1 **Biotransformation of flurbiprofen by *Cunninghamella* species**

2 Jessica Amadio, Katherine Gordon and Cormac D. Murphy*

3

4 School of Biomolecular and Biomedical Science, Centre for Synthesis and Chemical

5 Biology, Ardmore House, University College Dublin, Dublin 4, Ireland

6

7 *Corresponding author Fax: +353 (0)1 716 1183, Telephone: +353 (0)1 716 1311, email:

8 Cormac.d.murphy@ucd.ie

9

10 **Abstract**

11 The biotransformation of the fluorinated anti-inflammatory drug flurbiprofen was

12 investigated in *Cunninghamella* spp. Mono- and di-hydroxylated metabolites were detected

13 using gas chromatography-mass spectrometry and fluorine-19 nuclear magnetic resonance

14 spectroscopy, and the major metabolite 4'-hydroxyflurbiprofen was isolated by preparative

15 HPLC. *C. elegans* DSM 1908 and *C. blakesleeana* DSM 1906 also produced a phase II

16 (conjugated) metabolite, which was identified as the sulfated drug via deconjugation

17 experiments.

18   One of the objectives of the recent European Union legislation governing the testing and

19   evaluation of chemicals, REACH (Regulation, Evaluation, Authorisation and Restriction of

20   Chemicals), is to further reduce the need for animals in the testing process.   Some

21   microorganisms, such as the zygomycete fungus *Cunninghamella* and actinomycetes

22   bacteria, have been shown to metabolise xenobiotic compounds in an analogous fashion to

23   mammals (3, 5, 11, 17). It was suggested over three decades ago that microorganisms had

24   potential as models of mammalian metabolism (16), although there are concerns about their

25   predictive value (8). Nevertheless, certain microorganisms can be applied to the generation

26   of useful quantities of drug metabolic intermediates (13), which is more desirable than

27   isolation of these compounds from dosed animals, and avoids the concerns often associated

28   with chemical synthesis, such as the use of toxic reagents, and harsh reaction conditions.

29   Owing to the desirable physicochemical properties of the fluorine atom (small Van

30   der Waals radius, electronegativity, strength of the carbon-fluorine bond) approximately 25

31   % of drugs either currently on the market or in the pipeline are fluorinated (12). One such

32   example is flurbiprofen [(*RS*)-2-(2-fluoro-4-biphenyl) propionic acid], which is a non-

33   steroidal anti-inflammatory drug (NSAID) used in the treatment of inflammation caused by

34   arthritis.   In humans it is transformed to the phase I (oxidative) metabolites 4'-

35   hydroxyflurbiprofen, 3', 4'-dihydroxyflurbiprofen and 3'-hydroxy, 4'-methoxyflurbiprofen;

36   glucuronide and sulfate conjugates (phase II metabolites) have also been detected (9, 15). In

37   equine urine additional hydroxylated and methoxylated metabolites were detected (20).

38   Tracy et al. (18) demonstrated that only one cytochrome P450 isoform (2C9) is involved in

39   the oxidation of flurbiprofen, which makes the drug a potentially useful *in vivo* probe for this

40   particular isoform.   Despite the prevalence of fluorinated drugs, only a handful of

41   investigations have been undertaken to determine the microbial biotransformation of these

42   compounds (7, 21).   Here we describe the biotransformation of flurbiprofen by

43 *Cunninghamella* species and the determination of the metabolites by nuclear magnetic

44 resonance spectroscopy (^{1}H NMR and ^{19}F NMR), GC-MS and HPLC.

45   Three species of *Cunninghamella* were selected for the biotransformation

46 experiments: *C. elegans* (strains DSM 1908, DSM 8217, DSM 63299), *C. echinulata* DSM

47 1905 and *C. blakesleeana* DSM 1906. The fungi were grown on Sabouraud dextrose agar

48 plates (Sigma) for 5 days at 26 °C before being homogenized in 100 ml of sterile saline

49 solution. The homogenate (10 % v/v) was used to inoculate 50 ml of fresh Sabouraud

50 dextrose broth in 250 ml Erlenmeyer flasks, which were incubated at 28 °C with shaking at

51 150 rpm. Following previously established procedures (2), 5 mg of flurbiprofen (Sigma)

52 dissolved in dimethylformamide (20 µl) was added to the cultures after 72 h, and the

53 incubation continued up to a further 120 h. Control experiments were conducted in either the

54 absence of flurbiprofen or fungus. The cultures (supernatant and cells) were sonicated on ice

55 (Sonicator U200S control, IKA Labortechnik) for 5 minutes at 50 % amplitude, with intervals

56 of 30 seconds after each minute to prevent overheating. The sonicates were centrifuged and

57 the supernatant extracted with 50 ml of ethyl acetate, and the extracts evaporated to dryness.

58 **Analysis of fluorinated metabolites.** *C. elegans* DSM 1908 is well known as a model of

59 mammalian drug metabolism (10, 11) and analysis of the organically-extractable metabolites

60 by ^{19}F NMR spectroscopy using a Varian 400 MHz spectrometer revealed that flurbiprofen

61 was completely degraded to one fluorometabolite over three days (Figure 1). The

62 concentration of the metabolite was estimated at by using an internal standard (4-

63 fluorobiphenyl) in ^{19}F NMR analyses, and equated to 2 mg in the culture supernatant. No

64 fluorinated products were detected in uninoculated control flasks.

65   The fluorometabolite was isolated by preparative reversed phase HPLC using a

66 Varian Prostar HPLC system equipped with a Zorbax SB-C18 9.4 mm x 25 cm column

67 (Agilent Technologies). Compounds were eluted with a gradient of acetonitrile/water (20-60

68 % acetonitrile) over 30 minutes at a flow rate of 3.5 ml/min. The main metabolite, which

69 eluted at 19 minutes, was isolated and analysed by ^{1}H and ^{19}F NMR spectroscopy, and mass

70 spectrometry. The spectrum obtained with ^{1}H NMR analysis showed resonances at 1.56 ppm

71 (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

72 (C_6-H, ddd), 7.37 ppm (C_5-H, ddd) 7.43 ppm ($C_{2'}$ and $C_{6'}$-H, ddd). There was no resonance

73 for $C_{4'}$-H indicating that the hydroxylation occurred in this position. The spectrum obtained

74 by ^{19}F NMR analysis showed one signal with a chemical shift of – 117.85 ppm and splitting

75 pattern identical to the flurbiprofen, (dd, J= 11, 8 Hz), indicating that there were no changes

76 in the proximities of the fluorine atom. The metabolite was dried and further analysed by

77 GC-MS as the per-trimethylsilylated derivative, which was formed by adding 50 µl *N*-

78 methyl-*N*-(trimethyl-silyl) trifluoroacetamide (MSTFA) to the solid and heating at 100 °C for

79 1 hour. The derivatized compound was diluted in ethyl acetate (1 ml) and an aliquot (1 µl)

80 was injected onto a HP-1 column (12 m x 0.25 mm x 0.33 µm) and the oven temperature held

81 at 120 ºC for 2 min then raised to 300 ºC at 10 ºC min^{-1}. The mass and fragmentation pattern

82 of the metabolite, which had a retention time of 17.50 min, was composed of ions *m/z* 404

83 (M[+]), 389 (M[+]-CH_3), 287 (M[+]-COOTMS), 268 (M[+]-COOTMS, F), 253 (M[+]-COOTMS, F,

84 CH_3), and was identical to that described by (20) for 4'-hydroxyflurbiprofen.

85   Metabolic studies of flurbiprofen in human and different animal species reported the

86 presence of several metabolites excreted in urine (15). The major metabolite was identified as

87 4'-hydroxyflurbiprofen and two minor ones as 3',4'-dihydroxyflurbiprofen, 3'-hydroxy-4'-

88 methoxyflurbiprofen. In the present study only one metabolite was detectable by ^{19}F NMR,

89 but since this technique is relatively insensitive additional analyses of the organically

90 extractable metabolites were conducted by HPLC and GC-MS. HPLC analysis of time-

91 course experiments up to 120 hours confirmed that flurbiprofen was completely degraded

92 over three days to one polar metabolite with a retention time of 22.2 min (10-90% acetonitrile

93   over 30 min at 1 ml/min). GC-MS analyses of silylated organically soluble *C. elegans*

94   extracts revealed 4'-hydroxyflurbiprofen and other metabolites that could be tentatively

95   identified as hydroxylated and methoxylated flurbiprofen, based on their mass spectra (Table

96   1).

97   The microbial biotransformation of flurbiprofen was also investigated in selected

98   *Cunninghamella* strains previously shown to transform xenobiotics (4). GC-MS analysis of

99   the organically extractable metabolites, after derivatisation, demonstrated that they all

100 transformed the drug, to varying degrees, yielding the hydroxylated metabolites, and three of

101 the fungi generated hydroxylated methoxyflurbiprofen (Table 2). Using our methods, no

102 other organically soluble fluorometabolites were detected.

103   Phase II metabolism of flurbiprofen was studied since glucoronide and sulfate

104 flurbiprofen conjugates are reported to be important detoxification metabolites in mammals.

105 In humans, approximately 60-70% of flurbiprofen is excreted as conjugates (1), whereas less

106 than 30% of the flurbiprofen was reportedly conjugated in equine urine (20). Examination of

107 the aqueous extracts from *C. elegans* DSM 1908 by HPLC showed one peak at t_R 17.8 min

108 that was not present in the organic extract analysis, and the ^{19}F NMR spectrum of aqueous

109 phase showed a signal at -119.2 ppm, which had a concentration of 0.1 mg/ml by comparison

110 with an internal standard of sodium fluoride. No fluorometabolites were detected in control

111 experiments. Enzymatic deconjugation was carried out by incubating the aqueous phase with

112 sulfatase (from *Helix pomatia* type H-1), β-glucoronidase (from *Escherichia coli*) and β-

113 glucosidase (from almonds) (Sigma) in phosphate buffer at 37°C for 12 h. The deconjugated

114 reaction products were extracted into ethyl acetate and analysed by GC-MS; 3',4'-

115 dihydroxyflurbiprofen was detected after treatment with sulfatase, but no metabolites were

116 detected in extracts from deconjugation experiments with the other enzymes (Figure 2). The

117 other *Cunninghamella* species were examined for conjugated metabolites, and were only

observed 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-glucoronidase and β-glucosidase) and the reactions monitored by ^{19}F NMR spectroscopy between each addition (Figure 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'-dihydroxy-flubiprofen, respectively.

The anti-inflammatory activity 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*) (19). In order to evaluate differences in the rate 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 the racemic flurbiprofen (data not shown). (*R*)-flurbiprofen was biotransformed predominantly to 4'-hydroxyflurbiprofen that 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), 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 (0.1-5 mg/ml) added after 0, 6, 24 and 72 hours. Flurbiprofen completely inhibited germination starting at the lowest concentration administered at 0 and 6 hours; in the medium there was no presence of mature pellets of mycelium but only the

147   We have shown for the first time that flurbiprofen is converted by *Cunninghamella*

148 spp. to a variety of phase I and phase II metabolites (Figure 4) present in several mammalian

149 species including man**.** Among them, 4'-hydroxyflurbiprofen was confirmed to be the major

150 product being converted by both mammalian and microbial systems. This similarity is

151 remarkable considering that only one mammalian cytochrome P450 isoform can detoxify this

152 drug. In fact, previous chemical inhibition studies confirmed that only P450 2C9 was

153 involved in the 4'-hydroxylation of flurbiprofen in humans (18, 19). *C. elegans* DSM 1908

154 in particular would seem to be an appropriate microbial model of phase I metabolism in

155 mammals since all the major metabolites are produced, in addition to new hydroxy- and

156 hydroxy-methoxy isomers. The upscaling of the biotransformation may also have potential

157 as a method of generating the metabolites as analytical standards, in particular 4'-

158 hydroxyflurbiprofen.

159

Acknowledgement

164

References

166 1. **Adams, W. J., B. E. Bothwell, W. M. Bothwell, G. J. Vangiessen, and D. G.**
167   **Kaiser.** 1987. Simultaneous determination of flurbiprofen and its main metabolite in
168   physiological fluids using liquid chromatography with fluorescence detection. Anal.
169   Chem. **59:**1504-1509.

170   2.   **Amadio, J., and C. D. Murphy.** 2010. Biotransformation of fluorobiphenyl by
171   *Cunninghamella elegans*. Appl. Microbiol. Biotechnol. **86:**345-351.

172   3.   **Asha, S., and M. Vidyavathi.** 2009. *Cunninghamella* - A microbial model for drug
173   metabolism studies - A review. Biotechnol. Adv. **27:**16-29.

174   4.   **Azerad, R.** 1999. Microbial Models of Drug Metabolism. Adv. Biochem. Eng.
175   Biotechnol. **63:**169-218.

176   5.   **Cannell, R. J. P., A. R. Knaggs, M. J. Dawson, G. R. Manchee, P. J. Eddershaw,**
177   **I. Waterhouse, D. R. Sutherland, G. D. Bowers, and P. J. Sidebottom.** 1995.
178   Microbial biotransformation of the angiotensin II antagonist GR117289 by
179   *Streptomyces rimosus* to identify a mammalian metabolite. Drug Metab. Dispos.
180   **23:**724-729.

181   6.   **Chowdhury, B., M. Adak, and S. K. Bose.** 2003. Flurbiprofen, a unique non-
182   steroidal anti-inflammatory drug with antimicrobial activity against *Trichophyton*,
183   *Microsporum* and *Epidermophyton* species. Lett. Appl. Microbiol. **37:**158-161.

184   7.   **Corcoran, O., J. C. Lindon, R. Hall, I. M. Ismail, and J. K. Nicholson.** 2001. The
185   potential of F-19 NMR spectroscopy for rapid screening of cell cultures for models of
186   mammalian drug metabolism. Analyst **126:**2103-2106.

187   8.   **Jezequel, S. G.** 1998. Microbial models of mammalian metabolism: uses and misuses
188   (clarification of some misconceptions). J. Mol. Catal. B: Enzymatic **5:**371-377.

189   9.   **Knadler, M. P., and S. D. Hall.** 1989. High performance liquid chromatography of
190   the enantiomers of flurbiprofen and its metabolites in plasma and urine. J. Chromatog.
191   Biomed. Appl. **494:**173-182.

192   10.   **Moody, J. D., J. P. Freeman, P. P. Fu, and C. E. Cerniglia.** 2002.
193   Biotransformation of mirtazapine by *Cunninghamella elegans*. Drug Metab. Dispos.
194   **30:**1274-1279.

195   11.   **Moody, J. D., D. L. Zhang, T. M. Heinze, and C. E. Cerniglia.** 2000.
196   Transformation of amoxapine by *Cunninghamella elegans*. Appl. Environ. Microbiol.
197   **66:**3646-3649.

198   12.   **Murphy, C. D., B. R. Clark, and J. Amadio.** 2009. Metabolism of fluoroorganic
199   compounds in microorganisms: impacts for the environment and the production of
200   fine chemicals. Appl. Microbiol. Biotechnol. **84:**617-629.

201   13.   **Osorio-Lozada, A., S. Surapaneni, G. L. Skiles, and R. Subramanian.** 2008.
202   Biosynthesis of drug metabolites using microbes in hollow fiber cartridge reactors:
203   Case study of diclofenac metabolism by *Actinoplanes* species. Drug Metab. Dispos.
204   **36:**234-240.

205   14.   **Paik, M. J., Y. Lee, J. Goto, and K. R. Kim.** 2004. Chiral discrimination of multiple
206   profens as diastereomeric (R)-(+)-1-phenylethylamides by achiral dual-column gas
207   chromatography. J. Chrom. B **803:**257-265.

208   15.   **Risdall, P. C., S. S. Adams, E. L. Crampton, and B. Marchant.** 1978. Disposition
209   and metabolism of flurbiprofen in several species including man. Xenobiotica **8:**691-
210   703.

211   16.   **Smith, R. V., and J. P. Rosazza.** 1974. Microbial models of mammalian metabolism
212   – aromatic hydroxylation. Arch. Biochem. Biophys. **161:**551-558.

213   17.   **Sun, L., H. H. Huang, L. Liu, and D. F. Zhong.** 2004. Transformation of verapamil
214   by *Cunninghamella blakesleeana*. Appl. Environ. Microbiol. **70:**2722-2727.

215   18.   **Tracy, T. S., C. Marra, S. A. Wrighton, F. J. Gonzalez, and K. R. Korzekwa.**
216   1996. Studies of flurbiprofen 4'-hydroxylation - Additional evidence suggesting the
217   sole involvement of cytochrome P450 2C9. Biochem. Pharmacol. **52:**1305-1309.

218   19.   **Tracy, T. S., B. W. Rosenbluth, S. A. Wrighton, F. J. Gonzalez, and K. R.**
219   **Korzekwa.** 1995. Role of cytochrome P450 2C9 and an allelic variant in the 4'-

220   hydroxylation of (R)-flurbiprofen and (S)-flurbiprofen. Biochem. Pharmacol.
221   **49:**1269-1275.

222   20.   **Tsitsimpikou, C., M. H. E. Spyridaki, I. Georgoulakis, D. Kouretas, M.**
223   **Konstantinidou, and C. G. Georgakopoulos.** 2001. Elimination profiles of
224   flurbiprofen and its metabolites in equine urine for doping analysis. Talanta **55:**1173-
225   1180.

226   21.   **Williams, A. J., J. Deck, J. P. Freeman, M. P. Chiarelli, M. D. Adjei, T. M.**
227   **Heinze, and J. B. Sutherland.** 2007. Transformation of flumequine by the fungus
228   *Cunninghamella elegans*. Chemosphere **67:**240-243.

229
230
231
232
233
234

235

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 of M$^+$ (relative intensity)	m/z of fragment ions (relative intensity)
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 by (18)

[b] Approximately 80% of the total metabolites (by peak area) was 4'-OH-flurbiprofen.

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

Compound	*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	+	_	_	+	+

Figure legends

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

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

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

Fig. 4. Principal biotransformation reactions of flurbiprofen in *C. elegans* 1908.
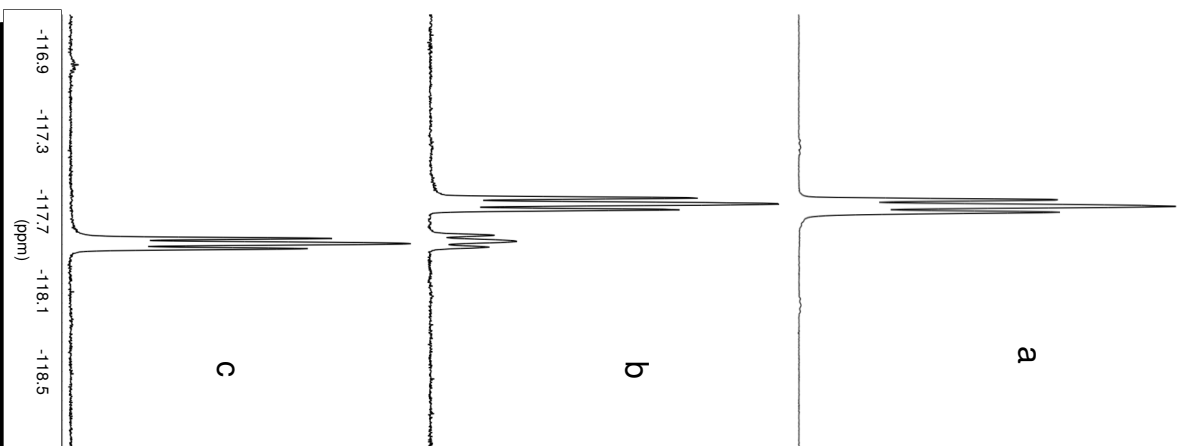
Figure 1

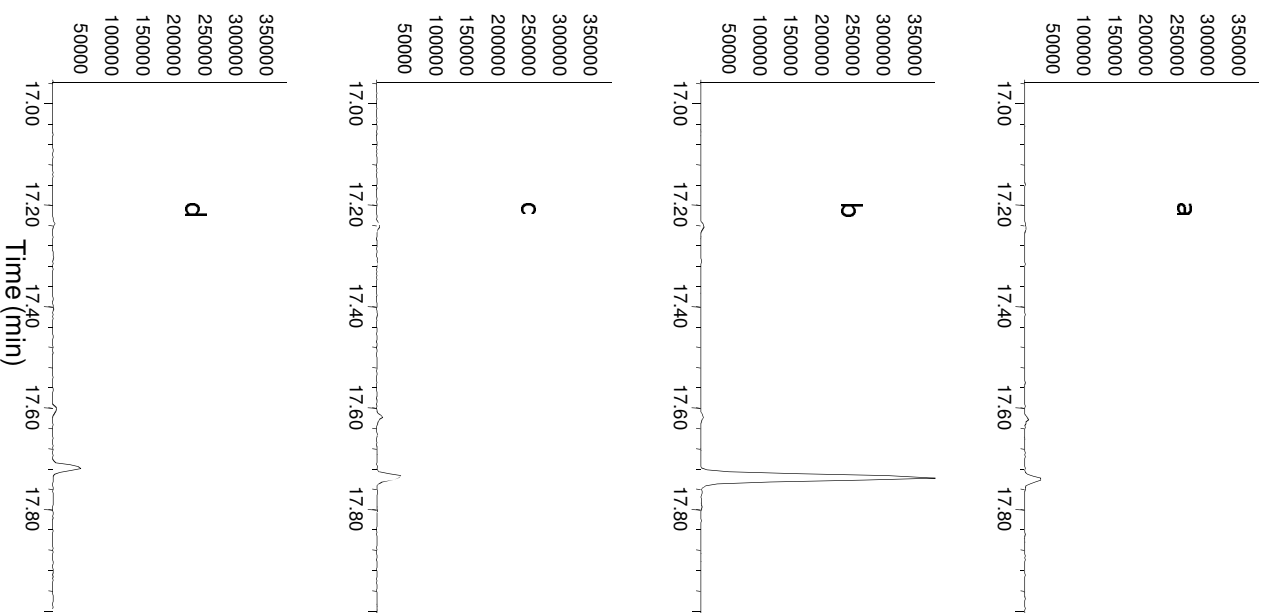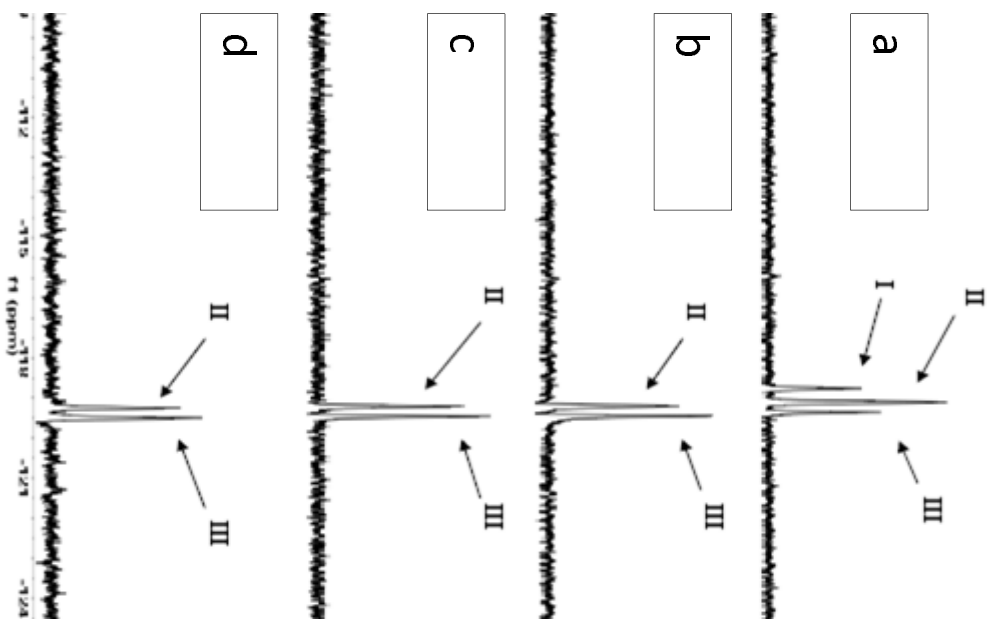-116.9   -117.3   -117.7   -118.1   -118.5
(ppm)

c

b

a

Fig 2

Abundance

a

b

c

d

Time (min)

Figure 3

Figure 4