Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting Information

Preparative regio- and stereoselective α-hydroxylation of medium chain fatty acids

Klara Bangert,[a] Alexander Swoboda,[b] Stephan Vrabl,[a] Haris Rudalija,[c] Mattia Lazzarotto,[c] Stefan Payer,[c] Anton Glieder,[d] Christian A. M. R. van Slagmaat,[e] Stefaan M. A. De Wildeman,[e] and Wolfgang Kroutil*[a,f,g]

[a]Department of Chemistry, Organic & Bioorganic Chemistry, University of Graz, Heinrichstraße 28, 8010 Graz, Austria. E-mail: wolfgang.kroutil@uni-graz.at; Tel: +43-316-380-5350.

[b]Austrian Centre of Industrial Biotechnology, c/o University of Graz, Heinrichstraße 28, 8010 Graz (Austria).
[c]Enzyan Biocatalysis GmbH, Stiftingtalstrasse 14, 8010 Graz (Austria).
[d]Bisy GmbH, Wünschendorf 292, 8200 Hofstätten an der Raab (Austria).
[e]B4Plastics BV, IQ-Parklaan2 A, 3650 Dilsen-Stokkem (Belgium).
[f]BioTechMed Graz, 8010 Graz (Austria).
[g]Field of Excellence BioHealth, University of Graz, 8010 Graz (Austria).

Table of Contents

General materials and methods

Reagents and organic solvents were obtained from chemical suppliers in reagent grade quality and applied without further purification. 5-Aminolevulinic acid HCl was obtained from ThermoFisher Scientific (Linz, Austria). All fatty acid substrates were obtained from Sigma-Aldrich if not stated otherwise. Hydrogen Peroxide was obtained from MERCK (Darmstadt, Germany). *Hsp*UPO was obtained from Bisy GmbH (Hofstätten an der Raab, Austria).

List of fatty acid substrates and products

Table S1: Substrates and products used in this study

Compound	**Compound number**
caproic acid	**1a**
α-hydroxy hexanoic acid	α-**2a**
β-hydroxy hexanoic acid	β-**2a**
(ω-1)-hydroxy hexanoic acid	(ω-1)-**2a**
caprylic acid	**1b**
α-hydroxy octanoic acid	α-**2b**
β-hydroxy octanoic acid	β-**2b**
γ-hydroxy octanoic acid	γ-**2b**
(ω-1)-hydroxy octanoic acid	(ω-1)-**2b**
capric acid	**1c**
α-hydroxy decanoic acid	α-**2c**
β-hydroxy decanoic acid	β-**2c**
(ω-1)-hydroxy decanoic acid	(ω-1)-**2c**
azelaic acid	**3a**
α-hydroxynonanedioic acid	α-**4a**
sebacic acid	**3b**
α-hydroxydecanedioic acid	α-**4b**
succinic acid	**3c**
adipic acid	**3d**
myristic acid	**3e**

Plasmids

The following plasmids were used in this study (pEG numbers indicate numbering for in-house plasmid data bank): pET28a$^+$-P450$_{CLA}$[1] (pEG306, NCBI Reference Sequence: WP_010966602.1[2]) and pDB-HisGST-P450$_{Spα}$[3] (pEG371, UniProtKB ID: O24782[4]) were already part of the in-house plasmid data bank. The plasmids, pET28a$^+$- P450$_{Jα}$ [5, 6] (pEG708, Genbank accession no.: MN737472), pET28a$^+$- P450$_{Exα}$, (pEG707, UniProtKB ID: C4L2G9)[7], pET24b$^+$- CYP152K6 (pEG705, UniprotKB ID: I3DZK9)[8] and pET28a$^+$- P450$_{Bsβ-\ F79L/G290F}$ [9] (pEG706, mutant of P450$_{Bsβ}$ (UniProtKB ID: O31440[10]), with the corresponding optimized genes for the enzymes were ordered from BioCat GmbH (Heidelberg Germany).

Gene and Protein Sequences

$P450_{Sp\alpha}$

Expression System: *Escherichia coli*

$P450_{Sp\alpha}$-Optimized Sequence

catATGCCGAAAACACCGCATACCAAAGGTCCGGATGAAACCCTGAGCCTGCTGGCAGATCCGTATCGTTTTATTAGC
CGTCAGTGTCAGCGTCTGGGTGCAAATGCCTTTGAAAGCCGTTTTCTGCTGAAAAAAACCAATTGTCTGAAAGGTGCA
AAAGCAGCCGAAATCTTTTATGATACCACCCGTTTTGAACGTGAAGGTGCAATGCCGGTTGCAATTCAGAAAACCCTG
CTGGGTCAGGGTGGTGTTCAGGGTCTGGATGGTGAAACCCATCGTCATCGTAAACAAATGTTTATGGGTCTGATGAC
ACCGGAACGTGTTCGTGCACTGGCACAGCTGTTTGAAGCAGAATGGCGTCGTGCAGTTCCGGGTTGGACCCGTAAAG
GTGAAATTGTTTTTTATGATGAACTGCATGAACCGCTGACCCGTGCAGTTTGTGCATGGGCAGGCGTTCCGCTGCCGG
ATGATGAAGCAGGTAATCGTGCCGGTGAACTGCGTGCACTGTTTGATGCAGCCGGTAGCGCAAGTCCGCGTCATCTG
TGGTCACGTCTGGCACGTCGTCGTGTTGATGCATGGGCCAAACGTATTATTGAAGGTATTCGTGCAGGTAGCATTGG
TAGCGGTAGCGGCACCGCAGCTTATGCAATTGCCTGGCATCGTGATCGTCATGATGATCTGCTGAGTCCGCATGTTGC
AGCAGTTGAACTGGTTAATGTTCTGCGTCCGACCGTTGCCATTGCAGTGTATATTACCTTTGTTGCACATGCACTGCAG
ACCTGTAGCGGTATTCGCGCAGCACTGGTTCAGCAGCCGGATTATGCAGAACTGTTTGTTCAAGAAGTGCGTCGCTTT
TATCCGTTTTTTCCGGCAGTTGTTGCACGTGCCAGCCAGGATTTTGAATGGGAAGGTATGGCATTTCCGGAAGGTCGT
CAGGTTGTTCTGGATCTGTATGGTAGCAATCATGATGCAGCAACCTGGGCTGATCCGCAAGAATTTCGTCCGGAACG
CTTTCGCGCATGGGATGAAGATAGCTTTAACTTTATTCCGCAGGGTGGCGGTGATCATTATCTGGGTCATCGTTGTCC
GGGTGAATGGATTGTTCTGGCAATTATGAAAGTTGCAGCACATCTGCTGGTGAATGCAATGCGTTATGATGTTCCGG
ATCAGGATCTGAGCATTGATTTTGCACGTCTGCCTGCACTGCCGAAAAGCGGTTTTGTTATGCGTAATGTTCATATCG
GTGGCTAAactcgag

$P450_{Sp\alpha}$- Protein

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGANAFESRFLLKKTNCLKGAKAAEIFYDTTRFEREGAMPVAIQKTLLGQGG
VQGLDGETHRHRKQMFMGLMTPERVRALAQLFEAEWRRAVPGWTRKGEIVFYDELHEPLTRAVCAWAGVPLPDDEAG
NRAGELRALFDAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGSIGSGSGTAAYAIAWHRDRHDDLLSPHVAAVELVNV
LRPTVAIAVYITFVAHALQTCSGIRAALVQQPDYAELFVQEVRRFYPFFPAVVARASQDFEWEGMAFPEGRQVVLDLYGSN
HDAATWADPQEFRPERFRAWDEDSFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDLSIDFARL
PALPKSGFVMRNVHIGG

$P450_{CLA}$

Expression System: *Escherichia coli*

$P450_{CLA}$-Optimized Sequence

ATGCTGCTGAAAGAAAACACCGCCAAAGATAAAGGTATTGATAGCACCCTGGATCTGCTGAAAGAGGGTTACCTGTT
TATCAAAAATCGTGCCGATCATTATCAGAGCGACCTGTTTGAAACCCGTCTGATGGGTCAGCGTATTATTTGTATGAC
CGGTGAAGAAGCAGCCCGTATCTTTTATGATAGCGATAAATTCAAACGTCAGGGTGCAGCACCGAAACGTGTTCAAG
AAACCCTGCTGGGTGAAAATGCAATTCAGACCCTGGATGGTGAAAGCCATCTGCATCGTAAAAAACTGTTTATGCTGC
TGACCAATCAGGTTCAGCAGAAACGTCTGGCAGAACTGACCACCGAAAAATGGGAAGCAAGCGCAAGCAAATGGCA
TACCAAAAGCATTGTGCTGTTTAACGAAGCCAATGAAATTCTGTGTCAGGTTGCATGTCATTGGGCAGGCGTTCCGCT
GATGGAAAGCGATATCAAAAACCGTGCGGAAGATTTTAGCAGCATGATTGATAGCTTTGGTGCAGTTGGTCCGCGTC
ATTGGAAAGGTAAAAAAGCACGTAATACCATTGAGGCCTGGATCAAAGAAATTATTGAAAATGTTCGTAGCGGTCGC
ATTCGTGCAGAAGAGGGTAGTCCGCTGCATGAAATTGCCTTTTATATCGATGTTAATGGCCAGCAGATGCCTGCAGA
AATGGCAGCAATTGAACTGATTAACATTCTGCGTCCGATTGTTGCAATTAGCACCTTTATTACCTTTAGCGCACTGGCA
CTGTATGAACATAGCGAATATCGCGAAAAACTGCAGAGCAAAGATATCCGTTATCTGGAAATGTTTACCCAAGAAGT
GCGTCGTTATTACCCGTTTGCACCGTTTGTTGGTGCACGTGTTCGTAAAGATTTTCTGTGGAATAACTGCGAGTTCAAA
AAAGAAATGCTGGTGCTGCTGGATATTTATGGCACCAATCATGATAGCCGTATTTGGCAGAAACCGTATGAATTTATT

CCGGATCGTTTCCGCAGCTATAAAGGTAACCTGTTCGATTTCATTCCGCAGGGTGGTGGTGATCCGAGCAGTACCCAT
CGTTGTCCGGGTGAAGGTATTACCCTGGAAATCATGAAAACCAGCCTGGATTTTCTGAGCACCAAAATTGATTTTACC
GTTCCGGATCAGGATCTGAGCTATAGCCTGAGCAAAATTCCGACCCTGCCGAAAAGCGGTTTTATCATTGATAACATC
AACCTGAAACTGTAA

$P450_{CLA}$-Protein

MLLKENTAKDKGIDSTLDLLKEGYLFIKNRADHYQSDLFETRLMGQRIICMTGEEAARIFYDSDKFKRQGAAPKRVQETLLG
ENAIQTLDGESHLHRKKLFMLLTNQVQQKRLAELTTEKWEASASKWHTKSIVLFNEANEILCQVACHWAGVPLMESDIKN
RAEDFSSMIDSFGAVGPRHWKGKKARNTIEAWIKEIIENVRSGRIRAEEGSPLHEIAFYIDVNGQQMPAEMAAIELINILRPI
VAISTFITFSALALYEHSEYREKLQSKDIRYLEMFTQEVRRYYPFAPFVGARVRKDFLWNNCEFKKEMLVLLDIYGTNHDSRI
WQKPYEFIPDRFRSYKGNLFDFIPQGGGDPSSTHRCPGEGITLEIMKTSLDFLSTKIDFTVPDQDLSYSLSKIPTLPKSGFIIDNI
NLKL

$P450_{Bs\beta\ F79L\ G290F}$

Expression System: *Escherichia coli*

$P450_{Bs\beta\ F79L\ G290F}$-Optimized Sequence

catatgAACGAACAGATTCCGCATGATAAAAGTCTGGATAATAGTCTGACCCTGCTGAAAGAAGGCTATCTGTTCATTA
AAAATCGTACCGAACGTTATAACAGTGATCTGTTCCAGGCCCGTCTGCTGGGCAAAAACTTCATCTGTATGACCGGTG
CAGAAGCAGCCAAAGTGTTCTATGATACCGATCGCTTCCAGCGCCAGAATGCCCTGCCGAAACGCGTTCAGAAAAGT
CTGCTGGGTGTGAATGCCATTCAGGGCATGGATGGTAGCGCACATATTCATCGTAAAATGCTGTTCCTGAGTCTGATG
ACCCCGCCGCATCAGAAACGCCTGGCAGAACTGATGACCGAAGAATGGAAAGCAGCCGTGACCCGCTGGGAAAAAG
CAGATGAAGTGGTTCTGTTCGAAGAAGCCAAAGAAATTCTGTGTCGCGTGGCATGCTATTGGGCCGGTGTTCCGCTG
AAAGAAACCGAAGTTAAAGAACGCGCAGATGACTTCATTGATATGGTTGATGCATTCGGCGCCGTGGGTCCGCGCCA
TTGGAAAGGTCGCCGCGCCCGTCCGCGCGCAGAAGAGTGGATTGAAGTTATGATTGAAGATGCACGCGCCGGTCTG
CTGAAAACCACCAGTGGCACCGCCCTGCATGAAATGGCCTTCCATACCCAGGAAGATGGTAGCCAGCTGGATAGCCG
CATGGCAGCCATTGAACTGATTAATGTGCTGCGTCCGATTGTGGCCATTAGTTACTTCCTGGTGTTCAGCGCACTGGC
ACTGCATGAACATCCGAAATATAAAGAATGGCTGCGTAGCGGCAATAGTCGTGAACGCGAAATGTTCGTGCAGGAA
GTTCGCCGTTATTATCCGTTCTTCCCGTTCCTGGGTGCCCTGGTTAAAAAAGACTTCGTGTGGAATAATTGCGAATTCA
AAAAAGGTACCAGTGTGCTGCTGGATCTGTATGGCACCAATCATGATCCGCGCCTGTGGGATCATCCGGATGAATTC
CGCCCGGAACGCTTCGCCGAACGTGAAGAAAATCTGTTCGATATGATTCCGCAGGGTGGTGGCCATGCAGAAAAAG
GCCATCGTTGTCCGGGCGAAGGCATTACCATTGAAGTTATGAAAGCAAGCCTGGACTTCCTGGTGCATCAGATTGAA
TATGATGTGCCGGAACAGAGTCTGCATTATAGTCTGGCCCGTATGCCGAGTCTGCCGGAAAGTGGCTTCGTGATGAG
CGGCATTCGTCGCAAAAGTCtcgagcaccaccaccaccaccactga

$P450_{Bs\beta\ F79L\ G290F}$- Protein

NEQIPHDKSLDNSLTLLKEGYLFIKNRTERYNSDLFQARLLGKNFICMTGAEAAKVFYDTDRFQRQNALPKRVQKSLLGVNA
IQGMDGSAHIHRKMLFLSLMTPPHQKRLAELMTEEWKAAVTRWEKADEVVLFEEAKEILCRVACYWAGVPLKETEVKER
ADDFIDMVDAFGAVGPRHWKGRRARPRAEEWIEVMIEDARAGLLKTTSGTALHEMAFHTQEDGSQLDSRMAAIELINVL
RPIVAISYFLVFSALALHEHPKYKEWLRSGNSREREMFVQEVRRYYPFFPFLGALVKKDFVWNNCEFKKGTSVLLDLYGTNH
DPRLWDHPDEFRPERFAEREENLFDMIPQGGGHAEKGHRCPGEGITIEVMKASLDFLVHQIEYDVPEQSLHYSLARMPSL
PESGFVMSGIRRKS

CYP152K6

Expression System: *Escherichia coli*

CYP156K6-Optimized Sequence

atacatATGAGTAACATCAACCAGATGCCGCGCGAAGAAGGTATTGATAGCACCTGGCGCCTGATGGAAGAAGGCTAT
ATGTATATTCTGAATCGTCGCCATAGCTTCAATAGTGATATCTTCGAAACCAGACTGCTGGGTAAAAAAGCAATCTGT

ATGGGTGGCAAAGAAGCCGCAGAAATCTTCTATGATACCGAAAAATTCAAGCGCAAAGATGCCGCCCCGAATCGTGT
TGTTCAGACCCTGTTCGGCAAAAATGGTGTTCAGGCCCTGGATGGTCAGACCCATAAACATCGCAAAGAAATGTTCAT
GAGCATTATGAGTCCGGATGAACTGGAAAAACTGACCGATATTACCAAAAAACAGTGGGAAATTGCCGTTGATAAAT
GGGAACAGATGGATAAAGTTATTCTGTATGAAGAAGCCAAAGAAATTATGTGCCGTACCGCCTGTCAGTGGGCCGGT
GTTCCGGTTCAGGAAAATGAAGTTAAACGCCTGACCAAAAATCTGGGTGCAATGTTCGAAAGTGCCGCCGCCGTGGG
TCTGAAACATTGGCTGGGTCGCCATGCCCGCAATTATGAAGAAATCTGGATTGAAGAACTGATTGATCGCGTTCGCG
ATGGTAAAGTTAATCCGCCGGAAAATACCACCCTGCATAAATTCAGCTGGTATCGCGATCTGGAAGGCAATCTGCTG
GATACCGAAACCGCCGCCGTTGAAGTTATTAATATTCTGCGTCCGATTGTTGCAATTGCCATCTTCATTAACTTCATTG
CACTGGCCCTGCATCATTATCCGGAAGAAAAAGAAAAACTGAAAAGCGGTGATAAAAAGTATAGCCAGATGTTCGTG
CAGGAAGTGCGTCGCTTCTATCCGTTCTTCCCGTTCGTTGTTGCCCTGGTGAAAAAAGACTTCACCTGGAAAGGCTAT
AAATTCGAAGAAGGCACCCTGACCCTGCTGGATCTGTATGGTACCAATCATGATCCGGAAATCTGGAAAAATCCGGA
TGTGTTCAGCCCGGATCGCTTCGCCAAATGGGAAGGTAGCCCGTTCAGCTTCATTCCGCAGGGTGGTGGCGATTACTT
CATGGGCCATCGTTGTGCCGGTGAATGGGTTACCATTGAAGTGATGAAAGTGAGTCTGGATTATCTGACCAATCGCA
TGGATTATGAAGTTCCGGATCAGGATCTGAGCTTCAGCATGGCCAGCATGCCGAGTATTCCGCATAGTAAAGTGGTG
ATTAAAAATGTTAAGAAGCGCATTgcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactga

CYP152K6- Protein

MSNINQMPREEGIDSTWRLMEEGYMYILNRRHSFNSDIFETRLLGKKAICMGGKEAAEIFYDTEKFKRKDAAPNRVVQTLF
GKNGVQALDGQTHKHRKEMFMSIMSPDELEKLTDITKKQWEIAVDKWEQMDKVILYEEAKEIMCRTACQWAGVPVQE
NEVKRLTKNLGAMFESAAAVGLKHWLGRHARNYEEIWIEELIDRVRDGKVNPPENTTLHKFSWYRDLEGNLLDTETAAVE
VINILRPIVAIAIFINFIALALHHYPEEKEKLKSGDKKYSQMFVQEVRRFYPFFPFVVALVKKDFTWKGYKFEEGTLTLLDLYGT
NHDPEIWKNPDVFSPDRFAKWEGSPFSFIPQGGGDYFMGHRCAGEWVTIEVMKVSLDYLTNRMDYEVPDQDLSFSMAS
MPSIPHSKVVIKNVKKRI

P450$_{Ex\alpha}$

Expression System: *Escherichia coli*

P450$_{Ex\alpha}$-Optimized Sequence

GgatccATGGGCAAAGTGATTCCGAAACAGGAAGGTCTGGATCATAGTGTGGACTTCCTGCGTGAAGGTTATCTGTTC
GTTGCCAATCGCCGTAAATCATTCCAGAGTAATATCTTCGAAAGTCGCCTGCTGGGCGAACGCGTGATCTGTCTGGGT
GGTGAAGAAGCCGCCGAAGTGTTCTATGATGCAAATAAATTCACCCGTCAGGATGCAGCACCGAAACGTCTGCTGAA
AACCTTATTCGGTGAAGGCGGCGTGCAGACCCTGGATGGTAGTGAACATACCCATCGTAAACAGATGTTCATGAGCC
TGATGACCAAAGAAAATATTGATCGCCTGCTGCGCCTGACCTATCGTGAATGGAATCAGATTGAACGCATGGGTGAA
GAAATTGTTCTGTATGATATTGCCCAGGAAGTGCTGATGAAAGCAGTGTGTGAATGGAGCGGCGTTCCGCTGGCAAA
AGAAGAAGTGGGCAAACGCACCGAAGAAATGCGCCTGCTGTTCGAAAGTGGTACCAGCCTGGGTCCGACCTATCTG
CAGGGTCGCAAAGCCCGCAGTAGCGCCGAAGTGTGGATTCGTCAGATGGTTAAAGAAGTGCGTAGTAATCGCCTGC
TGCCGAATGAACATACCGCCCTGTATGAATTCAGTTGGCATCGTGATGAAAGTGGCGAACTGCTGCCGGAAGAAGTT
GTTGCCGTTGAAGTGCTGAATATTCTGCGTCCGACCGTGGCCATTAGTGTGTATGTGCTGTTCACCGTGCTGGCCCTG
CATCAGTTCCCGGATGTGAAAGAACAGGTGGAACGCGGTGAAGTTAGCAAAACCGAATTCGTGCAGGAAGTTCGCC
GCTTCTATCCGTTCTTCCCGGTTGCAGCAGCACGTGTTAAAACCGACTTCGAATGGGATGGTTATGCCTTCCCGGAAG
GCACCCTGACCCTGCTGGATCTGTATGGTACCAATCATGATGTGAGCATCTGGACCGAACCGGATCGCTTCGATCCGA
GTCGCTTCAAAGATTGGAAAGAAAGCCCGTTCAACTTCATTCCGCAGGGTGGTGGTGATGTTGACTTCGGTCATCGTT
GCGCAGGTGAACATGTTACCATTGCCATTCTGGCCCAGGTTATTGAACTGTTCACCAAAGAATATGCATATACCGTTC
CGCCGCAGGATCTGAGCTATAGCTTCGTGGATATGCCGAGTCTGCCGAAAAGTAAACTGCGCCTGACACATCTGACC
CGTAATCAGaagcttgcggccgcactcgagcaccaccaccaccaccactga

P450$_{Ex\alpha}$- Protein

MGKVIPKQEGLDHSVDFLREGYLFVANRRKSFQSNIFESRLLGERVICLGGEEAAEVFYDANKFTRQDAAPKRLLKTLFGEG
GVQTLDGSEHTHRKQMFMSLMTKENIDRLLRLTYREWNQIERMGEEIVLYDIAQEVLMKAVCEWSGVPLAKEEVGKRTE
EMRLLFESGTSLGPTYLQGRKARSSAEVWIRQMVKEVRSNRLLPNEHTALYEFSWHRDESGELLPEEVVAVEVLNILRPTVA
ISVYVLFTVLALHQFPDVKEQVERGEVSKTEFVQEVRRFYPFFPVAAARVKTDFEWDGYAFPEGTLTLLDLYGTNHDVSIWT
EPDRFDPSRFKDWKESPFNFIPQGGGDVDFGHRCAGEHVTIAILAQVIELFTKEYAYTVPPQDLSYSFVDMPSLPKSKLRLT
HLTRNQ

P450$_{J\alpha}$

Expression System: *Escherichia coli*

P450$_{J\alpha}$–Optimized Sequence

ccatggccATGAATTCAAATATGCCAAATGATTCTGGTTTCGACAAGACTTTAAGCGTTCTTAAAGAGGGTTATGAATTC
GTCATGAACCGTGACAAGGAAATGCATACAAACATTTTCGAAACACGTATCCTCGTTGAAAAGACAATCTGCCTCACA
GGTAGTGAGCTCGCTGAACTATTTTACGACAACACCCGCTTCAGCCGCACTGATGCTGCACCTGCCAGAGTACAAAAA
ACACTTTTCGGTGAAGGTGGCGTACAAGGTCTCGATGGTGATGAACACAAAAACCGTAAAGCGATGTTCATGTCTTT
AATGGATAACAAAGCGATGGATGAAATAGAAGATTTAACACAGAAATATTGGCACGAATACTTTGAAGAAATTGATT
GGAACGACACCGTAGAGTTATATGAAGCAGCGAAAGTTGTATTTCTCAAAGTCGCTTGTGACTGGGTTGGAGTGTCT
CTAGAAGATGAAGACATCGAAACTCGTGCCAGTCAGATTGCCGATTTATACGAATCACCTGCAGCACTCGGAATCCAA
CACTGGAAGGGACGTAAATCTAGATCAGAAGCAGAAGACTGGATTGAGCGACTTATTGAAGGTGTTAGAAACGGTG
AGCTTGAAGCAGACAAAGATAGAGCACTCTATAAGTTCGCTATGCACAAAGATTTAAATGATGAACTCTTGGATGCTC
AAGTTGCTACAGTTGAGCTTTTAAACCTCATTCGTCCAATTAATGCTATCAGTGTATGGGTTGCAATGATTGGTCTCGC
TATTCATGAACACCCAGACGCTGCAGAGAAATTGAAAGATGCCAATCAACAACAATTAGAGTGGTTCATTCAAGAAG
TCAGACGTTATTATCCCTTCTTCCCATTCGCAGTGGCACGAGTGAAACTCGATTTCGAATGGCAAGGTTATGAGTTTAA
AGAAGGCACTTTAACACTATTAGACTTATACGGTACCAACCGTCATCCAGACGACTGGGTAGACCCTGATGAATTTAA
ACCCGAAAGATTTGAAGGTTGGCAAGAGACACCATTCAACTTTATACCACAAGGTGGTGGATCTTACGACTTCGGTCA
CCGCTGTGCAGGGGAGTTTATCACAATCGTCATGATGAGAACAACACTCGACTTTTTAGTCAATCACCTAGAATTCAA
AGTACCAGAACAAGATTTTGGCTTTGAATTTAACGATATTCCAGCTGTTCCTAATGACAAAGTGAAGATTAAGCCAAC
ACGTATCAAActcgagcaccaccaccaccaccactga

P450$_{J\alpha}$ -Protein

MNSNMPNDSGFDKTLSVLKEGYEFVMNRDKEMHTNIFETRILVEKTICLTGSELAELFYDNTRFSRTDAAPARVQKTLFGE
GGVQGLDGDEHKNRKAMFMSLMDNKAMDEIEDLTQKYWHEYFEEIDWNDTVELYEAAKVVFLKVACDWVGVSLEDE
DIETRASQIADLYESPAALGIQHWKGRKSRSEAEDWIERLIEGVRNGELEADKDRALYKFAMHKDLNDELLDAQVATVELL
NLIRPINAISVWVAMIGLAIHEHPDAAEKLKDANQQQLEWFIQEVRRYYPFFPFAVARVKLDFEWQGYEFKEGTLTLLDLY
GTNRHPDDWVDPDEFKPERFEGWQETPFNFIPQGGGSYDFGHRCAGEFITIVMMRTTLDFLVNHLEFKVPEQDFGFEFN
DIPAVPNDKVKIKPTRIK

Transformation into BL21(DE3)

E. coli BL21(DE3) was transformed with plasmid with pET28a$^+$-P450$_{CLA}$, pDB-HisGST-P450$_{Sp\alpha}$, pET28a$^+$-P450$_{J\alpha}$, pET28a$^+$- P450$_{Ex\alpha}$, pET24b$^+$- CYP152K6 and pET28a$^+$- P450$_{Bs\beta-\ F79L/G290F}$.

For transformation, competent cells (100 µl) were thawed on ice for 30 minutes. Plasmid solution (2 µl) was added, and cells were kept on ice for 30 minutes. Subsequently, cells were heat-shocked at 42 °C for 10 seconds and cooled down on ice for five minutes. LB medium (900 µl) was added and cells were incubated at 37 °C for one hour. Finally, a sample of cells (100 µl) was plated on LB$_{kan}$ plates (LB plates supplemented with 50 µg/mL kanamycin) and incubated at 37 °C over night.

Expression and Purification of P450 enzymes

Cultivation conditions

Table S2: Growth conditions for $P450_{CLA}$ and $P450_{Sp\alpha}$, $P450_{J\alpha}$, $P450_{Ex\alpha}$, CYP152K6 and $P450_{Bs\beta\text{- }F79L/G290F}$

Enzyme	*E. coli* host	Medium (3x 330 mL)	Growth Temp (°C)	Induction Temp (°C)	IPTG (mM)
$P450_{CLA}$	BL21	TB	37	30	0.1
$P450_{Sp\alpha}$	BL21	LB	37	20	0.1
$P450_{J\alpha}$	BL21	TB	37	25	0.1
$P450_{Ex\alpha}$	BL21	TB	37	25	0.1
CYP152K6	BL21	LB	37	25	0.1
$P450_{Bs\beta\ F79L/G290F}$	BL21	LB	37	25	0.1

$P450_{CLA}$, $P450_{Sp\alpha}$, $P450_{Ex\alpha}$, $P450_{J\alpha}$, CYP152K6 and $P450_{BS\beta\ F79L/G290F}$ were expressed in *E. coli* BL21(DE3) cells. For this purpose, overnight cultures (10 mL LB medium) of the transformants were prepared in 50 mL falcon tubes, kanamycin (50 μg/mL) was added and cells were incubated at 37 °C and shaken at 120 rpm. The shaking flasks (3x 1 L) were filled with 330 mL LB or TB medium and autoclaved. To achieve a higher protein concentration of $P450_{Sp\alpha}$ five shaking flasks (2 L) were used, filled with 660 mL LB medium and autoclaved. The medium was supplemented with kanamycin (50 μg/mL) and trace element solution[11] (330 mL medium =330 μL trace element solution) was added. The prepared medium was inoculated with 1 (for 330 mL cultures) or 2 mL (for 660 mL cultures) of overnight culture. At an OD_{600}= 0.7-0.9 the cultures were let cool to room temperature and 5-aminolevulinic acid was added [final conc. 0.5 mM (stock: 0.5 M)], the culture was incubated for 5 minutes at the respective induction temperature (37 °C) and expression was induced with IPTG [0.1 mM (stock: 1M)] and the cells incubated overnight at 30 °C ($P450_{CLA}$), 20 °C ($P450_{Sp\alpha}$) and 25 °C ($P450_{J\alpha}$, $P450_{Ex\alpha}$, CYP152K6 and $P450_{BS\beta\ F79L/G290F}$). Growth conditions of all enzymes are listed in **Table S2**. Cells were centrifuged (8000 rpm, 20 min, 4 °C). The pellet was washed in phosphate buffer (KPi) [pH 7.4, 100 mM (per g pellet 10 mL buffer)], transferred into a 50-mL flacon tubes and were frozen in liquid nitrogen and stored at -20 °C.

Enzyme purification procedure

Table S3: Purification buffers for $P450_{CLA}$, $P450_{J\alpha}$, $P450_{Ex\alpha}$ and CYP152K6

$P450_{CLA}$, $P450_{J\alpha}$, $P450_{Ex\alpha}$ and CYP152K6		
Lysis buffer:	Elution buffer:	Storage buffer:
100 mM KPi	100 mM KPi	100 mM KPi
300 mM KCl	300 mM KCl	300 mM KCl
50 mM imidazole	300 mM imidazole	pH 7.4
pH 7.5	pH 7.5	

Table S4: Purification buffers for $P450_{Sp\alpha}$

$P450_{Sp\alpha}$		
Lysis buffer:	Elution buffer:	Storage buffer:
100 mM KPi	100 mM KPi	100 mM KPi
300 mM NaCl	300 mM NaCl	15% glycerol
10 mM imidazole	250 mM imidazole	pH 7.4
1 mM PMSF	0.8% (w/v) cholate	
0.8% (w/v) cholate	15% glycerol	
15% glycerol	pH 7.5	
pH 7.5		

Pellets were resuspended in lysis buffer [10 mL per g pellet; $P450_{CLA}$, $P450_{J\alpha}$, $P450_{Ex\alpha}$, CYP152K6 and $P450_{BS\beta\ F79L/G290F}$: 300 mM KCl, 100 mM KPi and 50 mM imidazole pH 7.5 (degassed); $P450_{Sp\alpha}$: 100 mM NaCl, 100 mM KPi, 10 mM imidazole, 0.8% *w/v* cholate, 1 mM PMSF and 15% *v/v* glycerol pH 7.5 (degassed)]. For $P450_{Sp\alpha}$ samples were incubated for two hours in lysis buffer. Sonication was performed on ice [(30% amplitude, 2 seconds pulse on, 4 seconds pulse off for 2 minutes) x 2]. The insoluble fraction was removed by centrifugation at 4 °C for 20 minutes at 18000 rpm. For SDS-PAGE analysis a pellet sample was taken and dissolved in urea (900 µL, 6 M). The lysate was sterile filtered (0.45 µM Rotilabo® syringe filter).

Protein purification was conducted with a His-TrapTM column (5 mL) connected to a FPLC pumping system column (5 mL; GE Healthcare; Chicago, Illinois, United States). In the following the purification of $P450_{Sp\alpha}$ is described in detail and all the used buffers are listed in **Table S3** and **Table S4**.

For the lysate of 990 mL medium the column was washed with dH_2O (80 mL, filtered and degassed) and equilibrated with the respective lysis buffer (100 mL). After loading of the cleared lysate (70 mL), elution was conducted using an elution buffer (50 mL, until red color stopped) [$P450_{Sp\alpha}$: 100 mM NaCl, 100 mM KPi, 250 mM imidazole, 0.8% *w/v* cholate and 15% *v/v* glycerol, pH 7.5 (degassed)]. A cleaning buffer was used to clean the column of residual bound proteins with higher imidazole concentration [50 mL, 300 mM KCl, 100 mM KPi, 500 mM imidazole pH 7.5 (degassed)]. The column was then washed with dH_2O (50 mL) and stored in a 20% (v/v) EtOH solution at 4 °C. Sample and buffers were applied with a flow rate of 1-5 mL/min. Eluted fraction were collected and concentrated in VIVASPIN tubes (20 mL; Membrane: 10 000 MWCO PES; at 4 °C at 4000 rpm). After concentration, samples were desalted (PD-10 Desalting Columns, GE Healthcare), eluted in the respective storage buffer ($P450_{Sp\alpha}$: 100 mM KPi, 15% *v/v* glycerol, pH 7.4,) and stored at 4 °C.

Determination of P450 protein concentration based on CO-titration

Due to the high affinity of the Fe^{II} atom to CO, the concentration of the P450 enzymes can be measured. The protein sample (200 µL or 100 µL) was diluted with the respective buffer (1:5 or 1:10). Afterwards the samples were flushed with CO for 30 sec. The sample (1 mL) was blanked on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) (wavelength-range measured: 400-540 nm) and afterwards a spatula tip of sodium dithionate was added. The sample was measured again (for five minutes in steps of 30 seconds) and by applying the Lambert– Beer law the concentration of P450 enzyme could be calculated (**Table S5**).

$$c = \frac{Abs\ (448\ nm) - Abs\ (500\ nm)}{\varepsilon * d} * f$$

c = concentration [µM]
ε = molar attenuation coefficient [91 mM^{-1} cm^{-1} (for P450s)]
d = path length [1 cm] (standard cuvette)
f = dilution factor

Table S5: Active enzyme concentration of all P450 enzymes (after using the cell pellet of 3x 300 mL for enzyme purification) that were measured via CO titration: [a]Enzymes were stored in 100 mM KPi, 300 mM KCl, pH 7.4 ($P450_{Sp\alpha}$: 100 mM KPi, 15% glycerol, pH 7.4); [b]concentration of 3.5 mL enzyme solution ($P450_{Ex\alpha}$: 2x 3.5 mL solution)

Enzyme[a]	Concentration[b] [µM]	Concentration [mg/ mL]
$P450_{CLA}$	242	11.6
$P450_{Sp\alpha}$	35	1.7
$P450_{J\alpha}$	105	5
$P450_{Ex\alpha}$	209/ 228	10/ 10.9
CYP152K6	187	9
$P450_{Bs\beta\ F79L/G290F}$	215	10.3

SDS PAGE

For SDS PAGE prefabricated gels were used (GenScript,12% and 15 wells) and Tris-MOPS-SDS Running buffer (50 mM Tris Base, 50 mM MOPS, 0.1% SDS, 1 mM EDTA, pH 7.5, GenScript). The supernatant samples were mixed with the same volume Laemmli loading buffer 2x and heated (at 95 °C for five minutes) in a thermo shaker and loaded (total volume) in each slot. As reference, Page RulerTM Prestained Protein Ladder (5 µL) was loaded. The gels were run at 120 V. After that, the gels were stained with Colloidal Coomassie "Blue Silver" staining solution (58.82 mL 85% ortho- phosphoric acid, 50 g ammonium sulphate, 0.6 µg Coomassie G-250R, 100 mL EtOH, 500 mL deionized H_2O) for 2 hours. The gels were destained overnight in deionized H_2O and documented by scanning.

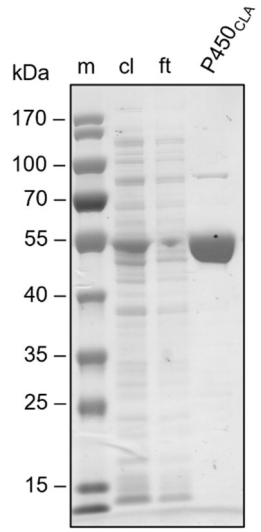

Fig. S1: SDS PAGE of the selected fractions before and after His-tag purification of $P450_{CLA}$: m = marker, cl = filtered cell lysate, ft = flow through, $P450_{CLA}$ after His-tag affinity chromatography and re-buffering.

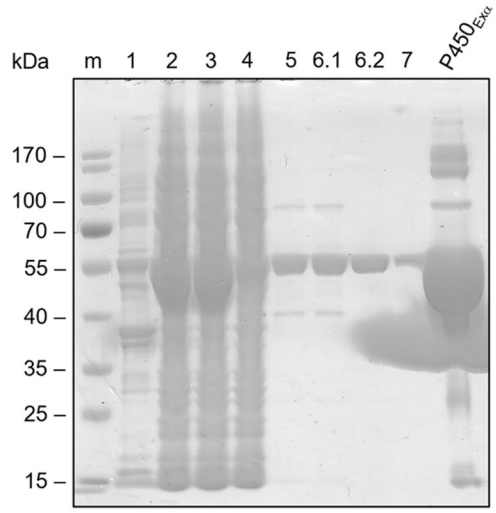

Fig. S2: SDS PAGE after His-tag purification of $P450_{Ex\alpha}$: m= marker; 1 cell pellet after harvesting; 2 cell lysate after sonification; 3 cell lysate after sterile filtration;4 fraction after loading onto column; 5 fraction of the flow through; 6.1/6.2 elution fraction; 7 column wash with cleaning buffer and purified $P450_{Ex\alpha}$ obtained after His-tag affinity chromatography and re-buffering.

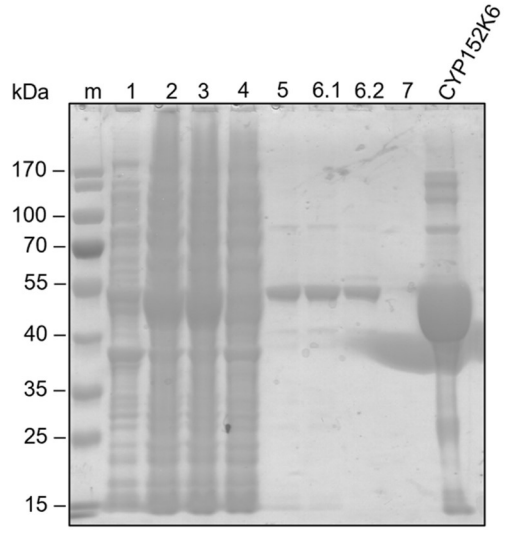

Fig. S3: SDS PAGE after His-tag purification of CYP152K6: m= marker; 1 cell pellet after harvesting; 2 cell lysate after sonification; 3 cell lysate after sterile filtration;4 fraction after loading onto column; 5 fraction of the flow through; 6.1/6.2 elution fraction; 7 column wash with cleaning buffer and purified CYP152K6 obtained after His-tag affinity chromatography and re-buffering.

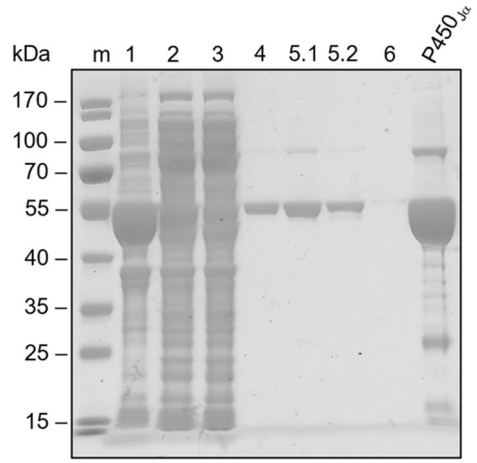

Fig. S4: SDS PAGE after His-tag purification of $P450_{J\alpha}$: m= marker; 1 cell pellet after harvesting; 2 cell lysate after sonification; 3 fraction after loading onto column; 4 fraction of the flow through; 5.1/5.2 elution fraction; 6 column wash with cleaning buffer and purified $P450_{J\alpha}$ obtained after His-tag affinity chromatography and re-buffering.

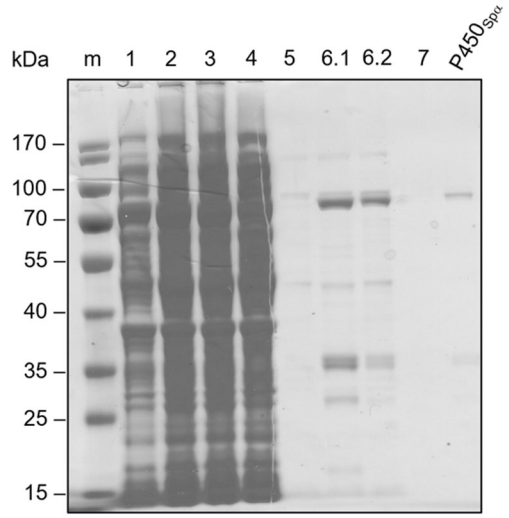

Fig. S5: SDS PAGE after His-tag purification of $P450_{Sp\alpha}$: m= marker; 1 cell pellet after harvesting; 2 cell lysate after sonification; 3 cell lysate after sterile filtration;4 fraction after loading onto column; 5 fraction of the flow through; 6.1/6.2 elution fraction; 7 column wash with cleaning buffer and purified $P450_{Sp\alpha}$ (2 µg was loaded) obtained after His-tag affinity chromatography and re-buffering.

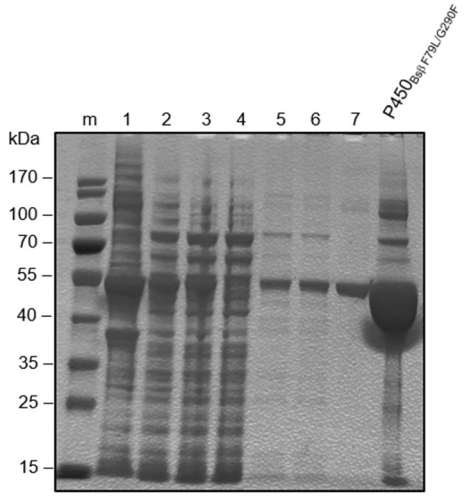

Fig. S6: SDS PAGE after His-tag purification of $P450_{Bs\beta\ F79L/G290F}$: m= marker; 1 cell pellet after harvesting; 2 cell lysate after sonification; 3 cell lysate after sterile filtration;4 fraction after loading onto column; 5 fraction of the flow through; 6 elution fraction; 7 column wash with cleaning buffer and purified $P450_{Bs\beta\ F79L/G290F}$ obtained after His-tag affinity chromatography and re-buffering.

α-Hydroxylation of fatty acids with CYP152 and *Hsp*UPO

Fatty acid substrates **1a**, **1b**, **1c**, **3a** and **3b**, [50 µL of stock solution [200 mM in EtOH for the biotransformations with CYP152 or MeCN for the biotransformations with *Hsp*UPO] were mixed with enzyme solution [950 µL of 3 µM or 7 µM of CYP152 or *Hsp*UPO in KPi buffer (100 mM, pH 7.4) respectively]. The reactions were performed in 1.5 mL glass grimp vials (21 °C, 400 rpm and for 15 h) containing the reaction mixture in a final volume of 1 mL. Syringes (1 mL) were used to continuously add H_2O_2 [1.6 mM/h (5 µL/h) over 12 hours to a final concentration of 20 mM (stock: 320 mM H_2O_2) for CYP152s and [1.25 mM/h (5 µL/h) over 12 hours to a final concentration of 15 mM (stock: 250 mM) for *Hsp*UPO]. The reactions were stopped with aqueous 5 M HCl (100 µL). The reaction suspension was extracted (2x 500 mL EtOAc with 5 mM lauric acid) and the organic phase was dried over Na_2SO_4. After centrifugation the dried organic phase (100 µl) was mixed with 1:1 BSTFA/TMCS: pyridine (200 µl) and the samples were incubated at room temperature for two hours. For biotransformation with **3a** and **3b** samples were incubated at 50 °C for 1 h and all samples were used for GC analysis.

GC-MS measurement was carried out on a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA), equipped with a 5975C mass selective detector and an HP-5MS column (5% phenylmethylsiloxane, 30 m x 320 µm, 0.25 µm film, J&W Scientific, Agilent Technologies) using helium as carrier gas. Following program was used: injector temperature: 250 °C; injection volume: 1 µL; flow rate: 0.7 mL/min; temperature program 1: 100 °C, hold 0.5 min, 10 °C/min to 300 °C.

For GC FID (on achiral phase) measurements were performed on an Agilent Technologies 7890 A GC system equipped with an FID detector and a 7693A Injector in combination with a 7693 Series Autosampler and using an HP-5 column (30 m x 320 µm, 0.25 µm film, J&W Scientific, Agilent Technologies) using helium as carrier gas. For **1a, 1c**, **3a** and **3b** the following GC-FID (on achiral phase) method was used: injector temperature 250 °C; detector 300 °C injection volume: 5 °µL; flow rate: 1.19 mL/min; temperature program: 1: 100 °C, hold 0.5 min, 10 °C/min to 300 °C, postrun: 325 °C. For **1b** the following GC-FID (on achiral phase) method was used: injector temperature 250 °C; detector 300 °C injection volume: 5 µL; flow rate: 1.19 mL/min; temperature program: 70 °C/hold 0.5 min; 20 °C/min to 300 °C; hold 2 min, postrun: 325 °C.

Fig. S7: Reaction setup for small scale reaction (1 mL): H_2O_2 was added continuously via syringes to vials that were adjusted to a bench shaker.

Calculation of product concentration (Table 1)

To calculate product concentrations calibration curves for all analytes (**1a, 1b, 1c and α-2a, α-2b, α-2c**) were established. The peak area and concentration of the analytes are used to calculate the response for each analyte. The Relative Response Factor (RRF) values between the fatty acid substrates and the internal standard (ISD) were determined to calculate product concentrations. Lauric acid (5 mM) served as ISD. The response factor for β-OH was assumed to be the same as for α-OH.

The relative response factor (RRF) can be used to calculate the unknown concentration of the fatty acid substrates in the presence of a known concentration of the internal standard.

To determine the RRF between the fatty acid substrates, **1a**, **1b** and **1c**, and the internal standard (FA and ISD); calibration curves for all analytes were established (**Fig. 6**). Firstly, the peak area and concentration of the analytes are used to calculate the response for each analyte, as in Equation 1.

$$Response\ factor\ = \frac{Peak\ area}{Concentration}$$

The response factors calculated for each analyte were then used to establish the RRF between the FA and ISD as in Equation 2.

$$Relative\ Response\ Factor\ (RRF) = \frac{Response\ Factor\ FA}{Response\ Factor\ ISD}$$

RRF values (FAs)	
1a	0.561288
α-2a	0.737332
1b	0.70813
α-2b	0.943538
1c	0.87875
α-2c	1.09382

The RRF can be used to calculate the unknown concentration of the FAs in the presence of a known concentration of ISD, as in Equation 3.

$$c(FA) = d\left(\left(\frac{area(FA)}{area(ISD)}\right) \times \frac{1}{RRF} \times c(ISD)\right)$$

d = dilution factor (=2)

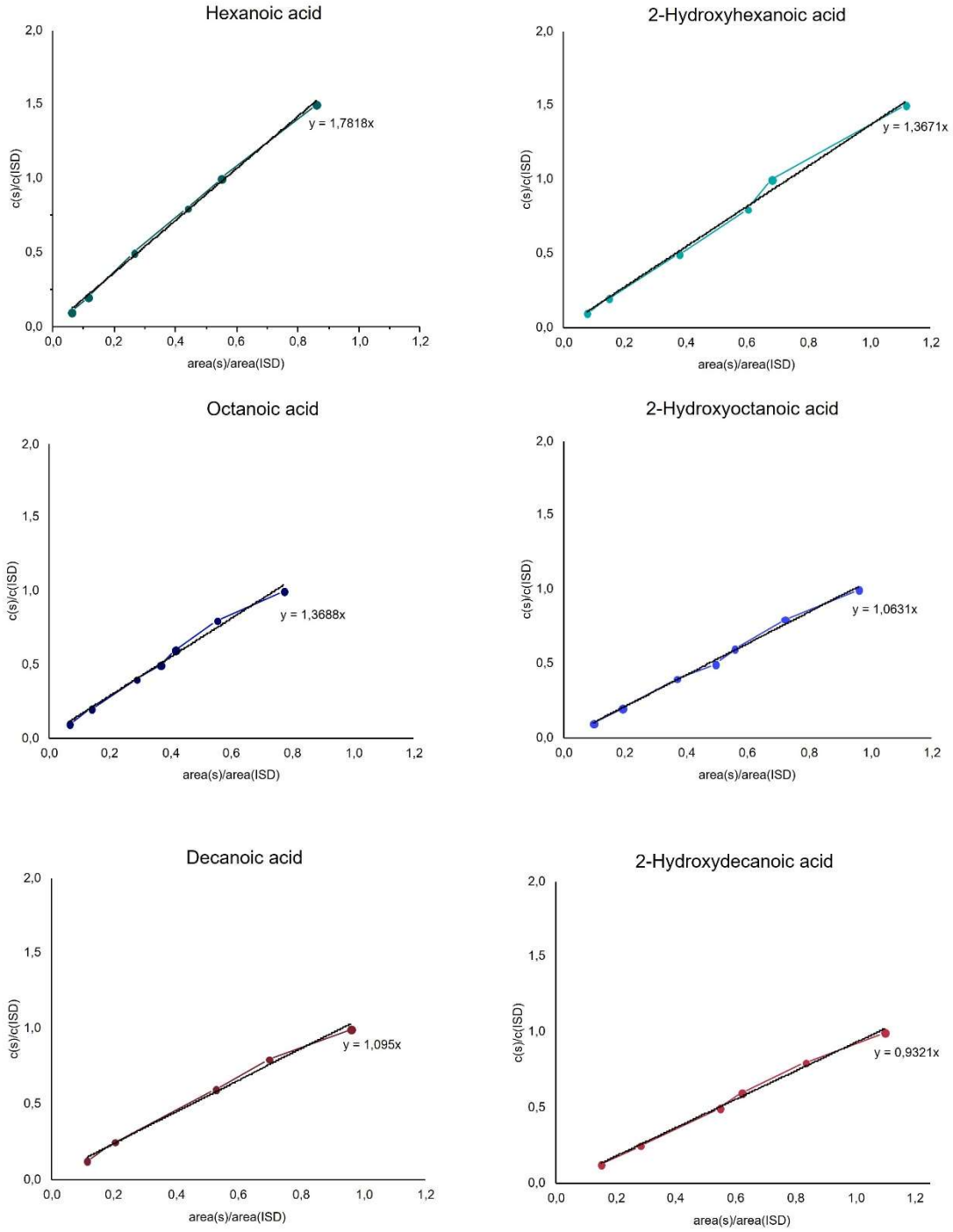

Fig. S8: Calibration curves of **1a**, **1b**, **1c** and **α-2a**, **α-2b**, **α-2c**: The respective standard was dissolved in EtOH (40 mM stock), for the calibration curves a serial dilution was performed in sample buffer (100 mM KPi, pH 7.4) (**1a** and **α-2a** 0.5 - 7.5 mM; **1b** and **α-2b** 0.5 – 5 mM and **1c** and **α-2c** 0.625 – 5 mM) and treated as described in "α-hydroxylation of fatty acids with CYP152 and *Hsp*UPO" for GC measurements.

Analysis of optical purity (Table 1)

After the biotransformation reaction samples had been extracted and the solvent was removed using a SpeedVac vacuum concentrator, the resulting residue was taken up in MeOH (700 µL) containing 5% DMAP, and ethyl chloroformate (150 µL) was added. The reaction was performed at 50 °C and 700 rpm for one hour. After solvent removal 2% aq. HCl (700 µL) was added and extraction was performed with EtOAc (2 x 500 µL). After drying of the combined organic phases over Na_2SO_4, samples were analyzed on GC-FID (chiral phase).

Retention times: Derivatized (*R*)-**α–2a**: 3.98 min; derivatized (*S*)- **α-2a**: 4.11 min; derivatized (*R*)- **α-2b**: 6.68 min; derivatized (*S*)- **α-2b**: 6.91 min; derivatized (*R*)- **α-2c**: 11.62 min; derivatized (*S*)- **α-2c**: 11.78 min.

GC measurements (on chiral phase) were performed on an Agilent Technologies 7890. A GC system equipped with an FID-detector and a 7693A Injector in combination with a 7693 Series Autosampler and using a Chirasil ChiralDexCB column (25 m x 320 µm, 0.25 µm film) using hydrogen as carrier gas. Injector temperature: 250 °C; injection volume: 1 µL; flow rate: 1.3 mL/min; detector temperature: 250 °C; temperature program: 100 °C, hold 1 min, 10 °C/ min to 130 °C, hold 5 min, 10 °C/min, 180 °C, hold 1 min.

GC-FID analysis for the conversion of **1a**,**1b** and **1c** with *Hsp*UPO

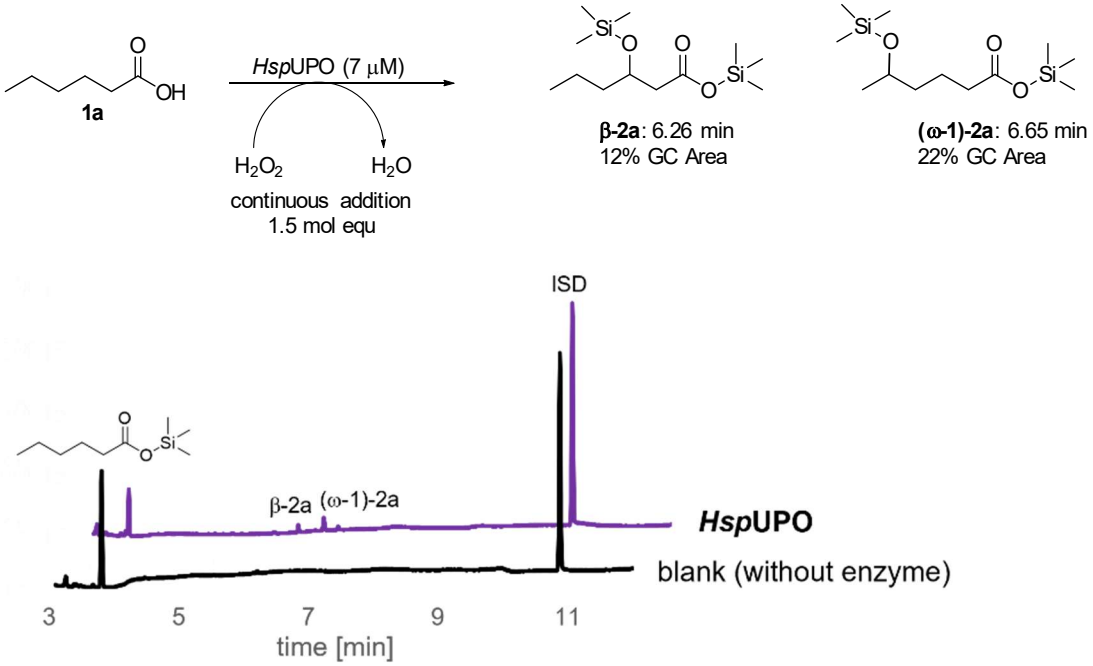

Fig. S9: Conversion of **1a** with *Hsp*UPO: Fatty acid was converted in 1.5 mL glass grimp vials (21 °C, 400 rpm and for 15 hours) containing reaction buffer (100 mM KPi buffer, pH 7.4) in a final volume of 1 mL, MeCN (5% v/v), fatty acid (10 mM) and enzyme (7 µM). H_2O_2 was added continuously via a syringe pump [1.25 mM/h (5 µL/h) over 12 hours to a final concentration of 15 mM (stock: 250 mM). The products are depicted with the corresponding retention time. GC traces are shown. On top the chromatogram of the enzyme reaction with *Hsp*UPO is shown and below the chromatogram of the blank reaction (without enzyme) is depicted. Ratios for monohydroxylated products were calculated based on GC-area. Lauric acid served as internal standard (ISD).

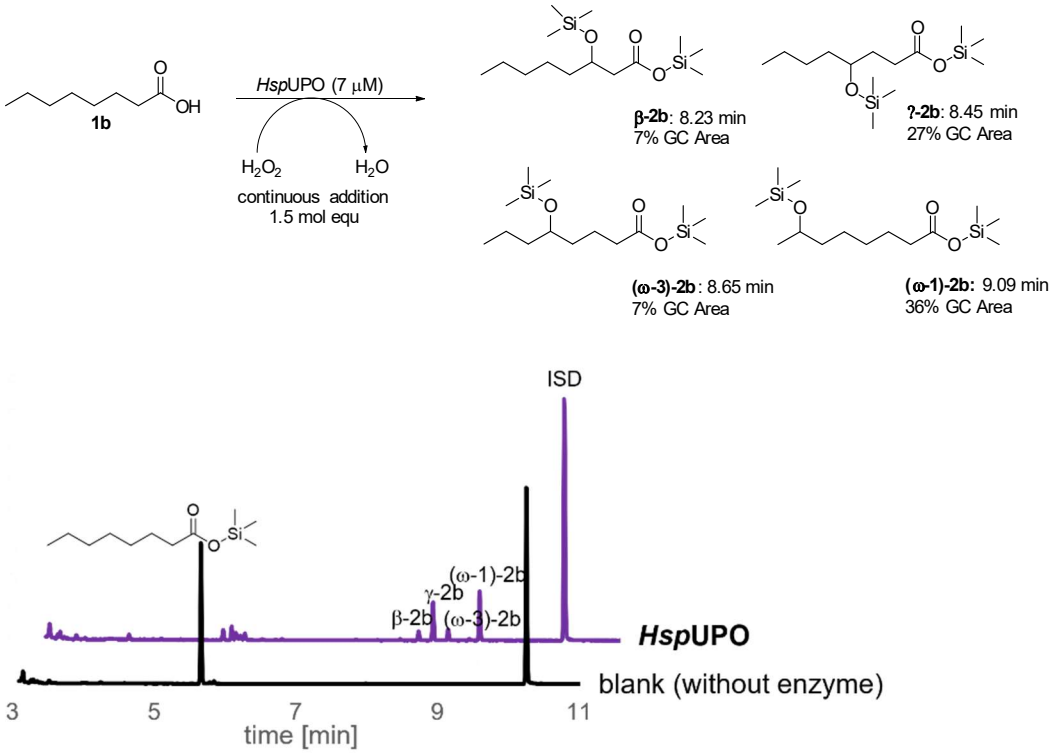

Fig. S10: Conversion of **1b** with *Hsp*UPO: Fatty acid was converted in 1.5 mL glass grimp vials (21 °C, 400 rpm and for 15 hours) containing reaction buffer (100 mM KPi buffer, pH 7.4) in a final volume of 1 mL, MeCN (5% v/v), fatty acid (10 mM) and enzyme (7 μM). H_2O_2 was added continuously via a syringe pump [1.25 mM/h (5 μL/h) over 12 hours to a final concentration of 15 mM (stock: 250 mM). The products are depicted with the corresponding retention time. GC traces are shown. On top the chromatogram of the enzyme reaction with *Hsp*UPO is shown and below the chromatogram of the blank reaction (without enzyme) is depicted. Ratios for monohydroxylated products were calculated based on GC-area. Lauric acid served as internal standard (ISD).

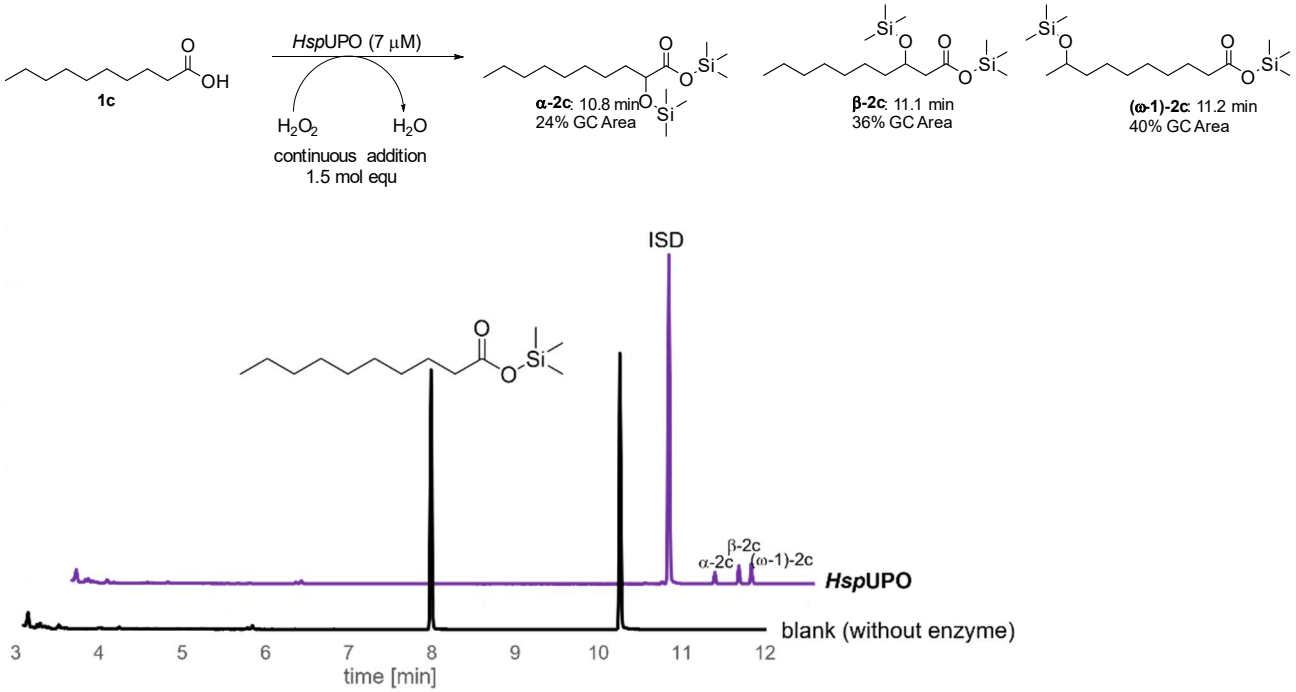

Fig. S11: Conversion of **1a**, **1b** and **1c** with *Hsp*UPO: Fatty acid was converted in 1.5 mL glass grimp vials (21 °C, 400 rpm and for 15 hours) containing reaction buffer (100 mM KPi buffer, pH 7.4) in a final volume of 1 mL, MeCN (5% v/v), fatty acid (10 mM) and enzyme (7 μM). H_2O_2 was added continuously via a syringe pump [1.25 mM/h (5 μL/h) over 12 hours to a final concentration of 15 mM (stock: 250 mM). The products are depicted with the corresponding retention time. GC traces are shown. On top the chromatogram of the enzyme reaction with *Hsp*UPO is shown and below the chromatogram of the blank reaction (without enzyme) is depicted. Ratios for monohydroxylated products were calculated based on GC-area. Lauric acid served as internal standard (ISD).

GC-FID analysis for the conversion of **1a**,**1b** and **1c** with P450 enzymes

GC analysis on achiral phase

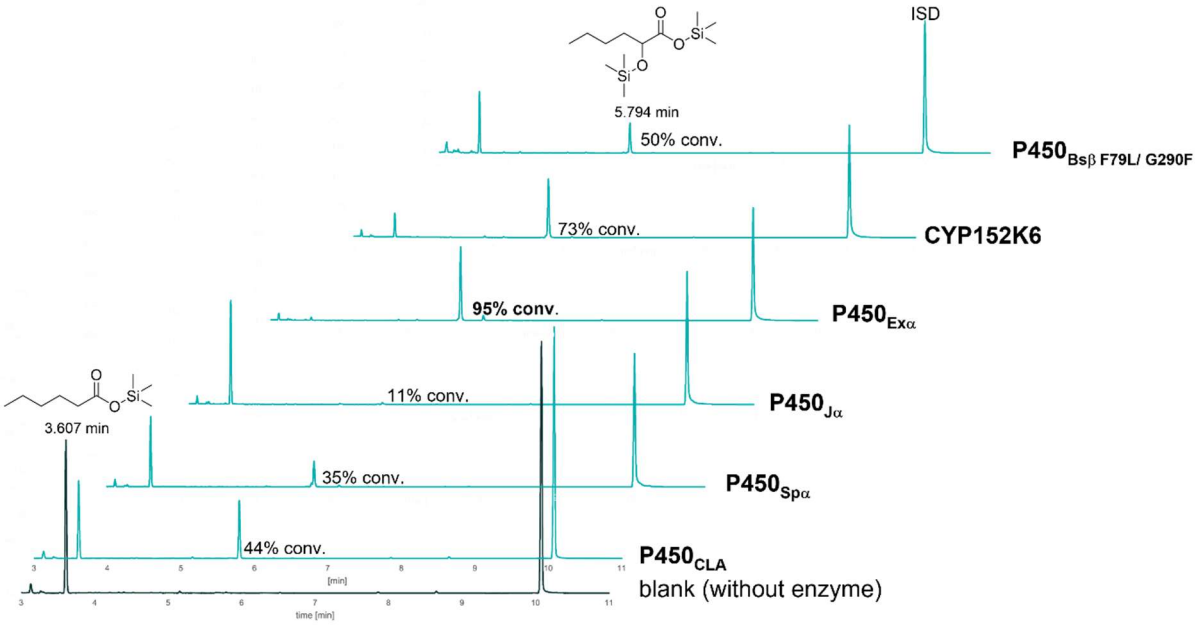

Fig. S12: Overlay of GC-FID (on achiral phase) chromatograms after conversion of **1a** with all CYP152 candidates: A blank reaction without enzyme was taken as comparison. Samples were derivatized with 1:1 BSTFA/TMCS: pyridine. Substrate and products are depicted with the corresponding retention time. The corresponding α-hydroxylated fatty acids or side products were only detected if the enzyme was added. Conversion [%] (i.e., consumption of substrate **1a**) was determined by GC using an int. standard (lauric acid) after derivatization.

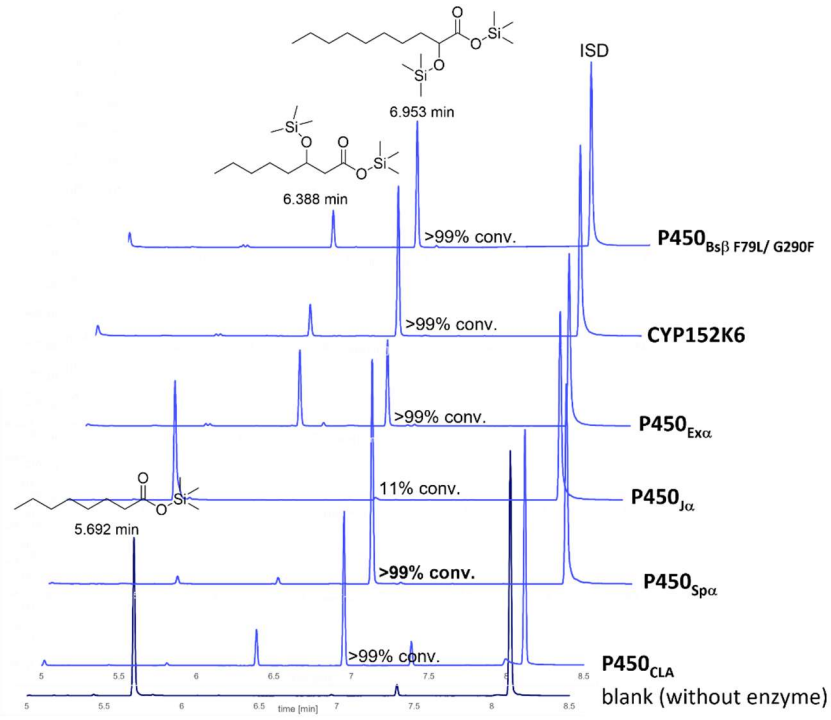

Fig. S13: Overlay of GC-FID (on achiral phase) chromatograms after conversion of **1b** with all CYP152 candidates: A blank reaction without enzyme was taken as comparison. Samples were derivatized with 1:1 BSTFA/TMCS: pyridine. Substrate and products are depicted with the corresponding retention time. The corresponding α-hydroxylated fatty acids or side products were only detected if the enzyme was added. Conversion [%] (i.e., consumption of substrate **1b**) was determined by GC using an int. standard (lauric acid) after derivatization.

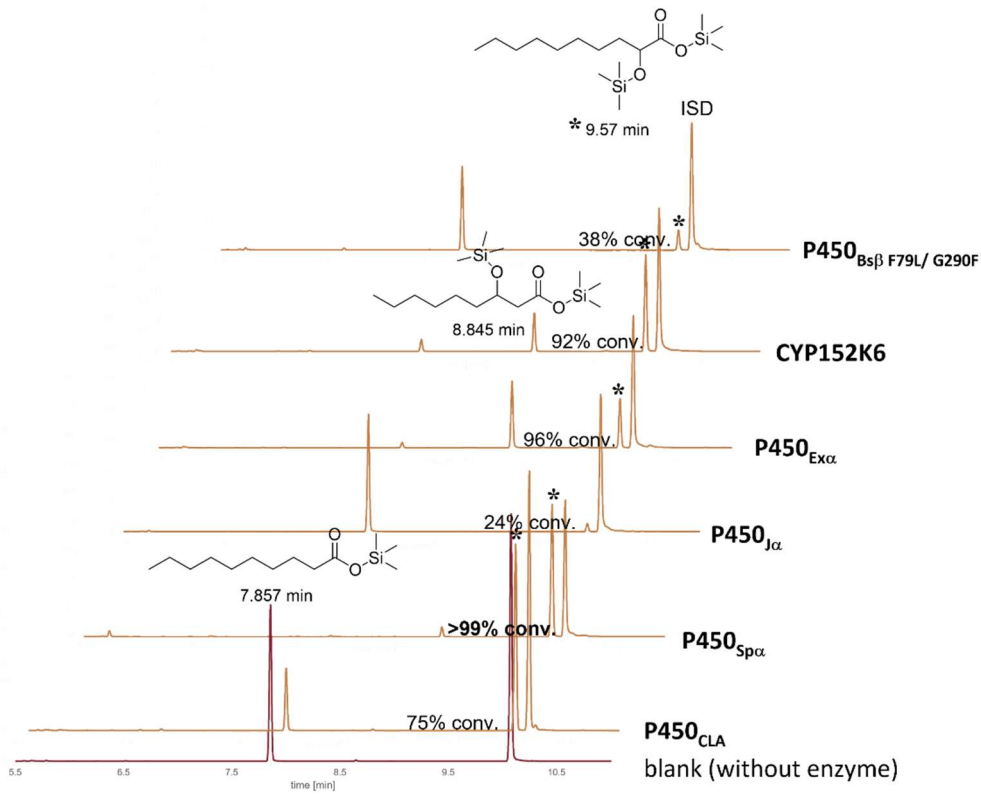

Fig. S14: Overlay of GC-FID (on achiral phase) chromatograms after conversion of **1c** with all CYP152 candidates: A blank reaction without enzyme was taken as comparison. Samples were derivatized with 1:1 BSTFA/TMCS: pyridine. Substrate and products are depicted with the corresponding retention time. The corresponding α-hydroxylated fatty acids or side products were only detected if the enzyme was added. Conversion [%] (i.e., consumption of substrate **1c**) was determined by GC using an int. standard (lauric acid) after derivatization.

GC analysis on chiral phase

Table S6: Determination of e.e. value using GC on chiral phase: e.e. values are given for the conversion of C6:0, C8:0 and C10:0 with all CYP152s is shown. *e.e. values are given in literature 71% e.e. (S)–α-2c and 36% e.e. (S)-α–2b[2]

CYP152s	Fatty acid	ee [%] α-OH
$P450_{CLA}$	**1a**	8 (*S*)
	1b	48 (*S*)*
	1c	62 (*S*)*
$P450_{Spα}$	**1a**	>99 (*S*)
	1b	>99 (*S*)
	1c	>99 (*S*)
$P450_{Exα}$	**1a**	81 (*S*)
	1b	>99 (*S*)
	1c	>99 (*S*)
CYP152K6	**1a**	93 (*S*)
	1b	>99 (*S*)
	1c	>99 (*S*)
$P450_{Bsβ\ F79L/G290F}$	**1a**	58 (*S*)
	1b	79 (*S*)
	1c	-

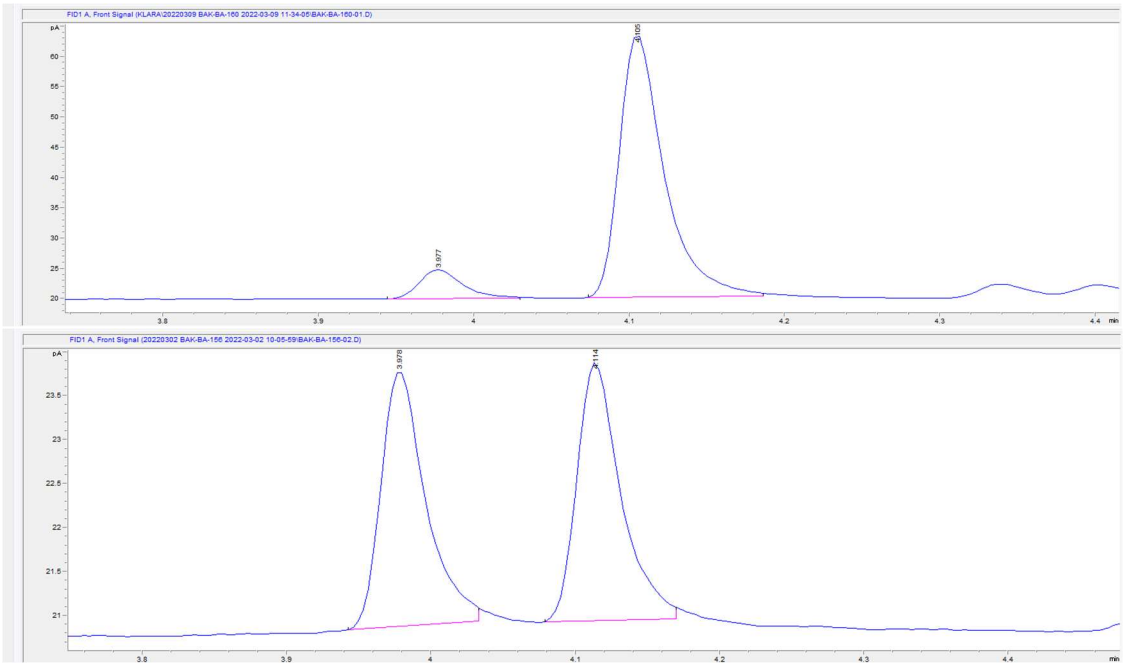

Fig. S15: GC-FID chromatogram on chiral phase for the conversion of **1a** with $P450_{Ex\alpha}$ (top): (*R*)-enantiomer at 3.977 min and *S*-enantiomer at 4.105 min. Bottom: racemic reference.

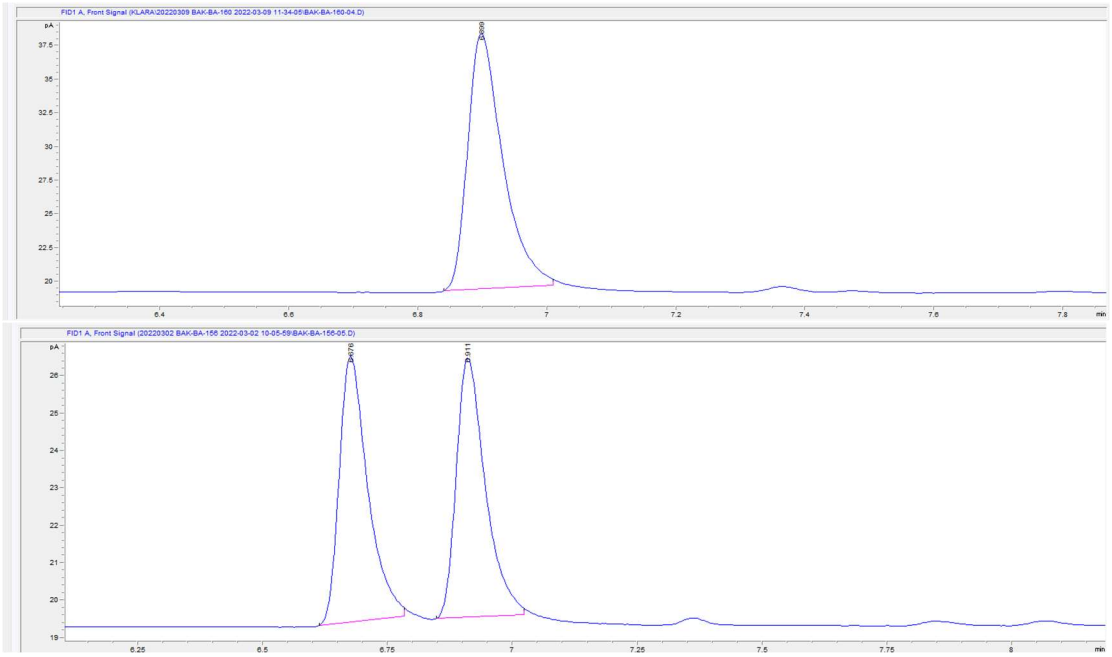

Fig. S16: GC-FID chromatogram on chiral phase for the conversion of **1b** with $P450_{Sp\alpha}$ [top, (*S*)-enantiomer at 6.899 min]. Bottom: racemic reference.

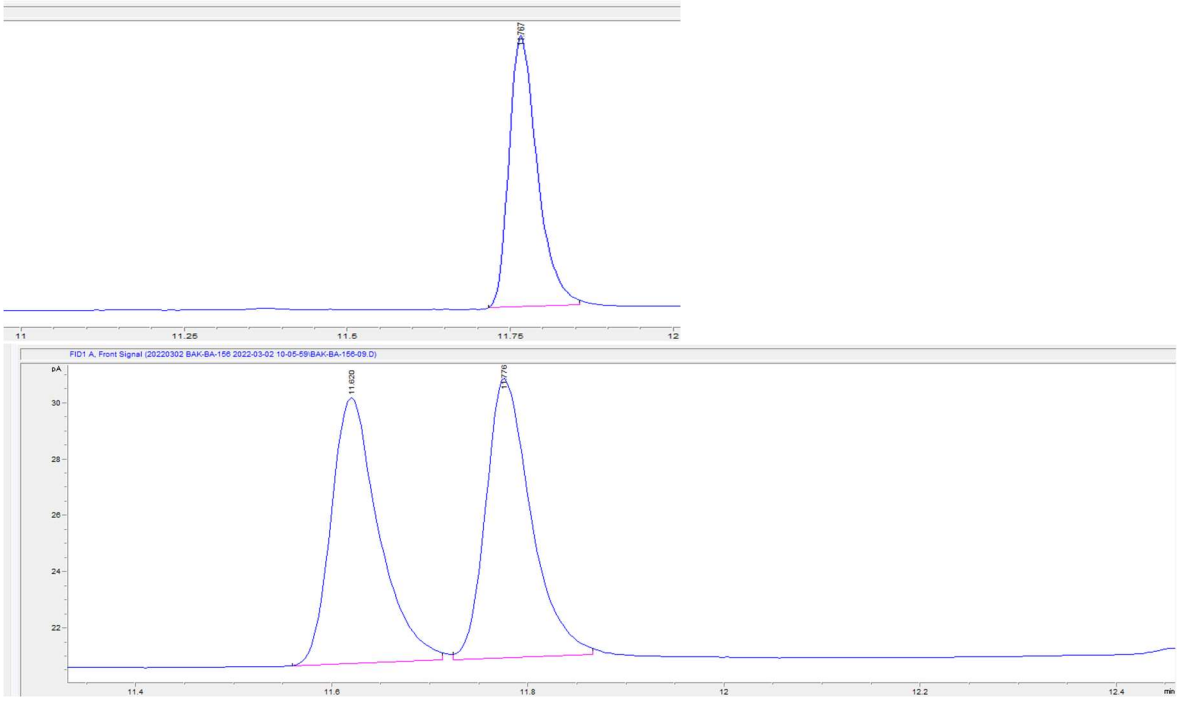

Fig. S17: GC-FID chromatogram on chiral phase for the conversion of **1c** with P450$_{Spα}$ [top, (*S*)-enantiomer after 11.764 min]. Bottom: racemic reference.

GC-FID analysis for the conversion of dicarboxylic acids with P450 enzymes

GC-FID analysis for the conversion of 3a and 3b with $P450_{Ex\alpha}$

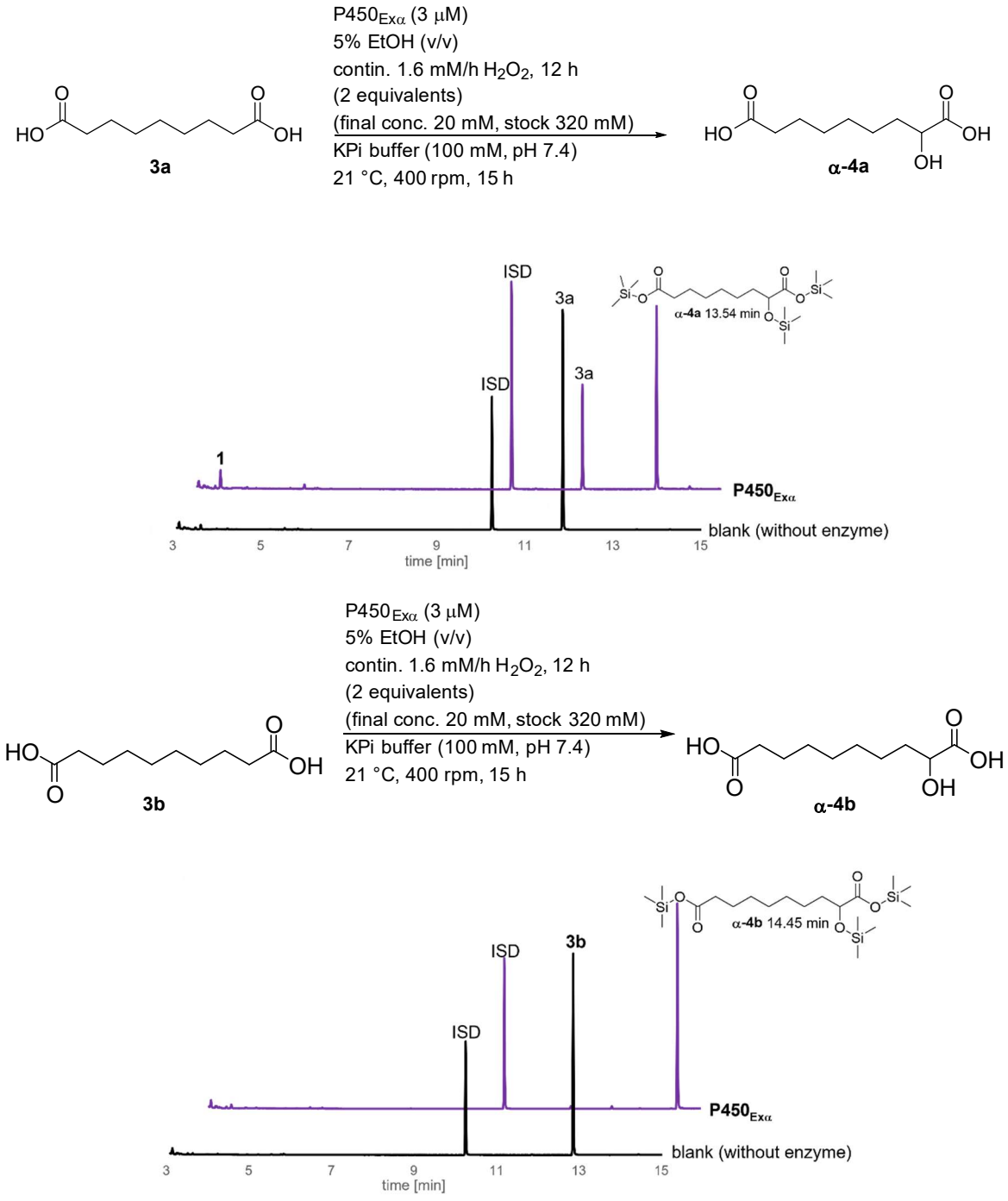

Fig. S18: Reaction conditions and GC chromatogram for the conversion of dicarboxylic acids with $P450_{Ex\alpha}$: Fatty acids were converted in 1.5 mL glass crimp vials (21 °C, 400 rpm and for 15 hours) containing reaction buffer (100 mM KPi buffer, pH 7.4) in a final volume of 1 mL, 5% EtOH (v/v), fatty acid (10 mM) and purified enzyme (3 μM). A blank reaction was carried out where no enzyme was added. **3a** and **3b** were converted to the corresponding monohydroxylated α-**4a** and α-**4b** with $P450_{Ex\alpha}$. The products are depicted with the corresponding retention time. Lauric acid served as internal standard (ISD).

GC-MS analysis

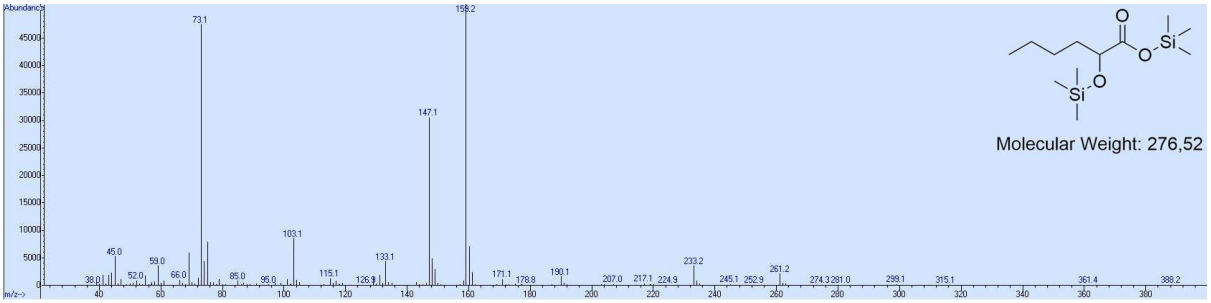

Fig. S19: GC-MS fragmentation of derivatized **α-2a**

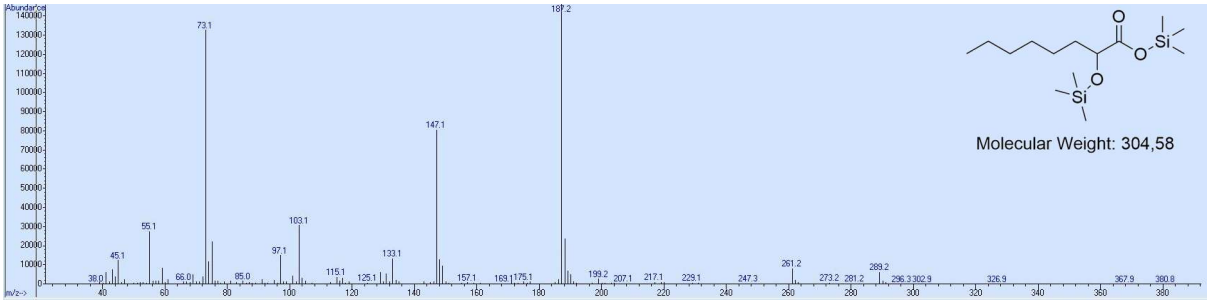

Fig. S20: GC-MS fragmentation of derivatized **α-2b**

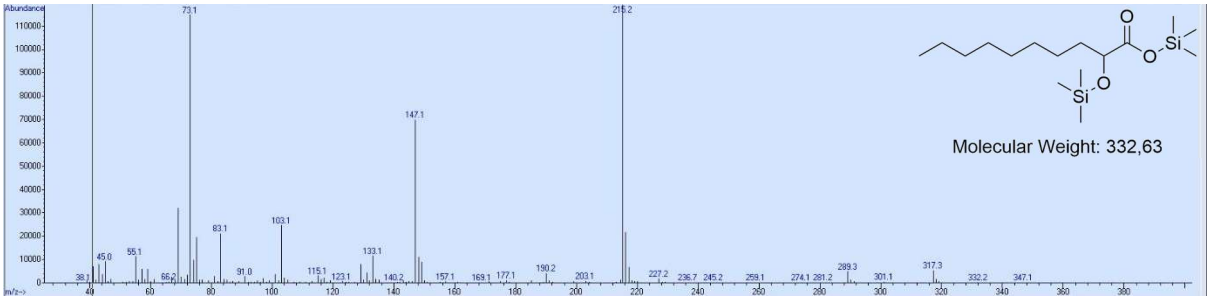

Fig. S21**:** GC-MS fragmentation of derivatized **α-2c**

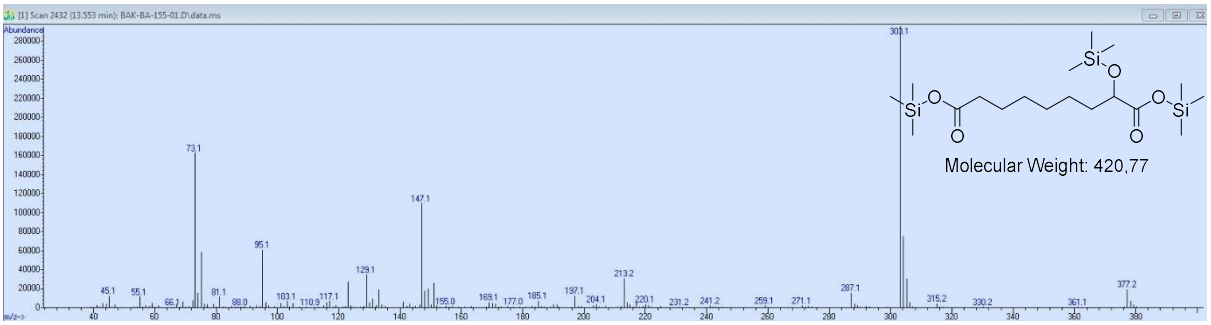

Fig. S22: GC-MS fragmentation of derivatized **α-4a**

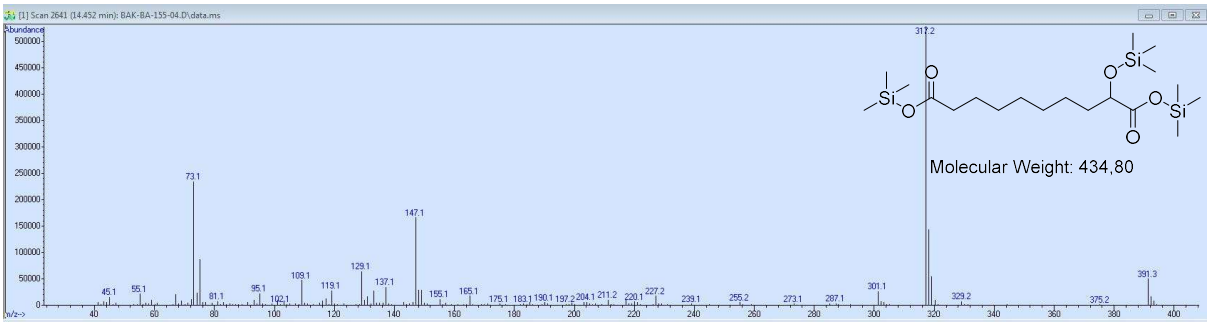

Fig. S23: GC-MS fragmentation of derivatized **α-4b**

Docking of **3a, 3b, 3d** and **3e** into the active site of $P450_{Ex\alpha}$ (pdb: 5YH1)

Docking simulations were executed using the YASARA Structure software,[12] incorporating the AutoDock Vina[13] algorithm. Energy minimization with a YASARA structure utilizing the AMBER03 Force Field and the standard YASARA minimization protocol was used for the ligands prior to docking. The simulation cell was defined at 4 Å around the iron atom of the heme and 100 docking trials were run. The results were visualized via the PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC.

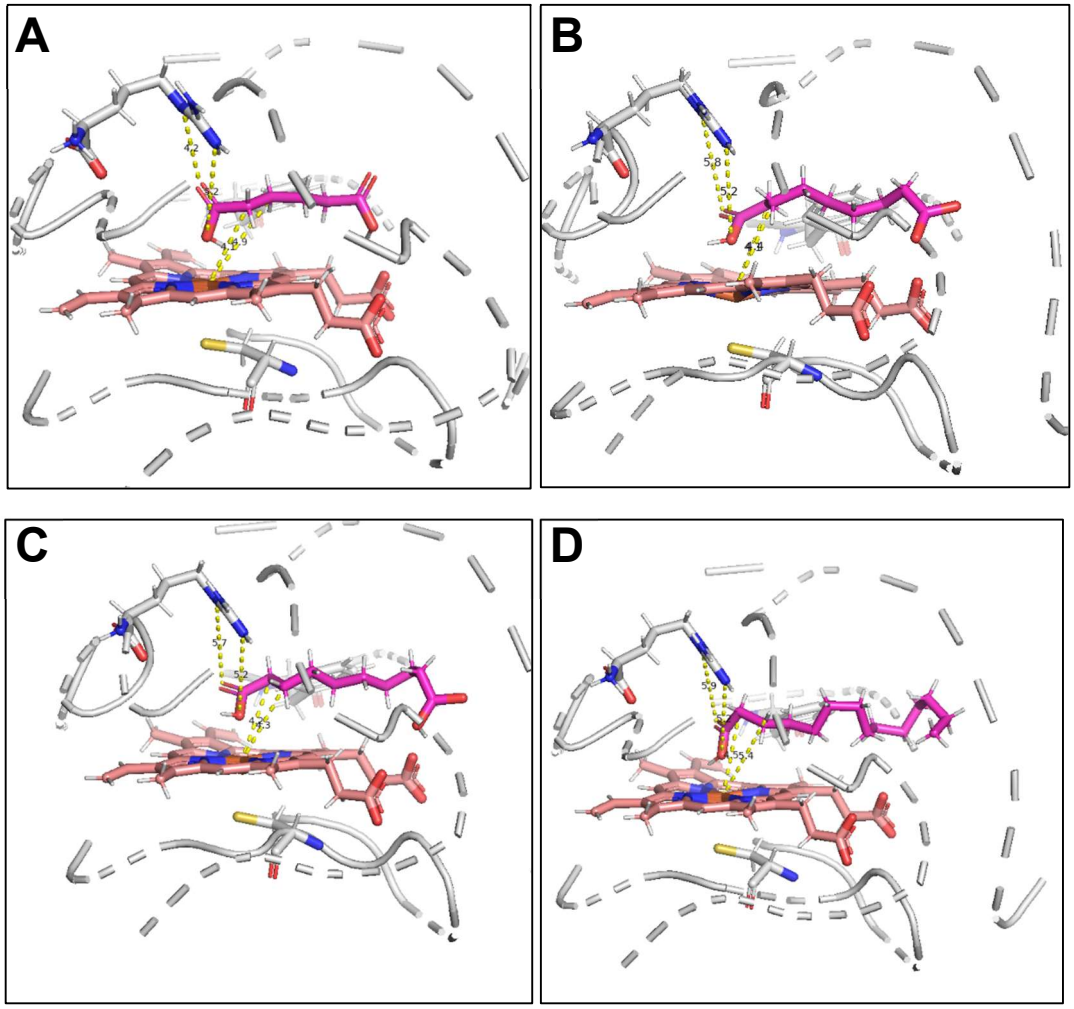

Fig. S24: Docking of **3e** (**A**), **3a** (**B**), **3b** (**C**) and **3e** (**D**) to the active site of $P450_{Ex\alpha}$. Salt bridges are shown in yellow.

Preparative scale transformations of 1b, 1c and 3b with $P450_{Sp\alpha}$ and $P450_{Ex\alpha}$

Table S7: Substrate and H_2O_2 concentrations for preparative scale transformations of **1b** with $P450_{Sp\alpha}$

Entry	1b	$[H_2O_2]$ stock	H_2O_2 addition/h	Final $[H_2O_2]$
1	10 mM	320 mM	1.6 mM/h	20 mM
2	50 mM	320 mM	8.3 mM/h	100 mM
3	100 mM	400 mM	12.5 mM/h	150 mM
4	150 mM	400 mM	12.5 mM/h	150 mM

Table S8: Substrate and H_2O_2 concentrations for preparative scale transformations of **1c** with $P450_{Sp\alpha}$

Entry	1c	$[H_2O_2]$ stock	H_2O_2 addition/h	Final $[H_2O_2]$
1	10 mM	320 mM	1.6 mM/h	20 mM

Table S9: Substrate and H_2O_2 concentrations for preparative scale transformations of **3b** with $P450_{Ex\alpha}$

Entry	3b	$[H_2O_2]$ stock	H_2O_2 addition/h	Final $[H_2O_2]$
1	10 mM	320 mM	1.6 mM/h	20 mM
2	50 mM	320 mM	8.3 mM/h	100 mM

In the following a representative procedure for the preparative scale transformations is given (**Table S4** entry 3). In **Table S4-S6** all substrate and H_2O_2 concentrations for preparative scale transformations of **1b**, **1c** and **3b** are listed.

Fatty acid substrate solution of **1b** [100 mM, (2 M stock solution in EtOH)] was prepared in KPi buffer (100 mM, pH 7.4) in a 250 mL reaction flask for a final volume of 50 mL. The pH was adjusted to 7.4 with aqueous 1 M KOH. Next, a stock solution of H_2O_2 was prepared by dissolving 35% concentrated H_2O_2 with the appropriated amount of dH_2O. The syringe pump was installed (20 mL syringe) and the flow rate was set to reach a final H_2O_2 over 12 hours [12.5 mM/h to a final concentration of 150 mM (stock: 400 mM)].

$P450_{Sp\alpha}$ solution [enzyme solution in KPi buffer 100°mM, pH 7.4] was added to the substrate solution to give a 3 μM enzyme solution. The reaction was initialized by starting a syringe pump and switching on the magnetic stirrer (speed adjusted accordingly – 700 rpm).

The reactions were stopped with aqueous 5 M HCl (5 mL) and centrifuged (4000 rpm, 40 min). The reaction suspension was extracted (3-4x 100 mL EtOAc) and the organic phase was washed with brine (200 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure at 40 °C. The white solid was dried under vacuum to yield **α-2b** [see Table 2 entry 1: 99% (80 mg); entry 2: 89%, (401 mg)], and **α-4b** [entry 6: 29% (26 mg)].

For entry 3 please look up "Purification of **2b** after re-extraction in heptane".

^{1}H NMR **α-2b** (300 MHz, MeOD) δ [ppm] = 4.08 (dd, 1H, *J* = 7.6, 4.5 Hz, C<u>H</u>OH), 1.81 – 1.55 (m, 2H, C<u>H$_2$</u>CHOH) 1.48 – 1.25 (m, 8H, C$_4$<u>H$_8$</u>), 0.89 (t, 3H, *J* = 7.5 Hz, C<u>H$_3$</u>)

^{13}C NMR **α-2b** (75 MHz, MeOD) δ [ppm] = 181.25, 78.39, 36.70, 32.89, 30.15, 25.53, 22.94, 12.96.

^{1}H NMR **α-2c** (300 MHz, MeOD) δ [ppm] = 4.05 (dd, 1H, *J* = 7.6 Hz, 4.5 Hz, C<u>H</u>OH), 1.77 – 1.52 (m, 2H, C<u>H$_2$</u>CHOH), 1.44 – 1.19 (m, 12H, C$_6$<u>H$_2$</u>), 0.85 (t, 3H, *J* = 7.5 Hz, C<u>H$_3$</u>).

^{13}C NMR **α-2c** (75 MHz, MeOD) δ [ppm] = 178.29, 71.71, 35.69, 33.32, 30.88, 30.76, 30.66, 26.40, 24.01, 14.72.

^{1}H NMR **α-4b** (300 MHz, MeOD) δ [ppm] = 4.02 (dd, 2H, *J* = 7.6, 4.4 Hz, C<u>H</u>OH,), 2.20 (t, 2H, *J* = 7.4 Hz, C<u>H$_2$</u>CHOH), 1.76 – 1.44 (m, 4H), 1.44 – 1.17 (m, 8H).

^{13}C NMR **α-4b** (75 MHz, MeOD) δ [ppm] = 178.29, 177.99, 71.69, 35.65, 35.21, 30.60, 30.56, 30.42, 26.34.

Fig. S25: Reaction setup for the preparative scale reaction on 50 mL scale: H_2O_2 was added continuously via a syringe to the reaction mixture in a 250 mL reaction flask. The reaction mixture was stirred using a magnet stirrer.

Purification of α-2b after re-extraction in heptane (adapted procedure from [14])

The residue after extraction of the biotransformation (Table 2 Entry 3, 100 mM **1b**) was reconstituted in *n*-heptane at 40 °C (approximately 5 mL/g crude solid). The clear, yellowish solution was then sealed in a round-bottom flask and placed at -21 °C for 1 hour. In parallel, a bottle with *n*-heptane was cooled to -21 °C. After that time at -21 °C, a thick, white slurry was formed. Most of the substance was taken out with a spoon-like spatula and placed on a Buechner funnel with a filter paper, and vacuum was applied to drain. The flask and the spoon were washed with ice-cold *n*-heptane (3 × 2 mL), and the suspension was transferred into the funnel with a Pasteur pipette to wash the filter cake. The filtrate was concentrated approximately 2× under reduced pressure (40 °C, 100 mbar) and again placed at -21 °C to precipitate the next fraction. Alternatively, the concentrated filtrate was aliquoted to 2 mL microcentrifuge tubes which were cooled down to -21 °C and centrifuged in a microcentrifuge for 30 sec at 4 °C and 14k rpm to separate the precipitated product. The supernatant was decanted and the solid was washed at -21 °C with *n*-heptane (2 × 500 µL) followed by centrifugation as before. All supernatant fractions were combined, concentrated under reduced pressure and the process was repeated until no precipitate formed upon cooling. The collected solid material was dried in a desiccator over anhydrous $CaCl_2$ overnight to yield **α-2b** [Table 2 Entry 3; 87%, (1260 mg) 90% e.e.].

Table S10: GC-MS composition after work-up and after re-extraction in heptane:

	GC-MS compositions [%]		
Compound	**1b** (5.81 min)	Glycerol (6.89 min)	**α-2b** (7.95 min)
Extract after work-up	10.9	0.7	88.4
Solid obtained after washing with heptane and filtration	0.2	0.5	99.4

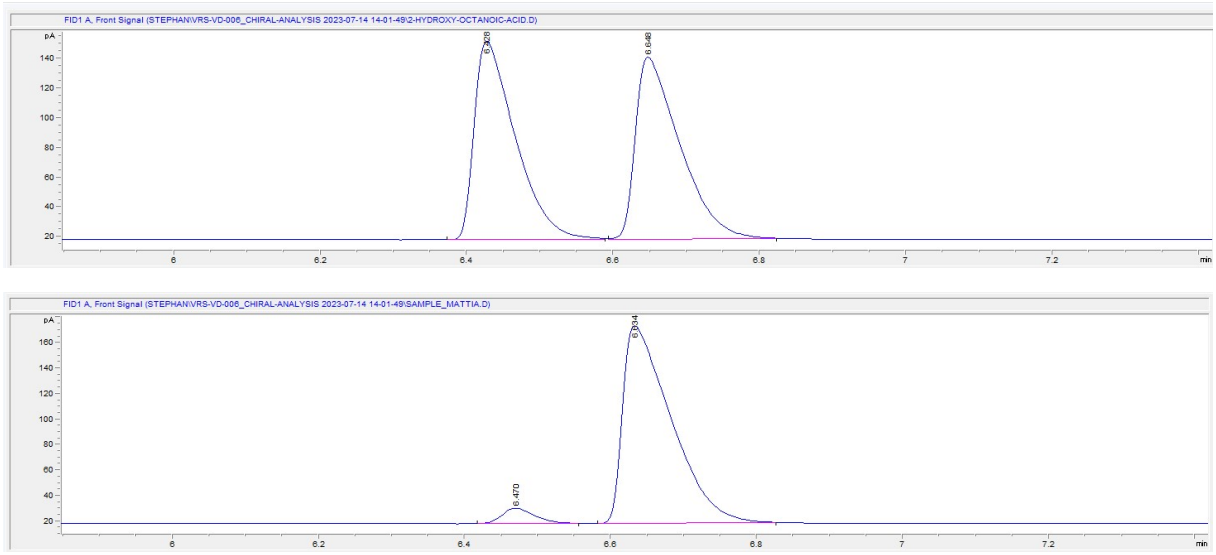

Fig. S26: GC-FID chromatogram on chiral phase for the conversion of **1b** with $P450_{Spα}$: (*S*)-enantiomer after 6.63 min.

^{1}H- and ^{13}C-NMR analysis

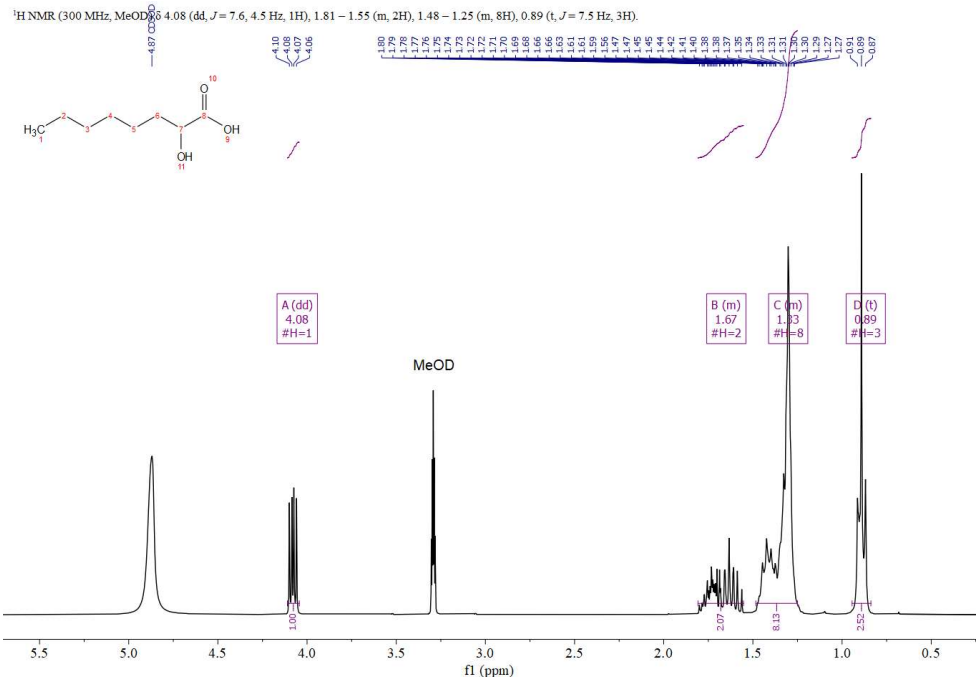

Fig. S27: ^{1}H NMR **α-2b** (300 MHz, MeOD) δ [ppm] = 4.08 (dd, 1H, J = 7.6, 4.5 Hz, C<u>H</u>OH), 1.81 – 1.55 (m,2H, C<u>H$_2$</u>CHOH) 1.48 – 1.25 (m, 8H, C$_4$<u>H$_8$</u>), 0.89 (t, 3H, J = 7.5 Hz, C<u>H$_3$</u>)
Data in accordance with literature.[15]

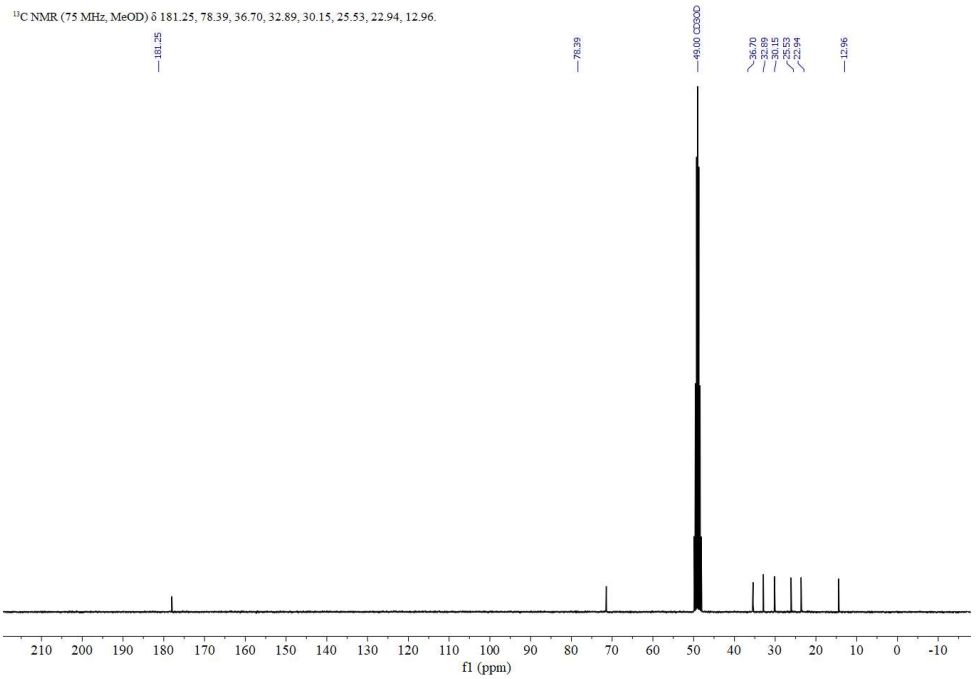

Fig. S28: ^{13}C NMR **α-2b** (75 MHz, MeOD) δ [ppm] = 181.25, 78.39, 36.70, 32.89, 30.15, 25.53, 22.94, 12.96.
Data is in accordance with literature.[15]

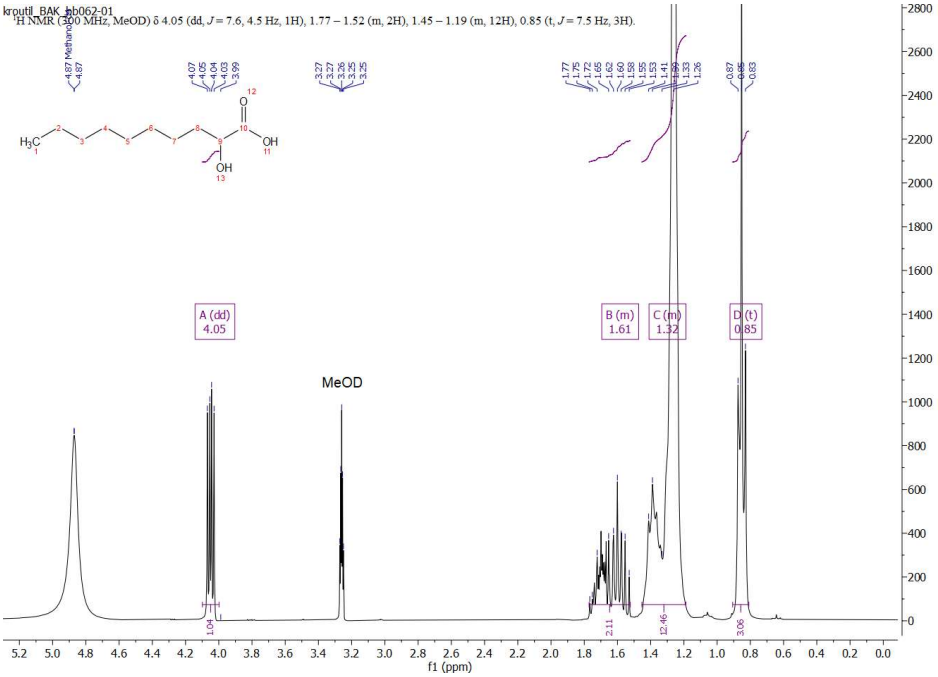

Fig. S29: ^{1}H NMR **α-2c** (300 MHz, MeOD) δ [ppm] = 4.05 (dd, 1H, J = 7.6 Hz, 4.5 Hz, C<u>H</u>OH), 1.77 – 1.52 (m, 2H, C<u>H$_2$</u>CHOH), 1.44 – 1.19 (m, 12H, C$_6$<u>H$_2$</u>), 0.85 (t, 3H, J = 7.5 Hz, C<u>H$_3$</u>).
Data is in accordance with literature.[15]

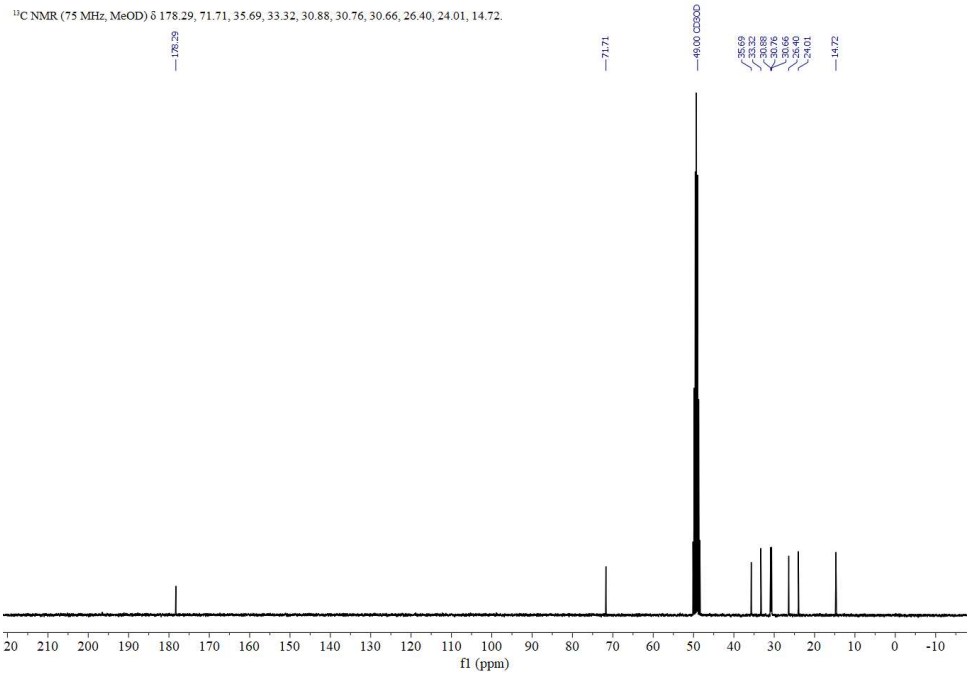

Fig. S30: ^{13}C NMR **α-2c** (75 MHz, MeOD) δ [ppm] = 178.29, 71.71, 35.69, 33.32, 30.88, 30.76, 30.66, 26.40, 24.01, 14.72.
Data is in accordance with literature.[15]

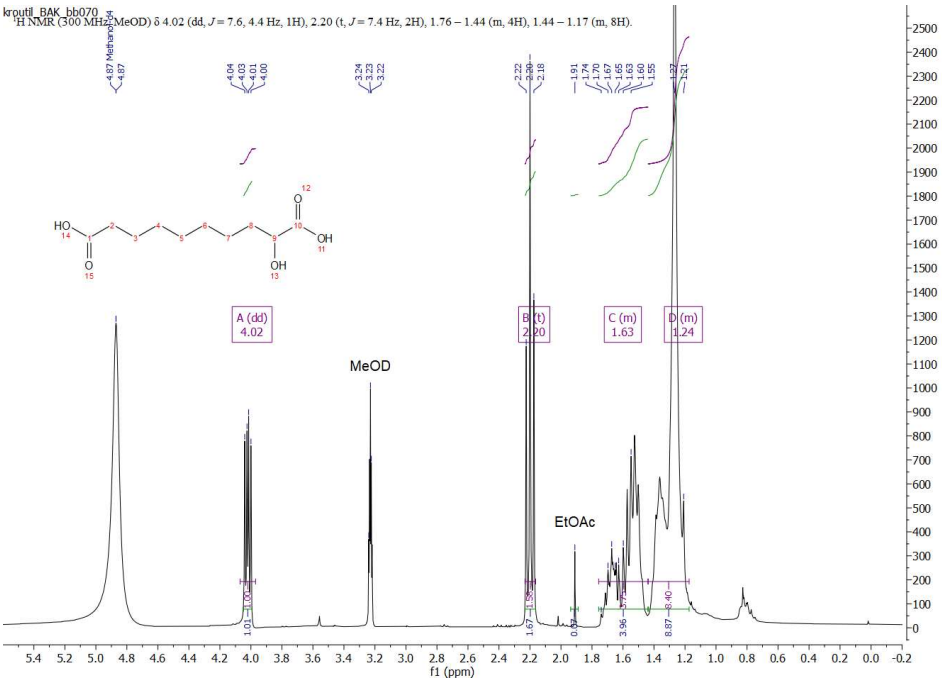

Fig. S31: ^{1}H NMR **α-4b** (300 MHz, MeOD) δ [ppm] = 4.02 (dd, 2H, J = 7.6, 4.4 Hz, C<u>H</u>OH,), 2.20 (t, 2H, J = 7.4 Hz, C<u>H$_2$</u>CHOH), 1.76 – 1.44 (m, 4H), 1.44 – 1.17 (m, 8H).

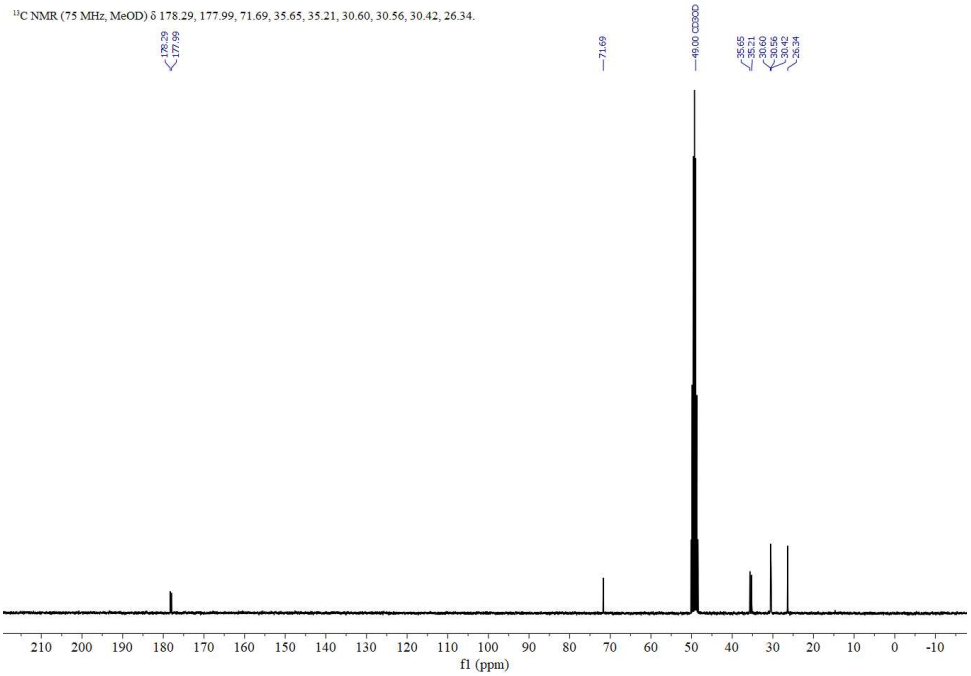

Fig. S32: ^{13}C NMR **α-4b** (75 MHz, MeOD) δ [ppm] = 178.29, 177.99, 71.69, 35.65, 35.21, 30.60, 30.56, 30.42, 26.34.

References

1. A. Dennig, S. Gandomkar, E. Cigan, T. C. Reiter, T. Haas, M. Hall and K. Faber, *Org. Biomol. Chem.*, 2018, **16**, 8030-8033.
2. M. Girhard, S. Schuster, M. Dietrich, P. Dürre and V. B. Urlacher, *Biochem. Biophys. Res. Commun.*, 2007, **362**, 114-119.
3. S. Gandomkar, A. Dennig, A. Dordic, L. Hammerer, M. Pickl, T. Haas, M. Hall and K. Faber, *Angew. Chem., Int. Ed.*, 2018, **57**, 427-430.
4. I. Matsunaga, E. Kusunose, I. Yano and K. Ichihara, *Biochem. Biophys. Res. Commun.*, 1994, **201**, 1554-1560.
5. J. Armbruster, M. Steinmassl, C. A. Müller Bogotá, G. Berg, B. Nidetzky and A. Dennig, *Chem. - Eur. J.*, 2020, **26**, 15910-15921.
6. J.-b. Wang, R. Lonsdale and M. T. Reetz, *Chem. Commun.*, 2016, **52**, 8131-8133.
7. H. Onoda, O. Shoji, K. Suzuki, H. Sugimoto, Y. Shiro and Y. Watanabe, *Catal. Sci. Technol.*, 2018, **8**, 434-442.
8. H. M. Girvan, H. Poddar, K. J. McLean, D. R. Nelson, K. A. Hollywood, C. W. Levy, D. Leys and A. W. Munro, *J. Inorg. Biochem.*, 2018, **188**, 18-28.
9. T. Fujishiro, O. Shoji, S. Nagano, H. Sugimoto, Y. Shiro and Y. Watanabe, *J. Biol. Chem.*, 2011, **286**, 29941-29950.
10. I. Matsunaga, A. Ueda, N. Fujiwara, T. Sumimoto and K. Ichihara, *Lipids*, 1999, **34**, 841-846.
11. J. Nazor, S. Dannenmann, R. O. Adjei, Y. B. Fordjour, I. T. Ghampson, M. Blanusa, D. Roccatano and U. Schwaneberg, *Protein Eng. Des. Sel.*, 2008, **21**, 29-35.
12. E. Krieger and G. Vriend, *J. Comput. Chem.*, 2015, **36**, 996-1007.
13. O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455-461.
14. E. Busto, N. Richter, B. Grischek and W. Kroutil, *Chem— Eur. J.*, 2014, **20**, 11225-11228.
15. A. Dennig, F. Blaschke, S. Gandomkar, E. Tassano and B. Nidetzky, *Adv. Synth. Catal.*, 2019, **361**, 1348-1358.