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Supporting Information

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Supporting Information

for

A Highly Active Single-Mutation Variant of P450Bm3 (CYP102A1)

Christopher J. C. Whitehouse, Stephen G. Bell, Wen Yang, Jake A. Yorke, Christopher F. Blanford, Anthony J. F. Strong, Edward J. Morse, Mark Bartlam, Zihe Rao and Luet-Lok Wong*

General, materials and equipment: General reagents and chemicals of an-alytical grade or higher quality were from Alfa-Aesar, Amresco, Fisher Sci-entific, Sigma-Aldrich and VWR International. Solvents of HPLC quality were from Rathburn Chemicals (UK), Sigma-Aldrich and Merck. NADPH (tetrasodi-um salt) was from Apollo Scientific. Horseradish peroxidase and glucose oxi-dase were from Sigma-Aldrich. Gold mesh was from Internet, Minneapolis, USA, and gold wire from Advent Research Materials, Eynsham, UK. Compe-tent *E. coli* strains were from Stratagene. Oligonucleotides were from Eurofins Genetic Services, UK. UV/visible spectra, in vitro assays and spectroelectro-chemical titrations were run on a Varian Cary 50 spectrophotometer. Poten-tials were fixed and currents recorded using a CompactStat (Ivium, Eindhoven, Netherlands). GC analysis was carried out on a Thermo Finnigan Trace instrument equipped with a 7 m DB-1 fused silica capillary column and a Thermo Finnigan 8000 Top instrument equipped with a 60 m SPB-1 column, with helium as the carrier gas. Injector temperatures were maintained at 200 °C and flame-ionization detectors (FIDs) at 250 °C.

DNA manipulations, protein expression and purification: Mutation I401P was introduced into the $P450_{BM3}$ gene in pET28 using the mutagenic primer 5′-GGAAACGGTCAGCGTGCGTGTCCCGGTCAGCAGTTCGCTCTTC-3′ and its reverse complement. The Stratagene Quik-Change kit protocol was followed, with transformation into *E. coli* XL1 Blue Supercompetent cells. To pre-pare the I401P haem domain, the I401P gene was subjected to PCR with the primers 5′-CCACCCATGGCAATTAAAG-

AAATGCCTCAGCC-3′ (NcoI site[1] underlined) and 5′-TGGATCCACTAGTTACGG-CGTATTATGAGCGTTTTC-3′ (SpeI site, incorporating stop codon,[2] underlined). Amplification was by 30 cycles of strand separation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 68 °C for 105 s + 2 s per cycle, concluding with a 74 °C soak (10 min). After purification with the QIAquick kit and digestion with NcoI and SpeI, the amplified gene fragment was incorporated into pET28 using NcoI and SpeI. Amplified genes were sequenced by Geneservice, UK on an ABI 3730-XL Prism DNA sequencer, and transformed into *E. coli* JM109(DE3) for protein expres-sion. Growth, expression, lysis and purification were as described.[2] For purification of the haem domain, 50 mM Tris buffer, pH 7.4 (Tris) was used on a Sephadex G-25 column, a linear gradient of 100-300 mM KCl in Tris on a fast-flow Sepharose column and a linear gradient of 50-200 mM KCl in Tris on a Source-Q column.

In vitro oxidation assays, kinetic titrations and peroxide assays: NADPH turn-overs were carried out at 30±0.1 °C in 1250 µL of oxygenated 50 mM Tris, pH 7.4 containing 0.1 µM enzyme (0.25 µM for WT with non-natural substrates to ensure complete NADPH consumption), 125 µg bovine liver catalase and 1 mM substrate (added as a 100 mM stock in DMSO). P450 enzyme concentra-tions were determined via reduced CO-difference spectra.[3] Assays were held at 30 °C for 1 min prior to NADPH addition as a 20 mg mL^{-1} stock to a final concentration equivalent to 2 AU (~320 µM). The NADPH consumption rate was derived using ε_{340} = 6.22 mM^{-1} cm^{-1}. No correction was made for the leak rates recorded using DMSO in place of sub-strate. All data are means of at least three experiments with standard deviations less than 5% of the mean. Peroxide uncoupling was assessed as described.[4] Kinetic ti-trations were run at 0.1 µM enzyme concentration with substrate concentrations rang-ing from 5 to 1250 µM, the volume of DMSO being held constant at 12.5 µL. K_M and k_{cat} values were derived by fitting initial NADPH consumption rates against substrate concentration to a hyperbolic function using Origin 8 software from Origin Labs.

Spectroelectrochemical titrations: Redox titrations were carried out in a custom-built optically transparent thin-layer electrode (OTTLE) cell construct-ed from a quartz cuvette of 0.1 cm path length. The working electrode was a gold mesh (500 wires per in, wire width 4.5 × 10^{-4} in, 60% transmission) at-tached to gold wire (0.2 mm diame-ter) with cyanoacrylate adhesive and insu-lated with paraffin film. A PMMA solution well was attached to the top of the cuvette by silicone sealant. A saturated calomel reference electrode was con-nected to the cell via a glass Luggin capillary placed in-

to the protein solution. The counter electrode was a platinum wire in solution. Anaerobic conditions were maintained by purging the system continually with argon that had first been sparged through ~10 cm of room-temperature water to hydrate the gas and keep cell solution levels constant and component concentrations steady. The cell was held at 25.0±0.1 ℃. A typical sample volume wa s 1300 µL, comprising ca. 30 µM protein, 5 µM methylene blue, 5 µM anthraquinone-1,5-disulfonate, 5 µM anthraquinone-2,6-disulphonate, 5 µM anthraquinone-2-sul-fonate, 5 µM methyl viologen, 10 mM glucose, 0.1 mg mL^{-1} glucose oxidase, 0.2 mg mL^{-1} catalase and 200 mM NaCl in 50 mM Tris, pH 7.4. Glucose, glucose oxidase and catalase were included to convert residual oxygen to water. Sample mixing and cell assembly took place in a glove box. Spectra were acquired for at least 80 min to allow equilibration. Longer periods produced no further changes to the spectrum within the spectrometer's sensitivity. Data were corrected for baseline drifts by a 0^{th} order adjustment based on the average optical density from 775–800 nm. Because the absorbance of the peaks in the Soret band was convoluted by contributions from methyl viologen (λ_{max} = 395 nm), the absorbances at 450, 465 and 570 nm were used for data analysis. Data were fitted to a sigmoid derived from the Nernst equation:

$$A(E) = \frac{A(E_{\max}) - A(E_{\min})}{1 + \exp[nF(E_m - E_{appl})/RT]} + A(E_{\min}) \qquad (1)$$

where $A(E)$ is the absorbance for a given potential, $A(E_{max})$ and $A(E_{min})$ are the optical densities for the fully oxidized and fully reduced chromophore, E_m and E_{appl} are the midpoint and applied potentials, R the gas constant, T the cell temperature, n the number of electrons transferred for the redox couple, and F Faraday's constant. The values of $A(E_{max})$, $A(E_{min})$ and E_m were determined by minimising the square of the difference between the measured value and the predicted value, assuming equal weighting of the data. The value of n was fixed to 1.

Derivatisation: The products from lauric acid turnovers were derivatised prior to analysis. 10 µL of decanoic acid (25 mM in ethanol) was added as internal standard to 990 µL of each completed turnover, together with 2 µL of concentrated HCl. The mixture was extracted three times into 400 µL of ethyl acetate, and the organic phases

were combined, dried over $MgSO_4$ and evaporated to dryness under a stream of dinitrogen. The sample was dissolved in 200 µL anhydrous acetonitrile and allowed to react with excess (25 µL) *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA + TMCS, 99:1) for at least 2 h to convert any carboxylic acid groups and alcohols present to the corresponding trimethylsilyl ethers. The resulting mixtures were subjected directly to gas chromatography (GC) analysis.

Product analysis: For substrates other than lauric acid, 3 µL of internal standard (100 mM in DMSO) was added to 1000 µL of each completed turnover prior to extraction into 400 µL of an organic phase prior to centrifugation at 19 000 *g* for 3½ min. For 3-methylpentane, the internal standard was 2-octanol, the organic phase was chloroform, the SPB-1 column was employed, and the oven temperature was raised from 70 °C to 90 °C at 1 °C min^{-1} and then from 90 °C to 220 °C at 65 °C min^{-1}. For the remaining substrates, the DB-1 column was employed. For lauric acid, the oven temperature was held at 100 °C for 1 min and then raised at 15 °C min^{-1} to 220 °C. For other substrates, it was held at 60 °C for 1 min and then raised at 15 °C min^{-1} to 150 °C, with 4-benzylphenol as the internal standard and ethyl acetate as the organic phase. Products from lauric acid turnovers were confirmed using GC-MS (Figures S5 – S9). Products from other substrates were identified by matching the GC elution times observed to those of authentic product samples. FID responses were calibrated byusing at least one representative product for each substrate on the assumption that isomeric products would give comparable responses. Samples containing a range of calibrant concentrations in 50 mM Tris, pH 7.4 and 1% *v/v* in DMSO were extracted as above. Integrated peak areas were expressed as ratios of internal standard peak areas and plotted against product concentration to derive calibration factors.

Table S1. In vitro oxidation activity and selectivity of wild-type $P450_{BM3}$ (WT) and variant I401P with lauric acid.

	N	C (%)	PFR	% *ω*-1	% *ω*-2	% *ω*-3	% *ω*-4
WT	2777	52	1439	33.5	29	37	0.5
I401P	3812	53	2012	36	30.5	33	0.5

N = NADPH turnover rate, C = coupling, PFR = product formation rate. Rates in nmol min^{-1} $(nmol\text{-}P450)^{-1}$. Products were 11-hydroxylauric acid (*ω*-1, 7.12 min), 10-hydroxylauric acid (*ω*-2, 7.03 min), 9-hydroxylauric acid (*ω*-3, 6.88 min) and 8-hydroxylauric acid (*ω*-4, 6.78 min).

Table S2. In vitro oxidation activity and selectivity of wild-type $P450_{BM3}$ (WT) and variant I401P with propylbenzene.

	N	C (%)	PFR	% 1-ol	% others
WT	894	71	635	98.5	1.5
I401P	4476	80	3578	98	2

N = NADPH turnover rate, C = coupling, PFR = product formation rate. Rates in nmol min^{-1} (nmol-P450)$^{-1}$. The principal product was 1-phenyl-1-propanol (3.85 min). The NADPH rate for WT exceeds that previously published by 28 min^{-1}[2] as leak rates were not subtracted in this study.

Table S3. In vitro oxidation activity and selectivity of wild-type $P450_{BM3}$ (WT) and variant I401P with 3-methylpentane.

	N	C (%)	PFR	% 3-ol	% 2-ol (A)	% 2-ol (B)
WT	126	22	28	12	65	23
I401P	2763	50	1378	15	63	22

N = NADPH turnover rate, PFR = product formation rate, both in nmol min^{-1} (nmol-P450)$^{-1}$. C = coupling. Products were 3-methyl-3-pentanol (3-ol, 11.81 min) and 3-methyl-2-pentanol (2-ol, 2 diastereomers, 14.12 min (A) and 14.32 min (B)).

Table S4. In vitro oxidation activity and selectivity of wild-type $P450_{BM3}$ (WT) and variant I401P with (+)-α-pinene.

	N	C (%)	PFR	% 2,3-epox	% 4-ol	% 10-ol	% 4-one	% others
WT	41	< 0.1	< 0.05	–	–	–	–	–
I401P	1229	19	238	19	73.5	2	0.5	5

N = NADPH turnover rate, C = coupling, PFR = product formation rate. Rates in nmol min^{-1} (nmol-P450)$^{-1}$. Products were (+)-α-pinene epoxide (2,3-epox, 2 isomers, 3.39 min and 3.46 min, 18% and 1% respectively), (+)-verbenol (4-ol, 2 isomers, 3.77 min and 3.81 min, 32% and 41.5% respectively), (+)-myrtenol (10-ol, 4.29 min) and verbenone (4-one, 4.41 min).

Table S5. In vitro oxidation activity and selectivity of wild-type $P450_{BM3}$ (WT) and variant I401P with fluorene.

	N	C (%)	PFR	% 9-one	% 9-ol
WT	7.9	0.9	0.1	29	71
I401P	1057	18	188	–	100

N = NADPH turnover rate, C = coupling, PFR = product formation rate. Rates in nmol min^{-1} (nmol-P450)$^{-1}$. Products were 9-fluorenone (9-one, 9.38 min) and 9-fluorenol (9-ol, 9.43 min).

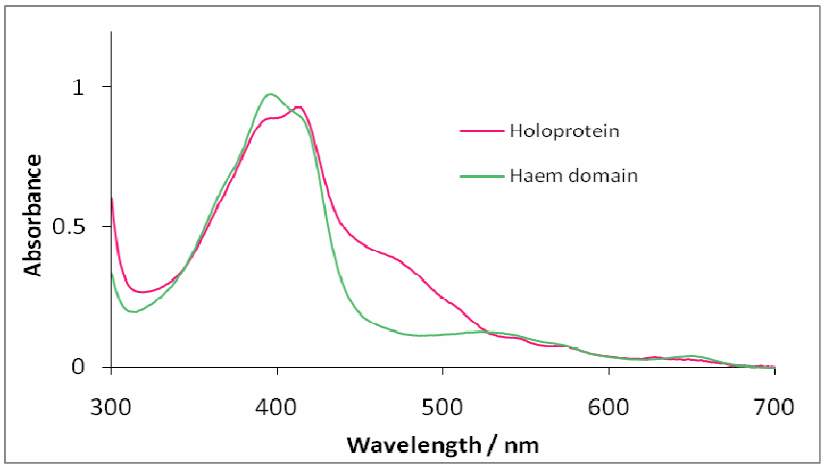

Figure S1. UV-visible absorption spectra for variant I401P and the corresponding haem domain. The low-spin peak is blue-shifted relative to WT at 414 nm while the α/β bands are red-shifted at 573/543 nm. The 650 nm absorbance band characteristic of Type I substrate-bound spectra is more clearly evident in the haem domain, which also has a greater high-spin haem content, this peak being red-shifted relative to substrate-bound WT at 395 nm.

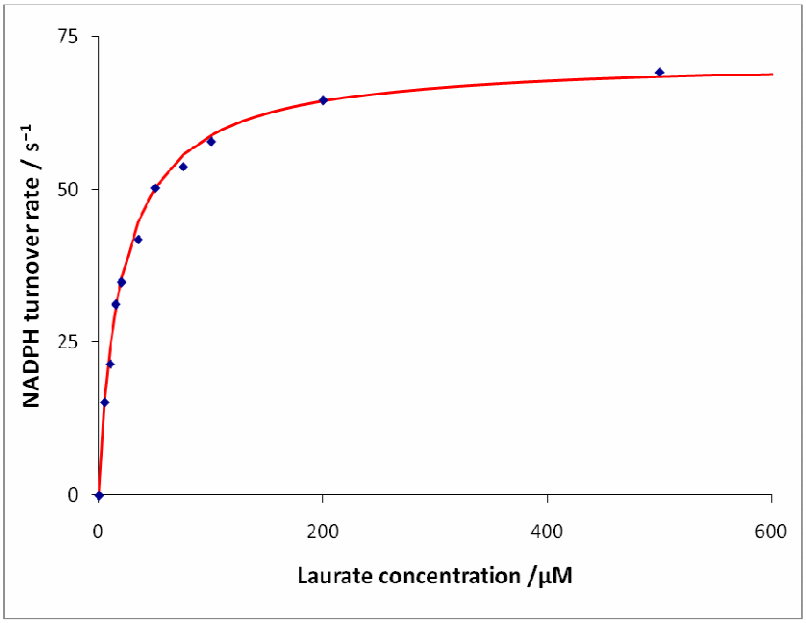

Figure S2a. Michaelis-Menten kinetics titrations for lauric acid oxidation by the I401P mutant in 50 mM Tris, pH 7.4 at 30 ℃. The enzyme concentration was 100 nM. The data were fitted to a hyperbolic function, with k_{cat} = 71 s^{-1}, K_M = 21 μM.

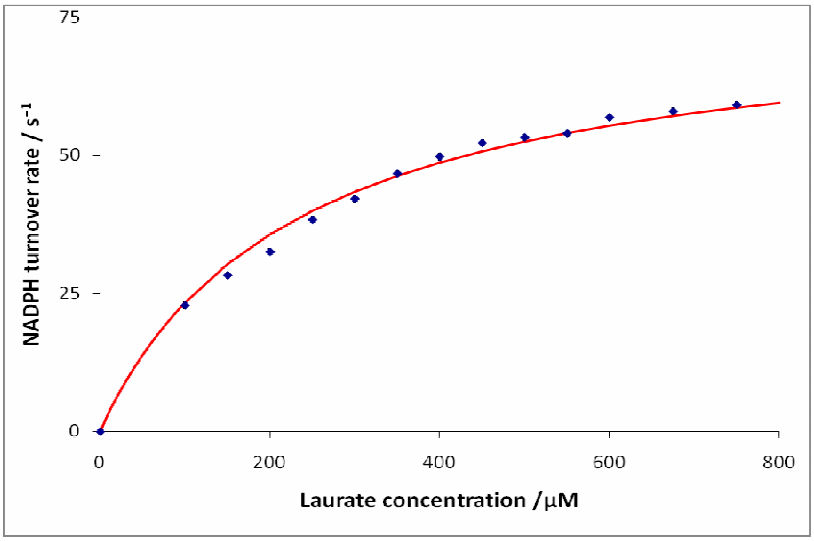

Figure S2b. Michaelis-Menten kinetics titrations for lauric acid oxidation by wild type $P450_{BM3}$ in 50 mM Tris, pH 7.4 at 30 ℃. The enzyme concentration was 100 nM. The data were fitted to a hyperbolic function, with k_{cat} = 77 s^{-1}, K_M = 230 μM.

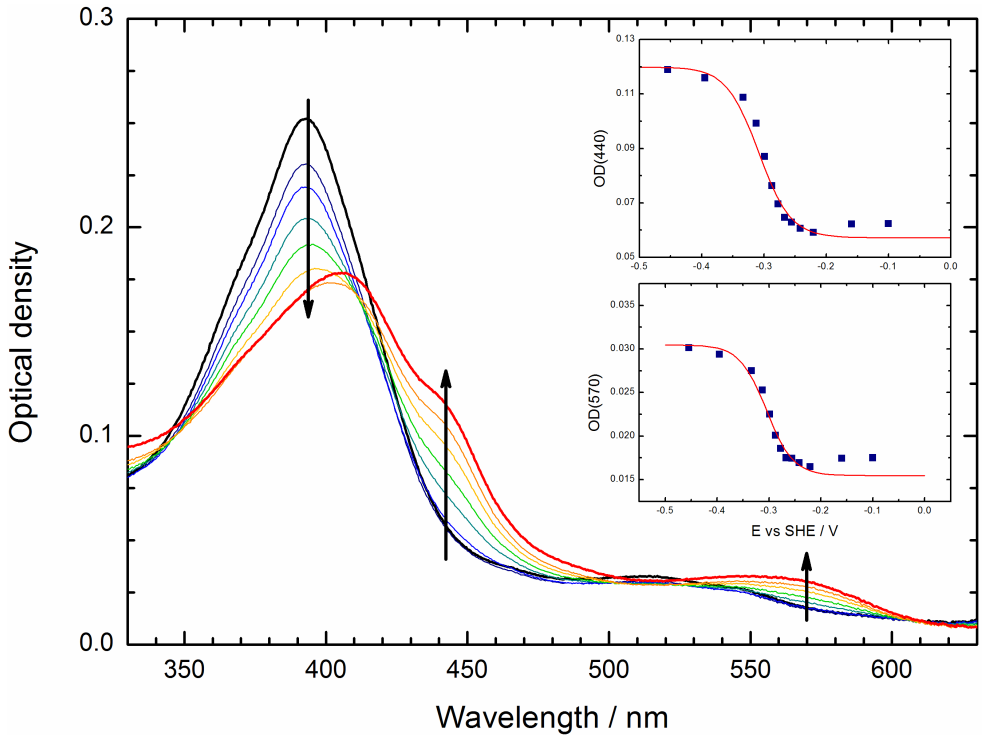

Figure S3. Potentiometric titration of the I401P haem domain.

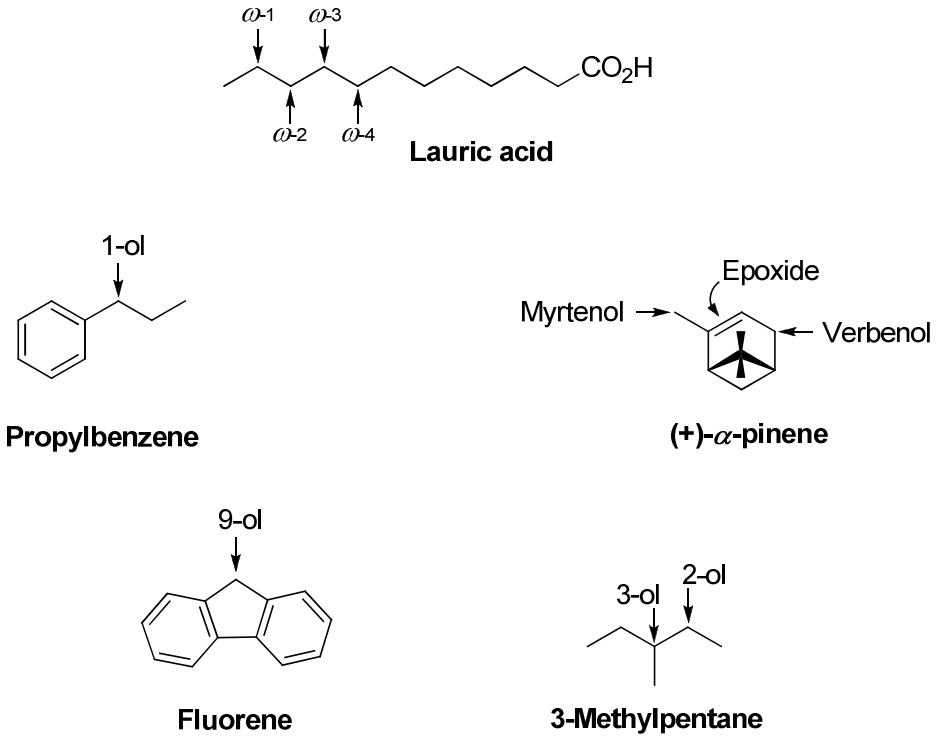

Figure S4. In vitro substrates and principal oxidation sites.

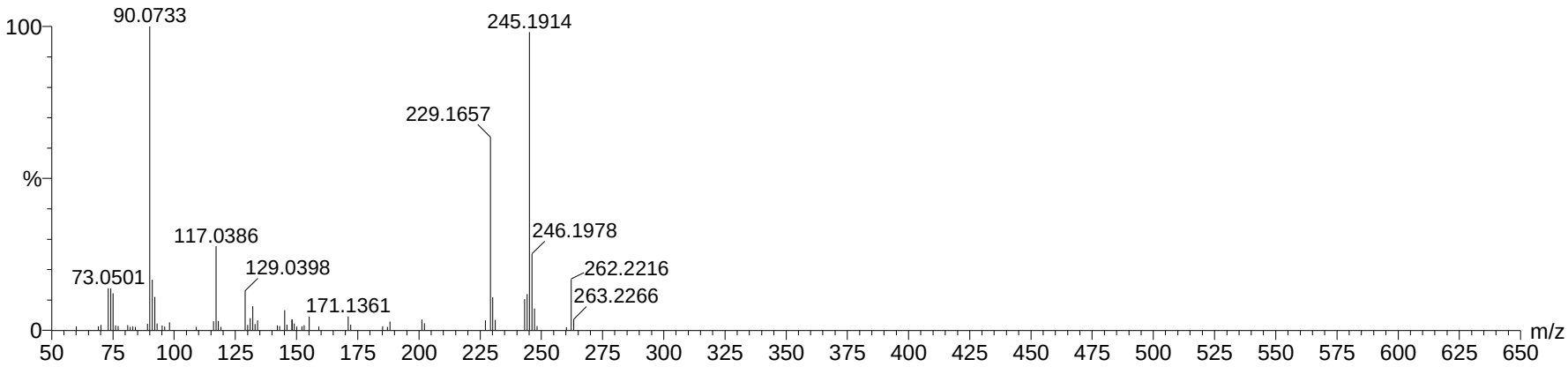

Figure S5. GC-MS analysis of trimethylsilyl derivatives of lauric acid turnovers by wild-type $P450_{BM3}$. Mass spectrum of the decanoic acid internal standard: calcd mass for $(M+H)^+$ $C_{13}H_{29}O_2Si$ 245.1937, observed mass 245.1914.

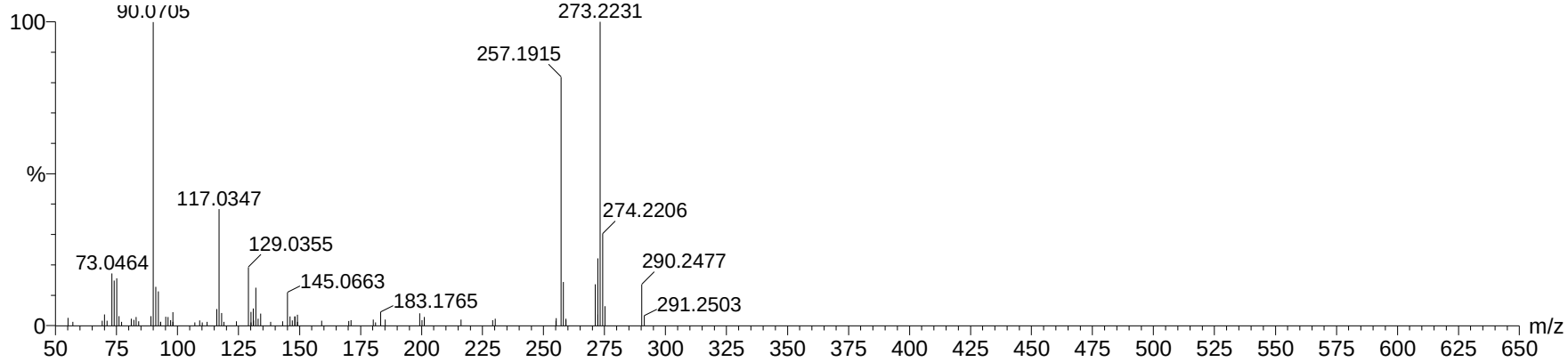

Figure S6. GC-MS analysis of trimethylsilyl derivatives of lauric acid turnovers by wild-type $P450_{BM3}$. Mass spectrum of the lauric acid substrate: calcd mass for $(M+H)^+$ $C_{15}H_{33}O_2Si$ 273.2250, observed mass 273.2231.

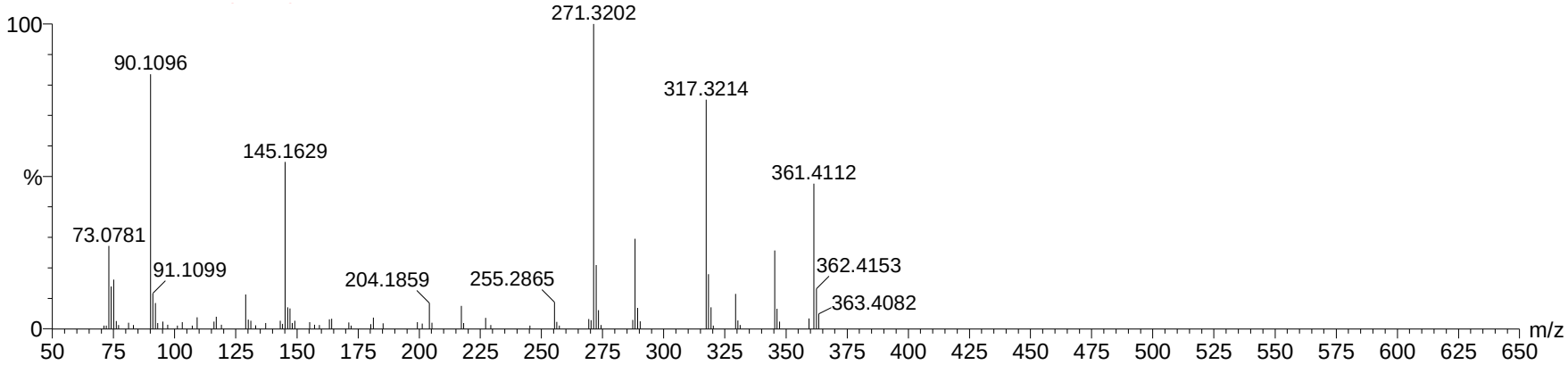

Figure S7. GC-MS analysis of trimethylsilyl derivatives of lauric acid turnovers by wild type $P450_{BM3}$. Mass spectrum of the 9-hydroxylauric acid (ω–3 hydroxylation) product: calculated mass for $(M+H)^+$ $C_{18}H_{41}O_3Si_2$, 361.2595, observed mass 361.4112. The peak at 317.3214 corresponds to loss of the ω, ω–1, ω–2 three-carbon unit as propane. The peak at 145.1629 corresponds to the $CH_3CH_2CH_2CHOSiMe_3$ fragment (calculated mass 145.1049).

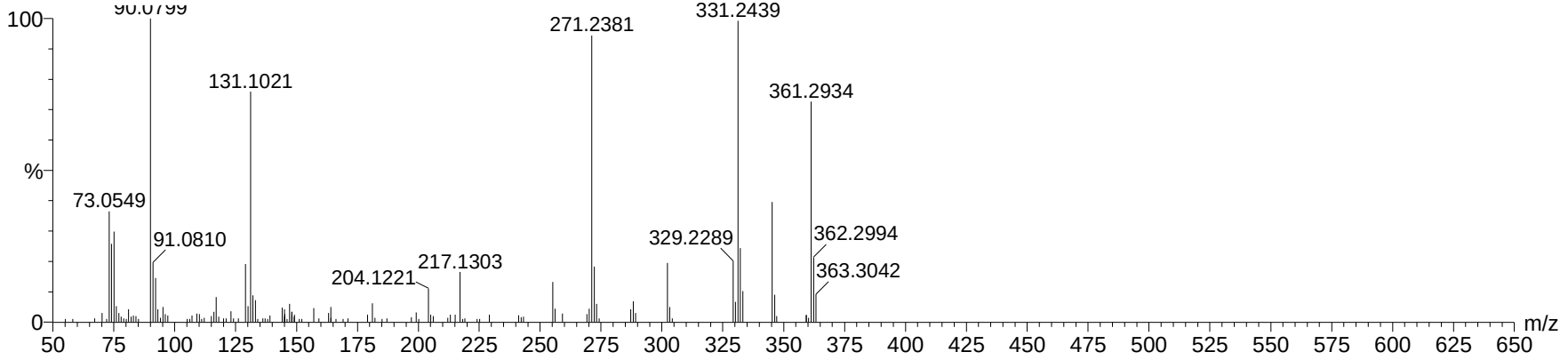

Figure S8. GC-MS analysis of trimethylsilyl derivatives of lauric acid turnovers by wild type $P450_{BM3}$. Mass spectrum of the 10-hydroxylauric acid (ω–2 hydroxylation) product: calculated mass for $(M+H)^+$ $C_{18}H_{41}O_3Si_2$, 361.2595, observed mass 361.2934. The peak at 331.2439 corresponds to loss of the ω, ω–1, two-carbon unit as ethane. The peak at 131.1021 corresponds to the $CH_3CH_2CHOSiMe_3$ fragment (calculated mass 131.0892).

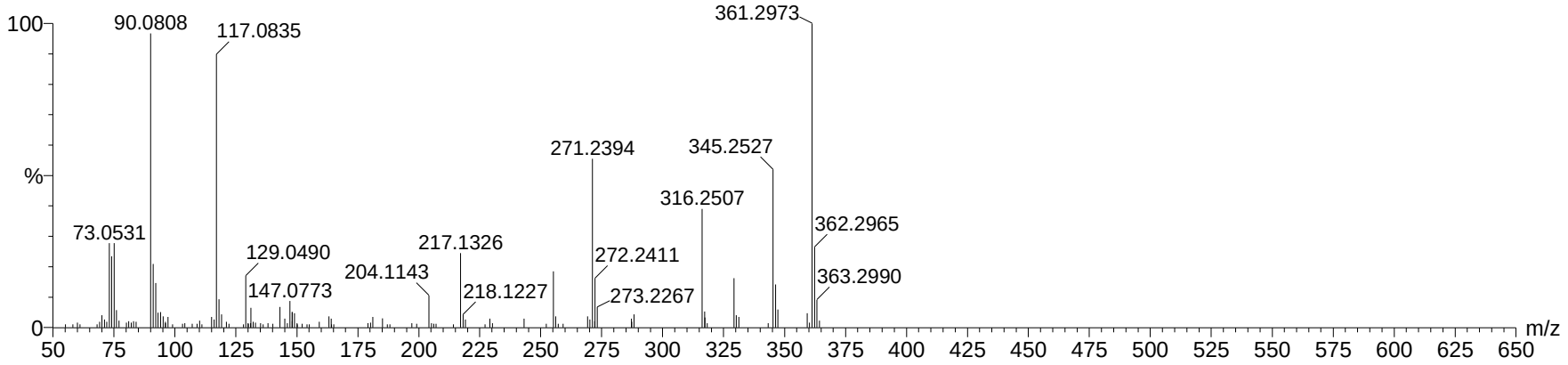

Figure S9. GC-MS analysis of trimethylsilyl derivatives of lauric acid turnovers by wild type $P450_{BM3}$. Mass spectrum of the 11-hydroxylauric acid (ω–1 hydroxylation) product: calculated mass for $(M+H)^+$ $C_{18}H_{41}O_3Si_2$, 361.2595, observed mass 361.2973. The peak at 345.2527 corresponds to loss of the ω carbon as methane. The peak at 117.0835 corresponds to the $CH_3CHOSiMe_3$ fragment (calculated mass 117.0736).

References

[1] A. B. Carmichael, L. L. Wong, *Eur J Biochem* **2001**, *268*, 3117-3125.

[2] C. J. Whitehouse, S. G. Bell, H. G. Tufton, R. J. Kenny, L. C. Ogilvie, L. L. Wong, *Chem Commun (Camb)* **2008**, 966-968.

[3] T. Omura, R. Sato, *J Biol Chem* **1964**, *239*, 2379-2385.

[4] F. Xu, S. G. Bell, Z. Rao, L. L. Wong, *Protein Eng Des Sel* **2007**, *20*, 473-480.