

Formation of conjugates from ciprofloxacin and norfloxacin in cultures of *Trichoderma viride*

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Abstract: The formation of conjugates from two antibacterial fluoroquinolone drugs, ciprofloxacin and norfloxacin, was observed in cultures of *Trichoderma viride* that had been grown in sucrose-peptone broth and extracted 16 d after dosing with the drugs. Both conjugates were purified by high-performance liquid chromatography and found to be optically active. They were identified by mass and proton nuclear magnetic resonance spectra as 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin and 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin. The transformation of veterinary fluoroquinolones in the presence of fungi may have ecological significance.

Key Words: biotransformation, fluoroquinolones

INTRODUCTION

At present, limited information is available about the ability of fungi to transform fluoroquinolones, such as the widely used antibacterial agents ciprofloxacin (FIG. 1A) and norfloxacin (FIG. 1B). Several fungi transform the fluoroquinolones enrofloxacin, danofloxacin, and sarafloxacin to various metabolites (Martens et al 1996, Chen et al 1997, Wetzstein et al 1997, Parshikov et al 2000, 2001a). *Mucor ramannianus* transforms ciprofloxacin to *N*-acetylciprofloxacin (Parshikov et al 1999). *Gloeophyllum striatum* and other wood-decaying basidiomycetes convert ciprofloxacin to at least 16 metabolites, including CO₂ (Wetzstein et al 1999). *Pestalotiopsis guepini* transforms both ciprofloxacin and norfloxacin to *N*-acetyl,

desethylene-*N*-acetyl, and *N*-formyl metabolites as well as to metabolites in which an amino group has replaced the piperazine ring (Parshikov et al 2001b).

Recently, the formation of two new products in cultures of *Trichoderma viride* was noted after dosing with ciprofloxacin and norfloxacin. The strain had been isolated during the screening of soil fungi for the ability to metabolize fluoroquinolones. Both ciprofloxacin and norfloxacin appeared to be conjugated with an unstable secondary metabolite, which had been previously reported in other *Trichoderma* spp. (Mukhopadhyay et al 1996).

MATERIALS AND METHODS

Strain T-58, isolated from a fruiting body of *Trametes versicolor* collected in a forest in Jefferson County, Arkansas, was identified as *Trichoderma viride* by Dr. S. N. Lekomtseva, Department of Mycology and Algology, Moscow State University, Moscow, Russia. Triplicate experimental cultures in flasks containing sucrose-peptone broth (Parshikov et al 1999) were incubated at 28 C with rotary shaking at 180 rpm. After 2 d, the cultures were dosed with 300 μM ciprofloxacin or 313 μM norfloxacin (Parshikov et al 2001b); in one experiment, 300 μM piperidine hydrochloride (Aldrich Chemical Co.) was substituted. The dosed cultures, control cultures, and noninoculated controls were incubated with shaking for another 16 d.

After harvesting, cultures were extracted with ethyl acetate (Parshikov et al 1999) and the residues were dissolved in methanol:acetonitrile:acetic acid (10:10:2) for analysis. Compounds were purified by collecting the peaks separated by high-performance liquid chromatography (HPLC), using the gradient described previously (Parshikov et al 2001a), and the relative concentrations were estimated from the peak areas at 280 nm. Circular dichroism spectra were obtained in methanol with a Jasco model 500A spectropolarimeter.

Direct exposure probe (DEP) mass spectrometry (MS) experiments were performed as previously described (Parshikov et al 1999), using the single quadrupole (Q1) and product-ion modes. The ion-source pressure for chemical ionization was 5.0–5.5 Torr, uncorrected. Product ions were generated with a collision-cell pressure of 0.5 mTorr of argon and a collision energy of 100 eV. Electrospray ionization (ESI) MS experiments (Parshikov et al 1999) were performed by either flow injection or LC/ESI MS. For flow injection, the mobile phase was 50% methanol with 0.1% trifluoroacetic acid. For LC/ESI MS, a procedure described previously (Parshikov et al 2000) was used except that the

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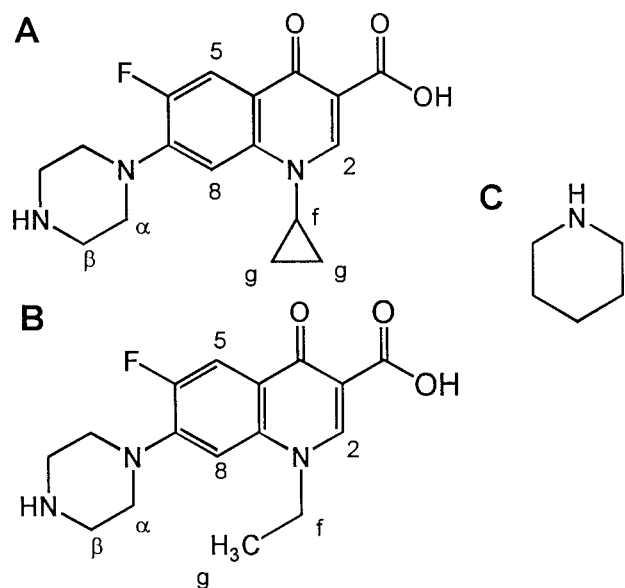


FIG. 1. Structures of compounds used for dosing cultures. A. Ciprofloxacin. B. Norfloxacin. C. Piperidine.

percent solvent B was 50% from 0 to 3 min and then was increased to 90% in a 15-min linear gradient. LC/ESI MS/MS experiments (Parshikov et al 1999) were performed with a collision energy of 25–50 eV.

¹H nuclear magnetic resonance (NMR) spectroscopy was performed at 500 MHz (Parshikov et al 1999) with the compounds dissolved in deuterated chloroform. ¹³C NMR spectroscopy was performed on one compound at 125.77 MHz.

RESULTS

Ciprofloxacin.—HPLC analysis of the ethyl acetate extracts from cultures of *T. viride* dosed with ciprofloxacin showed residual ciprofloxacin eluting at 11.1 min and an apparent metabolite at 21.7 min. Other peaks were found but were also detected in the controls. After 16 d, as shown by the peak areas at 280 nm, 31% of the ciprofloxacin had been transformed to the product and 69% remained unchanged. The ciprofloxacin product had a UV absorption spectrum with $\lambda_{\max} = 291$ and 332 nm. The circular dichroism

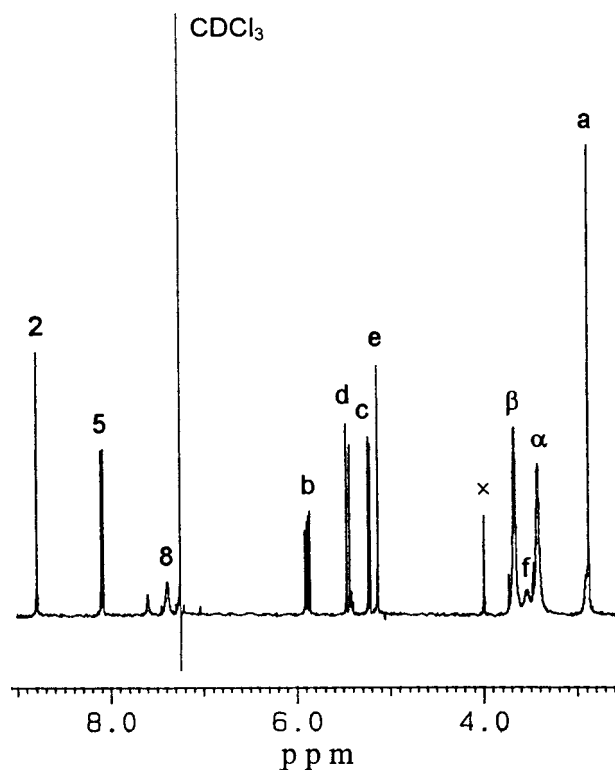


FIG. 2. ¹H NMR spectrum, obtained in CDCl₃ at 500 MHz, of the ciprofloxacin metabolite (4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin) produced by *T. viride*.

spectrum had a positive Cotton effect at 295 nm, indicating that the compound was optically active.

The DEP/NICI mass spectrum of the ciprofloxacin product (TABLE I) consisted of a molecular anion [M⁻] at *m/z* 453 and an oxygen adduct [M + O₂]⁻ at *m/z* 485. The product-ion (NICI MS/MS) mass spectrum (TABLE I) for the ion at *m/z* 453 had significant fragment ions at *m/z* 412 [M - 41]⁻, 368 [M - 85]⁻, and 246 [M - 207]⁻. The LC/ESI MS/MS mass spectrum (not shown) had an intense fragment ion at *m/z* 436 [MH-H₂O]⁺ and several smaller ions.

The ¹H NMR spectrum for the ciprofloxacin product is shown in FIG. 2; proton resonances that corresponded to those found in the spectrum of cipro-

TABLE I. Mass spectral data for ciprofloxacin, norfloxacin, and the conjugates found in cultures of *T. viride*

Compound	Mass spectral significant ions, <i>m/z</i> (% relative intensity)	
	DEP/NICI	NICI/MS/MS
Ciprofloxacin	363 ^a (16), 331 (100)	331, 290, 246, 201, 189, 182, 176, 161
Ciprofloxacin conjugate	485 ^a (26), 453 (100), 331 (8)	453, 412, 368, 350, 287, 246, 243, 203, 189, 182, 176, 166
Norfloxacin	351 ^a (100), 319 (42)	319, 290, 246, 201, 189, 182, 176, 161
Norfloxacin conjugate	473 ^a (56), 442 (44), 441 (100)	441, 412, 368, 350, 275, 246, 203, 201, 189, 182, 176, 166

^a Oxygen adducts [M + O₂]⁻.

TABLE II. ¹H NMR spectral data for ciprofloxacin, norfloxacin, and the conjugates found in cultures of *T. viride*^a

Compound	Chemical shifts, ppm	Coupling constants, Hz
Ciprofloxacin	8.89 (H2), 8.05 (H5), 7.34 (H8), 3.84, 3.79 (Hα), 3.65 (Hf), 3.48 (Hβ), 1.47, 1.27 (Hg)	$J_{5,F} = 12.8$, $J_{8,F} = 7.0$, $J_{b,c} = 10.6$, $J_{b,d} = 17.3$, $J_{e,g} = 7.3$
Ciprofloxacin conjugate	8.79 (H2), 8.10 (H5), 7.38 (H8), 5.88 (Hb), 5.44 (Hd), 5.22 (Hc), 5.13 (He), 3.66 (Hβ), 3.52 (Hf), 3.41 (Hα), 2.87 (Ha), 1.40, 1.20 (Hg)	$J_{5,F} = 12.7$, $J_{8,F} = 6.7$, $J_{b,c} = 10.6$, $J_{b,d} = 17.3$, $J_{e,g} = 7.3$
Norfloxacin	8.64 (H2), 8.10 (H5), 6.82 (H8), 4.27 (Hf), 3.94, 3.84 (Hα), 3.31 (Hβ), 1.34 (Hg)	$J_{5,F} = 12.8$, $J_{8,F} = 6.5$, $J_{b,c} = 10.6$, $J_{b,d} = 17.3$, $J_{e,g} = 7.3$
Norfloxacin conjugate	8.69 (H2), 8.14 (H5), 6.85 (H8), 5.88 (Hb), 5.44 (Hd), 5.22 (Hc), 5.13 (He), 4.31 (Hf), 3.66 (Hβ), 3.39 (Hα), 2.86 (Ha), 1.59 (Hg)	$J_{5,F} = 12.7$, $J_{8,F} = 6.7$, $J_{b,c} = 10.6$, $J_{b,d} = 17.3$, $J_{e,g} = 7.3$

^a Dissolved in CDCl₃.

floxacin were assigned accordingly (TABLE II). Five additional resonances (Ha–He) appeared to be part of an additional 123-Dalton moiety that had been detected by mass spectrometry (FIG. 3A). The resonances at 5.22, 5.44, and 5.88 (Hb–d) had multiplicities and coupling constants consistent with a vinyl group. In addition, there were two singlets at 2.87 and 5.13 ppm (Ha and He) that integrated as two

and one, respectively. Irradiation of each resonance resulted in a nuclear Overhauser effect (NOE) at the piperazine β resonance; irradiation of the resonance at 2.87 ppm (Ha) produced an NOE at 5.88 ppm (Hb), even though these protons were not coupled to one another. A proton-decoupled ¹³C NMR spectrum (not shown) was acquired from the ciprofloxacin conjugate and showed the same resonances (within 1.44 ppm) as those reported for a 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl moiety (Mukhopadhyay et al 1996), as well as those consistent with the carbons of the molecule. The NMR data show that the protons are arranged on a five-membered carbon ring. Based on the MS and NMR results, the ciprofloxacin product (FIG. 3A) was identified as a conjugate, 1-cyclopropyl-6-fluoro-7-[4-(4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl)piperazinyl]-4-oxohydroquinoline-3-carboxylic acid (= 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin).

Norfloxacin.—HPLC analysis of the ethyl acetate extracts from cultures of *T. viride* dosed with norfloxacin showed residual norfloxacin eluting at 10.9 min and an apparent metabolite at 21.5 min. Other peaks were found but were also detected in the controls. After 16 d, as shown by the peak areas at 280 nm, 42% of the norfloxacin had been transformed into the product and 58% remained unchanged. The norfloxacin product had a UV absorption spectrum with $\lambda_{\max} = 286, 321$ and 330 nm. The circular dichroism spectrum had a positive Cotton effect at 292 nm, indicating that the compound was optically active.

The DEP/NICI mass spectrum of the norfloxacin product (TABLE I) consisted of the molecular anion [M⁻] at *m/z* 441 and an oxygen adduct [M + O₂]⁻ at *m/z* 473. The production (NICI MS/MS) mass

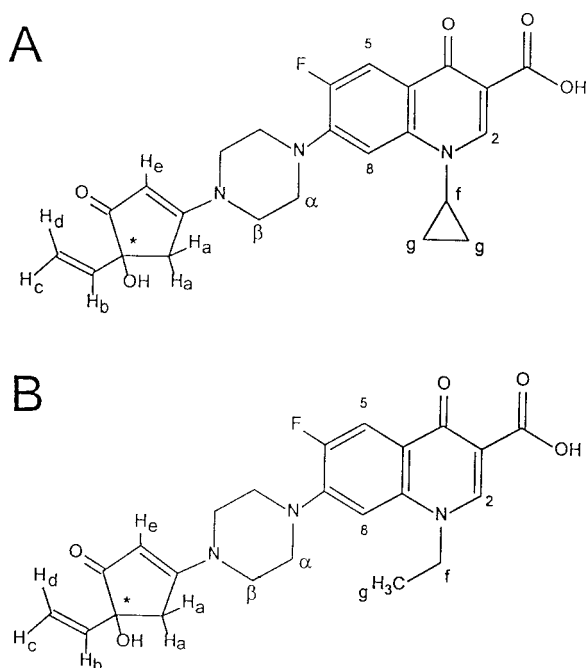


FIG. 3. Structures of metabolites produced by *T. viride* from fluoroquinolones. A. 4-Hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin. B. 4-Hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin. The carbon atoms are numbered as shown in the NMR data and the asymmetric carbon atom is shown by an asterisk.

spectrum (TABLE I) for the ion at m/z 441 had significant fragment ions at m/z 412 $[M - 29]^-$ and 368 $[M - 73]^-$. The LC/ESI MS/MS mass spectrum (not shown) had an intense fragment ion at m/z 424 $[MH-H_2O]^+$ and several smaller ions.

The 1H NMR spectrum of the norfloxacin product (TABLE II) was similar to that of norfloxacin for the H2, H5, H8, ethyl (Hf-g), and piperazine (H α - β) resonances. It also showed five additional resonances (Ha-e) with chemical shifts (FIG. 3B) that were the same as those of the substituted cyclopentenyl ring in the ciprofloxacin conjugate. Based on the MS and NMR results, the norfloxacin product was identified as a conjugate, 1-ethyl-6-fluoro-7-[4-(4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl)piperazinyl]-4-oxohydroquinoline-3-carboxylic acid (= 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin).

Piperidine.—To determine whether similar conjugates could be produced from other secondary amines, cultures of *T. viride* were grown with 300 μ M piperidine (FIG. 1C). Metabolites were extracted and analyzed directly by LC/ESI MS; one peak was found that was consistent with a conjugate similar to those seen for ciprofloxacin and norfloxacin. A positive-ion ESI mass spectrum with collision-induced dissociation showed ions at m/z 208 (3) $[MH]^+$, 190 (29) $[MH-H_2O]^+$, 162 (100) $[MH-H_2O-C_2H_4]^+$, and 134 (9) $[MH-H_2O-2C_2H_4]^+$. This spectrum is consistent with the structure of 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl piperidine.

DISCUSSION

Sulfate, formyl, and acetyl conjugates of ciprofloxacin (Zeiler et al 1987, Parshikov et al 1999, 2001b) and formyl, acetyl, and glucuronide conjugates of norfloxacin (Pauliukonis et al 1984, Parshikov et al 2001b) have previously been detected as products of various biological reactions. The compounds produced from these two fluoroquinolones in cultures of *T. viride*, in contrast, were optically active 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl conjugates.

The structures of the fluoroquinolone conjugates are reminiscent of 5-hydroxy-3-methoxy-5-vinyl-2-cyclopenten-1-one, derived from cultures of *T. album* (Strunz et al 1977), and 3-dimethylamino-5-hydroxy-5-vinyl-2-cyclopenten-1-one, derived from cultures of *T. koningii* (Mukhopadhyay et al 1996). When we dosed cultures of *T. viride* with piperidine, a secondary amine similar to the piperazine moiety of ciprofloxacin and norfloxacin, we found evidence of its conjugation with the same unstable fungal metabolite found by Mukhopadhyay et al. This showed that other secondary amines may also react with the me-

tabolite produced by *Trichoderma* spp. We suspect that the conjugation is a chemical process, because an enzymatic process would not be likely to work with all secondary amines.

Although the antibacterial activities of the conjugates produced by *T. viride* have not yet been investigated due to the minuscule amounts that have been available so far, those ciprofloxacin metabolites that have been tested have generally had significantly lower antibacterial activities than the parent drug (Zeiler et al 1987). Since species of *Trichoderma* and similar fungi are widespread on straw and other cellulose-rich debris in the environment (Cooke and Rayner 1984), the conjugation of fluoroquinolone residues with fungal metabolites may be ecologically important where these drugs are used for treatment of livestock and poultry.

3-Dimethylamino-5-hydroxy-5-vinyl-2-cyclopenten-1-one has no apparent antibacterial or antifungal activity (Mukhopadhyay et al 1996). However, the addition of the unstable *Trichoderma* spp. metabolite to other compounds may potentially be a useful reaction for the modification of secondary amines that are being investigated as antimicrobial agents, antidepressants, or anticancer drugs.

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