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1-Methoxypyrene and 1,6-dimethoxypyrene: two novel metabolites in fungal metabolism of polycyclic aromatic hydrocarbons

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Abstract The metabolism of pyrene by *Penicillium glabrum* strain TW 9424, a strain isolated from a site contaminated with polycyclic aromatic hydrocarbons (PAHs) was investigated in submerged cultures. The metabolites formed were identified as 1-hydroxypyrene, 1,6- and 1,8-dihydroxypyrene, 1,6- and 1,8-pyrenequinone, and 1-pyrenyl sulfate. In addition, two new metabolites were isolated and identified by UV, ^{1}H nuclear magnetic resonance, and mass spectroscopy as 1-methoxypyrene and 1,6-dimethoxypyrene. Experiments with [*methyl*-^{3}H]S-adenosyl-L-methionine (SAM) revealed that SAM is the coenzyme that provides the methyl group for the methyltransferase involved. To our knowledge, this is the first time that methoxylated metabolites of PAHs have been isolated from fungal cultures.

Key words Polycyclic aromatic hydrocarbons · Fungi · Biodegradation · Biotransformation · Methoxy-PAHs · Methyl conjugation · O-methylation · O-methyltransferase · Fungal secondary metabolism

Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent an important class of environmental pollutants since the compounds are among the most frequently found soil contaminants; several PAHs are known to be mutagenic and carcinogenic (Dipple 1976). Intensive studies of the microbial metabolism of PAHs have demonstrated that many species of bacteria and fungi are capable of oxidizing various PAHs (Cerniglia 1992).

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White-rot fungi such as *Phanerochaete chrysosporium* are able to oxidize PAHs by extracellular peroxidases to quinones, which subsequently can be oxidized to CO_2 (Hammel et al. 1986; Aust et al. 1994)

Metabolism of PAHs by non-white-rot fungi involves cytochrome P-450 monooxygenase enzyme systems similar to those observed in mammals (Smith and Rosazza 1974). The first steps of PAH oxidation by non-white-rot fungi result in the formation of monophenols, diphenols, dihydrodiols, and quinones as reported, e.g., for the zygomycete *Cunninghamella elegans* (Cerniglia and Gibson 1979; Cerniglia et al. 1986), the basidiomycete *Crinipellis stipitaria* (Lambert et al. 1994; Lange et al. 1994), and deuteromycetes of the genera *Aspergillus* (Datta and Samanta 1988; Wunder et al. 1994) and *Penicillium* (Launen et al. 1995). In a second step, water-soluble conjugates, which are detoxification products in fungi as well as in mammals, can be formed (Cerniglia et al. 1982; Lambert et al. 1995; Thakker et al. 1985). Among fungal PAH conjugates, sulfates (Cerniglia and Gibson 1979; Cerniglia 1982; Cerniglia et al. 1982; Lange et al. 1994; Wunder et al. 1994), glucuronides (Cerniglia et al. 1982), glucosides (Cerniglia et al. 1986, 1989), and xylosides (Sutherland et al. 1992) have been reported.

With the isolation and identification of two O-methyl conjugates of pyrene from cultures of *Penicillium glabrum*, we report on a new conjugation mechanism in the fungal metabolism of pyrene.

Materials and methods

Organism and growth conditions

The fungus used in this study was isolated from soil highly contaminated with polycyclic aromatic hydrocarbons (PAHs) and was determined as *Penicillium glabrum* strain TW 9424 according to the identification scheme of Pitt (1979). The strain belongs to the group of monoverticillate *Penicillium* species, subgenus *Aspergilloides*. Stock cultures of the organism were maintained on agar slants with a yeast malt glucose medium (YMG) consisting of (g/l): yeast extract (4.0), malt extract (10.0), glucose (4.0), and agar (15.0); the pH was 5.5. The same medium without agar was

used for metabolic experiments with pyrene in submerged cultures. For the quantification of pyrene transformation and the detection of metabolites the fungus was cultivated in 100-ml Erlenmeyer flasks containing 20 ml of YMG. The cultures were grown for 4 days on a rotary shaker at 25° C and 120 rpm before pyrene dissolved in dimethylformamide (DMF) was added aseptically to a final concentration of 20 mg/l. The cultures were incubated for another 10 days. Heat-treated (121° C, 10 min) cultures served as controls.

For labeling experiments with S-adenosylmethionine (SAM), the same experimental conditions were used as described above. [*Methyl*-^{3}H]SAM (2.5 μCi) and 1-hydroxypyrene (5 mg/l) were added to the cultures. For the isolation of metabolites, the fungus was grown in 5-1 Erlenmeyer flasks containing 2 1 YMG.

Analysis and identification of metabolites

The culture broth of the 20-ml cultures was separated from the mycelia by filtration on a Büchner funnel. The mycelia were extracted twice for 20 min with 100 ml acetone. After adjusting the pH to 2.0 (1 N HCl), the culture filtrate was extracted with three equal volumes of ethyl acetate. The extracts were combined, and the solvents were removed under reduced pressure at 40° C. The dry residues of the mycelial and culture filtrate extracts were dissolved in 2 ml and in 1 ml of methanol, respectively, and were analyzed by HPLC. In experiments with [*methyl*-^{3}H]SAM, cultures were extracted as described above. The residue of the mycelial extract was dissolved in 200 μl acetone, and 30 μl was used for HPLC analysis.

Reversed-phase HPLC was performed on a Merck-Hitachi system equipped with a L-3000 photo diode array detector. A 5 μm Nucleosil C_{18} PAH column (150 × 4 mm; Macherey & Nagel, Düren, Germany) and a 30 min linear gradient of acetonitrile-water (10 : 90 to 100 : 0, v/v; gradient A) at a solvent flow rate of 1 ml/min were used to separate the metabolites. For the separation and identification of [^{3}H]-labeled metabolites, a 20-min linear gradient (30 : 70 to 100 : 0, v/v; gradient B) was used, and 0.5-ml fractions were collected every 0.5 min in scintillation vials. After addition of 5 ml scintillation cocktail (Aquasafe 300 Plus; Zinsser Analytic, Frankfurt/Main, Germany), the radioactivity was determined on a Wallac 1410 liquid scintillation counter (Pharmacia, Wallac Oy, Turku, Finland).

For the isolation of metabolites, fungal mycelia from 2-l cultures were extracted with 1.5 l acetone. The filtrate was extracted as described above. The combined extracts were dissolved in 15 ml cyclohexane and then separated by solid phase extraction (SPE) using a vacuum manifold system equipped with five silica gel cartridges (1 g, 6 ml; Baker Chemikalien, Gross-Gerau, Germany). The crude extract was applied to the cartridge in 3-ml portions. Cyclohexane and cyclohexane-ethyl acetate (9 : 1, v/v) were used for the elution of metabolites. 1,6-Dimethoxypyrene was further purified by preparative HPLC using the system mentioned above.

UV absorbance spectra were obtained using the above mentioned diode array spectrophotometer attached to the HPLC system. Electron impact (EI) mass spectra of the metabolites were obtained with a Jeol SX102 mass spectrometer with an ionization voltage of 70 eV. The ion source temperature was 275° C. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded with a Bruker ARX500 spectrometer operating at 500 MHz. The spectra were recorded in acetone-d_6. Chemical shifts are reported in parts per million (ppm) with the solvent signal at 2.05 ppm as reference.

In deconjugation experiments, 200 μl of a crude mycelial extract solution in acetone was mixed with 5 ml of deionized water and diluted 1:1 with 0.2 M sodium acetate buffer (pH 5.0). Arylsulfatase (15 units, Type V, Sigma-Aldrich) was added. In order to inhibit β-glucuronidase activity, 15 μmol of D-saccharic acid-1,4-lactone was added. The samples were incubated at 37° C for 24 h on a rotary shaker operating at 120 rpm. Samples without enzyme served as controls.

Chemicals

Pyrene (purity > 99%) was purchased from Aldrich (Steinheim, Germany). [*Methyl*-^{3}H]S-adenosyl-L-methionine (SAM; specific activity 10–20 Ci/mmol, radiochemical purity > 95%) was obtained from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals used were of analytical grade. 1-Hydroxypyrene, 1-pyrenyl sulfate, the dihydroxypyrenes, and the pyrenequinones used as authentic standards were isolated and purified from fungal cultures (Lange et al. 1994; Wunder et al. 1994).

Results

When *Penicillium glabrum* strain TW 9424 was grown in the presence of pyrene (20 mg/l), the pyrene concentration decreased to 10.9 mg/l within 10 days. Elimination of pyrene was biologically mediated since the recovery of the compound in autoclaved cultures was 17.1 mg/l. About 37% of the pyrene added to the living fungal cultures was converted into metabolites, about 70% of which were located in the mycelial extracts. Analytical reversed-phase HPLC, as shown in Fig. 1, revealed the presence of at least seven pyrene metabolites formed by the fungus. Pyrene eluted at 22.6 min; the compounds eluting at 18.1, 13.7, 13.5, and 7.6 min were identified as 1-hydroxypyrene, 1,8-dihydroxypyrene, 1,6-dihydroxypyrene, and 1-pyrenyl sulfate, respectively, by comparison with authentic standards (Lambert et al. 1994; Lange et al. 1994; Wunder et al. 1994). None of the metabolites were detected in autoclaved controls. The peak eluting between 14.9 and 15.1 min corresponds to 1,6- and 1,8-pyrenequinones, which were not separated under these conditions. On-line UV spectral properties of the metabolites

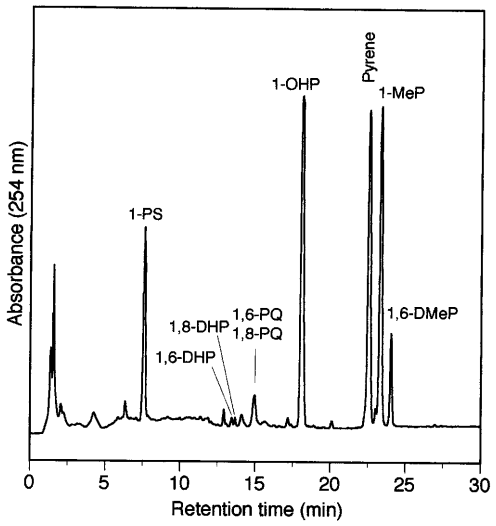

Fig. 1 HPLC chromatogram of the metabolites formed by *Penicillium glabrum* strain TW 9424 after 10 days of incubation with pyrene (20 mg/l) in yeast malt glucose medium. The extract was spiked with 1-MeP and 1,6-DMeP before HPLC analysis. The compounds were separated using gradient A (*1-MeP* 1-methoxypyrene; *1,6-DMeP* 1,6-dimethoxypyrene; *1-OHP* 1-hydroxypyrene; *1,6-PQ* 1,6-pyrenequinone; *1,8-PQ* 1,8-pyrenequinone; *1,6-DHP* 1,6-dihyrixyoyrene; *1,8-DHP* 1,8-dihydroxypyrene; *1-PS* 1-pyrenyl sulfate)

Table 1 Concentrations of pyrene (99 μM added) and its metabolites in cultures of *Penicillium glabrum* strain TW 9424 after 10 days of incubation and the percentage of the metabolites formed (*nd* not determined)

Metabolite	Concentration (μM)	Added pyrene metabolized (%)
Pyrene	54.0	–
1-Methoxypyrene	5.6	12.4
1,6 Dimethoxypyrene	2.1	4.6
1-Pyrenyl sulfate	8.5	18.9
1-Hydroxypyrene	14.0	31.1
1,6-, 1,8-Dihydroxypyrene + 1,6-, 1,8-Pyrenequinone	nd	≤ 1

Table 2 Mass spectral properties of 1-methoxypyrene and 1,6-dimethoxypyrene formed by *Penicillum glabrum* strain TW 9424

Metabolite	Fragment ions *m/z* (%)
1-Methoxypyrene	232(83) $[M]^+$, 217(100), 200(5), 189(49), 187(14), 153(5), 139(6), 125(12), 111(19), 97(27), 83(23), 71(26), 57(37), 43(20)
1,6-Dimethoxypyrene	262(100) $[M]^+$, 247(95), 232(23), 204(21), 187(11), 176(38), 149(15), 131(17), 64(16), 46(46)

Table 3 ^{1}H Nuclear magnetic resonance spectral data (500 Mhz) of 1-methoxypyrene and 1,6-dimethoxypyrene formed by *Penicillium glabrum* strain TW 9424. The spectra were recorded in acetone-d_6 with the solvent signal (2.05 ppm) as reference

Position	δ(ppm); Multiplicity; J(Hz)	
	1-Methoxypyrene	1,6-Dimethoxypyrene
$-OCH_3$	4.21; s	4.18; s
2-H	7.73; d; $J_{2,3}$ = 8.4	7.70; d; $J_{2,3}$ = 8.5
3-H	8.23; d; 8.4	8.15; d; 8.5
4-H	7.95; d; $J_{4,5}$ = 9.0	7.99; d; $J_{4,5}$ = 9.2
5-H	8.04; d; 9.0	8.22; d; 9.2
6-H	8.17; d; $J_{6,7}$ = 7.6	–
7-H	8.00; t; 7.6	7.70; d; $J_{7,8}$ = 8.5
8-H	8.18; d; $J_{7,8}$ = 7.6	8.15; d; 8.5
9-H	8.11; d; $J_{9,10}$ = 9.2	7.99; d; $J_{9,10}$ = 9.2
10-H	8.43; d; 9.2	8.22; d; 9.2

and their retention times were identical with authentic standards (Lambert et al. 1994; Lange et al. 1994; Wunder et al. 1994).

Two additional metabolites eluting at 23.4 and 24.1 min with pyrene like UV spectra were detected. Their chromatographic behavior suggested the presence of alkylated or perhaps dimeric products of fungal pyrene transformation. The UV spectral data showed characteristics nearly identical to those determined for 1-hydroxypyrene and 1,6-dihydroxypyrene in previous studies (Cerniglia et al. 1986; Lambert et al. 1994; Wunder et al. 1994). Thus, these metabolites were likely to be O-alkylated derivatives of their corresponding phenols. By UV, mass, and ^{1}H NMR spectroscopies, the compounds were identified as 1-methoxypyrene (1-MeP) and 1,6-dimethoxypyrene (1,6-DMeP), respectively. Table 1 summarizes the quantitative results of the pyrene transformation by *P. glabrum* strain TW 9424 after 10 days of incubation.

The cultivation of *P. glabrum* in a 2-l scale and the subsequent purification of the extracts by chromatography yielded 8.3 mg of pure 1-MeP and 0.7 mg of 1,6-DMeP. The UV spectrum of 1-MeP revealed maxima at λ_{max} 240, 266 (s = shoulder), 276, 334, 347, 362(s), and 382 nm. 1,6-DMeP gave UV absorbance peaks at λ_{max} 224(s), 234(s), 243, 267(s), 277, 320(s), 335, 350, 375, and 397 nm.

The mass spectral analysis of 1-MeP exhibited a molecular ion peak $[M]^+$ at *m/z* 232 (M) and fragment ion peaks at *m/z* 217 ($[M]^+$-CH_3), 200 (217-OH), and 189 (217-CO). The mass spectrum of 1,6-DMeP showed a base peak $[M]^+$ at *m/z* 262. Intense fragments at *m/z* 247 ($[M]^+$-CH_3), 232 ($[M]^+$-$2CH_3$), 204 (232-CO), and 176 (204-CO) were observed. The mass spectral analyses of the compounds were indicative of nono- and disubstituted methoxypyrenes. Table 2 summarizes the results obtained from mass spectral analysis.

The positions of the methoxy groups were determined by ^{1}H NMR spectral analysis. The NMR data of 1-MeP showed nine aromatic resonances and a characteristic three-proton singlet of a methoxy group (4.21 ppm). The spectrum was consistent with a pyrene ring system being substituted at the C-1 position (Lambert et al. 1994). 1,6-DMeP gave four aromatic signals, which all appeared as doublets, and one six-proton singlet for the methoxy substituents (4.18 ppm). The data clearly indicated a 1,6-disubstitution pattern. The ^{1}H chemical shifts and the coupling constants of both metabolites are given in Table 3.

When *P. glabrum* was incubated with 1-hydroxypyrene, the formation of the methoxypyrenes was also observed (Fig. 2A). In order to determine the coenzyme providing the methyl group, cultures of the fungus were grown in the presence of 1-hydroxypyrene and [*methyl*-^{3}H]SAM. When the cultures were analyzed for labeled metabolites after an incubation period of 10 days, both methoxypyrene peaks were labeled, as shown in Fig. 2B. In addition, a slight increase in the radioactivity content of some fractions collected between 15.0 and 17.5 min (as compared to the autoclaved control) was noted, suggesting the presence of more polar methylated derivatives (Fig. 2B).

In order to investigate whether 1,6-dimethoxypyrene is formed via hydroxylation and O-methylation of 1-methoxypyrene and in order to analyze for intermediary products, cultures of the fungus were incubated with 1-methoxypyrene (10 mg/l). After 10 days, 7.3 mg/l of the methoxypyrene added was recovered. As compared to the autoclaved control (recovery: 9.3 mg/l), 21.5% was metabolized by the fungus. As shown in Fig. 3A, analyses by HPLC confirmed that 1,6-dimethoxypyrene is one of the metabolic products formed from 1-methoxypyrene by *P. glabrum*. Furthermore, several other metabolites were observed; they eluted at 17.6 (I), 17.3, 8.2 (II), 7.9, and 6.4

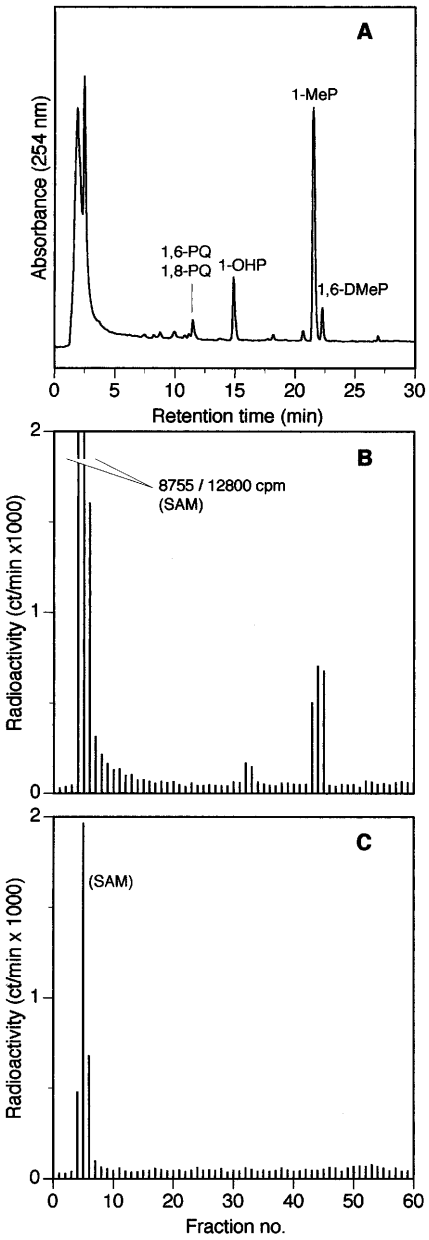

Fig. 2 **A** Chromatogram of a crude mycelial extract of *Penicillium glabrum* strain TW 9424 incubated with 5 mg/l 1-hydroxypyrene and 2.5 µCi [methyl-^{3}H]S-adenosylmethionine (SAM) for 10 days. The compounds were separated using gradient B. **B** Radioactivity determined in fractions from mycelial extracts collected during HPLC analysis. **C** Radioactivity present in fractions collected from mycelial extracts of heat-treated controls. The majority of the radioactivity was located in the culture filtrate. The total recovery rate of labeled SAM in the controls was 97.8%

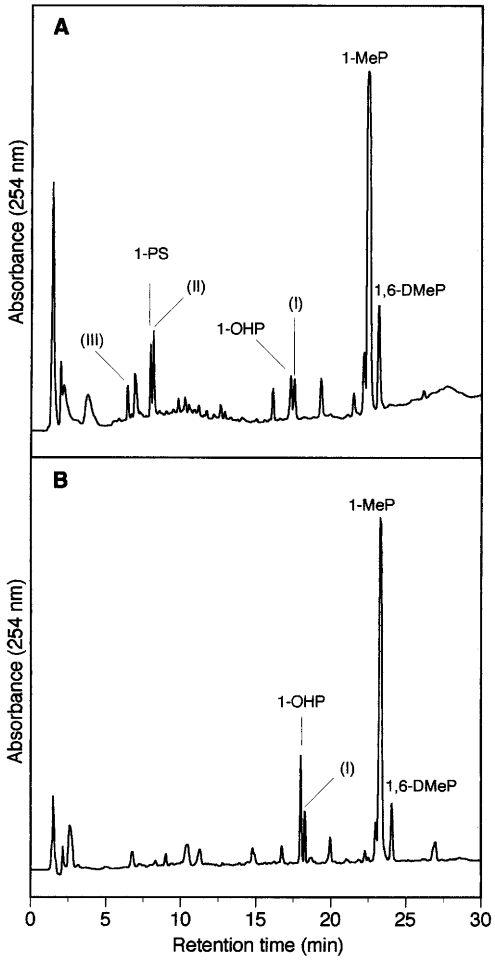

Fig. 3 **A** HPLC chromatogram of a crude mycelial extract of *Penicillium glabrum* strain TW 9424 incubated with 10 mg/l 1-methoxypyrene for 10 days. Concentrations (mg/l): 1-MeP (7.3); 1,6-DMeP (0.97); 1-OHP (0.35); 1-PS (0.53). **B** Chromatographic analysis of the same extract (diluted 1:1 with methanol) after treatment with arylsulfatase. Separation of the metabolites was achieved by using gradient A. Concentrations (mg/l): 1-MeP (3.82); 1,6-DMeP (0.41); 1-OHP (0.66) (*1-MeP* 1-methoxypyrene; *1,6 DMeP* 1,6-dimethoxypyrene; *1-OHP* 1-hydroxypyrene; *1-PS* 1-pyrenyl sulfate)

(III) min, respectively, and included 1-hydroxypyrene (17.3 min) and 1-pyrenyl sulfate (7.9 min) (Fig. 3A). The unidentified compounds designated (I), (II), and (III) revealed UV spectral properties nearly identical to those observed for 1,6-dihydroxypyrene and 1,6-dimethoxypyrene, suggesting that these metabolites might be derived from 1-methoxypyrene by further hydroxylation and substitution in the C-6 position. In the autoclaved control, no transformation products of 1-methoxypyrene were be detected.

As shown in the HPLC chromatogram in Fig. 3B, treatment of the crude mycelial extract with arylsulfatase type V resulted in the disappearance of the 1-pyrenyl sulfate peak and the peaks corresponding to (II) and (III), clearly indicating that these metabolites are sulfate conjugates (Cerniglia and Gibson 1979). At the same time, an increase in the amounts of 1-hydroxypyrene and metabolite (I) was noted.

Discussion

The deuteromycete *Penicillium glabrum* strain TW 9424 oxidizes pyrene at the C-1, C-6, and C-8 positions in a manner similar to that reported for zygomycetes, fungi

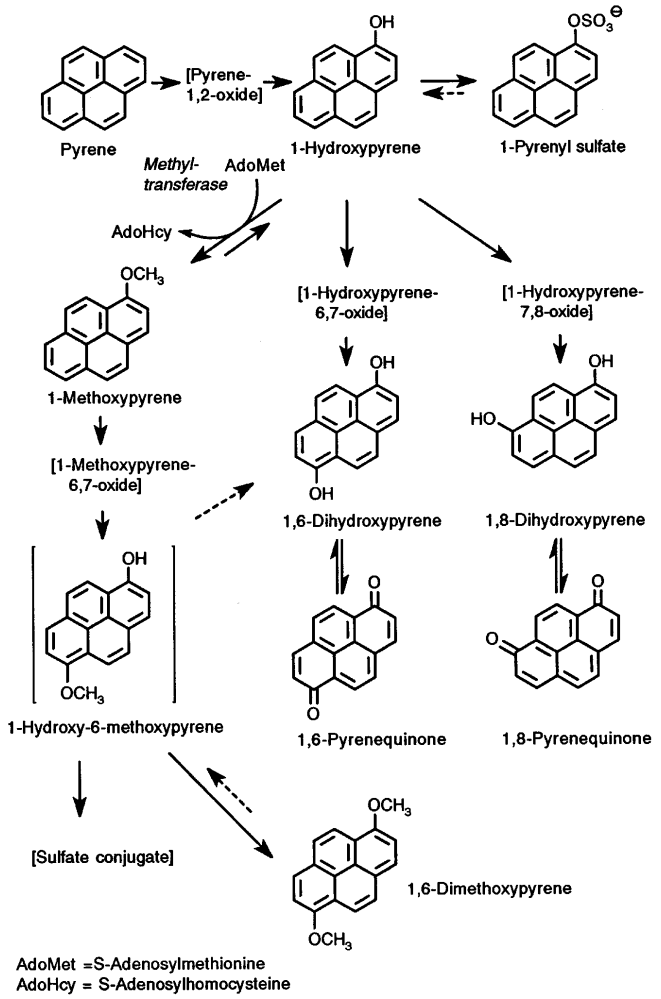

Fig. 4 Proposed pathways of the pyrene metabolism in *Penicillium glabrum* strain TW 9424

imperfecti, and non-white-rot basidiomycetes (Cerniglia et al. 1986; Lambert et al. 1994; Lange et al. 1994; Wunder et al. 1994, 1995; Launen et al. 1995).

The capacity of fungi to transform polycyclic aromatic hydrocarbon (PAH) phenols to water-soluble sulfate conjugates has been demonstrated in several studies (Cerniglia and Gibson 1979; Cerniglia 1982; Cerniglia et al. 1982; Lange et al. 1994; Wunder et al. 1994). Besides effecting sulfate conjugation, *Penicillium glabrum* transformed pyrene phenols by O-methylation, forming the novel pyrene metabolites 1-methoxypyrene and 1,6-dimethoxypyrene. The formation of the 1,6-dimethylated derivative most likely proceeds via hydroxylation of 1-methoxypyrene at the C-6 position with subsequent methylation of the second hydroxyl group. With its UV spectral properties (indicating a 1,6-disubstitution pattern) and the HPLC retention time of 17.6 min (matching a hydroxylated methoxypyrene), compound (I) might, therefore, be 1-hydroxy-6-methoxypyrene, the intermediary product in this pathway.

The possibility of O-methylation of the 1,6-dihydroxypyrene cannot be completely excluded. However, methylation of this compound is likely to occur only to a small extent since the dihyroxypyrenes and their respective quinones do not contribute much to the total amount of metabolites formed. On the assumption that 1-hydroxy-6-methoxypyrene is an intermediary product, it seems reasonable to suppose that one of the compounds (II) and (III) (Fig. 3A) represents a sulfate conjugate of 1-hydroxy-6-methoxypyrene. It will be clarified in the future whether two types of conjugation reactions, O-methylation and sulfation, can take place on the same PAH molecule.

The occurrence of 1-hydroxypyrene and 1-pyrenyl sulfate during the biotransformation of 1-methoxypyrene strongly suggests the presence of a demethylating enzyme. It is not clear whether the same enzyme is responsible for both methylation and demethylation. However, our findings show that fungal PAH metabolism has to be considered as a dynamic system of reactions that do not generally result in the formation of dead-end metabolites. Thus, the metabolic pathway of pyrene by *Penicillium glabrum* strain TW 9424 shown in Fig. 4 can be proposed.

The results obtained with labeling experiments imply a methylation mechanism catalyzed by a methyltransferase involving S-adenosyl-L-methionine (SAM) as the methyl group donor. This reaction is known from the methylation of phenolic compounds in mammalian tissues (Dingemanse et al. 1995; Tohgi et al. 1995) and in plants (Wollenweber and Dietz 1981; Ye and Varner 1995).

O-Methylation reactions occurring in fungal secondary metabolism result in the formation of numerous secondary metabolites with methoxy substituents, e.g., the fungicidal strobilurines (Anke et al. 1990) and oudemansines (Zapf et al. 1995) from basidiomycetes, or a variety of biologically active anthraquinones of the *Aspergillus glaucus* group (Anke et al. 1980). Fungal O-methyltransferases have been purified and characterized from *Aspergillus parasiticus* (Keller et al. 1993) and *Alternaria tenuis* (Stinson and Moreau 1986) both of which involve SAM in the biosynthesis of mycotoxins. In the biotransformation of xenobiotics, several species of the genus *Mycena* have been shown to methylate 2,3,5,6-tetrachlorohydroquinone, an intermediary product of the pentachlorophenol degradation pathway in these fungi, to tetrachloro-4-methoxyphenol (Kremer et al. 1992). This compound is also known as drosophilin A, a fungal secondary metabolite produced by many fungi (Schwarz et al. 1992). So far, O-methylation products in the metabolism of PAHs by fungi have not been isolated. The formation of 1-methoxyphenanthrene in phenanthrene-supplemented cultures of the marine cyanobacterium *Agmenellum quadruplicatum* has been reported by Narro et al. (1992). They have observed a distinct reduction of inhibitory effects of 9-methoxyphenanthrene (as compared to 9-hydroxyphenanthrene) when tested on the growth of *Agmenellum quadruplicatum* and have therefore suggested the methylation of the phenol to serve as a detoxification mechanism in this organism. The production of a methoxyphenanthrene from phenanthrene in cultures of a *Penicillium* species has been suggested, but without isola-

tion of the metabolite and without providing any data about the compound (Sack et al. 1995).

Since methylation of hydroxyl groups drastically lowers the water solubility of a phenolic compound (see chromatogram in Fig. 1), it is not clear whether this will result in less toxic products analogous to the sulfate, glycoside, and glucuronide conjugation reactions found in fungi. In several studies it has been shown that methylation of phenolic hydroxyl groups can result in a decrease of antibacterial and antifungal effects caused by the parent phenolic compound (Anke et al. 1980; Gupta et al. 1981; Narro et al. 1992; Buswell and Eridsson 1994). On the other hand, Buswell and Eriksson (1994) have demonstrated that O-methylation of the *para*-hydroxyl group of several benzoic and cinnamic acids enhances antifungal activities.

With respect to the important role of O-methylation reactions in the biosynthesis of fungal secondary metabolites, O-methylation of PAH phenols might be due to the activity of enzymes involved in secondary metabolism and does not represent a specific conjugation reaction in the fungal detoxification of PAHs. Recently Lange et al. (1995) have demonstrated the influence of PAHs on fungal secondary metabolism. Thus, O-methylation of PAHs could be a further indication of a close relationship between fungal secondary metabolism and fungal metabolism of PAHs.

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