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Metabolism of the veterinary fluoroquinolone sarafloxacin by the fungus *Mucor ramannianus*

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To investigate the microbial biotransformation of veterinary fluoroquinolones, *Mucor ramannianus* was grown in sucrose/peptone broth with sarafloxacin for 18 days. Cultures were extracted with ethyl acetate and extracts were analyzed by liquid chromatography. The two metabolites (26% and 15% of the A_{280}, respectively) were identified by mass and ^{1}H nuclear magnetic resonance spectra as *N*-acetylsarafloxacin and desethylene-*N*-acetylsarafloxacin. The biological formation of desethylene-*N*-acetylsarafloxacin has not been previously observed. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 140–144.

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Introduction

Sarafloxacin (Figure 1) is a fluoroquinolone antibacterial agent that is registered for veterinary use in the U.S. [1]. It is widely used in chickens and turkeys for the prevention and treatment of colibacillosis [5] and is also used in aquaculture in several countries [8,16,20]. Although it has not been shown to be carcinogenic, sarafloxacin has been associated with chromosomal aberrations and *in vitro* forward mutation of the *hprt* locus in Chinese hamster ovary cells in the presence of the S9 fraction from rat liver [2] and with unscheduled DNA synthesis in rat primary hepatocytes [2]. Furthermore, some isolates of *Escherichia coli, Salmonella* spp., and *Campylobacter jejuni* from poultry have developed resistance to sarafloxacin and other quinolones *in vitro* and *in vivo* [10,17].

Veterinary fluoroquinolones, including sarafloxacin, bind to organic matter [9] and persist unchanged for long times in soils [6] and marine sediments [4]. Due to this binding, sarafloxacin did not inhibit bacteria in an experiment using ocean sediments [2]. It inhibited *E. coli* and other bacteria in a simulated human gut model, but a dose five times higher than in broth cultures was required for equal effectiveness [9]. Microorganisms with the ability to biotransform fluoroquinolones have been found in the environment [3,7,12,18,19]. Danofloxacin is metabolized by a variety of fungi and bacteria *via* processes that include *N*-oxidation, *N*-demethylation, and degradation of the piperazine ring [3], and enrofloxacin is degraded by *Gloeophyllum striatum* and several other fungi to a large number of metabolites [7,13,18]. Both danofloxacin and enrofloxacin can be mineralized by some fungi [3,7,18]. Sarafloxacin is metabolized to a glucuronide by mice, rabbits, and dogs; to *N*-acetylsarafloxacin by mice and rabbits; to 3′-oxosarafloxacin by rabbits and humans; and to an ethylene diamine-substituted quinolone and an aminoquinolone by humans [2].

The aerobic incubation of sarafloxacin in soil produces a small amount of CO_2 [6]. To determine whether soil fungi might be able to biotransform sarafloxacin to other metabolites, we used a strain of *Mucor ramannianus* that we obtained while screening soil fungi for fluoroquinolone metabolism [12]. This strain is capable of *N*-oxidation, *N*-acetylation, *N*-deethylation, and breakdown of piperazine rings in other fluoroquinolones [12,13]. We hoped that the addition of sarafloxacin to cultures of saprobic fungi would shed additional light on the ability of these fungi to influence the amounts of veterinary fluoroquinolone residues in the environment.

Materials and methods

M. ramannianus strain R-56 [12] was maintained on agar slants [11,14]. Cultures were grown in triplicate flasks in a sucrose–peptone broth [12] with shaking at 180 rpm. Sarafloxacin [6-fluoro-1-(4-fluorophenyl)-4-oxo-7-piperazinyl-1,4-dihydroquinoline-3-carboxylic acid] hydrochloride (Figure 1) was a gift from Abbott Laboratories (North Chicago, IL). It was dissolved in 20 mM aqueous KOH and filter-sterilized; 1 ml was added to each flask to make a final concentration of 260 μM sarafloxacin. Cultures and controls [13] were incubated at 28°C with shaking at 180 rpm. They were harvested after 18 days and extracted with ethyl acetate [12]. Residues were dissolved in 2.0 ml of methanol:acetonitrile:acetic acid (10:10:2) for analysis.

Extracts were analyzed by high-performance liquid chromatography (HPLC) [13], using a Phenomenex Prodigy 5-μm ODS-3 column (10×250 mm) and a flow rate of 2.5 ml min^{-1}. The mobile phase consisted of solvent A (H_2O:methanol, 90:10) and solvent B (H_2O:methanol, 10:90); 2 ml l^{-1} acetic acid was added to each solvent, which was adjusted to pH 3.0 [12]. The percent solvent B was initially 10%; it was increased from 10% to 95% over 20 min and then held at 95% for 10 min. The metabolites were collected, concentrated [12], and dissolved in methanol:acetonitrile:acetic acid (10:10:2).

For direct exposure probe (DEP) mass spectrometry (MS), a Finnigan TSQ 700 triple quadrupole instrument [12] was operated in both the single quadrupole (Q1) and the product-ion modes. The ion source temperature was 150°C and the DEP current was

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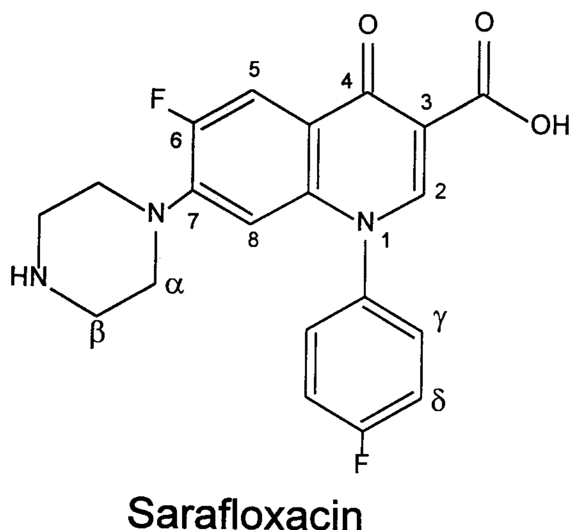

Figure 1 Structure of sarafloxacin.

ramped at 5 mA s^{-1}. The quadrupole was scanned from m/z 50 to 550 with a 0.5-s cycle time. Electron ionization (EI) mass spectrometry was performed with an electron energy of 70 V. Positive ion chemical ionization (PICI) and negative ion chemical ionization (NICI) mass spectrometry utilized 10% NH_3 (90% N_2) as the reagent gas with an uncorrected ion source pressure of 5.0-5.5 Torr. Product ions from the molecular anion (with NICI) and the protonated molecule (with PICI) were generated with a collision cell pressure of 0.5 mTorr of argon and a collision energy of 100 eV. Samples in methanol were applied to the rhenium wire of the DEP and the solvent was allowed to evaporate [12].

For electrospray ionization (ESI) mass spectrometry, a Finnigan TSQ 7000 instrument was operated at 200°C and 4.5 KeV in either the single-quadrupole (Q1) mode or the tandem (MS/MS) product-ion mode [12]. For full-scan ESI/MS, a Varian model 9012 HPLC pump was used with an autosampler [12] and a Vydac RP-18 Pharmaceutical 5-μm microbore column (1×250 mm) [13]. The mobile phase consisted of solvent A (H_2O:methanol, 95:5) and solvent B (H_2O:methanol, 5:95); 1 ml l^{-1} formic acid was added to each solvent [13]. The percent solvent B was 10% for 5 min; it was increased from 10% to 90% over 10 min. The flow rate was 70 μl min^{-1} and the absorbance was monitored at 280 nm. For MS/MS, argon was used with a collision energy of 50 eV. The apparent protonated molecules were mass-selected in Q1 and fragmented in the collision cell; the product ions were separated in Q3 [12,13].

For ^1H nuclear magnetic resonance (NMR) analysis, each metabolite was dissolved in 0.5 ml methanol-d_4 (99.96 at.% ^2H). The NMR measurements were carried out at 500.13 MHz on an AM500 spectrometer (Bruker Instruments, Billerica, MA). Chemical shifts are reported on the δ (ppm) scale by assigning the residual solvent peak at 3.30 ppm. Typical data acquisition parameters were: data size, 32 K; sweep width, 7042 Hz, filter width, 8900 Hz; acquisition time, 2.33 s; flip angle, 90°; relaxation delay, 0 s; temperature, 301 K. For spectra recorded under quantitative conditions, a 10- to 20-s relaxation delay was used. For measurement of coupling constants, the free induction decay (FID) was zero-filled to 64K, resulting in a final data point resolution of 0.215 Hz per point. Coupling constants reported are first order. Assignments were made *via* homonuclear decoupling experiments, nuclear Overhauser enhancement (NOE) experiments, integration, and analysis of substituent effects.

Results

HPLC analysis of the ethyl acetate extract from cultures of *M. ramannianus* dosed with sarafloxacin (Figure 2) shows that sarafloxacin eluted from the HPLC column at 12.0 min and two metabolites eluted at 21.6 and 23.1 min. After 18 days, as shown by the peak area (A_{280}), 59% of the starting material remained. The percentage of sarafloxacin remaining did not decrease with additional incubation (data not shown).

Sarafloxacin had a UV absorption spectrum with λ_{max}=282, 319 and 331 nm. The DEP/EI mass spectrum (Table 1) had significant ions at m/z 385 $[M]^{+\cdot}$, 343, and 341 $[M-CO_2]^{+\cdot}$. The base peak ion was m/z 299. The DEP/NICI mass spectrum had a molecular anion at m/z 385 $[M]^-$ and the DEP/PICI mass spectrum had a protonated molecule at m/z 386 $[M+H]^+$. The product-ion spectrum (NICI/MS/MS) for the molecular anion at m/z 385 included ions at m/z 290, 246, and 201 (Table 1). The ESI mass spectrum showed one prominent ion, the protonated molecule, at m/z 386. Because mass spectra vary somewhat depending upon conditions, none of these exactly match the previously published spectra for sarafloxacin [16]. The ^1H NMR spectral data for sarafloxacin are shown in Table 1.

Metabolite I eluted from the HPLC column at 21.6 min, with a yield of 15% of the total A_{280}, and had a UV spectrum with λ_{max}=284, 320 and 344 nm. The DEP/EI mass spectrum had significant, but low-intensity, ions at m/z 401 $[M]^{+\cdot}$ and 357 $[M-CO_2]^{+\cdot}$. The ESI mass spectrum showed a single prominent ion at m/z 402, corresponding to the protonated molecule. The product-ion spectrum from the protonated molecule, obtained by ESI/MS/MS, included an ion corresponding to water loss (m/z 384) and an ion at m/z 299 that was also observed in the product-ion spectrum from the protonated molecule of sarafloxacin. The DEP/PICI, DEP/NICI, and NICI/MS/MS spectra for metabolite I, shown in Table 1, are consistent with a compound with a molecular weight of 401.

The five resonances in the 6.0 to 9.0 ppm region of the ^1H NMR spectrum of metabolite I showed the same coupling patterns as those of sarafloxacin (Table 1), indicating that the aromatic ring system was unchanged. A methyl group was evidenced by a sharp singlet at 1.88 ppm that integrated as three in the upfield region of

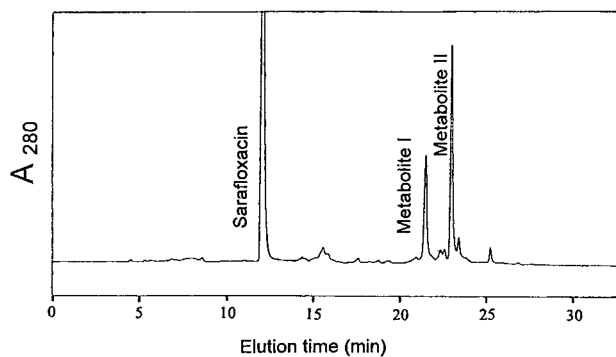

Figure 2 HPLC chromatogram, obtained at 280 nm, for the metabolites produced from sarafloxacin by *M. ramannianus*.

Table 1 Mass and ^{1}H NMR spectral data for sarafloxacin and the metabolites produced by *M. ramannianus*

Compound	Mass spectral significant ions, *m/z* (% relative intensity)						^{1}H NMR chemical shifts (ppm)[a]
	DEP/EI	DEP/PICI	DEP/NICI	NICI/MS/MS	PICI/MS/MS	ESI/MS	
Sarafloxacin[b]	385(62), 343(80), 341(72), 299(100), 284(12), 256(10), 184(10), 56(15)	386(100), 385(12), 343(8), 341(7), 299(4)	385(100)	385, 290, 246, 203, 201, 189, 182, 176	386, 368, 270	386(100)	8.76(H2), 8.11(H5), 7.65(Hγ), 7.45(Hδ), 6.53(H8), 3.35(Hα,β)
Metabolite I[c]	401(1), 357(4), 330(2)	402(100), 401(10), 357(6), 143(22), 137(16), 130(29), 113(56), 61(27)	401(100), 400(41), 381(7)	401, 381, 315, 306, 271, 262, 18, 176	402, 384, 299, 256, 86	402(100)	8.62(H2), 7.92(H5), 7.62(Hγ), 7.43(Hδ), 6.09(H8), 3.28(Hβ), 3.10(Hα), 1.88(CH_3)
Metabolite II[d]	427(26), 407(5), 383(100), 311(31), 298(117, 285(22), 283(31), 256(24), 137(62), 56(89)	428(100), 383(21)	427(100), 385(4)	427, 341, 332, 288, 246, 182	428, 410, 112, 70	428(100)	8.72(H2), 8.06(H5), 7.64(Hγ), 7.43(Hδ), 6.47(H8), 3.66, 3.69(Hβ), 3.10, 3.15(Hα), 2.09 (CH_3)

[a]Dissolved in deuterated methanol.
[b]Coupling constants are: $J_{H5,F}=12.9$ Hz, $J_{H8,F}=7.1$ Hz, $J_{\gamma,\delta}=8.6$ Hz.
[c]Coupling constants are: $J_{H5,F}=11.8$ Hz, $J_{H8,F}=7.3$ Hz, $J_{\gamma,\delta}=8.6$ Hz.
[d]Coupling constants are: $J_{H5,F}=13.1$ Hz, $J_{H8,F}=7.3$ Hz, $J_{\gamma,\delta}=8.8$ Hz.

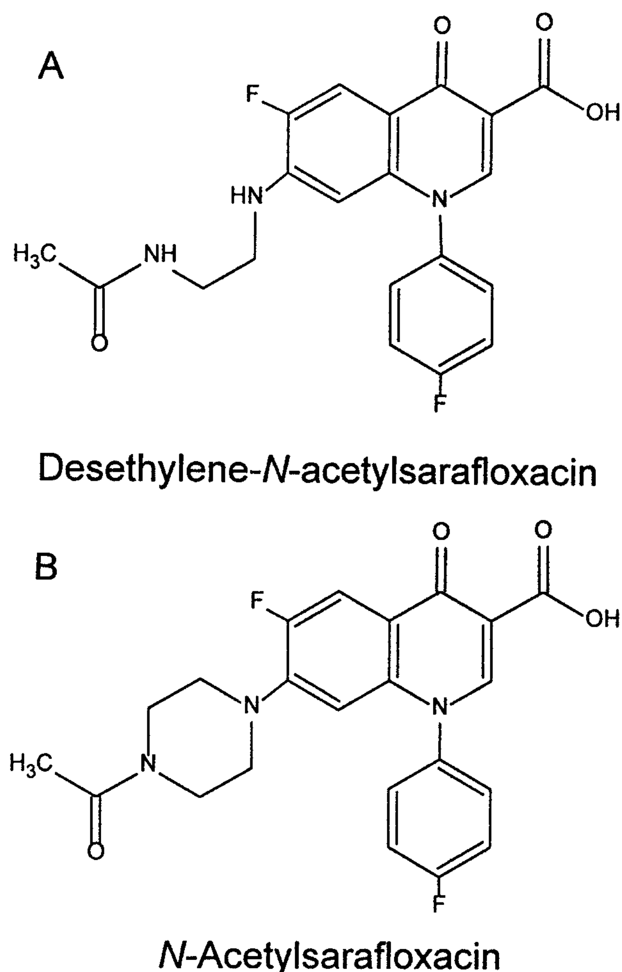

Figure 3 Structures of the two metabolites formed by *M. ramannianus* from sarafloxacin.

the spectrum (1.8 to 3.9 ppm). Two triplet resonances, representing protons associated with the piperazine moiety, were observed at 3.10 and 3.28 ppm. They were shifted upfield from the piperazine resonances of sarafloxacin and each one integrated as two, indicating that the piperazine ring had been degraded to an ethylidene-type structure. Upon irradiation of the resonance at 3.10 ppm, an NOE was detected at 6.09 ppm (H8), showing that the resonance at 3.10 ppm was the ethylidene α resonance. These ^{1}H NMR results are consistent with the assignment of metabolite I as 6-{[2-(acetylamino) ethyl] amino}-5-fluoro-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (=desethylene-*N*-acetylsarafloxacin, Figure 3).

Metabolite II eluted from the HPLC column at 23.1 min, with a yield of 26% of the total A_{280}, and had a UV spectrum with λ_{max}=286, 321 and 330 nm. The DEP/EI mass spectrum had significant ions at *m/z* 427 $[M]^{+\cdot}$, 407 $[M\text{-}HF]^{+\cdot}$, and 383 $[M\text{-}CO_2]^{+\cdot}$. The ESI mass spectrum showed the expected protonated molecule at *m/z* 428 and a single product ion, corresponding to water loss (*m/z* 410). Neither the ion at *m/z* 299 nor any other ion was above 5% relative abundance. The DEP/NICI, DEP/PICI and NICI/MS/MS mass spectra for metabolite II, shown in Table 1, are consistent with a compound with a molecular weight of 427.

The chemical shifts of the aromatic proton resonances in the ^{1}H NMR spectrum of metabolite II (Table 1) were all within 0.05 ppm of those of sarafloxacin. The chemical shifts and the multiplicities of the piperazine resonances were different, with two groups of two triplets. The resonances of the piperazine α protons were shifted upfield from those of sarafloxacin and those of the piperazine β protons were shifted downfield. The spectrum showed a sharp singlet, a methyl resonance that integrated as three and was shifted downfield from that of metabolite I. Irradiation of the methyl resonance produced an NOE to the group of triplets at 3.66 and 3.69 ppm, representing the piperazine β protons. When the H8 resonance at 6.47 ppm was irradiated, NOEs were detected to the resonances at 7.64 ppm and the group of triplets at 3.10 and 3.15 ppm, showing that these were the fluorophenyl α and piperazine α resonances, respectively. The ^{1}H NMR results are consistent with the assignment of metabolite II as 7-(4-acetylpiperazinyl)-6-fluoro-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (*N*-acetylsarafloxacin, Figure 3).

Discussion

N-Acetylation of the piperazine ring is a common reaction in the biotransformation of fluoroquinolones, as in the metabolism of sarafloxacin by mice and rabbits [2] and that of norfloxacin by humans [15]. It also occurs in the metabolism of enrofloxacin and ciprofloxacin by *M. ramannianus* [12,13]. In cultures of *M. ramannianus* with sarafloxacin, we found not only *N*-acetylsarafloxacin but also a previously undescribed metabolite produced by removing two carbons from the piperazine ring of *N*-acetylsarafloxacin. This step probably occurred rapidly after the acetylation step, because we did not find a desethylene–sarafloxacin metabolite without the acetyl group. In enrofloxacin, *G. striatum* and *M. ramannianus* remove an ethylene group to open the *N*-ethylpiperazine ring without forming an acetyl group [13,18]. During the metabolism of norfloxacin in humans, *N*-acetylation and breakdown of the piperazine ring may occur either singly or together [15].

N-Acetylsarafloxacin and three other potential metabolites are less active than sarafloxacin against several bacteria [2]. The transformation of sarafloxacin by *M. ramannianus*, which included *N*-acetylation and then the removal of two carbons from the piperazine ring, demonstrates the potential for environmental biotransformation of sarafloxacin by saprobic soil fungi.

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References

1 Brown SA. 1996. Fluoroquinolones in animal health. *J Vet Pharmacol Ther* 19: 1–14.

2 Chamberlain PL. 1998. Sarafloxacin. Toxicological Evaluation of Certain Veterinary Drug Residues in Food, prepared by the fiftieth meeting of the joint FAO/WHO expert committee on food additives (JECFA). World Health Organization, Geneva, Switzerland, pp. 37–68.
3 Chen Y, JPN Rosazza, CP Reese, H-Y Chang, MA Nowakowski and JP Kiplinger. 1997. Microbial models of soil metabolism: biotransformations of danofloxacin. *J Ind Microbiol Biotechnol* 19: 378–384.
4 Hektoen H, JA Berge, V Hormazabal and M Yndestad. 1995. Persistence of antibacterial agents in marine sediments. *Aquaculture* 133: 175–184.
5 Jones RN, ME Erwin and the Quality Control Study Group. 1998. *In vitro* susceptibility testing and quality control parameters for sarafloxacin (A-56620): a fluoroquinolone used for treatment and control of colibacillosis in poultry. *Diagn Microbiol Infect Dis* 32: 55–64.
6 Marengo JR, RA Kok, K O'Brien, RR Velagaleti and JM Stamm. 1997. Aerobic biodegradation of (^{14}C)-sarafloxacin hydrochloride in soil. *Environ Toxicol Chem* 16: 462–471.
7 Martens R, H-G Wetzstein, F Zadrazil, M Capelari, P Hoffmann and N Schmeer. 1996. Degradation of the fluoroquinolone enrofloxacin by wood-rotting fungi. *Appl Environ Microbiol* 62: 4206–4209.
8 Martinsen B and TE Horsberg. 1995. Comparative single-dose pharmacokinetics of four quinolones, oxolinic acid, flumequine, sarafloxacin, and enrofloxacin, in Atlantic salmon (*Salmo salar*) held in seawater at 10°C. *Antimicrob Agents Chemother* 39: 1059–1064.
9 McConville ML, JW Dijkstra, JM Stamm, JJM van Saene and JFM Nouws. 1995. Effects of sarafloxacin hydrochloride on human enteric bacteria under simulated human gut conditions. *Vet Q* 17: 1–5.
10 Medders WM, RE Wooley, PS Gibbs, EB Shotts and J Brown. 1998. Mutation rate of avian intestinal coliform bacteria when pressured with fluoroquinolones. *Avian Dis* 42: 146–153.
11 Modyanova LV, MR Duduchava, NF Piskunkova, GV Grishina, PB Terentyev and IA Parshikov. 1999. Microbial transformation of piperidine and pyridine derivatives [in Russian]. *Khim Geterotsikl Soed* 1999: 649–655.
12 Parshikov IA, JP Freeman, JO Lay, RD Beger, AJ Williams and JB Sutherland. 1999. Regioselective transformation of ciprofloxacin to *N*-acetylciprofloxacin by the fungus *Mucor ramannianus*. *FEMS Microbiol Lett* 177: 131–135.
13 Parshikov IA, JP Freeman, JO Lay, RD Beger, AJ Williams and JB Sutherland. 2000. Microbiological transformation of enrofloxacin by the fungus *Mucor ramannianus*. *Appl Environ Microbiol* 66: 2664–2667.
14 Parshikov IA, JP Freeman, AJ Williams, JD Moody and JB Sutherland. 1999. Biotransformation of *N*-acetylphenothiazine by fungi. *Appl Microbiol Biotechnol* 52: 553–557.
15 Pauliukonis LT, DG Musson and WF Bayne. 1984. Quantitation of norfloxacin, a new antibacterial agent in human plasma and urine by ion-pair reverse-phase chromatography. *J Pharm Sci* 73: 99–102.
16 Schilling JB, SP Cepa, SD Menacherry, LT Bavda, BM Heard and BL Stockwell. 1996. Liquid chromatography combined with tandem mass spectrometry for the confirmation of sarafloxacin in catfish tissue. *Anal Chem* 68: 1905–1909.
17 Smith KE, JM Besser, CW Hedberg, FT Leano, JB Bender, JH Wicklund, BP Johnson, KA Moore, MT Osterholm and the Investigation Team. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992–1998. *N Engl J Med* 340: 1525–1532.
18 Wetzstein H-G, N Schmeer and W Karl. 1997. Degradation of the fluoroquinolone enrofloxacin by the brown rot fungus *Gloeophyllum striatum*: Identification of metabolites. *Appl Environ Microbiol* 63: 4272–4281.
19 Wetzstein H-G, M Stadler, H-V Tichy, A Dalhoff and W Karl. 1999. Degradation of ciprofloxacin by basidiomycetes and identification of metabolites generated by the brown rot fungus *Gloeophyllum striatum*. *Appl Environ Microbiol* 65: 1556–1563.
20 WHO (World Health Organization). 1998. Use of quinolones in food animals and potential impact on human health: report of a WHO meeting. Geneva, Switzerland, 2–5 June 1998. 14 p. (http://www.who.int/emc-documents/zoonoses/docs/whoemczdi9810/html).