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Metabolism of cinnoline to *N*-oxidation products by *Cunninghamella elegans* and *Aspergillus niger*

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Cultures of the fungi *Cunninghamella elegans* and *Aspergillus niger* were grown in fluid Sabouraud medium at 28°C for 3 days and then dosed with cinnoline (1,2-diazanaphthalene). After 3 more days, metabolites were extracted from the cultures with ethyl acetate, separated by high-performance liquid chromatography, and identified by mass spectrometry and proton nuclear magnetic resonance spectroscopy. Both fungi oxidized 2–10% of the added cinnoline to mixtures of cinnoline 2-oxide and cinnoline 1-oxide.

Keywords: azaarenes; biotransformation; fungi; heterocyclic compounds; *N*-oxidation

Introduction

A variety of fungi oxidize polycyclic aromatic hydrocarbons [1,10,14]. Some of the same species also transform *N*-heterocyclic compounds, including indole, carbazole, quinoline, isoquinoline, and acridine [2,3,6,11,13].

Cinnoline ($C_8H_6N_2$) is a toxic air pollutant, containing two aza nitrogen atoms, that is produced in diesel engine exhaust [7]. Although it is oxidized to 4(1*H*)-cinnolinone by mammalian aldehyde oxidases [9] and by the quinaldine 4-oxidase of *Arthrobacter* sp [8], the fate of cinnoline in the environment is not known. Two fungi that are known to oxidize other azaarenes [3,11,13] were investigated for the ability to metabolize cinnoline.

Materials and methods

Cunninghamella elegans ATCC 36112 and *Aspergillus niger* NRRL 599 were obtained from the American Type Culture Collection (Manassas, VA, USA) and the National Center for Agricultural Utilization Research (Peoria, IL, USA), respectively. Cultures for use as inocula were grown on potato dextrose agar plates (Remel, Lenexa, KS, USA) and macerated in a sterile Waring blender cup with 30 ml of fluid Sabouraud medium (Remel). Triplicate 125-ml flasks, each containing 30 ml of fluid Sabouraud medium, were inoculated with 1 ml of mycelial suspension. The cultures and triplicate noninoculated controls were incubated at 28°C with shaking at 125 rpm. After 3 days, 25 mg of cinnoline hydrochloride hydrate (98%, Aldrich Chemical Co, Milwaukee, WI, USA) in 1.0 ml of deionized water was filter-sterilized and used to dose each of the cultures and controls, which then were incubated at 28°C with shaking at 125 rpm for 3 more days.

After incubation, the cultures were collected on glass wool. The mycelia and filtrates were extracted with three equal volumes of ethyl acetate. The solvent was dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residues were then dissolved in 2 ml of methanol.

The metabolites were analyzed by high-performance liquid chromatography (HPLC) [12], using a Hewlett-Packard (Palo Alto, CA, USA) HP-1100 liquid chromatograph. An analytical Prodigy-5 ODS-3 column (250 × 4.6 mm; Phenomenex, Torrance, CA, USA) was used with a mobile phase gradient of 18–58% methanol in buffer (50 mM ammonium acetate, pH 5.5) over 20 min at a flow rate of 1 ml min^{-1}.

Individual metabolites were isolated using HPLC peak collection methods [11–13], using a semipreparative column (250 × 10 mm) and a flow rate of 3 ml min^{-1}. UV/visible absorption spectra were obtained in methanol with a Shimadzu (Kyoto, Japan) UV-2101PC spectrophotometer.

For mass spectrometry, the metabolites were dissolved in acetone. Mass spectra were obtained by gas chromatography/electron ionization mass spectrometry (GC/EI-MS), using a Finnigan model TSQ 700 triple quadrupole instrument (Finnigan Corp, San Jose, CA, USA). The quadrupole was scanned from 50 to 650 daltons in 0.5 s.

^{1}H-NMR spectra were obtained on a Bruker AM500 spectrometer (Billerica, MA, USA) operated at 500.13 MHz; samples were dissolved in acetone-d_6 and the residual solvent signal was assigned as 2.04 ppm. Data were zero-filled to 64 K to measure coupling constants, giving data resolution of 0.121 Hz/point. Acquisition parameters were: data size, 32 K; sweep width, 3937 Hz; filter width, 5000 Hz; acquisition time, 4.16 s; pulse width, 86°; relaxation delay, 0 s; dummy scans, 2; number of scans for nuclear Overhauser enhancement (NOE) data, 168–1232. Assignments were made by integration, homonuclear decoupling, and NOE experiments.

Results

Both *C. elegans* and *A. niger* oxidized cinnoline to two metabolites (designated A and B), which eluted from the HPLC column at 11.0 ± 0.1 min and 11.7 ± 0.2 min, respectively (Figure 1). Residual cinnoline eluted at 14.2 ±

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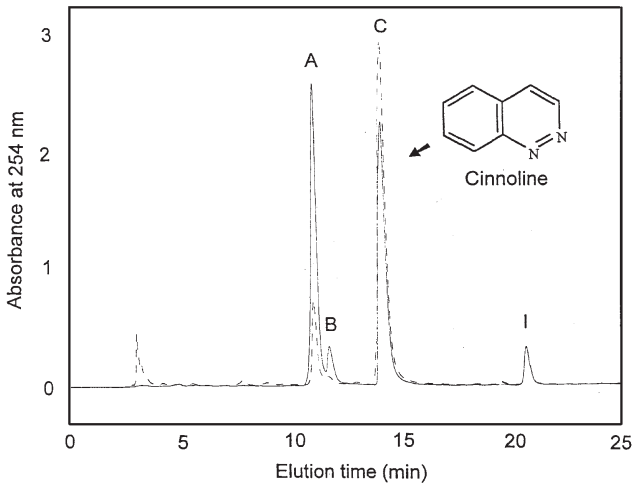

Figure 1 HPLC elution profiles (A_{254}) of the ethyl acetate extracts from cultures of fungi grown in fluid Sabouraud medium with cinnoline. (—) *Cunninghamella elegans*; (---) *Aspergillus niger*. A = metabolite A; B = metabolite B; C = residual cinnoline; I = impurity.

0.0 min (Figure 1) and an unidentified minor impurity (also found in the starting material) eluted at 20.6 min. The mean peak areas (A_{254}) obtained for the two metabolites produced by each fungus are shown in Table 1.

All of the HPLC, UV, and mass spectral data for metabolites A and B from *C. elegans* and *A. niger* indicated that the two fungi produced identical metabolites from cinnoline. Metabolite A had a UV/visible absorption spectrum with λ_{max} values of 261, 306, 351, and 360 nm. Metabolite B had a UV spectrum with λ_{max} values of 229, 304, 313, 351, and 366 nm.

When analyzed by GC/EI-MS, the retention times of metabolites A, B, and cinnoline on the GC column were 9.98, 9.55, and 7.52 min, respectively. The mass spectra of metabolites A and B, both of which had ions at *m/z* 146 [$M^{+\cdot}$], 130 (M^+-O), 116 (M^+-NO), and 102 (M^+-N_2O), were consistent with identification as cinnoline *N*-oxides. The relative intensities of the ions were different from those reported for the cinnoline *N*-oxides by Palmer *et al* [5], presumably because of the different ion-source temperatures and other instrumental parameters. The impurity, which eluted from the GC column at 16.23 min and had ions at *m/z* 258 [apparent $M^{+\cdot}$], 229, 202, 201, 200, 176, 174, 126, 100, 87, and 75, did not appear to be an azaarene.

^{1}H-NMR spectroscopy was used to make resonance assignments (Table 2) and confirm the structures of the two *N*-oxide isomers purified from cultures of *C. elegans*. The NMR chemical shifts for cinnoline 1-oxide and 2-oxide produced by *C. elegans* were reasonably similar to those for the chemically synthesized *N*-oxides published by Ogata *et al* [4]; the differences can be attributed to the use of different solvents (deuterated acetone instead of deuterated chloroform) and to the higher magnetic field strength (500 MHz instead of 60 MHz). The chemical shifts of cinnoline itself in acetone-d_6 were H-3, 7.82 ppm; H-4, 7.34 ppm; H-5, 6.72 ppm; H-6, 6.58 ppm; H-7, 6.52 ppm; and H-8, 7.07 ppm. The coupling constants of the *N*-oxides were in closer agreement with the published data [4].

Since cinnoline 2-oxide has 3.63 times the UV absorbance of cinnoline 1-oxide at 254 nm [4], the ratio of cinnoline 1-oxide to cinnoline 2-oxide produced by both *C. elegans* and *A. niger* was approximately 1.0 : 1.6.

Table 1 Production of metabolites from cinnoline by cultures of *Cunninghamella elegans* and *Aspergillus niger*

Peak designation	HPLC peak areas at 254 nm ($\times 10^3$ mAU)[a]		
	C. elegans	*A. niger*	Noninoculated
A (cinnoline 2-oxide)	48.8 ± 2.5	11.0 ± 2.0	<0.1
B (cinnoline 1-oxide)	8.4 ± 0.5	1.9 + 0.6	<0.1
C (residual cinnoline)	54.8 ± 5.4	71.3 ± 8.2	61.2 ± 3.4

[a]Injection volumes were 10 μl; all values are expressed as means ± standard errors of triplicate determinations.

Table 2 ^{1}H-NMR spectral parameters[a] for the metabolites produced from cinnoline by *Cunninghamella elegans*

Proton assignment	Metabolite A (cinnoline 2-oxide)		Metabolite B (cinnoline 1-oxide)	
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
H3	8.24	$J_{3,4} = 6.9$	8.33	$J_{3,4} = 6.2$
H4	8.32	$J_{4,8} = 1.0$	7.67	$J_{4,8} = 0.9$
H5	7.95	$J_{5,6} = 8.2$	8.06	$J_{5,6} = 8.0$
		$J_{5,7} = 1.3$		$J_{5,7} = 1.7$
H6	7.64	$J_{6,7} = 8.5$	7.90	$J_{6,7} = 8.6$
				$J_{6,8} = 1.3$
H7	7.85	$J_{7,8} = 8.5$	7.86	$J_{7,8} = 8.3$
H8	7.79		8.53	

[a]Samples were dissolved in acetone-d_6.

Discussion

Cunninghamella elegans was previously shown to oxidize two other azaarenes, quinoline and isoquinoline, to the corresponding *N*-oxides [13]. However, the same fungus oxidizes carbazole to a phenol [2] and acridine to a *trans*-dihydrodiol and a phenol [11]. *Aspergillus niger* metabolizes another azaarene, indole, apparently via hydroxylation of one of the carbons in the heterocyclic ring [3].

Oxidation of azaarenes by microorganisms may be an important step in the detoxification of air pollutants. The transformation of cinnoline to two isomeric *N*-oxides suggests that fungi have an enzymatic mechanism that is different from those of the bacteria and mammals that convert it to 4(1*H*)-cinnolinone [8,9]. Further work will be necessary to determine the types of enzymes involved in the fungal *N*-oxidation of quinoline, isoquinoline, and cinnoline as well as their distribution among various groups of fungi.

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