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Biotransformation of Flurbiprofen by *Cunninghamella* Species[▽]

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The biotransformation of the fluorinated anti-inflammatory drug flurbiprofen was investigated in *Cunninghamella* spp. Mono- and dihydroxylated metabolites were detected using gas chromatography-mass spectrometry and fluorine-19 nuclear magnetic resonance spectroscopy, and the major metabolite 4′-hydroxyflurbiprofen was isolated by preparative high-pressure liquid chromatography (HPLC). *Cunninghamella elegans* DSM 1908 and *C. blakesleeana* DSM 1906 also produced a phase II (conjugated) metabolite, which was identified as the sulfated drug via deconjugation experiments.

One of the objectives of the recent European Union legislation governing the testing and evaluation of chemicals, REACH (Regulation, Evaluation, Authorisation and Restriction of Chemicals), is to further reduce the need for animals in the testing process. Some microorganisms, such as the zygomycete fungus *Cunninghamella* and actinomycetes bacteria, have been shown to metabolize xenobiotic compounds in a fashion analogous to that of mammals (3, 5, 11, 17). It was suggested over 3 decades ago that microorganisms had potential as models of mammalian metabolism (16), although there are concerns about their predictive value (8). Nevertheless, certain microorganisms can be applied to the generation of useful quantities of drug metabolic intermediates (13), which is more desirable than isolation of these compounds from dosed animals, and avoids the concerns often associated with chemical synthesis, such as the use of toxic reagents and harsh reaction conditions.

Owing to the desirable physicochemical properties of the fluorine atom (small Van der Waals radius, electronegativity, and strength of the carbon-fluorine bond), approximately 25% of drugs either currently on the market or in the pipeline are fluorinated (12). One such example is flurbiprofen [(*RS*)-2-(2-fluoro-4-biphenyl)propionic acid], which is a nonsteroidal anti-inflammatory drug (NSAID) used in the treatment of inflammation caused by arthritis. In humans it is transformed to the phase I (oxidative) metabolites 4′-hydroxyflurbiprofen, 3′,4′-dihydroxyflurbiprofen, and 3′-hydroxy,4′-methoxyflurbiprofen; glucuronide and sulfate conjugates (phase II metabolites) have also been detected (9, 15). In equine urine additional hydroxylated and methoxylated metabolites were detected (20). Tracy et al. (18) demonstrated that only one cytochrome P450 isoform (2C9) is involved in the oxidation of flurbiprofen, which makes the drug a potentially useful *in vivo* probe for this particular isoform. Despite the prevalence of fluorinated drugs, only a few investigations have been undertaken to determine the microbial biotransformation of these compounds (7, 21). Here we describe the biotransformation of flurbiprofen by *Cunninghamella* species and the determination of the metabolites by nuclear magnetic resonance (NMR) spectroscopy (^{1}H and ^{19}F), gas chromatography-mass spectrometry (GC-MS), and high-pressure liquid chromatography (HPLC).

Three species of *Cunninghamella* were selected for the biotransformation experiments: *C. elegans* (strains DSM 1908, DSM 8217, and DSM 63299), *C. echinulata* DSM 1905, and *C. blakesleeana* DSM 1906. The fungi were grown on Sabouraud dextrose agar plates (Sigma) for 5 days at 26°C before being homogenized in 100 ml of sterile saline solution. The homogenate (10%, vol/vol) was used to inoculate 50 ml of fresh Sabouraud dextrose broth in 250-ml Erlenmeyer flasks, which were incubated at 28°C with shaking at 150 rpm. Following previously established procedures (2), 5 mg of flurbiprofen (Sigma) dissolved in dimethyl formamide (20 μl) was added to the cultures after 72 h, and the incubation was continued up to a further 120 h. Control experiments were conducted in the absence of either flurbiprofen or fungus. The cultures (supernatant and cells) were sonicated on ice (Sonicator U200S control; IKA Labortechnik) for 5 min at 50% amplitude, with intervals of 30 s after each minute to prevent overheating. The sonicates were centrifuged, the supernatant was extracted with 50 ml of ethyl acetate, and the extracts were evaporated to dryness.

Analysis of fluorinated metabolites. *C. elegans* DSM 1908 is well known as a model of mammalian drug metabolism (10, 11), and analysis of the organically extractable metabolites by ^{19}F NMR spectroscopy using a Varian 400-MHz spectrometer revealed that flurbiprofen was completely degraded to one fluorometabolite over 3 days (Fig. 1). The concentration of the metabolite was estimated by using an internal standard (4-fluorobiphenyl) in ^{19}F NMR analyses and equated to 2 mg in the culture supernatant. No fluorinated products were detected in uninoculated control flasks.

The fluorometabolite was isolated by preparative reversed-phase HPLC using a Varian Prostar HPLC system equipped with a Zorbax SB-C_{18} 9.4-mm by 25-cm column (Agilent Technologies). Compounds were eluted with a gradient of acetonitrile-water (20 to 60% acetonitrile) over 30 min at a flow rate of 3.5 ml/min. The main metabolite, which eluted at 19 min,

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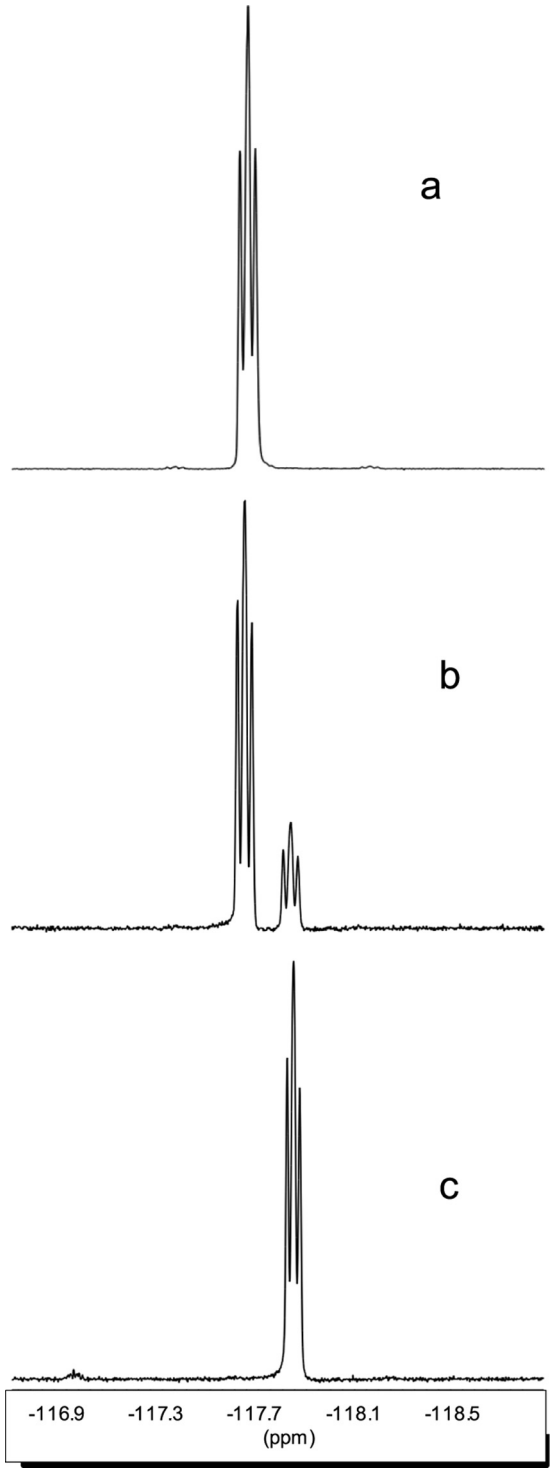

FIG. 1. Biotransformation of flurbiprofen and formation of its metabolite 4′-hydroxyflurbiprofen by *C. elegans* 1908 analyzed by ^{19}F NMR at 0 (a), 24 (b), and 72 h (c).

was isolated and analyzed by ^{1}H and ^{19}F NMR spectroscopy and mass spectrometry. The spectrum obtained with ^{1}H NMR analysis showed resonances at 1.56 ppm (CH_3, d), 3.78 ppm (CH, q), 6.9 ppm ($C_{3'}$ and $C_{5'}$-H, ddd), 7.14 ppm (C_2-H, ddd), 7.16 ppm (C_6-H, ddd), 7.37 ppm (C_5-H, ddd), and 7.43 ppm ($C_{2'}$ and $C_{6'}$-H, ddd). There was no resonance for $C_{4'}$-H, indicating that the hydroxylation occurred in this position. The spectrum obtained by ^{19}F NMR analysis showed one signal with a chemical shift of −117.85 ppm and splitting pattern identical to that of the flurbiprofen (dd, J = 11, 8 Hz), indicating that there were no changes in the proximities of the fluorine atom. The metabolite was dried and further analyzed by GC-MS as the pertrimethylsilylated derivative, which was formed by adding 50 μl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) to the solid and heating at 100°C for 1 h. The derivatized compound was diluted in ethyl acetate (1 ml), an aliquot (1 μl) was injected onto an HP-1 column (12 m by 0.25 mm by 0.33 μm), and the oven temperature was held at 120°C for 2 min and then raised to 300°C at 10°C min^{-1}. The mass and fragmentation pattern of the metabolite, which had a retention time of 17.50 min, was composed of ions m/z 404 (M^+), 389 (M^+-CH_3), 287 (M^+-COOTMS), 268 (M^+-COOTMS, F), and 253 (M^+-COOTMS, F, CH_3) and was identical to that described in reference 20 for 4′-hydroxyflurbiprofen.

Metabolic studies of flurbiprofen in humans and different animal species reported the presence of several metabolites excreted in urine (15). The major metabolite was identified as 4′-hydroxyflurbiprofen, and two minor ones were identified as 3′,4′-dihydroxyflurbiprofen and 3′-hydroxy-4′-methoxyflurbiprofen. In the present study only one metabolite was detectable by ^{19}F NMR, but since this technique is relatively insensitive, additional analyses of the organically extractable metabolites were conducted by HPLC and GC-MS. HPLC analysis of time course experiments up to 120 h confirmed that flurbiprofen was completely degraded over 3 days to one polar metabolite with a retention time of 22.2 min (10 to 90% acetonitrile over 30 min at 1 ml/min). GC-MS analyses of silylated organically soluble *C. elegans* extracts revealed 4′-hydroxyflurbiprofen and other metabolites that could be tentatively identified as hydroxylated and methoxylated flurbiprofen, based on their mass spectra (Table 1).

The microbial biotransformation of flurbiprofen was also investigated in selected *Cunninghamella* strains previously shown to transform xenobiotics (4). GC-MS analysis of the organically extractable metabolites, after derivatization, demonstrated that they all transformed the drug, to various degrees, yielding the hydroxylated metabolites, and three of the fungi generated hydroxylated methoxyflurbiprofen (Table 2). Using our methods, no other organically soluble fluorometabolites were detected.

Phase II metabolism of flurbiprofen was studied since glucuronide and sulfate flurbiprofen conjugates are reported to be important detoxification metabolites in mammals. In humans, approximately 60 to 70% of flurbiprofen is excreted as conjugates (1), whereas less than 30% of the flurbiprofen was reportedly conjugated in equine urine (20). Examination of the aqueous extracts from *C. elegans* DSM 1908 by HPLC showed one peak at a retention time (t_R) 17.8 min that was not present in the organic extract analysis, and the ^{19}F NMR spectrum of the aqueous phase showed a signal at −119.2 ppm, which had a concentration of 0.1 mg/ml by comparison with an internal standard of sodium fluoride. No fluorometabolites were detected in control experiments. Enzymatic deconjugation was carried out by incubating the aqueous phase with sulfatase

TABLE 1. GC-MS data for organic extracts of pertrimethylsilylated flurbiprofen and metabolites produced by *C. elegans* DSM 1908

Compound	t_R (min)	*m/z* (relative intensity) of:	
		M^+	Fragment ions
Flurbiprofen	12.85	316 (40)	301 (54), 198 (23), 180 (100), 165 (100), 73 (100)
4′-OH-flurbiprofen[a,b]	16.15	404 (94)	389 (43), 287 (43), 268 (72), 253 (28), 73 (100)
OH-flurbiprofen	16.64	404 (38)	389 (26), 313 (88), 285 (100), 158 (30), 73 (60)
OH-flurbiprofen	16.75	404 (25)	389 (13), 313 (100), 246 (17), 73 (46)
OH-flurbiprofen	17.34	404 (6)	389 (6), 298 (40), 179 (100), 73 (57)
3′,4′-DiOH-flurbiprofen[a]	17.50	492 (55)	477 (9), 375 (16), 267 (46), 73 (100)
OH-MeO-flurbiprofen	13.63	434 (31)	419 (7), 370 (32), 314 (80), 212 (50), 73 (100)
OH-MeO-flurbiprofen	14.68	434 (8)	419 (3), 337 (3), 129 (55), 73 (100)

[a] The mass spectra of these compounds were identical to those reported in reference 18.
[b] Approximately 80% of the total metabolites (by peak area) was 4′-OH-flurbiprofen.

(from *Helix pomatia* type H-1), β-glucuronidase (from *Escherichia coli*), and β-glucosidase (from almonds) (Sigma) in phosphate buffer at 37°C for 12 h. The deconjugated reaction products were extracted into ethyl acetate and analyzed by GC-MS; 3′,4′-dihydroxyflurbiprofen was detected after treatment with sulfatase, but no metabolites were detected in extracts from deconjugation experiments with the other enzymes (Fig. 2). The other *Cunninghamella* species were examined for conjugated metabolites, and such were observed only in *C. blakesleeana*. The aqueous fraction from a culture of this strain that had been incubated with flurbiprofen was treated with the sequential addition of deconjugation enzymes (added in the order sulfatase, β-glucuronidase, and β-glucosidase), and the reactions were monitored by ^{19}F NMR spectroscopy between each addition (Fig. 3), demonstrating that the sulfated metabolite was the only phase II compound present. Three resonances were initially observed, and metabolite I disappears after sulfatase treatment, with a concomitant increase in the height of metabolite III. Subsequent treatment with other deconjugative enzymes did not result in any further changes to the spectrum, and subsequent HPLC analysis led to the conclusion that metabolites II and III are most likely 4′-hydroxy- and 3′,4′-dihydroxyflurbiprofen, respectively.

The anti-inflammatory activities of profens, including flurbiprofen, are mainly ascribed to the active (*S*)-enantiomer (14). In human liver microsomes the (*S*)-enantiomer is transformed more rapidly than the (*R*)-enantiomer (19). In order to evaluate differences in the rates of degradation or metabolite formation between chiral and racemic flurbiprofen in fungi, biotransformation experiments were carried out using (*R*)-flurbiprofen. No difference in the formation of phase I and II metabolites was observed; the degradation was complete and comparable with that of the racemic flurbiprofen (data not

TABLE 2. Qualitative analysis by GC-MS of biotransformation of flurbiprofen by *Cunninghamella* species

Compound	Biotransformation by strain:				
	C. elegans 1908	*C. elegans* 8217	*C. elegans* 63299	*C. echinulata* 1905	*C. blakesleeana* 1906
Flurbiprofen	−	+	+	−	−
4′-OH-flurbiprofen	+	+	+	+	+
3′,4′-DiOH-flurbiprofen	+	+	+	+	+
OH-MeO-flurbiprofen	+	−	−	+	+

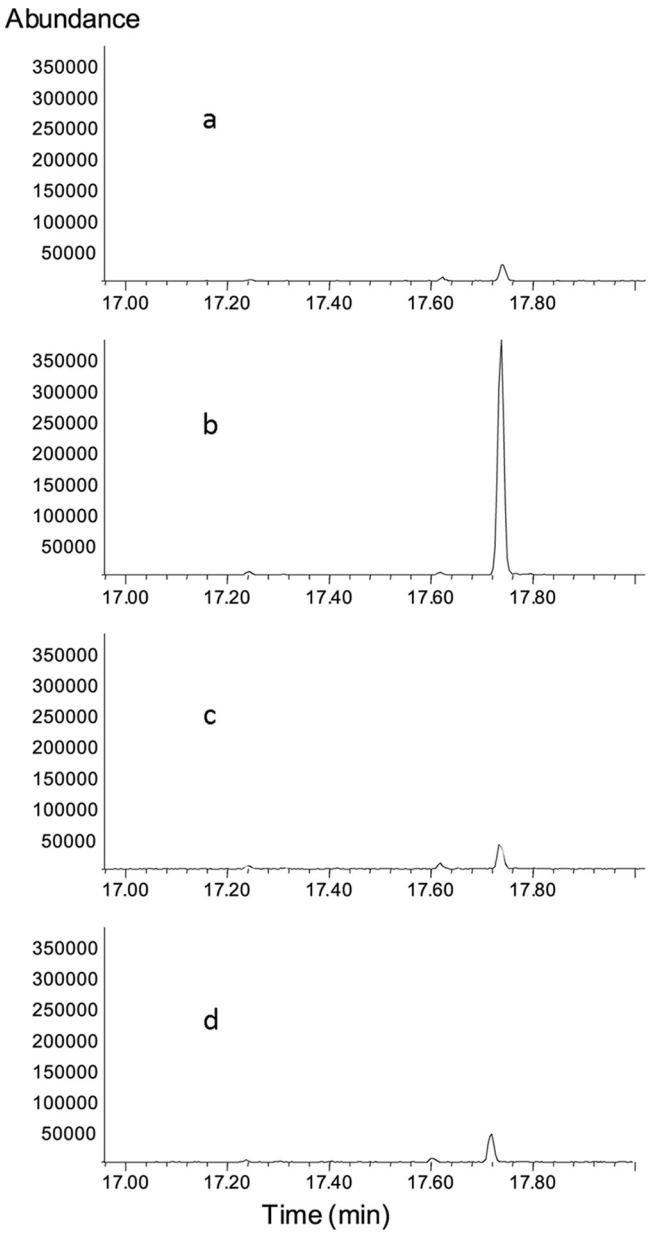

FIG. 2. Gas chromatograms of metabolites present in *C. elegans* 1908 aqueous extract after treatment without enzyme (a), with sulfatase (b), with β-glucuronidase (c), and with β-glucosidase (d).

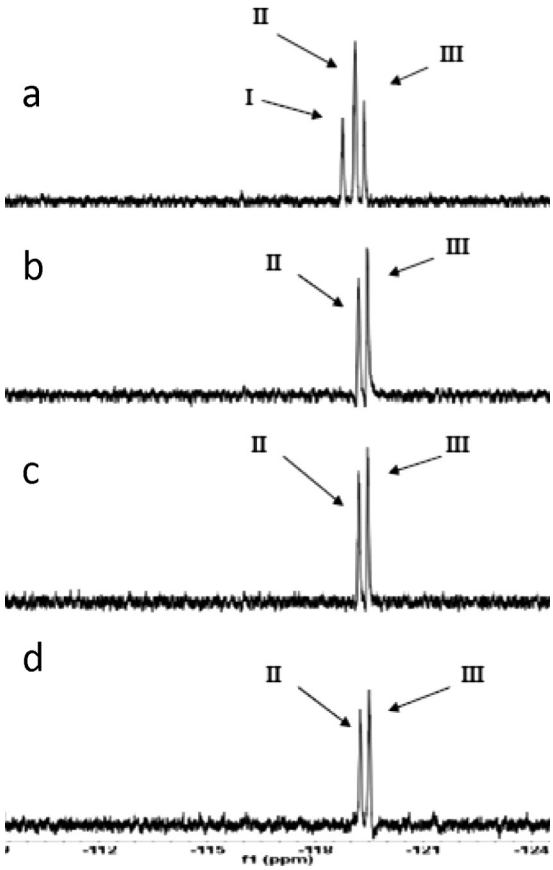

FIG. 3. ^{19}F NMR analysis of *C. blakesleeana* 1906 aqueous extracts after treatment without enzyme (a), with sulfatase (b), with β-glucuronidase (c), and with β-glucosidase (d). The enzymes were added sequentially to the same extract.

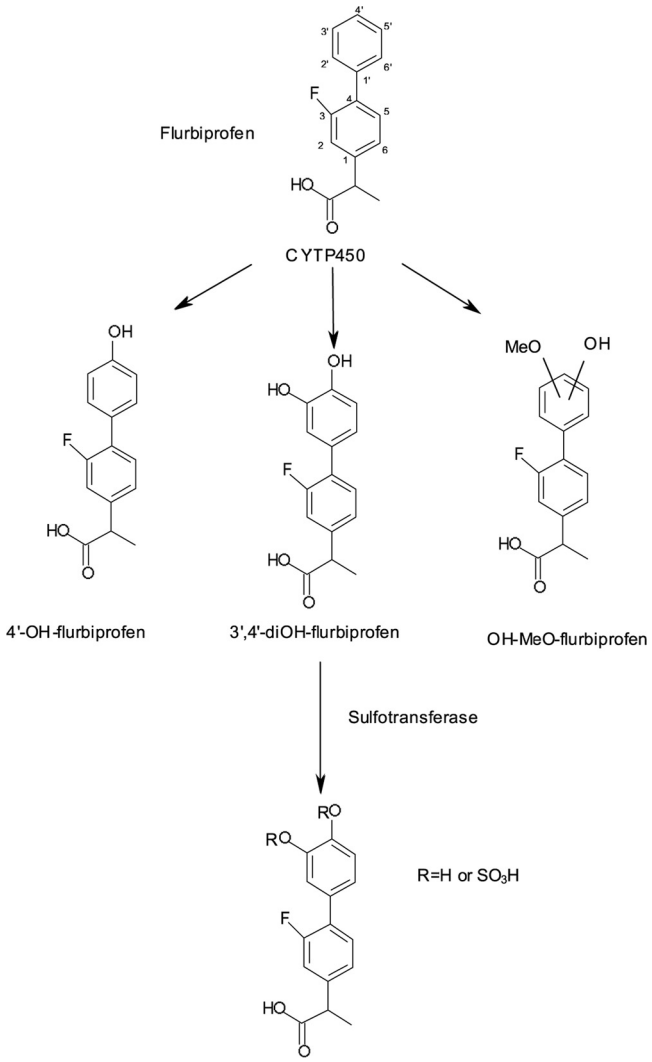

FIG. 4. Principal biotransformation reactions of flurbiprofen in *C. elegans* 1908.

shown). (*R*)-Flurbiprofen was biotransformed predominantly to 4′-hydroxyflurbiprofen, which was found to be identical to that produced by the racemate, based on ^{19}F NMR and GC-MS analysis.

Previous studies showed that flurbiprofen has strong antifungal activity (6), and so its effect on *C. elegans* DSM 1908 growth was investigated by incubating fungal spore suspension into Sabouraud dextrose liquid medium in 6-well plates (Sarstedt), with different concentrations of drug (0.1 to 5 mg/ml) added after 0, 6, 24, and 72 h. Flurbiprofen completely inhibited germination starting at the lowest concentration administered at 0 and 6 h; in the medium there was no presence of mature pellets of mycelium but only the fragments of the starting spore suspension. However, when flurbiprofen was added to cultures that were 24 to 72 h old, the fungal growth was not inhibited, and there was no difference in the biomasses collected from culture flasks used in the biotransformation experiments that had been exposed to flurbiprofen and those from the control cultures to which no drug was added.

We have shown for the first time that flurbiprofen is converted by *Cunninghamella* spp. to a variety of phase I and phase II metabolites (Fig. 4) present in several mammalian species, including humans. Among them, 4′-hydroxyflurbiprofen was confirmed to be the major product being converted by both mammalian and microbial systems. This similarity is remarkable considering that only one mammalian cytochrome P450 isoform can detoxify this drug. In fact, previous chemical inhibition studies confirmed that only P450 2C9 was involved in the 4′-hydroxylation of flurbiprofen in humans (18, 19). *C. elegans* DSM 1908 in particular would seem to be an appropriate microbial model of phase I metabolism in mammals since all the major metabolites are produced, in addition to new hydroxy and hydroxymethoxy isomers. The upscaling of the biotransformation may also have potential as a method of generating the metabolites as analytical standards, in particular 4′-hydroxyflurbiprofen.

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