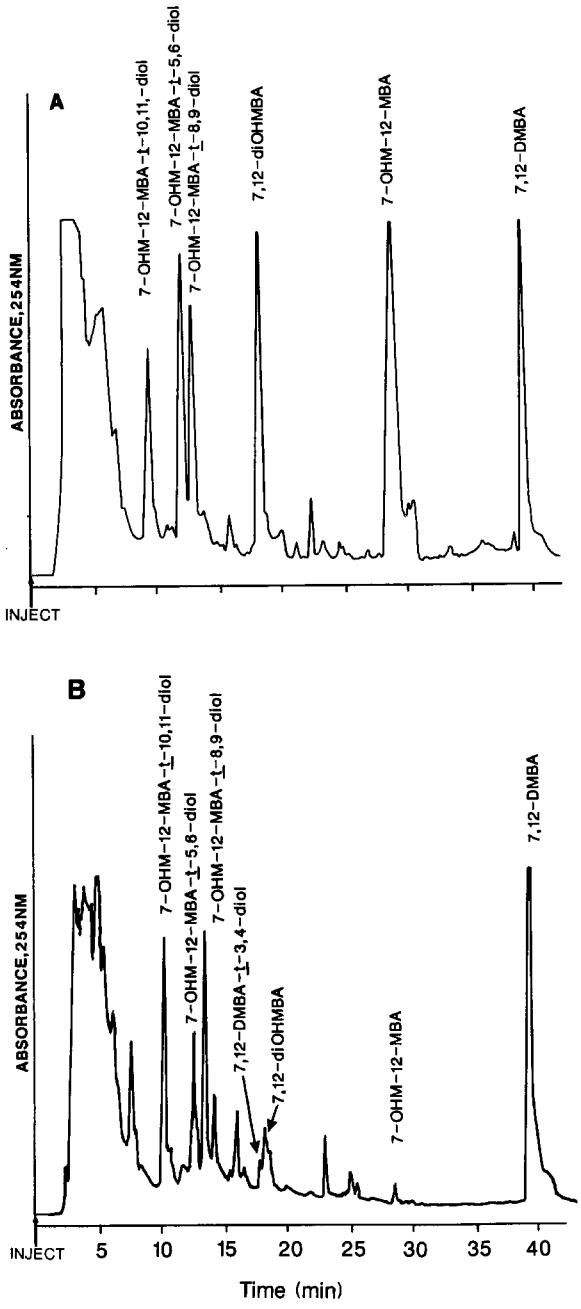

Fig. 2. HPLC elution profile of DMBA metabolites isolated from incubations of (A) *S. racemosum* strain UT-70 and (B) *C. elegans* ATCC 36112.

MBA and 7,12-diOH-MBA standards, respectively. UV-visible absorbance spectra of these metabolites (Table 1) revealed that the DMBA chromophore had been retained, which further indicated that metabolism had occurred at the methyl groups. Mass spectral analysis of the metabolite with the retention time of 29.0 min (Table 1) gave a molecular ion (M^+) at *m/z* 272 and fragment ions at *m/z* 255 ($M^+ - 17$, OH loss) and *m/z* 243 ($M^+ - 29$), which indicated the presence of a monohydroxy-derivative of DMBA. 500 MHz ^{1}H-NMR spectral analysis of this compound revealed that hydroxylation occurred at the 7-position methyl moiety. Comparison of these data with DMBA indicates that the structure of this metabolite is consistent with 7-OHM-12-MBA (Table 2).

Mass spectral analysis of the metabolite which eluted at 18.5 min gave an M^+ at *m/z* 288 and fragment ions at *m/z* 252, *m/z* 241 and *m/z* 239, which indicated that this compound was a dihydroxylated derivative of DMBA. Analysis of the 500 MHz ^{1}H-NMR spectral data of the dihydroxylated metabolite revealed that hydroxylation had occurred at the 7- and 12-position methyl moieties. Comparison of the methylene proton resonances of this metabolite with DMBA confirmed the identification of this metabolite as 7,12-diOHMBA (Table 2).

The major ethyl acetate-soluble metabolites produced by *C. elegans* eluted at 9.5, 12.5 and 13.0 min (Fig. 2B). These metabolites were also isolated as minor components from the spent medium after *S. racemosum* incubations (Fig. 2A). UV-visible absorbance spectral analysis of these metabolites revealed a loss of the DMBA chromophore, which indicated that metabolism had occurred at the aromatic double bonds. Further inspection of the UV-visible absorbance spectra of the above metabolites indicated that they were similar to previously published spectra of DMBA-*trans*-10,11-, 5,6-, and 8,9-dihydrodiols, respectively [11].

Mass spectral analysis revealed that each compound gave an M^+ at *m/z* 306 and fragment ions at *m/z* 288 ($M^+ - 18$, H_2O loss) and *m/z* 259 ($M^+ - 47$, H_2O and HCO loss). The mass spectral fragmentation pattern indicated that these metabolites were hydroxymethyl dihydrodiol derivatives of DMBA.

The 500 MHz ^{1}H-NMR spectral data for the

Table 1

HPLC retention times, UV-visible absorption and mass spectral properties of DMBA metabolites formed from DMBA by *S. racemosum* and *C. elegans*

HPLC retention time (min)	UV-visible absorbance maxima (nm)	Mass spectra *m/z* (%)	Assignment
9.5	375,303,275,268,225,215	306(M^+,90)288(28)275(74) 270(30)260(81)259(85)257(28) 242(40)229(53)215(100)202(73) 189(41)113(59)107(63)101(26)	7-OHM-12-MBA-*trans*-10,11-dihydrodiol
12.5	307,285,268,256,246,215	306(M^+,49)288(20)270(57) 259(100)242(87)228(47)215(82) 202(33)189(10)107(51)101(21)	7-OHM-12-MBA-*trans*-5,6-dihydrodiol
13.0	370,355,335,320,275,264,215	306(M^+,42)288(14)273(15)259(100) 245(36)242(47)226(22)215(54) 202(36)189(18)119(30)113(17) 107(20)101(18)	7-OHM-12-MBA-*trans*-8,9-dihydrodiol
17.9	425,401,270	290(M^+,100)272(20)257(43) 244(30)243(58)229(78) 228(37)215(28)	DMBA-*trans*-3,4-dihydrodiol
18.5	373,356,340,325,290,280,271,262,235,215	288(M^+,65)269(5)252(7)241(100) 239(34)228(36)226(23)215(9) 202(5)119(36)	7,12-diOHMBA
29.0	373,356,340,325,290,282,273,233,215	272(M^+,100)255(29)243(43)239(39) 229(31)228(86)226(18)202(5) 119(36)	7-OHM-12-MBA
39.5	375,357,341,290,260,230,215	256(M^+,100)241(37)239(30)226(5) 215(3)202(2)126(8)119(24) 113(8)106(4)	DMBA

metabolite peak eluting at 9.5 min were consistent with the compound being 7-OHM-12-MBA-*trans*-10,11-dihydrodiol. NOE enhancements were observed for H_1 (8.65 ppm) and H_{11} (5.21 ppm) upon irradiation of the three-proton resonance at 3.13 ppm (12-position, CH_3). In addition, the coupling constant between the carbinol protons ($J_{10,11}$) of the *trans*-10,11-dihydrodiol is small (2.6 Hz). Similarly, the coupling constant between the non-benzylic olefinic and non-benzylic carbinol proton, H_{10} ($J_{9,10}$) is small (5.6 Hz). These data indicate that this dihydrodiol is in the *trans* configuration with both hydroxy groups preferentially in the quasidiaxial positions [31].

The NMR spectral assignments shown in Table 2 for the compounds which eluted at 12.5 and 13.0 min were also based on NOE difference spectra, homonuclear decoupling, and comparison to DMBA, and are consistent with 7-OHM-12-MBA-5,6-dihydrodiol and 7-OHM-12-MBA-8,9-dihydrodiol, respectively. For the K-region 5,6-dihydrodiol, the small coupling constant between the carbinol protons ($J_{5,6}$ = 3.4 Hz) is indicative of a *trans*-dihydrodiol with its hydroxy groups preferentially in the quasidiaxial conformation. This confirmation avoids steric hindrance from the 7-OHM group [15]. Similarly, for the 8,9-dihydrodiol, the coupling constant between the carbinol protons ($J_{8,9}$) with a small value of 2.6 Hz also indicates that it is a *trans*-dihydrodiol with its hydroxyl groups preferentially in the quasidiaxial positions [31].

Table 2

500 MHz ^{1}H-NMR spectral parameters of DMBA metabolites isolated from extracts of *S. racemosum* and *C. elegans*

Compound	Ring system proton assignments (ppm)[a]										Acyclic proton assignments (ppm)	
	H_1	H_2	H_3	H_4	H_5	H_6	H_8	H_9	H_{10}	H_{11}	7-CH_2OH	12-CH_3
7-OHM-12-MBA-*trans*-10,11-diol[b]	8.65	7.60	7.60	7.96	7.80	8.24	7.29	6.30	4.37	5.21	5.19	3.13
7-OHM-12-MBA-*trans*-5,6-diol[c]	8.15	7.50–7.57	7.29	7.45	4.83	5.36	8.34	7.50–7.57	7.40	7.62	5.17 5.33	2.90
7-OHM-12-MBA-*trans*-8,9-diol[d]	8.59	7.57–7.62	7.57–7.62	7.96	7.78	8.24	5.30	4.38	6.33	7.19	5.35	3.60
7,12-diOHMBA[e]	9.23	7.63–7.71	7.63–7.71	7.93	7.71	8.29	8.60	7.63–7.71	7.63–7.71	8.75	5.61	5.54[f]
7-OHM-12-MBA[g]	8.56	7.65–7.71	7.65–7.71	7.92	7.69	8.27	8.59	7.65–7.71	7.65–7.71	8.44	5.59	3.39
DMBA[h]	8.54	7.63–7.70	7.63–7.71	7.90	7.66	8.11	8.41	7.59–7.63	7.59–7.63	8.42	3.07[i]	3.35

[a] In acetone-d_6 downfield from tetramethylilane (0.00 ppm).
[b] The coupling constants are: $J_{5,6}$ = 9.0, $J_{8,9}$ = 9.9, $J_{9,10}$ = 5.6 and $J_{10,11}$ = 2.6 Hz.
[c] The coupling constants are: $J_{1,2}$ = 7.7, $J_{3,4}$ = 7.7, $J_{5,6}$ = 3.4, $J_{8,9}$ = 9.4, $J_{10,11}$ = 7.7, $J_{7\text{-}CH_2,gem}$ = 12.9 Hz.
[d] The coupling constants are: $J_{5,6}$ = 9.5, $J_{8,9}$ = 2.6, $J_{9,10}$ = 5.6 and $J_{10,11}$ = 9.9 Hz.
[e] The coupling constants are: $J_{1,2}$ = 8.2, $J_{3,4}$ = 6.9, $J_{5,6}$ = 9.5, $J_{8,9}$ = 8.2 and $J_{10,11}$ = 8.2 Hz.
[f] The 12-position acyclic resonance integrates for two protons (CH_2OH moiety).
[g] The only observable first-order coupling was $J_{5,6}$ = 9.5 Hz.
[h] The only observable first-order coupling was $J_{5,6}$ = 9.0 Hz.
[i] The 7-position acyclic resonance integrates for three protons (CH_3 moiety).

The HPLC elution profile of *C. elegans* DMBA metabolites (Fig. 2B) also shows a small peak which eluted at 17.9 min. Since this metabolite coeluted with 7,12-diOHMBA, a definitive identification could not be made. However, the UV-visible absorbance spectrum of this compound with a maximum at 270 nm is characteristic of a 3,4-diol derivative [10]. Mass spectral analysis of this compound showed an M^+ at *m/z* 290 and fragment ions at *m/z* 272 ($M^+ - 18$, H_2O loss) and *m/z* 243 ($M^+ - 47$, H_2O and HCO loss) (Table 1). Thus the results obtained from UV and mass spectral analyses suggested that this compound is DMBA-*trans*-3,4-dihydrodiol.

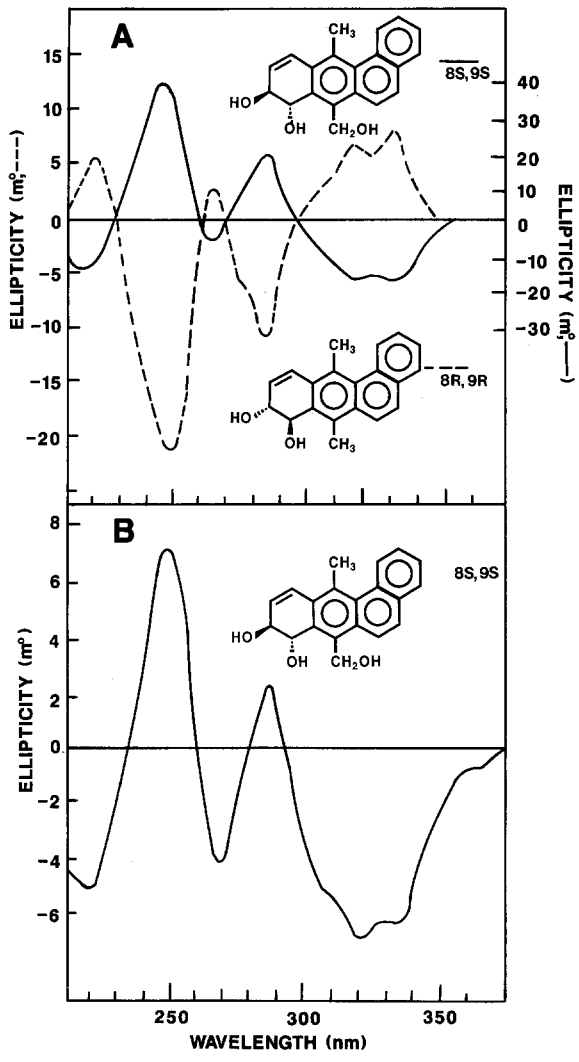

Fig. 3. CD spectra of (A) 7-OHM-12-MBA-*trans*-8,9-dihydrodiol from *C. elegans* (———) and DMBA-8*R*,9*R*-dihydrodiol from rat liver microsomes (– – – –), and (B) 7-OHM-12-MBA-*trans*-8,9-dihydrodiol from *S. racemosum*.

Absolute stereochemistry and enantiomeric purity of DMBA-dihydrodiol metabolites

CD spectral analysis and enantiomeric resolution were performed on the dihydrodiol metabolites produced by *S. racemosum* and *C. elegans* to compare the absolute configuration and enantiomeric purity of these compounds with previously characterized mammalian DMBA-dihydrodiols [30]. Fig. 3 compares the CD spectra of 7-OHM-12-MBA-*trans*-8,9-dihydrodiol from *S. racemosum* and *C. elegans* with the DMBA-8*R*,9*R*-dihydrodiol obtained from the incubation of DMBA with liver microsomes of rats pretreated with 3-methylcholanthrene [30]. The CD spectra of the fungal 8,9-dihydrodiols shown in Fig. 3 are mirror images of the DMBA-8*R*,9*R*-dihydrodiol spectrum, which indicates that the major enantiomers isolated from *S. racemosum* and *C. elegans* possess an 8*S*,9*S* absolute configuration.

Partial resolution of the *trans*-8,9-dihydrodiol enantiomers from *S. racemosum* was achieved using a covalently bonded chiral column, and the HPLC elution profile is shown in Fig. 4A. The UV-visible absorbance spectra of both enantiomers were identical to 7-OHM-12-MBA-*trans*-8,9-dihydrodiol (Fig. 4B). Further analysis of the HPLC elution profile indicated that the first peak was slightly larger than the second one. Since CD spectral analysis indicated that the 8*S*,9*S*-dihydrodiol was the predominant enantiomer, the peaks were assigned as the 8*S*,9*S* and 8*R*,9*R* enantiomers, respectively, in order of elution. In addition, the elution order of these two enantiomers is consistent with the observation that DMBA-8*S*,9*S*-dihydrodiol has a shorter retention time than DMBA-8*R*,9*R*-dihydrodiol under identical chromatographic conditions [30]. Since baseline resolution of the 8,9-dihyrodiol enantiomers isolated from *S. racemosum* incubations could not be obtained, the percentage of the 8*S*,9*S* and 8*R*,9*R* enantiomers was based on comparison of peak height rather than

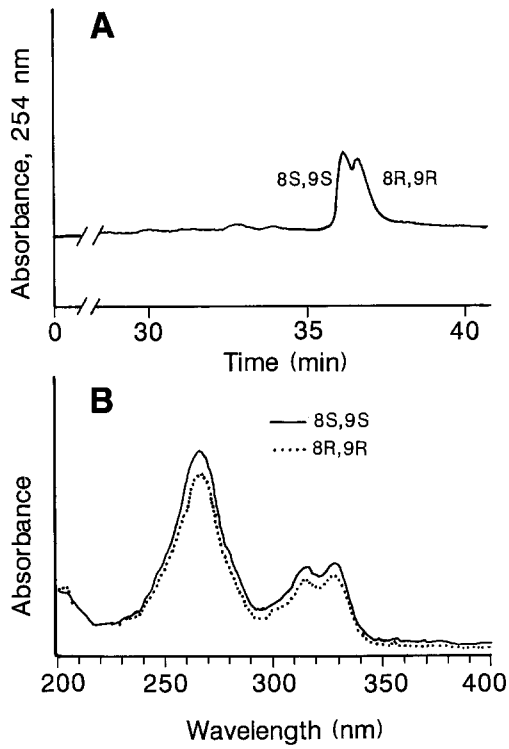

Fig. 4. HPLC elution profile of (A) 7-OHM-12-MBA-*trans*-8,9-dihydrodiol enantiomers from *S. racemosum* and (B) UV-visible absorption spectra of the resolved enantiomers.

integration and was estimated to be 55 and 45%. The enantiomeric purity of the 8,9-dihydrodiol from *C. elegans* was then calculated using the CD spectral data from *S. racemosum* (Fig. 3B). The percentage of 8*S*,9*S* and 8*R*,9*R* enantiomers isolated from *C. elegans* was estimated to be about 75 and 25%, respectively, and the enantiomeric purity about 50%.

The absolute configurations of the 7-OHM-12-MBA-*trans*-5,6-dihydrodiols isolated from *S. racemosum* and *C. elegans* were also determined. CD spectral analysis of the 5,6-dihydrodiols formed from DMBA by both organisms indicated that they are mirror images of the 5*S*,6*S*-dihydrodiol from rat liver microsomes (Fig. 5). Therefore, the major enantiomers from both organisms had an *R,R* absolute configuration. Direct resolution of the 7-OHM-12-MBA-*trans*-5,6-dihydrodiols from *S. racemosum* and *C. elegans* indicated that the ratio of *S,S*:*R,R* was 12:88 and 23:77, respectively (Fig.

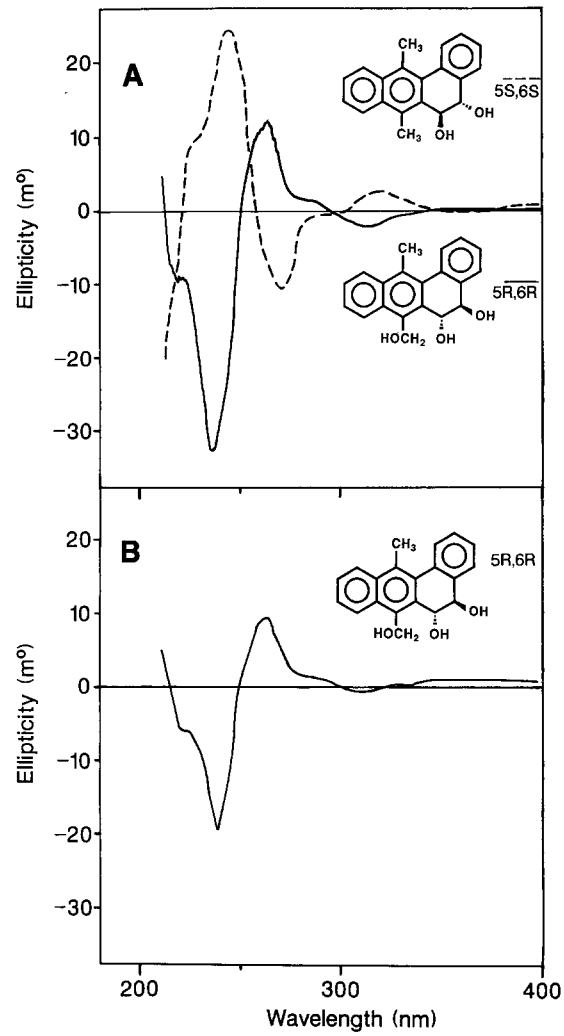

Fig. 5. CD spectra of (A) 7-OHM-12-MBA-*trans*-5,6-dihydrodiol from *S. racemosum* (———) and DMBA-5*S*,6*S*-dihydrodiol from rat liver microsomes (– – – –), and (B) 7-OHM-12-MBA-*trans*-5,6-dihydrodiol from *C. elegans*.

6A). Their UV-visible absorption spectra are identical to that of the original 7-OHM-12-MBA-*trans*-5,6-dihydrodiol (Fig. 6B). The enantiomeric purities of the 5,6-dihydrodiols from *S. racemosum* and *C. elegans* were 76% and 54%, respectively.

Both 7-OHM-12-MBA-*trans*-10,11- and 3,4-dihydrodiols gave weak CD spectral Cotton effects. Their CD spectra were similar to that of DMBA-10*R*,11*R*-dihydrodiol and DMBA-3*R*,4*R*-dihydrodiol, respectively (data not shown), which suggested that the predominant enantiomers of both metabolites had an *R,R* absolute configuration. Attempts

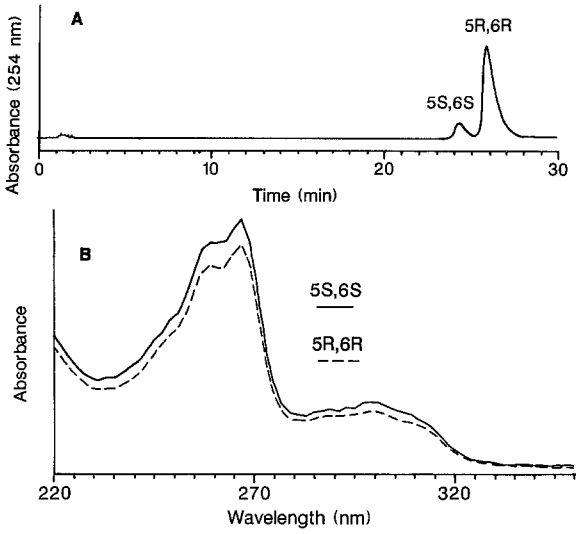

Fig. 6. HPLC elution profile of (A) 7-OHM-12-MBA-*trans*-5,6-dihydrodiol enantiomers from *S. racemosum* and (B) UV-visible absorption spectra of the resolved enantiomers.

at direct resolution of the 10,11-dihydrodiol enantiomers were not successful. Based on the magnitude of the Cotton effects observed in the CD spectra, the optical purity of the 10,11-dihydrodiol from *C. elegans* is slightly higher than that from *S. racemosum*. Direct resolution of the DMBA-3,4-dihydrodiol isolated from *C. elegans* was not achieved.

Detection of water-soluble glucuronide and sulfate conjugates of DMBA

After the contents of the flask containing *S. racemosum* cells exposed to [^{14}C]DMBA for 5 days had been filtered and extracted with ethyl acetate, the aqueous phase was divided into three fractions and analyzed for the presence of glucuronide and sulfate conjugates as described in 'Materials and Methods'. Fig. 7 shows the HPLC radioactivity histograms of extracts of the aqueous fractions after treatment with β-glucuronidase (Fig. 7A), arylsulfatase (Fig. 7B), or no enzyme (Fig. 7C). Both of the enzyme-treated fractions showed the liberation of oxidized derivatives of DMBA. These data indicate that the derivatives liberated by deconjugation enzymes are probably phenolic metabolites of 7-OHM-12-MBA and/or DMBA. In addition, quantitation of the radioactivity which appeared in enzyme-treated fractions revealed that the ratio of glucuronide to sulfate conjugates was approximately 1:1.

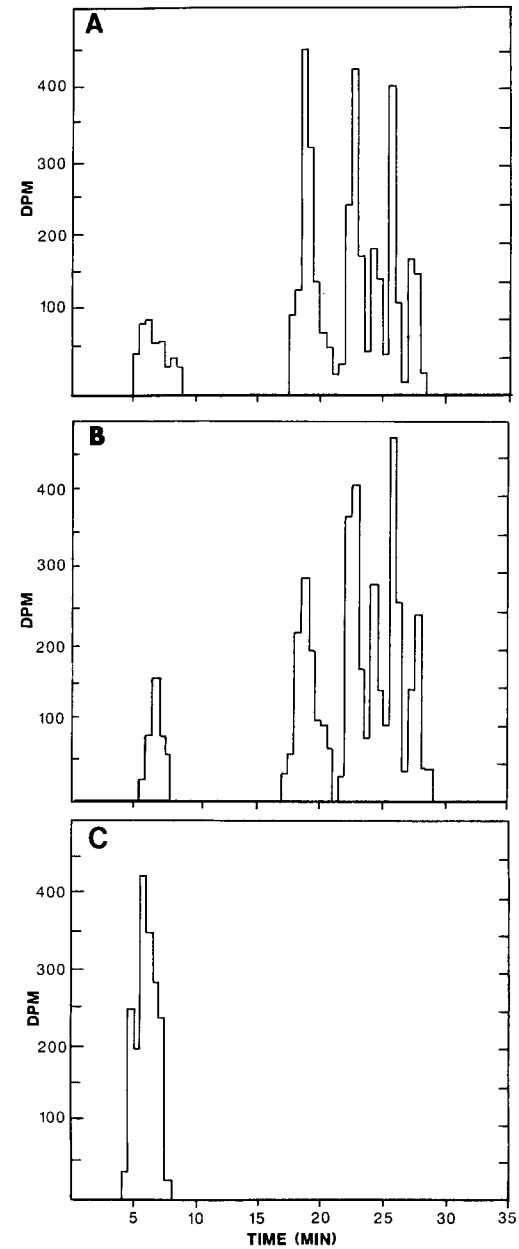

Fig. 7. HPLC elution profiles of extracts of the aqueous fractions from *S. racemosum* cultures containing [^{14}C]DMBA after treatment with (A) β-glucuronidase, (B) arylsulfatase, or (C) no enzyme.

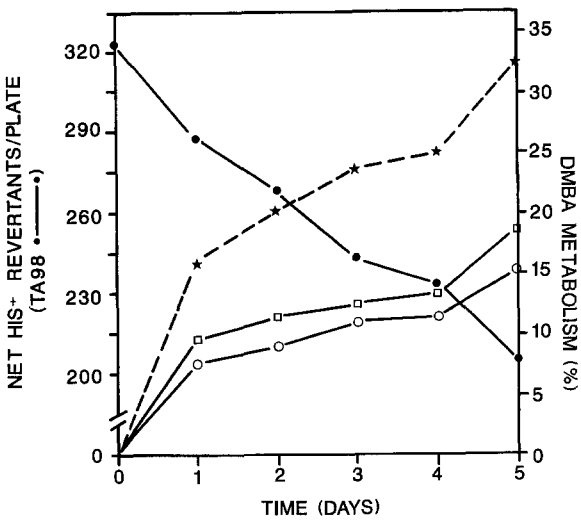

Fig. 8. Mutagenicity of extracts from cultures of *S. racemosum* exposed to DMBA for various time intervals in *Salm. typhimurium* TA98 with metabolic activation. Percent metabolism is also indicated. Mean net His^+ revertants/plate (●——●); % total metabolism (★- - -★); % organic-soluble metabolites (□——□); % aqueous-soluble metabolites (○——○).

Time course of DMBA metabolism and detoxification by S. racemosum

The kinetics of [^{14}C]DMBA metabolism by *S. racemosum* was examined along with the time-dependent loss of mutagenic activity of ethyl acetate extracts in the *Salm. typhimurium* reversion assay. Fig. 8 shows the time course of DMBA metabolism and the corresponding mutagenicity of the extracts in tester strain TA98 with metabolic activation. As the parent compound was metabolized, a corresponding decrease in the number of His^+ revertants was observed. No mutagenic activity was observed in cultures without metabolic activation. These data indicate that *S. racemosum* metabolizes DMBA to detoxified derivatives.

Screening experiment

Four other strains of fungi as well as *C. elegans* and *S. racemosum* strains were screened for their ability to metabolize DMBA, and the amount of organic-soluble and aqueous-soluble metabolites produced by the different organisms is shown in Table 3. These results indicate that both *C. elegans* strains and *S. racemosum* exhibited the highest total metabolic activity among the organisms surveyed. With the exception of *C. elegans* ATCC 36112, the ratio of organic-soluble metabolites to water-soluble metabolites was generally greater than 2:1.

There are also qualitative similarities and differences in the major organic-soluble metabolites produced by each organism (Table 3). Metabolites produced by *S. racemosum* and *C. elegans* ATCC 36112 were identified directly, and their HPLC retention times were used as standards for the tentative identification of the major metabolites formed by the other organisms (Table 3). The HPLC retention times of the major metabolites produced by *Mucor* sp. and *P. chrysogenum* were similar to retention times of phenols extracted from deconjugation enzyme-treated *S. racemosum* incubations.

DISCUSSION

The aims of this study were to establish whether fungi have the ability to metabolize the most potent aryl hydrocarbon carcinogen, DMBA, to detoxified products and to determine whether the fungal monooxygenase(s) has a stereoselective preference similar to that shown by the reactions catalyzed by rat liver microsomes. In this investigation, we showed that six fungal strains have the ability to metabolize DMBA, presumably via a cytochrome *P*-450 monooxygenase(s), at various ring carbons as well as on the methyl groups.

S. racemosum metabolized DMBA primarily at the methyl groups to form 7-OHM-12-MBA and 7,12-diOHMBA. Further metabolism of 7-OHM-12-MBA by *S. racemosum* and *C. elegans* resulted in the formation of optically active hydroxymethyl-*trans*-5,6-, 8,9- and 10,11-dihydrodiols. Isolation of a K-region (5,6-positions) dihydrodiol from both organisms was unexpected because the absence of metabolism at the K-region of PAHs by fungi was previously thought to be a general phenomenon [3,5,6,9]. In addition, Wong and coworkers [27] reported that one of the major DMBA metabolites produced by *C. elegans* ATCC 9245 was the 3,4-dihydrodiol, which is a bioactivated

Table 3

Fungal survey of [^{14}C] DMBA metabolism

Organism	% Metabolism[a]			Major organic-soluble metabolites[b] (≈%)
	organic-soluble metabolites	DMBA	aqueous-soluble metabolites	
Candida lipolytica 37-1	8.5	65.0	1.0	7-OHM-12-MBA (60) 7,12-diOHMBA (30)
Cunninghamella elegans ATCC 36112	28.0	0.7	33.0	7-OHM-12-MBA-*trans*-8,9-diol (25) 7-OHM-12-MBA-*trans*-10,11-diol (30)
Cunninghamella elegans ATCC 9245	24.0	4.6	11.0	7-OHM-12-MBA-*trans*-8,9-diol (15) 7-OHM-12-MBA-*trans*-10,11-diol (25)
Mucor sp.	11.0	0.4	2.0	DMBA phenol (100)
Penicillium chrysogenum	6.0	2.6	1.6	DMBA phenol (100)
Syncephalastrum racemosum UT-70	25.0	1.8	6.1	7-OHM-12-MBA (45) 7,12-diOHMBA (35)

[a] Quantitation of DMBA metabolism was determined as described in 'Materials and Methods'. The amount of radioactivity remaining with cells varied among organisms and ranged from 25.5 to 89.0%.

[b] Identification of metabolites determined by comparing the HPLC retention time of metabolites identified from *S. racemosum* and *C. elegans* ATCC 36112 experiments.

form of DMBA [10]. The 3,4-dihydrodiol was also the major 7-MBA metabolite isolated from *C. elegans* ATCC 36112 incubations [5,14]. However, DMBA-*trans*-3,4-dihydrodiol was only a minor metabolite of *C. elegans* ATCC 36112, and was not detected in spent medium which contained *S. racemosum* which had been grown in the presence of DMBA. Possible explanations for the observed differences in metabolism of DMBA and 7-MBA could be steric hindrance exerted by the 12-methyl group or instability of the DMBA-*trans*-3,4-dihydrodiol and DMBA-3,4-oxide which could dehydrate or isomerize to the phenol with subsequent conjugation to water-soluble metabolites.

CD spectral analysis and enantiomeric resolution of the fungal hydroxymethyldihydrodiols revealed both similarities and differences in the stereoselective metabolism of DMBA by fungal and mammalian cytochrome *P*-450 monooxygenases. The absolute configuration of the major fungal 8*S*,9*S*- and 5*R*,6*R*-dihydrodiol enantiomers was opposite to that of the corresponding mammalian DMBA metabolites. In contrast, the enantiomers of the 10*R*,11*R*- and 3*R*,4*R*-dihydrodiols isolated from fungal incubations showed the same absolute configuration as the corresponding mammalian metabolites. Interestingly, the absolute configuration of the 10,11-dihydrodiols isolated from fungal incubations with DMBA was opposite to that of previously characterized 10,11-dihydrodiols from fungal incubations with 4-MBA [6]; this may be due to the steric effect of the 12-methyl group. Direct resolution of DMBA dihydrodiol enantiomers isolated from rat liver microsomes has shown that the process is highly stereoselective [30]. For example, the enantiomeric purity of the 5*S*,6*S*-dihydrodiol from liver microsomes of rats treated with 3-methylcholanthrene was >95%. Enantiomeric resolution of dihydrodiol enantiomers from *S. racemosum* and *C. elegans* indicated that metabolism of PAHs by fungi may not be as highly stereoselective as mammalian metabolism of PAHs. With the exception of the 5,6-dihydrodiol, the optical purity of the dihydrodiols isolated from *C. elegans* was generally higher than that of *S. racemosum*.

Enzymatic formation of hydroxymethyl *trans*-dihydrodiols involves three transformations: (1) oxidation of the methyl group, (2) epoxidation of an aromatic double bond to form an epoxide, and (3) enzymatic hydration of the epoxide to form the *trans*-dihydrodiol [6]. Steps 2 and 3 could be highly stereoselective and the fungal cytochrome *P*-450 monooxygenase and/or epoxide hydrolase could have opposite stereoselective preferences during *trans*-dihydrodiol formation to those of the enzymes in rat liver microsomes.

Metabolism of DMBA by *S. racemosum* resulted in detoxification of the parent compound, as measured in the *S. typhimurium* reversion assay. These results are consistent with those observed from previous experiments with carcinogenic PAHs metabolized by *C. elegans* [8]. Although *C. elegans* produced the proximate carcinogen DMBA-3*R*,4*R*-dihydrodiol, the amount produced was relatively low compared to the amount of detoxified metabolites. Watabe et al. [23] have shown 7-OHM-12-MBA sulfate to be a potent mutagen in *Salm. typhimurium* TA98 in the absence of hepatic soluble supernatant and a phosphoadenosine phosphosulfate-generating system. Formation of hepatic DNA and RNA adducts from 7-OHM-12-MBA has also been reported recently [22]. Although 7-OHM-12-MBA is a major metabolite of *S. racemosum*, deconjugation experiments with arylsulfatase indicated that sulfate conjugation occurs on phenolic derivatives rather than at the 7-OHM group, which results in the production of detoxified rather than bioactivated metabolites.

If microorganisms are to be employed in the detoxification of carcinogenic PAHs in the environment, further research is necessary to examine the mechanisms by which bacteria and fungi stereoselectively metabolize PAHs. Studies such as these are particularly important because the genotoxicity of PAHs is highly dependent upon the enantiomeric purity of the dihydrodiol metabolites.

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