

## Supporting information

# The Cytochrome P450 CYP199A4 from *Rhodopseudomonas palustris* catalyses heteroatom dealkylations, sulfoxidation and amide and cyclic hemiacetal formation

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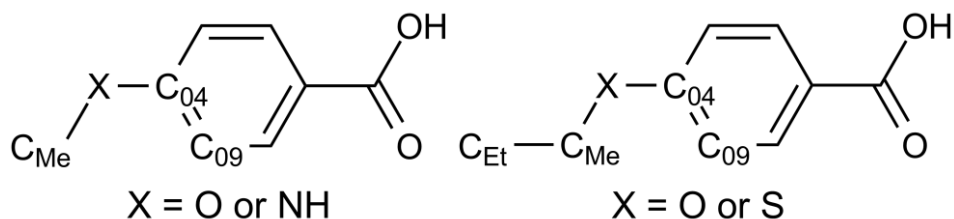
**Table S1** Substrate binding parameters and catalytic turnover activity data for CYP199A4 with various 4-aminomethylbenzoic acid derivatives. The data are given as mean  $\pm$  S.D. with  $n \geq 3$ . The reaction mixtures (in 50 mM Tris, pH 7.4) contained 0.5  $\mu$ M P450, 5  $\mu$ M HaPux and 0.5  $\mu$ M HaPuR. Rates are given as nmol.nmol-CYP<sup>-1</sup>.min<sup>-1</sup>. The leak rate of the system in the absence of the substrate was 9.0 nmol.nmol-CYP<sup>-1</sup>.min<sup>-1</sup>.

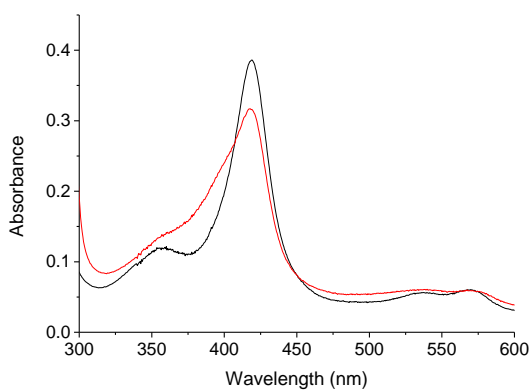
Substrate	% HS	$N^{[a]}$	PFR <sup>[b]</sup>	C (%) <sup>[c],[d]</sup>
4-aminomethylbenzoic acid	20%	13 $\pm$ 1	0.3 $\pm$ 0.003	2 $\pm$ 0.02
4-(methylamino)methylbenzoic acid	*(420 nm) <sup>[e]</sup>	24 $\pm$ 3	1.3 $\pm$ 0.06	5.6 $\pm$ 0.8
4-(dimethylamino)methylbenzoic acid	10%	24 $\pm$ 2	2.9 $\pm$ 0.2	12.2 $\pm$ 0.6
4-(ethylamino)methylbenzoic acid	<5%	15 $\pm$ 1	0.4 $\pm$ 0.05	2.9 $\pm$ 0.04

[a] NADH oxidation activity. [b] PFR: product formation rate defines the number of product forming catalytic cycles completed over the time period. [c] C is the coupling efficiency which is the percentage of NADH consumed in the reaction that led to the formation of products. H<sub>2</sub>O<sub>2</sub> is the percentage of NADH converted to hydrogen peroxide via this uncoupling pathway. [d] The coupling values are based on the total amount of identified products in the turnovers. There were small amounts of both products (terephthalic acid and 4-formylbenzoic acid) in control turnovers containing no NADH or CYP199A4. However the overall product formations rates are significantly lower for these substrates compared to those in Table 1. [e] On addition of substrate the maximum absorption of the Soret peak is red shifted from 418 to 420 nm, however a Type I difference spectrum is still obtained.

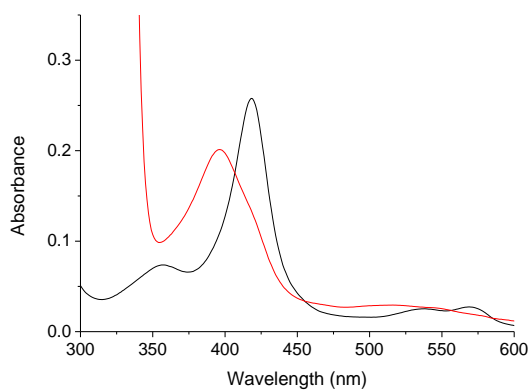
**Table S2** Overview of the distances (angstroms Å) and angles of pertinent substrate atoms from the heme iron in the crystal structure of CYP199A4 for various substrates. Also included are distances and angles from the likely position of the reactive oxygen of compound I.

Distance Å	4-methoxy PDB:4DO1	4-ethoxy PDB:5U6T	4-methylamino PDB: 5U6W	4-methylthio PDB:5KT1	4-ethylthio PDB:5U6U
C Me - Fe	4.1	4.2	4.1	4.4	5.2
C Me - O=Fe	2.7	2.8	3.1	3.3	4.0
C Et - Fe	-	4.3	-	-	6.7
C Et - O=Fe	-	3.0	-	-	5.5
X - Fe	(X=O) 5.2	(X=O) 5.1	(X=N) 4.2	(X=S) 4.9	(X=S) 4.7
X - O=Fe	3.6	3.6	3.0	3.4	3.3
Cx - closest F298 C	(X=Me) 3.8	(X=Me) 3.6	(X=Me) 3.5	(X=Me) 3.2	(X=Et) 4.0
<b>Angle</b>					
C <sub>04</sub> -X-C Me	118.6	119.9	120.3	104.3	106.1
Dihedral C <sub>09</sub> -C <sub>04</sub> -X-C <sub>Me</sub>	2.1	9.2	50.0	33.9	55.2
Fe=O-C <sub>Me</sub>	140.7	144.8	133	131.9	133.7
Fe=O-X	158.3	163.4	158.4	162.5	159.2
Fe=O-C <sub>Et</sub>	-	137.3	-	-	137.9

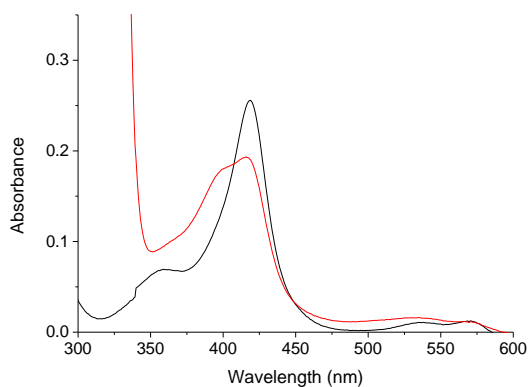




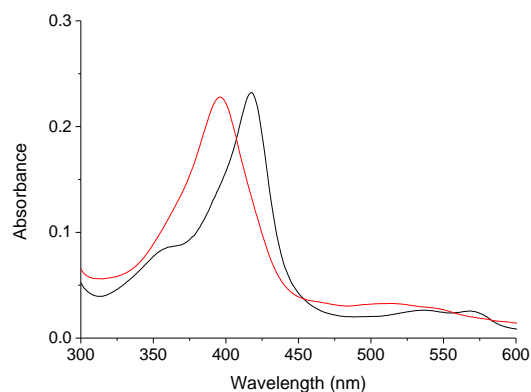
a) 4-aminomethylbenzoic acid



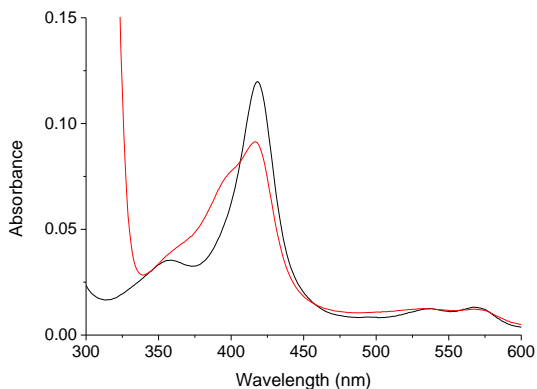
b) 4-diethylaminobenzoic acid



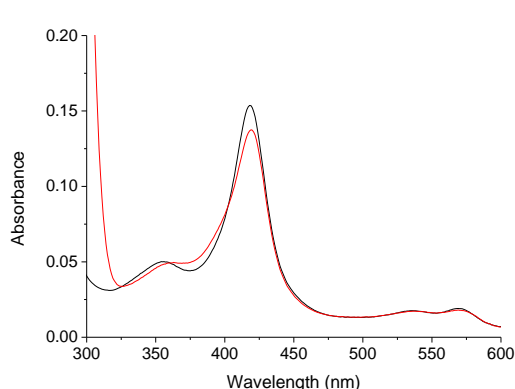
c) 4-dimethylaminobenzoic acid



d) 4-ethoxybenzoic acid



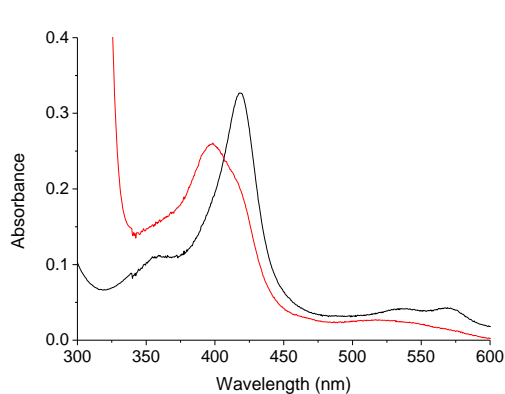
e) 4-ethylaminobenzoic acid



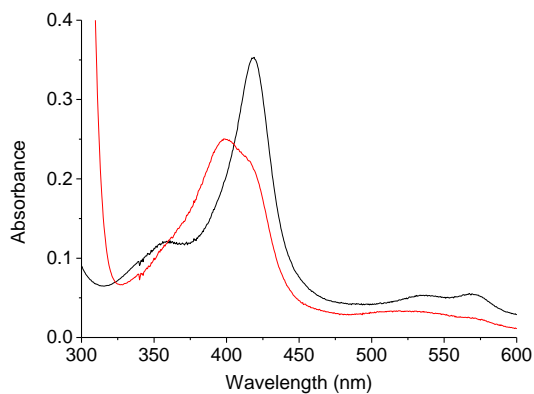
f) 4-ethylthiobenzoic acid

Note Soret Maxima shifted slightly from 418 to 419 nm for 4-ethylthiobenzoic acid (a type I difference spectrum is still obtained – Figure S2).

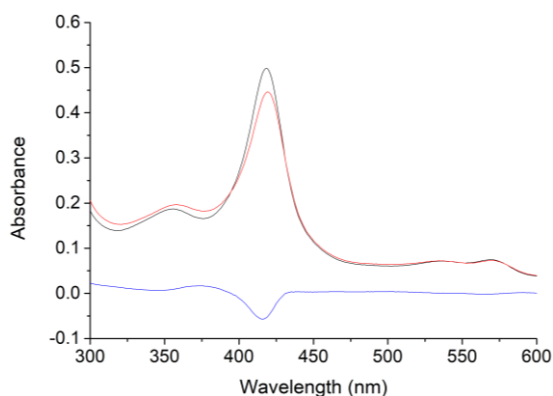
**Figure S1** Spin state shift assays of CYP199A4 and a selection of substrates. The substrate free form is shown in black and the substrate bound form in red.



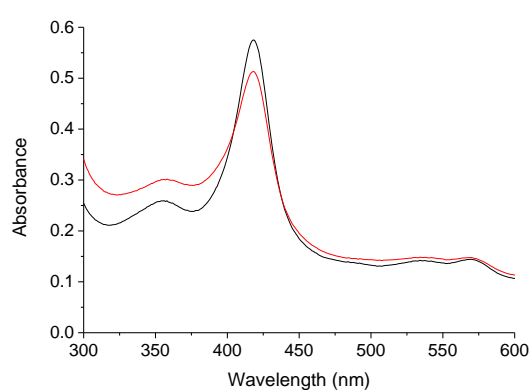
g) 4-methylaminobenzoic acid



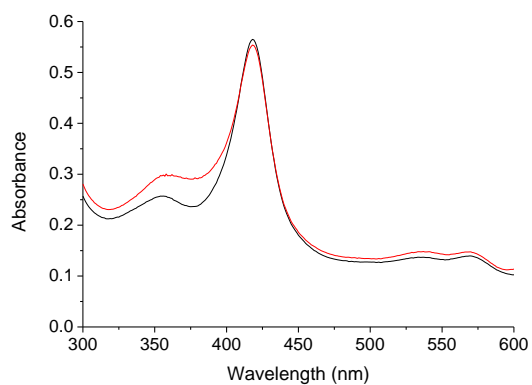
h) 4-methylthiobenzoic acid



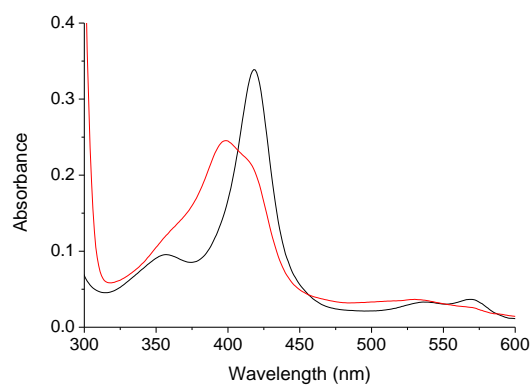
i) 4-(methylamino)methylbenzoic acid  
Note Soret Maxima shifted slightly from 418 to 420 nm but a type I difference spectrum is still obtained – blue spectrum).



j) 4-(dimethylamino)methylbenzoic acid

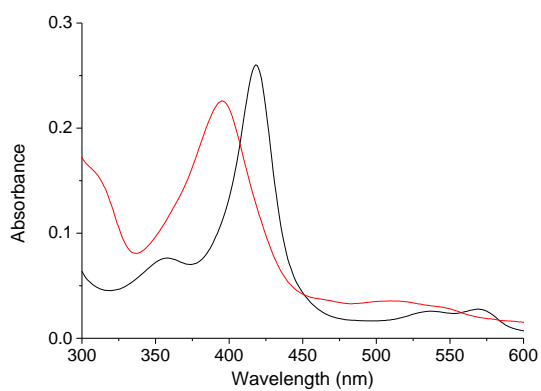


k) 4-(ethylamino)methylbenzoic acid

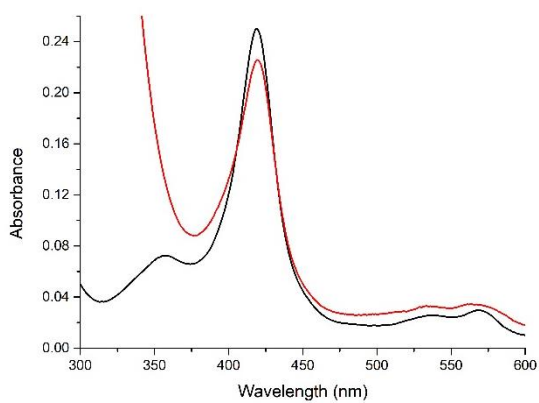


l) 3,4-ethylenedioxybenzoic acid

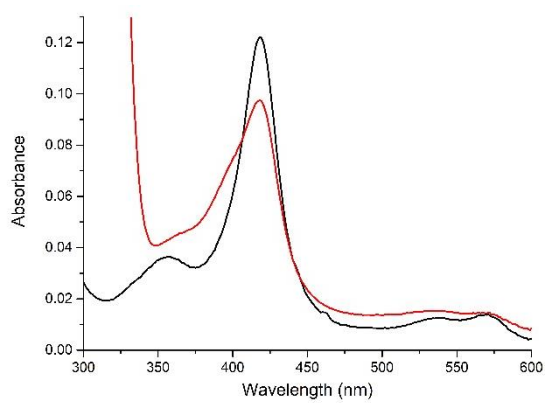
**Figure S1 (continued)** Spin state shift assays of CYP199A4 and a selection of substrates. The substrate free form is shown in black and the substrate bound form in red.



m) 4-trifluoromethoxybenzoic acid

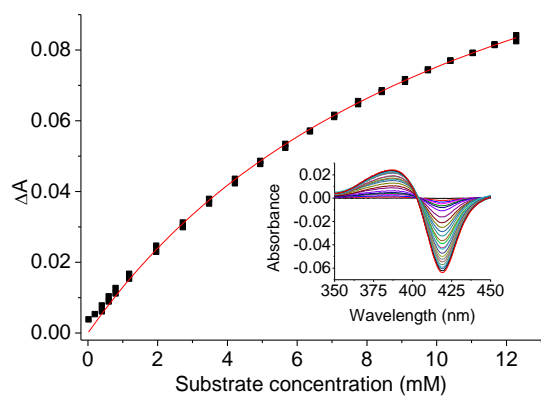


n) 3-methylaminobenzoic acid

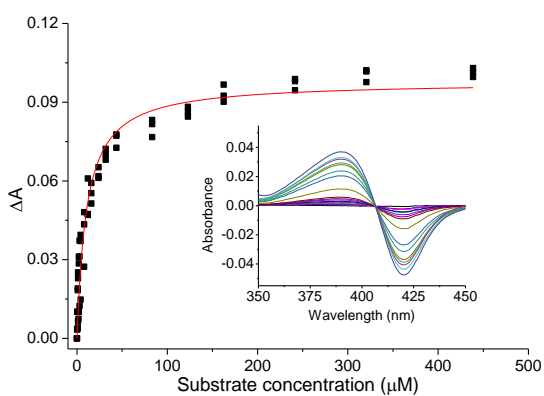


o) 3-methylthiobenzoic acid

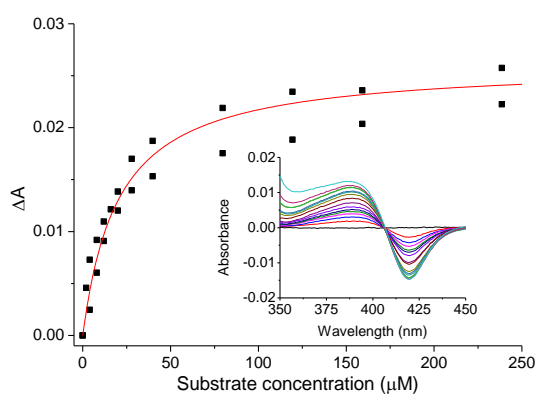
**Figure S1 (continued)** Spin state shift assays of CYP199A4 and a selection of substrates. The substrate free form is shown in black and the substrate bound form in red.



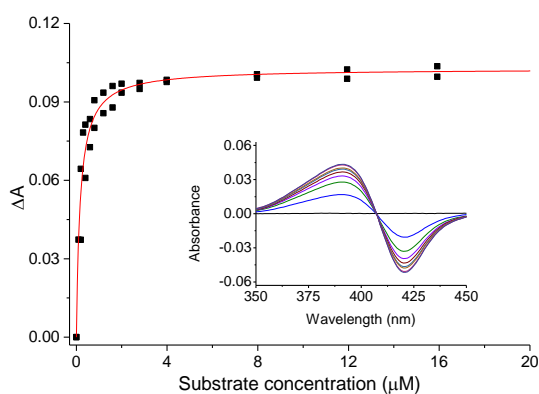
a) 4-aminomethylbenzoic acid  
1.69  $\mu\text{M}$   $A_{419}/A_{386}$



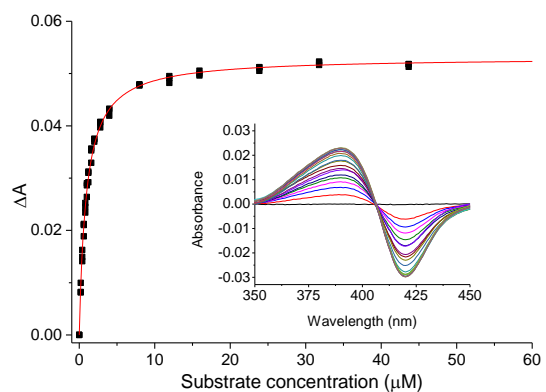
b) 4-diethylaminobenzoic acid  
1.44  $\mu\text{M}$   $A_{420}/A_{390}$



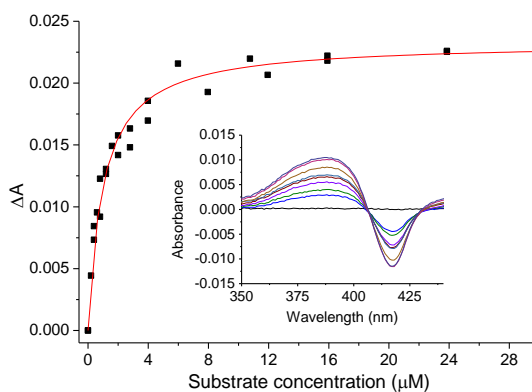
c) 4-dimethylaminobenzoic acid  
0.39  $\mu\text{M}$   $A_{419}/A_{388}$



d) 4-ethoxybenzoic acid  
0.89  $\mu\text{M}$   $A_{421}/A_{391}$

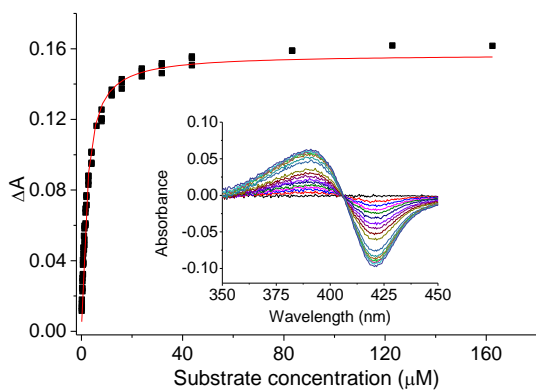


e) 4-ethylaminobenzoic acid  
1.01  $\mu\text{M}$   $A_{420}/A_{390}$

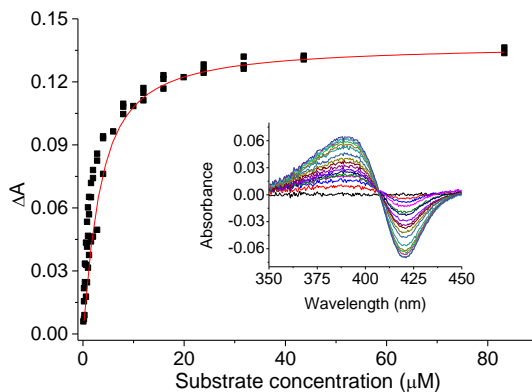


f) 4-ethylthiobenzoic acid  
1.01  $\mu\text{M}$   $A_{417}/A_{387}$

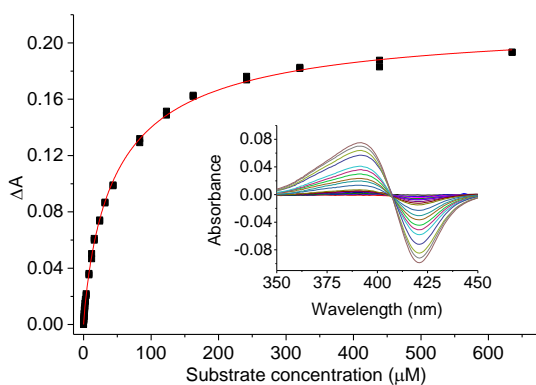
**Figure S2** Dissociation constant analysis of CYP199A4 with selected substrates. The concentration of enzyme used is given as are the absorbances of the trough and the peak.



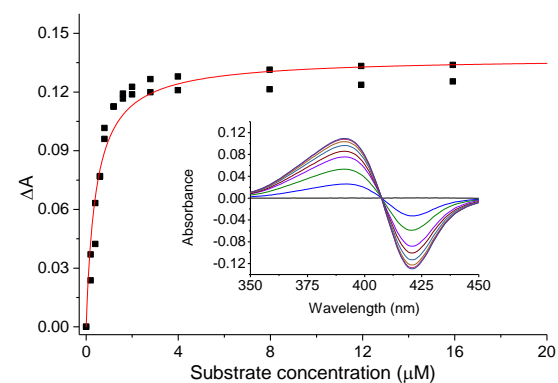
g) 4-methylaminobenzoic acid  
1.41  $\mu\text{M}$   $A_{421}/A_{390}$



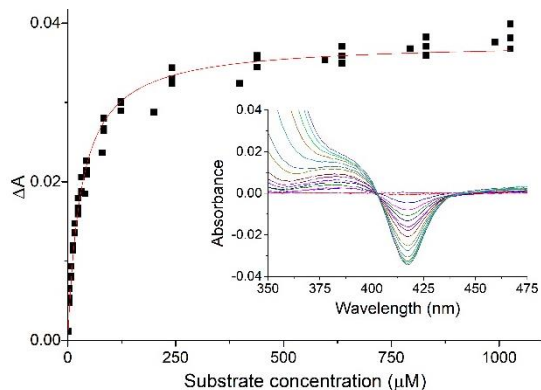
h) 4-methylthiobenzoic acid  
1.50  $\mu\text{M}$   $A_{421}/A_{389}$



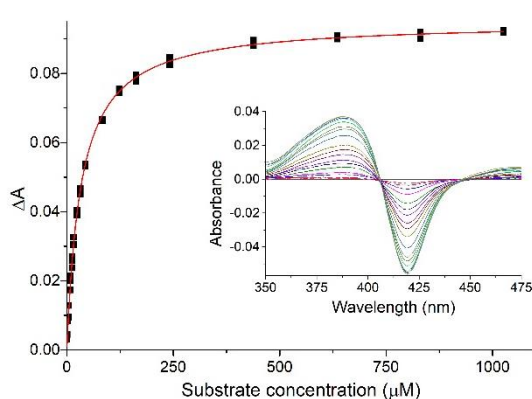
i) 3,4-ethylenedioxybenzoic acid  
2.0  $\mu\text{M}$   $A_{421}/A_{392}$



j) 4-trifluoromethoxybenzoic acid  
1.50  $\mu\text{M}$   $A_{421}/A_{391}$



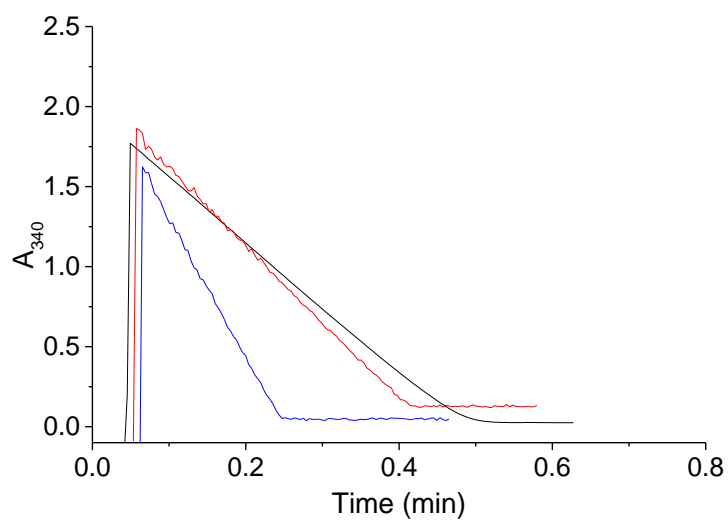
k) 3-methylaminobenzoic acid  
2.0  $\mu\text{M}$   
Due to interference of the substrate  
with the peak at 390 nm the reduction  
in the trough at 417 nm was used



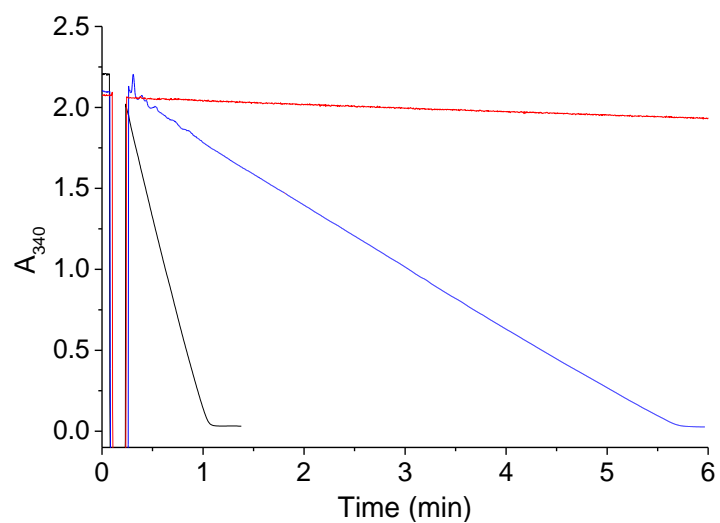
l) 3-methylthiobenzoic acid  
2.2  $\mu\text{M}$   $A_{419}/A_{389}$

**Figure S2 (continued)** Dissociation constant analysis of CYP199A4 with selected substrates. The concentration of enzyme used is given as are the absorbances of the trough and the peak.

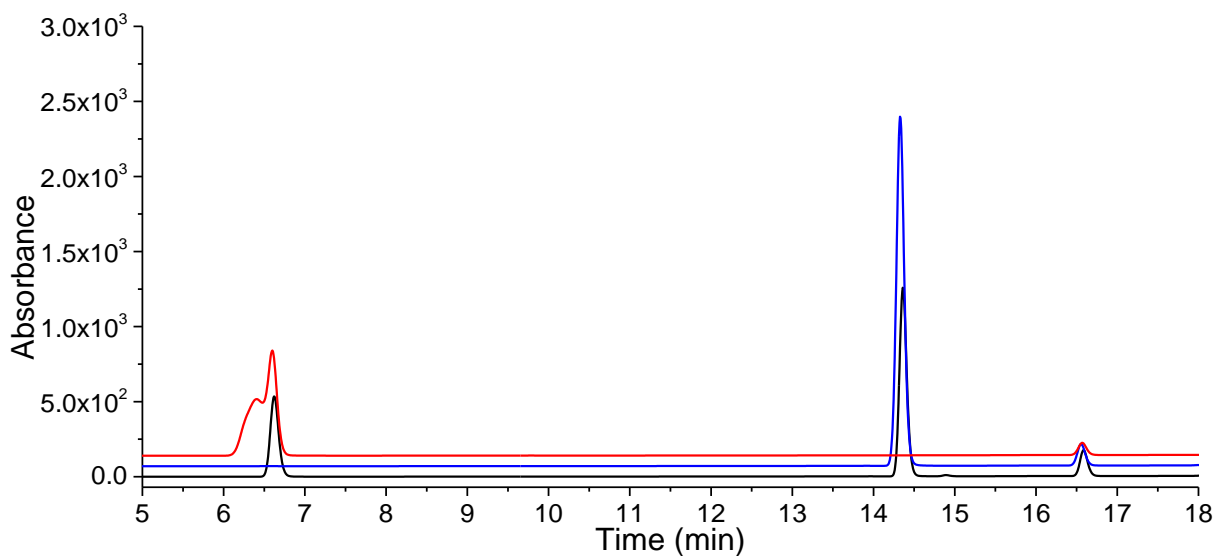
Black = 4-methoxyBA, red = 4-methylaminoBA, blue = 4-methylthioBA



Black = 4-ethoxyBA, red = 4-aminomethylBA, blue = 4-ethylthioBA



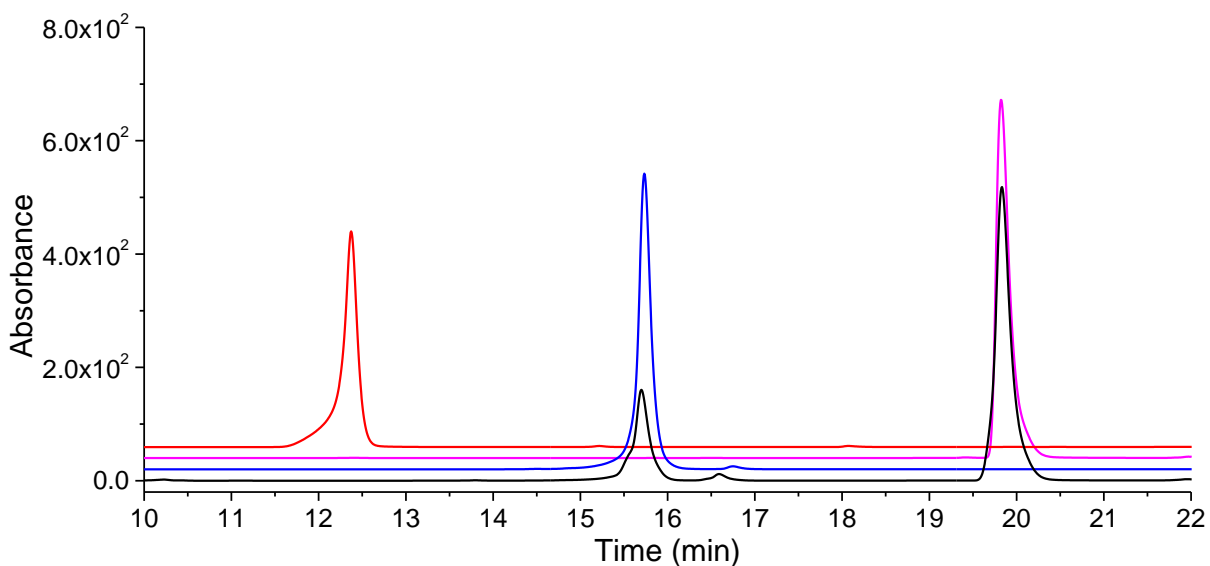
**Figure S3** Examples of CYP199A4 catalysed NADH oxidation assays of selected substrates (Table 1).



**Figure S4 (a)** HPLC analysis of the *in vitro* turnovers of 4-ethoxybenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Ethoxybenzoic acid, 254 nm, 20-95% acetonitrile.

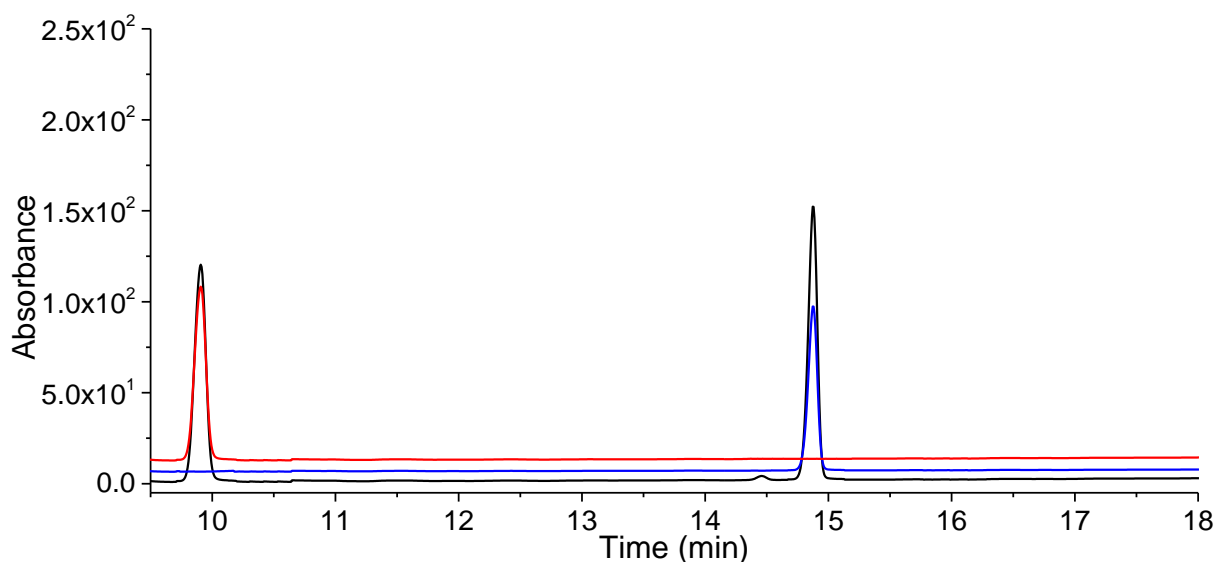
Black = *in vitro* turnover, blue = 4-ethoxyBA control, red = 4-hydroxyBA control. Internal standard shown at 16.6 min.



**Figure S4 (b)** HPLC analysis of the *in vitro* turnovers of 3,4-ethylenedioxybenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

3,4-ethylenedioxybenzoic acid, 254 nm, 20-95% acetonitrile.

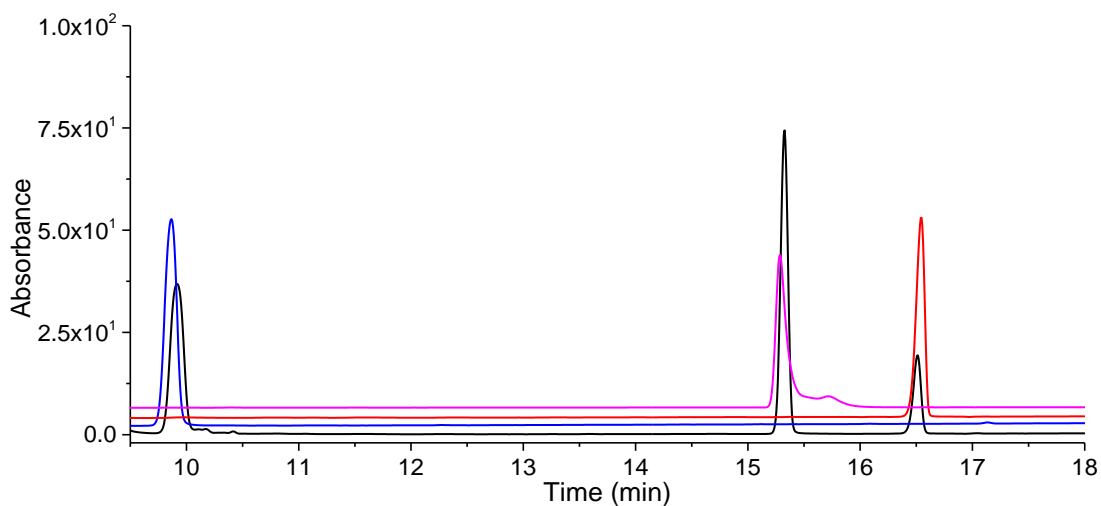
black = *in vitro* turnover, blue = isolated hydroxylation product, pink = substrate control, red = 3,4-dihydroxyBA control.



**Figure S4 (c)** HPLC analysis of the *in vitro* turnovers of 4-methylaminobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Methylaminobenzoic acid, 240 nm, 0-50% acetonitrile.

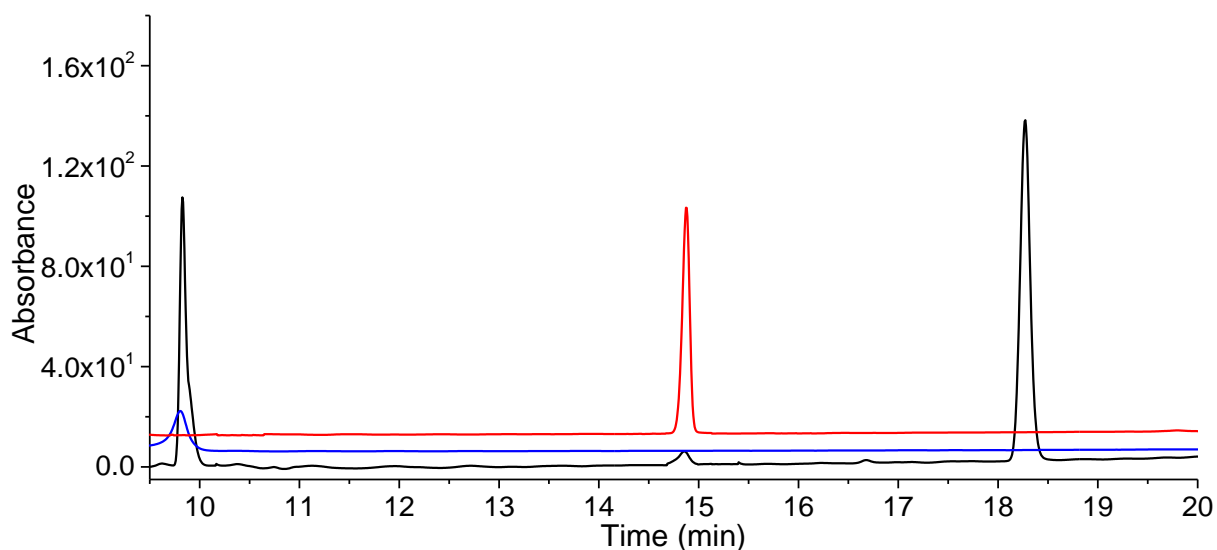
black = *in vitro* turnover, blue = 4-methylaminoBA control, red = 4-aminoBA control



**Figure S4 (d)** HPLC analysis of the *in vitro* turnovers of 4-methylaminobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Ethylaminobenzoic acid, 254 nm, 0-50% acetonitrile.

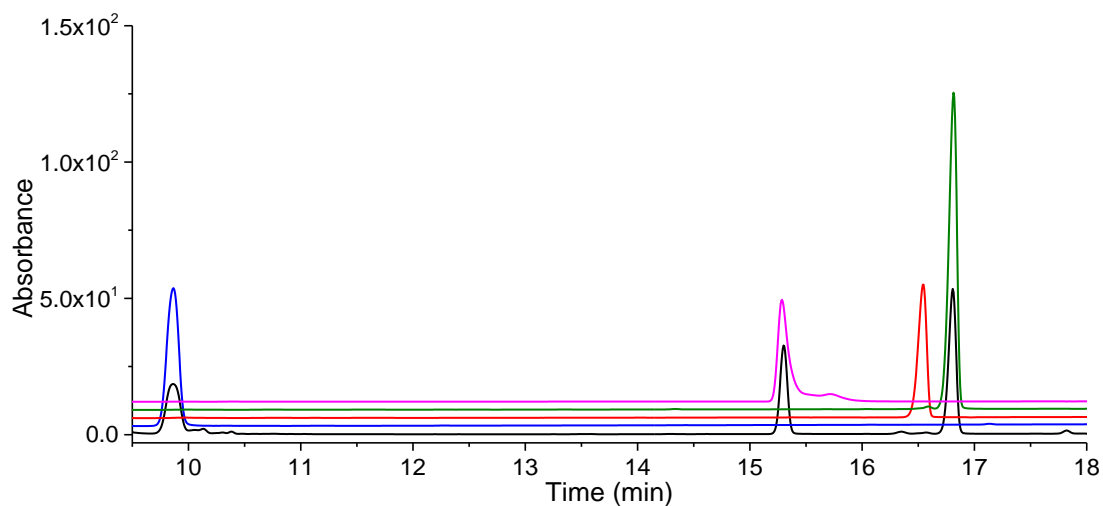
black = *in vitro* turnover, blue = 4-aminoBA control, red = 4-ethylaminoBA control, pink = 4-acetamidoBA control.



**Figure S4 (e)** HPLC analysis of the *in vitro* turnovers of 4-dimethylaminobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Dimethylaminobenzoic acid, 240 nm, 0-50% acetonitrile.

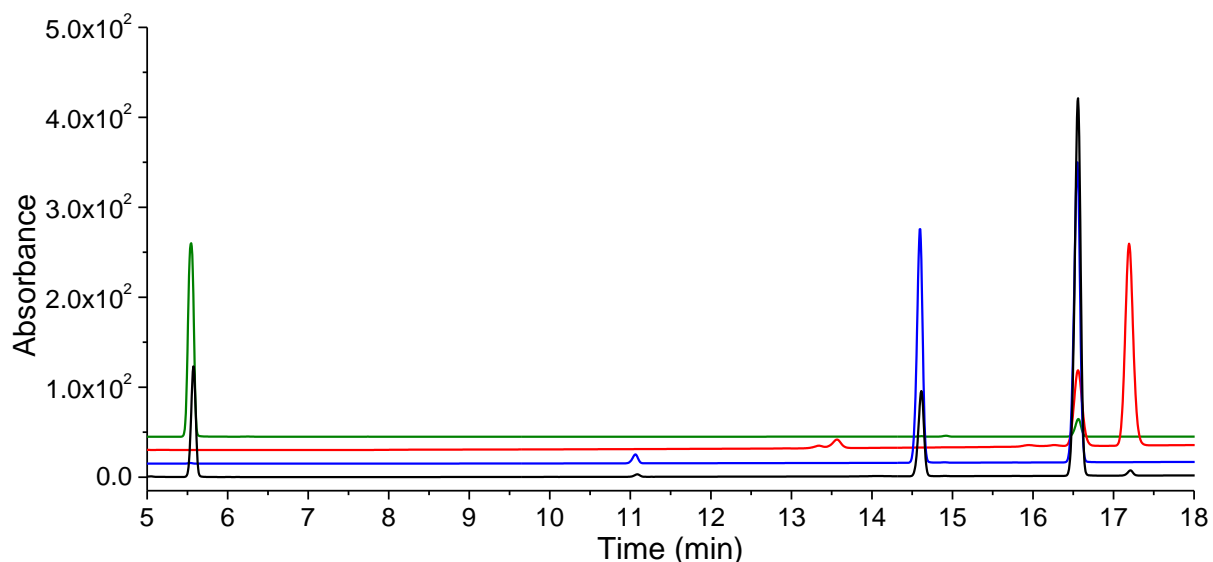
black = *in vitro* turnover, blue = 4-aminoBA control, red = 4-methylaminoBA control



**Figure S4 (f)** HPLC analysis of the *in vitro* turnovers of 4-diethylaminobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Diethylaminobenzoic acid, 254 nm, 0-50% acetonitrile.

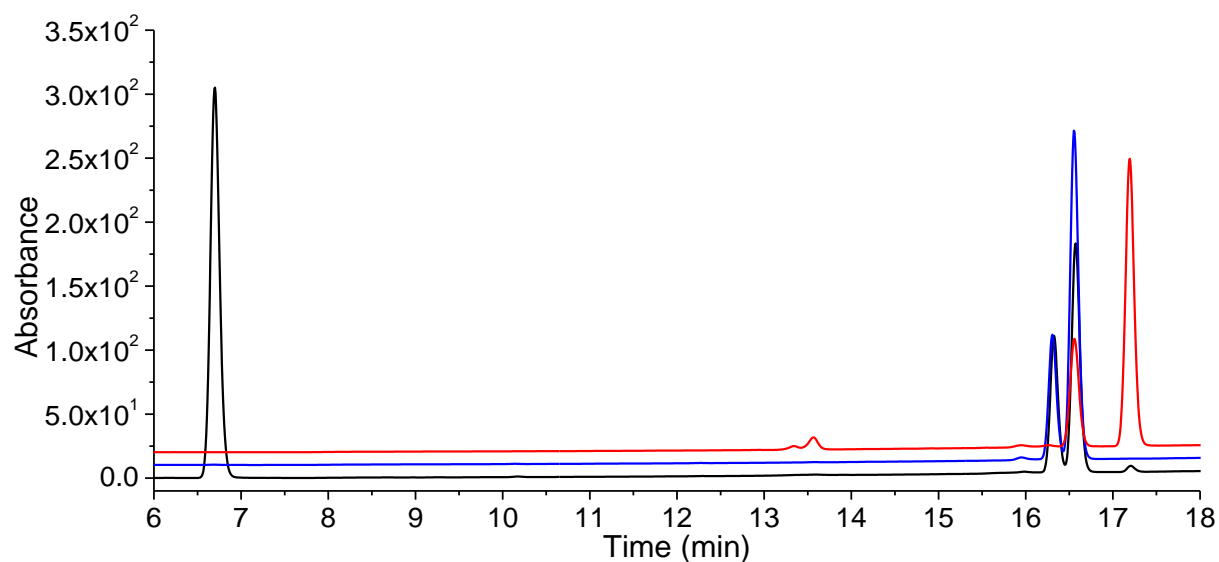
black = *in vitro* turnover, blue = 4-aminoBA control, red = 4-ethylaminoBA control, green = 4-diethylaminoBA control, pink = 4-acetamidoBA control.



**Figure S4 (g)** HPLC analysis of the *in vitro* turnovers of 4-methylthiobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Methylthiobenzoic acid, 254 nm, 20-95% acetonitrile.

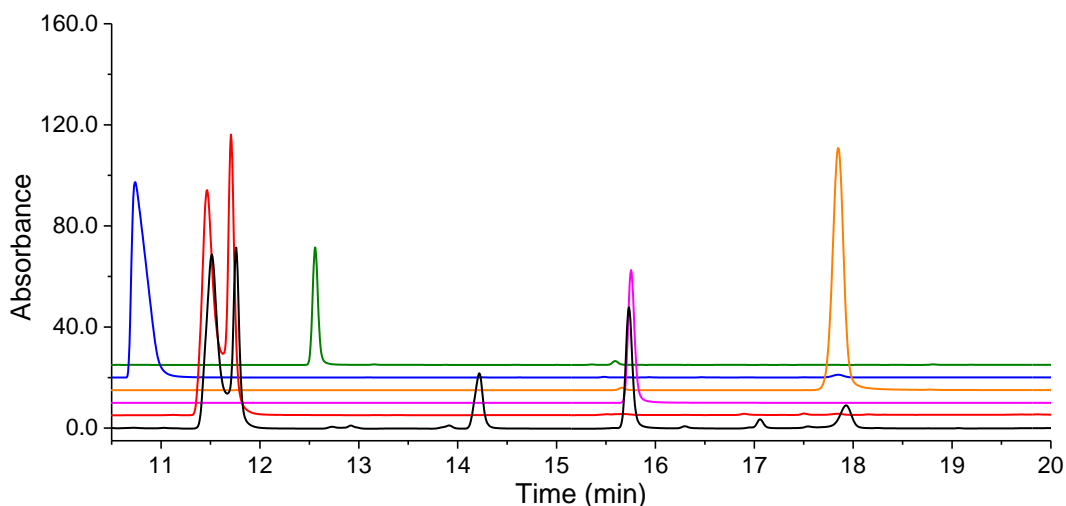
black = *in vitro* turnover, blue = 4-methylthioBA control, red = 4-mercaptobenzoic acid control, green = 4-methylsulfinyl benzoic acid control.



**Figure S4 (h)** HPLC analysis of the *in vitro* turnovers of 4-ethylthiobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-Ethylthiobenzoic acid, 254 nm, 20-95% acetonitrile.

black = *in vitro* turnover, blue = 4-ethylthioBA control, red = 4-mercaptoBA control. Internal standard shown at 16.6 min.

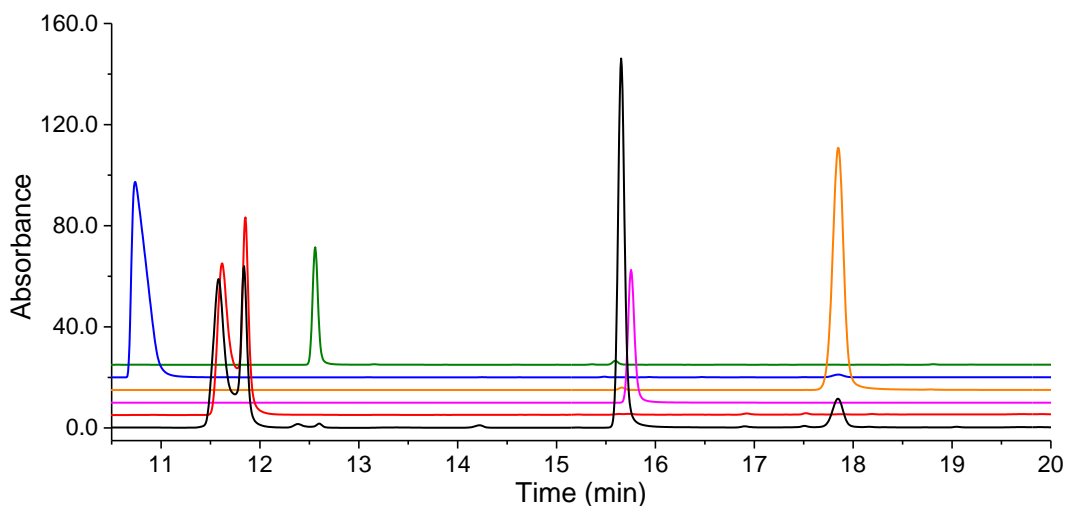


**Figure S4 (i)** HPLC analysis of the *in vitro* turnovers of 4-(methylamino)methylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-(methylamino)methylbenzoic acid, 254 nm, 0-50% acetonitrile,

black = *in vitro* turnover, red = substrate control, blue = 4-aminomethylbenzoic acid control, pink = terephthalic acid control, orange = 4-formylbenzoic acid control and green = monoamide terephthalic acid control.

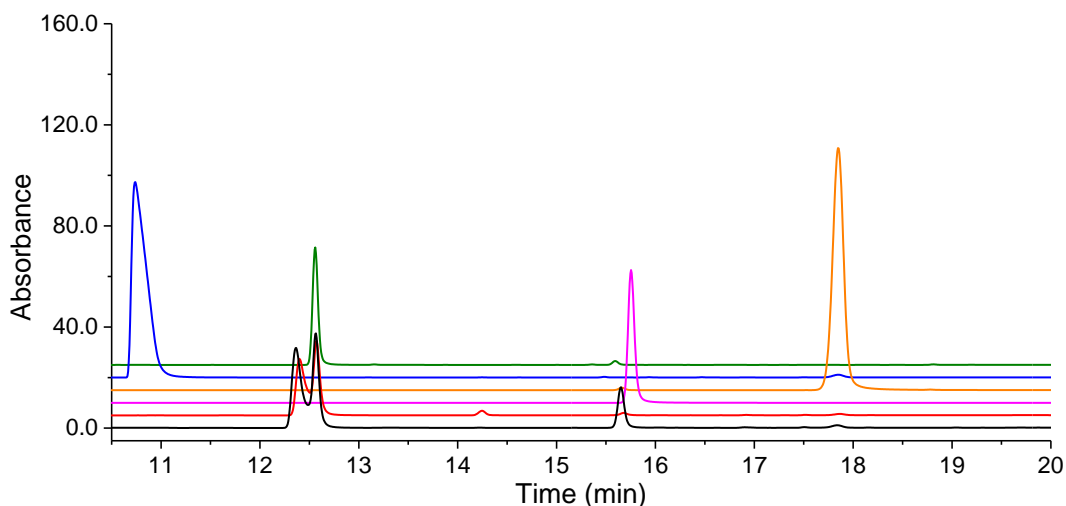
The peak at 14.2 min is uncharacterised no equivalent additional peak could be detected/assigned in the GC-MS analysis.



**Figure S4 (j)** HPLC analysis of the *in vitro* turnovers of 4-(dimethylamino)methylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-(dimethylamino)methylbenzoic acid, 254 nm, 0-50% acetonitrile,

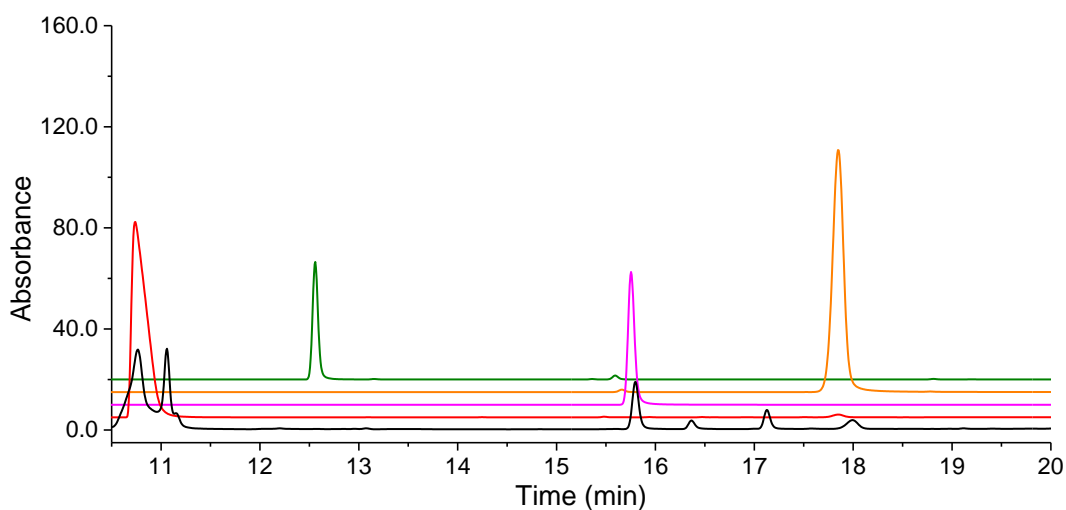
black = turnover, red = substrate control, blue = 4-aminomethylbenzoic acid control, pink = terephthalic acid control, orange = 4-formylbenzoic acid control and green = monoamide terephthalic acid control.



**Figure S4 (k)** HPLC analysis of the *in vitro* turnovers of 4-(ethylamino)methylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-(ethylamino)methylbenzoic acid, 254 nm, 0-50% acetonitrile,

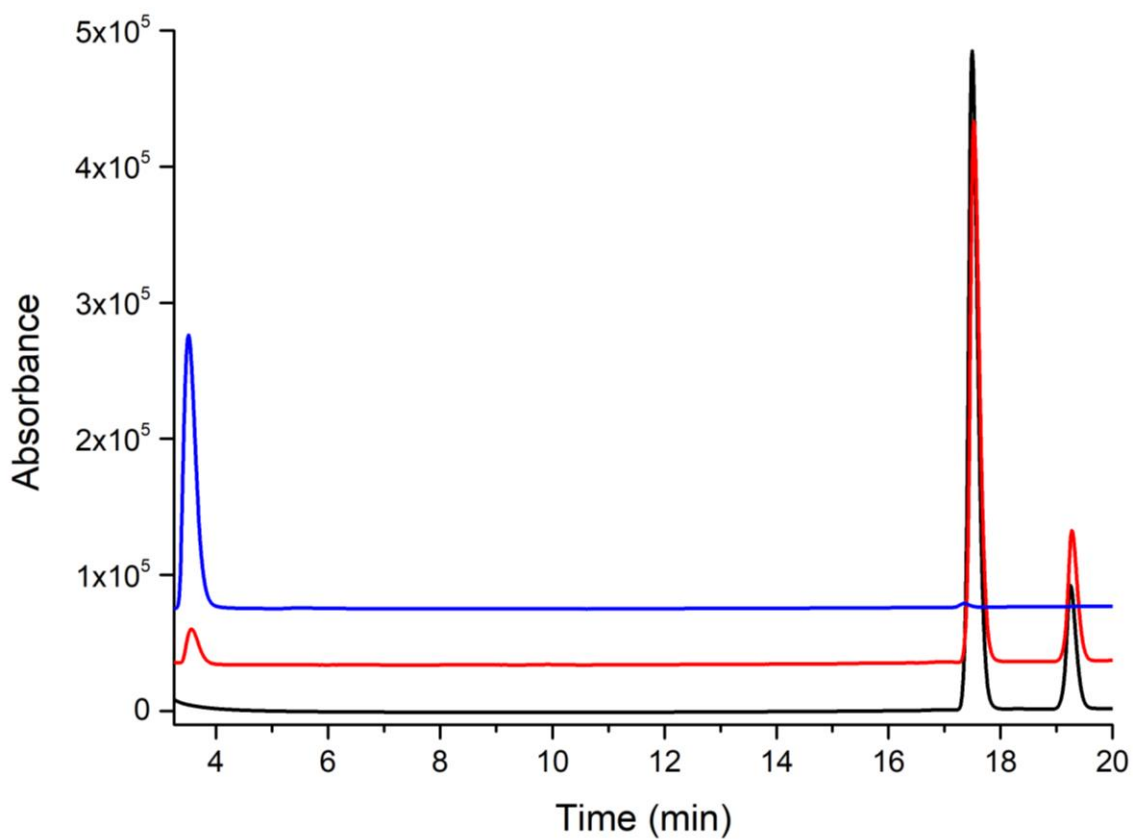
black = turnover, red = substrate control, blue = 4-aminomethylbenzoic acid control, pink = terephthalic acid control, orange = 4-formylbenzoic acid control and green = monoamide terephthalic acid control.



**Figure S4 (l)** HPLC analysis of the *in vitro* turnovers of 4-aminomethylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

4-aminomethylbenzoic acid, 254 nm, 0-50% acetonitrile,

black = turnover, red = substrate control, pink = terephthalic acid control, orange = 4-formylbenzoic acid control and green = monoamide terephthalic acid control. Note minor unidentified peaks at 16.4 min and 17.2 min.

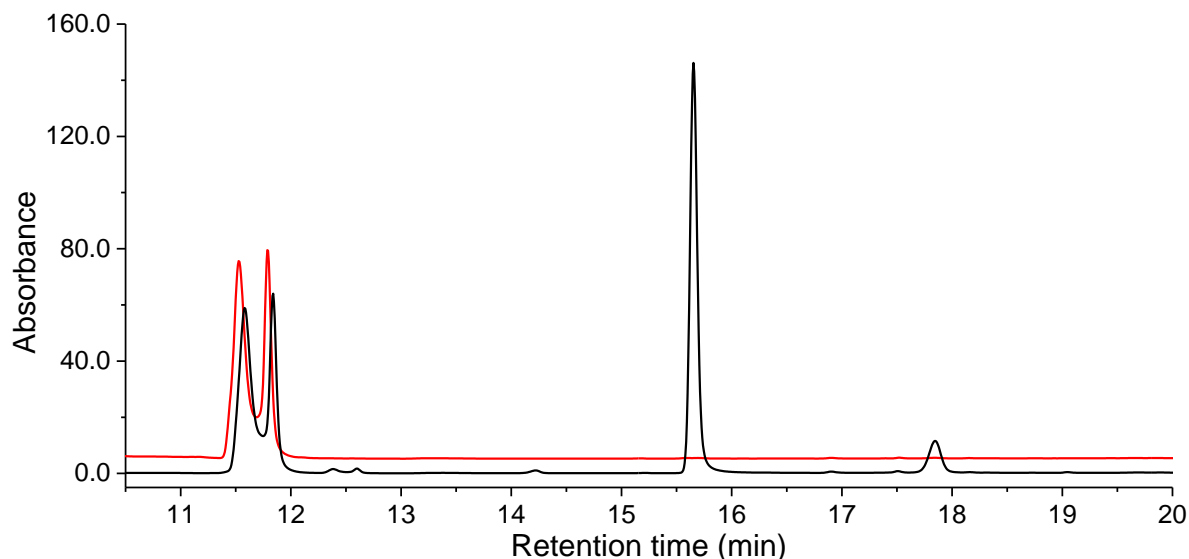


**Figure S4 (m)** HPLC analysis of the *in vitro* turnovers of 3-methylthiobenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis.

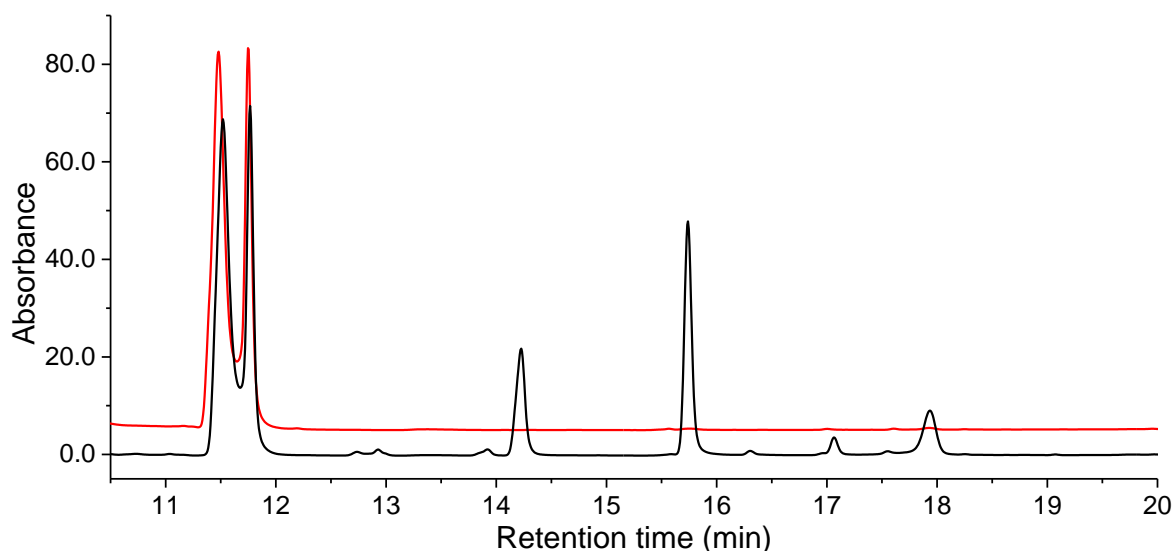
3-methylthiobenzoic acid HPLC, 254 nm, 20-95% acetonitrile

red = *in vitro* turnover, blue = chemically synthesised 3-(methylsulfinyl)benzoic acid standard and black = no P450 control reaction (**black**). The chromatograms have been offset along the y-axis.

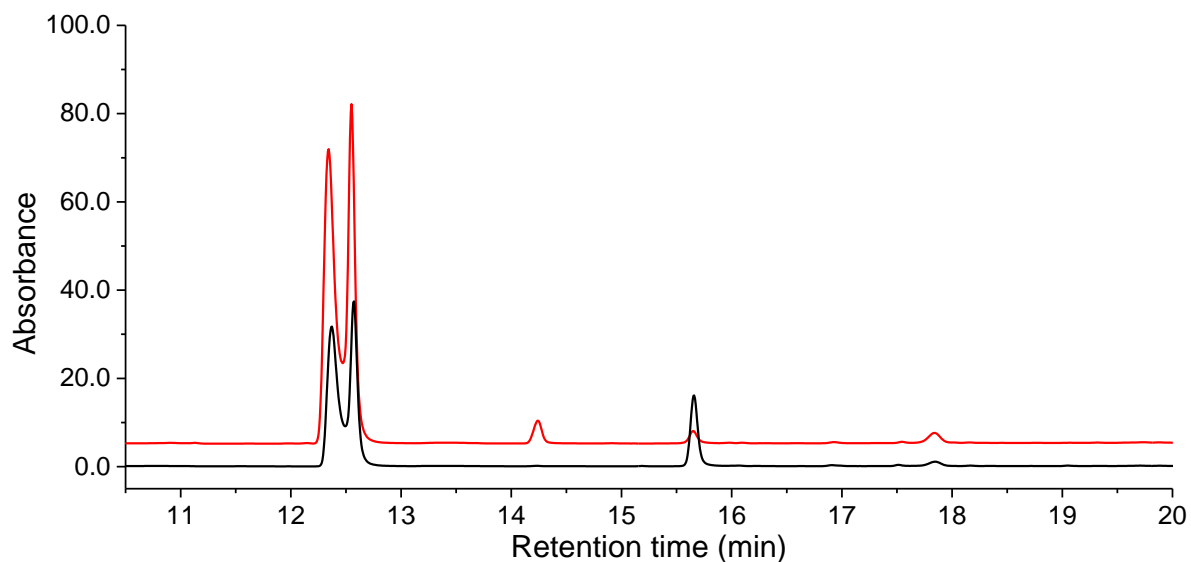
Note for the 4-(methylamino)methylbenzoic acid, 4-(dimethylamino)methylbenzoic acid, 4-(ethylamino)methylbenzoic acid and 4-aminomethylbenzoic acid turnovers no peaks could be assigned to potential products before 10 minutes which were not present in controls (both no P450 and no NADH controls were run). It is important to note that peaks corresponding to terephthalic acid (RT 15.7 min) and formylbenzoic acid (RT 17.9 min) were also present in some of these controls but in much lower quantities than observed in the turnovers.



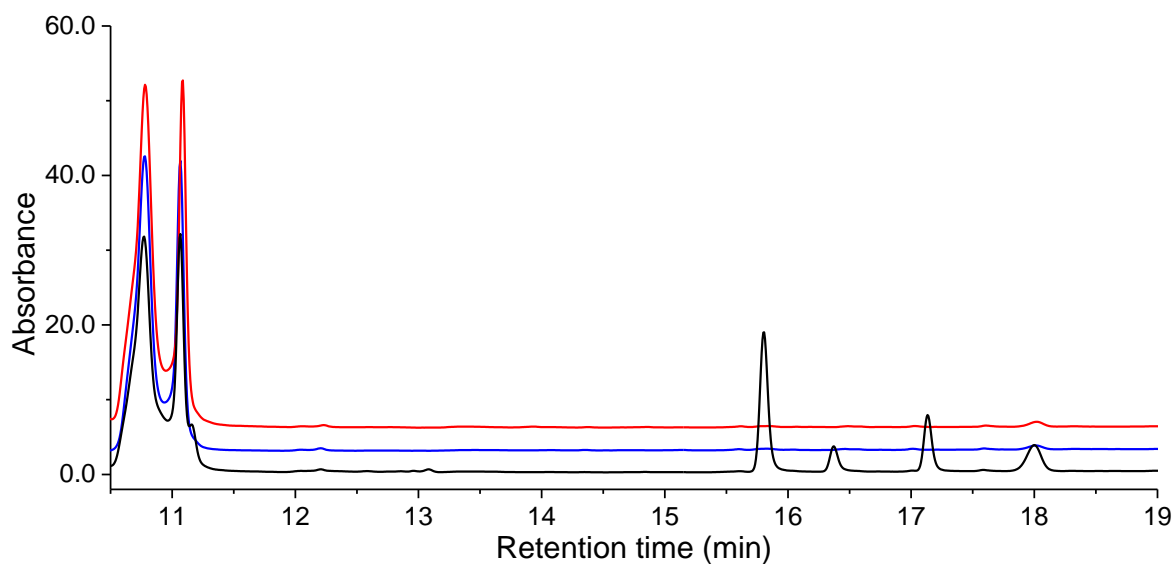
**Figure S4 (n)** HPLC analysis of the *in vitro* turnovers of 4-(dimethylamino)methylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis. black = *in vitro* turnover, red = no p450 control.



**Figure S4 (o)** HPLC analysis of the *in vitro* turnovers of 4-(methylamino)methylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis. black = *in vitro* turnover, red = no p450 control.



**Figure S4 (p)** HPLC analysis of the *in vitro* turnovers of 4-(ethylamino)methylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis. black = *in vitro* turnover, red = no p450 control.



**Figure S4 (q)** HPLC analysis of the *in vitro* turnovers of 4-aminomethylbenzoic acid with CYP199A4. For clarity the chromatograms have been offset along the y axis. black = *in vitro* turnover, red = no p450 control.

List of reverse phase HPLC retention times. A: H<sub>2</sub>O, B: acetonitrile. The gradient used is given in parentheses.

Internal standard – 9-hydroxyfluorene: 16.6 min (20-95%)

4-Ethoxybenzoic acid: 14.3 min (20-95%)

4-Hydroxybenzoic acid: 6.6 min (20-95%)

4-Methylthiobenzoic acid: 14.7 min (20-95%)

4-Methylsulfinylbenzoic acid: 5.3 min (20-95%)

4-Ethylthiobenzoic acid: 16.3 min (20-95%)

4-Ethylsulfinylbenzoic acid: 6.7 min (20-95%)

3,4-Ethylenedioxybenzoic acid: 19.9 min (20-95%)

4-Mercaptobenzoic acid: 17.2 min (20-95%)

6-Carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin: 15.6 min (20-95%)

3-(Methylthio)benzoic acid: 17.5 min (20-95%)

3-(Methylsulfinyl)benzoic acid: 3.5 min (20-95%)

Internal standard – 9-hydroxyfluorene: 26.1 min (0-50%)

4-Diethylaminobenzoic acid: 16.8 min (0-50%)

4-Ethylaminobenzoic acid: 16.5 min (0-50%)

4-Acetamidobenzoic acid: 15.3 min (0-50%)

4-Dimethylaminobenzoic acid: 18.3 min (0-50%)

4-Methylaminobenzoic acid: 14.9 min (0-50%)

4-Aminobenzoic acid: 9.4 min (0-50%)

4-Aminomethylbenzoic acid: 10.8 min (0-50%) (second peak at 11.1 min)

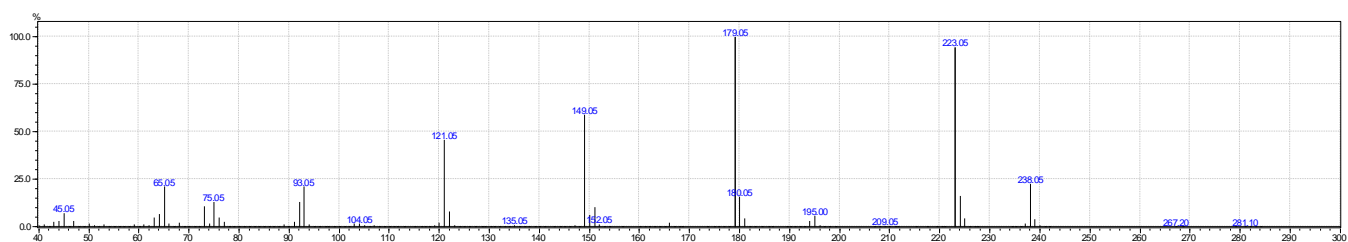
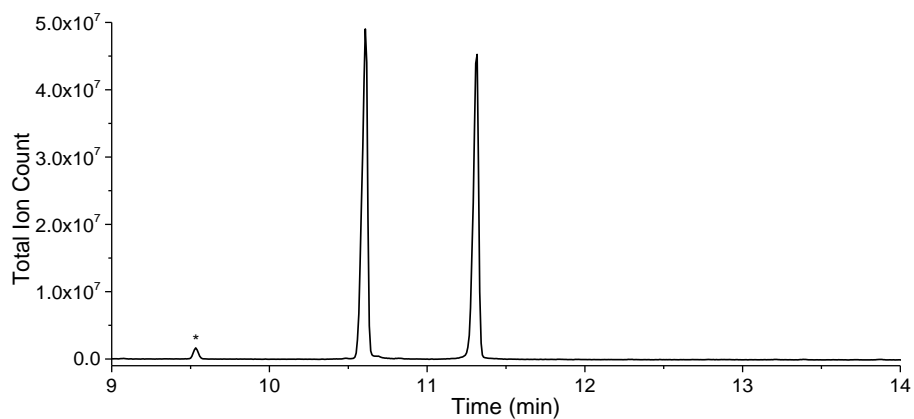
Terephthalic acid: 15.7 min (0-50%)

4-Formylbenzoic acid: 17.9 min (0-50%)

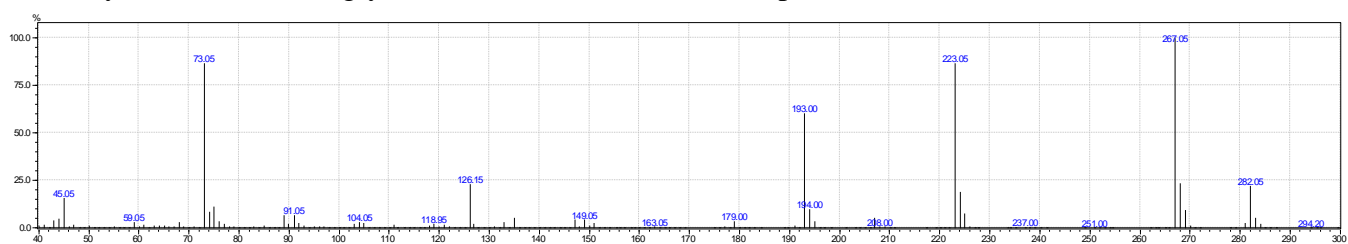
4-(Methylamino)methylbenzoic acid: Double peak 11.5 min & 11.8 min (0-50%)

4-(Dimethylamino)methylbenzoic acid: Double peak 11.6 min & 11.9 min (0-50%)

4-(Ethylamino)methylbenzoic acid: Double peak 12.4 min & 12.6 min (0-50%)

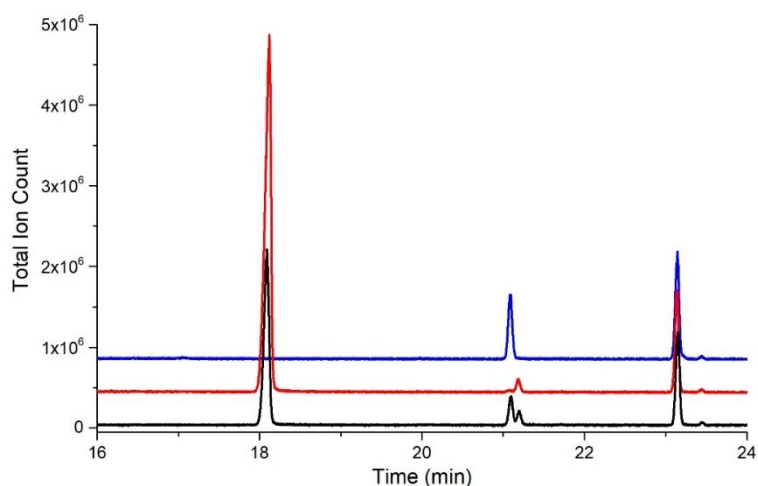


4-EthoxyBA substrate (singly derivatised):  $m/z = 238.05$  (exp.  $m/z = 238.1025$ ).

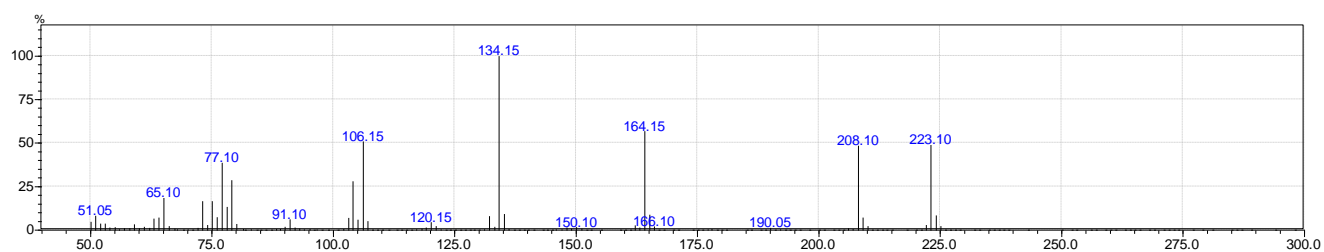


4-Hydroxybenzoic acid (doubly derivatised):  $m/z = 282.05$  (exp.  $m/z = 282.1107$ ).

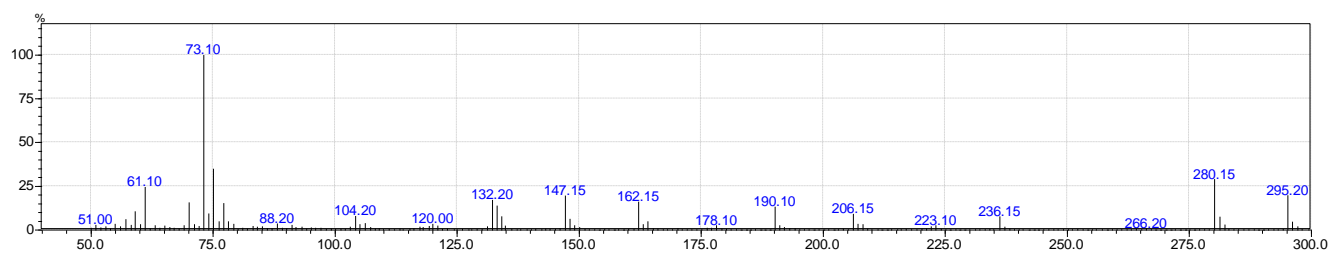
**Figure S5** (a) GC-MS chromatogram of the *in vitro* turnover of 4-ethoxybenzoic acid with CYP199A4 and the MS data. GC retention times of the trimethylsilyl chloride derivatised substrates and products; 4-ethoxybenzoic acid: 10.6 min; 4-hydroxybenzoic acid: 11.3 min.



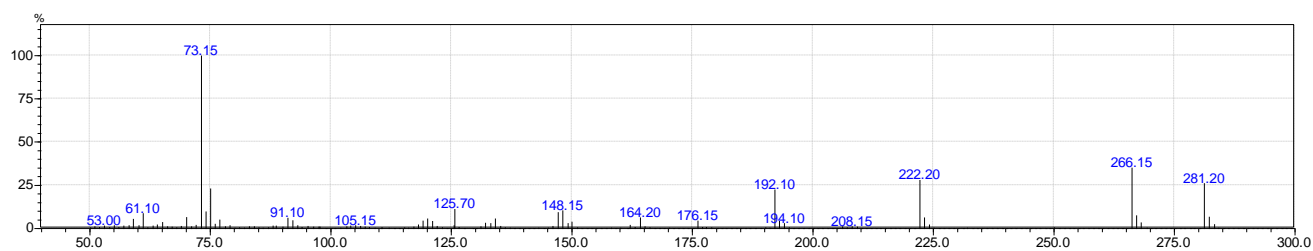
Black = *in vitro* turnover, red = no P450 control, blue = 3-aminoBA control (doubly derivatised)



3-Methylaminobenzoic acid substrate (singly derivatised):  $m/z = 223.10$  (exp.  $m/z = 223.1029$ )

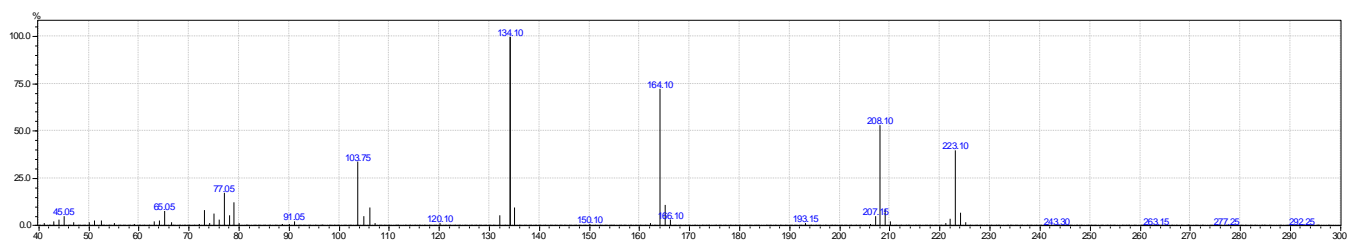


3-Methylaminobenzoic acid substrate (doubly derivatised):  $m/z = 295.20$  (exp.  $m/z = 295.1424$ ).

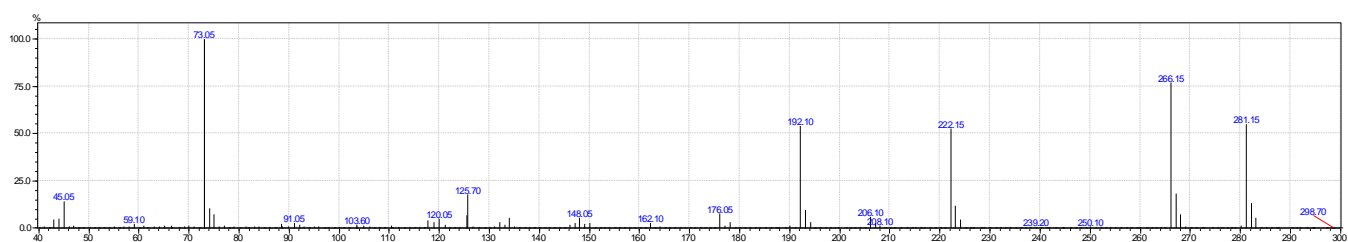


3-Aminobenzoic acid product (doubly derivatised):  $m/z = 281.20$  (exp.  $m/z = 281.1267$ ).

**Figure S5 (b)** GC-MS chromatogram of the *in vitro* turnover of 3-methylaminobenzoic acid with CYP199A4 and the MS data. GC retention times of the trimethylsilyl chloride derivatised substrates and products; 3-methylaminobenzoic acid substrate (singly derivatised): 18.1 min; 3-aminobenzoic acid product (doubly derivatised): 21.1 min; 3-methylaminobenzoic acid substrate (doubly derivatised): 21.2 min; internal standard (derivatised 9-hydroxyfluorene): 23.2 minutes.

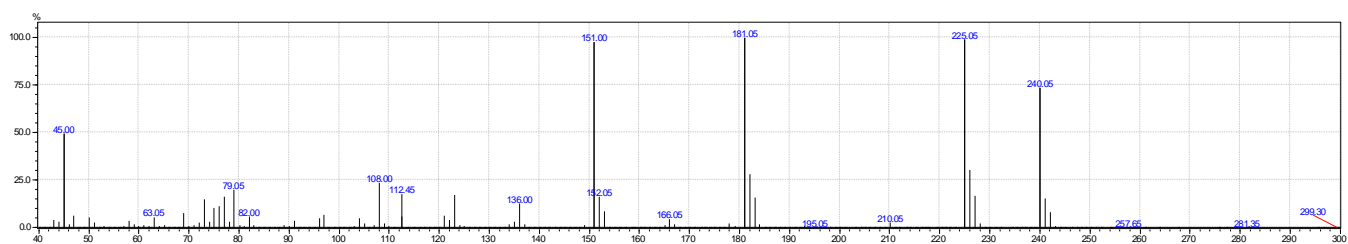


4-methylaminobenzoic acid substrate (singly derivatised):  $m/z = 223.10$  (exp.  $m/z = 223.3437$ ),  
RT = 13.5 min

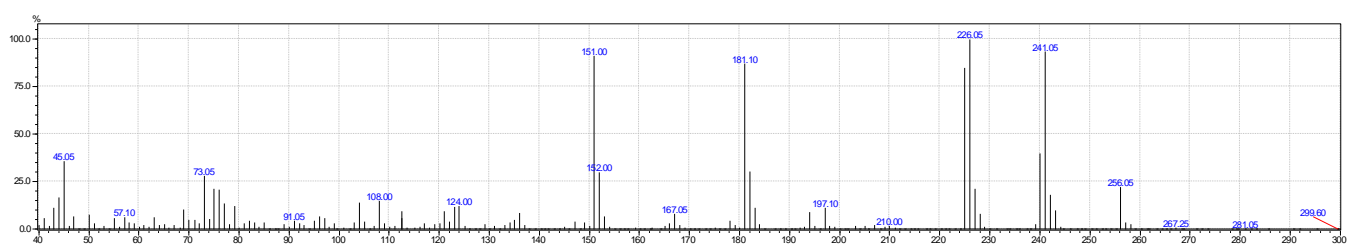


4-aminobenzoic acid (doubly derivatised):  $m/z = 281.15$  (exp.  $m/z = 281.1267$ ),  
RT = 15.1 min

**Figure S5 (c)** MS data for 4-methylaminobenzoic acid turnovers with CYP199A4.

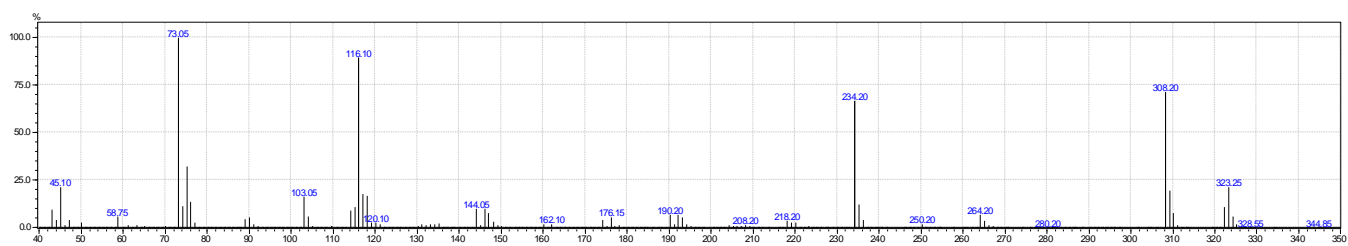


4-methylthiobenzoic acid substrate (singly derivatised):  $m/z = 240.05$  (exp.  $m/z = 240.0640$ ),  
RT = 12.6 min

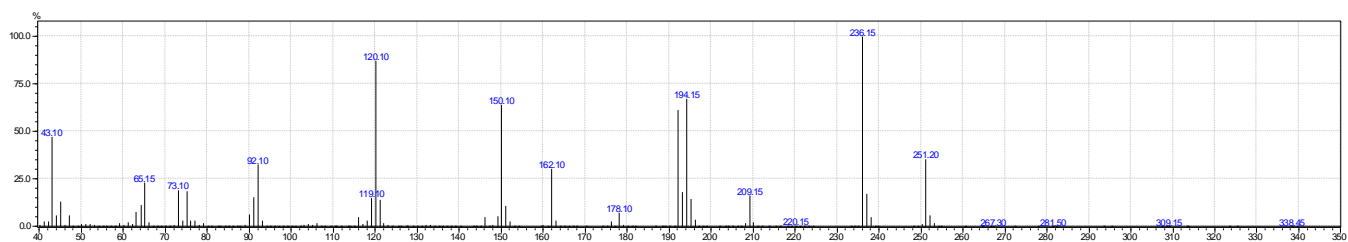


4-methylsulfinylbenzoic acid (singly derivatised):  $m/z = 256.05$  (exp.  $m/z = 256.0589$ ),  
RT = 16.2 min

**Figure S5 (d)** MS data for 4-methylthiobenzoic acid turnovers with CYP199A4.

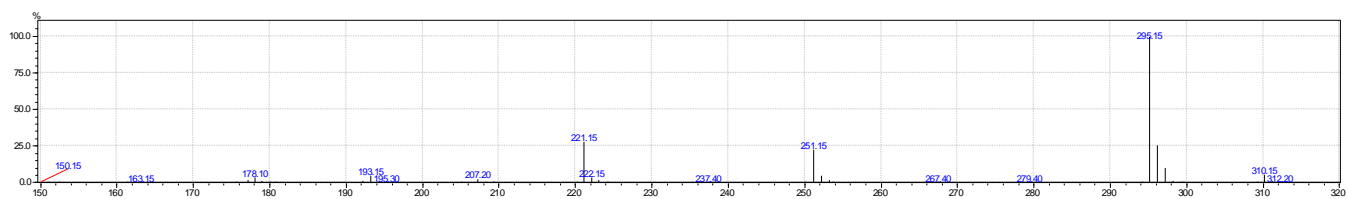


4-acetamidobenzoic acid substrate (doubly derivatised):  $m/z = 323.25$  (exp. 323.1373),  
 RT = 15.4 min



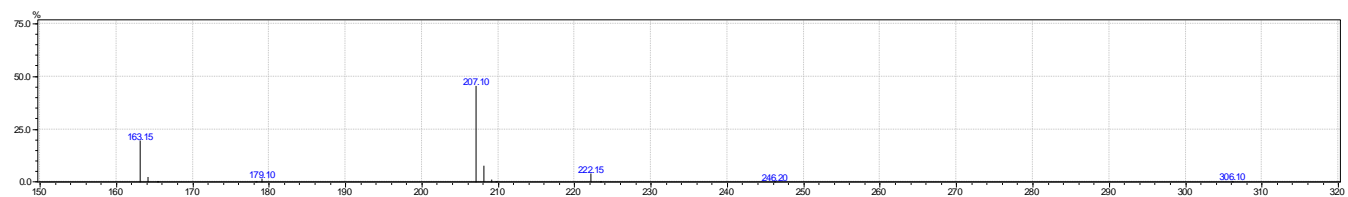
4-acetamidobenzoic acid substrate (singly derivatised):  $m/z = 251.20$  (exp. 251.0978),  
 RT = 17.8 min

**Figure S5 (e)** MS data for 4-acetamidobenzoic acid product arising from the turnovers with CYP199A4.



terephthalic acid product, doubly derivatised  $m/z = 310.15$  (-Me  $\rightarrow$  295.15; vs exp. 310.1057)

RT 14.2 min

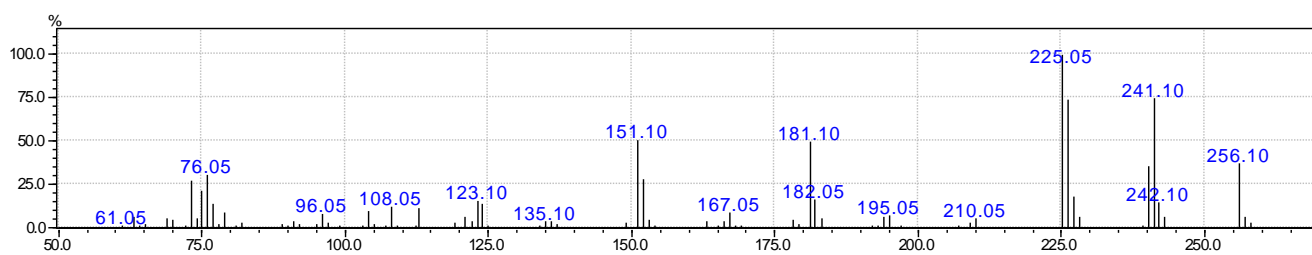


4-formylbenzoic acid, derivatised  $m/z = 222.15$  (exp. 222.0712)

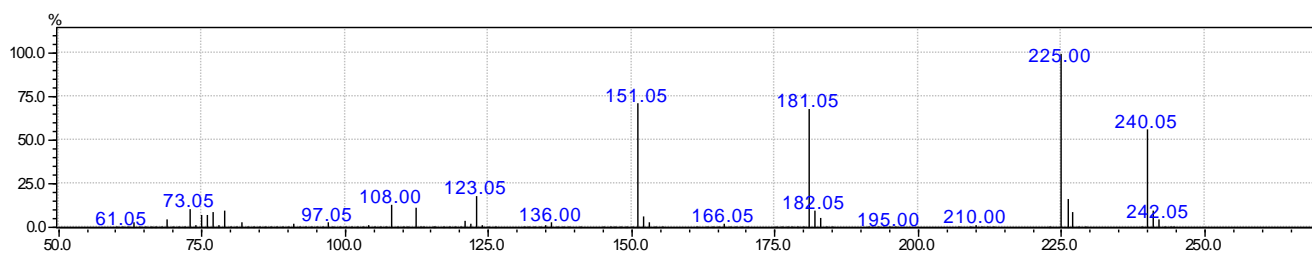
RT 7.8 min

**Figure S5 (f)** MS data for 4-aminomethylbenzoic acid products arising from the turnovers with CYP199A4.

### 3-Methylthiobenzoic acid MS data

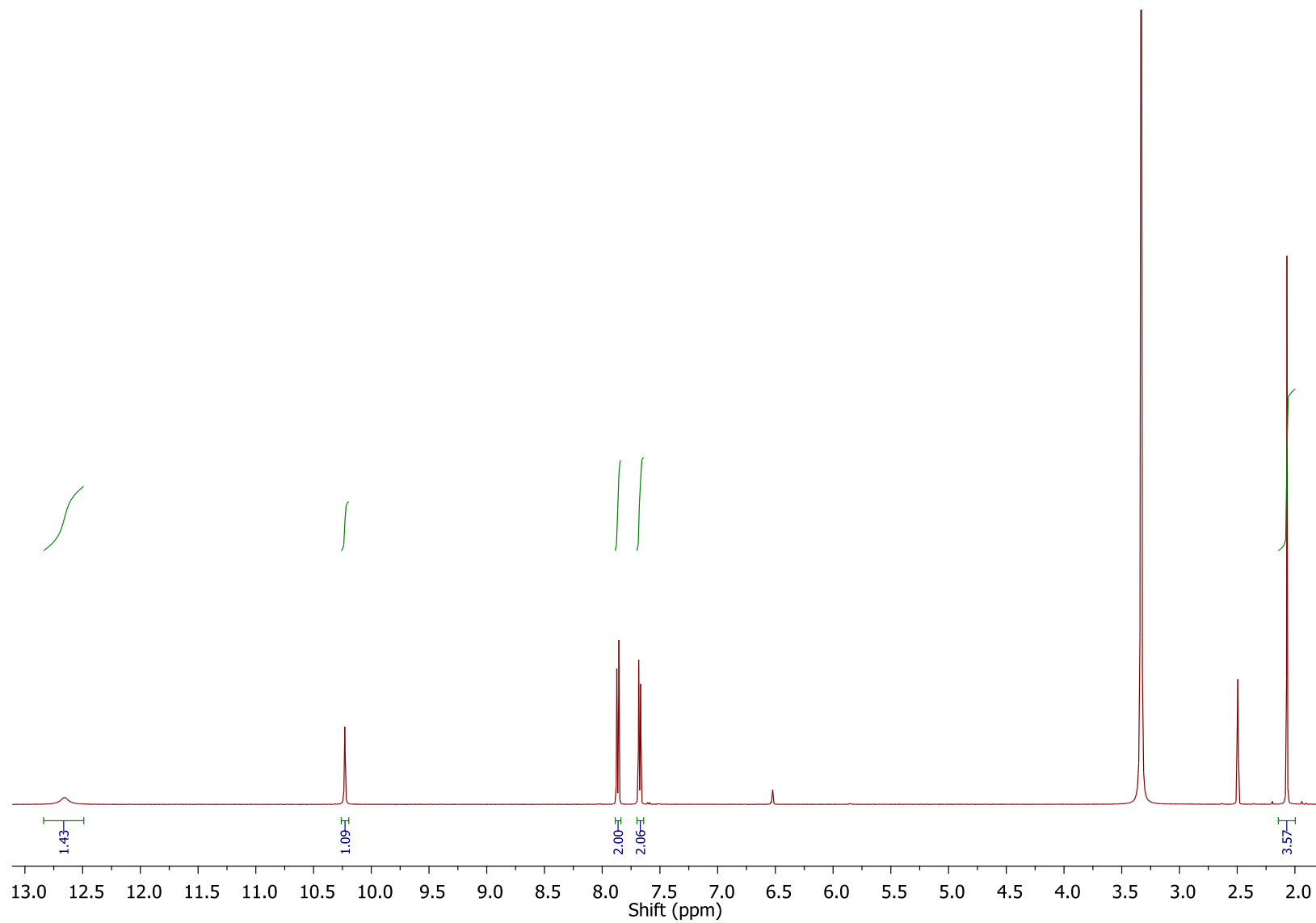


3-(Methylsulfinyl)benzoic acid (singly derivatised): m/z = 256.10 (exp. 256.0589),  
RT = 17.0 min

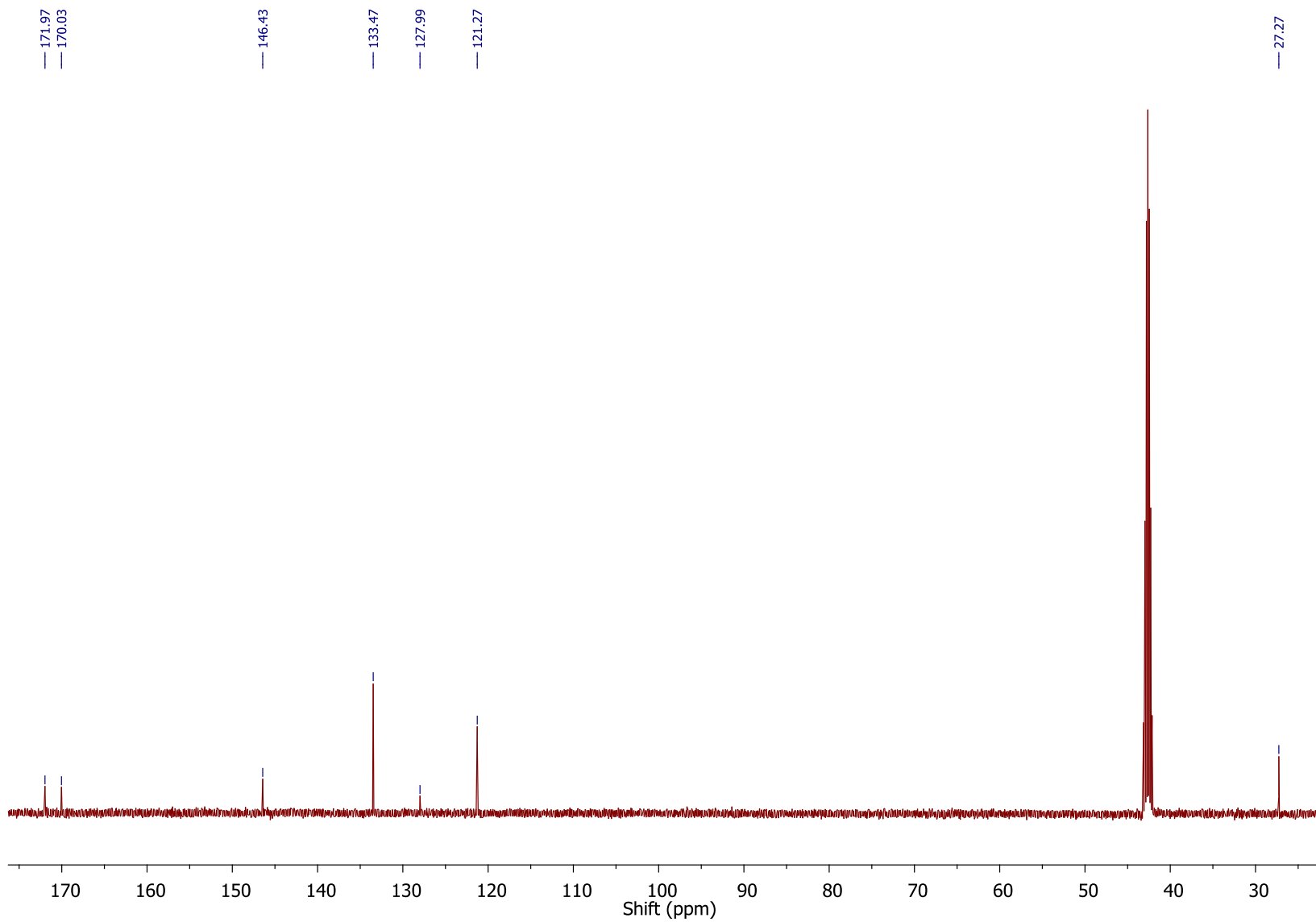


3-(Methylthio)benzoic acid substrate (singly derivatised): m/z = 240.05 (exp. 240.0640),  
RT = 12.2 min

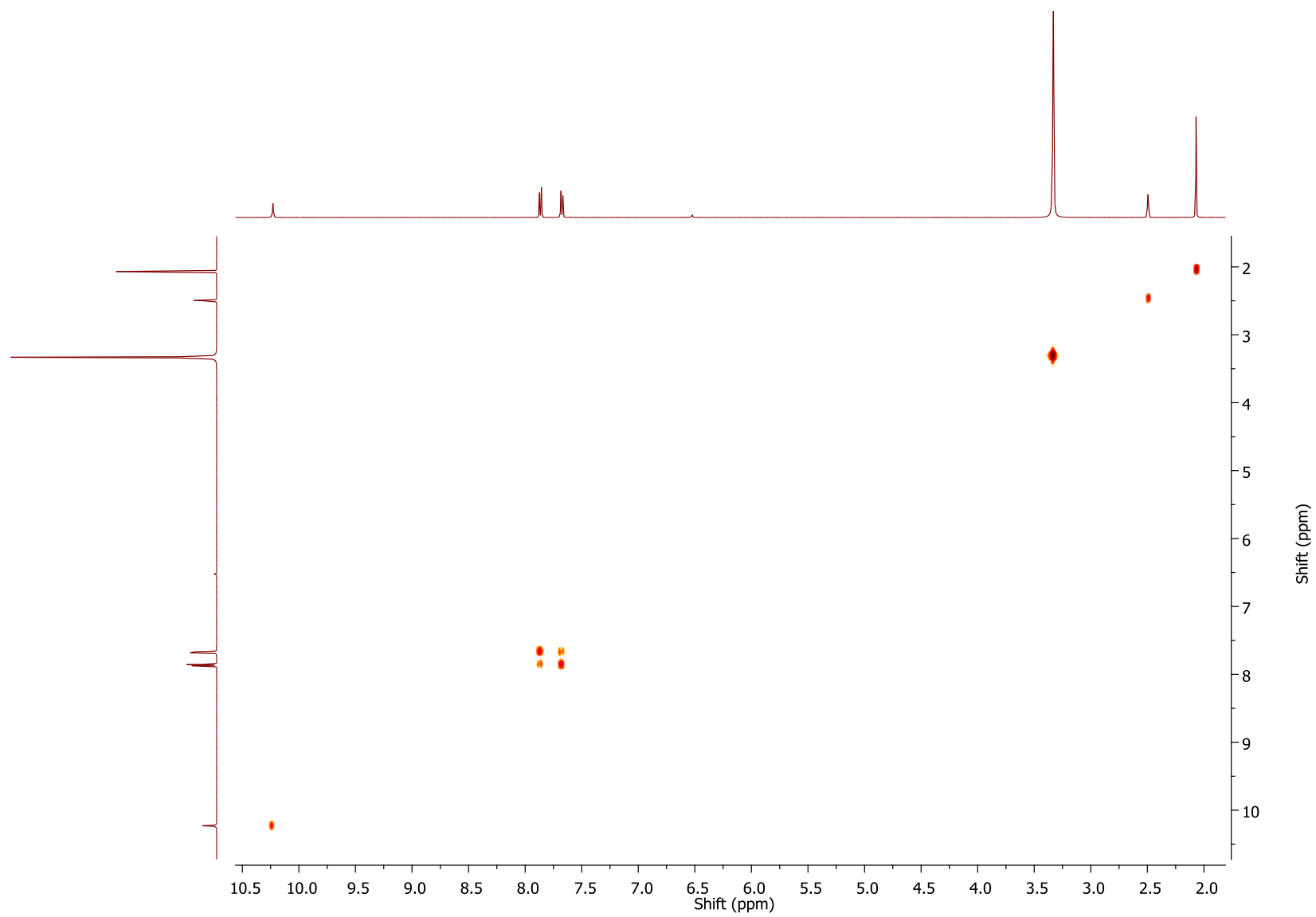
**Figure S5 (g)** MS data for 3-methylthiobenzoic acid turnovers with CYP199A4.



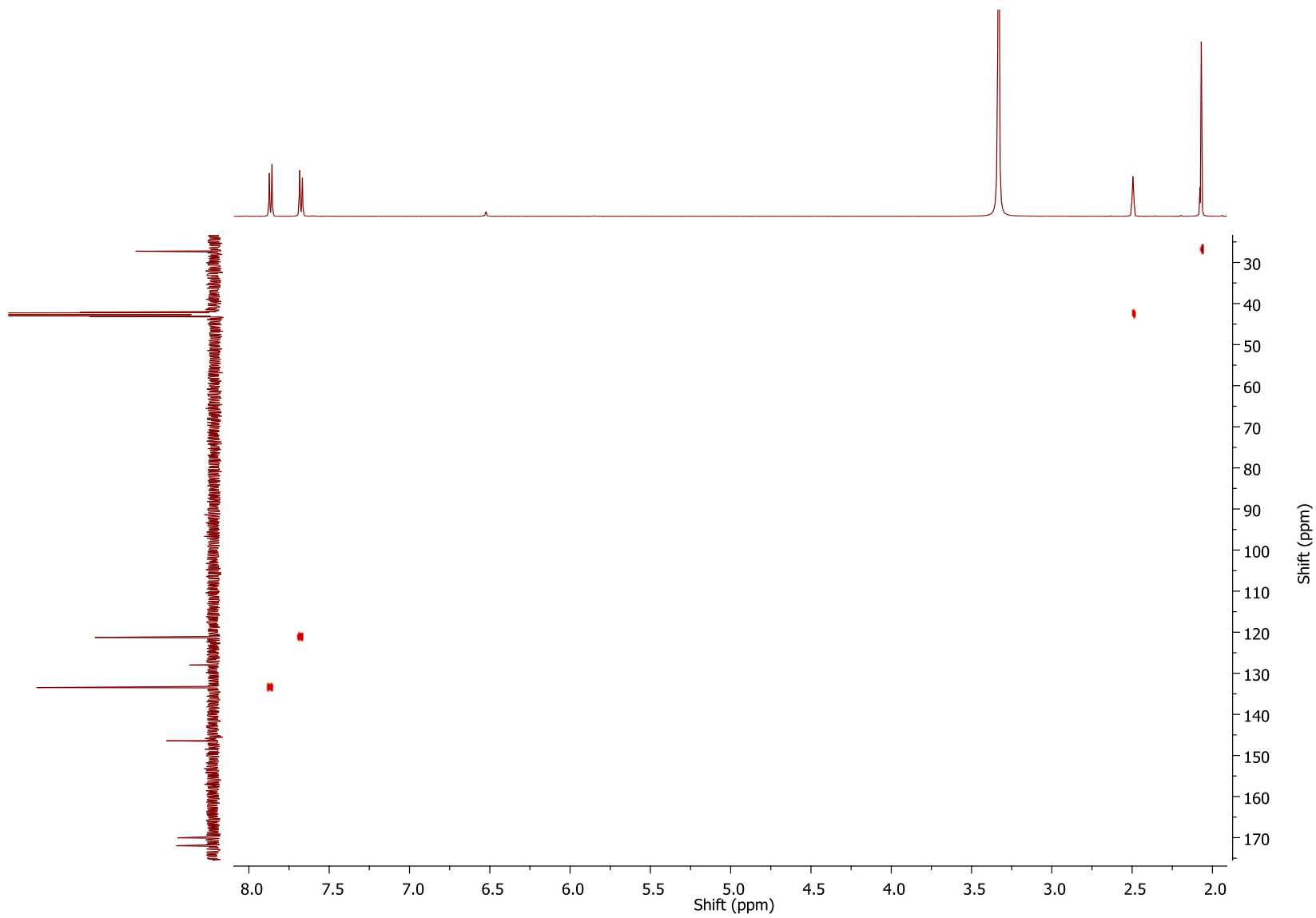
**Figure S6 (a)**  $^1\text{H}$  NMR of 4-acetamidobenzoic acid.



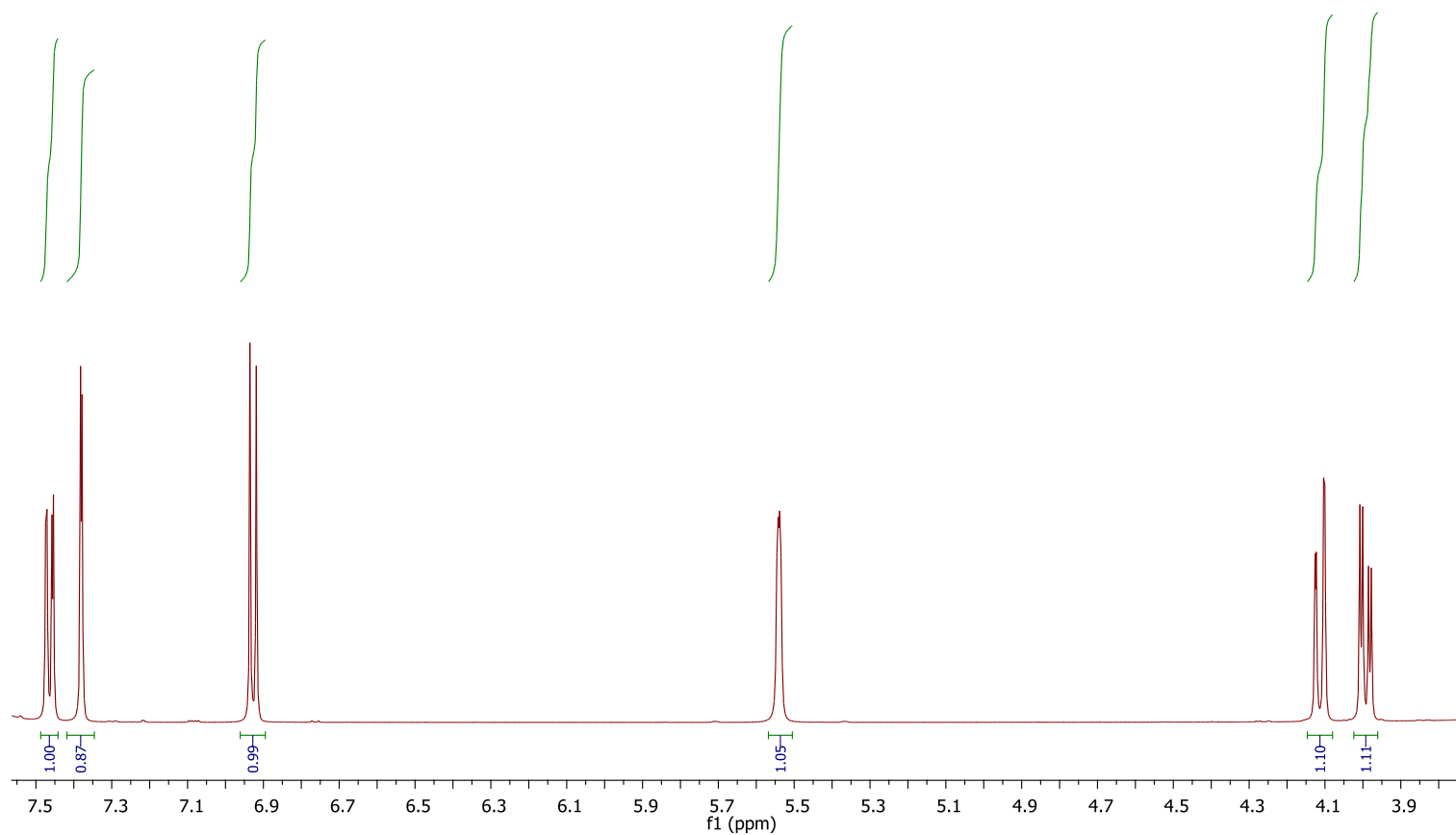
**Figure S6 (b)**  $^{13}\text{C}$  NMR of 4-acetamidobenzoic acid.



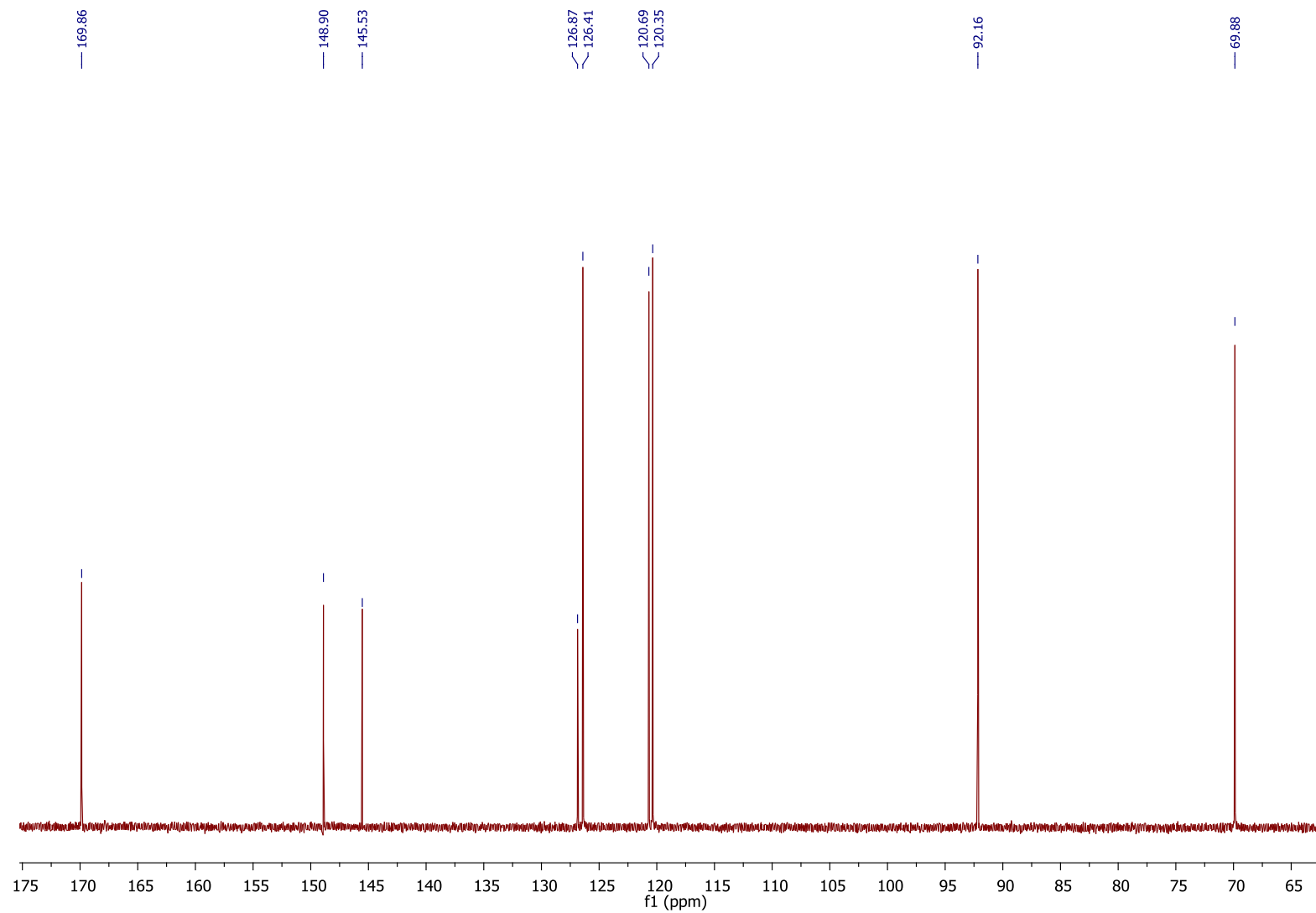
**Figure S6 (c)** COSY NMR of 4-acetamidobenzoic acid.



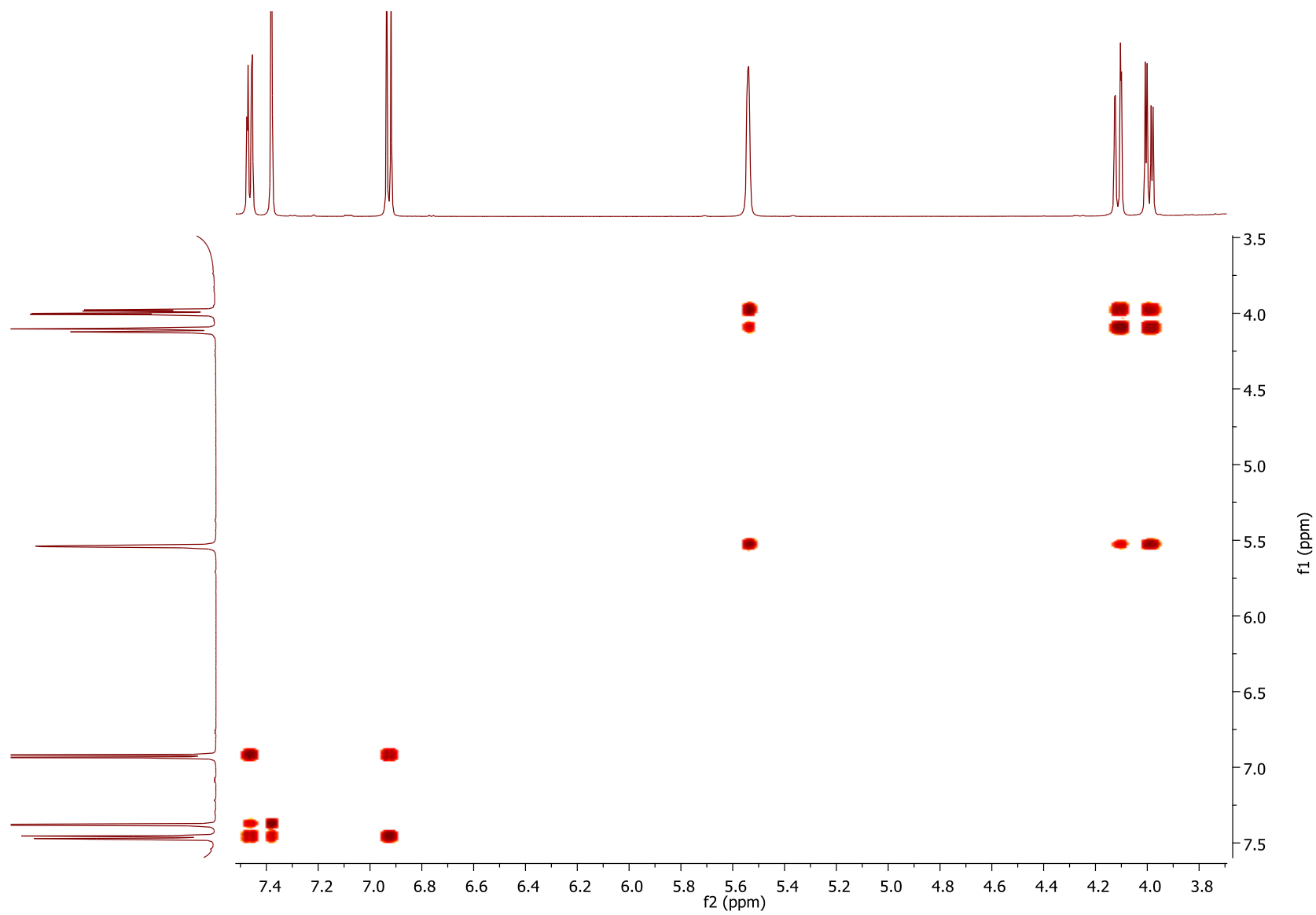
**Figure S6 (d)** HSQC NMR of 4-acetamidobenzoic acid



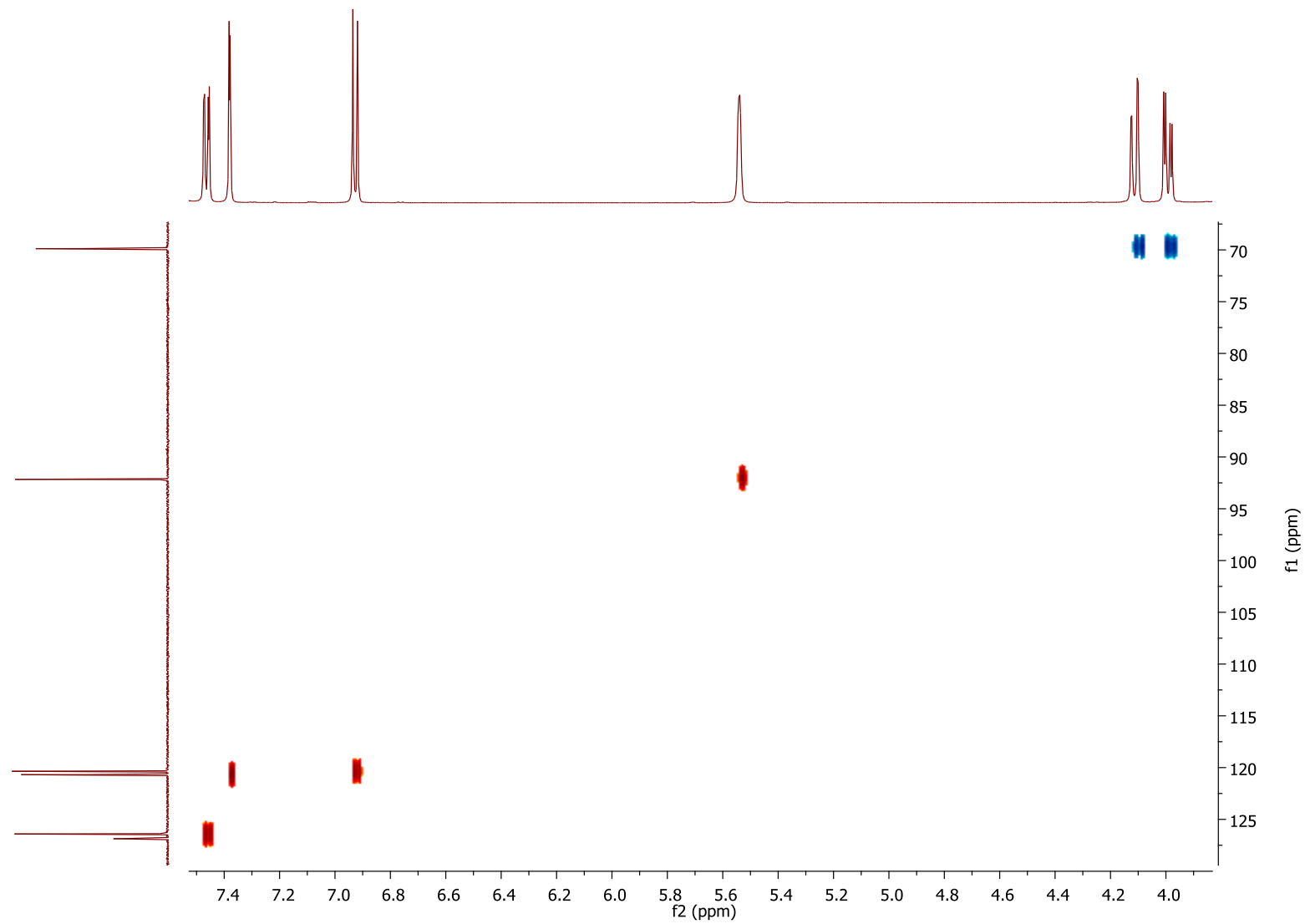
**Figure S6 (e)** NMR data for product from *in vitro* turnover of 3,4-ethylenedioxybenzoic acid to 6-carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin. <sup>1</sup>H NMR of 4-- 6-carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin. <sup>1</sup>H NMR (500 MHz, dmsO) δ 7.46 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.38 (d, *J* = 2.0 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 5.54 (dd, *J* = 3.7, 1.7 Hz, 1H), 4.11 (dd, *J* = 11.3, 1.7 Hz, 1H), 3.99 (dd, *J* = 11.3, 3.7 Hz, 1H).



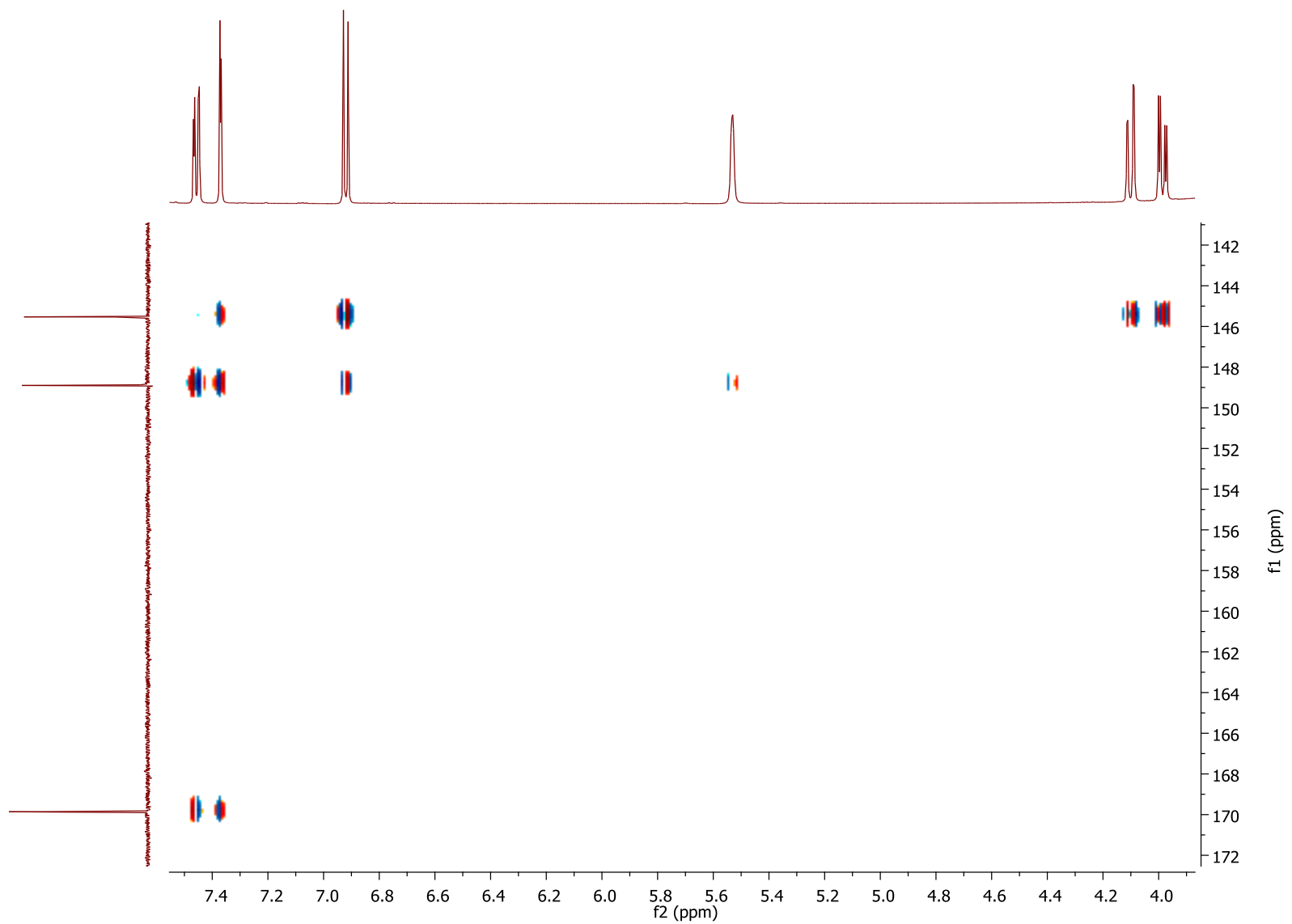
**Figure S6 (f)**  $^{13}\text{C}$  NMR of 6-carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin  
(500 MHz, DMSO)  $\delta$  169.86, 148.90, 145.53, 126.87, 126.41, 120.69, 120.35, 92.16, 69.88.



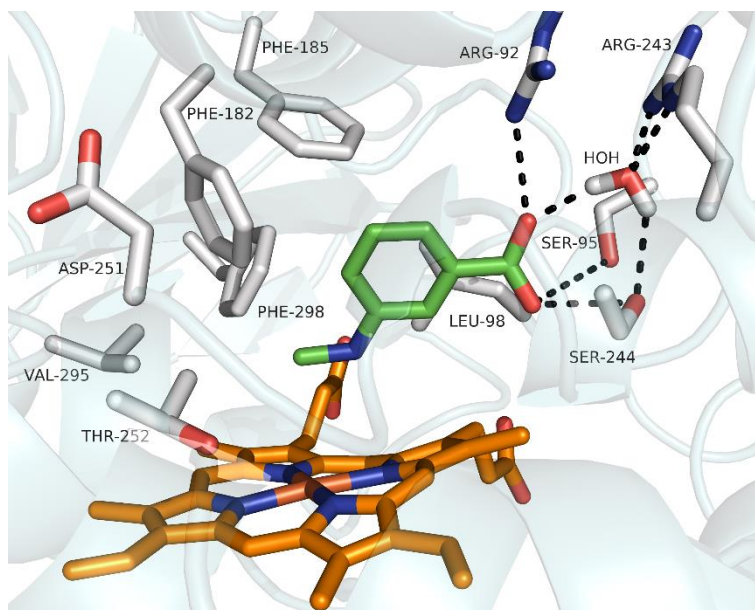
**Figure S6 (g)** <sup>1</sup>H-<sup>1</sup>H COSY of 6-carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin.



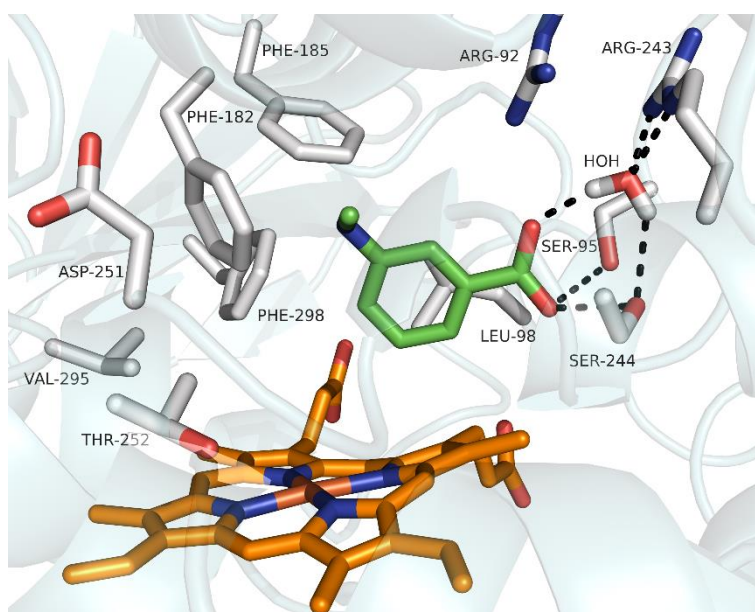
**Figure S6 (h)**  $^1\text{H}$ - $^{13}\text{C}$  HSQC 6-carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin



**Figure S6 (i)**  $^1\text{H}$ - $^{13}\text{C}$  HMBC of 6-carboxy-2,3-dihydro-2-hydroxy-1,4-benzodioxin.



The lowest energy orientation of 3-methylaminobenzoic acid after docking in the active site of CYP199A4 and energy minimisation.



A higher energy orientation of 3-methylaminobenzoic acid after docking in the active site of CYP199A4 in the 'up' orientation.

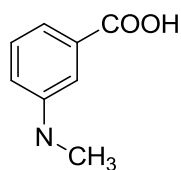
**Figure S7** Docking studies with 3-methylaminobenzoic acid

3-Methylaminobenzoic acid, heme and active site residues are depicted in green, orange and grey respectively, with nitrogen atoms shown in blue, oxygen in red and the heme iron in brown. Hydrogen bonds are represented by black dashed lines. Comparative distances and scores from ICM-Pro docking program are given overleaf.

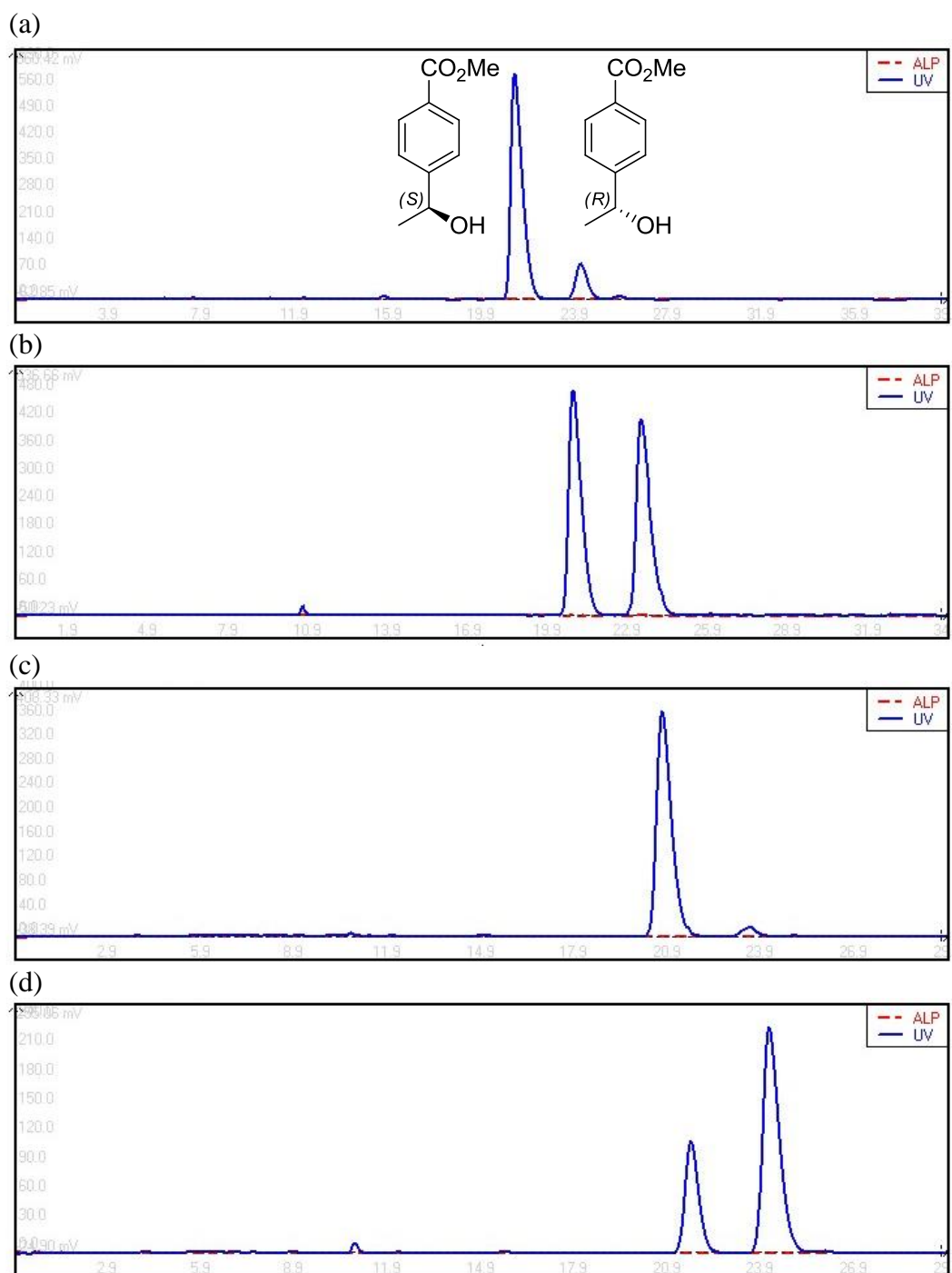
Pose	Score	Vls Score	Strain kcal/mol	Steric	Torsion	Electro	Hbond kcal/mol	Hydroph kcal/mol	Surface
'Down' orientation	-38.03	-40.46	2.421	-16.86	1	-4.597	-9.406	-3.178	6.355
'Up' orientation	-26.73	-36.42	9.698	-6.917	1	-11.1	-10.08	-3.049	6.341

Selected distances between 3-methylaminobenzoic acid in the up or down position and the heme or active site residues of CYP199A4.

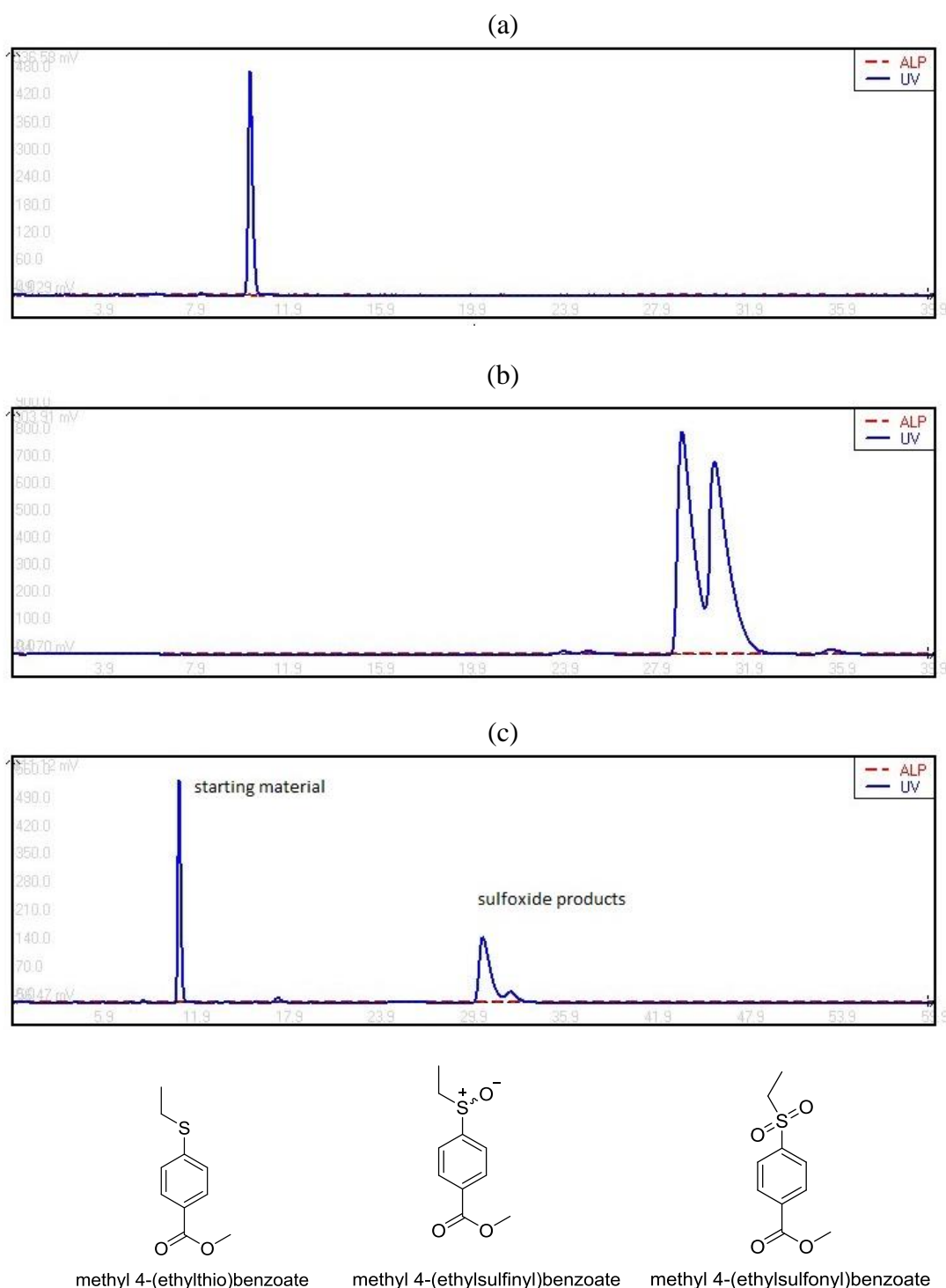
	Down	Up
CH <sub>3</sub> – Fe	3.9 Å	7.4 Å
N – Fe	4.2 Å	7.2 Å
N – nearest heme N	3.6 Å	n.m.
CH <sub>3</sub> – nearest heme N	3.3 Å	n.m.
CH <sub>3</sub> – nearest heme C	3.4 Å	n.m.
CH <sub>3</sub> – closest C of Phe182	4.1 Å	2.8 Å
N – closest C of Phe182	n.m.	3.1 Å
CH <sub>3</sub> – closest C of Phe298	3.8	5.0 Å
CH <sub>3</sub> – closest C of Phe185	n.m.	3.2 Å



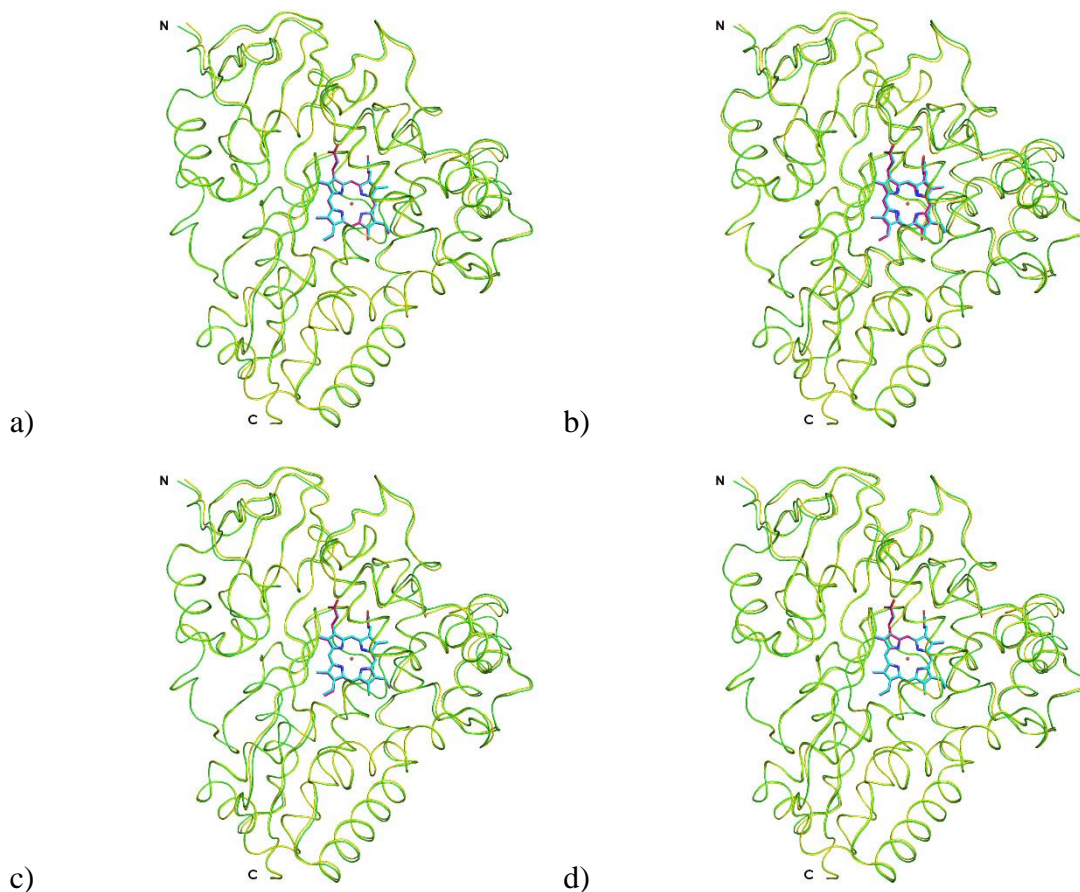
**Figure S7 (continued)** Docking studies with 3-methylaminobenzoic acid



**Figure S8 (a)** Chiral HPLC data for 4-ethylbenzoic acid. Chiral HPLC analysis of the 4-ethylbenzoic acid alcohol product (methyl ester derivatised). Retention times of the two enantiomers are 20.92 (*S*) and 23.44 (*R*) min. (a) The CYP199A4 turnover of 4-ethylbenzoic acid (86:13 (*S*):(*R*)); (b) the racemic mixture of the alcohol product; (c) e turnover products spiked with the purified (*S*)-alcohol and (d) The turnover products spiked with the purified (*R*)-alcohol.



**Figure S8** (b) Chiral HPLC data for 4-ethylthiobenzoic acid (methyl ester derivatised); (a) HPLC analysis of methyl 4-(ethylthio)benzoate (substrate, RT 10.3 min); HPLC analysis of methyl 4-(ethylsulfinyl)benzoate (RT 29.0 and 31.5 min). The retention time of methyl 4-(ethylsulfinyl)benzoate was 35.6 min and (c) HPLC analysis of the CYP199A4 turnover of 4-(ethylthio)benzoate (91:9 ratio of the enantiomers).



**Figure S9** Superimposed structures of the C $\alpha$  chains of different CYP199A4 structures. The 4-methoxybenzoic acid-bound backbone is shown as green with the heme unit shown as blue in all; the overlaid structure is shown as yellow with a magenta heme unit. Shown are (a) 4-ethoxybenzoic acid, (b) 4-methylaminobenzoic acid, (c) 4-methylthiobenzoic acid and (d) 4-ethylthiobenzoic acid. The N and C terminus is indicated on each. All structures displayed r.m.s.d.  $<0.60$  Å.