Bacterial production of hydroxylated and amidated metabolites of flurbiprofen

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Abstract

Several *Streptomyces* and *Bacillus* strains were examined for their ability to transform the anti-inflammatory drug flurbiprofen **1** to the hydroxylated metabolites that are found in humans after ingestion of this compound. Of the seven *Streptomyces* spp. examined, all but one transformed flurbiprofen to the main mammalian metabolite 4'-hydroxyflurbiprofen **2**, and the majority also produced 3',4'-dihydroxyflurbiprofen **3**. Three strains, *Streptomyces griseus* DSM40236 and ATCC13273, and *S. subrutilis* DSM40445, also elaborated 3'-methoxy, 4'-hydroxy-flurbiprofen **4**. None of the *Bacillus* spp. examined yielded these metabolites. Examination of the extracted supernatants of *S. lavenduligriseus* and *S. rimosus* by fluorine-19 nuclear magnetic resonance (^{19}F NMR), indicated new resonances and these new fluorometabolites were purified by HPLC and revealed to be flurbiprofenamide **5** and 7-hydroxyflurbiprofenamide **6** after MS and NMR analyses. Subsequent re-examination of the culture supernatants from *Bacillus subtilis* IM7, *B. megaterium* NCIMB8291 and *B. megaterium* ATTC14581 showed that these strains also produced **5** and **6**. Resting cell investigations suggested that the amidation reaction employed nitrogen from an as yet unidentified amino acid.

Keywords: Biotransformation; Fluorometabolite; F-19 NMR

1. Introduction

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

Approximately 25% of the drugs available or in the pipeline contain fluorine, which 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 of fluorinated drugs, such as danofloxacin [10] and flutamide [11]. Monitoring the catabolism of organofluorine compounds in bacteria and fungi has been improved with the development of fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F NMR), which has been applied in several studies on the microbial transformation of fluoro-aryl compounds [12], including fluorinated drugs [13]. We are interested in the microbial metabolism of fluorinated drugs to enable the production of mammalian metabolites as an alternative to chemical synthesis [14, 15]. Here, we describe experiments on the biotransformation of the fluorinated, non-steroidal anti-inflammatory drug flurbiprofen [(*RS*)-2-(2-fluoro-4-biphenyl) propionic acid] **1** by *Streptomyces* and *Bacillus* spp., and the characterisation of the products formed.

2. Experimental

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.

2.2. Culture conditions

The microorganisms were maintained on agar slants of ISP4 or tryptone soya agar. The *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 inoculated into 250 ml Erlenmeyer flasks containing 50 ml medium, which was either tryptic soy broth (tsb) or soy bean medium composed of soya bean meal (5 g/l), glycerol (20 g/l), yeast extract (5 g/l) and K_2HPO_4 (5 g/l), with the pH adjusted to 7.0 [17]. Cultures were 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 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 microorganism were established. Incubation was then continued for a further 72 h. Cultures were sonicated on ice (Sonicator U200S control, IKA Labortechnik) for 1 min at 50% amplitude, and the sonicate was centrifuged at 18,000 x *g* for 15 min and the pellet discarded. The supernatant was extracted twice with 30 ml of ethyl acetate. *Bacillus* spp. were cultured

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.

2.3. Analysis of metabolites

Fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F 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 $CDCl_3$. Aqueous fractions were freeze dried using a LSL Secfroid freeze drier and solubilised in 800 µl D_2O.

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].

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

To identify the new fluorometabolites observed in *S. lavenduligriseus*, the biotransformation products were isolated from the supernatant from 18 flasks (30 ml per flask) by semi-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. ^{1}H and ^{13}C NMR spectra ($CDCl_3$) 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. ^{13}C resonances are singlets unless otherwise specified.

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

Compound **6** ^{1}H NMR (δ, ppm): H-2'/6', 7.49 (dt, *J* = 8.0, 1.4 Hz, 2H); H-6, 7.41 (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); N*H*$_2$ 6.83 (brs); N*H*$_2$ 5.88 (brs); H-8, 1.78 (s). ^{13}C NMR (δ, ppm): C-9, 177.6; C-3, 159.5 (d, *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', 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, *J* = 3.4 Hz); C-2, 113.3 (d, *J* = 24.9 Hz); C-7, 75.7; C-8, 27.0. ^{19}F NMR (δ, ppm): -117.81 (dd, *J* = 12.2, 7.6Hz). MS (HRESI (-) MS): *m/z* 258.0942 $[M - H]^-$, $C_{15}H_{13}NO_2F$ requires 258.0930. GC-EIMS for per-trimethylsilylated derivative: *m/z* = 73 (100), 198 (53), 288 (44), 388 (2)

3. Results and discussion

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

In mammals flurbiprofen **1** is metabolised to the phase I metabolites 4'-hydroxyflurbiprofen **2**, 3',4'-dihydroxyflurbiprofen **3** and 3'-hydroxy, 4'-methoxyflurbiprofen **4** (Figure 1), in addition to glucuronide and sulphate conjugates. We have recently reported the fungal metabolism of flurbiprofen **1** to these metabolites [14], and extended this study to include *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 drug. After 72 h incubation the biotransformation products were extracted and analysed by GC-MS to determine the presence of the human metabolites.

Of the *Streptomyces* spp. examined, only *S. griseolus* did not produce any of the 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 metabolite in the two *S. griseus* strains, indicating the presence of a methyl transferase activity. Dhar et al. [19] isolated an *S*-adenosyl methionine-dependent *O*-methyl transferase enzyme from *S. griseus* ATCC 13273 that specifically methylates catechol substrates, and might be expected to methylate dihydroxyflurbiprofen. Cytochrome P450 10105D1 from *S. griseus* was overexpressed in *E. coli* and shown to transform a number of xenobiotics [5]. This enzyme is analogous to the major xenobiotic-metabolising cytochrome P450 in 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 unusual, since, for example, *S. coelicolor* A3 (2) has 18 cytochromes P450 [20]. None of the *Bacillus* strains investigated (*B. subtilis* IM7, *B. subtilis* ATCC6633, *B. licheniformis* NCIMB8549, *B. megaterium* NCIMB8291 and *B. megaterium* ATCC14581) produced phase I metabolites. This was surprising since cytochromes P450 are known in this genus [21].

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 attempt to obtain consistency in the biotransformations. A greater degree of transformation was observed when the concentration of flurbiprofen was lowered to 0.1 mg/ml and added to a 24 h culture that had not been subcultured. Furthermore, some strains responded better to a different culture medium, for example, *S. griseolus*, which did not transform flurbiprofen when cultured in the original soybean medium described by [17], produced **2** when cultured in tryptone soy broth.

3.2 Identification of new metabolites from S. lavenduligriseus

Routinely, organic extracts were analysed by ^{19}F NMR, which revealed the presence of two resonances in the extracts of *S. lavenduligriseus* and *S. rimosus* at δ -116.97 and -117.1 ppm, which were distinct from the resonances of flurbiprofen **1** and 4'-hydroxybiprofen **2** (Figure 2). To identify these compounds *S. lavenduligriseus* was cultured in a large volume in order to obtain enough material for detailed analyses to be conducted. Metabolites were purified 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* 244.1138, $[M+H]^+$) was consistent with a molecular formula of $C_{15}H_{14}NOF$. The most noticeable change in the ^1H NMR of **5** compared to **1** was the presence of an additional broad singlet in the ^1H NMR spectrum (δ 5.83 ppm, 2H). These protons showed no correlations in a HSQC experiment, suggesting that they were not connected to a carbon atom. These observations suggested the presence of a primary amide at C-9. Other significant differences 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 (δ_C 176.4), consistent with such a structure. Thus, **5** was identified as flurbiprofenamide

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

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

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

3.3 Resting cell studies

Maruyama et al. [26] observed that the nitrogen atom involved in the amidation of 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. lavenduligriseus* resting cultures were incubated with flurbiprofen and a nitrogen source (glycine, $(NH_4)_2SO_4$ and peptone). Table 2 shows the extent of flurbiprofen **1** biotransformation under these conditions, and reveals that while some biotransformation does occur in resting cells in the absence of added nitrogen, the presence of peptone resulted in a greater transformation. A similar observation was made with *B. subtilis* resting cells, which transformed 63 % of **1** to the amidated metabolites when peptone was included, but only 30 % when no nitrogen source was added.

4. Conclusion

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 *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. Surprisingly, no hydroxylated flurbiprofen metabolites were detected in *Bacillus* cultures that 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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Table 1. Mammalian metabolites of flurbiprofen **1** observed in *Streptomyces* species

Microorganism/Fluorometabolite	4'-OH-flurbiprofen **2**	3',4'-diOH-flurbiprofen **3**	3'-OMe, 4'-OH-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	++	-	-
Streptomyces subrutilis DSM 40445 [c]	+	-	+

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

[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

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 from the GC peak areas of the metabolites.

	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
$(NH_4)_2SO_4$ (20 mM)	75.3	21.4	3.3
Peptone (2 % w/v)	41.9	52.5	5.6

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

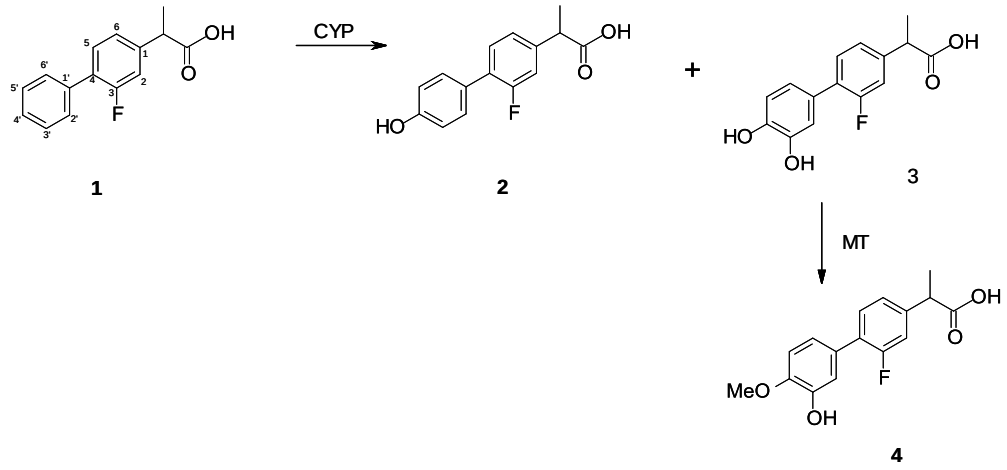
307 flurbiprofen **1**; flubiprofenamide **5** and 7-hydroxyflurbiprofenamide **6** were detected by

308 comparison of their retention times (A) and mass spectra (B and C) with the compounds

309 purified from *S. lavenduligriseus*.

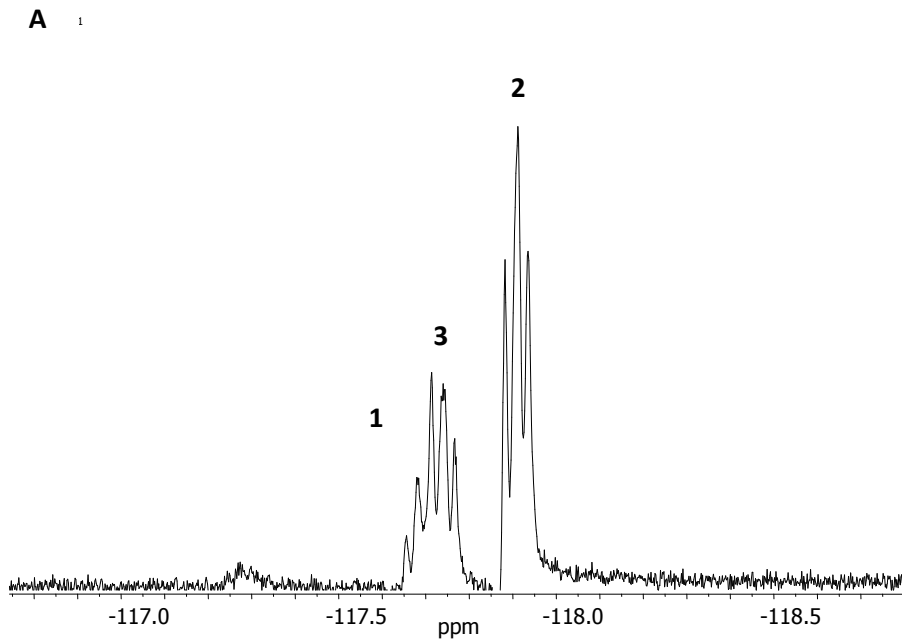
310

Figure 1



315 Figure 2

316



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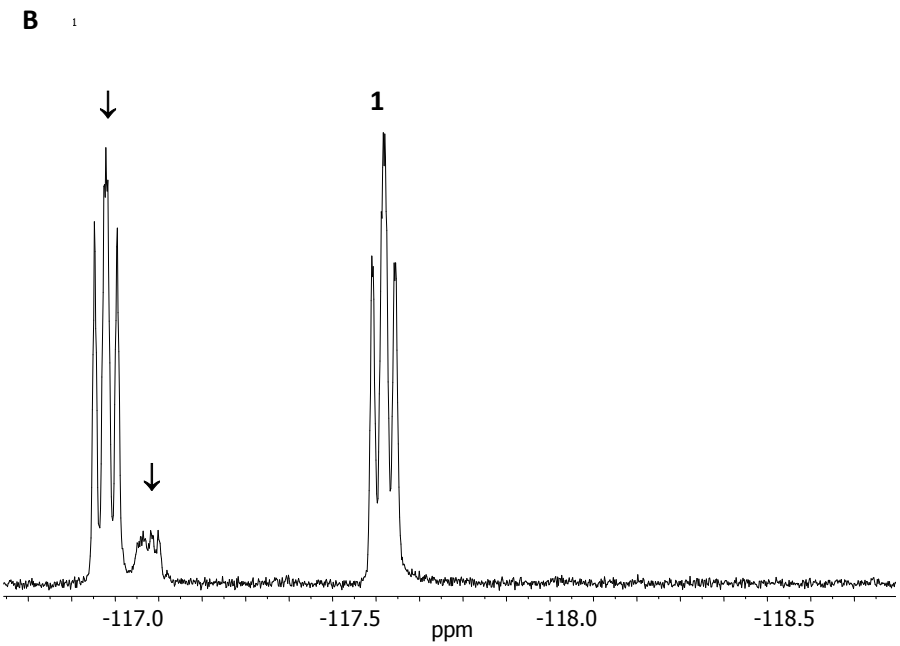

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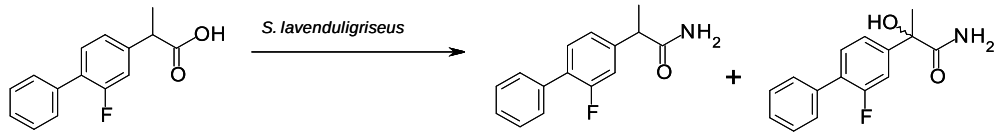
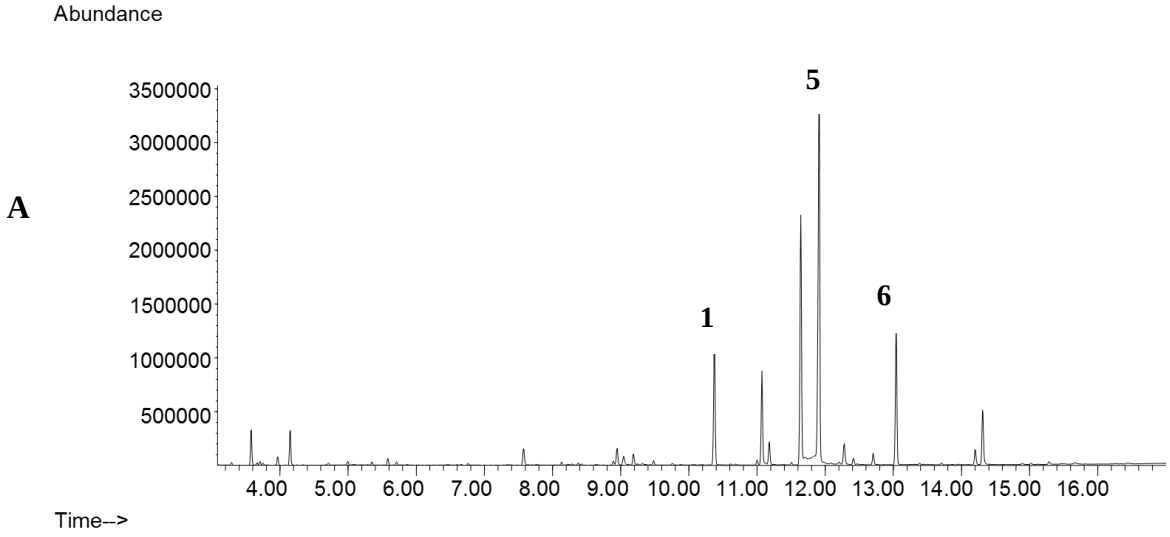
Figure 3



1 **5** **6**

Figure 4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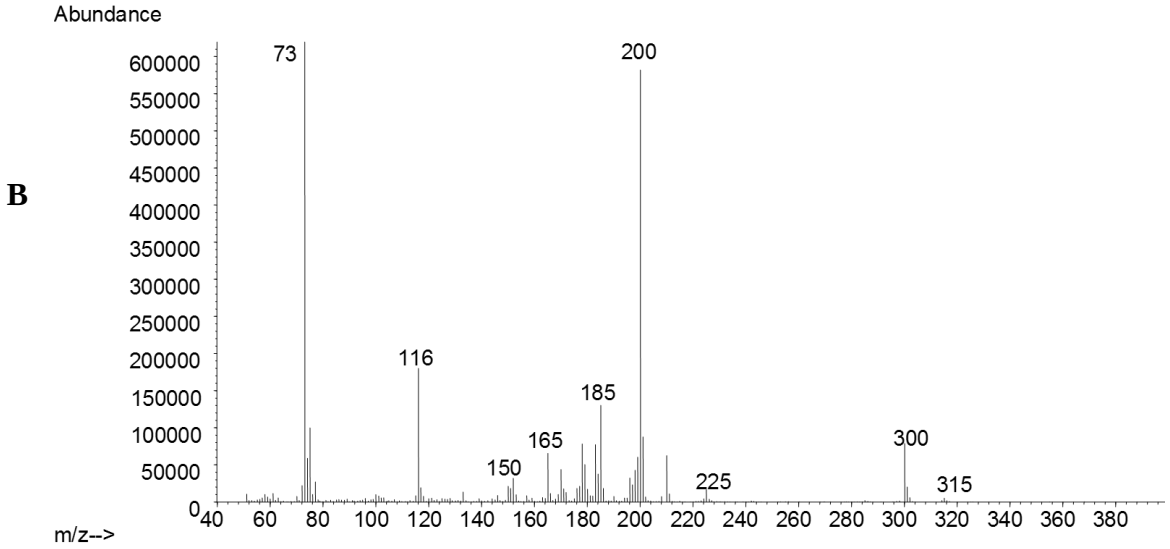
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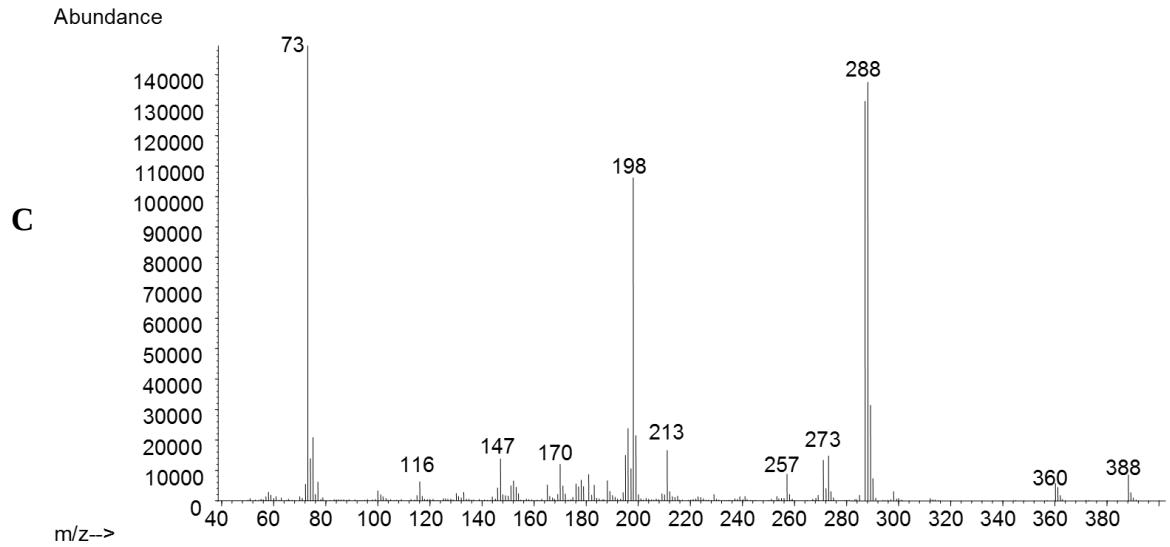
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213
147
170
116
273
257
360
388