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Transformation of cinoxacin by *Beauveria bassiana*

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Abstract

The ability of the fungus *Beauveria bassiana* ATCC 7159 to transform the antibacterial agent cinoxacin was investigated. Cultures in sucrose–peptone broth were dosed with cinoxacin, grown for 20 days, and then extracted with ethyl acetate. Two metabolites were detected and purified by high-performance liquid chromatography. The major metabolite was identified by mass and proton nuclear magnetic resonance spectra as 1-ethyl-1,4-dihydro-3-(hydroxymethyl)[1,3]dioxolo[4,5-*g*]cinnolin-4-one and the minor metabolite was identified as 1-ethyl-1,4-dihydro-6,7-dihydroxy-3-(hydroxymethyl)cinnolin-4-one. *B. bassiana* also reduced quinoline-3-carboxylic acid to 3-(hydroxymethyl)quinoline.

Keywords: Antibacterial agent; *Beauveria bassiana*; Biotransformation; Cinoxacin

1. Introduction

Cinoxacin (1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-*g*]-cinnoline-3-carboxylic acid) is a synthetic antibacterial agent [1] that is structurally similar to both nalidixic acid and the quinolone oxolinic acid. It has been used to treat Gram-negative urinary tract pathogens, including strains of *Escherichia*, *Proteus*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter* [2,3], and it is used as an antibacterial agent in aquaculture [4]. In humans, cinoxacin is metabolized to a dioxolo ring-cleavage product, two glucuronide conjugates, and an acid-labile conjugate [5].

Selected fungi have often been found useful in the laboratory for performing oxidative, reductive, and hydrolytic biotransformations of organic compounds [6]. Furthermore, the ability of fungi to biotransform antimicrobial agents in the environment may be ecologically significant [7]. We have investigated the transformation of cinoxacin by *Beauveria bassiana* ATCC 7159, a strain that has been used for the preparative biotransformation of a large variety of organic compounds [6,8,9].

2. Materials and methods

2.1. Strain and cultural conditions

B. bassiana ATCC 7159 was maintained on potato dextrose agar. Triplicate experimental cultures in flasks, each containing 100 ml of sucrose–peptone broth [10], were incubated at 28°C with rotary shaking at 180 rpm. After 2 days, each of the cultures (dry weight 1.2 g) and triplicate non-inoculated controls was dosed (final concentration = 308 μM) with 8.1 mg of cinoxacin (Sigma Chemical Co., St. Louis, MO, USA) that had been dissolved in 20 mM KOH and filter-sterilized. The dosed cultures, triplicate control cultures, and non-inoculated controls were incubated with shaking for another 20 days.

2.2. Extraction and purification of metabolites

After harvest, cultures were extracted with ethyl acetate [10] and the residues were dissolved in methanol: acetonitrile: acetic acid (10:10:2). Metabolites were purified by high-performance liquid chromatography (HPLC) [11]; since the molar extinction coefficients of the metabolites are not known, the relative concentrations of metabolites were not determined.

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2.3. Mass spectrometry

Direct exposure probe/positive-ion chemical ionization (DEP/PICI) mass spectrometry analyses of the metabolites were performed in the single quadrupole (Q1) and product-ion modes [10] on a ThermoFinnigan TSQ 700 triple quadrupole mass spectrometer. The ion-source pressure (10% NH_3 in N_2) for chemical ionization was 5.0–5.5 Torr, uncorrected. Product ions were generated with a collision-cell pressure of 0.5 mTorr Ar and a collision energy of 100 eV.

Gas chromatography (GC)/PICI mass spectrometry was performed on the same mass spectrometer with the same ionization and fragmentation conditions, except that the collision energy was 50 eV. The gas chromatographic separation was provided by a DB-5ms column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), using 103 kPa He as the carrier gas.

Liquid chromatography/electrospray ionization (LC/ESI) mass spectrometry was performed using a Hewlett-Packard 1090L/M HPLC system with a 2.0 × 250 mm Prodigy 5-μm ODS-3 column and a Hewlett-Packard 5989B quadrupole mass spectrometer operated in the positive-ion electrospray mode. A linear 40-min gradient from 95% water/5% acetonitrile to 5% water/95% acetonitrile, with constant 0.1% formic acid, at a flow rate of 0.2 ml min^{-1} was used. Molecular masses of analytes were confirmed by positive-ion electrospray mass spectrometry.

2.4. Nuclear magnetic resonance (NMR) spectroscopy

Proton NMR spectroscopy was performed at 500 MHz [10] with the compounds dissolved in deuterated methanol or deuterated dimethyl sulfoxide. Chemical shifts are reported on the δ (ppm) scale by assigning a shift of 3.30 ppm (methanol) or 2.49 ppm (dimethylsulfoxide) to the residual solvent signal. Assignments were made based on integration, nuclear Overhauser effect (NOE) experiments, homonuclear decoupling experiments, and analysis of substituent effects.

3. Results

3.1. HPLC analysis of metabolites

HPLC analysis of the ethyl acetate extracts from cultures of *B. bassiana* dosed with cinoxacin and grown for 20 days showed a minor metabolite (I) eluting at 15.1 min, a major metabolite (II) at 17.3 min, and residual cinoxacin at 19.3 min (data not shown). Neither of the two metabolite peaks was found in the controls, although several minor peaks were found in both the dosed cultures and the controls. As shown by the HPLC peak area at 264 nm, only 47.3% of the added cinoxacin remained.

3.2. Identification of metabolites

The major metabolite (II), 47% of the total peak area at 264 nm, had an UV absorption spectrum with $\lambda_{max} = 264$, 351, and 364 nm. The DEP/PICI product-ion mass spectrum of metabolite II (Fig. 1A) and the positive-ion ESI mass spectrum indicated a molecular mass of 248 (cinoxacin = 262). The ^1H NMR spectrum of metabolite II (Table 1) showed that the ethyl protons were present and that the carbocyclic and dioxolo rings were both intact. Also, a singlet at 4.71 ppm (Hc), which integrated as two, when irradiated did not produce an NOE to any other proton. This indicated that the carboxyl group had been reduced to a hydroxymethyl; if the carbonyl group of the ring had been reduced to a methylene group, an NOE would have been produced to the proton at Hd. From these data, metabolite II was identified as 1-ethyl-1,4-dihydro-3-(hydroxymethyl)[1,3]dioxolo[4,5-*g*]cinnolin-4-one (Fig. 2).

The minor metabolite (I), 5.6% of the total peak area at 264 nm, had an UV absorption spectrum with $\lambda_{max} = 264$, 355, and 367 nm. The DEP/PICI product-ion mass spectrum of metabolite I (Fig. 1B) and the positive-ion ESI mass spectrum indicated a molecular mass of 236. The ^1H NMR spectrum of metabolite I (Table 1) showed all of the protons of the ethyl group (Ha and Hb) and the singlets from the carbocyclic ring (Hd and He), but the protons of the dioxolo ring (Hf) were absent. The singlets of the carbocyclic ring exhibited upfield shifts compared to those of cinoxacin, consistent with the presence of hydroxyl groups. The singlet at 4.71 ppm (Hc) integrated as two and showed that the carboxyl group had been reduced to a hydroxymethyl; irradiation of this singlet did not produce an NOE to any other proton. From these data, metabolite I was identified as 1-ethyl-1,4-dihydro-6,7-dihydroxy-3-(hydroxymethyl)cinnolin-4-one (Fig. 2).

3.3. Transformation of quinoline-3-carboxylic acid

To see whether other heterocyclic carboxylic acids

Table 1
^1H NMR parameters for cinoxacin and the metabolites produced by *B. bassiana*

Proton[a]	Chemical shifts (ppm)[b]		
	Metabolite I[c]	Metabolite II[c]	Cinoxacin[c]
Ha	1.48	1.47	1.56
Hb	4.48	4.53	4.72
Hc	4.71	4.71	
Hd	7.51	7.51	7.66
He	7.01	7.27	7.55
Hf		6.17	6.29

[a]NOE experiments were used to make assignments for the protons in the carbocyclic ring. When the resonances for the *N*-ethyl protons (Ha and Hb) were irradiated, an NOE was produced to the singlet at He and the remaining aromatic singlet could be assigned as Hd.
[b]Samples dissolved in deuterated methanol.
[c]$J_{a,b} = 7.3$ Hz.

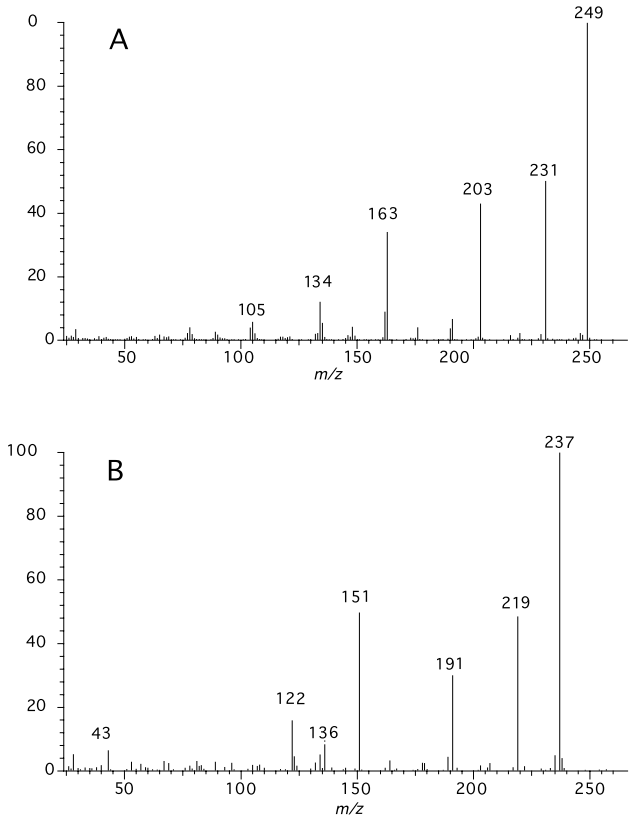

Fig. 1. Product-ion mass spectra (percent relative intensity using DEP/PICI) of the metabolites produced from cinoxacin by *B. bassiana*. A: Metabolite II. B: Metabolite I.

would also be reduced, we incubated *B. bassiana* with quinoline-3-carboxylic acid [12]. A single metabolite comprising 80% of the total peak area at 264 nm was purified by HPLC. The DEP/EI mass spectrum (Q1) had ions at *m/z* 159 [M^+], 158, 131, and 130, and the GC/PICI production mass spectrum for *m/z* 160 (9.015 min) had ions at *m/z* 143, 130 and 31. The ^{1}H NMR assignments and coupling constants, obtained in deuterated dimethyl sulfoxide, were 8.85 (H2; $J_{2,4} = 2.2$ Hz), 4.71 (–CH_2–), 5.43 (–OH), 8.22 (H4), 7.96 (H5; $J_{5,6} = 8.2$ Hz, $J_{5,7} = 1.3$ Hz), 7.59 (H6; $J_{6,7} = 7.7$ Hz, $J_{6,8} = 1.3$ Hz), 7.71 (H7; $J_{7,8} = 8.4$ Hz), and 8.00 (H8). These spectra indicated that the carboxyl group had been reduced to yield 3-(hydroxymethyl)-quinoline (Fig. 3).

4. Discussion

4.1. Carboxyl-group reduction

The reduction of the carboxyl group of cinoxacin by *B. bassiana* to a hydroxymethyl group, as found in the structures proposed for both metabolites I and II, was unexpected. The same strain of *B. bassiana* is known to hydroxylate 1-ethyl-1,4,6,7,8,9-hexahydro-4-oxobenzo[*g*]-quinoline-3-carboxylic acid at the 6-position without altering the carboxyl group [13]. In quinoline-3-carboxylic acid, the carboxyl group was also reduced. Although the reduction of the carboxyl groups of 1-naphthoic acid and quinoline-6-carboxylic acid has been demonstrated previously with a different fungus, *Cunninghamella elegans* [14], the enzymatic mechanism is not yet understood.

4.2. Ring cleavage

The structure proposed for metabolite I requires cleavage of the dioxolo ring to produce a catechol. In insects and mammals, the metabolism of 1,2-(methylenedioxy)-benzene compounds in the presence of $NADPH_2$ yields catechols with the release of formate [15]. One of the hydroxyl groups of the catechol may subsequently be meth-

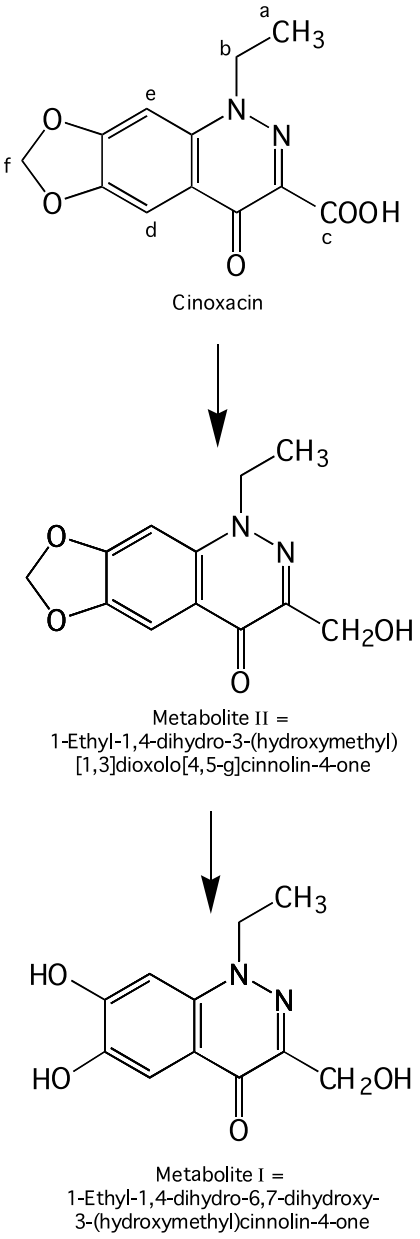

Fig. 2. Structures of cinoxacin and the two metabolites produced by *B. bassiana*.

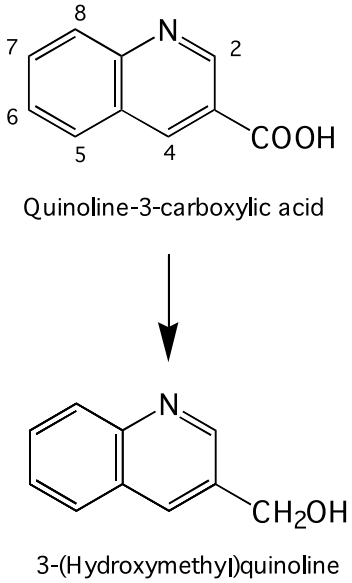

Fig. 3. Structures of quinoline-3-carboxylic acid and 3-(hydroxymethyl)-quinoline.

ylated [16], as in the methoxylated and glucuronide-conjugated human cinoxacin metabolite detected in urine [5]. However, *B. bassiana* cleaved the dioxolo ring without methylating either of the resulting hydroxyl groups.

4.3. Proposed metabolic pathway

Both products of cinoxacin transformation by *B. bassiana* had a 3-hydroxymethyl group, suggesting that the carboxyl group had been reduced to hydroxymethyl before the opening of the dioxolo ring. Thus it seems most likely that the major product, metabolite II, was produced first in the pathway and then the minor product, metabolite I (Fig. 2). The results show the ability of *B. bassiana*, a soil fungus that is also an insect pathogen [17], to reduce a carboxyl group to hydroxymethyl and to cleave a dioxolo ring with release of the methylene group.

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