Supporting Information

Bacterial CYP153A monooxygenases for the synthesis of omega-hydroxy fatty acids

Sumire Honda Malca, Daniel Scheps, Lisa Kühnel, Elena Venegas-Venegas, Alexander Seifert, Bettina M. Nestl and Bernhard Hauer*

Institute of Technical Biochemistry, Universitaet Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
**E-mail bernhard.hauer@itb.uni-stuttgart.de*

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Materials and Methods

1 Chemicals, enzymes, vectors and strains

Solvents and buffer components were obtained from Sigma-Aldrich (Schnelldorf, Germany). Saturated and monounsaturated fatty acids were purchased from Sigma-Aldrich or Nu-Check Prep, Inc. (Minnesota, USA). 12-Hydroxydodecanoic acid, 16-hydroxyhexadecanoic acid and 1,12-dodecanedioic acid were purchased from Sigma-Aldrich. *Pfu* DNA polymerase, endonucleases, T4 DNA ligase and isopropyl β-*D*-thiogalactopyranoside (IPTG) were obtained from Fermentas (St. Leon-Rot, Germany). NADH disodium salt was purchased from Codexis (Jülich, Germany). Glucose-6-phosphate dehydrogenase (1000 U) from *Leuconostoc mesenteroides* was obtained from Roche Diagnostics (Mannheim, Germany). Plasmid pET-28a(+) and *E. coli* strain BL21(*DE3*) originated from Novagen (Madison, Wisconsin, USA). *E. coli* strain DH5α was purchased from Invitrogen (Darmstadt, Germany).

2 Cloning, expression and quantification of purified proteins

2.1 CYP153A enzymes

We previously cloned CYP153A (Bpro_5301) from *Polaromonas* sp. strain JS666 and CYP153A16 (MMAR_3154) from *Mycobacterium marinum* M. ATCC BAA-535 into vectors pET-28a(+) and pKK223-3, respectively.[1] CYP153A (Maqu_0600) from *Marinobacter aquaeolei* VT8 DSM 11845 (CYP153A *M. aq.*) was amplified by PCR with oligonucleotides 5'-ATT CAT ATG CAT CAT CAT CAT CAT CAT CCA ACA CTG CCC AGA A-3' (encoding a N-terminal His_6-tag sequence) and 5'- CGC AAG CTT TTA ACT GTT CGG TGT CAG TTT GAC-3' for its insertion into the *Nde*I and *Hind*III cloning sites of pET-28a(+). After digestion and ligation, the plasmid was used to transform competent *E. coli* DH5α cells. Successful cloning was verified by DNA sequencing (GATC-Biotech, Konstanz, Germany). Expression and purification of the His_6-tagged CYP153A enzymes were carried out as described elsewhere.[1] Concentrations of the P450 enzymes were determined by the carbon monoxide (CO) differential spectral assay described by Omura and Sato.[2,3]

2.2 Site-directed mutagenesis of CYP153A M. aq.

Plasmid pET28a(+) harbouring His_6-tagged CYP153A *M. aq.* was mutated using the QuikChange standard protocol. Variants G307A/V were created by PCR amplification with oligonucleotide 5'-GGT AAT TTG ACG CTG CTC ATA GTC GYG GGC AAC GAT ACG ACG CGC-3' and its complementary sequence 5'-GCG CGT CGT ATC GTT GCC CRC GAC TAT GAG CAG CGT CAA ATT ACC-3', which contained the degenerate codon GYG covering Ala (GCG) and Val (GTG). Variants L354I/F were generated by amplification with oligonucleotide 5'-G GTG TCG GAA ATC ATC CGC TGG CAA ACG CCG WTT GCC TAT ATG CGC CGA ATC GCC GCC AAG CAG

GAT GTC GAA CTG-3' and its complementary sequence 5'-CAG TTC GAC ATC CTG CTT GGC GGC GAT TCG GCG CAT ATA GGC AAW CGG CGT TTG CCA GCG GAT GAT TTC CGA CAC C-3'. These primers contained the degenerate codon WTT which coded for Ile (ATT) and Phe (TTT). Competent *E. coli* DH5α cells were transformed with the *Dpn*I-treated PCR mixtures. Isolated plasmids with the desired mutations (sequencing by GATC-Biotech, Konstanz, Germany) were used to transform competent *E. coli* BL21(DE3). Protein expression, purification and determination of P450 concentration were carried out as described in the previous section.

2.3 CamA and CamB

Putidaredoxin reductase (CamA) and putidaredoxin (CamB) from *Pseudomonas putida* ATCC 17453 have previously been shown to support the activity of CYP153A enzymes.[1,4] CamA and CamB were expressed and purified as described previously.[5] Protein concentrations were determined spectrophotometrically as described elsewhere.[6]

3 *In vitro* oxidation assays towards fatty acids

3.1 Reaction setup

The activity of each CYP153A enzyme was reconstituted with CamA and CamB and assayed *in vitro* using $C_{8:0}$-$C_{20:0}$ saturated and $C_{14:1}$-$C_{18:1}$ *n*-9 monounsaturated fatty acids as substrates. Biotransformations were performed using a final volume of 0.5 ml in 50 mM potassium phosphate buffer pH 7.5 containing 3 µM CYP153A, 15 µM CamA, 30 µM CamB and the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system (1 mM $MgCl_2$, 5 mM G6P and 12 U/ml G6PDH) for cofactor regeneration. For studying the activity, selectivity and substrate range of the different CYP153A enzymes, fatty acids were added in a final concentration of 0.2 mM (from a 10 mM stock solution in DMSO). The reaction was started by the addition of 0.2 mM NADH.

For reactions with wild-type and the four variants of CYP153A *M. aq.*, 1 mM fatty acids (from a 100 mM stock solution in DMSO), 1 mM NADH and cofactor regeneration were used. In all cases, samples were incubated at 30°C and 500 rpm for 4 h.

For the determination of the apparent kinetic constants, 0.75 – 1 µM CYP153A *M. aq* plus CamA and CamB (CYP-CamA-CamB in a 1:5:10 ratio), 2% DMSO, 1 mM NADH and cofactor regeneration were used. $C_{8:0}$, $C_{9:0}$ and $C_{14:0}$ fatty acids were added in concentrations not exceeding their water solubility limit. Samples were pre-incubated at 30°C for 2 minutes prior to addition of NADH. Reactions were run at 30°C and 500 rpm and stopped after reaction times within the constant rate range (steady-state).

3.2 Sample treatment

Conversion was stopped with 20 µl 37% HCl, followed by the addition of internal standard in a final concentration of 0.1 mM (octanoic acid for $C_{9:0}$-$C_{10:0}$ substrates; decanoic acid for $C_{12:0}$ and $C_{13:0}$ substrates; tridecanoic acid for $C_{8:0}$ and substrates > $C_{14:0}$). The reaction mixtures were extracted twice with 0.5 ml diethyl ether. The organic phases were collected, dried with $MgSO_4$ (anhydrous) and evaporated. Samples were resuspended in 40 µl of 1% trimethylchlorosilane in *N*,*O*-bis(trimethylsilyl) trifluoroacetamide and incubated at 75 °C for 30 min for derivatization.

3.3 Analysis of substrates and formed products

Samples were analyzed on a GC/MS QP-2010 instrument (Shimadzu, Japan) equipped with a FS-Supreme-5 column (30 m×0.25 mm×0.25 µm, Chromatographie Service GmbH, Langerwehe, Germany) and with helium as carrier gas (flow rate, 0.69 ml/min; linear velocity 30 cm/s). Mass spectra were collected using electrospray ionization. The injector and detector temperatures were set at 250°C and 285°C, respectively.

For analysis of the $C_{8:0}$-$C_{13:0}$ fatty acids, the column oven was set at 130°C for 2 min, raised to 250°C at a rate of 10°C/min, held isotherm for 3 min, and then raised to 300°C at 40°C/min. For the $C_{14:0}$-$C_{20:0}$ compounds, the temperature was maintained at 180°C for 1 min, then raised to 300°C at 8°C/min and held isotherm for 5 min. Reaction products were identified by their characteristic mass fragmentation patterns.[7] The identity of ω-OHFA and α,ω-DCA products was also verified by comparison of their retention times with those of standards (e.g. 12-hydroxydodecanoic acid, 16-hydroxyhexadecanoic acid and 1,12-dodecanedioic acid). Substrate conversions were quantified using calibration curves estimated from a series of standard solutions (0.01 – 1.0 mM fatty acids) treated in the same manner as the samples. Product distributions were calculated from the relative peak areas. ω-Regioselectivities were estimated from the total hydroxylated product.

Kinetic parameters were determined by GC analysis of substrate conversion using the methodology described above. In case of the $C_{8:0}$ substrate, samples were injected with a lower split ratio in order to detect product formation at low substrate concentrations. The adjusted method was used for all $C_{8:0}$ concentrations with both wild-type and mutant. Data were fit to the Michaelis–Menten equation by non-linear regression using the "OriginPro 8G SR1" software (OriginLab Corporation, USA).

4 *In vitro* oxidation assays towards *n*-alkanes and primary alcohols

4.1 Reaction setup

CYP153A *P.* sp. and CYP153A16 monooxygenases were previously tested towards 1 mM C_5-C_{12} *n*-alkanes and 0.2 mM C_8-C_{12} primary alcohols.[1] To make alkane conversion values comparable with those of primary alcohols and fatty acids, *in vitro* biotransformations of *n*-alkanes were conducted

under the conditions described in 3.1. The oxidation activity of *M. aq*. towards both *n*-alkanes and primary alcohols was also evaluated following the same protocol. In all cases, substrates were added in a final concentration of 0.2 mM from a 20 mM stock solution in ethanol.

4.2 Sample treatment

Samples were treated as described previously.[1]

4.3 Analysis of substrates and formed products

GC/MS analysis was used according to previously described parameters.[1] Substrate conversions were quantified using calibration curves estimated from a series of standard solutions (0.01 – 0.5 mM *n*-alkanes or primary alcohols) treated in the same manner as the samples. Product distributions were calculated from the relative peak areas.

Supporting Tables

Table S1. Substrate conversions and product distributions in reactions catalyzed by CYP153A monooxygenases

CYP153[a]		Fatty acid (0.2 mM)													
		Saturated									9-Monounsaturated				
		8:0	9:0	10:0	12:0	13:0	14:0	16:0	18:0	20:0	(*Z*)-14:1	(*Z*)-16:1	(*E*)-16:1	(*Z*)-18:1	(*E*)-18:1
P. sp.	**Conv.**	**1.5**	**4.5**	**1.9**	**<1.0**	**<1.0**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
	α-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	β-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(ω-1)-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	ω-OH	100	100	100	100	100	-	-	-	-	-	-	-	-	-
	α,ω -DCA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A16	**Conv.**	**-**	**-**	**39.3**	**71.1**	**91.8**	**56.1**	**6.3**	**-**	**-**	**34.1**	**32.4**	**34.6**	**3.5**	**1.9**
	α-OH	-	-	-	1.3	1.9	1.9	-	-	-	2.1	-	-	-	-
	β-OH	-	-	-	0.3	0.7	0.7	-	-	-	0.7	-	-	-	-
	(ω-1)-OH	-	-	-	5.1	18.8	11.4	-	-	-	24.2	40.5	47.0	-	-
	ω-OH	-	-	100	88.3	68.8	75.1	100	-	-	65.6	59.5	53.0	100	100
	α,ω -DCA	-	-	-	5.0	9.8	10.9	-	-	-	7.4	-	-	-	-
M. aq.	**Conv.**	**-**	**2.0**	**52.2**	**63.7**	**78.1**	**83.2**	**39.8**	**25.2**	**4.2**	**34.2**	**75.4**	**93.2**	**66.3**	**73.1**
	α-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	β-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(ω-1)-OH	-	-	-	0.9	2.9	2.7	1.0	3.0	-	8.3	4.1	-	0.8	-
	ω-OH	-	100	100	97.4	95.2	96.1	99.0	97.0	100	90.1	95.9	100	99.2	100
	α,ω -DCA	-	-	-	1.7	1.9	1.2	-	-	-	1.6	-	-	-	-

[a] CYP153A from *Polaromonas* sp. (*P.* sp.), *Mycobacterium marinum* (A16) and *Marinobacter aquaeolei* (*M. aq.*). Preliminary activity assays with A16 indicated that reaction times longer than 4 h did not contribute to increased conversions. Although CO differential spectra measurements indicated that *M. aq.* and *P.* sp. were more stable (>24 h, room temperature) than A16 (data not shown), all reactions were stopped after 4 h for the sake of comparison.

Substrate conversions (Conv.) and product distributions are given in percentage. Conversions were quantified using substrate calibration curves. Product distributions were calculated from the relative peak areas.

- not detected

Table S2. Hydrocarbon substrates yielding maximum ratios of ω- and/or α,ω-oxidized products in reactions catalyzed by CYP153A monooxygenases

CYP153A[a]	Substrate (0.2 mM)	Conv. [%]	Product distribution [%]		
			ω-OH	α,ω[b]	others[c]
P. sp.	*n*-octane	+	91	3	6
	1-octanol[d]	+	n.a.	60	40
	nonanoic acid	+	100	–	–
	MUFA	–	–	–	–
A16	*n*-octane	+	85	12	3
	n-nonane	+	18	73	9
	1-nonanol[d]	++	n.a.	90	10
	dodecanoic acid	+++	88	5	7
	9(*Z*)-tetradecenoic acid	++	66	7	27
M. aq.	*n*-octane	+	85	14	1
	n-nonane	+	74	25	1
	1-nonanol[d]	++	n.a.	92	8
	dodecanoic acid	+++	97	2	1
	9(*E*)-hexadecenoic acid	++++	100	–	–

– (no conversion/not detected), + (1 - 30%), ++ (31 - 60%), +++ (61 - 90%), ++++ (> 90%)

[a] CYP153A from *Polaromonas* sp. (*P.* sp.), *Mycobacterium marinum* (A16) and *Marinobacter aquaeolei* (*M. aq.*). [b] α,ω-products: α,ω-diols from *n*-alkanes and 1-alcohols; α,ω-DCAs from fatty acids. [c] Other products: 2-alcohols, aldehydes and fatty acids from alkanes; aldehydes and fatty acids from 1-alcohols; hydroxylated regioisomers from fatty acids. [d] as reported in reference 1. Abbreviations: MUFA monounsaturated fatty acid; n.a. not applicable.

Supporting Figures

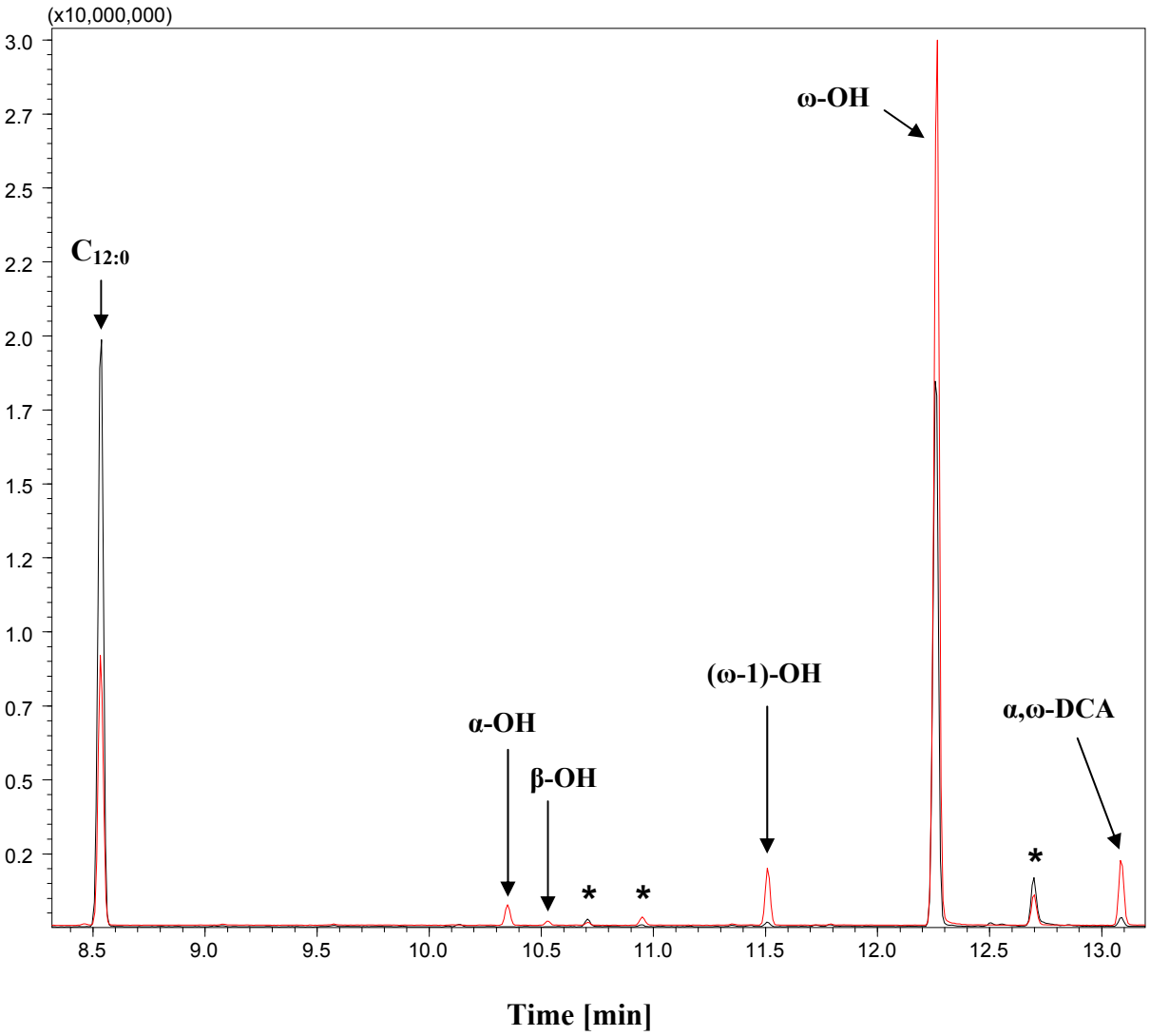
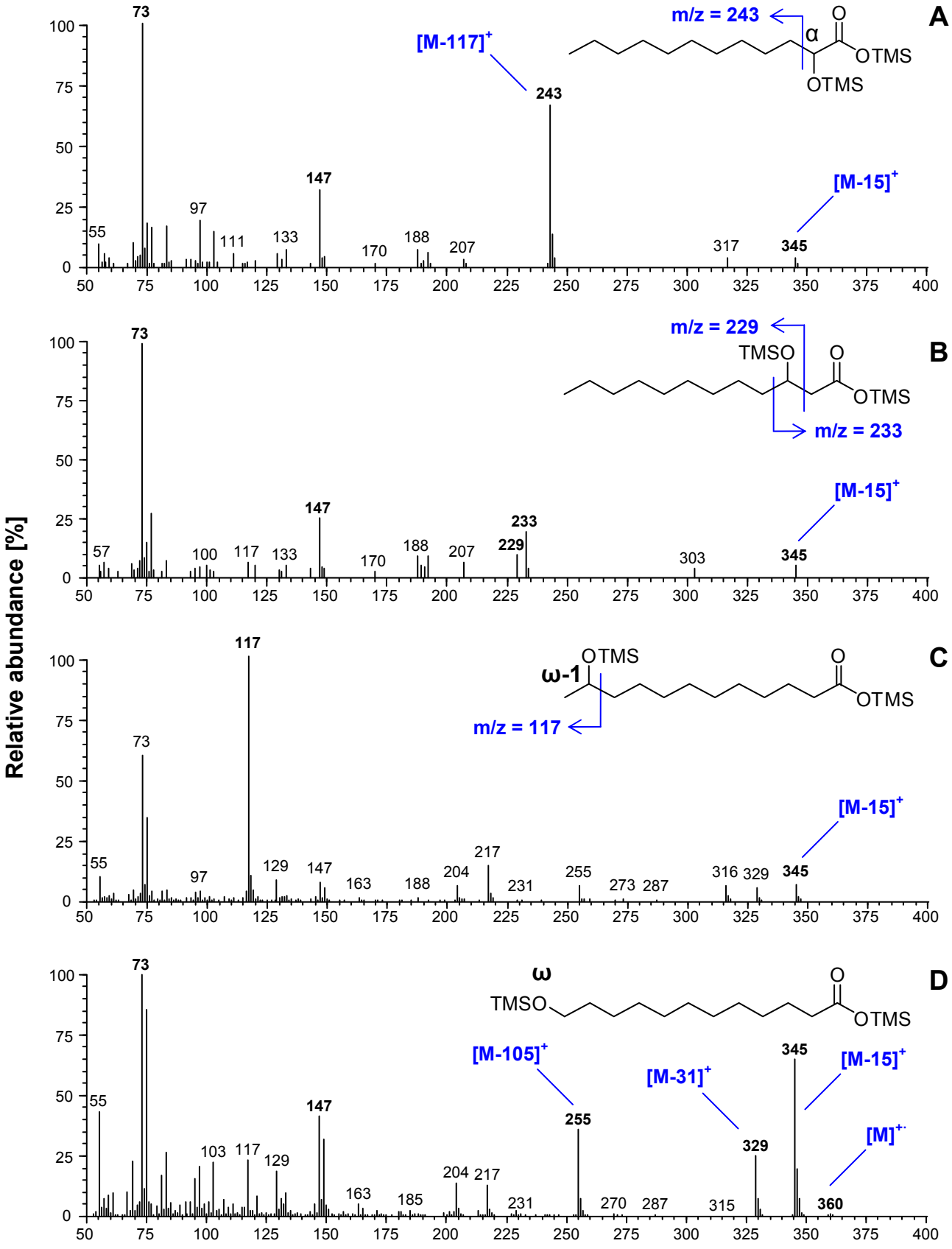


Figure S1. Gas chromatograms for CYP153A-catalyzed reactions with dodecanoic acid. CYP153A16 (red line) and CYP153A *M. aq.* (black line). The substrate and formed products were measured as TMS derivatives. Abbreviations: $C_{12:0}$, dodecanoic acid, α-OH, 2-hydroxydodecanoic acid, β-OH, 3-hydroxydodecanoic acid; (ω-1)-OH, 11-hydroxydodecanoic acid; ω-OH ,12-hydroxydodecanoic acid; α,ω-DCA, 1,12-dodecanedioic acid; * impurity.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α
O
OTMS
OTMS
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147
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170
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207
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345
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TMSO
O
OTMS
m/z = 233
[M-15]+
73
147
233
229
57
100
117
133
170
188
207
303
345
C
117
OTMS
ω-1
O
OTMS
m/z = 117
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55
97
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329
345
D
ω
TMSO
O
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[M-15]+
[M]+·
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231
270
287
315
360
Relative abundance [%]

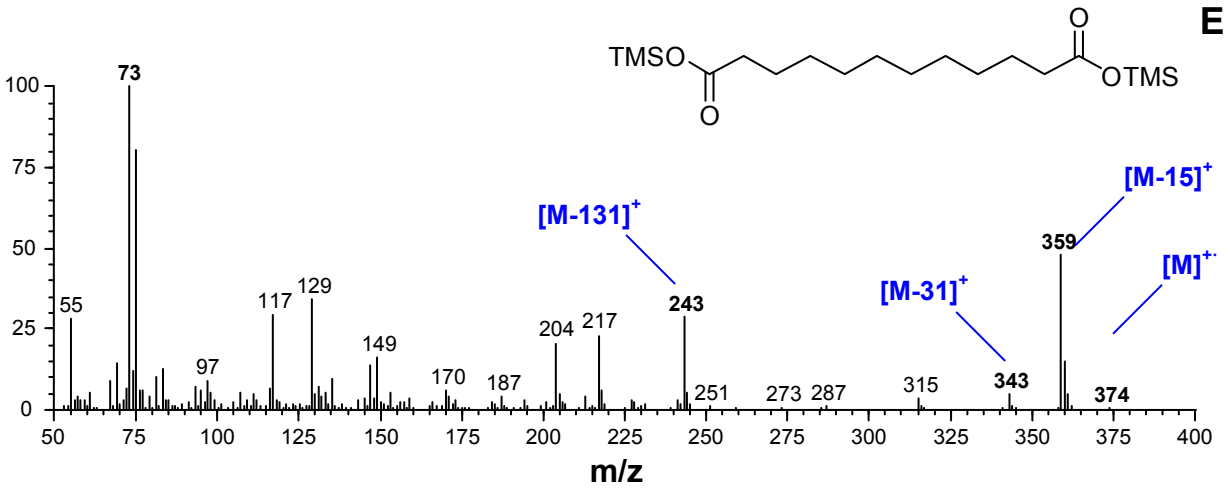

Figure S2. Electron ionization mass spectral fragmentations of trimethylsiloxyl (TMS) derivatives of dodecanoic acid. The compounds were identified as α-hydroxydodecanoic acid (A), β-hydroxydodecanoic acid (B), (ω-1)-hydroxydodecanoic acid (C), ω-hydroxydodecanoic acid (D), and α,ω-dodecanedioic acid (E). The common fragment ion at m/z = 73 occurs upon the loss of one TMS ester group. The fragment ion at m/z = 147, typical of polysylated compounds, involves the loss of a methyl radical from one silyl group and its interaction with another TMS ester group.[7] Characteristic peaks of each hydroxycarboxylic acid and the dicarboxylic acid derivative are indicated in the corresponding mass spectrum.

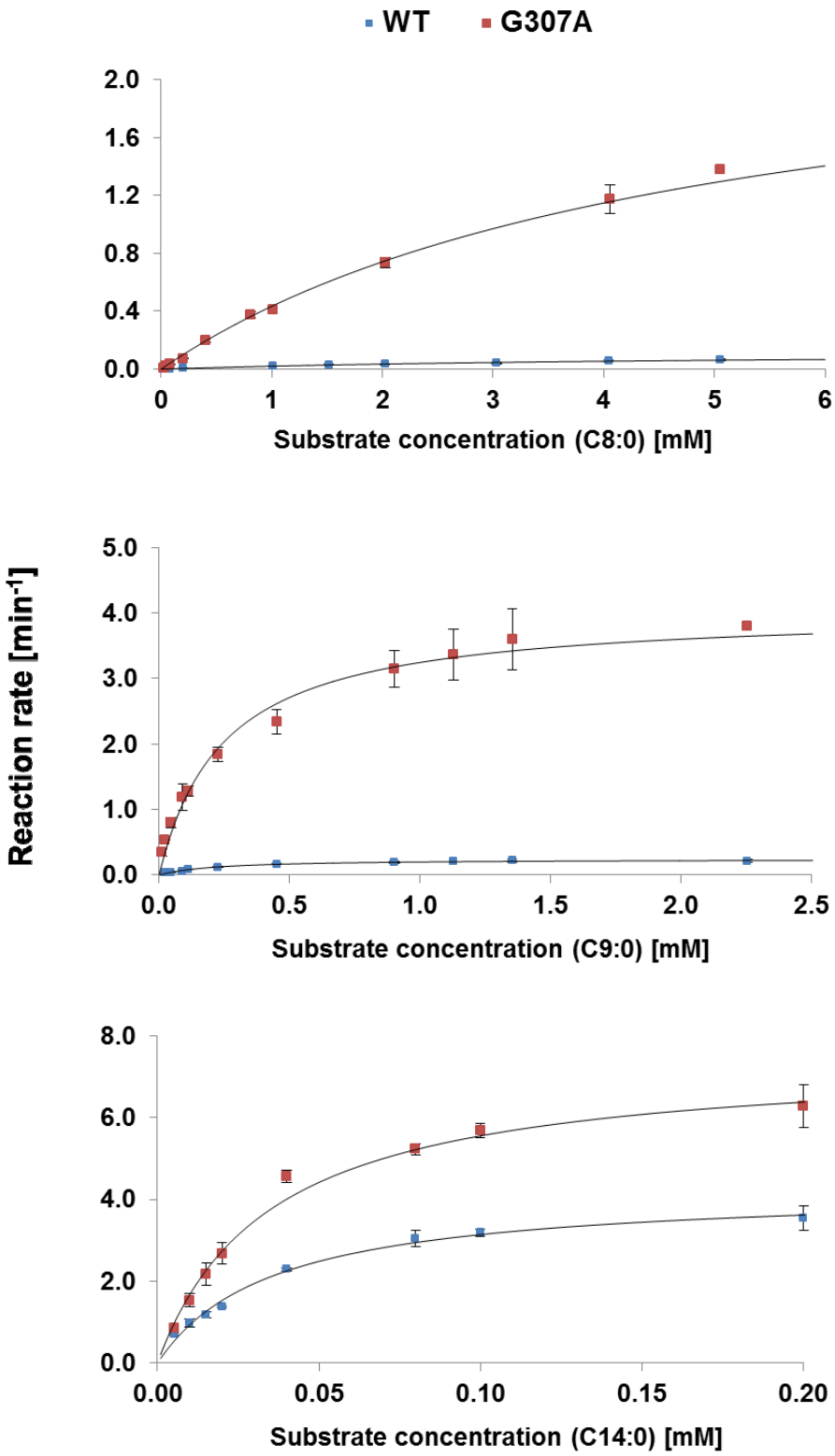

Figure S3. Michaelis-Menten plots of octanoic, nonanoic and tetradecanoic acids in reactions catalyzed by CYP153A *M. aq.* wild-type and variant G307A.

References

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