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Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi

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Three filamentous fungi were examined for the ability to biotransform phenanthrene to oxidative (phase I) and conjugative (phase II) metabolites. Phenanthrene metabolites were purified by high-performance liquid chromatography (HPLC) and identified by UV/visible absorption, mass, and ^{1}H NMR spectra. *Aspergillus niger* ATCC 6275, *Syncephalastrum racemosum* UT-70, and *Cunninghamella elegans* ATCC 9245 initially transformed [9-^{14}C]phenanthrene to produce metabolites at the 9,10-, 1,2-, and 3,4- positions. Subsequently, sulfate conjugates of phase I metabolites were formed by *A. niger, S. racemosum*, and *C. elegans*. Minor glucuronide conjugates of 9-phenanthrol and phenanthrene *trans*-9,10-dihydrodiol were formed by *S. racemosum* and *A. niger*, respectively. In addition, *C. elegans* produced the glucose conjugates 1-phenanthryl β-D-glucopyranoside and 2-hydroxy-1-phenanthryl β-D-glucopyranoside, a novel metabolite. [9-^{14}C]Phenanthrene metabolites were not detected in organic extracts from biotransformation experiments with the yeasts, *Candida lipolytica* 37-1, *Candida tropicalis* ATCC 32113, and *Candida maltosa* R-42.

Keywords: phenanthrene; fungi; degradation; conjugates; metabolism

Introduction

Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) found in aquatic and terrestrial oil-contaminated sediments [12, 16]. Although many PAHs can be bioactivated to form electrophilic compounds, which produce tumorigenic and toxic effects [20, 22], phenanthrene is considered noncarcinogenic [18] and nonmutagenic [10, 25]. Phenanthrene, which has a fused-ring structure resembling higher molecular weight carcinogenic PAHs, has received interest as a useful model PAH for mammalian [15, 18, 30] and fungal [14, 24, 32, 33] studies of metabolism.

Filamentous fungi and yeasts have the ability to oxidize PAHs by oxidative (phase I) mechanisms which are similar to mammalian systems [5, 14, 31, 36]. However, the enantiomeric composition of fungal *trans*-dihydrodiols indicates differences in enantiomeric selectivity between fungal and mammalian enzymes [9, 13, 26, 32]. The fungal transformation of phenanthrene to *trans*-dihydrodiols initially involves a cytochrome P-450 monooxygenase-catalyzed ring epoxidation of phenanthrene to form an unstable arene oxide [14]. Epoxide hydrolase, which has been found in *Cunninghamella elegans* [35], catalyzes hydration of the arene oxide to form a *trans*-dihydrodiol [14]. Phenanthrols are expected to form by the nonenzymatic rearrangement of unstable arene oxides [3, 17].

Although most studies involving biotransformation by fungi have emphasized phase I metabolism of PAHs, fungi are able to form sulfate, glucuronide, glucoside, and xyloside conjugates of PAH phenols and *trans*-dihydrodiols by enzymatic reaction mechanisms that are similar to mammalian phase II mechanisms [32]. Wackett and Gibson [35] also reported the presence of glutathione *S*-transferase (GST) activity in cell extracts of *Cunninghamella elegans*, but the physiological role of GST in fungi is not known [23,34].

Previous studies of phenanthrene metabolism by fungi have shown that the major phenanthrene metabolites are *trans*-1,2-, 3,4-, and 9,10-dihydrodiols, 3-, 4- and 9-phenanthrols [9, 32, 33], and glucoside conjugates of 9- and 1-hydroxyphenanthrene [11, 33]. Although rabbits [2, 29], rats [2, 29], and marine animals [30] produce sulfate and glucuronide conjugates from phenanthrene, studies with fungi have not demonstrated the presence of sulfate or glucuronide conjugates. The present paper broadens the initial studies of the fungal transformation of phenanthrene to identify sulfate, glucuronide, and glucoside conjugates of primary oxidative metabolites.

Materials and methods

Chemicals

Phenanthrene and [9-^{14}C]phenanthrene (10.9 mCi mmol^{-1}; radiochemical purity, 99%) were purchased from Sigma Chemical Co, St Louis, MO, USA. All other chemicals were of reagent grade, HPLC-grade, or the highest purity commercially available.

Organisms and biotransformation conditions

All cultures were maintained on Sabouraud dextrose (Sab) agar (Difco Laboratories, Detroit, MI, USA) plates and stored at 4°C. The yeasts, *Candida lipolytica* 37-1, *Candida tropicalis* ATCC 32113, and *Candida maltosa* R-42, were obtained from the Georgia State University Department of Biology culture collection, Atlanta, Georgia. The filamen-

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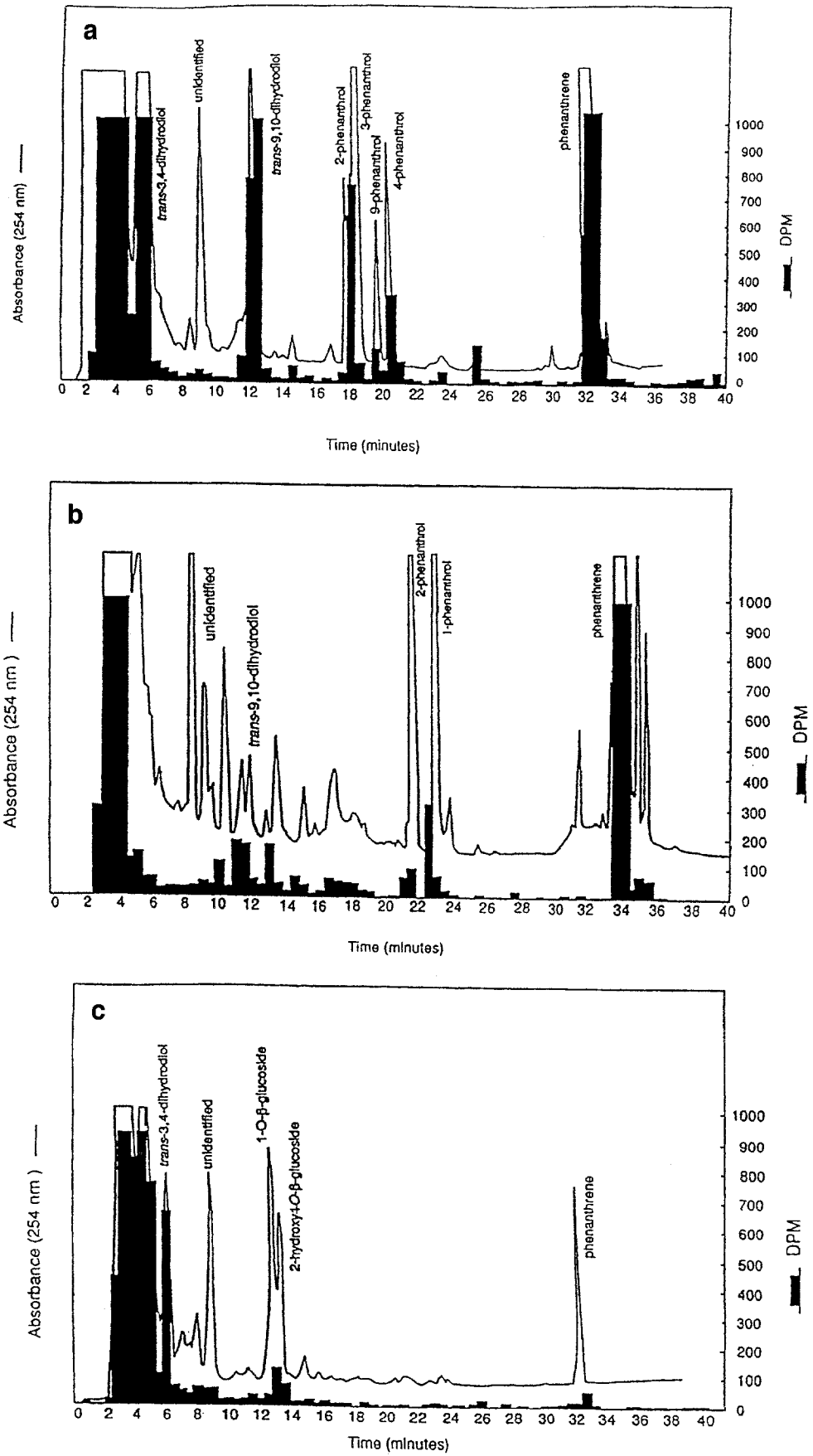

Figure 1 HPLC elution profile and radiochemical histogram of the ethyl acetate-extractable metabolites formed by incubation of [9-^{14}C]phenanthrene with (a) *S. racemosum* UT-70, (b) *A. niger* ATCC 6275, and (c) *C. elegans* ATCC 9245.

Table 1 Percentages of organic-soluble metabolites and unmetabolised parent compound after 192-h incubation with [9-^{14}C]phenanthrene

Organism	Organic soluble metabolites by region (HPLC retention time)				
	Polar region (2.0–5.5 min)	Dihydrodiol region (6.0–12.5 min)	Glucoside region (12.5–15.5 min)	Phenanthrol region (20–26 min)	Unmetabolized Phenanthrene (34 min)
A. niger ATCC 6275	28.6	2.3	0.37	1.9	66.4
C. elegans ATCC 9245	92.5	5.1	1.0	0.3	0.4
S. racemosum UT-70	41.5	10.8	0.17	2.4	44.6
Candida maltosa R-42	ND[a]	ND	ND	ND	98.3
Candida tropicalis ATCC 32113	ND	ND	ND	ND	97.7
Candida lipolytica 37-1	ND	ND	ND	ND	97.1

[a]ND – Phenanthrene metabolites were not detected from these organisms.

Table 2 Percentage total radioactivity and HPLC retention times for organic solvent-extractable phenanthrene dihydrodiols, phenols, and glucoside metabolites

Metabolite	*C. elegans* ATCC 9245	*S. racemosum* UT-70	*A. niger* ATCC 6275	HPLC retention time (min)
Phenanthrene-*trans*-3,4-dihydrodiol	3.6	14.0	–	6.0
Phenanthrene-*trans*-9,10-dihydrodiol	–	4.6	1.5	12.0
1-Phenanthryl β-D-glucopyranoside	0.4	–	–	12.5
2-Hydroxy-1-phenanthryl β-D-glucopyranoside	0.6	–	–	13.0
2-Phenanthrol	–	0.6	1.1	22.8
3-Phenanthrol	–	1.6	–	23.0
1-Phenanthrol	–	–	2.7	24.0
9-Phenanthrol	–	0.5	–	24.4
4-Phenanthrol	–	1.1	–	25.0

tous fungi, *Aspergillus niger* ATCC 6275, *Syncephalastrum racemosum* UT-70, and *Cunninghamella elegans* ATCC 9245 were obtained from the culture collection of the Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas.

Cells from the three *Candida* yeasts were taken from Sab agar plates and used to inoculate aseptically (t_0 $A_{594} = 0.1$) 125-ml Erlenmeyer flasks containing 30 ml of 0.067% yeast nitrogen base (YNB) (Difco Laboratories) supplemented with 0.45% $(NH_4)_2SO_4$ and 0.5% hexadecane (Aldrich Chemical Co, Milwaukee, WI, USA) as the sole carbon and energy source (HexYNB). Cultures were grown to late-log phase ($A_{594} = 1.0$) at 25°C on a rotary shaker (adapted cell culture). Biotransformations were performed by aseptically transferring 10 ml of the adapted HexYNB cell culture to sterile 125-ml Erlenmeyer flasks containing 30 ml fresh 0.5% HexYNB broth. Duplicate flasks were dosed with 0.14 mM nonradioactive phenanthrene dissolved in *N,N*-dimethylformamide (DMF) and 37 kBq per flask [9-^{14}C]phenanthrene dissolved in dimethylsulfoxide (DMSO) for experiments using the radioactive compound. Cultures were incubated at 25°C on a rotary shaker at 140 rpm. After 192 h of incubation, yeast cells were pelleted by centrifugation at 8000 × *g* for 10 min. Each supernatant fraction was collected and combined with the supernatant fraction from cell pellets washed with ethyl acetate. Combined supernatant fractions were then extracted with three equal volumes of ethyl acetate. Organic extracts were dried over anhydrous sodium sulfate, concentrated to dryness under reduced pressure at 40°C, and redissolved in methanol for analysis by HPLC.

Fungal spores and mycelia from *A. niger*, *S. racemosum*, and *C. elegans* cultured on Sab agar plates were used to inoculate duplicate 125-ml Erlenmeyer flasks containing 30 ml of Sab broth. After 120 h, cultures were dosed with 0.28 mM nonradioactive phenanthrene (in DMF) and 37 kBq per flask [9-^{14}C]phenanthrene (in DMSO) for experiments using the radioactive compound. Cultures were incubated at 25°C on a rotary shaker at 140 rpm. Control experiments were performed by incubating phenanthrene in the presence of autoclaved cultures (121°C for 40 min), and by incubating the cultures without phenanthrene. After 192 h of incubation, flask contents from filamentous fungal cultures were filtered through glass wool and rinsed with ethyl acetate. Culture filtrates were extracted with three

Table 3 UV absorption λ_{max} values and electron impact mass spectra of the metabolites produced from phenanthrene by *C. elegans*, *S. racemosum*, and *A. niger*

Metabolite	UV-visible absorption λ_{max} (nm)	Characteristic mass ion fragments with relative abundance, m/z (%)
Phenanthrene *trans*-3,4-dihydrodiol[a,b]	252, 261, 308	212 $M^{+\cdot}$(20), 194[M-H_2O]$^{+\cdot}$(75), 181[M-CH_2OH]$^+$(36), 166(49), 165[M-H_2O-CHO]$^+$(71), 153(58), 152[M-HOCHCHOH]$^{+\cdot}$(40), 140(28), 139(21), 115(21)
Phenanthrene *trans*-9,10-dihydrodiol[b,c]	271	212 $M^{+\cdot}$(75), 194[M-H_2O]$^{+\cdot}$(38), 181[M-CH_2OH]$^+$(73), 166(65), 165[M-H_2O-CHO]$^+$(100), 153(16), 152[M-HOCHCHOH]$^{+\cdot}$(28), 139(8), 115(8)
1-Phenanthryl β-D-glucopyranoside[a]	250, 287, 299, 334, 350	356 $M^{+\cdot}$ (0.6), 195(16), 194(100), 166(28), 165(65)
Acetylated derivative of 1-phenanthryl β-D-glucopyranoside[a]		524 $M^{+\cdot}$(0.6), 331(37), 194(23), 169(100), 165(22), 127(21), 109(61)
2-Hydroxy-1-phenanthryl β-D-glucopyranoside[a]	259, 289, 301, 334, 350	372 $M^{+\cdot}$(1.5), 211(15), 210(100), 194(2), 182(7), 181(14), 166(6), 165(6), 153(7), 152(11)
2-Phenanthrol[c]	255, 278, 290	195(8), 194 $M^{+\cdot}$(100), 166[M-CO]$^{+\cdot}$6(8), 165[M-CHO]$^+$(35), 139(5), 115(3), 97(11), 82(10)
3-Phenanthrol[b]	252, 277, 300, 339, 356	195(15), 194 $M^{+\cdot}$(100), 166[M-CO]$^{+\cdot}$(10), 165[M-CHO]$^+$(44), 139(5), 115(3), 97(12), 82(18)
1-Phenanthrol[c]	248, 293, 305, 338, 354	195(13), 194 $M^{+\cdot}$(100), 166[M-CO]$^{+\cdot}$(24), 165[M-CHO]$^+$(90), 139(8), 115(3), 97(15), 82(21)
9-Phenanthrol[b]	248, 275, 293, 305, 338, 356	195(15), 194 $M^{+\cdot}$(100), 166[M-CO]$^{+\cdot}$(20), 165[M-CHO]$^+$(98), 139(9), 115(10), 97(29), 82(27)
4-Phenanthrol[b]	245, 274, 293, 302, 338, 354	195(13), 194 $M^{+\cdot}$(100), 166[M-CO]$^{+\cdot}$(30), 165[M-CHO]$^+$(75), 139(5), 115(0.5), 97(4), 82(19)

[a]*C. elegans.*
[b]*S. racemosum.*
[c]*A. niger.*

equal volumes of ethyl acetate and prepared for HPLC analysis as described above.

Analytical methods

Reversed-phase HPLC was performed using Shimadzu model LC-600 pumps (Shimadzu Scientific Instruments, Kyoto, Japan) fitted with a C_{18} column (Beckman 5-μm Ultrasphere-ODS column, 25 cm × 4.6 mm inner diameter; Altex Scientific, Berkeley, CA, USA). The mobile phase was a 40-min linear gradient of methanol : water (from 50 : 50 to 95 : 5, vol/vol) with a flow rate of 1.0 ml min^{-1}. A Shimadzu model SPD-6AV UV-VIS spectrophotometric detector set at 254 nm was used to monitor phenanthrene metabolites which eluted from the column. A Shimadzu model SPD-M6A UV-VIS photodiode array detector linked to an Acma 486 computer (loaded with Shimadzu SPD-M6A Utility software) was used to obtain and analyze UV absorption spectral data of phenanthrene metabolites separated by HPLC. Repeated injections of the culture extracts were made, and compounds with similar retention times and UV-visible absorption spectra were pooled and concentrated *in vacuo* in a Savant Speed Vac concentrator (Savant Instruments, Hicksville, NY, USA).

In experiments using [9-^{14}C]phenanthrene, fractions were collected in scintillation vials at 30-s intervals. Ultima Gold scintillation cocktail (Packard Instrument Co, Meriden, CT, USA) was added to each vial and the radioactivity was determined on a Packard 2000CA Tri-Carb liquid scintillation counter (Packard Instrument Co, Downers Grove, IL, USA). Radioactivity remaining in the aqueous phase after ethyl acetate extraction (non-extractable metabolites) was determined by measuring the volume and then removing a 1-ml aliquot for liquid scintillation counting. Radioactivity associated with yeast cells after washing with ethyl acetate was determined by adding scintillation fluid to pelleted cells and counting it by liquid scintillation.

HPLC-purified phenanthrene metabolites were analyzed by electron ionization (EI) mass spectrometry on a Finnigan MAT 4023 quadrupole mass spectrometer (Finnigan MAT Corp, San Jose, CA, USA) using a platinum wire direct exposure probe [13]. A Vacuumetrics DCI programmer (Vacuumetrics Corp, Ventura, CA, USA) was used to generate an 80-s ramp from 0 to 3 amps for the direct exposure probe. Phenanthrene metabolites isolated from the glucoside region were acetylated in 0.5 ml ethyl acetate, 1.0 ml pyridine, and 0.2 ml acetic anhydride at 60°C for 16 h.

Proton nuclear magnetic resonance (^{1}H NMR) measurements were obtained at 500 MHz using a Bruker AM500 spectrometer (Bruker Instruments, Billerica, MA, USA). Spectral data were obtained using typical data acquisition parameters described previously [11]. Resonance assignments were based on the analysis of chemical shifts, coupling patterns, and nuclear Overhauser effect (NOE) measurements.

Deconjugation experiments

The residue from the [9-^{14}C]phenanthrene ethyl acetate extract was dissolved in 1 ml of methanol and then divided into 0.1-ml portions. The first sample was incubated with 50 units β-D-glucuronidase, type VII-A, from *Escherichia coli* (EC 3.2.1.31; Sigma). The second sample was incubated with one unit arylsulfatase, type VI, from *Aerobacter aerogenes* (EC 3.1.6.1, Sigma). The third sample was incubated with 3.4 units β-D-glucoside glucohydrolase, type I, from almonds (EC 3.2.1.21, Sigma). The fourth sample, which served as a control, had no enzyme. The reaction mixtures were diluted to 1.0 ml with phosphate buffer (pH 7.0) and incubated for 16 h at 37°C. Samples were then

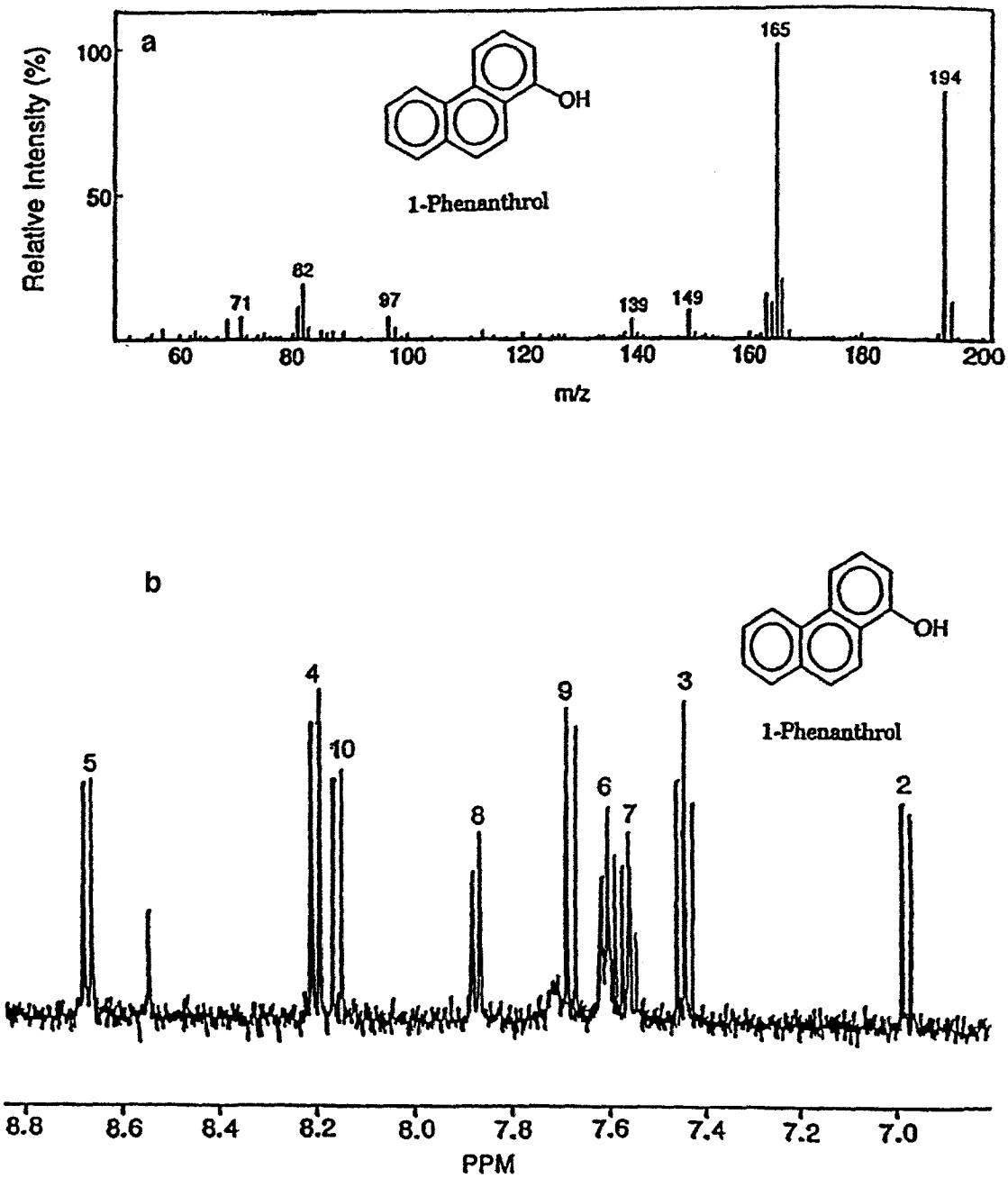

Figure 2 (a) Mass and (b) proton NMR spectra of 1-phenanthrol produced by an *A. niger* ATCC 6275 culture incubated with phenanthrene.

extracted with three equal volumes of ethyl acetate and prepared for HPLC analysis. Fractions were collected in scintillation vials at 30-s intervals and the radioactivity was determined by the methods described above. Radioactivity remained in the aqueous phase after ethyl acetate extraction was counted directly by liquid scintillation.

Results

Identification of oxidative metabolites

The HPLC elution profiles and radiochemical histograms obtained from the metabolism of [9-^{14}C]phenanthrene by *S. racemosum*, *C. elegans*, and *A. niger* are shown in Figure 1. [9-^{14}C]Phenanthrene was metabolized to radioactive metabolites that could be separated into polar (water-soluble), dihydrodiol, glucoside, and phenanthrol regions based on HPLC retention times (Table 1). The percentage of total radioactivity and the HPLC retention time for each identified oxidative [9-^{14}C]phenanthrene metabolite is shown in Table 2. Although the 2- and 3-phenanthrol produced from *S. racemosum* were resolved according to their UV spectral characteristics, the percentage of radioactivity associated with 2- and 3-phenanthrol was difficult to differentiate, therefore values represent an estimate.

Phenanthrene *trans*-9,10 and 3,4-dihydrodiols, and 3-, 4- and 9-phenanthrols were characterized, initially, by comparison of their relative retention times and UV-visible absorption spectra with previously published results [1,

Table 4 ^{1}H NMR chemical shifts (δ) of phenanthrene metabolites produced by *A. niger* and *C. elegans*

Chemical shift (ppm)	1-phenanthrol[a]	1-phenanthryl β-D-glucopyranoside[b]	2-hydroxy-1-phenanthryl β-D-glucopyranoside[b]
2	6.97	7.41	
3	7.44	7.60	7.30
4	8.20	8.46	8.53
5	8.68	8.78	8.68
6	7.60	7.68	7.63
7	7.56	7.64	7.54
8	7.87	7.96	7.90
9	7.68	7.80	7.76
10	8.15	8.37	8.50
1′[c]		5.19	4.77
2′		3.72	3.72
3′		3.59	3.58
4′		3.53	3.58
5′		3.59	3.40
6a′		3.91	3.88
6b′		3.74	3.78

[a]Metabolite produced by *A. niger*, δ scale is based on methanol-d_4 at 3.30 ppm.
[b]Metabolites produced by *C. elegans*, δ scale is based on acetone-d_6 at 2.05 ppm.
[c]Chemical shifts (ppm) for the glucose moiety are designated by 'primes'.

Table 5 ^{1}H NMR coupling constants (J) of phenanthrene metabolites produced by *A. niger* and *C. elegans*

Coupling constant (Hz)	1-phenanthrol[a]	1-phenanthryl β-D-glucopyranoside[b]	2-hydroxy-1-phenanthryl β-D-glucopyranoside[b]
$J_{2,3}$	7.7	8.0	
$J_{2,4}$	0.9	0.9	
$J_{3,4}$	8.2	8.6	9.0
$J_{5,6}$	8.2	8.2	8.2
$J_{5,7}$	1.5	1.3	1.1
$J_{6,7}$	7.7	7.7	7.7
$J_{6,8}$	1.5	1.5	1.3
$J_{7,8}$	8.0	7.7	8.0
$J_{9,10}$	9.0	9.0	9.2

[a]Metabolite produced by *A. niger*.
[b]Metabolites produced by *C. elegans*.

31]. EI mass spectral data for the *trans*-9,10 and 3,4-dihydrodiols and the 3-,4- and 9-phenanthrols revealed molecular and fragment ions (Table 3) that are consistent with those previously reported for these phenanthrene metabolites [33]. The UV-visible absorption spectrum for a phenanthrol region metabolite (22.8 min) produced by *A. niger* and *S. racemosum* revealed λ_{max} values (Table 3) that were identical to those of authentic 2-phenanthrol and similar to the published spectrum for 2-phenanthrol [1]. In addition, an EI mass spectrum of the metabolite produced by *A. niger* (Table 3) shows a molecular ion at m/z 194 [$M^{+\cdot}$] and a fragment ion at m/z 165 [M-CHO]$^+$, which are typical of a phenanthrol [21]. *A. niger* produced another metabolite (23.0 min) which eluted in the phenanthrol region. The UV absorption spectrum is similar to the published spectrum for 1-phenanthrol [1]. Its EI mass spectrum (Figure 2a) showed a molecular ion at m/z 194 [$M^{+\cdot}$] and a fragment ion at m/z 165 [M-CHO]$^+$, which is expected for a phenanthrol [21]. Proton NMR confirmed the site of hydroxyl substitution as the C-1 position on phenanthrene (Figure 2b). Resonance assignments were determined from nuclear Overhauser effects, chemical shifts (Table 4), and coupling patterns (Table 5).

[9-^{14}C]Phenanthrene metabolites were not detected in organic extracts from biotransformation experiments with yeasts. The total radioactivity recovered as unmetabolized phenanthrene indicated that the yeasts had metabolized less than 3% of the phenanthrene, whereas *C. elegans*, *S. racemosum*, and *A. niger* had transformed 99.6%, 55.4%, and 33.6% of the phenanthrene, respectively (Table 1).

Identification of conjugative metabolites

C. elegans produced a glucoside region metabolite (12.5 min) with UV absorption λ_{max} and EI mass spectral characteristics (Table 3) similar to the published data for 1-phenanthryl β-D-glucopyranoside [11]. The identification of this compound was confirmed by ^{1}H NMR spectral data (Tables 4 and 5) which are consistent with data previously reported for 1-phenanthryl β-D-glucopyranoside [11]. Acetylation of the metabolite and subsequent EI mass spectral analysis gave a spectrum (Figure 3a) with fragment ions at m/z 331, 194, 169, 165, 127, and 109 which are characteristic of phenanthrol and a peracetylated glucose moiety [11].

C. elegans produced another metabolite that eluted in the glucoside region (13.0 min), but its UV absorption spectrum exhibited different λ_{max} values (Table 3) than those previously reported for phenanthrene glucoside conjugates [11, 31]. The EI mass spectrum of this metabolite (Figure 3b) shows a weak molecular ion at m/z 372 [$M^{+\cdot}$] and strong fragment ions at m/z 210 and 181, which would be expected for a glucoside conjugate of dihydroxyphenanthrene. The glucose moiety and a hydroxyl substitution on the phenanthrene ring were confirmed by NMR (Figure 4). Tables 4 and 5 show the chemical shifts and coupling constants, which indicated substitution at the C-1 and C-2 positions. An NOE found between the H-1′ on the glucose and the H-10 of phenanthrene confirmed the substitution of the glucose at the C-1 position, and the identification of the novel phenanthrene metabolite as 2-hydroxy-1-phenanthryl β-D-glucopyranoside.

The percentages of radioactivity for individual phenanthrene conjugative metabolites following treatment of the 192-h [9-^{14}C]phenanthrene ethyl acetate extracts from *C. elegans*, *S. racemosum*, and *A. niger* with arylsulfatase, β-D-glucuronidase, and β-D-glucosidase are shown in Table 6. HPLC elution profiles and radiochemical histograms (data not shown) indicated that treatment with either arylsulfatase or β-glucuronidase resulted in a significant decrease in the percentage of radioactivity associated with polar region (water-soluble) metabolites. This decrease corresponded to an increase in the radioactivity determined for dihydrodiol and phenanthrol region hydrolysis products. These observations suggest that sulfate and glucuronide conjugates were associated with the polar region metabolites. Sulfate conjugates produced by *C. elegans*, *S. racemosum*, and *A. niger* accounted for 11.7%, 29.0%, and 24.1%, respectively, of the polar region metabolites

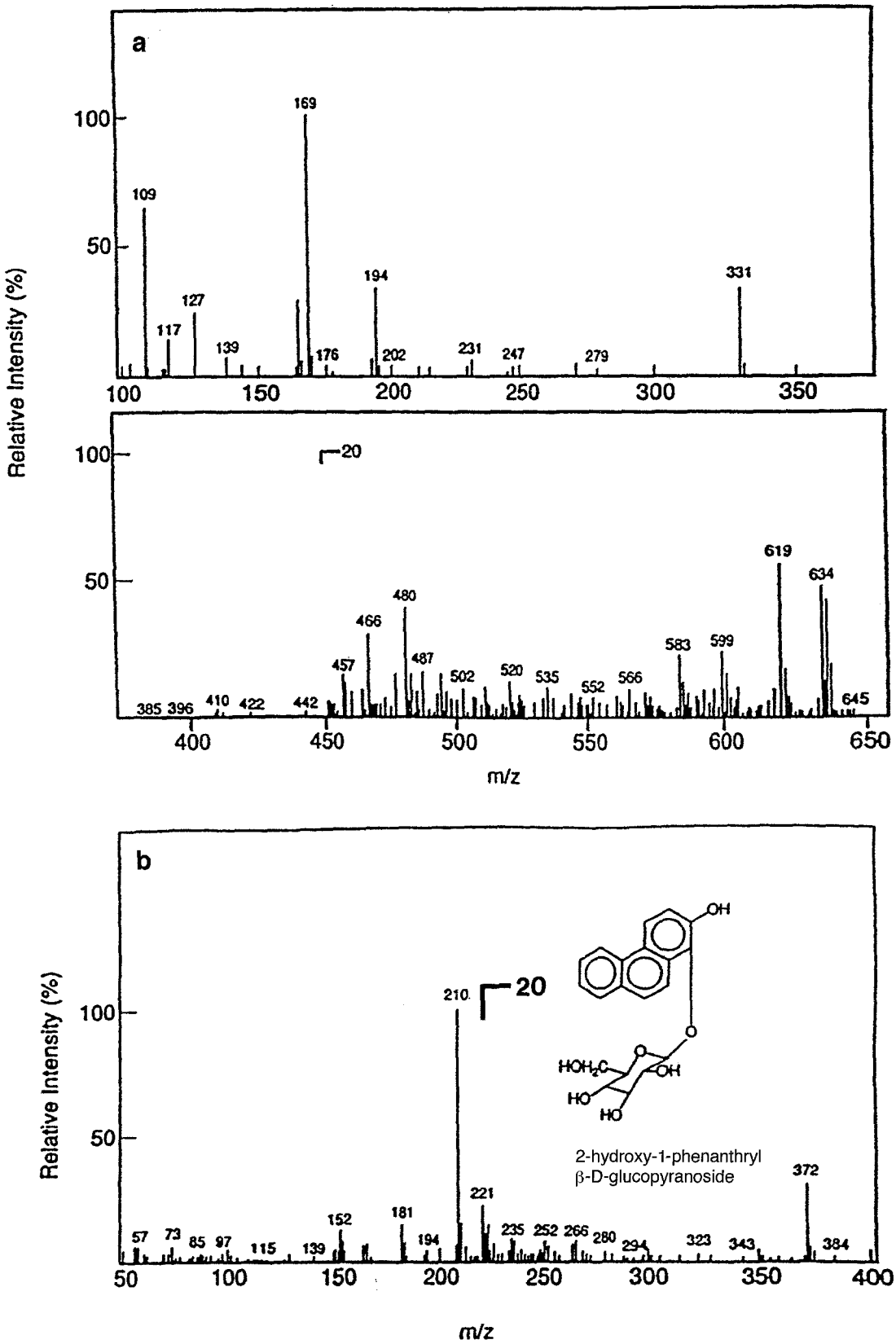

Figure 3 Mass spectra of the acetylated derivative of the phenanthryl-β-D-glucopyranoside (a) and the nonderivatized metabolite 2-hydroxy-1-phenanthryl-β-D-glucopyranoside (b), produced by *C. elegans* ATCC 9245 grown in the presence of phenanthrene.

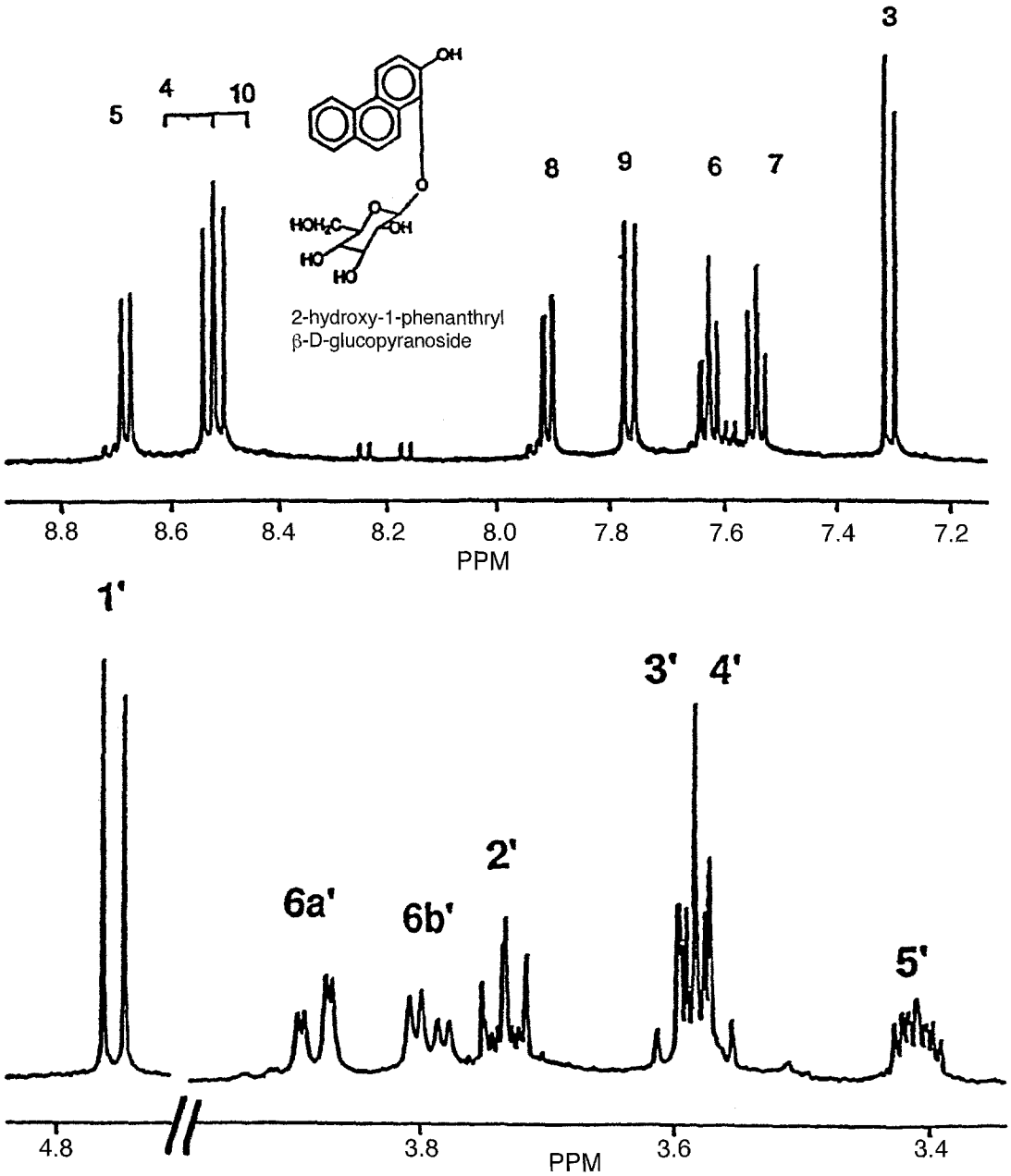

Figure 4 Proton NMR spectrum with assignments for 2-hydroxy-1-phenanthryl-β-D-glucopyranoside, produced by *C. elegans* ATCC 9245 grown in the presence of phenanthrene.

(Table 6). The ratios of sulfate conjugates to initial oxidative metabolites were 3 : 1, 1 : 1, and 4 : 1 for *C. elegans*, *S. racemosum*, and *A. niger*, respectively. Treatment with β-D-glucuronidase revealed minor glucuronide conjugates of 9-phenanthrol (0.5%) and *trans*-9,10-dihydrodiol (0.3%), produced from *S. racemosum* and *A. niger*, respectively. Treatment of the ethyl acetate extract from *C. elegans* with β-D-glucosidase yielded the hydrolysis products 1-phenanthrol (0.4%) and 1,2-dihydroxyphenanthrene (0.6%). These data strongly support the previously described identification of the 1-phenanthryl β-D-glucopyranoside and the novel metabolite, 2-hydroxy-1-phenanthryl β-D-glucopyranoside produced by *C. elegans*.

Discussion

Results for the metabolism of phenanthrene presented here demonstrate parallels between microbial and mammalian xenobiotic-metabolizing enzyme mechanisms and pathways. The present study identifies initial oxidative and subsequent conjugative phenanthrene K-region and bay-region metabolites which have not previously been isolated from filamentous fungi. The proposed pathways for the initial oxidation and subsequent conjugation of phenanthrene at the 1,2-, 3,4- (bay-region), and 9,10-positions (K-region) by *A. niger*, *S. racemosum*, and *C. elegans* are shown in Figure 5. The principal metabolic pathways for the fungal

Table 6 Percentage total radioactivity for conjugated metabolites produced from phenanthrene

Conjugate	Sulfate			Glucoside	Glucuronide	
	C. elegans	*S. racemosum*	*A. niger*	*C. elegans*	*S. racemosum*	*A. niger*
Phenanthrene *trans*-3,4-dihydrodiol	0.4	–	–	–	–	–
Phenanthrene *trans*-9,10-dihydrodiol	6.3	0.8	1.9	–	–	0.3
2-Phenanthrol	0.4	5.1[a]	4.3	–	–	–
3-Phenanthrol	0.3	17.5[a]	–	–	–	–
1-Phenanthrol	4.5	–	17.9	0.4	–	–
1,2-Dihydroxyphenanthrene	0.3	–	–	0.6	–	–
9-Phenanthrol	–	4.9	–	–	0.5	–
4-Phenanthrol	–	0.7	–	–	–	–
Total conjugates	11.7	29.0	24.1	1.0	0.5	0.3

[a]Values are estimated because 2- and 3-phenanthrol eluted close together.

metabolism of PAHs have been recently reviewed [14, 31]. Although the bay-region of phenanthrene was previously shown to be the main site of enzymatic attack, fungi are now also known to transform phenanthrene at the K-region [33], which is a major site of mammalian enzymatic attack [4].

The inability of the yeast strains described in this paper to metabolize [9-^{14}C]phenanthrene is consistent with the findings of MacGillivray and Shiaris [24]. In their study of the yeast biotransformation of PAH-contaminated coastal sediments, the yeast *Candida lipolytica* 37-1 was unable to metabolize [9-^{14}C]phenanthrene. This is the same yeast strain reported in this paper, which, in addition to two other hydrocarbonoclastic yeasts, did not exhibit the ability to metabolize [9-^{14}C]phenanthrene.

The five possible isomeric phenanthrols, 1-, 2-, 3-, 4- and 9-phenanthrols, were identified as fungal oxidative metabolites in this study. *S. racemosum* produced all of the phenanthrols except for 1-phenanthrol. *A. niger* produced only the 1- and 2-phenanthrol. This is the first report on the direct isolation of 1- and 2-phenanthrols produced from the fungal metabolism of phenanthrene. The urinary excretion of predominantly 1- and 2-phenanthrols by rats, following oral, intraperitoneal and intrapulmonary administration of phenanthrene [18], indicates that the regiospecificity for the formation of phenanthrols by *A. niger* corresponds with that of primary phenanthrol formation in rats.

The fungi are known to biotransform phenanthrene to *trans*-dihydrodiol metabolites that are generally the mirror images of the corresponding *trans*-dihydrodiols obtained from rat liver microsomes [9, 13, 26]. The *trans*-3,4- and 9,10-dihydrodiols, produced by *C. elegans* and *A. niger*, respectively, were not isolated in sufficient amounts to determine their enantiomeric composition. Phenanthrene *trans*-1,2-dihydrodiol was not isolated from the culture media of *A. niger*, *S. racemosum*, and *C. elegans* ATCC 9245, similar to the results observed for *Phanerochaete chrysosporium* [33], but different from *C. elegans* ATCC 36112 [9]. The enantiomeric composition and optical purities of *trans*-dihydrodiols produced during the metabolism of phenanthrene by *S. racemosum*, *C. elegans*, and *P. chrysosporium* have recently been published [32]. The results indicate interspecific differences in the stereoselectivity among fungal enzymes, in addition to the differences in the enantiomeric selectivities of fungal and mammalian enzymes.

Fungi form sulfate and glucuronide conjugates of PAH phenols and *trans*-dihydrodiols by enzymatic reaction mechanisms that are similar to mammalian detoxication enzyme mechanisms [31]. Sulfate and glucuronide conjugates produced during the fungal metabolism of PAHs are commonly found in the aqueous phase (water-soluble metabolites) following organic extraction of the culture broth [7, 8, 27]. Previous studies on the fungal metabolism of phenanthrene did not identify sulfate or glucuronide conjugates. However, unidentified polar (water-soluble) metabolites were reported but not characterized [5, 31]. Sulfate conjugates of 1-, 2-, and 3-phenanthrols and 1,2-dihydroxyphenanthrene were detected following enzymatic hydrolysis of the initial organic extract from *C. elegans*, which suggests that phenanthrene was initially oxidized to these primary phenol metabolites and subsequently conjugated with sulfate. Sulfate, glucuronide, and glucose conjugation of PAHs by fungi is an important detoxication mechanism [32]. In this paper, data are shown for the isolation and identification of 1-phenanthryl β-D-glucopyranoside and 2-hydroxy-1-phenanthryl β-D-glucopyranoside, a novel phenanthrene glucoside conjugate. Both metabolites were produced by *C. elegans* ATCC 9245. Although the exact mechanism for the formation of 2-hydroxy-1-phenanthryl β-D-glucopyranoside was not determined, Boyland and Sims [2] reported the production of large amounts of 1,2-dihydroxyphenanthrene conjugates in the urine of animals given phenanthrene, and indicated that 1,2-dihydroxyphenanthrene (the primary oxidative metabolite) arose from the dehydrogenation of the *trans*-1,2-dihydrodiol.

Phenanthrene 1,2-, 3,4-, and 9,10-oxides were not isolated from the fungal culture medium; however, the presence of 1-, 2-, 3-, 4- and 9-phenanthrols and their conjugates indirectly provides evidence that arene oxides were primary oxidative intermediates in the metabolism of phenanthrene by *A. niger*, *S. racemosum*, and *C. elegans*. Since the *trans*-9,10-dihydrodiol, but not 9-phenanthrol, was detected in the culture medium of *A. niger*, it can be suggested that either there is little or no conversion of the 9,10-oxide to 9-phenanthrol, or else 9-phenanthrol is produced but further metabolized to polar (water-soluble) metabolites.

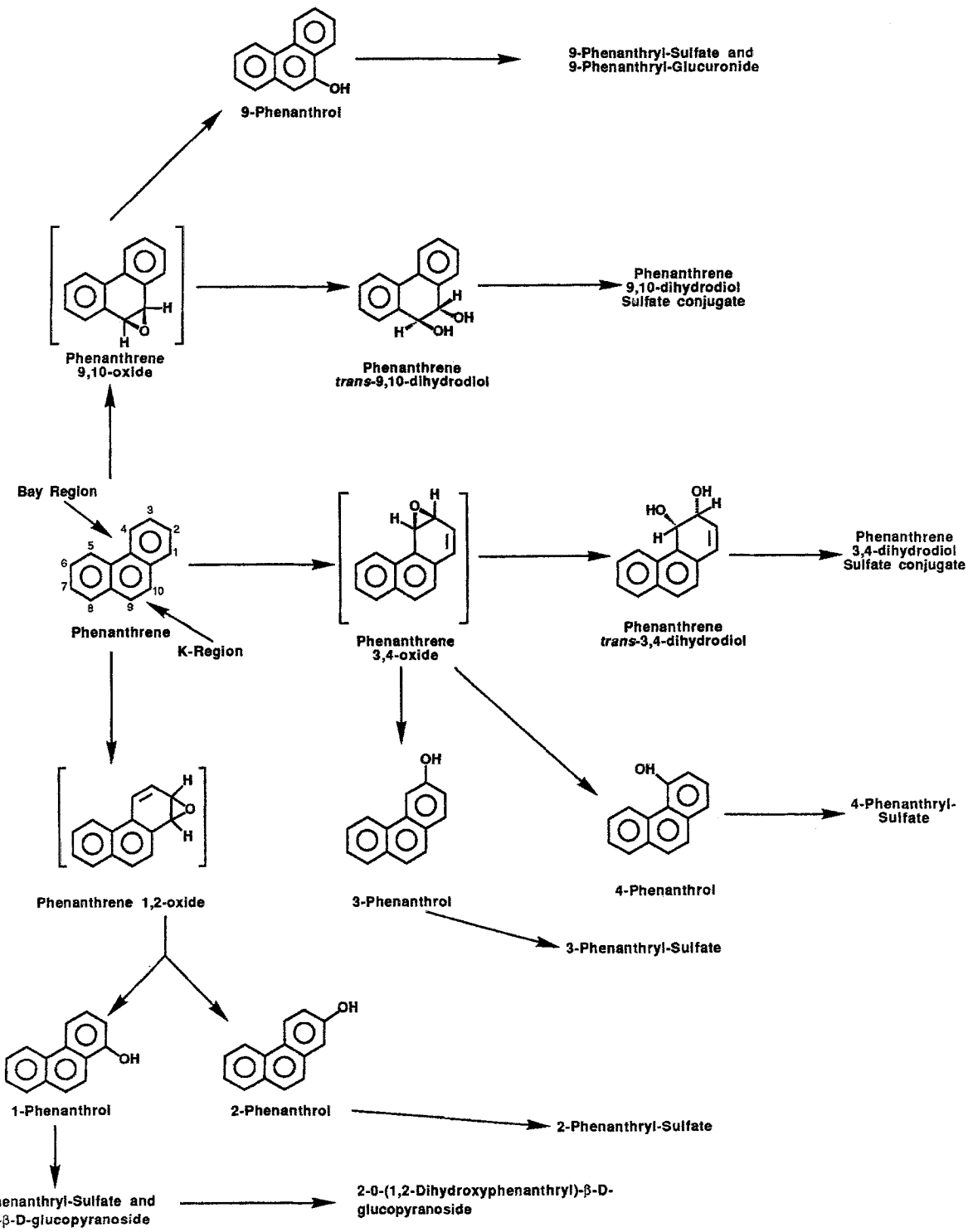

Figure 5 Proposed pathways for the phase I (oxidative) and phase II (conjugative) metabolism of phenanthrene by *A. niger* ATCC 6275, *C. elegans* ATCC 9245, and *S. racemosum* UT-70.

The formation of phenanthrene *trans*-dihydrodiols and hydroxyphenanthrenes by *A. niger*, *S. racemosum*, and *C. elegans* ATCC 9245 is consistent with the expected fungal [14] and mammalian [19, 28] oxidative metabolism of unsubstituted PAHs. Furthermore, the metabolic pathways and biochemical reaction mechanisms for the biotransformation of phenanthrene by these fungi closely resemble phase I (oxidation) and subsequent phase II (conjugation) metabolic pathways documented for terrestrial [2, 29] and aquatic animals [30]. The results therefore suggest the involvement of cytochrome P-450, epoxide hydrolase, UDP-glucuronyltransferase, glycosyltransferase, and arylsulfotransferase activities in the oxidation and subsequent conjugation of phenanthrene by these fungi. Although we were unable to demonstrate these enzymes directly or detect their *in vitro* activities, enzymic deconjugation

experiments provided indirect evidence that these conjugative enzymes must be present. Since conjugates of PAHs, including phenanthrene, produced by fungi are generally nonmutagenic [10, 32], the ability of *A. niger*, *S. racemosum*, and *C. elegans* to metabolize phenanthrene to water-soluble conjugates further supports the use of selected fungi for the bioremediation of PAH-contaminated sites.

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