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Biotransformation of phthalazine by *Fusarium moniliforme* and *Cunninghamella elegans*

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Abstract: *Fusarium moniliforme* and *Cunninghamella elegans* were grown in fluid Sabouraud medium at 28 C. They were dosed with 6.2 mM phthalazine and then incubated for 3 d with shaking at 125 rpm. After extraction with ethyl acetate, the metabolites were purified by HPLC and identified by the UV/visible, mass, and NMR spectra. The results show that *F. moniliforme* oxidized about 42% of the phthalazine to 1(2*H*)-phthalazinone (= 1-hydroxyphthalazine) and that *C. elegans* oxidized about 6% of the phthalazine to a novel metabolite, phthalazine *N*-oxide (= phthalazine 2-oxide).

Key Words: azaarenes, heterocyclic compounds, *N*-oxides, phthalazinone

Several fungi are known to metabolize polycyclic azaarenes, such as carbazole (Holland et al 1986), indole (Kamath and Vaidyanathan 1990), and acridine, isoquinoline, and quinoline (Sutherland et al 1994a, b). One azaarene that has pharmacological relevance is phthalazine (= 2,3-diazanaphthalene), which is produced during the in vivo metabolism of the antihypertensive drug hydralazine (Lesser et al 1974). It is also found in certain specialized paper products (Lande et al 1987).

Phthalazine is slightly toxic to aquatic microorganisms and moderately toxic to plants (Schultz and Cajina-Quezada 1982, Lande et al 1987). It induces aldehyde oxidase and xanthine oxidase activity in rabbits (Johnson et al 1984). Although phthalazine is oxidized to 1(2*H*)-phthalazinone by enzymes found in bacteria (Lehmann et al 1994, Stephan et al 1996, Sutherland et al 1998) and mammals (Stubley et al 1979), it is not known whether any fungi can metabolize it.

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During an investigation of the fungal production of *trans*-dihydrodiols, phenols, *N*-oxides, and other potential metabolites from various azaarenes, we tested *Fusarium moniliforme* Sheldon and *Cunninghamella elegans* Lendner with phthalazine. We found that both of these fungi oxidized phthalazine, but they converted it to different metabolites.

Cultures of *F. moniliforme* strain 279 (Sutherland et al 1983) and *C. elegans* ATCC 36112 were grown on potato dextrose agar plates (Remel, Inc., Lenexa, Kansas). The agar containing the mycelia was macerated in sterile blender cups with 30 mL fluid Sabouraud medium (Remel). Quadruplicate 125-mL Erlenmeyer flasks, each containing 30 mL fluid Sabouraud medium, were inoculated with 1 mL of one of the mycelial suspensions. The cultures and quadruplicate uninoculated controls were incubated at 28 C with shaking at 125 rpm. After 3 d, phthalazine (98%, Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in water, filter-sterilized, and added aseptically to the cultures and controls at a final concentration of 6.2 mM. The flasks were incubated at 28 C with shaking at 125 rpm for another 3 d.

Following incubation, the cultures and controls were extracted with three equal volumes of ethyl acetate. The extracts from each culture were combined and the solvent was evaporated in vacuo. Methanol (2 mL) was added to each extract before analysis.

The fungal metabolites were analyzed by reversed-phase HPLC, using a Hewlett-Packard HP1100 chromatograph (Palo Alto, California) with a Phenomenex (Torrance, California) Prodigy 5-μm ODS-3 column. The mobile phase consisted of a 20-min gradient of 18% to 58% methanol in buffer (50 mM potassium phosphate, pH 7.5) at a flow rate of 1 mL/min. The UV detector was at 254 nm. The individual metabolites were isolated by HPLC methods (Sutherland et al 1994b, 1998) and concentrated in vacuo. UV/visible absorption spectra were obtained in methanol with a Shimadzu UV-2101PC spectrophotometer (Kyoto, Japan).

Gas chromatography/mass spectrometry (GC/MS) was performed using a Finnigan model 4500 instrument (Finnigan Corp., San Jose, California) at a pressure of 1.5×10^{-6} torr; the quadrupole was scanned from *m/z* 60 to 460 in 1.0 s. The source temperature was 150 C and the injector was at 250 C; the electron voltage was 70 eV. A J&W capillary DB5ms column (30 m × 0.25 mm diam × 0.25 μm thick; J&W Scientific, Folsom, California) was used. Direct exposure probe mass spectrometry (DEP/MS) was done with a Finnigan model TSQ700 instrument; the source temperature was 150 C and the quadrupole was scanned from *m/z* 35 to 635 in 0.5 s.

^{1}H-NMR spectroscopy was performed using a Bruker

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TABLE I. HPLC elution times and peak areas measured at 254 nm for phthalazine and the two metabolites produced by *Fusarium moniliforme* and *Cunninghamella elegans*

Peak	Time (min)	Peak areas at 254 nm ($\times 10^3$ mAU)[a]		
		F. moniliforme	*C. elegans*	Control
Phthalazine *N*-oxide	7.2	0.0	4.2 ± 0.6	0.0
Phthalazine	12.5	69.2 ± 7.4	112.8 ± 25.0	119.9 ± 5.7
1(2*H*)-Phthalazinone	13.3	102.2 ± 9.0	0.0	0.0

[a] Injection volumes were 10 μL.

AM500 NMR spectrometer (Billerica, Massachusetts) at 500.13 MHz with metabolites dissolved in acetone-d_6 (Sutherland et al 1994a). The residual acetone signal was assigned as 2.04 ppm. Assignments were made by integration, homonuclear decoupling experiments, and nuclear Overhauser enhancement (NOE) experiments.

The results of the HPLC analysis (TABLE I) show that *F. moniliforme* removed about 42% of the added phthalazine and *C. elegans* about 6%. The metabolites produced by the two fungi eluted at different times; the times and peak areas (A_{254}) are shown in TABLE I. Extracts from the noninoculated controls had no peaks that corresponded to either of these metabolites.

Fusarium moniliforme produced one metabolite from phthalazine, with a peak area that represented 60% of the total A_{254} (TABLE I). The retention times of phthalazine and the metabolite were 12.5 min and 13.3 min, respectively (TABLE I). The elution time and UV/visible spectrum of the metabolite were identical to those of authentic 1(2*H*)-phthalazinone (99%, Aldrich Chemical Co.). The mass spectrum (FIG. 1A), which was obtained by DEP/MS and shows ions at *m/z* 146 [$M^{+\cdot}$], 118 (M^+-CO), 90, 89 (M^+-CON_2H), and 63, was the same as that of the standard. Since the ^{1}H-NMR chemical shifts and coupling constants [7.84 (1 H, tr, $J_{7,8}$ = 7.8 Hz, H7), 7.90–7.94 (2 H, m, $J_{5,7}$ = 2.4, $J_{6,7}$ = 7.2, H5 and H6), 8.26 (1 H, s, H4), 8.30 (dm, $J_{4,8}$ = 0.9 Hz, H8), 11.69 (1 H, bs, NH)] were also identical to those of the standard, the *F. moniliforme* metabolite was identified as 1(2*H*)-phthalazinone.

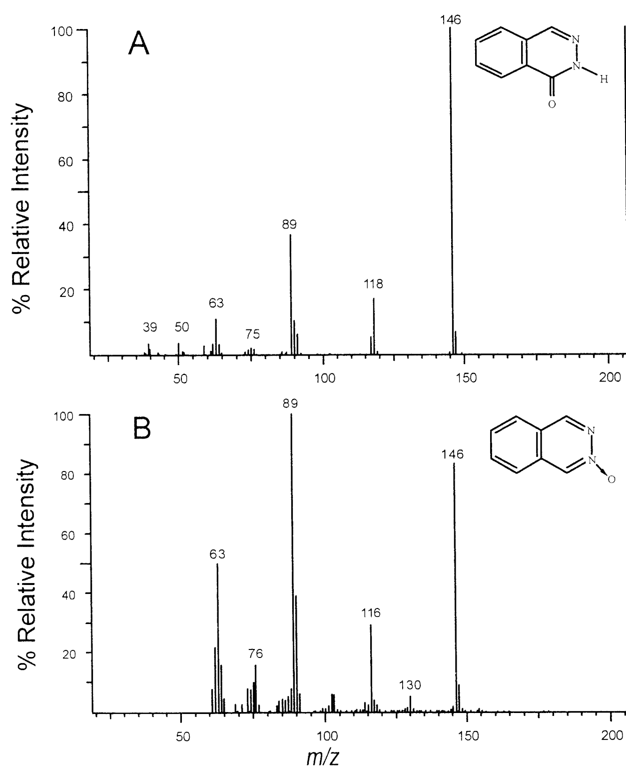

FIG. 1. Electron ionization mass spectra of the metabolites produced from phthalazine by A. *Fusarium moniliforme*, B. *Cunninghamella elegans*.

Cunninghamella elegans produced a different metabolite from phthalazine, with a peak area representing 3.6% of the total A_{254} and a retention time of 7.2 min (TABLE I). The UV/visible spectrum had λ_{max} values of 214, 254, 296, and 306 nm. The mass spectrum (FIG. 1B), obtained by GC/MS, showed ions at *m/z* 146 [$M^{+\cdot}$], 130 (M^+-O), 116 (M^+-NO), 90, 89 (M^+-CHN_2O), 76, and 63. The ^{1}H-NMR spectrum, with tentative assignments [7.72 (1 H, tr, $J_{7,8}$ = 8.4 Hz, H7), 7.85–7.91 (2 H, m, $J_{5,7}$ = 1.7, $J_{6,7}$ = 7.3, H5 and H6), 8.11 (1 H, dm, H8), 8.67 (1 H, s, $J_{4,8}$ = 0.9 Hz, H4), 9.24 (1 H, s, H1)], was more complete than that published by Tori et al (1963). The coupling patterns of the resonances of H5–H8 were exactly the same as those in phthalazine. Since the small 0.9 Hz coupling constant mentioned by Tori et al (1963) was present in the resonance at 8.11 ppm (H8) and in the singlet at 8.67 ppm (H4), the other singlet at 9.24 ppm could be assigned as H1. NOE experiments agreed with these tentative assignments; when the H8 resonance was irradiated, an enhancement was detected at 9.24 ppm (H1), and when the resonance at 8.67 ppm (H4) was irradiated, an enhancement of H5 was detected in the multiplet at 7.85–7.91 ppm. Since the H1 resonance had been shifted to a lower field than the others, it was most likely *ortho* to the N-O group. The differences between the NMR spectra can be explained by the use

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of different solvents, magnetic field strengths, and chemical shift scales. Thus, the *C. elegans* metabolite was identified as phthalazine *N*-oxide.

1(2*H*)-Phthalazinone, the metabolite produced by *F. moniliforme,* is also produced from phthalazine by bacteria (Lehmann et al 1994, Stephan et al 1996, Sutherland et al 1998) and mammals (Stubley et al 1979). Although 1(2*H*)-phthalazinone induces aldehyde oxidase and xanthine oxidase in rabbits, it is not a substrate for either of these enzymes (Johnson et al 1984). It has been tested for mutagenicity to bacteria but found to be negative (Zeiger et al 1987).

Phthalazine *N*-oxide, the metabolite produced by *C. elegans,* has not been reported previously as a biological metabolite, although the same fungus oxidizes isoquinoline and quinoline to the corresponding *N*-oxides (Sutherland et al 1994b). Phthalazine *N*-oxide has also been tested for mutagenicity to bacteria and found to be negative (Morita et al 1995).

Thus, both *F. moniliforme* and *C. elegans* oxidized the heterocyclic ring of phthalazine; *F. moniliforme* produced 1(2*H*)-phthalazinone and *C. elegans* produced phthalazine *N*-oxide. The enzymes used for the metabolism of phthalazine by these two fungi presumably belong to different groups but have not yet been studied.

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