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Microbiological Transformation of Enrofloxacin by the Fungus *Mucor ramannianus*

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Enrofloxacin metabolism by *Mucor ramannianus* was investigated as a model for the biotransformation of veterinary fluoroquinolones. Cultures grown in sucrose-peptone broth were dosed with enrofloxacin. After 21 days, 22% of the enrofloxacin remained. Three metabolites were identified: enrofloxacin *N*-oxide (62% of the total absorbance), *N*-acetylciprofloxacin (8.0%), and desethylene-enrofloxacin (3.5%).

Fluoroquinolones are synthetic antimicrobial agents that are active against a broad spectrum of pathogenic gram-negative bacteria as well as some gram-positive bacteria and mycoplasmas (10). Several fluoroquinolones are used in clinical medicine, and enrofloxacin and sarafloxacin have been approved in the United States for veterinary use (4). The major metabolite of enrofloxacin in animals is ciprofloxacin, produced by N deethylation of the ethylpiperazine ring (21).

The persistence of veterinary fluoroquinolones and the types of metabolites that result from their microbial conversion in the environment have been little known until recent years (6, 12, 22, 23; H. G. Wetzstein, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. abstr. Q-262, p. 583, 1999). Currently, there is a question as to whether the use of fluoroquinolones in poultry causes the replacement of fluoroquinolone-sensitive coliform bacteria by resistant strains (2, 13).

Cultures of wood-decaying basidiomycetes, including strains found in manure, have been shown to convert enrofloxacin to CO_2 and at least 11 other metabolites (12, 22). Since other pathways are likely to be used by different organisms involved in the bioconversion of fluoroquinolones in the environment, we investigated the transformation of enrofloxacin by a typical zygomycetous soil fungus.

Mucor ramannianus strain R-56, which had been isolated from a mushroom collected in a forest in Arkansas (17), was maintained on agar slants (14, 18). Triplicate cultures for experiments were grown in sucrose-peptone broth (17) for 2 days. Enrofloxacin [1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid], as the hydrochloride, was a gift from Bayer Corp., Shawnee, Kans. Enrofloxacin hydrochloride was dissolved in 20 mM aqueous KOH and filter sterilized; 1.0 ml was added to each flask to make a final concentration of 253 μM enrofloxacin. The cultures were then incubated for an additional 21 days. Cultures without enrofloxacin and dosed, noninoculated controls were also incubated.

Methylene chloride extracts of cultures and controls were analyzed by high-performance liquid chromatography (HPLC) (17), using a Phenomenex (Torrance, Calif.) Prodigy 5-μm ODS-3 column (10 by 250 mm) with a water-acetic acid-methanol gradient (17). The flow rate was 2.5 ml min^{-1}; metabolites were quantified by the peak areas at 280 nm.

For direct exposure probe-electron ionization (DEP/EI) mass spectrometry (17), the quadrupole was scanned from *m/z* 50 to 650. For liquid chromatography-electrospray ionization (LC/ESI) mass spectrometry (17), a Vydac RP-18 pharmaceutical (5-μm) microbore column (1 by 250 mm; Separations Group, Hesperia, Calif.) was used. The mobile-phase components consisted of 5% methanol with 0.1% formic acid (A) and 95% methanol with 0.1% formic acid (B). The mobile phase was 10% B for 5 min, followed by a linear gradient of 10 to 90% B over 10 min at a flow rate of 70 μl min^{-1}. For tandem mass spectrometry (MS/MS) (17), the apparent protonated molecules were mass selected in Q1 and fragmented in the collision cell, with the product ions separated in Q3. With one metabolite, in-source collision-induced dissociation (CID) at 10 eV was used in conjunction with LC/ESI/MS to increase the yield of fragment ions.

Metabolites to be analyzed by ^1H nuclear magnetic resonance (NMR) spectrometry (17) were dissolved in D_2O containing 20 mM KOD.

HPLC analysis of the methylene chloride extracts from cultures of *M. ramannianus* dosed with enrofloxacin (Fig. 1) showed that enrofloxacin eluted from the HPLC column at 13.9 min and three metabolites eluted at 13.3, 16.2, and 33.2 min. By 21 days, the levels of all three metabolites had reached plateaus (data not shown). Then, as shown by the peak area (A_{280}), only 22% of the enrofloxacin remained.

Enrofloxacin had a UV spectrum nearly identical to that reported by Wetzstein et al. (22). The DEP/EI mass spectrum had significant ions at *m/z* 359 $[M]^{+}\cdot$, 344, and 315, and the negative-ion chemical ionization (DEP/NICI) mass spectrum had a radical anion at *m/z* 359 with an apparent O_2 adduct at *m/z* 391. The LC/ESI/MS and ESI/MS/MS mass spectra for enrofloxacin are shown in Table 1, and the ^1H NMR spectral data appear in Table 2.

Metabolite I eluted from the HPLC column at 13.3 min (Fig. 1), with a peak area of 3.5% of the total A_{280} and a UV spectrum nearly identical to that of the desethylene-enrofloxacin metabolite F-4 of Wetzstein et al. (22). The positive-ion chemical ionization (DEP/PICI) mass spectrum of metabolite I had an apparent protonated molecule at *m/z* 334 $[MH]^{+}$ and a fragment ion at *m/z* 276; the DEP/NICI mass spectrum consisted primarily of a molecular anion at *m/z* 333. The LC/

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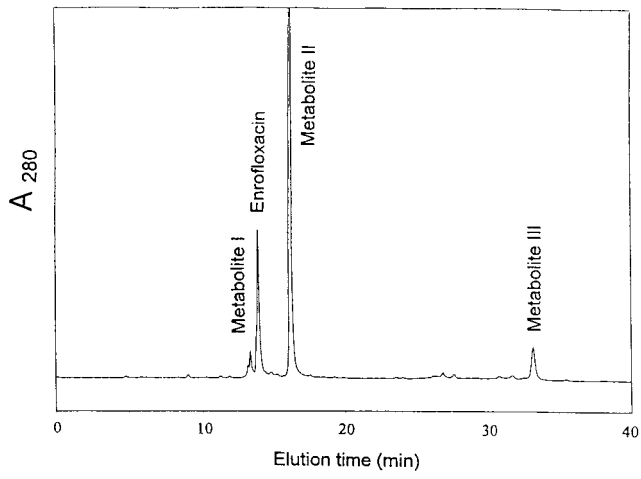

FIG. 1. HPLC chromatogram, obtained at 280 nm, for the metabolites (I to III) produced from enrofloxacin by *M. ramannianus*.

ESI/MS and ESI/MS/MS mass spectra for metabolite I are shown in Table 1.

In the ^{1}H NMR data for metabolite I (Table 2), all of the aromatic resonances were shifted with respect to enrofloxacin. The resonance of proton H8 was shifted to 7.15 ppm from 7.56 ppm for enrofloxacin. Four of the piperazine protons of enrofloxacin were missing in metabolite I. Two of the remaining piperazine protons, now called the ethylidene α protons, were shifted to 3.09 ppm from 3.28 ppm for enrofloxacin. The other two, now called the ethylidene β protons, were shifted to 2.86 ppm from 2.57 ppm for enrofloxacin. The CH_2 protons of the ethyl group were shifted to 3.60 ppm from 2.40 ppm for enrofloxacin, and the CH_3 protons were shifted to 1.17 ppm from 1.06 ppm for enrofloxacin. The results are consistent with the assignment of metabolite I as 1-cyclopropyl-7-{[2-(ethylamino)-ethyl]-amino}-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (desethylene-enrofloxacin) (Fig. 2).

Metabolite II eluted from the HPLC column at 16.2 min (Fig. 1), with a peak area of 62% of the total A_{280}. The UV spectrum of metabolite II had λ_{max} values of 284, 319, and 331 nm. A background-subtracted, full-scan LC/ESI/MS mass spectrum of metabolite II (Table 1) showed three significant ions at *m/z* 376 $[MH]^+$, 360, and 316. The ion at *m/z* 360, corresponding to the loss of an oxygen atom from the protonated molecule, was consistent with an N-oxide. The ion at *m/z* 376 was selected for ESI/MS/MS, and a product-ion mass spectrum was obtained (Table 1); a major fragment ion appeared at *m/z* 315 $[MH\text{-}CH_3CH_2N(O)H_2]^+$.

The ^{1}H NMR data for metabolite II (Table 2) show the same number of protons as in enrofloxacin. In metabolite II, six of the eight piperazine proton resonances were degenerate at 3.61 ppm and the other two were at 3.37 ppm. The CH_2 protons of the ethyl group of metabolite II were shifted to 3.46 ppm from 2.40 ppm for enrofloxacin. The CH_3 of the ethyl group of metabolite II was shifted to 1.40 ppm from 1.06 ppm for enrofloxacin. The resonance of proton H8 was shifted to 7.67 ppm from 7.56 ppm for enrofloxacin. All the results of integration, dipolar coupling, and nuclear Overhauser effect difference experiments were consistent with 1-cyclopropyl-7-(4-ethyl-4-oxopiperazinyl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (enrofloxacin *N*-oxide), with the oxygen attached to the terminal nitrogen of the piperazine ring (Fig. 2).

Metabolite III eluted from the HPLC column at 33.2 min (Fig. 1), with a peak area of 8.0% of the total A_{280}. The UV and LC/ESI mass spectra were identical to those of *N*-acetylciprofloxacin (17). When in-source CID was used to produce fragments in an LC/ESI/MS experiment (Table 1), the signal for the protonated molecule at *m/z* 374 decreased and a single major fragment was observed at *m/z* 356, corresponding to a loss of water $[MH\text{-}H_2O]^+$ from the carboxylic acid moiety.

The ^{1}H NMR chemical shifts for metabolite III (Table 2) were the same as those of *N*-acetylciprofloxacin (17).

The proton-decoupled ^{19}F NMR spectrum of enrofloxacin and that of each of the metabolites (not shown) had only one resonance peak, representing a single F atom.

The biotransformation of drugs by different organisms may proceed by different pathways. In animals, fluoroquinolones are usually attacked at the piperazine or the *N*-methylpiperazine ring, if either is present, or at the carboxyl group (3). For example, ofloxacin, pefloxacin, and fleroxacin may be modified by N oxidation of the methylpiperazine rings (11, 16, 20). Enoxacin may be metabolized in animals by N acetylation, by the removal of two ethylene carbons from the piperazine ring, and by other reactions (15). The N dealkylation of enrofloxacin to ciprofloxacin in animals (21) may occur by the monooxygenase-mediated α-hydroxylation of the ethyl group via an unstable hemiaminal intermediate (1).

Several fungi already have been shown to metabolize fluoroquinolones. For instance, the zygomycete *Rhizopus arrhizus* demethylates the *N*-methylpiperazine ring of danofloxacin (6). *Gloeophyllum striatum* and other wood-decaying basidiomycetes metabolize enrofloxacin and ciprofloxacin by hydroxylation, decarboxylation, defluorination, and removal of part or all of the piperazine ring (12, 22, 23). One of the enrofloxacin metabolites of *G. striatum*, produced by removing two ethylene

TABLE 1. Mass spectral data for enrofloxacin and the metabolites produced by *M. ramannianus*

Compound	Method of analysis	Mass spectrum, *m/z* (% relative intensity)
Enrofloxacin	LC/ESI/MS	360 $[MH]^+$ (100), 316 $[MH\text{-}CO_2]^+$ (12)
Enrofloxacin	ESI/MS/MS[a] (*m/z* 360)	342 $[MH\text{-}H_2O]^+$ (100), 316 $[MH\text{-}CO_2]^+$ (15), 286 (65), 245 (49), 217 (25), 203 (29), 84 (20), 72 (14), 70 (13), 57 (10)
Metabolite I	LC/ESI/MS	335 (16), 334 $[MH]^+$ (100), 316 $[MH\text{-}H_2O]^+$ (10)
Metabolite I	ESI/MS/MS[a] (*m/z* 334)	316 $[MH\text{-}H_2O]^+$ (100), 296 $[MH\text{-}H_2O, HF]^+$ (11), 245 $[MH\text{-}H_2O, C_2H_4NHC_2H_4]^+$ (10), 72 $[NC_4H_{10}]^+$ (12), 58 $[NC_3H_8]^+$ (17)
Metabolite II	LC/ESI/MS	376 $[MH]^+$ (100), 360 $[MH\text{-}O]^+$ (18), 316 $[MH\text{-}O\text{-}CO_2]^+$ (28)
Metabolite II	ESI/MS/MS[a] (*m/z* 376)	358 $[MH\text{-}H_2O]^+$ (15), 344 $[MH\text{-}CH_3OH]^+$ (17), 330 $[MH\text{-}HCOOH]^+$ (24), 315 $[MH\text{-}CH_3CH_2N(O)H_2]^+$ (100), 300 (32), 84 (12), 70 (11)
Metabolite III	LC/ESI/MS[b]	374 $[MH]^+$ (62), 356 $[MH\text{-}H_2O]^+$ (100)

[a] 1-millitorr argon collision gas and 50-eV collision energy.
[b] In-source CID with 10-eV collision energy.

TABLE 2. ^{1}H NMR spectral parameters of enrofloxacin and the metabolites produced by *M. ramannianus*[a]

Proton(s)	Enrofloxacin				Metabolite I				Metabolite II				Metabolite III			
	δ (ppm)	Int.	Mult.	*J* (Hz)	δ (ppm)	Int.	Mult.	*J* (Hz)	δ (ppm)	Int.	Mult.	*J* (Hz)	δ (ppm)	Int.	Mult.	*J* (Hz)
H2	8.65	1	s		8.41	1	s		8.48	1	s		8.49	1	s	
H5	7.91	1	d	13.4[b]	7.78	1	d	11.6[b]	7.91	1	d	13.5[b]	7.92	1	d	13.5[b]
H8	7.56	1	d	7.5[b]	7.15	1	d	—[c]	7.67	1	d	7.5[b]	7.63	1	d	7.7[b]
Piperazine, H-α	3.28	4	m										3.79	4	m	
Piperazine, H-β	2.57	4	m						3.37	2	m					
Piperazine, 4H-α + 2H-β									3.61	6	m					
Piperazine, H-β-1													3.37	2	m	
Piperazine, H-β-2													3.31	2	m	
CH_2 ethylidene H-α					3.59	2	m									
CH_2 ethylidene H-β					3.09	2	m									
Ethyl, CH_2	2.40	2	q	7.2	2.86	2	m		2.34	2	q	7.2				
Ethyl, CH_3	1.06	3	t	7.1	1.17	3	t		1.40	3	t	7.1				
Acetyl, CH_3													2.19	3	s	
Cyclopropyl, CH	3.83	1	m		3.60	1	m		3.61	1	m		3.65	1	m	
Cyclopropyl, CH_2	1.33	2	m		1.34	2	m		1.34	2	m		1.34	2	m	
Cyclopropyl, CH_2	1.18	2	m		1.13	2	m		1.13	2	m		1.13	2	m	

[a] Int., integrals. Multiplicities (Mult.) are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.
[b] $J_{H,F}$.
[c] —, not measurable.

carbons from the piperazine ring (22), was also a metabolite of *M. ramannianus*. Ciprofloxacin is metabolized by *M. ramannianus* to *N*-acetylciprofloxacin (17), which has now also been identified as a metabolite of enrofloxacin. We propose that enrofloxacin is first converted to ciprofloxacin by N dealkylation and that the resulting ciprofloxacin is N acetylated to give *N*-acetylciprofloxacin. Ciprofloxacin was not detected in our experiments, presumably because the acetylation step (17) occurred quickly.

In addition to *R. arrhizus* and *M. ramannianus*, other zygomycetous fungi perform drug biotransformations. For example, four species of *Cunninghamella* transform the *N*-methylpiperidine ring of codeine by demethylation (19), and *Cunninghamella elegans* transforms pyrilamine maleate, thenyldiamine, methapyrilene, and tripelennamine by N oxidation and N demethylation (5, 9). It is noteworthy that during the transformation of (−)-deprenyl and pargyline by *Cunninghamella echinulata* (7), N dealkylation is followed by N acetylation.

The transformation of enrofloxacin by *M. ramannianus*, including N oxidation, N dealkylation, N acetylation, and the breakdown of the piperazine ring, is similar to the mammalian metabolism of other fluoroquinolones (3, 15, 20). For ciprofloxacin, the major mammalian metabolites have significantly less antibacterial activity than the parent compound (24). The fungal enrofloxacin metabolites previously reported also appear to have less antibacterial activity (22).

Since *Mucor* and *Rhizopus* species are common in soil and decaying organic matter (8), the biotransformations of veterinary drug residues by zygomycetous fungi are likely to be ecologically significant.

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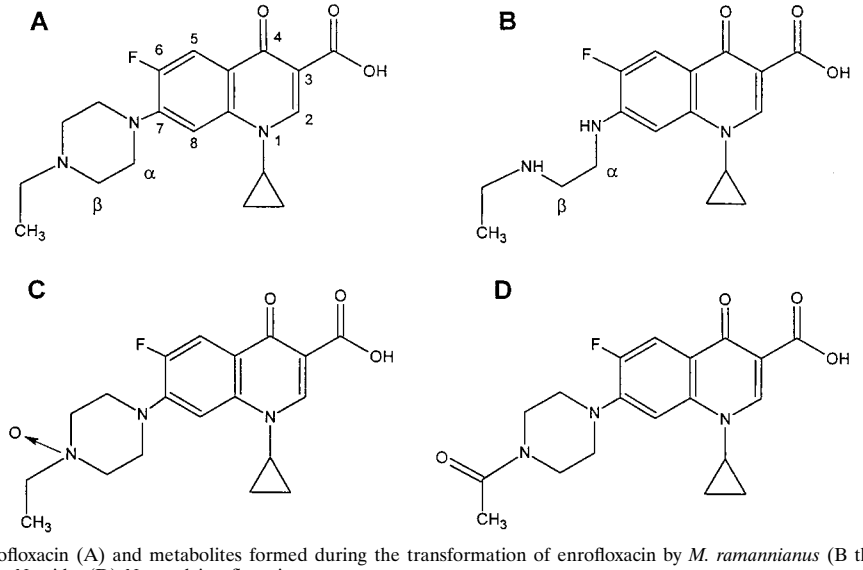

FIG. 2. Structures of enrofloxacin (A) and metabolites formed during the transformation of enrofloxacin by *M. ramannianus* (B through D) (B) Desethyleneenrofloxacin; (C) enrofloxacin *N*-oxide; (D) *N*-acetylciprofloxacin.

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