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1	Bacterial production of hydroxylated and amidated metabolites of flurbiprofen
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8	
9	Abstract
10	Several Streptomyces and Bacillus strains were examined for their ability to transform the
11	anti-inflammatory drug flurbiprofen 1 to the hydroxylated metabolites that are found in
12	humans after ingestion of this compound. Of the seven Streptomyces spp. examined, all but
13	one transformed flurbiprofen to the main mammalian metabolite 4'-hydroxyflurbiprofen 2,
14	and the majority also produced 3',4'-dihydroxyflurbiprofen 3 . Three strains, <i>Streptomyces</i>
15	griseus DSM40236 and ATCC13273, and S. subrutilis DSM40445, also elaborated 3'-
16	methoxy, 4'-hydroxy-flurbiprofen 4. None of the Bacillus spp. examined yielded these
17	metabolites. Examination of the extracted supernatants of S. lavenduligriseus and S. rimosus

- 18 by fluorine-19 nuclear magnetic resonance (¹⁹F NMR), indicated new resonances and these
- 19 new fluorometabolites were purified by HPLC and revealed to be flurbiprofenamide **5** and 7-
- 20 hydroxyflurbiprofenamide 6 after MS and NMR analyses. Subsequent re-examination of the
- culture supernatants from *Bacillus subtilis* IM7, *B. megaterium* NCIMB8291 and *B.*
- 22 *megaterium* ATTC14581 showed that these strains also produced **5** and **6**. Resting cell
- 23 investigations suggested that the amidation reaction employed nitrogen from an as yet
- 24 unidentified amino acid.
- 25 Keywords: Biotransformation; Fluorometabolite; F-19 NMR

26 **1. Introduction**

Microorganisms can metabolise pharmaceutical compounds in a similar fashion to animals, 27 and thus can act as models of drug metabolism [1]. Furthermore, the ease of scaling-up 28 microbial cultures has the potential of generating sufficient quantities of drug metabolites that 29 might also be required for in vivo testing [2, 3]. The fungus *Cunninghamella elegans* has 30 been a particular focus for investigations on drug transformations [4], as it is known to 31 generate oxidative (phase I) and conjugative (phase II) metabolites. Studies have also been 32 conducted in bacteria belonging to the genus Streptomyces and Bacillus, which have 33 cytochrome P450 activity [5, 6] and can transform drugs such as irbesartan and diazepam [7]. 34 Pospisil et al. (1996) [8] reported that the biotransformation of salicylate by Streptomyces 35 spp. resulted in oxidation and amidation, yielding gentisate and salicamide. 36

Approximately 25% of the drugs available or in the pipeline contain fluorine, which 37 38 confers attractive properties, such as improved lipophilicity and slower metabolism compared with the non-fluorinated analogue [9]. There are some studies on the microbial metabolism 39 40 of fluorinated drugs, such as danofloxacin [10] and flutamide [11]. Monitoring the 41 catabolism of organofluorine compounds in bacteria and fungi has been improved with the development of fluorine-19 nuclear magnetic resonance spectroscopy (¹⁹F NMR), which has 42 been applied in several studies on the microbial transformation of fluoro-aryl compounds 43 [12], including fluorinated drugs [13]. We are interested in the microbial metabolism of 44 fluorinated drugs to enable the production of mammalian metabolites as an alternative to 45 chemical synthesis [14, 15]. Here, we describe experiments on the biotransformation of the 46 fluorinated, non-steroidal anti-inflammatory drug flurbiprofen [(RS)-2-(2-fluoro-4-biphenyl) 47 propionic acid] 1 by *Streptomyces* and *Bacillus* spp., and the characterisation of the products 48 49 formed.

51 **2. Experimental**

52 2.1. Chemicals and microorganisms

Flurbiprofen 1 and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased
from Sigma. ISP4 medium and tryptic soy broth were supplied from Difco and soya bean
meal was obtained from BDH chemicals. Bacterial strains were obtained from DSMZ,
Germany, LGC Standards, UK and NCIMB, Aberdeen, UK. *Bacillus subtilis* IM7 was
obtained from the culture collection, School of Biomolecular and Biomedical Science,
University College Dublin.

59

60 2.2. Culture conditions

The microorganisms were maintained on agar slants of ISP4 or tryptone soya agar. The 61 62 Streptomyces spp. were grown in a two stage fermentation procedure similar to that described by Griffiths et al. [16]. For the initial screening of the Streptomyces the strains were firstly 63 inoculated into 250 ml Erlenmeyer flasks containing 50 ml medium, which was either tryptic 64 soy broth (tsb) or soy bean medium composed of soya bean meal (5 g/l), glycerol (20 g/l), 65 yeast extract (5 g/l) and K₂HPO₄ (5 g/l), with the pH adjusted to 7.0 [17]. Cultures were 66 67 incubated for 72 h with rotary agitation (200 rpm) at 27 °C. Seed cultures (3 ml) were then transferred into 250 ml flasks containing 30 ml medium and incubated under the same 68 69 conditions. After 24 h flurbiprofen 1 (5 mg) solubilised in 40 µl dimethylformamide was added to each flask; control experiments in which flasks contained either no drug or 70 microorganism were established. Incubation was then continued for a further 72 h. Cultures 71 were sonicated on ice (Sonicator U200S control, IKA Labortechnik) for 1 min at 50% 72 amplitude, and the sonicate was centrifuged at 18,000 x g for 15 min and the pellet discarded. 73 The supernatant was extracted twice with 30 ml of ethyl acetate. Bacillus spp. were cultured 74

in 50 ml Lauria Bertani (LB) broth at 37 °C for 24 h; flurbiprofen 1 (5 mg) was added and the
cultures incubated for a further 48 h. Metabolites were extracted as described above.

Resting cultures were prepared by harvesting cells that were grown in either tsb (*S. lavenduligriseus*) or LB (*B. subtilis*) for 48 h, by centrifugation. The cells were washed in
either phosphate buffer (pH 7, 50 mM) or water, centrifuged, resuspended to the original
culture volume in buffer or water containing the desired nitrogen source, and incubated with
flurbiprofen for 48-72 h. The metabolites in the supernatant were extracted as previously
described.

83

84 2.3. Analysis of metabolites

Fluorine-19 nuclear magnetic resonance spectroscopy (¹⁹F NMR) on a Varian Inova 400
MHz spectrometer was used to analyse the aqueous and organic extracts. Organic extracts
were dried under a stream of nitrogen and solubilised in 800 µl CDCl₃. Aqueous fractions
were freeze dried using a LSL Secfroid freeze drier and solubilised in 800 µl D₂O.

The organic extracts were also analysed by gas chromatography-mass spectrometry (GC-MS) after the extracts (100 µl) were silylated by adding MSTFA (50 µl) and heating at 100 °C for 1 h. Derivatised extracts were diluted in 100 µl and samples (1 µl) were injected onto a HP5 MS column and the oven temperature was held at 150 °C for 2 min then raised to 300 °C over 8 min with a run time of 17 min. The hydroxylated and hydroxy, methoxylated flurbiprofen metabolites were identified via retention time and mass spectra [14, 18].

95

96 2.4. Isolation and identification of new fluorometabolites from S. lavenduligriseus

97 To identify the new fluorometabolites observed in *S. lavenduligriseus*, the biotransformation

98 products were isolated from the supernatant from 18 flasks (30 ml per flask) by semi-

99 preparative HPLC using a Zorbax SB-C18 (9.4 mm x 25 cm, 5 μm particles) column with a

gradient of acetonitrile/water (10-100% acetonitrile) over 30 min at a flow rate of 4 ml/min; 6
mg of 5 and 5 mg of 6 were recovered. ¹H and ¹³C NMR spectra (CDCl₃) were recorded on a
Varian Inova 400 MHz spectrometer and high resolution mass spectra were measured on a
Micromass LCT time-of-flight mass spectrum coupled to a Waters Alliance 2695 solvent
delivery system. ¹³C resonances are singlets unless otherwise specified.

Compound **5** ¹H NMR (δ , ppm): H-2[']/6['], 7.49 (d, J = 8.2 Hz, 2H); H-3[']/5['], 7.39 (t, J105 = 7.5Hz, 2H); H-5, 7.35 (dd, J = 8.5, 7.9 Hz); H-4', 7.32 (t, J = 7.3 Hz); H-6, 7.13 (dd, J = 7.5Hz); H-6, 7.5Hz); H-6, 7.13 (dd, J = 7.5Hz); H-6, 7.13 (dd, J = 7.5Hz); H-6, 7.5Hz); H-6, 7.13 (dd, J = 7.5Hz); H-6, 106 7.9, 1.7 Hz); H-2, 7.10 (dd, J = 11.5, 1.7 Hz); NH₂, 5.83 (brs, 2H); H-7, 3.58 (q, J = 7.6); H-107 8, 1.50 (d, J = 7.1). ¹³C NMR (δ , ppm): C-9, 176.4; C-3, 159.8 (d, J = 249.3 Hz); C-1, 142.5; 108 C-1', 135.0; C-5, 131.1 (d, J = 3.8 Hz); C-2'/6', 128.9 (d, J = 2.8 Hz); C-3'/5', 128.5; C-109 4,128.0; C-4', 127.7; C-6, 123.6 (d, *J* = 3.2 Hz); C-2, 115.25 (d, *J* = 23.5 Hz); C-7, 46.0; C-8, 110 18.23. ¹⁹F NMR (δ, ppm): -117.3 (dd, J = 11.5, 8.5 Hz). MS (HRESI (+) MS): m/z 244.1138 111 $[M+H]^+$, C₁₅H₁₅NOF requires 244.1138. GC-EIMS for per-trimethylsilylated derivative: m/z112 = 73 (100), 200 (76), 116 (26), 185 (18), 300 (8), 315 (0.006). 113 Compound **6** ¹H NMR (δ , ppm): H-2²/6², 7.49 (dt, J = 8.0, 1.4 Hz, 2H); H-6, 7.41 114 (ovl); H-2, 7.39 (d, *J* = 12.2 Hz); H-3[']/5['], 7.39 (ovl, 2H); H-5, 7.38 (ovl); H-4['], 7.32 (m); 115

116 NH_2 6.83 (brs); NH_2 5.88 (brs); H-8, 1.78 (s). ¹³C NMR (δ , ppm): C-9, 177.6; C-3, 159.5 (d,

117 J = 247 Hz); C-1, 145.1 (d, J = 7.3 Hz); C-1', 135.4; C-5, 130.4 (d, J = 3.8 Hz); C-2'/6',

118 128.9 (d, *J* = 3.0 Hz); C-3', 128.4; C-4, 128.1 (d, *J* = 13.7 Hz); C-4'/5', 127.6; C-6, 121.3 (d,

119 J = 3.4 Hz); C-2, 113.3 (d, J = 24.9 Hz); C-7, 75.7; C-8, 27.0. ¹⁹F NMR (δ , ppm): -117.81

120 (dd, J = 12.2, 7.6Hz). MS (HRESI (-) MS): m/z 258.0942 [M – H]⁻, C₁₅H₁₃NO₂F requires

121 258.0930. GC-EIMS for per-trimethylsilylated derivative: m/z = 73 (100), 198 (53), 288

122 (44), 388 (2)

123

125 **3. Results and discussion**

126 3.1 Screening of bacteria for mammalian metabolites of flurbiprofen 1

In mammals flurbiprofen 1 is metabolised to the phase I metabolites 4'-hydroxyflurbiprofen 127 2, 3',4'-dihydroxyflurbiprofen 3 and 3'-hydroxy, 4'-methoxyflurbiprofen 4 (Figure 1), in 128 addition to glucuronide and sulphate conjugates. We have recently reported the fungal 129 metabolism of flurbiprofen 1 to these metabolites [14], and extended this study to include 130 131 *Streptomyces* and *Bacillus*, which are known to generate oxidative metabolites of other drugs. The strains were cultured as described in Materials and Methods, and incubated with the 132 133 drug. After 72 h incubation the biotransformation products were extracted and analysed by GC-MS to determine the presence of the human metabolites. 134 Of the Streptomyces spp. examined, only S. griseolus did not produce any of the 135 136 phase I metabolites, and S. griseus DSM 40236 and ATCC 13273 produced all three (Table 1). Interestingly, 3'-methoxy, 4'-hydroxy-flurbiprofen was also observed as a major 137 metabolite in the two S. griseus strains, indicating the presence of a methyl transferase 138 activity. Dhar et al. [19] isolated an S-adenosyl methionine-dependent O-methyl transferase 139 enzyme from S. griseus ATCC 13273 that specifically methylates catechol substrates, and 140 might be expected to methylate dihydroxyflurbiprofen. Cytochrome P450 10105D1 from S. 141 griseus was overexpressed in E. coli and shown to transform a number of xenobiotics [5]. 142 143 This enzyme is analogous to the major xenobiotic-metabolising cytochrome P450 in 144 mammals, CYP3A4; interestingly, flurbiprofen is metabolised by a different isoform in humans, 2C9, suggesting that S. griseus has other cytochromes P450. This would not be 145 unusual, since, for example, S. coelicolor A3 (2) has 18 cytochromes P450 [20]. None of the 146 Bacillus strains investigated (B. subtilis IM7, B. subtilis ATCC6633, B. licheniformis 147 NCIMB8549, B. megaterium NCIMB8291 and B. megaterium ATCC14581) produced phase 148 I metabolites. This was surprising since cytochromes P450 are known in this genus [21]. 149

150 It was observed that the extent of flurbiprofen transformation and the relative amounts of metabolites varied in the replicate flasks, thus different conditions were employed in an 151 attempt to obtain consistency in the biotransformations. A greater degree of transformation 152 was observed when the concentration of flurbiprofen was lowered to 0.1 mg/ml and added to 153 a 24 h culture that had not been subcultured. Furthermore, some strains responded better to a 154 different culture medium, for example, S. griseolus, which did not transform flurbiprofen 155 when cultured in the original soybean medium described by [17], produced 2 when cultured 156 in tryptone soy broth. 157

158

159 3.2 Identification of new metabolites from S. lavenduligriseus

Routinely, organic extracts were analysed by ¹⁹F NMR, which revealed the presence of two 160 resonances in the extracts of S. lavenduligriseus and S. rimosus at δ -116.97 and -117.1 ppm, 161 which were distinct from the resonances of flurbiprofen 1 and 4'-hydroxybiprofen 2 (Figure 162 2). To identify these compounds S. lavenduligriseus was cultured in a large volume in order 163 to obtain enough material for detailed analyses to be conducted. Metabolites were purified 164 165 by reversed-phase HPLC and their structures determined using the NMR and mass spectroscopic analyses. High-resolution mass spectrometry analysis of compound 5 (m/z) 166 244.1138, $[M+H]^+$) was consistent with a molecular formula of $C_{15}H_{14}NOF$. The most 167 noticeable change in the ¹H NMR of **5** compared to **1** was the presence of an additional broad 168 singlet in the ¹H NMR spectrum (δ 5.83 ppm, 2H). These protons showed no correlations in a 169 HSQC experiment, suggesting that they were not connected to a carbon atom. These 170 observations suggested the presence of a primary amide at C-9. Other significant differences 171 included changes in the chemical shift of H-7 (δ_H 3.58, q, J = 7.6 Hz), C-7 (δ_C 46.0), and C-9 172 $(\delta_{\rm C} 176.4)$, consistent with such a structure. Thus, **5** was identified as flurbiprofenamide 173

(Figure 3), which has been reported previously [22] although only limited spectral data werereported.

Compound 6 possessed a molecular formula of $C_{15}H_{14}NO_2F$, as determined by HR-176 MS $(m/z 258.0942 [M - H]^{-})$. The resonance for the H-7 proton of 5 was absent in the ¹H 177 NMR spectrum of **6**, and the resonance of the H-8 methyl group ($\delta_{\rm H}$ 1.78) changed from a 178 doublet to a singlet, indicating substitution at C-7. Based on the HR-MS and the dramatic 179 change in the ¹³C chemical shift of the resonance for C-7 (δ_C 75.7), in comparison to the 180 equivalent carbon of flurbiprofenamide 5, compound 6 was tentatively identified as 7-181 hydroxyflurbiprofenamide (Figure 3). Two dimensional NMR analysis supported such a 182 structure, with HMBC correlations from H-8 to C-6, C-7 and C-9 (δ_C 145.1, 75.7, and 177.6) 183 Interestingly, unlike 5, in the ¹H NMR spectrum of 6 there are two resonances for the amide 184 protons ($\delta_{\rm H}$ 6.83, brs; and 5.88, brs), which can be attributed to hydrogen bonding of the 185 amide protons to the C-7 hydroxyl group; similar patterns have been observed in the ¹H NMR 186 spectra of other primary amides in which hydrogen bond acceptors are available [23]. To 187 188 determine the order of the amidation and hydroxylation reactions in *S. lavenduligriseus* production of the metabolites was followed with time: flurbiprofenamide 5 was observed 189 within 1 h of incubation with flurbiprofen by GC-MS and 7-hydroxyflurbiprofenamide 6 190 could be detected after 3 h, indicating that amidation occurred first. The possibility that the 191 highly activated C-7 of 5 underwent spontaneous oxidation was examined by standing the 192 compound in soybean medium for 72 h; however, no hydroxylated product was observed, 193 thus it was concluded that the transformation is biological. Kergomard and Renard [24] 194 reported amidation of a range of substituted benzoic acids by S. violaceoniger and among 22 195 196 strains of actinomycetes that transformed cinnamic acid to cinnamamide, S. halstedii demonstrated 95 % molar conversion [25]; however, in neither study was the enzyme 197 responsible identified. 198

199 Amidation of aryl carboxylic acids is also known to occur in *Bacillus cereus* [26, 27], thus the analyses of the silvlated products from the biotransformation experiments conducted 200 with the various Bacillus spp. were re-examined to determine if amidation of flurbiprofen had 201 202 occurred. Flurbiprofenamide 5 and 7-hydroxyflurbiprofenamide 6 were detected in B. subtilis IM7, B. megaterium NCIMB 8291 and B. megaterium ATCC14581 (Figure 4). As 203 with S. lavenduligriseus, in experiments with B. subtilis the amidated metabolite 5 was 204 205 detected in the culture first, after 3 h; the hydroxyamidated metabolite 6 was not detected until 48 h. 206

207

208 *3.3 Resting cell studies*

209 Maruyama et al. [26] observed that the nitrogen atom involved in the amidation of 210 polyaromatic carboxylic acids by resting cells of *B. cereus* originated from the amino group of amino acids. Therefore, to examine further the amidation reaction occurring in S. 211 lavenduligriseus resting cultures were incubated with flurbiprofen and a nitrogen source 212 (glycine, $(NH_4)_2SO_4$ and peptone). Table 2 shows the extent of flurbiprofen 1 213 biotransformation under these conditions, and reveals that while some biotransformation does 214 occur in resting cells in the absence of added nitrogen, the presence of peptone resulted in a 215 greater transformation. A similar observation was made with B. subtilis resting cells, which 216 transformed 63 % of 1 to the amidated metabolites when peptone was included, but only 30 217 218 % when no nitrogen source was added.

219

220 *4. Conclusion*

221 Microorganisms have been investigated as models of drug metabolism owing to the

expression of a wide range of cytochromes P450. Here we screened a small selection of

223 Streptomyces and Bacillus spp. for their ability to oxidise the non-steroidal anti-inflammatory

- drug flurbiprofen to metabolites detected in mammals. *Streptomyces griseus* ATCC13273
- was observed to be the most effective strain examined in the production of the hydroxylated
- metabolites of flurbiprofen, which are the predominant metabolites generated in mammals.
- 227 Surprisingly, no hydroxylated flurbiprofen metabolites were detected in *Bacillus* cultures that
- 228 were incubated with the drug. Two new fluorometabolites were detected in culture extracts
- of *S. lavenduligriseus* and *S. rimosus*, flurbiprofenamide **5** and 7-hydroxy-flurbiprofenamide
- **6**, and in three strains of *Bacillus*, probably via the action of an amidotransferase.

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- 235
- 236 **References**

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- 283 284

Microorganism/Fluorometabolite	4'-OH-	3',4'-diOH-	3'-OMe, 4'-OH-	
	flurbiprofen 2	flurbiproten 3	flurbiprofen 4	
Streptomyces griseolus DSM 40854	-	-	-	
Streptomyces griseus ATCC 13273	++	++	+++	
Streptomyces griseus DSM 40226	+	-	-	
Streptomyces griseus DSM 40236 ^a	++	+++	+++	
Streptomyces lavenduligriseus DSM 40487 ^b	+	+	-	
Streptomyces rimosus DSM 40260	++	-	-	

+

_

+

Table 1. Mammalian metabolites of flurbiprofen 1 observed in Streptomyces species 286

287 288

+ 1-10%, ++ 11-50%, +++ 50-100% by GC-MS

Streptomyces subrutilis DSM 40445 ^c

^a There were noticeable variations in the proportions of the metabolites in each flask ^b 3',4'-diOH-flurbiprofen was only observed in one flask ^c 3'-OMe,4'-OH-flurbiprofen was only observed in one flask 289

290

291

292

- 294 Table 2. The conversion of flurbiprofen to amidated metabolites by *S. lavenduligriseus*
- resting cells in the presence of different nitrogen sources. The percentages were determined

296	from the	GC peak	areas of	the m	etabolites.
-----	----------	---------	----------	-------	-------------

		Fluorometabolite (%)	
Nitrogen source	Flurbiprofen 1	Flurbiprofenamide 5	7-hydroxy- flurbiprofenamide 6
No nitrogen source	72.8	23.1	4.1
Glycine (10 mM)	67.3	28.1	4.6
(NH ₄) ₂ SO ₄ (20 mM)	75.3	21.4	3.3
Peptone (2 % w/v)	41.9	52.5	5.6

- 299 Figure legends
- Figure 1. Phase I metabolism of flurbiprofen 1 (CYP: cytochrome P450; MT: methyl
 transferase)
- Figure 2. NMR spectra showing (A) 4'-hydroxy- 2, 3',4'-dihydroxy-flurbiprofen 3 and
- flurbiprofen 1 (overlapping peak) from *S.griseus* ATCC 13273, and (B) new
- 304 fluorometabolites from *S.lavenduligriseus* indicated by arrows.
- Figure 3. Metabolism of flurbiprofen **1** in *S. lavenduligriseus*.
- Figure 4. GC-MS analysis of supernatant from *B. subtilis* IM7 that was incubated with
- 307 flurbiprofen 1; flubiprofenamide 5 and 7-hydroxyflurbiprofenamide 6 were detected by
- 308 comparison of their retention times (A) and mass spectra (B and C) with the compounds
- 309 purified from *S. lavenduligriseus*.



312 Figure 1



315 Figure 2







Figure 4

Abundance







