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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, *Streptomyces*  
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 (<sup>19</sup>F 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  
21 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**

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

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

50

## 51 **2. Experimental**

### 52 *2.1. Chemicals and microorganisms*

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

59

### 60 *2.2. Culture conditions*

61 The microorganisms were maintained on agar slants of ISP4 or tryptone soya agar. The  
62 *Streptomyces* spp. were grown in a two stage fermentation procedure similar to that described  
63 by Griffiths et al. [16]. For the initial screening of the *Streptomyces* the strains were firstly  
64 inoculated into 250 ml Erlenmeyer flasks containing 50 ml medium, which was either tryptic  
65 soy broth (tsb) or soy bean medium composed of soya bean meal (5 g/l), glycerol (20 g/l),  
66 yeast extract (5 g/l) and K<sub>2</sub>HPO<sub>4</sub> (5 g/l), with the pH adjusted to 7.0 [17]. Cultures were  
67 incubated for 72 h with rotary agitation (200 rpm) at 27 °C. Seed cultures (3 ml) were then  
68 transferred into 250 ml flasks containing 30 ml medium and incubated under the same  
69 conditions. After 24 h flurbiprofen **1** (5 mg) solubilised in 40 µl dimethylformamide was  
70 added to each flask; control experiments in which flasks contained either no drug or  
71 microorganism were established. Incubation was then continued for a further 72 h. Cultures  
72 were sonicated on ice (Sonicator U200S control, IKA Labortechnik) for 1 min at 50%  
73 amplitude, and the sonicate was centrifuged at 18,000 x g for 15 min and the pellet discarded.  
74 The supernatant was extracted twice with 30 ml of ethyl acetate. *Bacillus* spp. were cultured

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

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

83

### 84 2.3. Analysis of metabolites

85 Fluorine-19 nuclear magnetic resonance spectroscopy (<sup>19</sup>F NMR) on a Varian Inova 400  
86 MHz spectrometer was used to analyse the aqueous and organic extracts. Organic extracts  
87 were dried under a stream of nitrogen and solubilised in 800 µl CDCl<sub>3</sub>. Aqueous fractions  
88 were freeze dried using a LSL Secfroid freeze drier and solubilised in 800 µl D<sub>2</sub>O.

89 The organic extracts were also analysed by gas chromatography-mass spectrometry  
90 (GC-MS) after the extracts (100 µl) were silylated by adding MSTFA (50 µl) and heating at  
91 100 °C for 1 h. Derivatized extracts were diluted in 100 µl and samples (1 µl) were injected  
92 onto a HP5 MS column and the oven temperature was held at 150 °C for 2 min then raised to  
93 300 °C over 8 min with a run time of 17 min. The hydroxylated and hydroxy, methoxylated  
94 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

100 gradient of acetonitrile/water (10-100% acetonitrile) over 30 min at a flow rate of 4 ml/min; 6  
101 mg of **5** and 5 mg of **6** were recovered.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra ( $\text{CDCl}_3$ ) were recorded on a  
102 Varian Inova 400 MHz spectrometer and high resolution mass spectra were measured on a  
103 Micromass LCT time-of-flight mass spectrum coupled to a Waters Alliance 2695 solvent  
104 delivery system.  $^{13}\text{C}$  resonances are singlets unless otherwise specified.

105 Compound **5**  $^1\text{H}$  NMR ( $\delta$ , ppm): H-2'/6', 7.49 (d,  $J = 8.2$  Hz, 2H); H-3'/5', 7.39 (t,  $J$   
106 = 7.5 Hz, 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,  $J =$   
107 7.9, 1.7 Hz); H-2, 7.10 (dd,  $J = 11.5, 1.7$  Hz);  $\text{NH}_2$ , 5.83 (brs, 2H); H-7, 3.58 (q,  $J = 7.6$ ); H-  
108 8, 1.50 (d,  $J = 7.1$ ).  $^{13}\text{C}$  NMR ( $\delta$ , ppm): C-9, 176.4; C-3, 159.8 (d,  $J = 249.3$  Hz); C-1, 142.5;  
109 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-  
110 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,  
111 18.23.  $^{19}\text{F}$  NMR ( $\delta$ , ppm): -117.3 (dd,  $J = 11.5, 8.5$  Hz). MS (HRESI (+) MS):  $m/z$  244.1138  
112  $[\text{M}+\text{H}]^+$ ,  $\text{C}_{15}\text{H}_{15}\text{NOF}$  requires 244.1138. GC-EIMS for per-trimethylsilylated derivative:  $m/z$   
113 = 73 (100), 200 (76), 116 (26), 185 (18), 300 (8), 315 (0.006).

114 Compound **6**  $^1\text{H}$  NMR ( $\delta$ , ppm): H-2'/6', 7.49 (dt,  $J = 8.0, 1.4$  Hz, 2H); H-6, 7.41  
115 (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);  
116  $\text{NH}_2$  6.83 (brs);  $\text{NH}_2$  5.88 (brs); H-8, 1.78 (s).  $^{13}\text{C}$  NMR ( $\delta$ , 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.  $^{19}\text{F}$  NMR ( $\delta$ , ppm): -117.81  
120 (dd,  $J = 12.2, 7.6$  Hz). MS (HRESI (-) MS):  $m/z$  258.0942  $[\text{M}-\text{H}]^-$ ,  $\text{C}_{15}\text{H}_{13}\text{NO}_2\text{F}$  requires  
121 258.0930. GC-EIMS for per-trimethylsilylated derivative:  $m/z = 73$  (100), 198 (53), 288  
122 (44), 388 (2)

123

124

125 **3. Results and discussion**

126 *3.1 Screening of bacteria for mammalian metabolites of flurbiprofen 1*

127 In mammals flurbiprofen **1** is metabolised to the phase I metabolites 4'-hydroxyflurbiprofen  
128 **2**, 3',4'-dihydroxyflurbiprofen **3** and 3'-hydroxy, 4'-methoxyflurbiprofen **4** (Figure 1), in  
129 addition to glucuronide and sulphate conjugates. We have recently reported the fungal  
130 metabolism of flurbiprofen **1** to these metabolites [14], and extended this study to include  
131 *Streptomyces* and *Bacillus*, which are known to generate oxidative metabolites of other drugs.  
132 The strains were cultured as described in Materials and Methods, and incubated with the  
133 drug. After 72 h incubation the biotransformation products were extracted and analysed by  
134 GC-MS to determine the presence of the human metabolites.

135 Of the *Streptomyces* spp. examined, only *S. griseolus* did not produce any of the  
136 phase I metabolites, and *S. griseus* DSM 40236 and ATCC 13273 produced all three (Table  
137 1). Interestingly, 3'-methoxy, 4'-hydroxy-flurbiprofen was also observed as a major  
138 metabolite in the two *S. griseus* strains, indicating the presence of a methyl transferase  
139 activity. Dhar et al. [19] isolated an *S*-adenosyl methionine-dependent *O*-methyl transferase  
140 enzyme from *S. griseus* ATCC 13273 that specifically methylates catechol substrates, and  
141 might be expected to methylate dihydroxyflurbiprofen. Cytochrome P450 10105D1 from *S.*  
142 *griseus* was overexpressed in *E. coli* and shown to transform a number of xenobiotics [5].  
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  
145 humans, 2C9, suggesting that *S. griseus* has other cytochromes P450. This would not be  
146 unusual, since, for example, *S. coelicolor* A3 (2) has 18 cytochromes P450 [20]. None of the  
147 *Bacillus* strains investigated (*B. subtilis* IM7, *B. subtilis* ATCC6633, *B. licheniformis*  
148 NCIMB8549, *B. megaterium* NCIMB8291 and *B. megaterium* ATCC14581) produced phase  
149 I metabolites. This was surprising since cytochromes P450 are known in this genus [21].

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

158

### 159 3.2 Identification of new metabolites from *S. lavenduligriseus*

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



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

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

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

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  
211 of amino acids. Therefore, to examine further the amidation reaction occurring in *S.*  
212 *lavenduligriseus* resting cultures were incubated with flurbiprofen and a nitrogen source  
213 (glycine, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and peptone). Table 2 shows the extent of flurbiprofen **1**  
214 biotransformation under these conditions, and reveals that while some biotransformation does  
215 occur in resting cells in the absence of added nitrogen, the presence of peptone resulted in a  
216 greater transformation. A similar observation was made with *B. subtilis* resting cells, which  
217 transformed 63 % of **1** to the amidated metabolites when peptone was included, but only 30  
218 % when no nitrogen source was added.

219

### 220 4. Conclusion

221 Microorganisms have been investigated as models of drug metabolism owing to the  
222 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

224 drug flurbiprofen to metabolites detected in mammals. *Streptomyces griseus* ATCC13273  
225 was observed to be the most effective strain examined in the production of the hydroxylated  
226 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  
229 of *S. lavenduligriseus* and *S. rimosus*, flurbiprofenamide **5** and 7-hydroxy-flurbiprofenamide  
230 **6**, and in three strains of *Bacillus*, probably via the action of an amidotransferase.

231

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235

### 236 **References**

237

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

Microorganism/Fluorometabolite	4'-OH- flurbiprofen <b>2</b>	3',4'-diOH- flurbiprofen <b>3</b>	3'-OMe, 4'-OH- flurbiprofen <b>4</b>
<i>Streptomyces griseolus</i> DSM 40854	-	-	-
<i>Streptomyces griseus</i> ATCC 13273	++	++	+++
<i>Streptomyces griseus</i> DSM 40226	+	-	-
<i>Streptomyces griseus</i> DSM 40236 <sup>a</sup>	++	+++	+++
<i>Streptomyces lavenduligriseus</i> DSM 40487 <sup>b</sup>	+	+	-
<i>Streptomyces rimosus</i> DSM 40260	++	-	-
<i>Streptomyces subutilis</i> DSM 40445 <sup>c</sup>	+	-	+

287 + 1-10%, ++ 11-50%, +++ 50-100% by GC-MS

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289 <sup>a</sup> There were noticeable variations in the proportions of the metabolites in each flask

290 <sup>b</sup> 3',4'-diOH-flurbiprofen was only observed in one flask

291 <sup>c</sup> 3'-OMe,4'-OH-flurbiprofen was only observed in one flask

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294 Table 2. The conversion of flurbiprofen to amidated metabolites by *S. lavenduligriseus*  
295 resting cells in the presence of different nitrogen sources. The percentages were determined  
296 from the GC peak areas of the metabolites.

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

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299 Figure legends

300 Figure 1. Phase I metabolism of flurbiprofen **1** (CYP: cytochrome P450; MT: methyl  
301 transferase)

302 Figure 2. NMR spectra showing (A) 4'-hydroxy- **2**, 3',4'-dihydroxy-flurbiprofen **3** and  
303 flurbiprofen **1** (overlapping peak) from *S.griseus* ATCC 13273, and (B) new  
304 fluorometabolites from *S.lavenduligriseus* indicated by arrows.

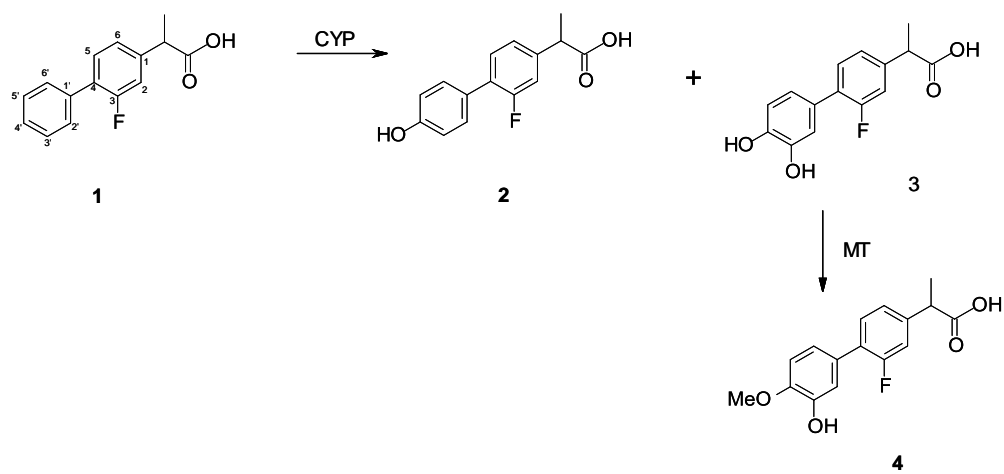
305 Figure 3. Metabolism of flurbiprofen **1** in *S. lavenduligriseus*.

306 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*.

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311

312 Figure 1



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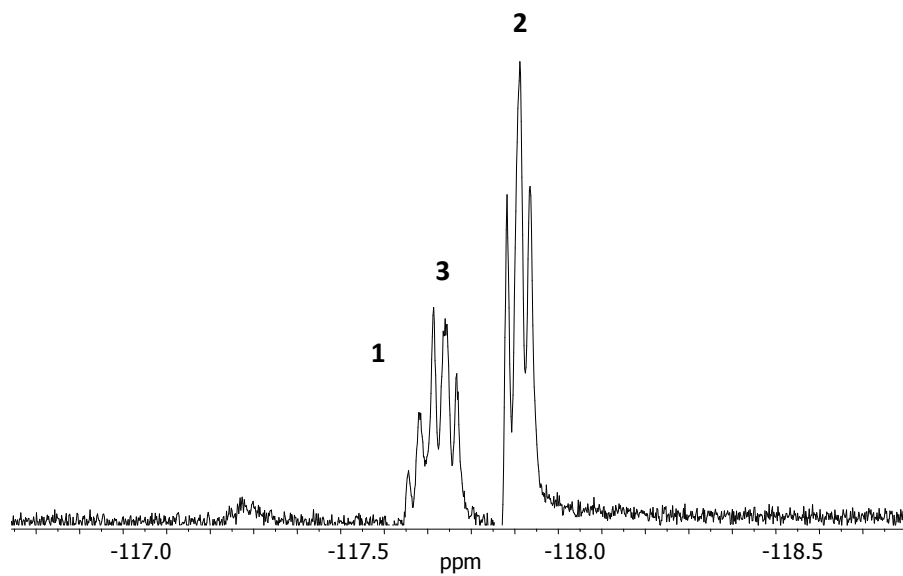
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315 Figure 2

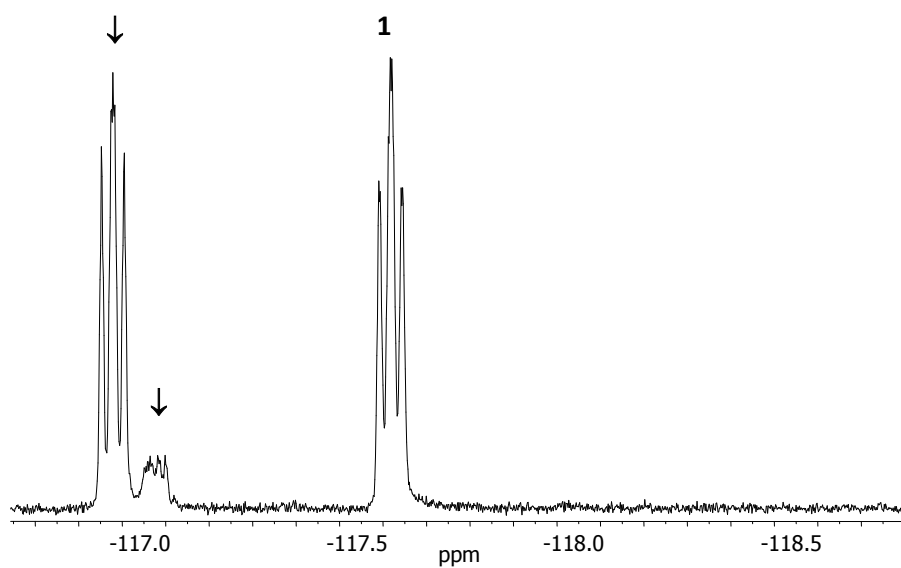
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**A** <sub>1</sub>



317

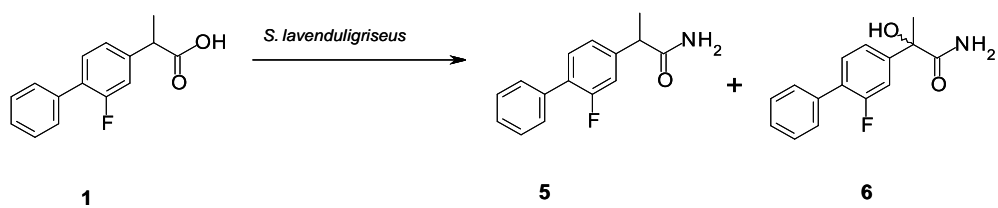
**B** <sub>1</sub>



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319

320 Figure 3



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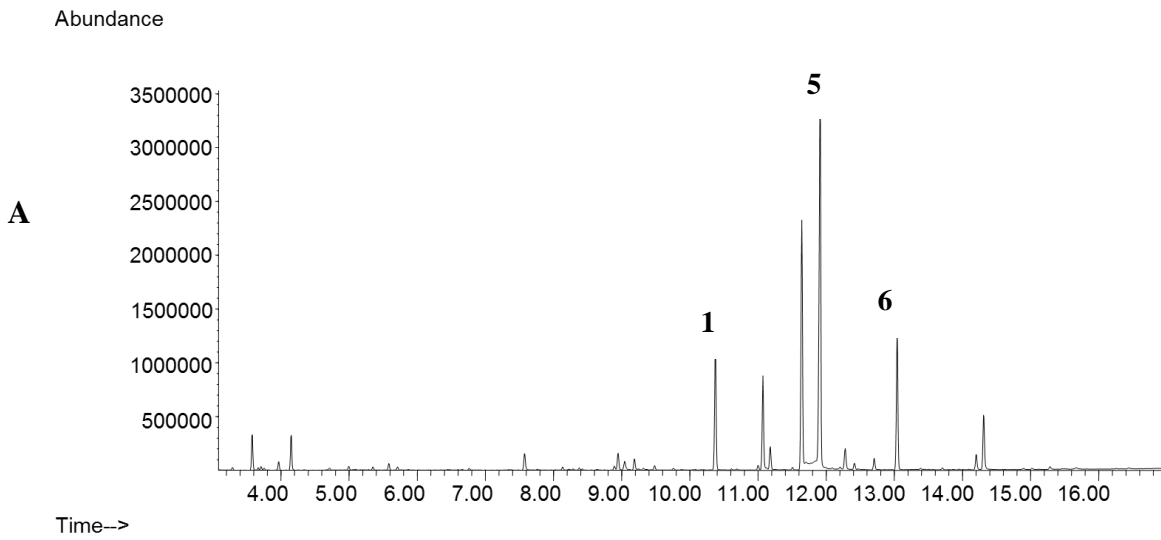
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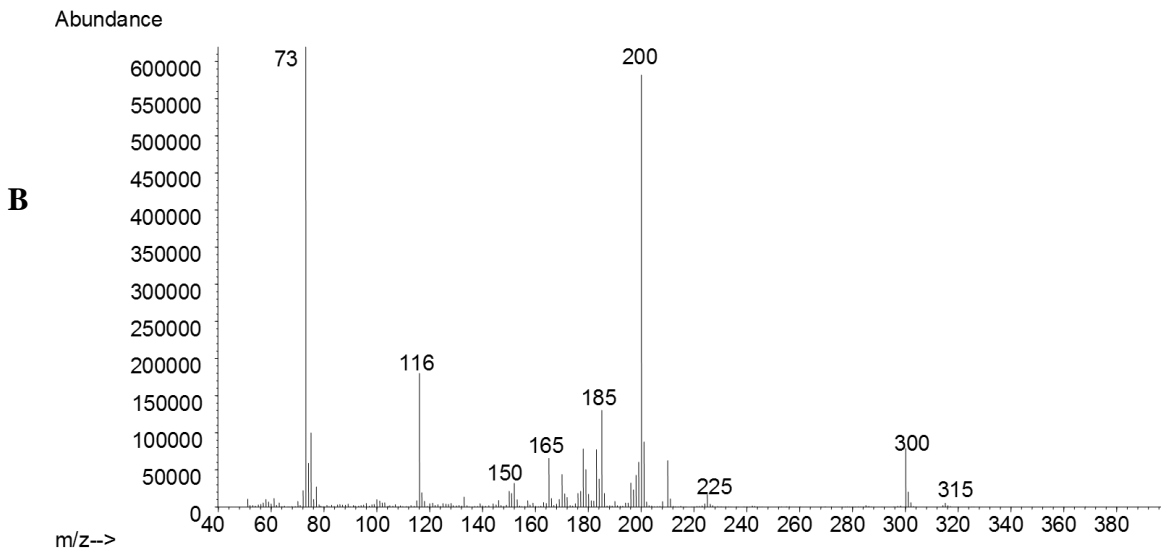
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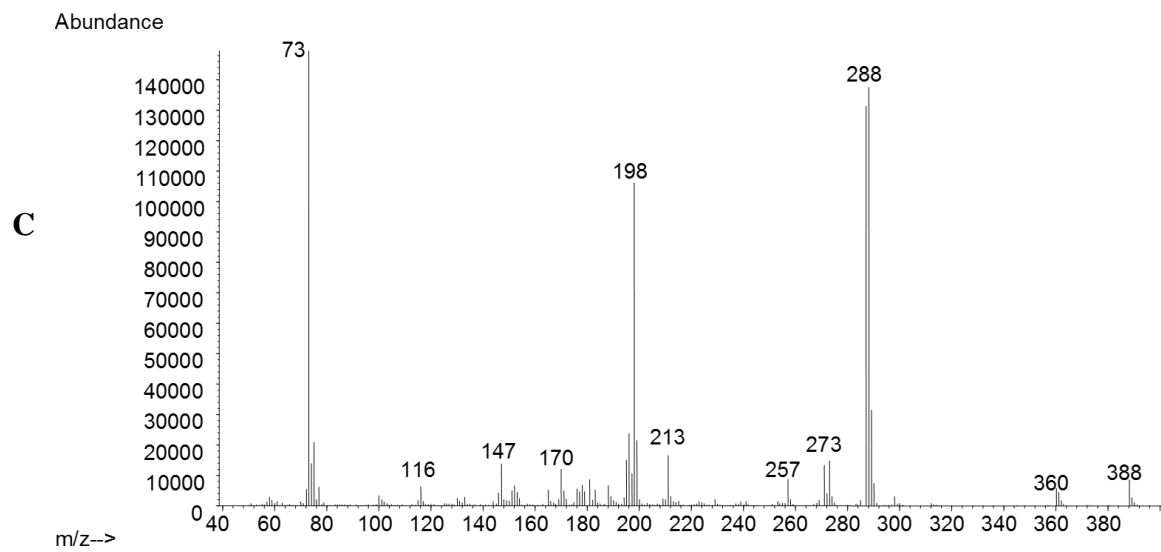
327 Figure 4



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