Electronic Supporting Information

CYP199A4 catalyses the efficient demethylation and demethenylation of para-substituted benzoic acid derivatives

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Scheme S1 Detection of formaldehyde product by purpald: (a) Formation of formaldehyde by CYP199A4 with 4-methoxybenzoic acid and (b) reaction of formaldehyde with purpald (I) to form intermediate (II) which is oxidised to form purple formaldehyde-purpald adduct (III)

(a)

(b)
Figure S1 Spin state shift of CYP199A4 with the substrates (a) 2-methoxybenzoic acid (b) 3,5-dimethoxybenzoic acid (c) 2,5-dimethoxybenzoic acid (d) 2-hydroxy-4-methoxybenzoic acid (e) 3-hydroxy-4-methoxybenzoic acid, (f) 6-methoxynicotinic acid and (g) benzoic acid; red; substrate-bound CYP199A4, black; substrate-free CYP199A4.
Figure S2 Dissociation constant analysis of various substrates with CYP199A4 (a) 2,4-dimethoxybenzoic acid (b) 3,4-(methylenedioxy)benzoic acid (c) 3-hydroxy-4-methoxybenzoic acid, (d) 6-methoxynicotinic acid and (e) benzoic acid.
Figure S3 HPLC analysis of CYP199A4 substrate turnovers. Shown are (a) 2-methoxybenzoic acid (b) 2,5-dimethoxybenzoic acid (c) 3-methoxybenzoic acid (d) 2,4-dimethoxybenzoic, (e) 2-hydroxy-4-methoxybenzoic acid and (f) benzoic acid. A gradient, 20 – 95%, of acetonitrile (with trifluoroacetic acid, 0.1%) in water (TFA, 0.1%) was used and the absorbance was monitored at 254 nm with the exception of the benzoic acid turnover where the gradient used was 0 – 50% and the absorbance was monitored at 240 nm.

(a) 2-methoxybenzoic acid (turnover in black and substrate control in blue)

(b) 2,5-dimethoxybenzoic acid (turnover in black and substrate control in blue)
(c) 3-methoxybenzoic acid (turnover in black, substrate control in blue, 3-hydroxybenzoic acid control in red, 3-methoxy-4-hydroxybenzoic acid control in green). No product was observed via GC-MS analysis.

(d) 2,4-dimethoxybenzoic acid (turnover in black, substrate control in blue)
(e) 2-hydroxy-4-methoxybenzoic acid (turnover in black, substrate control in blue and 2,4-
dihydroxybenzoic acid in red).

(f) benzoic acid (turnover in black, substrate control in blue, 2-hydroxybenzoic acid control in red, 3-
hydroxybenzoic acid in magenta and 4-hydroxybenzoic acid control in green). No product was observed via GC-MS analysis.
Figure S4 Mass spec analysis of the BSTFA/TMSCI derivatised products from the CYP199A4 turnover with (a) 3,5-dimethoxybenzoic acid and (b) 2,4-dimethoxybenzoic acid.

(a) 3,5-dimethoxybenzoic acid product (3-hydroxy-5-methoxybenzoic acid)

(b) 2,4-dimethoxybenzoic acid product (2-methoxy-4-hydroxybenzoic acid)
**Figure S5** Structures of CYP199A4 with different substrates docked in the active site after energy minimisation. Two orientations are shown for substrates with substitutions at the 2- and 3-positions.

2,4-dimethoxybenzoic acid

![Image of structures with distances](image)

**Distances**

**2,4-dimethoxybenzoic acid – down orientation**

- Fe – 4-methoxy carbon 3.82
- Fe – 2-methoxy carbon 6.49
- 2-methoxy oxygen – S244-oxygen 4.09
- 2-methoxy carbon – closest heme carbon 2.52

**2,4-dimethoxybenzoic acid – up orientation**

- Fe – 4-methoxy carbon 4.02
- Fe – 2-methoxy carbon 9.28
- 2-methoxy carbon – F185 carbon 3.43
2-hydroxy-4-methoxybenzoic acid

Distances

2-hydroxy-4-methoxybenzoic acid – down orientation

Fe – 4-methoxy carbon 3.88
Fe – 2-hydroxy oxygen 6.35
2-hydroxy oxygen – S244 oxygen 4.77
2-hydroxy oxygen – closest heme carbon 3.05

2-hydroxy-4-methoxybenzoic acid – up orientation

Fe – 4-methoxy carbon 3.73
Fe – 2-hydroxy oxygen 8.41
2-hydroxy oxygen – S247 oxygen 3.94
2-hydroxy oxygen – S247 carbon 3.24
2-hydroxy oxygen – F185 carbon 3.98
3,4-(methylenedioxy)benzoic acid (See Fig. 6)

**Distances**

**3,4-(methylenedioxy)benzoic acid – up orientation**

Fe – 3,4-methylenedioxy carbon 5.71
3,4-methylenedioxy carbon – F182 carbon 2.71
3,4-methylenedioxy carbon – F185 carbon 4.04
3-oxygen – F182 carbon 3.2
3-oxygen – F185 carbon 3.92

**3,4-(methylenedioxy)benzoic acid – down orientation**

Fe – 3,4-methylenedioxy carbon 3.95
Fe – 3-oxygen 4.18
3-oxygen – closest heme carbon 3.2
4-oxygen – F182 carbon 3.21
4-oxygen – F298 carbon 3.87
4-oxygen – D251 nitrogen 8.21
6-indolecarboxylic acid

A comparison of the distances observed in the crystal structure of 6-indolecarboxylic acid-bound CYP199A4 (PDB: 4EGO) with those in the docking study with 3,4-(methylenedioxy)benzoic acid.

Fe – nitrogen 4.48
Fe – indole 1-carbon 4.08
Indole nitrogen – closest heme carbon 3.22
Indole 1-carbon – closest heme carbon 3.41
Indole 2-carbon – F182 carbon 3.54
Indole 2-carbon – F185 carbon 4.49
Indole 2-carbon – F298 carbon 3.50
3-hydroxy-4-methoxybenzoic acid

Distances

3-hydroxy-4-methoxybenzoic acid – down orientation

Fe – 4-methoxy carbon 3.93
Fe – 3-hydroxy oxygen 4.26
3-hydroxy oxygen – closest heme nitrogen 3.05
4-methoxy oxygen – F182 carbon 3.16
4-methoxy carbon – F182 carbon 3.82
4-methoxy carbon – F298 carbon 3.79

3-hydroxy-4-methoxybenzoic acid – up orientation

Fe – 4-methoxy carbon 3.86
Fe – 3-hydroxy oxygen 7.36
3-hydroxy oxygen – F182 carbon 3.25
3-hydroxy oxygen – F185 carbon 3.38
4-methoxybenzoic acid (PDB: 4DO1)
Distances for Figure 6 (a-b)

3,5-dimethoxybenzoic acid

Fe - 3-methoxy carbon 5.03
Fe - 3-methoxy oxygen 4.83
3-methoxy carbon – closest heme carbon 3.03
3-methoxy carbon – closest heme nitrogen 3.02
3-methoxy carbon – L98 carbon 2.78
Fe - 5-methoxy carbon 8.84
Fe - 5-methoxy oxygen 7.52
5-methoxy carbon – F182 carbon 2.88
5-methoxy oxygen – F182 carbon 2.9

3,4-dimethoxybenzoic acid (PDB: 4EGN)

Fe – 4-methoxy carbon 3.63
4-methoxy carbon – closest heme carbon 3.42
Fe – 4-methoxy oxygen 4.79
3-methoxy carbon – F182 carbon 2.92

Distances

6-methoxynicotinic acid – down orientation
Fe – 4-methoxy carbon 3.88
Fe – 6- nitrogen 5.17

6-methoxynicotinic acid – up orientation
Fe – 4-methoxy carbon 3.92
Fe – 6- nitrogen 6.73
**Figure S6** Calibration of formaldehyde levels using the purpald assay