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# Novel Metabolites in Phenanthrene and Pyrene Transformation by *Aspergillus niger*

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***Aspergillus niger*, isolated from hydrocarbon-contaminated soil, was examined for its potential to degrade phenanthrene and pyrene. Two novel metabolites, 1-methoxyphenanthrene and 1-methoxypyrene, were identified by conventional chemical techniques. Minor metabolites identified were 1- and 2-phenanthrol and 1-pyrenol. No $^{14}CO_2$ evolution was observed in either [$^{14}$C]phenanthrene or [$^{14}$C]pyrene cultures.**

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic environmentally persistent compounds and are genotoxic at up to four or five fused aromatic rings (6). Several wood-decaying fungi are capable of detoxifying and mineralizing PAHs (3, 4, 19, 21, 22). Little is known about the ability of soil fungi to metabolize PAHs. Most attention has been focused on deuteromycetes, such as *Penicillium* spp. (14, 18) and *Aspergillus* spp. (5, 20, 23). The zygomycete *Cunninghamella elegans* has been studied extensively to investigate possible pathways for the detoxification of PAHs. In the case of phenanthrene and pyrene, *C. elegans* is able to transform these PAHs to *trans*-dihydrodiols, phenols, and quinones and to form glycoside conjugates (7–9). Wunder et al. (23) reported that an *Aspergillus niger* strain transformed pyrene to 1-pyrenol, 1,6- and 1,8-pyrenequinone, 1,6- and 1,8-dihydroxypyrene, 1-pyrenyl sulfate, and 1-hydroxy-8-pyrenyl sulfate. In soil, such ring oxidation products are able to form bound residues through enzymatic or spontaneous reactions with soil organic matter (17). Ring oxidation products of PAHs are also substrates for bacteria or other indigenous soil microorganisms to mineralize and thus enhance mineralization of PAHs (10). This paper describes novel metabolites of phenanthrene and pyrene degradation by an *A. niger* strain isolated from mineral oil-contaminated soil in La Plata, Argentina.

**Metabolism of [$^{14}$C]phenanthrene and [$^{14}$C]pyrene.** Kinetics of [ring U-$^{14}$C]phenanthrene (4.33 GBq/mmol; radiochemical purity after thin-layer chromatography in benzene, 96.5%; Amersham Buchler, Braunschweig, Germany) and [4,5,9,10-$^{14}$C]pyrene (2.07 GBq/mmol; radiochemical purity, 98%; Amersham Buchler) metabolism were determined with 300-ml Erlenmeyer flasks containing 100 ml of Czapek-Dox medium (12) that were inoculated with 15-mycelium 1-cm$^2$ plugs of malt agar cultures of *A. niger* van Tieghem (strain b52, DSM 11167) isolated from mineral oil-contaminated soil of an oil refinery in La Plata, Argentina, as described previously (1). After 24 h, 100 μl of phenanthrene or pyrene solution (37 kBq of radiolabelled and unlabelled PAH with a final concentration of 5 mg/100 ml, dissolved in dimethylformamide) was added to each sample. The flasks were aerated continually with $CO_2$-free, humidified air. The air was passed through dimethylformamide to trap volatile organic substances. The liberated $^{14}CO_2$ was trapped in 2 N NaOH and quantified as described previously (19). Sterile controls to detect abiotic PAH decrease were prepared. The experiments were conducted in triplicate in the dark at 24°C. For mass balance analysis, culture fluid and mycelia were extracted with ethyl acetate after an incubation time of 63 days by the procedure of Sack et al. (19). The combined extracts were dried and analyzed by high-pressure liquid chromatography (HPLC) as described previously (19). One-milliliter HPLC fractions were collected every 1 min in scintillation vials and mixed with scintillation cocktail, and the radioactivity of each fraction was determined by liquid scintillation counting (19). After extraction, mycelia were air dried, homogenized, and combusted in an oxidizer (Biological oxidizer OX 500; Zinsser Analytic) (19).

Table 1 illustrates the mass balance analysis of [$^{14}$C]phenanthrene and [$^{14}$C]pyrene after 63 days of incubation. No $^{14}CO_2$ evolution in either phenanthrene or pyrene cultures was observed; since *A. niger* does not contain extracellular radical generating enzymes, such as ligninases, the lack of $CO_2$ liberation is not surprising. In phenanthrene metabolism, 3.1% (abiotic control, 19.4%) of radioactivity was from volatile organic substances, and in pyrene metabolism, 0.1% (abiotic control, 1.1%) was from volatile organic substances. Less than 10% of initial radioactivity was detected in the aqueous phases. A total of 33.9% (phenanthrene) and 10.1% (pyrene) of radioactivity was cell associated in mycelia (final dry weight, 3.5 g/liter) and was considered to be unreacted phenanthrene and pyrene. When *A. niger* was grown in the presence of [$^{14}$C]phenanthrene, 51.4% of the radioactivity initially added was found in the ethyl acetate extract; 20% of this extract was from polar metabolites, and 10% was from metabolites with a retention time higher than that of phenanthrene. A total of 88.2% of the radioactivity initially added was ethyl acetate extractable after cultivation of *A. niger* in the presence of [$^{14}$C]pyrene; 2.5% of this extract was polar metabolites, and 3% was less polar than pyrene.

**Isolation, detection, and identification of metabolites.** To identify phenanthrene and pyrene metabolites, the fungus was cultivated in 1,000-ml Erlenmeyer flasks containing 400 ml of Czapek-Dox medium and inoculated with 50-mycelium plugs. Phenanthrene or pyrene (20 mg) was dissolved in 500 μl of dimethylformamide and added to one flask after 24 h of incu-

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TABLE 1. Mass balance analysis of radioactivity initially added (37 kBq) as [$^{14}$C]phenanthrene and [$^{14}$C]pyrene to liquid cultures of *A. niger* after a period of 63 days[a]

| Source of radioactivity | Radioactivity (%) | | | |
|---|---|---|---|---|
| | [ring U-$^{14}$C] phenanthrene | | [4,5,9,10-$^{14}$C] pyrene | |
| | *A. niger* | Control | *A. niger* | Control |
| $^{14}CO_2$ | 0.7 | 0.3 | 0.2 | 0.1 |
| Volatile organic substances | 3.1 | 19.4 | 0.1 | 1.1 |
| Ethyl acetate extract | 51.4 | 67.9 | 88.2 | 97.3 |
| Aqueous phase | 9.6 | 7.8 | 1.2 | 0.1 |
| Mycelia | 33.9 | 0 | 10.1 | 0 |
| Balance | 98.7 | 95.4 | 99.8 | 98.6 |

[a] All values are means ($n$ = 3) with a standard deviation of <3%.

bation in darkness. The combined extracts were dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure at 40°C. The residue was dissolved in methanol and analyzed by HPLC (Merck, Darmstadt, Germany) with a model L 4500 diode array detector operating at 254 nm as described previously (19). Gas chromatography-mass spectrometry analyses were performed with a Varian Saturn II (Varian Instrument Group, Walnut Creek, Calif.) ion trap mass spectrometer (electron ionization mode with an electron energy of 70 eV; manifold temperature, 210°C). The instrument was scanned from 50 to 450 Da in 1 s. A DB 5 ms capillary column (0.25 by 30 m, 0.25 μm thick) (Durabond) was used for gas chromatography separation. $^1$H-nuclear magnetic resonance ($^1$H-NMR) spectra were obtained at ambient temperature with a Bruker AM500 spectrometer (Bruker Instruments, Billerica, Mass.). Samples were dissolved in deuterated acetone, with the residual proton signal assigned as 2.04 ppm. Data acquisition conditions were as follows: spectrometer frequency, 500 MHz; data size, 32K; sweep width, 7,042 Hz; filter width, 8,900 Hz. Data were zero filled to 64K for determination of coupling constants. $^1$H-NMR resonance assignments were made by proton integration, homonuclear decoupling experiments, and nuclear Overhauser effect experiments.

Phenanthrene was metabolized to small amounts of 1- and 2-phenanthrol by *A. niger*. The metabolites were identified by comparing their relative HPLC retention times (23.1 and 21.7 min), UV-visible absorption spectra, and mass spectra to previously published spectra and electron-impact mass-spectral properties (2, 5) after 63 days of incubation. The major metabolite (which made up 8.2% of the radioactivity among the metabolite fraction of the ethyl acetate extract) identified had a retention time of 36.7 min. This indicated a metabolite less polar than phenanthrene. It had a UV absorption spectrum with maximum wavelength ($\lambda_{max}$) values at 251, 292, 302, 331, and 346 nm, and a mass spectrum (Fig. 1) with a molecular ion ($M^{+\cdot}$) at $m/z$ 208 and fragment ions at $m/z$ 193 ($M^+ - {}^{\cdot}CH_3$) and $m/z$ 165 ($M^+ - {}^{\cdot}CH_3$ and $-CO$), which is similar to the spectrum reported for 1-methoxyphenanthrene, formed by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6 (15). $^1$H-NMR spectroscopy analysis was conducted to determine the configuration of this metabolite. Resonance assignments (parts per million) and coupling constants (hertz) for the metabolite are as follows: 4.05 (s, 3, $-OCH_3$), 7.15 (d, 1, $J_{2,3}$ = 8.0, $J_{2,4}$ = 0.6, H2), 7.61 (t, 1, $J_{3,4}$ = 8.2, H3), 7.62 (m, 1, $J_{7,8}$ = 7.8, H7), 7.67 (m, 1, $J_{6,8}$ = 1.4, H6), 7.80 (d, 1, $J_{9,10}$ = 9.2, H9), 7.96 (d, 1, H8), 8.21 (d, 1, H10), 8.37 (d, 1, H4), 8.77 (d, 1, $J_{5,6}$ = 8.2, $J_{5,7}$ = 1.4, H5). The metabolite was identified as 1-methoxyphenanthrene.

In pyrene metabolism by *A. niger*, two major metabolites and one minor metabolite were identified. The minor metabolite had an HPLC retention time of 13.5 min and a UV absorption spectrum with $\lambda_{max}$ values at 244, 267, 275, 333, 345, 372, and 389 nm, which were comparable to those published for 1,6-dihydroxypyrene (13, 23). The metabolite was not formed in quantities sufficient for isolation or further structural characterization. Major metabolite I had an HPLC retention time (27.8 min), a UV absorption (with $\lambda_{max}$ at 241, 276, and 341 nm), and a mass spectrum consistent with those of synthetic 1-pyrenol. Major metabolite II (which made up 10.2% of ra-

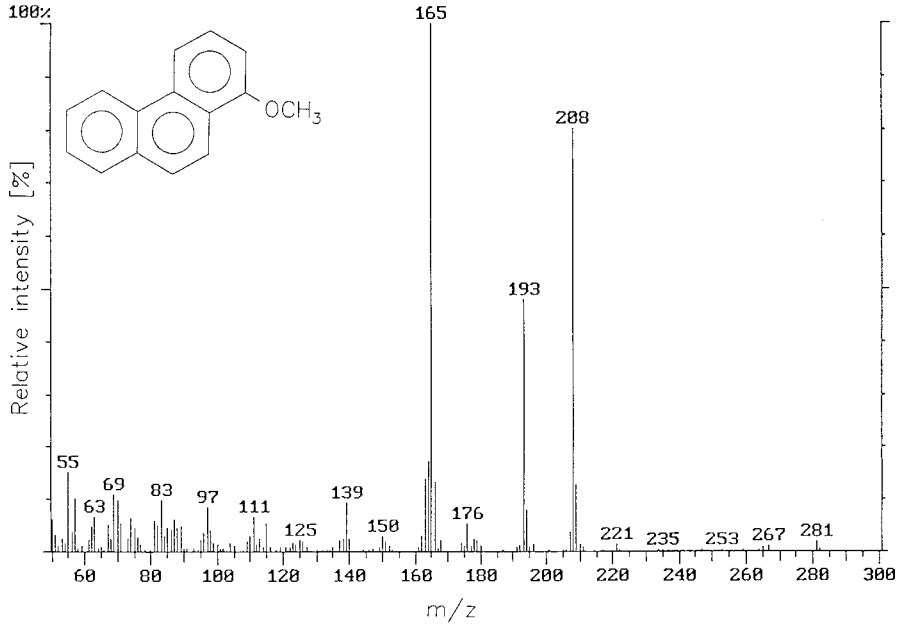


FIG. 1. Mass spectrum of 1-methoxyphenanthrene produced by *A. niger*.

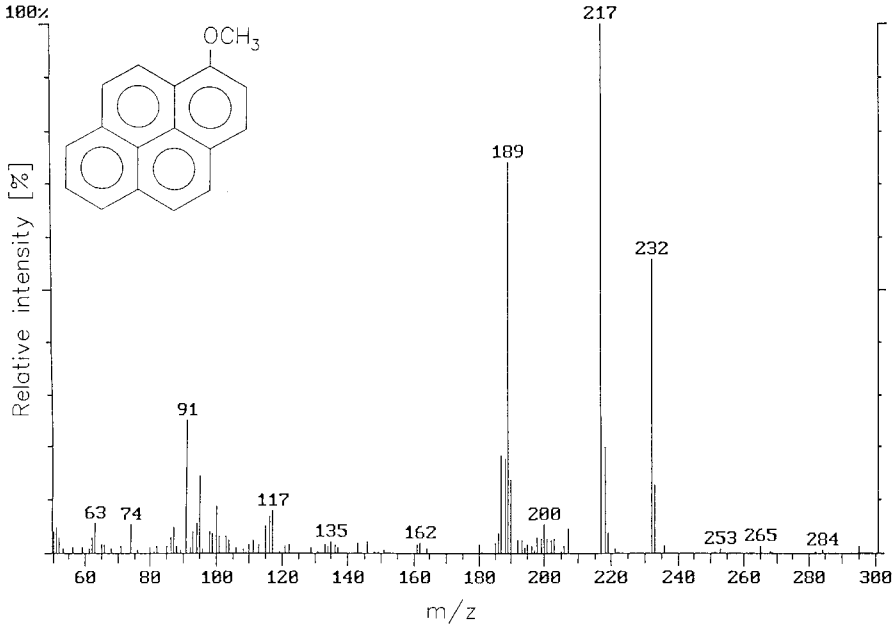


FIG. 2. Mass spectrum of 1-methoxypyrene produced by *A. niger*.

dioactivity among the metabolite fraction of the ethyl acetate extract) had an HPLC retention time of 40.6 min and a UV absorption spectrum with $\lambda_{max}$ values at 232, 266, 275, 341, and 372 nm. The mass spectrum (Fig. 2) had a molecular ion ($M^{+\cdot}$) at *m/z* 232 and fragment ions at *m/z* 217 ($M^+ - {}^{\cdot}CH_3$) and *m/z* 189 ($M^+ - {}^{\cdot}CH_3$ and $-CO$). The similarity of the UV spectrum to that of 1-pyrenol and the similarity of the mass-spectral fragmentation to that of methoxyphenanthrene indicated that the metabolite was methoxypyrene. The structure was identified by $^1$H-NMR spectroscopy as 1-methoxypyrene. Resonance assignments (parts per million) and coupling constants (hertz) for the metabolite are as follows: 4.20 (s, 3, $-OCH_3$), 7.73 (d, 1, $J_{2,3}$ = 8.4, H2), 7.94 (d, 1, H5), 7.99 (t, 1, $J_{7,8}$ = 7.5, H7), 8.03 (d, 1, $J_{4,5}$ = 9.0, H4), 8.10 (d, 1, $J_{9,10}$ = 9.2, H9), 8.15 (d, 1, $J_{6,7}$ = 7.7, $J_{6,8}$ = 1.1, H6), 8.18 (d, 1, H8), 8.23 (d, 1, H3), 8.43 (d, 1, H10).

To our knowledge, 1-methoxyphenanthrene and 1-methoxypyrene are novel metabolites in the transformation of PAHs by fungi which have not previously been isolated. Narro et al. (15) described the formation of 1-methoxyphenanthrene by the marine cyanobacterium *A. quadruplicatum* PR-6 and postulated a methylation of 1-phenanthrol. 1-Phenanthrol and 1-pyrenol production by *A. niger* were identified, and these PAHs could be precursors of the methoxylated PAH derivatives. To date, methylation reactions of hydroxyl groups of monoaromatic compounds (chloro- and nitrocatechols) have been reported only for fungi such as *Penicillium frequentans* (11) and *Phanerochaete chrysosporium* (16).

*A. niger* metabolized phenanthrene and pyrene like other soil fungi. Casillas et al. (5) reported 1-phenanthrol, 2-phenanthrol, and phenanthrene *trans*-9,10-dihydrodiol as major metabolites from the metabolism of phenanthrene by *A. niger*. Glycoside, glucuronide, sulfate, or xyloside conjugate formation, as described by Wunder et al. (23), was not observed with *A. niger* after a cultivation period of 63 days. The formation of the conjugates may be growth medium dependent. Cerniglia et al. measured degradation of phenanthrene by *C. elegans*; they (7) reported that a maximum accumulation of the glycoside conjugate of 1-phenanthrol occurred 72 h after the addition of phenanthrene and subsequently declined.

The formation of methoxylated metabolites may be one way for fungi to detoxify 1-phenanthrol and 1-pyrenol, since Narro et al. (15) have demonstrated inhibition of *A. quadruplicatum* growth by 9-phenanthrol with an alga lawn bioassay. However, no effect on growth after addition of 9-methoxyphenanthrene has been observed.

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