Are branched chain fatty acids the natural substrate for $P450_{BM3}$?

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Supplementary Information

Enzymatic Turnover of Single Substrates with $P450_{BM3}$. Turnovers with all substrates were performed in triplicate using the following protocol: $P450_{BM3}$ (2 μM), fatty acid (500 μM) and catalase (1 μM) were combined in pH 7.4 Kpi Buffer (100 mM) to a final volume of 500 μL in a 1.5 mL Eppendorf vial. NADPH (300 μM) was added to the solution that was left to incubate for 1 hour at 37°C. Phenyl acetic acid (final concentration 50 μM) was then added as an internal standard. The turnover was applied to solid phase extraction (SPE) cartridges (Phenomenex, strata-X reverse phase absorbent, 30 mg/mL capacity) that had been washed with methanol (1 mL) and equilibrated with Millipore distilled water (1 mL). The sample was washed with Millipore distilled water (0.5 mL) before being dried under vacuum for 30 minutes. The sample was then eluted with diethyl ether (0.5 mL) and treated with a solution of ethereal diazomethane. Blank turnovers were performed under identical conditions with the exception of either NADPH or $P450_{BM3}$. Mixed substrate turnovers were performed in duplicate with a further set of experiments using a final NADPH concentration of 100 μM (relative processing and regiochemical composition were identical in both sets of experiments). The turnover mixtures were then analysed by GC/MS.

Compound Numbering.

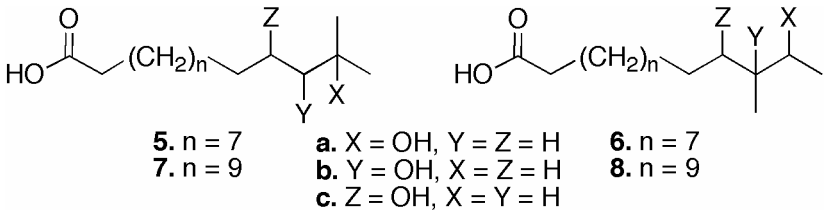


Figure 1. Hydroxylated fatty acid standards **5-8**.

GC Analysis of Single Substrate Turnovers. GC Program for turnover analysis of **1-4** and standard identification on a Varian Factor-4 VF-23ms column: Splitless mode; Column Flow 2.5 mL / minute; Total Flow 46.4 mL / minute; Injector 250°C; Detector 250°C; Oven 100°C (1.0 minutes equilibration) hold for 2.0 minutes, ramp 16°C / minute to 250°C and hold for 18.6 minutes (total program time 30.0 minutes). Retention times (methyl esters): **1** 6.75 minutes, **2** 6.78 minutes, **3** 7.82 minutes, **4** 7.87 minutes, **5a** 10.08 minutes, **5b** 10.35 minutes, **5c** 10.21 minutes, **6a** 10.49/10.52 minutes, **6b** 10.08 minutes, **6c** 10.29/10.33 minutes, **7a** 10.91 minutes, **7b** 11.20 minutes, **7c** 11.05 minutes, **8a** 11.32/11.35 minutes, **8b** 10.93 minutes, **8c** 11.13/11.17 minutes.

GC Analysis of Diasteromeric Composition of 6a-8a. GC Program for turnover analysis of **2** and **4** plus racemic **6a** and *erythro* **6a** standard identification on a J&W DB-WAX column: Split mode; Column Flow 1.8 mL / minute; Split Ratio 35; Total Flow 67.4 mL / minute; Injector 250°C; Detector 250°C; Oven 80°C (1.0 minutes equilibration) hold for 2.0 minutes, ramp 5°C / minute to 230°C and hold for 15.0 minutes (total program time 47.0 minutes). Retention times (methyl esters): *rac* **6a** 32.82 & 32.97 minutes, *erythro* **6a** 32.81 (major – 65%) & 32.96 (minor – 35%) minutes, **6a** from $P450_{BM3}$ turnover of **2** 32.81 (major ≥ 98%) & 32.97 (minor ≤ 2%) minutes, **8a** from $P450_{BM3}$ turnover of **4** 37.48 (major ≥ 98%) & 37.66 (minor ≤ 2%) minutes.

GC Analysis of Mixed Substrate Turnovers. GC Program for mixed C_{14}, **1** and **2** turnover analysis and standard identification on a Varian Factor-4 VF-23ms column: Splitless mode; Column Flow 3.3 mL / minute; Total Flow 46.4 mL/min; Injector 250°C; Detector 250°C; Oven 40°C (1.0 minutes equilibration) hold for 2.0 minutes, ramp 5°C / minute to 120°C and hold for 10.0 minutes, ramp 1°C / minute to 160°C and hold for 5.0 minutes, ramp 15°C / minute to 250°C and hold for 10.3 minutes (total program time 80.0 minutes). Retention times (methyl esters): C_{14} minutes, **1** minutes, **2** minutes, **C_{14}-OH_{11}** 67.18 minutes, **C_{14}-OH_{12}** 68.62 minutes, **C_{14}-OH_{13}** 70.48 minutes, **5a** 67.08 minutes, **5b** 72.05 minutes, **5c** 70.06 minutes, **6a** 75.90 minutes (single diastereomer), **6b** 67.65 minutes, **6c** 73.16 minutes (single diastereomer). Determination of the moles of product produced was by integration of the single ion peaks and comparison to the relative response, shown in the table below.

GC/MS Response Factors for Hydroxylated Standards:

GC Slope	**5a**	**5b**	**5c**	**6a**	**6b**	**6c**	**C_{14} ω-3**	**C_{14} ω-2**	**C_{14} ω-1**
TIC / Internal Std	0.225	0.100	0.105	0.088	0.137	0.093	0.162	0.137	0.126
Single Ion	214	197	183	228	211	183	183	197	214
SIM / Internal Std	0.0041	0.0058	0.0045	0.0012	0.0029	0.0042	0.0050	0.0031	0.0016
Rel. %	1.8%	5.8%	4.3%	1.4%	2.1%	4.5%	3.1%	2.3%	1.3%

[a] Relative GC/MS response for both total ion current (TIC) and single ion monitoring (SIM) for a 1:1 molar ratio of compound (methyl ester) to phenyl acetic acid (methyl ester). Response factors generated in relation to a standard curve for each compound against the internal standard.

C_{15} Branched Chain Fatty Acid Methyl Ester Fragmentation Patterns:

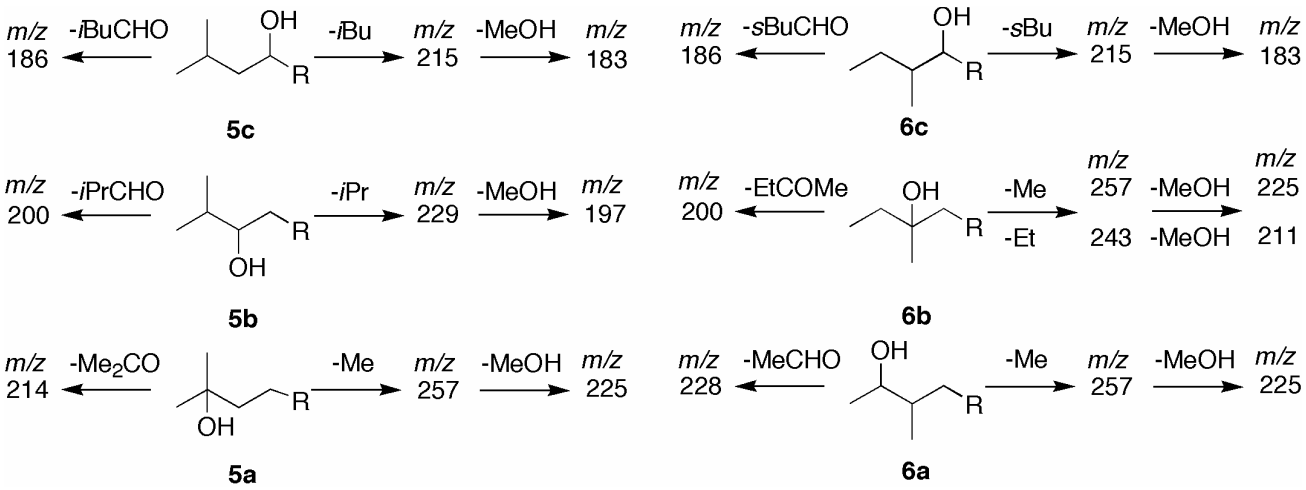


Figure 2. Mass spectral fragmentation loss patterns of **5a-5c** and **6a-6c** (R = $(CH_2)_9CO_2Me$).

Analysis of the Stereochemistry of the Products of Oxidation of 1 and 3 by $P450_{BM3}$.

Isolation of hydroxyfatty acids from turnover. Crude extracts were purified by preparative TLC (plates conditioned in 10% MeOH in $CHCl_3$) using 10% ethyl acetate in hexanes.

Benzoylation of hydroxyfatty acids. To a solution of hydroxyfatty acid (1 mg) in CH_2Cl_2 (0.2 mL) stirring at room temperature under nitrogen was added sequentially pyridine (5 mg) and benzoyl chloride (5 mg). After 3 h the reaction was concentrated *in vacuo* and the crude product purified by preparative TLC (10% ethyl acetate in hexanes) to yield the benzoylated hydroxyfatty acid which was then analyzed by enantiomeric HPLC.

HPLC Program for turnover analysis and standard identification of 5b. Column (Chiralcel OD) Flow 1.0 mL / minute; 0.25% isopropanol in hexanes; PDA detector (230 nm).

Retention times (Benzoylated standards):

Compound	1st Enantiomer	2nd Enantiomer
Racemic Synthesized **5b**	18.18 minutes	30.97 minutes
$P450_{BM3}$ Product **5b**	18.24 minutes	N/D
Coinjection	18.22 minutes	30.95 minutes

HPLC Program for turnover analysis and standard identification of 7b. Column (Chiralcel OD) Flow 1.0 mL / minute; 0.10% isopropanol in hexanes; PDA detector (230 nm).

Retention times (Benzoylated standards):

Compound	1st Enantiomer	2nd Enantiomer
Racemic Synthesized **7b**	25.92 minutes	32.35 minutes
$P450_{BM3}$ Product **7b**	25.96 minutes	N/D
Coinjection	25.92 minutes	32.37 minutes

Postulated Stereochemistry of Products. To date we have analysed the stereochemistry of eight benzoylated hydroxyfatty acids using enantiomeric HPLC (Chiralcel OD column), with the *R*-isomer eluting first in all cases. Based upon this trend, and the fact that the major isomer is the first to elute for the $P450_{BM3}$ oxidised products of both **1** and **3**, the stereochemistry of hydroxylation of **1** and **3** by $P450_{BM3}$ appears to be highly selective for the formation of the *R*-isomer.[1]

Enantiomeric HPLC Retention Profile for Synthesised Hydroxy Fatty Acids (Benzoylated):

Compound (Benzoylated)	1st Eluting Isomer	Compound (Benzoylated)	1st Eluting Isomer
Methyl 11-hydroxytetradecanoate	*R*	Methyl 12-hydroxyhexadecanoate	*R*
Methyl 12-hydroxytetradecanoate	*R*	Methyl 13-hydroxyhexadecanoate	*R*
Methyl 13-hydroxytetradecanoate	*R*	Methyl 14-hydroxyhexadecanoate	*R*
Methyl 11-hydroxyhexadecanoate	*R*	Methyl 15-hydroxyhexadecanoate	*R*

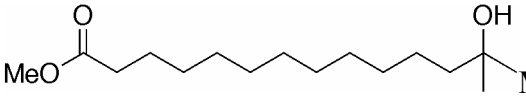
[a] All samples analysed on a Chiralcel OD column using the following conditions: Hydroxylated C_{14} fatty acids: Flow 1.0 mL / minute; 0.25% isopropanol in hexanes; PDA detector (230 nm); Hydroxylated C_{16} fatty acids: Flow 1.0 mL / minute; 0.10% isopropanol in hexanes; PDA detector (230 nm).

Determination of the Binding Constant of 1-4, C_{14} and C_{16} Acids with $P450_{BM3}$. Binding constants were determined by observing the absorption difference between high and low spin forms of the enzyme (417 and 392 nm) with increasing concentrations of substrate. Two identical solutions (500 μL of 5 μM $P450_{BM3}$, 100 mM potassium phosphate buffer pH 7.4) were placed in a dual beam UV/Visible spectrometer. In order to observe substrate binding, 1 μL aliquots of 0.05 mM substrate (later aliquots switched concentration to 1 mM) in water was added to the front solution, with 1 μL of pure water added to the rear solution to account for dilution. The spectrum was then acquired, with the difference between the absorbance at 417 and 392 nm recorded. Further additions were performed, with the total change in solution volume never exceeding 5% of the original volume. A graph was then plotted of absorption change versus concentration of substrate added, with the

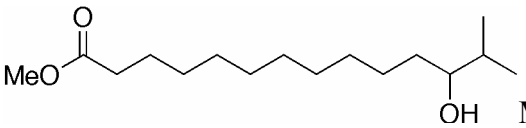
binding constant determined by applying the relevant curve fitting equation. Duplicate binding constant determinations were prepared for all substrates.

Determination of the Initial Rate of Oxidation of 1-4, C_{14} and C_{16} Acids by $P450_{BM3}$. The initial rate of reaction was measured by observing the disappearance of NADPH (extinction coefficient at 340 nm, $\varepsilon = 6.22\ cm^{-1}\ mM^{-1}$) and calculating the slope of the first 3 seconds of the reaction. Two identical solutions (450 μL of 2 μM $P450_{BM3}$, 100 mM potassium phosphate buffer pH 7.4, 500 μM substrate, 1 μM catalase) were placed in a dual beam UV/Visible spectrometer. 50 μL of pure water was added to the rear solution and mixed, followed by addition of 50 μL of NADPH solution (concentration such that the final solution concentration was 300 μM) to the front solution. This was quickly mixed and the absorption change at 340 nm followed over 30 seconds. Triplicate experiments were performed for all probes, with an extra experiment each for C_{16}, **2** and **3**.

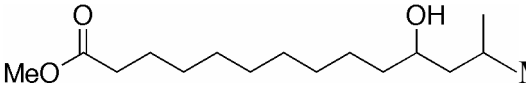
Compound Characterisation of 5a-5c, 6a-6c and 7b.



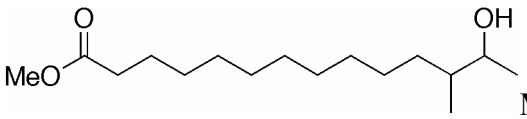
Methyl 13-hydroxy-13-methyltetradecanoate (5a). Colourless oil. ^{1}H NMR (400MHz, $CDCl_3$) δ 1.20-1.35 (23H, m), 1.57 (4H, m), 2.27 (2H, t, J_1 = 7.5 Hz), 3.63 (3H, s). ^{13}C NMR (100MHz, $CDCl_3$) δ 23.3, 24.8, 25.7 (2C), 29.0, 29.10, 29.28, 29.41, 29.45, 29.49, 30.1, 34.1, 39.2, 51.3, 68.1, 174.5. GC/MS: 225 (10.9), 214 (10.4), 87 (39.8), 74 (31.8), 59 (100), 55 (34.2), 43 (68.6), 41 (46.4). Anal. Calcd. For $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found C, 70.69; H, 11.99.



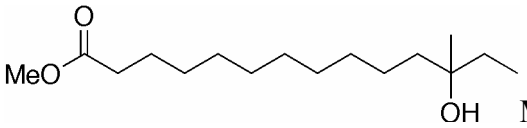
Methyl 12-hydroxy-13-methyltetradecanoate (5b). Colourless oil. ^{1}H NMR (400MHz, $CDCl_3$) δ 0.87 (6H, 2 d, J_1 = 4.9 Hz), 1.20 (18H, m), 1.58 (3H, m), 2.25 (2H, t, J_1 = 7.6 Hz), 3.62 (3H, s). ^{13}C NMR (100MHz, $CDCl_3$) δ 16.9 (2C), 18.7, 24.6, 24.8, 25.9, 28.95, 28.96, 29.0, 29.25, 29.37, 29.44, 29.6, 33.3, 34.0, 51.3, 76.9, 174.3. GC/MS: 229 (16.9), 197 (66.3), 95 (43.5), 74 (31.6), 69 (47.5), 55 (93.9), 43 (100), 41 (94.6). Anal. Calcd. For $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found C, 70.41; H, 12.08.



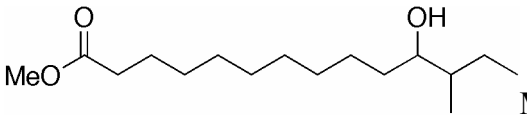
Methyl 11-hydroxy-13-methyltetradecanoate (5c). Colourless oil. ^{1}H NMR (400MHz, $CDCl_3$) δ 0.89 (6H, 2 d, J_1 = 5.16 Hz), 1.26 (14H, m), 1.40 (2H, m), 1.59 (2H, m), 1.74 (1H, m), 2.24 (1H, br s), 2.28 (2H, t, J_1 = 7.56), 3.64 (3H, s), 3.65 (1H, m). ^{13}C NMR (100MHz, $CDCl_3$) δ 21.9 (2C), 23.4, 24.5, 24.8, 25.5, 29.0, 29.2, 29.4, 29.5, 34.0, 37.9, 46.7, 51.3, 69.9, 174.2. GC/MS: 215 (13.9), 183 (48.4), 87 (79.8), 74 (49.6), 69 (75.3), 55 (67.9), 43 (100), 41 (98.8). Anal. Calcd. For $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found C, 70.29; H, 12.02.



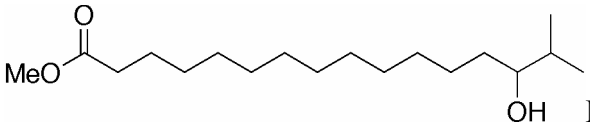
Methyl 12-methyl-13-hydroxytetradecanoate (6a). Colourless oil, two diastereomers. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.83 (1.5H, d, J_1 = 6.9 Hz), 0.85 (1.5H, d, J_1 = 6.9 Hz), 1.09 (1.5H, d, J_1 = 6.3 Hz), 1.12 (1.5H, d, J_1 = 6.3 Hz), 1.14-1.40 (15H, m), 1.49 (1H, br s), 1.59 (2H, m), 2.27 (2H, t, J_1 = 7.6 Hz), 3.61 (3H, s), 3.60-3.70 (1H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 14.5, 19.3, 20.3, 24.9, 27.2, 27.3, 29.1, 29.2, 29.4, 29.5, 29.6, 29.9, 32.5, 32.6, 39.8, 40.0, 51.4, 71.4, 71.7, 174.3. GC/MS: 228 (26.7), 199 (7.2), 185 (17.8), 143 (29.8), 129 (13.7), 101 (10.3), 87 (100), 74 (78.4), 55 (80.6), 45 (84.2). Anal. Calcd. For $C_{16}H_{30}O_3$: C, 70.54; H, 11.84. Found C, 70.48; H, 11.98.



Methyl 12-hydroxy-12-methyltetradecanoate (6b). Colourless oil. ^{1}H NMR (400MHz, $CDCl_3$) δ 0.86 (3H, t, J_1 = 7.5 Hz), 1.12 (3H, s), 1.20-1.35 (17H, m), 1.45-1.65 (4H, m), 2.28 (2H, t, J_1 = 7.4 Hz), 3.65 (3H, s). ^{13}C NMR (100MHz, $CDCl_3$) δ. 8.2, 23.8, 24.9, 26.4, 29.1, 29.2, 29.4, 29.5, 29.6. 30.2. 34.1, 34.2, 41.3, 51.4, 72.9, 174.3. GC/MS: 225 (6.2), 211 (27.9), 200 (6.5), 87 (35.6), 74 (25.1), 73 (92.7), 55 (59.7), 43 (100). Anal. Calcd. For $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found C, 70.59; H, 12.06.



Methyl 11-hydroxy-12-methyltetradecanoate (6c). Colourless oil. ^{1}H NMR (400MHz, $CDCl_3$) δ 0.83-0.91 (6H, 2 t (J_1 = 7.2 Hz) and 2 d (J_1 = 6.2 Hz)), 1.23-1.61 (19H, m), 2.28 (2H, t, J_1 = 7.5 Hz), 3.46 (2H, m), 3.64 (3H, s). ^{13}C NMR (100MHz, $CDCl_3$) δ 24.6, 24.9, 26.0, 26.2, 29.1, 29.2, 29.4, 29.5, 29.68, 29.70, 33.4, 34.5, 40.5, 51.4, 74.9, 174.4. GC/MS: 215 (19.3), 183 (72.5), 95 (28.0), 81 (38.8), 74 (23.8), 69 (42.5), 55 (69.0), 41 (100). Anal. Calcd. For $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found C, 70.40; H, 11.90.



Methyl 14-hydroxy-15-methylhexadecanoate (7b). White solid (Melting Point 35.0-36.0ºC). ^{1}H NMR (500MHz, $CDCl_3$) δ 0.89 (6H, t, J_1 = 6.5 Hz), 1.15-1.40 (21H, m), 1.52-1.59 (3H, m), 2.28 (2H, t, J_1 = 7.5 Hz), 3.33 (1H, m), 3.64 (3H, s). ^{13}C NMR (125MHz, $CDCl_3$) δ 17.0, 18.9, 25.0, 26.0, 29.1, 29.2, 29.4, 29.55, 29.58, 29.62, 29.7, 33.6, 34.1, 34.2, 51.4, 67.5, 174.3. GC/MS: 257 (8.1), 225 (21.2), 207 (10.4), 189 (11.5), 123 (9.2), 95 (34.9), 74 (32.3), 69 (43.2), 55 (100), 43 (73.2). Anal. Calcd. For $C_{18}H_{36}O_3$: C, 71.95; H, 12.08. Found C, 72.05; H, 12.28.

(1) Cryle, M. J.; Matovic, N. J.; De Voss, J. J. *Organic Letters* **2003**, *5*, 3341-3344.