Induction of Secondary Metabolism by Environmental Pollutants: Metabolization of Pyrene and Formation of 6,8-Dihydroxy-3-methylisocoumarin by *Crinipellis stipitaria* JK 364

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In submerged cultures, when grown in the presence of pyrene, the saprophytic and plant inhabiting basidiomycete *Crinipellis stipitaria* was found to produce 6,8-dihydroxy-3-methylisocoumarin. The amount of 6,8-dihydroxy-3-methylisocoumarin formed depended on the concentration of pyrene in the medium. In cultures incubated without pyrene or with less than 2.5 mg/l no formation of 6,8-dihydroxy-3-methylisocoumarin was observed. Besides pyrene, chrysene, another four ring polycyclic aromatic hydrocarbon, induced isocoumarin formation whereas anthracene, phenanthrene or fluoranthene were not effective. During metabolization experiments with [4,5,9,10-^{14}C]pyrene (0.4 mCi (14.8 kBq)/mmol), labelled 6,8-dihydroxy-3-methylisocoumarin (specific activity 15 µCi (0.6 kBq)/mmol) was isolated, in addition labelled acetic acid (specific activity 0.1 µCi (4 Bq)/mmol) was identified. When *C. stipitaria* JK 364 was incubated for 14 days with 5 µCi (0.2 kBq) sodium [2-^{14}C]acetate and 10 mg/l pyrene, labelled 6,8-dihydroxy-3-methylisocoumarin was isolated from the culture. The results are in agreement with the polyketide origin of the isocoumarin and proof the breakdown of pyrene to acetate which is a central intermediate in the pathway from pyrene to 6,8-dihydroxy-3-methylisocoumarin.

Introduction

The environmental and toxicological significance of polycyclic aromatic hydrocarbons (PAHs) causes an increasing interest in the potential of fungi to metabolize and detoxify these compounds (Cerniglia and Heitkamp, 1989; Cerniglia *et al.*, 1992; WHO, 1983). Many investigations have shown the ability of fungi to transform a wide range of PAHs under cometabolic conditions (Cerniglia, 1984; Lambert *et al.*, 1994; Lange *et al.*, 1994; Wunder *et al.* 1994; Sutherland, 1992). Fungi metabolize PAHs by cytochrome P450 monooxygenase and epoxide hydrolase enzyme systems to oxidation and conjugation products (Cerniglia *et al.*, 1985; Cerniglia *et al.*, 1986). The conjugation products like glucosides and sulfates are less toxic than the parent compounds, whereas the oxidation products like quinones or hydroxy-derivatives are more toxic (Cerniglia *et al.*, 1982; Lambert *et al.*, 1995; Okamoto and Yoshida, 1981). The white rot fungus *Phanerochaete chrysosporium* was shown to mineralize several polycyclic aromatic compounds like phenanthrene and pyrene (Hammel *et al.*, 1986; Hammel *et al.*, 1992).

In contrast to the number of studies on the oxidation and mineralization of PAHs by fungi, there are, to our knowledge, no reports about the role of secondary metabolism during microbial metabolization and detoxification of PAHs. Although fungi are known to produce a wide variety of secondary metabolites in response to environmental conditions (Elander and Lowe, 1992; Turner, 1971), nothing is known on the impact of environmental pollutants on secondary metabolism.

The basidiomycete *Crinipellis stipitaria* belongs to the order *Agaricales*, family *Tricholomataceae* and occurs worldwide on grasses and rotting plant material (Singer, 1986). Whereas all strains of the species produce crinipellins, antimicrobially active diterpenes with a tetraquinane skeleton (Anke *et al.*, 1985; Kupka *et al.*, 1979), metabolization of pyrene is strain specific. Strain JK 375 transformed

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pyrene to pyrenequinones and their hydroxylated precursors (Lambert *et al.*, 1994) and strain JK 364 to 1-pyrenylsulfate and *trans*-4,5-dihydro-4,5-dihydroxypyrene (Lange *et al.*, 1994).

During metabolization experiments, HPLC analyses revealed that cultures with pyrene produced a metabolite which could not be detected in the absence of pyrene and which was not related to the PAH or its metabolization products.

The present investigation describes the isolation and identification of 6,8-dihydroxy-3-methylisocoumarin, the induction of secondary metabolism in *C. stipitaria* by pyrene and other PAHs, and the incorporation of parts of the carbon skeleton of pyrene *via* acetate into 6,8-dihydroxy-3-methylisocoumarin.

Materials and Methods

Chemicals

[4,5,9,10-^{14}C]pyrene (specific activity 56 mCi/mmol) was obtained from Amersham-Buchler, Braunschweig, Germany. Chemical analyses of [^{14}C]pyrene by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with radioactivity detection showed that radiochemical purity exceeded 99%. Sodium [2-^{14}C]acetate (specific activity 53 mCi/mmol, radiochemical purity 97.5%) was also obtained from Amersham-Buchler. Unlabelled pyrene, fluoranthene and anthracene were purchased from Aldrich Chemie, Steinhofen, Germany. Phenanthrene was obtained from ICN Biochemicals, Cleveland, Ohio, USA and chrysene from Sigma Chemie, Deisenhofen, Germany.

HPLC grade solvents were purchased from Heraeus, Karlsruhe, Germany. All other chemicals were of reagent grade and the highest purity available.

General

Quantification of pyrene and detection of 6,8-dihydroxy-3-methylisocoumarin were performed by HPLC equipped with a Merck-Hitachi photo diode array detector operated at 254 nm and fitted with a Nucleosil 5 C-18 PAH column (inner dimensions: 150 mm × 4 mm; Macherey-Nagel, Düren, Germany). The HPLC elution program consisted of a 20-min linear gradient of acetonitrile/water (10:90 to 100:0 [vol/vol]) followed by isocratic elution with 100% acetonitrile for 5 min. The flow rate was 1 ml/min. The retention time of pyrene under these conditions was 21.7 min, the retention time of 6,8-dihydroxy-3-methylisocoumarin 11.0 min.

HPLC analyses of organic acids were done with an Aminex HPX-87H column (Biorad, München, Germany) eluted isocratically at 35 °C with phosphoric acid (0.1%)/acetonitrile (95:5). Retention time of acetic acid under these conditions was 14.7 min.

The UV spectrum of 6,8-dihydroxy-3-methylisocoumarin was determined in methanol with a Cary 17 UV spectrometer (Cary Instruments, Peckroad/Monrovia, California). Electron impact (EI) mass spectral analyses (ionizing energy 70 eV) were performed with a Joel SX 102 spectrometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance (NMR) measurements were carried out in $CDCl_3/CD_3OD$ (95 : 5) on a Bruker ARX 500 spectrometer with the solvent signals (7.26 and 77.0 ppm respectively) as reference.

Radioactivity was determined with a liquid scintillation counter (type Wallac 1410, Pharmacia, Wallac Oy, Turku, Finland).

Microorganism and culture conditions

Crinipellis stipitaria JK 364 (Kupka *et al.* 1979) was maintained on a MM1 agar composed of (g/l): glucose 10, yeast extract 0.3, $(NH_4)_2SO_4$ 1, $CaCl_2$ 0.1, NaCl 0.1, $MgSO_4 \times 7\,H_2O$ 0.5, K_2HPO_4 0.125, KH_2PO_4 0.875, trace salt solution 10 ml/l (Wedding and Kendrick, 1959). The pH value was adjusted to 5.5 before sterilization.

Fermentation and isolation of metabolites

Fermentations were carried out in a Biostat S apparatus (Braun, Melsungen, Germany) containing 10 liters of MM1 medium with agitation (120 rpm) and aeration (3.0 l air/min) at 25 °C. A well grown seed culture of *C. stipitaria* JK 364 (250 ml) in the same medium was used as inoculum. 6,8-Dihydroxy-3-methylisocoumarin was isolated from a fermentation in a 140-liter tank (E. Jachan Apparatebau, Bad Wimpfen, Germany) containing 100 liters of MM1 medium (inoculum 10%, 100 rpm, 25 °C).

After 6 days of incubation, pyrene dissolved in dimethylformamide was added to a final concentration of 20 mg/l. During the fermentation, 50 ml samples of the culture were withdrawn to follow the decrease of pyrene and the appearance of metabolites.

Samples were filtered to separate mycelia from the culture filtrate. The filtrate was extracted with two equal volumes of ethyl acetate, the combined extracts were dried with anhydrous sodium sulfate and evaporated under reduced pressure at 45 °C. The mycelia were extracted twice with acetone by stirring for 20 min each. The acetone extracts were combined and evaporated *in vacuo*. The dry residues of the crude extracts were dissolved in methanol and analyzed by HPLC.

For the isolation of 6,8-dihydroxy-3-methylisocoumarin, the fermentation was interrupted after 20 days, when the largest amount of 6,8-dihydroxy-3-methylisocoumarin was detected in the culture filtrate. The culture filtrate was passed through a column containing Mitsubishi HP 21 adsorber resin (Mitsubishi Chemical Industries, Tokyo, Japan) which was eluted with acetone. The extract was concentrated. The crude extract was purified by chromatography on silica gel (Merck silica gel 60, elution with cyclohexane/ethyl acetate, 2:8). The enriched product was further purified by solid phase extraction on a RP-18 phase. Final separation was achieved by preparative HPLC at RP-18 eluted isocratically with H_2O/methanol (45:55).

Incubation of *C. stipitaria* JK 364 with varying pyrene concentrations and with different PAHs (10 mg/l each) was carried out in 500-ml flasks containing 250 ml of MM1 medium. After 14 days of incubation, cultures were extracted and analyzed as described above for the fermentation samples.

Metabolization experiments with [^{14}C]pyrene and [^{14}C]acetate

Cultures of *C. stipitaria* (250 ml) were preincubated for 7 days before 5 μCi/2.5 mg of [4,5,9,10-^{14}C]pyrene (specific activity 0.4 mCi/mmol) or 5 μCi/0.5 mg sodium [2-^{14}C] acetate (specific activity 0.6 mCi/mmol) were added. To determine the release of $^{14}CO_2$ from labelled pyrene, cultures were ventilated with sterile air (1.0 l/min) and the effluent gas passed through a vial containing 20 ml of a 2 N KOH solution.

After 14 days of incubation, cultures were filtered to separate mycelia from the culture filtrate. The culture filtrate (240 ml) was adjusted to a pH value below 1.0 by adding 2.5 N sulfuric acid and extracted twice with 125 ml ethyl acetate. The extracts were combined and concentrated to a volume of 10 ml. The acetic acid was extracted with 1.0 ml of a 1.0 M K_2CO_3 solution. The carbonate solution was separated from the organic phase and passed through a membrane filter. Extraction of mycelia was performed as described above for the fermentaion samples.

For liquid scintillation counting aliquots were removed from each extract and added to vials containing 5 ml of scintillation fluid (Quickzint 501, Zinsser Analytic, Frankfurt, Main, Germany). To determine the radioactivity of single compounds, crude extracts were analyzed by HPLC with liquid scintillation counting of the eluate collected every 0.5 min.

Isolation of 6,8-dihydroxy-3-methylisocoumarin from cultures incubated with [^{14}C]pyrene was performed by preparative TLC on silica gel 60 $WF_{254}S$ (E. Merck, Darmstadt, Germany). The solvent used for TLC was cyclohexane/ethyl acetate/acetic acid (120 : 40 : 5). After separation silica gel containing 6,8-dihydroxy-3-methylisocoumarin was eluted with methanol and the isolated metabolite analyzed by HPLC.

Results

Isolation and structural elucidation of 6,8-dihydroxy-3-methylisocoumarin

A typical fermentation of *Crinipellis stipitaria* JK 364 in MM1 in presence of pyrene is shown in Fig. 1. The strain was preincubated for 6 days before 20 mg/l of pyrene were added. HPLC analyses revealed that pyrene disappeared completely within 32 days, on day 38, pyrene was no longer detectable. Five days after the addition of pyrene, 6,8-dihydroxy-3-methylisocoumarin appeared in the culture filtrate. During the following days concentration of 6,8-dihydroxy-3-methylisocoumarin increased and reached its maximum at the 20th day of fermentation. The maximum was followed by a fast decrease of the isocoumarin until, after 25 days, 6,8-dihydroxy-3-methylisocoumarin was no longer detectable.

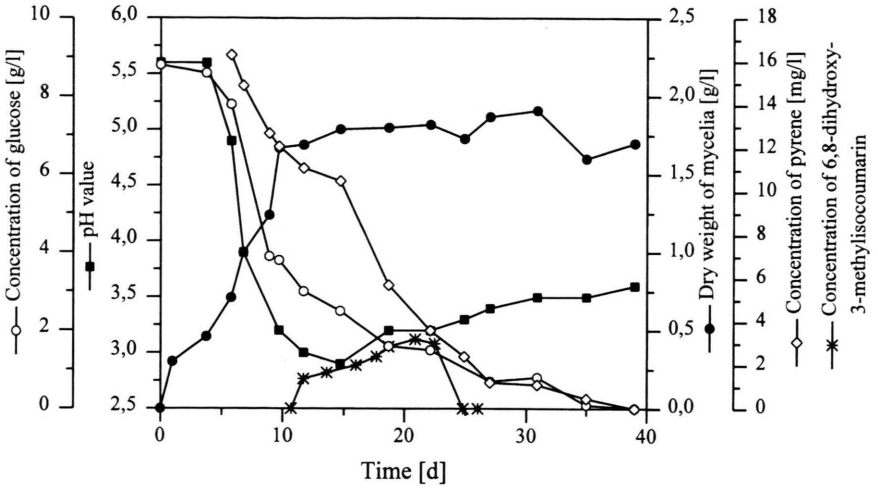

Fig. 1. Fermentation of *C. stipitaria* JK 364 in the presence of pyrene. The fungus was pregrown for 6 days before 20 mg of pyrene per liter were added. The pyrene concentration reflects the sum of mycelia and culture filtrate. ■ pH value, ● dry weight of mycelia, ○ glucose, ◇ pyrene, * 6,8-dihydroxy-3-methylisocoumarin.

For the isolation of 6,8-dihydroxy-3-methylisocoumarin, the fermentation was stopped after 20 days when the concentration was at its highest level of 3.2 mg/l. Isolation was performed as described in the experimental section.

6,8-Dihydroxy-3-methylisocoumarin exhibits a molecular ion at *m/z* 192 in the EI mass spectrum corresponding to the molecular formula $C_{10}H_8O_4$. The ^1H NMR spectrum reveals the presence of two isolated aromatic protons (δ_H 6.19 and 6.31) and one olefinic proton (δ_H 6.11) at the C-4 position of the lactone ring. The presence of the methyl group attached to the olefinic region was ascribed to the resonances δ_C 12.2 and δ_H 2.17. Furthermore ^{13}C NMR analysis showed the lactone carbonyl resonance at δ_C 165.2. All assignments of ^1H and ^{13}C NMR signals as well as the UV spectrum with maxima at 242 nm (log ε 3.98) and 320 nm (log ε 3.07) were consistent with those, described by Kumagai and coworkers (1994) for 6,8-dihydroxy-3-methylisocoumarin.

Induction of isocoumarin production by pyrene and other PAH

The influence of the pyrene concentration on the formation of 6,8-dihydroxy-3-methylisocoumarin is shown in Fig. 2. In cultures without pyrene or with 2.5 mg/l the isocoumarin could not be detected. The highest yield (4.6 mg) of 6,8-dihydroxy-3-methylisocoumarin/l was observed at 5 mg/l pyrene.

Besides pyrene, chrysene also induced the production of the isocoumarin. Anthracene, phenantrene and fluoranthene were not effective. In cultures containing 10 mg/l chrysene, 2.8 mg/l 6,8-dihydroxy-3-methylisocoumarin were formed.

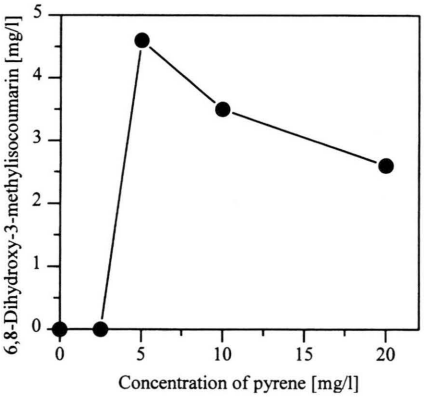

Fig. 2. Influence of pyrene on the production of 6,8-dihydroxy-3-methylisocoumarin in cultures of *C. stipitaria* JK 364.

Metabolization experiments with [^{14}C]pyrene and sodium [^{14}C]acetate

When *C. stipitaria* was incubated for 14 days with 5 μCi [4,5,9,10-^{14}C]pyrene (0.4 mCi/mmol) the total recovery rate of the radioactivity in the mycelium and culture filtrate was 97%. 67% of the labelled substrate were converted into metabolites extractable with ethyl acetate; unmetabolized

pyrene accounted for 29% of the radioactivity (Table I). 1% was recovered as $^{14}CO_2$ in the KOH solution. The formation of $^{14}CO_2$ was confirmed by acidification of the solution and reprecipitation of the labelled carbon dioxide in an adjacent washing bottle. 6,8-Dihydroxy-3-methylisocoumarin (0.07 μCi) was only detected in the ethyl acetate extract of the culture filtrate, the concentration was 3.6 mg/l. The labelled compound was purified by TLC and quantified by HPLC and liquid scintillation counting of the eluate. Fig. 3A shows the HPLC elution profile at 240 nm, Fig. 3B shows the radioactive labelling of 6,8-dihydroxy-3-methylisocoumarin. The specific radioactivity of 6,8-dihydroxy-3-methylisocoumarin was determined as 15 μCi/mmol. This clearly demonstrates, that parts of the carbon skeleton of pyrene were incorporated into the methylisocoumarin molecule.

Methylisocoumarins are synthesized *via* the polyketide pathway (Turner, 1971; Kumagai *et al.*, 1994). Therefore cultures of *C. stipitaria* incubated with [^{14}C]pyrene were analyzed for the presence of radioactive labelled acetic acid, the precursor of the ^{14}C-labelled isocoumarin. Radioactivity of the K_2CO_3 extract of organic acids accounted for 1.7% of the total radioactivity recovered. HPLC analysis of the extract revealed the presence of acetic acid which was identified by comparing the retention time and UV spectrum with an authentic standard. The total amount of acetic acid in the culture filtrate was 240 mg/l with a specific activity of 0.1 μCi/mmol.

To confirm that acetic acid is the precursor of 6,8-dihydroxy-3-methylisocoumarin in cultures of *C. stipitaria*, the organism was incubated with 5 μCi (0.5 mg) sodium [2-^{14}C]acetate (specific activity 0.6 mCi/mmol) and with 10 mg/l unlabelled pyrene. After 14 days of incubation *C stipitaria* had produced 3.5 mg/l 6,8-dihydroxy-3-methylisocoumarin which was radioactively labelled with a specific activity of 16 μCi/mmol.

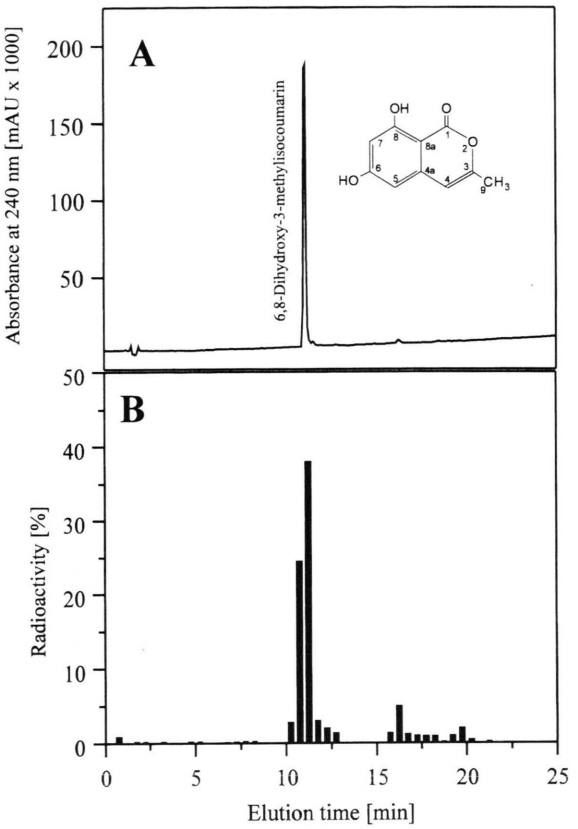

Fig. 3. HPLC chromatogram (A) and radioactivity elution profile (B) of the TLC fraction containing 3-methyl-6,8-dihydroxyisocoumarin. The sample (20 μl) applied to the HPLC contained 0.04 μCi/ml. Fractions eluting from the column were collected at 0.5 min intervals.

Table I. Distribution of radioactivity among pyrene and pyrene metabolites in cultures of *C. stipitaria* JK 364 incubated for two weeks with 5 μCi (2.5 mg) [^{14}C]pyrene (total recovery rate: 97 %).

Compound	Radioactivity [%]
Pyrene	29.9
1-Pyrenylsulfate	19.6
trans-4,5-Dihydro-4,5-dihydroxypyrene	4.1
1-Hydroxypyrene	3.1
6,8-Dihydroxy-3-methylisocoumarin	1.4
Not identified	39.2
K_2CO_3 solution	1.7
Carbon dioxide	1

Discussion

C. stipitaria JK 364 was found to produce 6,8-dihydroxy-3-methylisocoumarin during the metabolization of pyrene. In absence or at low concentrations of pyrene no formation of the isocoumarin derivative was observed. In addition to pyrene, chrysene also induced the formation of 6,8-dihydroxy-3-methylisocoumarin, whereas anthracene, phenanthrene, and fluoranthene were not effective.

Formation of crinipellins (Anke *et al.*, 1985; Kupka *et al.*, 1979) was not observed in cultures of *C. stipitaria* JK 364 during the metabolization of pyrene.

6,8-Dihydroxy-3-methylisocoumarin is known as a metabolite of the ascomycetes *Ceratocystis minor* and *Ceratocystis ulmi* (Ayer *et al.*, 1986; Claydon *at al.*, 1974; McGraw and Hemingway, 1977) and was shown to exhibit phytotoxic activities, while stimulating the transpiration in pine seedlings (DeAngelis *et al.*, 1985; Hemingway *et al.*, 1977).

From cultures of *C. stipitaria* JK 364 which were incubated with [^{14}C]pyrene, radioactive labelled 6,8-dihydroxy-3-methylisocoumarin and labelled acetic acid were isolated. This demonstrates the breakdown of the pyrene molecule to acetate and that parts of the carbon skeleton of pyrene were incorporated into the isocoumarin molecule which is formed *via* the polyketide pathway as shown by the incorporation of [^{14}C]acetate. Therefore, acetyl-CoA is a central intermediate in the pathway from pyrene to 6,8-dihydroxy-3-methylisocoumarin. Concentration of labelled acetic acid in the culture filtrate of *C. stipitaria* was quite high (240 mg/l). This explains the low specific activity of acetic acid (0.1 μCi/mmol) in comparison with the labelling of 6,8-dihydroxy-3-methyl-isocoumarin (15 μCi/mmol). Incubation of *C. stipitaria* with highly labelled acetate (0.6 mCi/mmol) resulted in the formation of 6,8-dihydroxy-3-methylisocoumarin with a significantly lower labelling (16 μCi/mmol) than expected from the condensation of five acetate units. This also demonstrates that labelled acetate in the culture is diluted by unlabelled acetic acid arising from the metabolization of glucose. The excretion of acetate into the culture filtrate during the metabolization of glucose is in accordance with the fast decrease of the pH value in the beginning of the fermentation (Fig. 1). As shown in the fermentation diagram, formation of 6,8-dihydroxy-3-methylisocoumarin by *C. stipitaria* started in the late growth phase of the fungus shortly after the beginning of pyrene metabolization which indicates that degradation of pyrene to acetate is closely connected with the synthesis of 6,8-dihydroxy-3-methylisocoumarin from acetyl-CoA units. The high specific activity of 6,8-dihydroxy-3-methylisocoumarin shows that acetyl-CoA, the building block for the isocoumarin, which remains within the cells is less diluted than acetic acid which is excreted into the culture filtrate and diluted by previously formed, unlabelled acetic acid.

Little is known about the mechanism of ring fission of PAHs containing more than three rings (Guerin and Jones, 1988), although some bacterial ring cleavage products of pyrene, like 4-phenanthroic acid and 4-hydroxyperinaphthenone, have been identified (Heitkamp *et al.*, 1988; Walter *et al.*, 1991). At present the mechanism of acetate release from the pyrene molecule as well as ring cleavage products formed by *C. stipitaria* are still unknown and remain to be elucidated.

Although there have been many reports on the biological activity of PAHs and their transformation products formed by fungi (Cerniglia *et al.*, 1982; Okamoto and Yoshida, 1981), no attention has been paid to secondary metabolism and metabolites produced during PAH metabolization and the toxicity of these secondary metabolites. The present study shows that secondary metabolism of *C. stipitaria* can be induced by particular PAHs which results in the formation of a phytotoxic metabolite. Furthermore results of experiments with radioactive labelled pyrene demonstrate, that not only transformation and mineralization of PAHs contribute to the degradation of these compounds, secondary metabolism as well has to be taken into consideration when fungal PAH metabolism is investigated.

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