Combining Rational Design and Computational Tools in Multi-Parameter Enzyme Engineering to Increase the Fitness of a CYP152 Peroxygenase for α-Hydroxylation of Fatty Acids

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SUPPLEMENTARY INFORMATION

Contents

Supplementary data

Calculation of relative per-residue solvent accessible surface area

The relative per-residue solvent accessible surface area (SASA) was calculated for cysteine-, methionine- and tyrosine residues of $PO_{SP\alpha}$ wt [Protein Data Bank (PDB): 3AWM] using PyMOL (version 2.5.4).

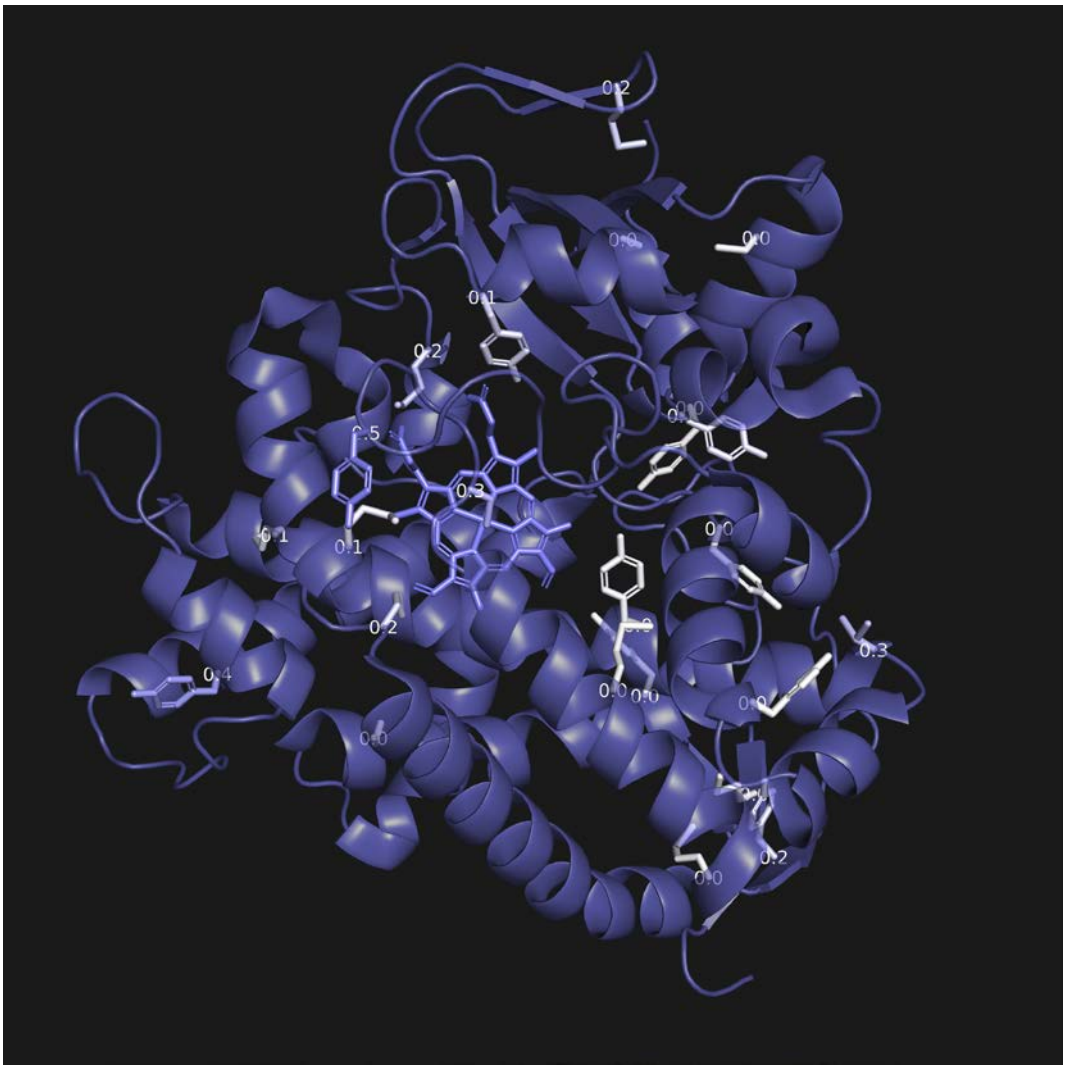


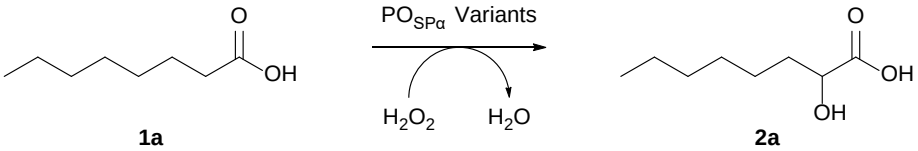
Figure S1: Relative per-residue solvent accessible surface area for cysteine-, methionine-, and tyrosine residues of $PO_{SP\alpha}$ (PDB: 3AWM) calculated in PyMOL. Relative SASA values are displayed for each residue. Colour gradient indicates degree of solvent exposure (white = low SASA, blue = high SASA).

Table S1: Relative per-residue solvent accessible surface area for cysteine-, methionine-, and tyrosine residues of $PO_{SPα}$ (PDB: 3AWM) given as percentages and sorted from largest to smallest.

Entry	Amino Acid	Position	SASA [%]
01	Tyr	356	46
02	Tyr	214	36
03	Cys	361	31
04	Cys	260	25
05	Met	69	23
06	Tyr	384	18
07	Met	102	16
08	Met	304	16
09	Tyr	58	14
10	Tyr	317	13
11	Met	97	10
12	Met	99	8
13	Tyr	285	5
14	Cys	28	3
15	Tyr	249	3
16	Met	382	2
17	Tyr	21	1
18	Tyr	134	1
19	Tyr	273	1
20	Met	1	0
21	Cys	47	0
22	Cys	146	0
23	Met	371	0
24	Met	408	0

Analytical-scale biotransformation of **1a** in batch

To compare the performance, the α-hydroxylation of octanoic acid was investigated in batch for selected variants (Scheme S1). The wildtype enzyme as well as variants V3 and V3-P04 were tested (Table S2). Interestingly, while variant V3-P04 portrayed higher H_2O_2 tolerance and outperformed the parent for biotransformations conducted under continuous oxidant addition, the variant yielded the lowest amounts of product in the batch setup at both 25- and 50 mM substrate loading. Furthermore, V3, which had shown the highest H_2O_2 tolerance under the investigated conditions, only reached marginally higher product concentrations than the wildtype.



1a **2a**

*Scheme S1: $PO_{SPα}$-catalyzed hydroxylation of octanoic acid **1a** to 2-hydroxyoctanoic acid **2a**.*

*Table S2: Product formation for the $PO_{SPα}$-mediated hydroxylation of **1a** in batch.*

Batch setup				
Substrate Loading	Variant	**2a** [mM]	*ee* [%]	wt-Productivity [%]
25 mM	wt	7.0	98 (*S*)	100
	V3	7.2	98 (*S*)	103
	V3-P04	5.6	97 (*S*)	81
50 mM	wt	9.6	98 (*S*)	100
	V3	9.8	98 (*S*)	103
	V3-P04	7.7	97 (*S*)	80

*Reaction conditions: 3 µM peroxygenase, 25-50 mM **1a**, 1 eq H_2O_2, in KPi buffer [1 mL, 100 mM, pH 7.4, 5% (v/v) ethanol] inside 2 mL microcentrifuge tubes under shaking (400 rpm, 25 °C, 15 h). Biotransformations were prepared in duplicate.*

Supplementary methods

Gene synthesis

All genes were obtained from commercial suppliers in the desired vectors and codon optimised for expression in *E. coli*. Genes for $PO_{SP\alpha}$ (UniProtKB ID: O24782) and its variants were sub-cloned into pDB-HisGST vectors due to poor soluble expression. The gene for PO_{CLA} (NCBI Reference Sequence: WP_010966602.1) was subcloned into a pET28a(+) vector.

Protein expression and purification

Overnight cultures were prepared in LB-medium (10 mL, 50 µg/mL kanamycin) in 50 mL conical tubes using single colonies for inoculation. Inoculated cultures were incubated at 37 °C and 120 rpm overnight. Main cultures were prepared in LB-/TB-medium (660 mL, 50 µg/mL kanamycin) in 2 L baffled cultivation flasks using overnight cultures (5 mL). Main cultures were incubated at 30-37 °C and 120 rpm until they reached an OD_{600} of 0.6-0.8. Cultures were then cooled to 20 °C before addition of 5-aminolevulinic acid (0.5 mM, 500 mM stock). After incubating the cultures at 20 °C and 120 rpm for ten minutes, protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.4-1 mM final, 1 M stock). Induced cultures were incubated at 20 °C and 120 rpm overnight. Cells were harvested by centrifugation (8,000 rpm, 4 °C, 20 min), supernatant was discarded, and pellets were resuspended in KPi buffer (10 mL/g pellet, 100 mM, pH 7.4). Cells were transferred to 50 mL conical tubes and again centrifuged (4,500 rpm, 4 °C, 20 min). Pellets were stored at -20 °C.

For purification, pellets were resuspended in binding buffer I/II and incubated for two hours on ice. Cells were then disrupted by sonication (30% amplitude; pulse: 2 sec ON, 4 sec OFF; 2 min total; twice for each). Insoluble cell components were removed by centrifugation (18,000 rpm, 4 °C, 30 min) and supernatant was sterile filtered (0.45 µm syringe filter). Protein purification was done using a HisTrap™ column (5 mL) connected to a peristaltic pump system. The column was washed with dH_2O (5 column volumes) and then equilibrated with binding buffer I/II. After loading the sterile filtered lysate, unbound protein was removed with binding buffer I/II (8-10 column volumes). Then, bound protein was eluted using elution buffer I/II. Elution was clearly visible due to the protein's red colour. Any remaining protein on the column was removed with cleaning buffer [25 mL, 300 mM KCl, 100 mM KPi, 500 mM imidazole pH 7.5 (degassed)]. The column was then washed with dH_2O (10 column volumes) and stored

in 20% (v/v) ethanol at 4 °C. Eluted protein was concentrated in VIVASPIN tubes (30,000 MWCO PES), desalted with PD-10 columns, and eluted in KPi buffer [100 mM, pH 7.4, 15% (v/v) glycerol]. Purified protein was shock-frozen in liquid nitrogen and stored at -20 °C. Protein expression was investigated by SDS-PAGE (Figures S2-S6) and CO difference spectroscopy.

Buffers for protein purification:

Binding buffer I: 100 mM KPi, 300 mM KCl, 50 mM imidazole, pH 7.5 (filtered and degassed)

Binding buffer II: 100 mM NaCl, 100 mM KPi, 40 mM imidazole, 0.8% w/v cholate, 1 mM PMSF, and 15% (v/v) glycerol, pH 7.5 (filtered and degassed)

Elution buffer I: 100 mM KPi, 300 mM KCl, 300 mM imidazole, pH 7.5 (filtered and degassed)

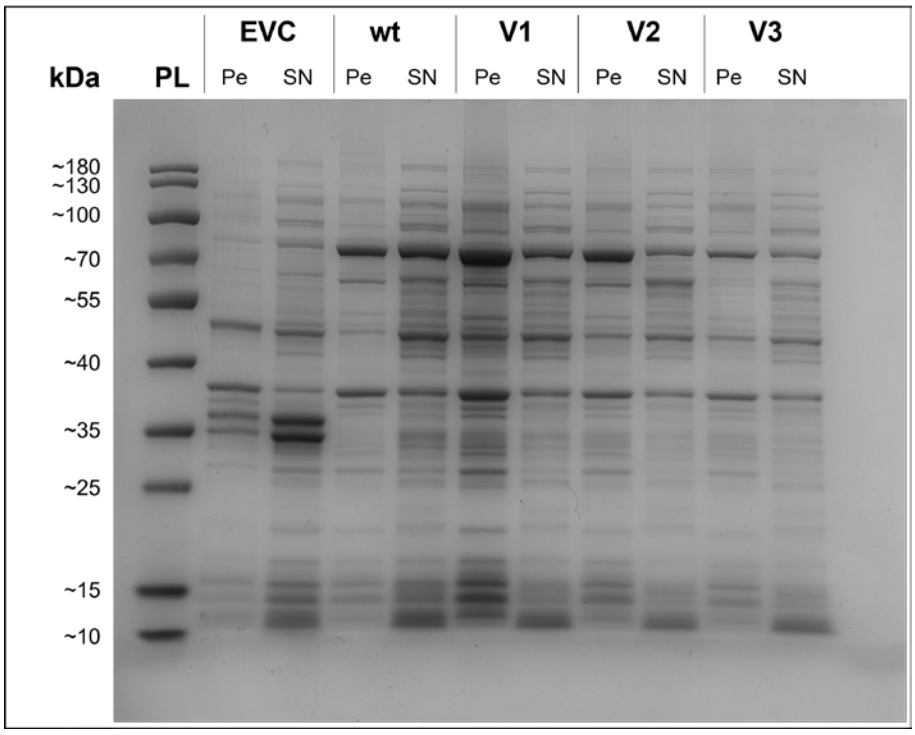
Elution buffer II: 100 mM NaCl, 100 mM KPi, 250 mM imidazole, 0.8% (w/v) cholate, and 15% (v/v) glycerol, pH 7.5 (filtered and degassed)

For $PO_{SP\alpha}$ variants:

Main cultures were prepared in LB-medium and incubated at 37 °C. Protein production was induced with IPTG (1 mM final concentration). Protein purification was done using binding buffer II and elution buffer II.

For PO_{CLA}:

Main cultures were prepared in TB-medium and incubated at 30 °C. Protein production was induced with IPTG (0.4 mM final concentration). Protein purification was done using binding buffer I and elution buffer I.



FigureS2: SDS-PAGE of $PO_{SP\alpha}$ variants heterologously expressed in E. coli BL21(DE3). PL: PageRuler™ Prestained Protein Ladder, Pe: pellet, SN: supernatant. EVC: Empty vector control (pDB-HisGST). $PO_{SP\alpha}$ variants: ~72.5 kDa.

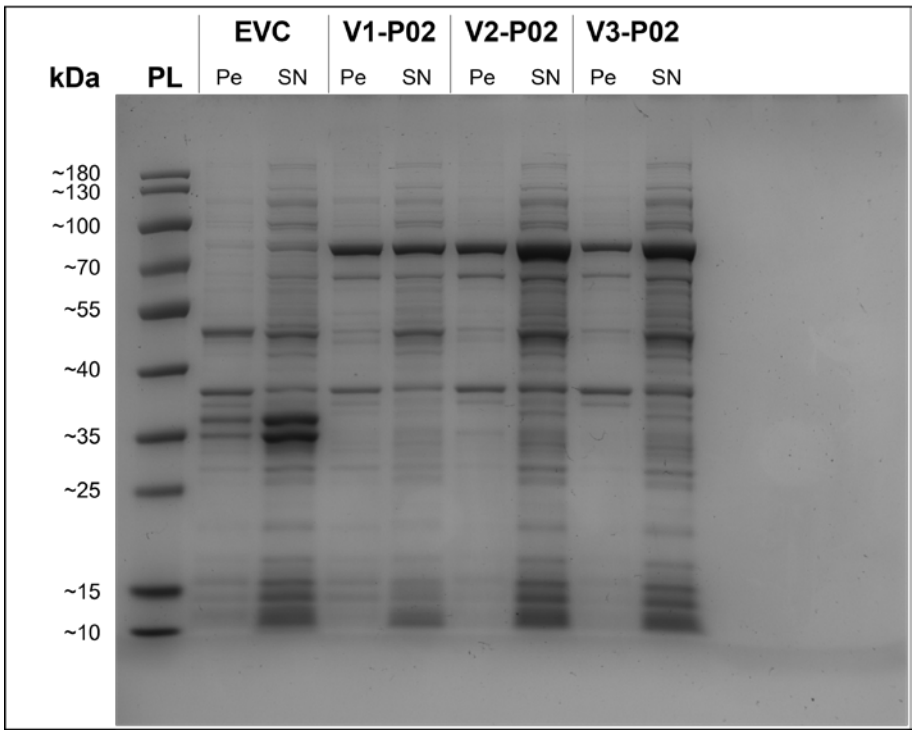


Figure S3: SDS-PAGE of $PO_{SP\alpha}$ variants heterologously expressed in E. coli BL21(DE3). PL: PageRuler™ Prestained Protein Ladder, Pe: pellet, SN: supernatant. EVC: Empty vector control (pDB-HisGST). $PO_{SP\alpha}$ variants: ~72.5 kDa.

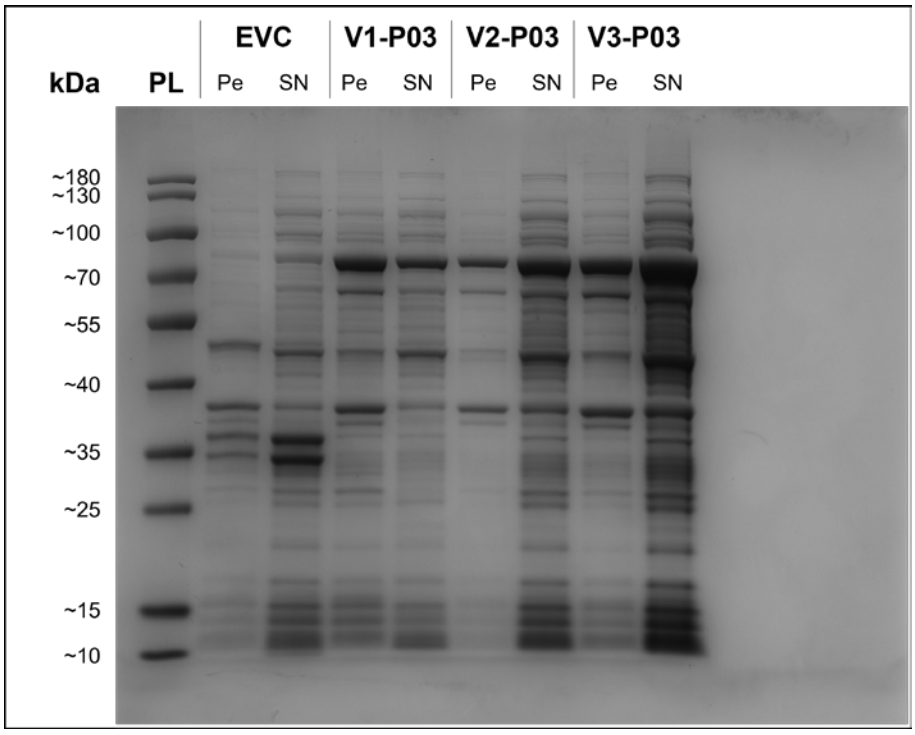


Figure S4: SDS-PAGE of $PO_{SP\alpha}$ variants heterologously expressed in E. coli BL21(DE3). PL: PageRuler™ Prestained Protein Ladder, Pe: pellet, SN: supernatant. EVC: Empty vector control (pDB-HisGST). $PO_{SP\alpha}$ variants: ~72.5 kDa.

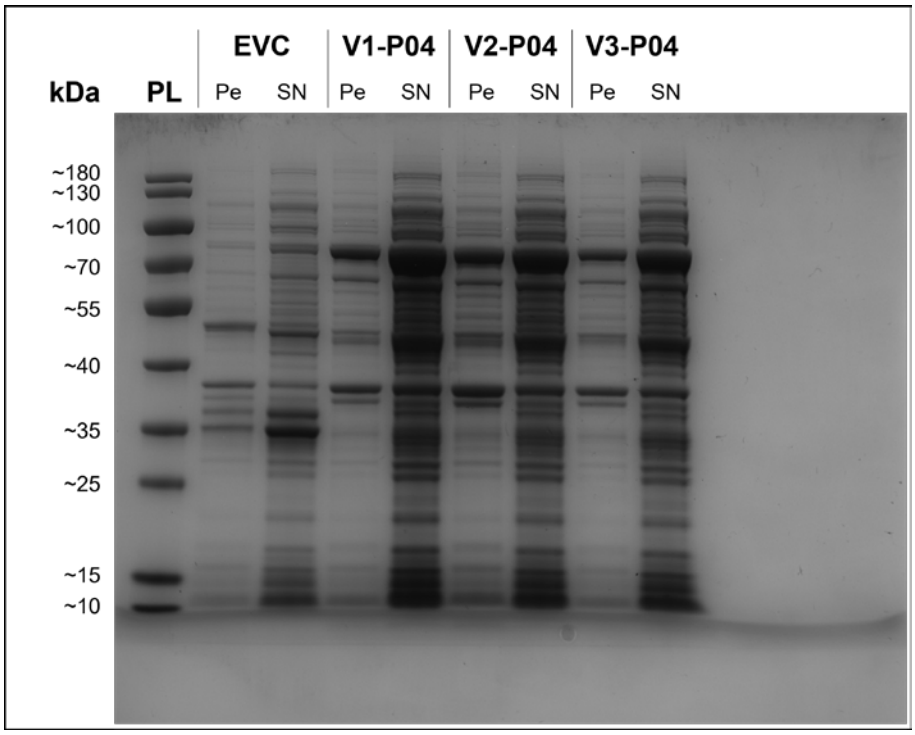


Figure S5: SDS-PAGE of $PO_{SP\alpha}$ variants heterologously expressed in E. coli BL21(DE3). PL: PageRuler™ Prestained Protein Ladder, Pe: pellet, SN: supernatant. EVC: Empty vector control (pDB-HisGST). $PO_{SP\alpha}$ variants: ~72.5 kDa.

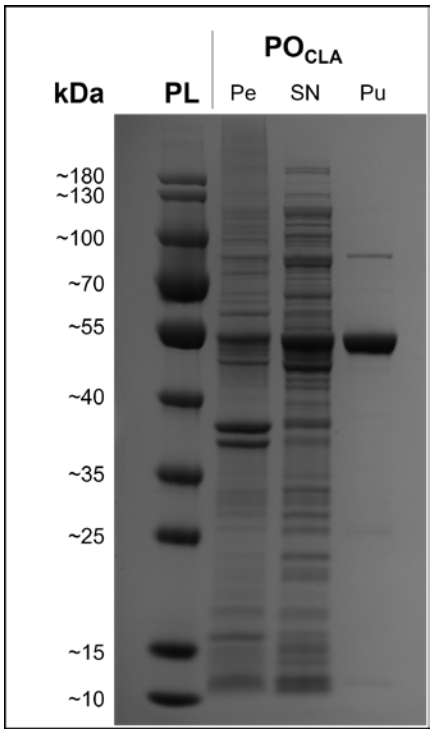


Figure S6: SDS-PAGE of PO_{CLA} heterologously expressed in E. coli BL21(DE3). PL: PageRuler™ Prestained Protein Ladder, Pe: pellet, SN: supernatant, Pu: purified fraction (affinity chromatography). PO_{CLA}: ~48.2 kDa.

CO-spectral assay (difference spectroscopy)

CYP concentration was determined by measuring the absorbance of ferrous CO-complexes according to the protocol by Guengerich *et al*.[1]

CYP solutions were prepared in KPi buffer [2 mL, 100 mM, pH 7.4, 15% (v/v) glycerol] at concentrations ≤ 5 µM to remain within the linear range of the assay. Samples were divided equally to two separate polystyrene cuvettes (10 x 4 x 45 mm), one of which was subsequently saturated with carbon monoxide by bubbling for approximately 60 seconds. Reference sample and CO-saturated sample were then reduced by addition of a spatula tip of sodium dithionite ($Na_2S_2O_4$) leading to the ferrous form of the iron complex. Absorbance spectra of both samples were recorded every 60 seconds over a range of 400-540 nm using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) until the absorbance maximum at ~450 nm of the CO-saturated ferrous species no longer increased. CYP concentration was determined from the absorbance difference (Figure S7) according to Equation S1.

Equation S1: Determination of CYP concentration using the CO-spectral assay.

$$\frac{(A_{450}-A_{490})_{saturated}-(A_{450}-A_{490})_{reference}}{0.091}=\mu mol\ CYP\ per\ L$$

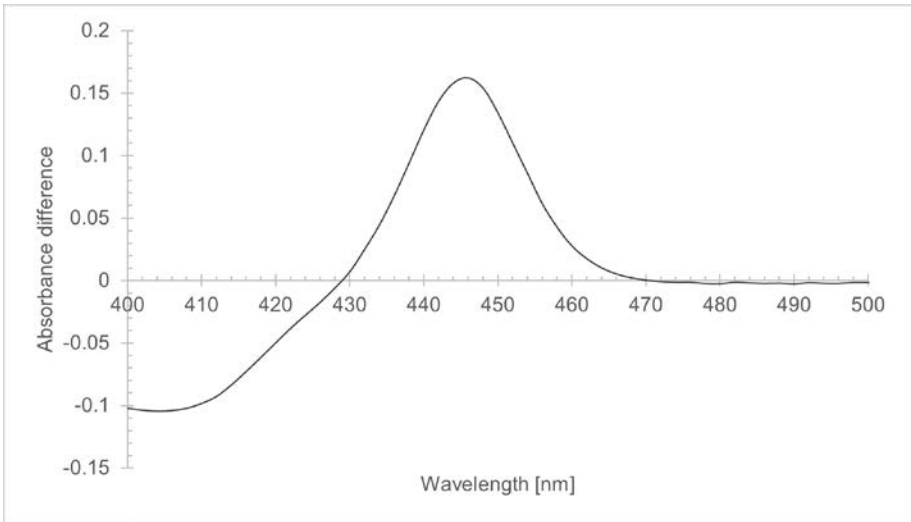


Figure S7: Exemplary difference-spectrum of the CO-saturated ferrous species (saturated) versus ferrous species (reference) of $PO_{SPα}$ wt.

H_2O_2-Mediated enzyme inactivation studies

For investigating the H_2O_2-tolerance of $PO_{SPα}$ variants, peroxygenases were incubated for two hours with 20 mM H_2O_2 in potassium phosphate buffer [100 mM, pH 7.4, 15% (v/v) glycerol]. Peroxygenase stocks were thawed and diluted with KPi buffer [100 mM, pH 7.4, 15% (v/v) glycerol] to obtain solutions of 4 µM enzyme (3.5 mL). In addition, a H_2O_2 stock solution (40 mM) was prepared in KPi buffer [100 mM, pH 7.4, 15% (v/v) glycerol]. Enzyme solutions (4 µM, 500 µL) were mixed with the H_2O_2 stock solution (500 µL) inside 2 mL microcentrifuge tubes to obtain samples with final concentrations of 2 µM enzyme and 20 mM H_2O_2. Additionally, reference samples were prepared in KPi buffer without hydrogen peroxide. Samples were incubated for two hours (25 °C, 400 rpm) before remaining H_2O_2 was removed by addition of an aqueous catalase solution [50 µL, 2 mg/mL catalase (≥10.000 U/mg, bovine liver, Sigma-Aldrich, C40), 100 mM KPi, pH 7.4]. Samples were put on ice and haem-bound P450 concentration was determined by CO-spectral assay.

Determination of specific activity

To determine specific activities of $PO_{SPα}$-variants for the α-hydroxylation of **1a**, biotransformations were conducted at analytical scale in 2 mL microcentrifuge tubes. Purified enzyme was thawed, desalted using a PD-10 column, then eluted in KPi buffer (100 mM, pH 7.4) to obtain enzyme solutions with the desired concentration (0.4 µM). In addition, a stock of **1a** (1 M) was prepared in ethanol and a H_2O_2 stock (20 mM) was prepared in KPi buffer (100 mM, pH 7.4). H_2O_2 stock (250 µL), KPi buffer (200 µL, 100

mM, pH 7.4), **1a** stock (50 µL), and finally enzyme stock (500 µL) were transferred to 2 mL microcentrifuge tubes leading to biotransformation samples with final concentrations of 0.2 µM enzyme, 50 mM **1a**, and 5 mM H_2O_2 in KPi buffer [1 mL, 100 mM, pH 7.4, 5% (v/v) ethanol]. Exact enzyme concentrations were afterwards determined using the CO-spectral assay. Biotransformations were shaken for precisely 20 minutes (25 °C, 400 rpm) before reactions were quenched by the addition of an aqueous HCl solution (5 M, 100 µL). Samples were extracted with ethyl acetate (2 x 500 µL, 5 mM dodecanoic acid).

Specific enzyme activities were calculated based on Equation S2 using the amount of formed product (**2a**).

Equation S2: Determination of the specific activity of $PO_{SPα}$-variants for the hydroxylation of octanoic acid. Amount of formed product was used instead of the amount of consumed substrate. Enzyme amounts were calculated based on CO-spectral assay data using the molecular weight with GST-tag (72,490 Da).

$$Specific\ Activity\ \left[\frac{U}{mg}\right] = \frac{Product\ [\mu M]}{Enzyme\ \left[\frac{mg}{mL}\right] * 20\ min}$$

Scoring of $PO_{SPα}$ variants (performance scores)

To deduce the overall best-performing catalyst from all the collected data, partial scores (s_p) were assigned for each of the four investigated parameters (H_2O_2-stability, expression yield, specific activity for oxidation of **1a**, stereoselectivity for formation of **2a**) according to Equation S3. By choosing a relative scale where m_p and M_p are the respective minimum- and maximum values of each category, each partial score was mapped into a range of $s_p \in [0, 1]$ and therefore all parameters were weighted equally. The overall score (performance score, s) was defined as the sum of all partial scores (Equation S3, **b.**). The respective partial scores and overall scores of each $PO_{SPα}$ variant are given in Table S3.

*Equation S3: **a.** Determination of partial scores (s_p) assigned to $PO_{SPα}$ variants for each of the four experimental parameters. x_p = experimental value, $m_p = min\{x_p\}$; $M_p = max\{x_p\}$. **b.** Determination of overall scores (performance scores, s) based on partial scores.*

$$\boldsymbol{a}.\ s_p = \frac{x_p - m_p}{M_p - m_p} \qquad\qquad \boldsymbol{b}.\ s = \sum_p s_p$$

Table S3: Partial scores (s_p) and performance scores (s) of all investigated $PO_{SPα}$ variants.

Variant	Partial scores (s_p)				Performance Score (s)
	H_2O_2-Stability	Expression Yield	Spec. Activity	Stereo-selectivity	
V3-P04	0.65	0.64	1.00	0.56	**2.85**
V1	0.76	0.45	0.53	0.85	**2.59**
V3-P03	0.50	0.45	0.79	0.69	**2.43**
V3-P02	0.39	0.75	0.78	0.45	**2.38**
V2-P03	0.23	0.85	0.27	0.97	**2.32**
V1-P04	0.18	1.00	0.42	0.68	**2.27**
V2-P04	0.41	0.83	0.26	0.61	**2.11**
wt	0.12	0.46	0.41	1.00	**1.99**
V1-P02	0.48	0.76	0.43	0.30	**1.96**
V3	1.00	0.00	0.43	0.41	**1.84**
V1-P03	0.73	0.58	0.20	0.23	**1.74**
V2	0.77	0.05	0.12	0.35	**1.31**
V2-P02	0.00	0.61	0.00	0.00	**0.61**

Analytical-scale biotransformations conducted in fed-batch

Biotransformations were prepared in KPi buffer [100 mM, pH 7.4, 5% (v/v) ethanol] inside glass GC-vials (1.5 mL). Purified enzyme was thawed, desalted using a PD-10 column, then eluted in KPi buffer (3.5 mL, 100 mM, pH 7.4) to obtain enzyme solutions with the desired concentrations (6 µM). Substrate stocks (100-200 mM) were prepared in KPi buffer [100 mM, pH 7.4, 10% (v/v) ethanol]. Enzyme stocks (6 µM, 500 µL) were then transferred to glass GC-vials with substrate stocks (500 µL) leading to final concentrations of 3 µM enzyme and 50-100 mM substrate in KPi buffer [1 mL, 100 mM, pH 7.4, 5% (v/v) ethanol]. Samples were shaken overnight (15 h, 400 rpm, RT) while an H_2O_2 solution (300-400 mM, 100 mM KPi, pH 7.4) was continuously added (21-31 µL/h) over 12 hours *via* a syringe pump leading to the addition of 1.5 equivalents of H_2O_2. Afterwards, reactions were quenched by addition of an aqueous HCl solution (150 µL, 5 M) and the product was extracted with ethyl acetate (2 x 500 µL, 100 mM dodecanoic acid). The total volume of 1 mL of ethyl acetate implies that the obtained analyte concentrations correspond to the one at the start of the reaction.

50 mM substrate loading: 100 mM stock, 300 mM H_2O_2 solution, 21 µL/h feeding rate, 252 µL of total volume.

75 mM substrate loading: 150 mM stock, 300 mM H_2O_2 solution, 31 µL/h feeding rate, 372 µL of total volume.

100 mM substrate loading: 200 mM stock, 400 mM H_2O_2 solution, 31 µL/h feeding rate, 372 µL of total volume.

Analytical-scale biotransformations conducted in batch

Biotransformations were prepared in KPi buffer [100 mM, pH 7.4, 5% (v/v) ethanol] in microcentrifuge tubes (2 mL). Purified enzyme was thawed, desalted using a PD-10 column, then eluted in KPi buffer (3.5 mL, 100 mM, pH 7.4) to obtain enzyme solutions with the desired concentrations (3.53 µM). In addition, substrate stocks (250-500 mM) were prepared in a KPi buffer (100 mM, pH 7.4)/ethanol mixture (1:1) and hydrogen peroxide stocks (0.5-1 M) were prepared in KPi buffer (100 mM, pH 7.4). Enzyme stocks (850 µL) were then transferred to 2 mL microcentrifuge tubes with substrate stocks (100 µL) and H_2O_2-stocks (50 µL) leading to final concentrations of 3 µM enzyme, 25-50 mM substrate, and 25-50 mM H_2O_2 (1 eq) in KPi buffer [1 mL, 100 mM, pH 7.4, 5% (v/v) ethanol]. Samples were shaken overnight inside a tilted (90°) benchtop shaker (15 h, 400 rpm, 25 °C). Afterwards, reactions were quenched by the addition of an aqueous HCl solution (100 µL, 5 M) and the product was extracted with ethyl acetate (2 x 500 µL, 100 mM dodecanoic acid).

Analytical-scale biotransformations for the over-oxidation of **2a**

Biotransformations were prepared in KPi buffer [100 mM, pH 7.4, 5% (v/v) ethanol] inside glass GC vials (1.5 mL). Purified enzyme was thawed, desalted using a PD-10 column, then eluted in KPi buffer (3.5 mL, 100 mM, pH 7.4) to obtain enzyme solutions with the desired concentrations (3.16 µM). A stock of *rac*-**2a** (200 mM) was prepared in ethanol. Enzyme stocks (3.16 µM, 950 µL) were then transferred to glass GC vials with substrate stocks (50 µL) leading to final concentrations of 3 µM enzyme and 10 mM **2a** in KPi buffer [1 mL, 100 mM, pH 7.4, 5% (v/v) ethanol]. Samples were shaken at 400 rpm and room temperature overnight (15 h) while an H_2O_2 solution (133 mM, 100 mM KPi, pH 7.4) was continuously added (12.5 µL/h) over 12 hours *via* a syringe pump leading to 1.5 equivalents of H_2O_2. Afterwards, reactions were

quenched by the addition of an aqueous HCl solution (100 µL, 5 mM) and the product was extracted with ethyl acetate (2 x 500 µL, 10 mM dodecanoic acid).

Molecular docking of (*S*)- and (*R*)-**2a** in the active site of $PO_{SP\alpha}$

Molecular docking of (*S*)- and (*R*)-**2a** in the active site of $PO_{SP\alpha}$ was performed in Maestro using rigid receptor docking with Glide.[2] Ligands were prepared in Maestro with LigPrep using the OPLS4 force field and a target pH of 7.00 +/- 2.00 for ionisation. The crystal structure of $PO_{SP\alpha}$ (pdb: 3AWM) was used as receptor and the protein preparation was performed with Maestro using chain A and substructures HEM 501, HEM 501 Fe, and HOH 481. Missing loops were filled in using Prime. The bond length between HEM 501 Fe and HOH 481 was adjusted to 1.66 Å, bond order was set to 0, and the charge of the oxygen of HOH 481 was adjusted to -2 to simulate a reactive Fe(IV) oxo species.[3] The receptor grid was centred around HEM 501, HEM 501 Fe, and ARG 241 with a grid length of 15 Å. Rigid receptor docking was performed in Maestro, generating ten poses for each of the two ligands.

Preparative-scale biotransformations with V3-P04

Preparative-scale biotransformations were prepared in KPi buffer (20 mL, total volume) inside round-bottom flasks (100 mL). Purified enzyme ($PO_{SP\alpha}$ variant V3-P04) was thawed, desalted using a PD-10 column, then eluted in KPi buffer (3.5 mL, 100 mM, pH 7.4) to obtain enzyme amounts needed to reach the desired final concentration (3 µM). Meanwhile, substrate stock solutions were prepared in ethanol [1 mL, 1-3 M (**1a**-**d**)] and transferred to a round-bottom flask containing KPi buffer (15.5 mL, 100 mM, pH 7.4). An aqueous NaOH solution (10 M) was then used to readjust the pH of the reaction mixture to 7.4. Finally, the prepared enzyme solution was added to the round-bottom flask under stirring (400 rpm) leading to final concentrations of 3 µM of $PO_{SP\alpha\ PROSS4\text{-}Q}$ and 50-150 mM of substrate (**1a**: 150 mM, **1b**: 50 mM, **1c**: 100 mM, **1d**: 75 mM). The reaction was stirred overnight (15 h) while continuously adding H_2O_2 *via* a syringe pump [250-600 mM (**1a**: 600 mM, **1b**: 250 mM, **1c**: 500 mM, **1d**: 375 mM), in KPi buffer (100 mM, pH 7.4), 500 µL/h, 12 h, 6 mL total volume, 1.5 eq (1.2 eq for **1a**)]. To observe the reaction progress, samples (500 µL) were taken after 2, 4, 6, 8, and 12 h (for **1a**); as well as immediately before the reaction work up. These samples were extracted with ethyl acetate (2 x 250 µL, 100 mM dodecanoic acid), organic phases were dried with Na_2SO_4 and 100 µL were mixed with BSTFA-TMCS (100 µL) and pyridine (100 µL) for derivatization. Samples were then shaken for two hours (700

rpm, 25 °C) before being analyzed by GC-FID. As the reaction volume increased over time due to H_2O_2-feeding and samples were taken throughout the reaction, overall analyte concentrations decreased over time. However, as all analytes were quantified by linear calibration with authentic standards, concentrations were corrected based on the total analyte recovery of each sample.

Afterwards, reactions were acidified (pH 3.0) with an aqueous HCl solution (36%) leading to precipitation of product and catalyst. Ethyl acetate (20 mL) was added, and the resulting suspensions were filtered through a bed of celite to remove precipitated protein. Formed product was extracted with ethyl acetate (3x 20 mL) using a separating funnel, combined organic phases were dried with Na_2SO_4, and solvent was removed under reduced pressure. Conversion was checked by GC-MS and when necessary, product was purified *via* flash column chromatography. Product formation was confirmed by NMR.

2a

Yield: 385 mg (2.4 mmol, 80%) white solid

94% *ee* (*S*)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.30 (dd, *J* = 7.5, 4.2 Hz, 1H), 1.97 – 1.81 (m, 1H), 1.81 – 1.61 (m, 1H), 1.54 – 1.25 (m, 8H), 0.89 (t, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.1, 70.3, 34.2, 31.6, 28.9, 24.7, 22.6, 14.1.

2b

Yield: 36 mg (0.25 mmol, 25%) slightly yellow solid

89% *ee* (*S*), $[\alpha]_D^{20}$ = +3.24° [c = 1.7, $CHCl_3$]

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.30 (dd, *J* = 7.7, 4.0 Hz, 1H), 1.95 – 1.79 (m, 1H), 1.79 – 1.63 (m, 1H), 1.56 – 1.41 (m, 2H), 1.40 – 1.24 (m, 4H), 0.97 – 0.86 (m, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.0, 70.3, 34.1, 31.4, 24.4, 22.5, 14.0.

2c

Yield: 302 mg (1.7 mmol, 87%) white solid

95% *ee* (*S*), $[\alpha]_D^{25}$ = +4.32° [c = 3.15, $CHCl_3$]

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.30 (dd, *J* = 7.5, 4.2 Hz, 1H), 1.96 – 1.80 (m, 1H), 1.80 – 1.63 (m, 1H), 1.56 – 1.41 (m, 2H), 1.41 – 1.25 (m, 8H), 0.95 – 0.85 (m, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.1, 70.3, 34.2, 31.8, 29.2, 29.1, 24.8, 22.6, 14.1.

2d

Yield: 253 mg (1.4 mmol, 91%) white solid

94% *ee* (*S*), $[\alpha]_D^{20}$ = +4.50° [c = 1.00, $CHCl_3$]

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.83 (ddt, *J* = 16.9, 10.1, 6.7 Hz, 1H), 5.08 – 4.90 (m, 2H), 4.30 (dd, *J* = 7.6, 4.1 Hz, 1H), 2.15 – 1.98 (m, 2H), 1.96 – 1.61 (m, 2H), 1.60 – 1.25 (m, 8H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.0, 139.0, 114.3, 70.3, 34.2, 33.7, 29.1, 28.9, 28.8, 24.7.

Preparative-scale biotransformations with PO_{CLA}

As authentic standards for racemic **2c**-**d** were not commercially available, preparative-scale biotransformations of **1c**-**d** were conducted with PO_{CLA} to obtain the desired α-hydroxy acids. PO_{CLA} was chosen as catalyst due to its low stereoselectivity, enabling the synthesis of both enantiomers of **2b**-**d**, which was important for establishing chiral analytics.

Preparative-scale biotransformations were prepared in KPi buffer (20 mL, total volume) inside round-bottom flasks (100 mL). Purified enzyme (PO_{CLA}) was thawed, desalted using a PD-10 column, then eluted in KPi buffer (3.5 mL, 100 mM, pH 7.4) to obtain enzyme amounts needed to reach the desired final concentration (6 µM). Meanwhile, substrate stock solutions were prepared in ethanol [1 mL, 400 mM (**1b**-**d**)] and transferred to a round-bottom flask containing KPi buffer (15.5 mL, 100 mM, pH 7.4). An aqueous NaOH solution (10 M) was then used to readjust the pH of the reaction mixture to 7.4. Finally, the prepared enzyme solution was added to the round-bottom flask under stirring (400 rpm) leading to final concentrations of 6 µM PO_{CLA} and 20 mM

substrate. The reaction was stirred overnight (15 h) while continuously adding H_2O_2 *via* a syringe pump [400 mM, in KPi buffer (100 mM, pH 7.4), 167 µL/h, 12 h, 2 eq].

Afterwards, reactions were acidified (pH 3.0) with an aqueous HCl solution (36%) leading to precipitation of product and catalyst. Ethyl acetate (20 mL) was added, and the resulting suspensions were filtered through a bed of celite to remove precipitated protein. Formed product was extracted with ethyl acetate (3x 20 mL) using a separating funnel, combined organic phases were dried with Na_2SO_4, and solvent was removed under reduced pressure. Conversion was checked by GC-MS and when necessary, product was purified *via* flash column chromatography. Product formation was confirmed by NMR.

2b

Yield: 57 mg (0.39 mmol, 98%) slightly yellow solid

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.30 (dd, *J* = 7.5, 4.2 Hz, 1H), 1.96 – 1.61 (m, 2H), 1.58 – 1.24 (m, 6H), 1.01 – 0.86 (m, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.0, 70.3, 34.1, 31.4, 24.4, 22.5, 14.0.

2c

Yield: 52 mg (0.3 mmol, 75%) white solid

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.30 (dd, *J* = 7.5, 4.2 Hz, 1H), 1.96 – 1.62 (m, 2H), 1.57 – 1.21 (m, 10H), 0.95 – 0.85 (m, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.0, 70.3, 34.2, 31.8, 29.2, 29.1, 24.7, 22.6, 14.1.

2d

Yield: 33 mg (0.18 mmol, 44%) slightly yellow solid

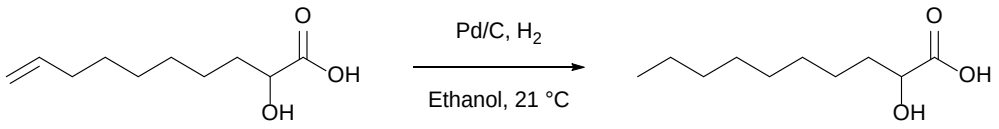
Crude directly employed for the hydrogenation with Pd/C

Hydrogenation of **2d**

Pd/C (3 mg, 2.8 µmol) was added to an oven-dried two-necked round-bottom flask (10 mL) with a stirring bar. The flask was equipped with a septum and a two-way valve. **2d** (30 mg, 161 µmol) was dissolved in dry ethanol (4 mL) and transferred to the flask. The system was flushed with argon (5 minutes) and afterwards hydrogen was added under vigorous stirring using a balloon for increased pressure. The reaction was stirred

overnight at room temperature (21 °C). The hydrogen balloon had to be refilled several times to maintain a high-pressure environment. The following day, conversion was checked by TLC (cyclohexane/ethyl acetate/acetic acid, 25:25:1) and the system was again flushed with argon (10 minutes) to remove remaining hydrogen. The reaction mixture was then filtered through a bed of celite. The filtration residue was washed with ethanol (10 mL). Solvent was removed under reduced pressure.

Crude product: 27 mg (145 µmol, 90%) white solid



2d **2e**

*Scheme S2: Reaction scheme for the hydrogenation of 2-hydroxy-9-decenoic acid (**2d**) to 2-hydroxydecanoic acid (**2e**).*

Derivatisation for GC-analysis

For GC-MS analysis and quantitative analysis on GC-FID, analytes were derivatised with BSTFA-TMCS. After extraction with ethyl acetate, combined organic phases were dried with Na_2SO_4 and 100 µL were mixed with BSTFA-TMCS (100 µL) and pyridine (100 µL) for derivatisation. These samples were shaken for two hours (25 °C, 700 rpm) before being analysed by GC-FID (method GC-01). Analyte concentrations were determined by linear calibration using either commercial reference material (for **1a**-**d** and **2a**) or authenticated isolated material (for **2b**-**d**).

For chromatographic separation of hydroxy fatty acids on a chiral phase, analytes were derivatised with MeOH/ethyl chloroformate. After extraction with ethyl acetate, solvent of combined organic phases was removed under air flow and samples were resuspended in methanol [700 µL, 5% (w/v) DMAP]. Ethyl chloroformate (150 µL) was added to the samples which were then shaken for one hour (50 °C, 700 rpm). Solvent was removed under reduced pressure (GeneVac EZ-2 plus) and samples were resuspended in an aqueous HCl solution (0.2%, 700 µL). Product was extracted with ethyl acetate (2 x 500 µL), organic phases were combined and dried with Na_2SO_4. Samples were analysed by GC-FID (method GC-02).

Calculation of green metrics

For a more meaningful comparison of the synthetic approaches for the preparation of α-hydroxy acids discussed within this work, green chemistry metrics were determined. The E-Factor (Environmental Factor), which provides information on the amount of waste produced in a process, and the Atom Economy, describing the relative mass of reactants of a given reaction that are incorporated in the final product, were chosen for this purpose.

E-Factor calculations:

While for E-Factor calculations the entire process should in general be considered up to the isolation of the desired final product, this was unfortunately not feasible as often no quantifiable amounts were provided in literature for the work up- and/or purification steps of the respective processes. Consequnetly, exclusively components used in the actual reactions (including solvents and buffer salts) were considered for the E-factor calculations (Table S4). Furthermore, only the reactions yielding the highest amounts of α-hydroxylated carboxylic acid product were considered for this comparison (reaction schemes are shown in Scheme S3). E-Factors were calculated according to Equation S4 where all material that is not incorporated into the desired product is defined as waste.

Equation S4: E-Factor calculation based on the amounts of components in the reaction and the amount of final product obtained.

$$E\ Factor = \frac{m(waste)}{m(desired\ product)} = \frac{\sum m(reaction\ components) - m(desired\ product)}{m(desired\ product)}$$

TableS4: Amounts of reaction components, amount of product, and calculated E-factors for the α-hydroxylation approaches discussed within this work.

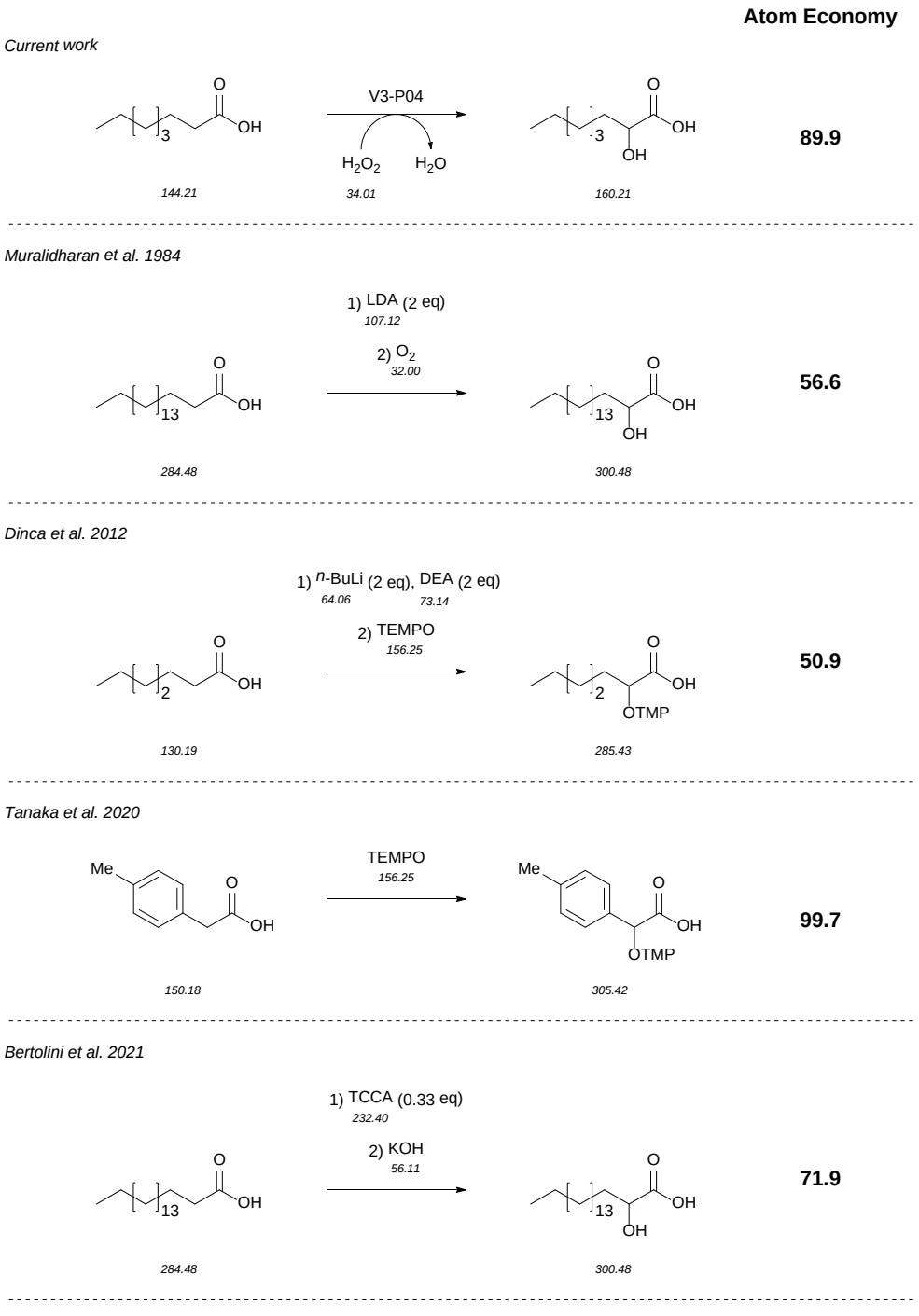
Entry	Article	Reaction components	Product [g]	E-Factor
01	Current work	Water (26 g) Ethanol (0.789 g) K_2HPO_4/KH_2PO_4 (0.433 g) Substrate (0.433 g) H_2O_2 (0.122 g)	0.385	71.1
02	Muralidharan et al. 1984	THF (2.67 g) LDA (0.268 g) HMPA (0.205 g) Substrate (0.028 g)	0.015	210.4
03	Dinca et al. 2012	THF (17.8 g) *n*-BuLi (1.6 M in hexane, 1.818 g) $[Fc]PF_6$ (0.99 g) LiCl (0.4 g) TEMPO (0.374) Diethylamine (0.321 g) Substrate (0.26 g)	0.291	74.5
04	Tanaka et al. 2020	THF (17.8 g) Molecular sieve (4 Å, 2 g) TEMPO (1.31 g) Substrate (0.601 g) Ligand L5 (0.011 g) $Fe(OAc)_2$ (0.007 g)	1.19	17.3
05	Bertolini et al. 2021	Water (200 g) Substrate (9.957 g) KOH (7.85 g) TCCA (3.78 g) PCl_3 (0.165 g)	7.151	30.0

Atom Economy calculations:

Atom Economies for each of the discussed reactions were calculated based on the total mass of reactants and the mass of the final product according to Equation S5.

Equation S5: Atom economy calculation based on the product mass and the total mass of reactants.

$$Atom\ Economy = \frac{m(desired\ product)}{m(reactants)} * 100$$



Scheme S3: Atom Economy values for synthetic processes for the preparation of α-hydroxy acids.

Gene and protein sequences

DNA sequences are shown from restriction site to restriction site (restriction sites are underlined), bases of backbone are shown in lower case letter, bases of gene of interest in upper case letters. The pEG number refers to the group internal plasmid collection (plasmid of the elk group).

The protein sequence is shown as expressed and being present in the CFE including amino acids resulting from the expression vector (printed in lower case letters). Amino acids of the gene of interest are in upper case letters.

PO$_{SP\alpha}$ wt (pEG 371)

Optimised DNA sequence:

catATGCCGAAAACACCGCATACCAAAGGTCCGGATGAAACCCTGAGCCTGCTG
GCAGATCCGTATCGTTTTATTAGCCGTCAGTGTCAGCGTCTGGGTGCAAATGCC
TTTGAAAGCCGTTTTCTGCTGAAAAAAACCAATTGTCTGAAAGGTGCAAAAGCAG
CCGAAATCTTTTATGATACCACCCGTTTTGAACGTGAAGGTGCAATGCCGGTTG
CAATTCAGAAAACCCTGCTGGGTCAGGGTGGTGTTCAGGGTCTGGATGGTGAA
ACCCATCGTCATCGTAAACAAATGTTTATGGGTCTGATGACACCGGAACGTGTT
CGTGCACTGGCACAGCTGTTTGAAGCAGAATGGCGTCGTGCAGTTCCGGGTTG
GACCCGTAAAGGTGAAATTGTTTTTTATGATGAACTGCATGAACCGCTGACCCG
TGCAGTTTGTGCATGGGCAGGCGTTCCGCTGCCGGATGATGAAGCAGGTAATC
GTGCCGGTGAACTGCGTGCACTGTTTGATGCAGCCGGTAGCGCAAGTCCGCGT
CATCTGTGGTCACGTCTGGCACGTCGTCGTGTTGATGCATGGGCCAAACGTATT
ATTGAAGGTATTCGTGCAGGTAGCATTGGTAGCGGTAGCGGCACCGCAGCTTAT
GCAATTGCCTGGCATCGTGATCGTCATGATGATCTGCTGAGTCCGCATGTTGCA
GCAGTTGAACTGGTTAATGTTCTGCGTCCGACCGTTGCCATTGCAGTGTATATTA
CCTTTGTTGCACATGCACTGCAGACCTGTAGCGGTATTCGCGCAGCACTGGTTC
AGCAGCCGGATTATGCAGAACTGTTTGTTCAAGAAGTGCGTCGCTTTTATCCGT
TTTTTCCGGCAGTTGTTGCACGTGCCAGCCAGGATTTTGAATGGGAAGGTATGG
CATTTCCGGAAGGTCGTCAGGTTGTTCTGGATCTGTATGGTAGCAATCATGATG
CAGCAACCTGGGCTGATCCGCAAGAATTTCGTCCGGAACGCTTTCGCGCATGG
GATGAAGATAGCTTTAACTTTATTCCGCAGGGTGGCGGTGATCATTATCTGGGT
CATCGTTGTCCGGGTGAATGGATTGTTCTGGCAATTATGAAAGTTGCAGCACAT
CTGCTGGTGAATGCAATGCGTTATGATGTTCCGGATCAGGATCTGAGCATTGAT
TTTGCACGTCTGCCTGCACTGCCGAAAAGCGGTTTTGTTATGCGTAATGTTCATA
TCGGTGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGANAFESRFLLKKTNCLKGAKAAEIF
YDTTRFEREGAMPVAIQKTLLGQGGVQGLDGETHRHRKQMFMGLMTPERVRALA
QLFEAEWRRAVPGWTRKGEIVFYDELHEPLTRAVCAWAGVPLPDDEAGNRAGELR
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HDDLLSPHVAAVELVNVLRPTVAIAVYITFVAHALQTCSGIRAALVQQPDYAELFVQE
VRRFYPFFPAVVARASQDFEWEGMAFPEGRQVVLDLYGSNHDAATWADPQEFRP
ERFRAWDEDSFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQ
DLSIDFARLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V1 (pEG 690)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAAGCCTGCTGG
CAGATCCGTATCGCTTCATTAGTCGCCAGTGTCAGCGTCTGGGTGCCAATGCAT
TCGAAAGCCGCTTCCTGCTGAAAAAAACCAATTGTCTGAAAGGTGCAAAAGCCG
CAGAAATCTTCTATGATACCACCCGCTTCGAACGTGAAGGCGCCATGCCGGTTG
CAATTCAGAAAACCTTACTGGGCCAGGGCGGTGTGCAGGGCCTGGATGGTGAA
ACACATCGTCATCGTAAACAGATGTTCATGGGTCTGATGACCCCGGAACGCGTT
CGCGCCCTGGCCCAACTGTTCGAAGCCGAATGGCGTCGTGCAGTGCCGGGCT
GGACCCGTAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAACCGCTGACCC
GTGCAGTGTGTGCCTGGGCCGGCGTTCCGCTGCCTGATGATGAAGCAGGCAAT
CGTGCAGGTGAACTGCGCGCACTGTTCGATGCAGCCGGTAGCGCCAGTCCGC
GCCATCTGTGGAGTCGCCTGGCACGCCGTCGTGTTGATGCATGGGCAAAACGC
ATTATTGAAGGCATTCGTGCAGGCAGCATTGGTAGCGGTAGCGGCACCGCCGC
ATACGCTATTGCATGGCATCGCGATCGCCATGATGATCTGCTGAGCCCGCATGT
TGCCGCAGTGGAACTGGTTAATGTGCTGCGCCCGACCGTGGCCATTGCCGTGT
ATATTACCTTCGTTGCACATGCCCTGCAGACCCATAGTGGTATTCGCGCCGCAC
TGGTGCAGCAGCCGGATTATGCAGAACTGTTCGTGCAGGAAGTTCGTCGCTTCT
ATCCGTTCTTCCCGGCAGTGGTGGCCCGTGCAAGCCAGGACTTCGAATGGGAA
GGCATGGCCTTCCCGGAAGGTCGTCAGGTGGTTCTGGATCTGTATGGCAGTAA
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GCGCATGGGATGAAGATAGCTTCAACTTCATTCCGCAGGGTGGTGGTGATCATT
ATCTGGGCCATCGCTGTCCGGGCGAATGGATTGTTCTGGCCATTATGAAAGTTG
CAGCACATCTGCTGGTTAATGCCATGCGCTATGATGTGCCGGATCAGGATCTGA
GTATTGACTTCGCCCGCCTGCCGGCCCTGCCGAAAAGCGGCTTCGTGATGCGC
AATGTTCATATTGGCGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGANAFESRFLLKKTNCLKGAKAAEIF
YDTTRFEREGAMPVAIQKTLLGQGGVQGLDGETHRHRKQMFMGLMTPERVRALA
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HDDLLSPHVAAVELVNVLRPTVAIAVYITFVAHALQTHSGIRAALVQQPDYAELFVQE
VRRFYPFFPAVVARASQDFEWEGMAFPEGRQVVLDLYGSNHDAATWADPQEFRP
ERFRAWDEDSFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQ
DLSIDFARLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V2 (pEG 692)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAAGTCTGCTGG
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CCGAAATCTTCTATGATACCACCCGCTTCGAACGTGAAGGTGCAATGCCGGTGG
CAATTCAGAAAACCTTACTGGGTCAGGGCGGTGTGCAGGGCCTGGATGGTGAA
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GGACCCGCAAAGGTGAAATTGTGTTCTATGATGAACTGCATGAACCGCTGACCC
GCGCAGTGTGTGCATGGGCCGGTGTGCCGCTGCCGGATGATGAAGCCGGCAA
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AGTTGCAATTGCATGGCATCGTGATCGTCATGATGATCTGCTGAGTCCGCATGT
GGCCGCCGTGGAACTGGTGAATGTTCTGCGTCCGACCGTTGCAATTGCGGTGT
ATATTACCTTCGTGGCCCATGCCCTGCAGACCCATAGTGGTATTCGTGCAGCAC
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ATCCGTTCTTCCCGGCAGTGGTTGCCCGCGCAAGCCAGGACTTCGAATGGGAA
GGTATGGCCTTCCCGGAAGGCCGTCAGGTGGTGCTGGATCTGTATGGTAGTAA
TCATGATGCAGCAACCTGGGCCGATCCGCAGGAATTCCGTCCGGAACGCTTCC
GTGCATGGGATGAAGATAGCTTCAACTTCATTCCGCAGGGTGGTGGTGATCATG
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CAGCACATCTGCTGGTGAATGCCATGCGCTATGATGTTCCGGATCAGGATCTGA
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Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGANAFESRFLLKKTNCLKGAKAAEIF
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HDDLLSPHVAAVELVNVLRPTVAIAVYITFVAHALQTHSGIRAALVQQPDYAELFVQE
VRRFYPFFPAVVARASQDFEWEGMAFPEGRQVVLDLYGSNHDAATWADPQEFRP
ERFRAWDEDSFNFIPQGGGDHVLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQ
DLSIDFARLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V3 (pEG 691)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAAGTCTGCTGG
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TCGAAAGCCGCTTCCTGCTGAAAAAAACCAATTGTCTGAAAGGTGCAAAAGCAG
CAGAAATCTTCTATGATACCACCCGCTTCGAACGTGAAGGCGCCATGCCGGTTG
CCATTCAGAAAACCTTACTGGGCCAGGGCGGCGTTCAGGGCCTGGATGGTGAA

ACACATCGTCATCGTAAACAGATGTTCATGGGCCTGATGACCCCGGAACGCGTG
CGTGCACTGGCCCAGCTGTTCGAAGCAGAATGGCGCCGTGCAGTTCCGGGTTG
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CAGCTGGGCCATCGCTGTCCGGGTGAATGGATTGTGCTGGCAATTATGAAAGT
GGCAGCCCATCTGCTGGTTAATGCCATGCGCTATGATGTTCCGGATCAGGATCT
GAGCATTGACTTCGCCCGTCTGCCGGCACTGCCGAAAAGTGGCTTCGTTATGC
GTAATGTTCATATTGGTGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGANAFESRFLLKKTNCLKGAKAAEIF
YDTTRFEREGAMPVAIQKTLLGQGGVQGLDGETHRHRKQMFMGLMTPERVRALA
QLFEAEWRRAVPGWTRKGEIVFYDELHEPLTRAVCAWAGVPLPDDEAGNRAGELR
ALFDAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGSIGSGSGTAAQAIAWHRDR
HDDLLSPHVAAVELVNVLRPTVAIAVYITFVAHALQTHSGIRAALVQQPDYAELFVQE
VRRFYPFFPAVVARASQDFEWEGMAFPEGRQVVLDLYGSNHDAATWADPQEFRP
ERFRAWDEDSFNFIPQGGGDHQLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQ
DLSIDFARLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V1-P02 (pEG 696)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGTCCGGATGAAACCTTAAGTCTGCTGG
CAGATCCGTATCGCTTCATTAGTCGTCAGTGCCAGCGTCTGGGCAGCAATGCAT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGAAAGGCGCAAAAGCAG
CAGAAATCTTCTATGATACCACCCGCTTCGAACGCGAAGGTGCCATGCCGAAAG
CCATTCAGAAAACCTTACTGGGTCAGGGCGGTGTTCAGGGTCTGGATGGTGAA
GCACATCGTCATCGCAAACAGATGTTCATGAGTCTGATGACCCCGGAACGTGTT
CGCGCCCTGGCACAGCTGTTCGAAGCCGAATGGCGTCGTGCCGTTGAAGATTG
GACCCGCAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCCG
TGCCGTGTGCGCCTGGGCCGGTGTTCCTCTGCCGGATGATGAAGCCAAAAATC
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CGCCAGGATCCGGATTATGCCGAACTGTTCGTGCAGGAAGTTCGCCGCTTCTAT
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ATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGCGGCGGCGATCATTATC
TGGGTCATCGTTGTCCGGGTGAATGGATTGTGCTGGCAATTATGAAAGTTGCCG
CACATCTGCTGGTTAATGCCATGCGCTATGATGTTCCGGATCAGGATCTGAGCA
TTGACTTCAGCCGTCTGCCGGCCCTGCCGAAAAGCGGCTTCGTTATGCGTAATG
TGCATATTGGTGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGSNAFETRFLLKKTICLKGAKAAEIFY
DTTRFEREGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQL
FEAEWRRAVEDWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAKNRARELRALF
DAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGKIPAPEGTAAYAIAWHRDLHGKL
LSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVRRF
YPFFPAVVARARQDFEWEGMAFPEGRQVVLDLYGTNHDAATWEDPQEFRPERFR
DWDEDPFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDLSID
FSRLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V2-P02 (pEG 698)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAAGTCTGCTGG
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TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGAAAGGTGCAAAAGCAG
CCGAAATCTTCTATGATACCACCCGCTTCGAACGCGAAGGTGCCATGCCGAAAG
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CGCAGTGTGCGCATGGGCCGGTGTTCCGCTGCCGGATGATGAAGCAAAAAATC
GCGCACGTGAACTGCGCGCCCTGTTCGATGCCGCCGGCTCAGCAAGTCCGCG
CCATCTGTGGAGCCGTCTGGCACGCCGCCGTGTTGATGCCTGGGCCAAACGCA
TTATTGAAGGTATTCGCGCCGGCAAAATTCCGGCCCCGGAAGGTACCGCAGCA
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GCCGCCGTTGAACTGGTGAATGTTCTGCGTCCGACCGTGGCAATTGCAGTGTAT
ATTACCTTCGTGGCACATGCACTGCATACCCATCCGGGTATTCGCGAAGCCCTG
CGCCAGGATCCGGATTATGCAGAACTGTTCGTTCAGGAAGTGCGCCGCTTCTAT
CCGTTCTTCCCGGCCGTGGTGGCCCGTGCACGTCAGGACTTCGAATGGGAAGG
CATGGCATTCCCGGAAGGTCGCCAGGTTGTGCTGGATCTGTATGGTACCAATCA
TGATGCCGCCACCTGGGAAGATCCGCAGGAATTCCGTCCGGAACGCTTCCGTG
ATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGTGGCGGCGATCATGTTC
TGGGCCATCGCTGTCCGGGCGAATGGATTGTGCTGGCCATTATGAAAGTGGCC
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ATTGACTTCAGTCGTCTGCCGGCACTGCCGAAAAGTGGCTTCGTTATGCGTAAT
GTTCATATTGGCGGTTAActcgag

Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGSNAFETRFLLKKTICLKGAKAAEIFY
DTTRFEREGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQL
FEAEWRRAVEDWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAKNRARELRALF
DAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGKIPAPEGTAAVAIAWHRDLHGKL
LSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVRRF
YPFFPAVVARARQDFEWEGMAFPEGRQVVLDLYGTNHDAATWEDPQEFRPERFR
DWDEDPFNFIPQGGGDHVLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDLSID
FSRLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V3-P02 (pEG 697)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAAGTCTGCTGG
CAGATCCGTATCGCTTCATTAGTCGTCAGTGCCAGCGTCTGGGCAGTAATGCCT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGAAAGGTGCAAAAGCAG
CCGAAATCTTCTATGATACCACCCGCTTCGAACGTGAAGGTGCCATGCCGAAAG
CAATTCAGAAAACCTTACTGGGTCAGGGCGGTGTTCAGGGCCTGGATGGTGAA
GCACATCGCCATCGTAAACAGATGTTCATGAGTCTGATGACCCCGGAACGCGTG
CGTGCCCTGGCCCAACTGTTCGAAGCAGAATGGCGTCGTGCAGTGGAAGATTG
GACCCGTAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCCG
TGCAGTGTGTGCATGGGCAGGTGTGCCGCTGCCGGATGATGAAGCCAAAAATC
GTGCACGCGAACTGCGCGCACTGTTCGATGCAGCCGGTAGTGCAAGTCCGCGC
CATCTGTGGAGTCGTCTGGCACGTCGTCGCGTGGATGCATGGGCCAAACGTAT
TATTGAAGGCATTCGTGCAGGCAAAATTCCGGCCCCGGAAGGTACCGCCGCCC
AGGCAATTGCATGGCATCGTGATCTGCATGGCAAACTGCTGAGCCCGCATGTTG
CCGCAGTTGAACTGGTTAATGTTCTGCGTCCGACCGTTGCCATTGCAGTGTATA
TTACCTTCGTGGCCCATGCCCTGCATACCCATCCGGGTATTCGTGAAGCCCTGC
GCCAGGATCCGGATTATGCCGAACTGTTCGTTCAGGAAGTGCGCCGCTTCTATC
CGTTCTTCCCGGCCGTGGTGGCACGCGCACGTCAGGACTTCGAATGGGAAGGC
ATGGCATTCCCGGAAGGCCGTCAGGTTGTTCTGGATCTGTATGGTACCAATCAT
GATGCCGCCACCTGGGAAGATCCGCAGGAATTCCGCCCGGAACGCTTCCGTGA
TTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGCGGTGGCGATCATCAGCT
GGGTCATCGCTGTCCGGGTGAATGGATTGTTCTGGCAATTATGAAAGTGGCCG
CACATCTGCTGGTGAATGCAATGCGTTATGATGTTCCGGATCAGGATCTGAGTA
TTGACTTCAGTCGCCTGCCGGCCCTGCCGAAAAGCGGCTTCGTTATGCGTAATG
TGCATATTGGCGGTTAActcgag

Protein sequence:

MPKTPHTKGPDETLSLLADPYRFISRQCQRLGSNAFETRFLLKKTICLKGAKAAEIFY
DTTRFEREGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQL
FEAEWRRAVEDWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAKNRARELRALF
DAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGKIPAPEGTAAQAIAWHRDLHGK
LLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVRR

FYPFFPAVVARARQDFEWEGMAFPEGRQVVLDLYGTNHDAATWEDPQEFRPERF
RDWDEDPFNFIPQGGGDHQLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDLSI
DFSRLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V1-P03 (pEG 699)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAGCACTGCTGG
CCGATCCGTATCGCTTCATTAGTCGCCAGTGCCAGCGTCTGGGTAGTAATGCCT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGCGTGGCGCCAAAGCAG
CCGAAATCTTCTATGATACCACCCGCTTCCAGCGCGAAGGCGCCATGCCGAAA
GCCATTCAGAAAACCTTACTGGGCCAGGGTGGTGTTCAGGGTCTGGATGGCGA
AGCCCATCGCCATCGCAAACAGATGTTCATGAGTCTGATGACCCCGGAACGCG
TTCGTGCACTGGCCCAGCTGTTCGAAGCCGAATGGCGCCGCGCAGTTGAACGT
TGGACCCGCAAAGGTGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACC
CGCGCCGTGTGTGCCTGGGCAGGTGTTCCGCTGCCGGATGATGAAGCCAAAAA
TCGCGCCCGCGAACTGCGTGCCCTGTTCGATGCAGCAGGTAGTGCAAGCCCGC
GTCATCTGTGGAGTCGCCTGGCACGTCGCCGCGTTGATGCCTGGGCAAAACGT
ATTATTGAAGGTATTCGCGCAGGCAAAATTCCGGCCCCGGAAGGCACCGCAGC
ATACGCTATTGCATGGCATCGTGATCTGCATGGCAAACTGCTGAGTCCGCATGT
TGCAGCAGTTGAACTGGTGAATGTGCTGCGTCCGACCGTGGCAATTGCAGTGT
ATATTACCTTCGTGGCACATGCCCTGCATACCCATCCGGGTATTCGTGAAGCCC
TGCGTCAGGATCCGGATTATGCAGAACTGTTCGTGCAGGAAGTGCGTCGCTTCT
ATCCGTTCTTCCCGGCAGTTGTTGCACGCGCACGCCAGGACTTCGAATGGGAA
GGCATGGCATTCCCGGAAGGTCGTCAGGTTGTGCTGGATCTGTATGGCACCAA
TCATGATGCCGCAACCTGGGAAGATCCGCAGGAATTCCGTCCGGAACGCTTCC
GCGATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGTGGCGGCGATCATT
ATCTGGGCCATCGTTGTCCGGGTGAATGGATTGTTCTGGCAATTATGAAAGTGG
CAGCCCATCTGCTGGTTAATGCCATGCGTTATGATGTGCCGGATCAGGATCTGA
GCATTGACTTCAGTCGCCTGCCGGCCCTGCCGAAAAGTGGCTTCGTTATGCGTA
ATGTGCATATTGGTGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLALLADPYRFISRQCQRLGSNAFETRFLLKKTICLRGAKAAEIFY
DTTRFQREGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQ
LFEAEWRRAVERWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAKNRARELRAL
FDAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGKIPAPEGTAAYAIAWHRDLHGK
LLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVRR
FYPFFPAVVARARQDFEWEGMAFPEGRQVVLDLYGTNHDAATWEDPQEFRPERF
RDWDEDPFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDLSI
DFSRLPALPKSGFVMRNVHIGG

$PO_{SP\alpha}$ variant V2-P03 (pEG 701)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGTCCGGATGAAACCTTAGCACTGCTGG
CCGATCCGTATCGCTTCATTAGTCGCCAGTGTCAGCGTCTGGGTAGCAATGCCT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGCGCGGTGCAAAAGCAG
CAGAAATCTTCTATGATACCACCCGCTTCCAGCGTGAAGGTGCAATGCCGAAAG
CCATTCAGAAAACCTTACTGGGCCAGGGTGGTGTGCAGGGTCTGGATGGTGAA
GCACATCGCCATCGCAAACAGATGTTCATGAGCCTGATGACCCCGGAACGTGT
GCGTGCCCTGGCACAGCTGTTCGAAGCCGAATGGCGTCGCGCAGTTGAACGCT
GGACCCGTAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCC
GTGCCGTGTGTGCATGGGCAGGTGTGCCGCTGCCGGATGATGAAGCCAAAAAT
CGCGCACGTGAACTGCGTGCCCTGTTCGATGCCGCAGGTAGTGCCAGCCCGC
GTCATCTGTGGAGTCGTCTGGCACGTCGTCGCGTTGATGCCTGGGCCAAACGT
ATTATTGAAGGCATTCGTGCCGGTAAAATTCCGGCACCGGAAGGTACCGCCGC
CGTTGCAATTGCATGGCATCGCGATCTGCATGGTAAACTGCTGAGCCCGCATGT
TGCAGCCGTTGAACTGGTGAATGTGCTGCGCCCGACCGTGGCAATTGCCGTGT
ATATTACCTTCGTGGCACATGCCCTGCATACCCATCCGGGTATTCGTGAAGCCC
TGCGCCAGGATCCGGATTATGCCGAACTGTTCGTTCAGGAAGTTCGCCGCTTCT
ATCCGTTCTTCCCGGCAGTTGTGGCCCGTGCACGCCAGGACTTCGAATGGGAA
GGCATGGCCTTCCCGGAAGGTCGCCAGGTTGTGCTGGATCTGTATGGTACCAA
TCATGATGCAGCCACCTGGGAAGATCCGCAGGAATTCCGTCCGGAACGCTTCC
GTGATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGTGGCGGCGATCAT
GTTCTGGGCCATCGTTGTCCGGGTGAATGGATTGTTCTGGCCATTATGAAAGTG
GCAGCCCATCTGCTGGTGAATGCCATGCGCTATGATGTTCCGGATCAGGATCTG
AGCATTGACTTCAGCCGCCTGCCGGCACTGCCGAAAAGCGGCTTCGTTATGCG
CAATGTGCATATTGGTGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLALLADPYRFISRQCQRLGSNAFETRFLLKKTICLRGAKAAEIFY
DTTRFQREGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQ
LFEAEWRRAVERWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAKNRARELRAL
FDAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGKIPAPEGTAAVAIAWHRDLHGK
LLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVRR
FYPFFPAVVARARQDFEWEGMAFPEGRQVVLDLYGTNHDAATWEDPQEFRPERF
RDWDEDPFNFIPQGGGDHVLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDLSI
DFSRLPALPKSGFVMRNVHIGG

$PO_{SP\alpha}$ variant V3-P03 (pEG 700)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGCCCGGATGAAACCTTAGCACTGCTGG
CCGATCCGTATCGCTTCATTAGTCGTCAGTGCCAGCGCCTGGGTAGTAATGCAT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGCGCGGTGCCAAAGCAG
CAGAAATCTTCTATGATACCACCCGCTTCCAGCGCGAAGGTGCAATGCCGAAAG
CCATTCAGAAAACCTTACTGGGTCAGGGCGGTGTGCAGGGCCTGGATGGTGAA

GCACATCGCCATCGCAAACAGATGTTCATGAGCCTGATGACCCCGGAACGTGTT
CGTGCACTGGCACAGCTGTTCGAAGCAGAATGGCGCCGTGCAGTGGAACGTTG
GACCCGTAAAGGTGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCCGT
GCAGTGTGTGCCTGGGCAGGTGTTCCGCTGCCGGATGATGAAGCAAAAAATCG
TGCCCGTGAACTGCGCGCACTGTTCGATGCCGCAGGTAGCGCAAGCCCGCGC
CATCTGTGGAGCCGCCTGGCTCGTCGCCGTGTGGATGCATGGGCAAAACGTAT
TATTGAAGGTATTCGCGCAGGCAAAATTCCGGCACCGGAAGGCACCGCCGCAC
AGGCAATTGCATGGCATCGCGATCTGCATGGCAAACTGCTGAGTCCGCATGTG
GCCGCAGTGGAACTGGTGAATGTTCTGCGCCCGACCGTTGCAATTGCAGTGTA
TATTACCTTCGTGGCACATGCACTGCATACCCATCCGGGTATTCGTGAAGCCCT
GCGTCAGGATCCGGATTATGCCGAACTGTTCGTGCAGGAAGTTCGCCGCTTCTA
TCCGTTCTTCCCGGCAGTTGTGGCACGCGCCCGCCAGGACTTCGAATGGGAAG
GTATGGCCTTCCCGGAAGGCCGTCAGGTGGTGCTGGATCTGTATGGCACCAAT
CATGATGCCGCAACCTGGGAAGATCCGCAGGAATTCCGTCCGGAACGCTTCCG
TGATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGCGGTGGTGATCATCA
GCTGGGCCATCGCTGTCCGGGTGAATGGATTGTTCTGGCCATTATGAAAGTTGC
CGCACATCTGCTGGTGAATGCCATGCGTTATGATGTTCCGGATCAGGATCTGAG
CATTGACTTCAGTCGCCTGCCGGCACTGCCGAAAAGCGGCTTCGTGATGCGCA
ATGTTCATATTGGCGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLALLADPYRFISRQCQRLGSNAFETRFLLKKTICLRGAKAAEIFY
DTTRFQREGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQ
LFEAEWRRAVERWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAKNRARELRAL
FDAAGSASPRHLWSRLARRRVDAWAKRIIEGIRAGKIPAPEGTAAQAIAWHRDLHG
KLLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVR
RFYPFFPAVVARARQDFEWEGMAFPEGRQVVLDLYGTNHDAATWEDPQEFRPER
FRDWDEDPFNFIPQGGGDHQLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPDQDL
SIDFSRLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V1-P04 (pEG 702)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGTCCGGATGAAACCTTAGCACTGCTGG
CAGATCCGTATCGCTTCATTAGTCGTCAGTGCCAGCGCCTGGGTACCAATGCAT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGCGCGGTGCCAAAGCCG
CAGAAATCTTCTATGATACCACCCGCTTCCAGCGTCATGGCGCAATGCCGAAAG
CAATTCAGAAAACCTTACTGGGTCAGGGCGGCGTTCAGGGTCTGGATGGTGAA
GCACATCGCCATCGCAAACAGATGTTCATGAGCCTGATGACCCCGGAACGTGTT
CGCGCACTGGCACAGCTGTTCGAAGCAGAATGGCGTCGCGCAGTTGAACGCTG
GACCCGCAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCCG
CGCCGTGTGTGCATGGGCCGGTGTTCCGCTGCCGGATGATGAAGCAGAAAAAC
GCGCACGTGAACTGCGTGCACTGTTCGATGCCGCAGGCAGCGCAAGTCCGCGT
CATCTGTGGAGTCGTTGGGCCCGTCGCCGTGTGGATGCATGGGCCAAACGTAT
TATTGAAGGTATTCGTGCAGGCAAAATTCCGGCACCGGAAGGCACCGCCGCAT
ACGCTATTGCCTGGCATCGTGATCTGGATGGTCGTCTGCTGAGTCCGCATGTTG
CAGCAGTGGAACTGGTTAATGTGCTGCGTCCGACCGTTGCCATTGCCGTGTATA

TTACCTTCGTGGCACATGCACTGCATACCCATCCGGGTATTCGCGAAGCACTGC
GCCAGGATCCGGATTATGCCGAACTGTTCGTTCAGGAAGTTCGTCGCTTCTATC
CGTTCTTCCCGGCAGTTGTTGCACGTGCACGCCAGGACTTCGAATGGGAAGGT
TATGCATTCCCGGAAGGCCGCCAGGTTGTGCTGGATCTGTATGGTACCAATCAT
GATGCAGCCATCTGGGAAGATCCGCAGGAATTCCGTCCGGAACGCTTCCGTGA
TTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGTGGTGGCGATCATTATCT
GGGTCATCGCTGCCCGGGTGAATGGATTGTGCTGGCCATTATGAAAGTTGCCG
CACATCTGCTGGTTAATGCAATGCGTTATGATGTTCCGCCGCAGGATCTGAGCA
TTGACTTCAGTCGTCTGCCGGCACTGCCGAAAAGCGGCTTCGTTATGCGTAATG
TGCATATTGGTGGCTAActcgag

Protein sequence:

MPKTPHTKGPDETLALLADPYRFISRQCQRLGTNAFETRFLLKKTICLRGAKAAEIFY
DTTRFQRHGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQ
LFEAEWRRAVERWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAEKRARELRAL
FDAAGSASPRHLWSRWARRRVDAWAKRIIEGIRAGKIPAPEGTAAYAIAWHRDLDG
RLLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVR
RFYPFFPAVVARARQDFEWEGYAFPEGRQVVLDLYGTNHDAAIWEDPQEFRPERF
RDWDEDPFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPPQDLSI
DFSRLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V2-P04 (pEG 704)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGTCCGGATGAAACCTTAGCACTGCTGG
CAGATCCGTATCGCTTCATTAGTCGTCAGTGCCAGCGTCTGGGTACCAATGCCT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGCGCGGCGCCAAAGCCG
CCGAAATCTTCTATGATACCACCCGCTTCCAGCGCCATGGCGCCATGCCGAAAG
CAATTCAGAAAACCTTACTGGGTCAGGGTGGTGTGCAGGGTCTGGATGGCGAA
GCACATCGTCATCGTAAACAGATGTTCATGAGCCTGATGACCCCGGAACGTGTT
CGTGCACTGGCACAGCTGTTCGAAGCCGAATGGCGTCGCGCAGTGGAACGTTG
GACCCGTAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCCG
CGCAGTGTGTGCATGGGCCGGTGTTCCGCTGCCGGATGATGAAGCCGAAAAAC
GTGCCCGTGAACTGCGTGCCCTGTTCGATGCCGCCGGCAGTGCCAGTCCGCGT
CATCTGTGGAGTCGTTGGGCCCGCCGCCGCGTTGATGCATGGGCTAAACGCAT
TATTGAAGGTATTCGTGCCGGCAAAATTCCGGCACCGGAAGGCACCGCCGCCT
ATGCAATTGCCTGGCATCGCGATCTGGATGGTCGCCTGCTGAGTCCGCATGTT
GCCGCCGTTGAACTGGTGAATGTGCTGCGTCCGACCGTTGCCATTGCAGTGTA
TATTACCTTCGTGGCACATGCCCTGCATACCCATCCGGGTATTCGCGAAGCACT
GCGTCAGGATCCGGATTATGCAGAACTGTTCGTGCAGGAAGTGCGCCGCTTCT
ATCCGTTCTTCCCGGCCGTTGTGGCACGCGCCCGCCAAGACTTCGAATGGGAA
GGTTATGCATTCCCGGAAGGCCGCCAGGTTGTTCTGGATCTGTATGGTACCAAT
CATGATGCAGCCATCTGGGAAGATCCGCAGGAATTCCGTCCGGAACGCTTCCG
CGATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGTGGTGGTGATCATTA
TCTGGGCCATCGCTGCCCGGGCGAATGGATTGTGCTGGCAATTATGAAAGTTG
CAGCCCATCTGCTGGTGAATGCCATGCGTTATGATGTGCCGCCGCAGGATCTG

AGTATTGACTTCAGTCGCCTGCCGGCCCTGCCGAAAAGTGGCTTCGTGATGCG
TAATGTGCATATTGGTGGTTAActcgag

Protein sequence:

MPKTPHTKGPDETLALLADPYRFISRQCQRLGTNAFETRFLLKKTICLRGAKAAEIFY
DTTRFQRHGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQ
LFEAEWRRAVERWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAEKRARELRAL
FDAAGSASPRHLWSRWARRRVDAWAKRIIEGIRAGKIPAPEGTAAYAIAWHRDLDG
RLLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVR
RFYPFFPAVVARARQDFEWEGYAFPEGRQVVLDLYGTNHDAAIWEDPQEFRPERF
RDWDEDPFNFIPQGGGDHYLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPPQDLSI
DFSRLPALPKSGFVMRNVHIGG

PO$_{SP\alpha}$ variant V3-P04 (pEG 703)

Optimised DNA sequence:

catATGCCGAAAACACCTCATACCAAAGGTCCGGATGAAACCTTAGCCCTGCTGG
CAGATCCGTATCGCTTCATTAGCCGTCAGTGCCAGCGTCTGGGTACCAATGCCT
TCGAAACCAGATTCCTGCTGAAAAAAACCATCTGTCTGCGCGGTGCCAAAGCAG
CCGAAATCTTCTATGATACCACCCGCTTCCAGCGTCATGGCGCAATGCCGAAAG
CAATTCAGAAAACCTTACTGGGCCAGGGCGGTGTGCAGGGTCTGGATGGTGAA
GCCCATCGTCATCGCAAACAGATGTTCATGAGCCTGATGACCCCGGAACGTGTT
CGCGCCCTGGCCCAGCTGTTCGAAGCCGAATGGCGCCGCGCAGTGGAACGCT
GGACCCGTAAAGGCGAAATTGTGTTCTATGATGAACTGCATGAAATTCTGACCC
GTGCCGTGTGTGCATGGGCCGGTGTGCCGCTGCCGGATGATGAAGCAGAAAAA
CGCGCACGCGAACTGCGCGCACTGTTCGATGCAGCCGGCAGTGCCAGTCCGC
GCCATCTGTGGAGCCGCTGGGCCAGACGTCGCGTTGATGCATGGGCCAAACG
CATTATTGAAGGCATTCGTGCCGGTAAAATTCCGGCCCCGGAAGGCACCGCAG
CACAGGCAATTGCATGGCATCGCGATCTGGATGGCCGCCTGCTGAGTCCGCAT
GTTGCCGCAGTGGAACTGGTGAATGTTCTGCGCCCGACCGTTGCAATTGCAGT
GTATATTACCTTCGTTGCCCATGCACTGCATACCCATCCGGGCATTCGCGAAGC
ACTGCGCCAGGATCCGGATTATGCAGAACTGTTCGTGCAGGAAGTGCGTCGCT
TCTATCCGTTCTTCCCGGCCGTTGTTGCCCGCGCCCGCCAAGACTTCGAATGG
GAAGGTTATGCCTTCCCGGAAGGTCGTCAGGTTGTGCTGGATCTGTATGGTACC
AATCATGATGCCGCCATCTGGGAAGATCCGCAGGAATTCCGCCCGGAACGCTT
CCGTGATTGGGATGAAGATCCGTTCAACTTCATTCCGCAGGGTGGTGGCGATCA
TCAGCTGGGCCATCGTTGCCCGGGTGAATGGATTGTGCTGGCAATTATGAAAGT
TGCAGCACATCTGCTGGTTAATGCCATGCGCTATGATGTTCCGCCGCAGGATCT
GAGCATTGACTTCAGTCGCCTGCCGGCCCTGCCGAAAAGTGGCTTCGTGATGC
GCAATGTTCATATTGGTGGTTAActcgag

Protein sequence:

MPKTPHTKGPDETLALLADPYRFISRQCQRLGTNAFETRFLLKKTICLRGAKAAEIFY
DTTRFQRHGAMPKAIQKTLLGQGGVQGLDGEAHRHRKQMFMSLMTPERVRALAQ
LFEAEWRRAVERWTRKGEIVFYDELHEILTRAVCAWAGVPLPDDEAEKRARELRAL
FDAAGSASPRHLWSRWARRRVDAWAKRIIEGIRAGKIPAPEGTAAQAIAWHRDLDG
RLLSPHVAAVELVNVLRPTVAIAVYITFVAHALHTHPGIREALRQDPDYAELFVQEVR

RFYPFFPAVVARARQDFEWEGYAFPEGRQVVLDLYGTNHDAAIWEDPQEFRPERF
RDWDEDPFNFIPQGGGDHQLGHRCPGEWIVLAIMKVAAHLLVNAMRYDVPPQDLSI
DFSRLPALPKSGFVMRNVHIGG

PO$_{CLA}$ (pEG 306)

Optimised DNA sequence

catATGCTGCTGAAAGAAAACACCGCCAAAGATAAAGGTATTGATAGCACCCTGG
ATCTGCTGAAAGAGGGTTACCTGTTTATCAAAAATCGTGCCGATCATTATCAGAG
CGACCTGTTTGAAACCCGTCTGATGGGTCAGCGTATTATTTGTATGACCGGTGA
AGAAGCAGCCCGTATCTTTTATGATAGCGATAAATTCAAACGTCAGGGTGCAGC
ACCGAAACGTGTTCAAGAAACCCTGCTGGGTGAAAATGCAATTCAGACCCTGGA
TGGTGAAAGCCATCTGCATCGTAAAAAACTGTTTATGCTGCTGACCAATCAGGTT
CAGCAGAAACGTCTGGCAGAACTGACCACCGAAAAATGGGAAGCAAGCGCAAG
CAAATGGCATACCAAAAGCATTGTGCTGTTTAACGAAGCCAATGAAATTCTGTGT
CAGGTTGCATGTCATTGGGCAGGCGTTCCGCTGATGGAAAGCGATATCAAAAAC
CGTGCGGAAGATTTTAGCAGCATGATTGATAGCTTTGGTGCAGTTGGTCCGCGT
CATTGGAAAGGTAAAAAAGCACGTAATACCATTGAGGCCTGGATCAAAGAAATT
ATTGAAAATGTTCGTAGCGGTCGCATTCGTGCAGAAGAGGGTAGTCCGCTGCAT
GAAATTGCCTTTTATATCGATGTTAATGGCCAGCAGATGCCTGCAGAAATGGCA
GCAATTGAACTGATTAACATTCTGCGTCCGATTGTTGCAATTAGCACCTTTATTA
CCTTTAGCGCACTGGCACTGTATGAACATAGCGAATATCGCGAAAAACTGCAGA
GCAAAGATATCCGTTATCTGGAAATGTTTACCCAAGAAGTGCGTCGTTATTACCC
GTTTGCACCGTTTGTTGGTGCACGTGTTCGTAAAGATTTTCTGTGGAATAACTGC
GAGTTCAAAAAAGAAATGCTGGTGCTGCTGGATATTTATGGCACCAATCATGATA
GCCGTATTTGGCAGAAACCGTATGAATTTATTCCGGATCGTTTCCGCAGCTATAA
AGGTAACCTGTTCGATTTCATTCCGCAGGGTGGTGGTGATCCGAGCAGTACCCA
TCGTTGTCCGGGTGAAGGTATTACCCTGGAAATCATGAAAACCAGCCTGGATTT
TCTGAGCACCAAAATTGATTTTACCGTTCCGGATCAGGATCTGAGCTATAGCCT
GAGCAAAATTCCGACCCTGCCGAAAAGCGGTTTTATCATTGATAACATCAACCT
GAAACTGTAActcgag

Protein sequence:

MLLKENTAKDKGIDSTLDLLKEGYLFIKNRADHYQSDLFETRLMGQRIICMTGEEAAR
IFYDSDKFKRQGAAPKRVQETLLGENAIQTLDGESHLHRKKLFMLLTNQVQQKRLAE
LTTEKWEASASKWHTKSIVLFNEANEILCQVACHWAGVPLMESDIKNRAEDFSSMID
SFGAVGPRHWKGKKARNTIEAWIKEIIENVRSGRIRAEEGSPLHEIAFYIDVNGQQM
PAEMAAIELINILRPIVAISTFITFSALALYEHSEYREKLQSKDIRYLEMFTQEVRRYYP
FAPFVGARVRKDFLWNNCEFKKEMLVLLDIYGTNHDSRIWQKPYEFIPDRFRSYKG
NLFDFIPQGGGDPSSTHRCPGEGITLEIMKTSLDFLSTKIDFTVPDQDLSYSLSKIPTL
PKSGFIIDNINLKL

Analytical methods

Gas chromatography coupled with flame ionization detector

For quantitative and chiral analysis of biotransformations for the α-hydroxylation of **1a-d**, samples were measured by gas chromatography coupled with a flame ionization detector (GC-FID) using an Agilent 7890A GC system.

Method GC-01:

Column:	HP-5ms (30 m x 0.25 mm x 0.25 µm)
Carrier gas:	Helium
Inlet temperature:	250 °C
Detector temperature:	300 °C
Injection volume:	2 µL
Split ratio:	50:1
Oven temperature program:	100 °C, 0.5 min; 10 °C/min to 300 °C
Total run time:	20.5 min

Method GC-02:

Column:	CP-Chirasil-Dex CB (25 m x 0.32 mm x 0.25 µm)
Carrier gas:	Hydrogen
Inlet temperature:	250 °C
Detector temperature:	250 °C
Injection volume:	2 µL
Split ratio:	50:1
Oven temperature program:	100 °C, 1 min; 10 °C/min to 130 °C, 5 min; 10 °C/min to 180 °C, 1 min
Total run time:	15 min

Gas chromatography coupled with mass spectrometer

For qualitative information, biotransformations were analysed *via* gas chromatography coupled with a mass spectrometer (GC-MS) using an Agilent 8890 GC system with a mass-selective Agilent 5977C detector (electron impact ionization, 70 eV, quadrupole mass selection, mass scan = 33-400 m/z). An HP-5ms column (Agilent J&W, 30 m x 250 µm x 0.25 µm, stationary phase: bonded and cross-linked 5% phenyl methyl polysiloxane) was used with helium as carrier gas at a flow rate of 0.7 mL/min.

Method GC-03:

Column:	HP-5ms (30 m x 0.25 mm x 0.25 µm)
Inlet temperature:	250 °C
MS transfer line temperature:	300 °C
MS source temperature:	230 °C
MS quadrupole temperature:	150 °C
MS scan range:	m/z = 33-400
Split ratio:	90:1
Oven temperature program:	100 °C, 0.5 min; 10 °C/min to 300 °C
Total run time:	20.5 min

GC-chromatograms & MS-spectra

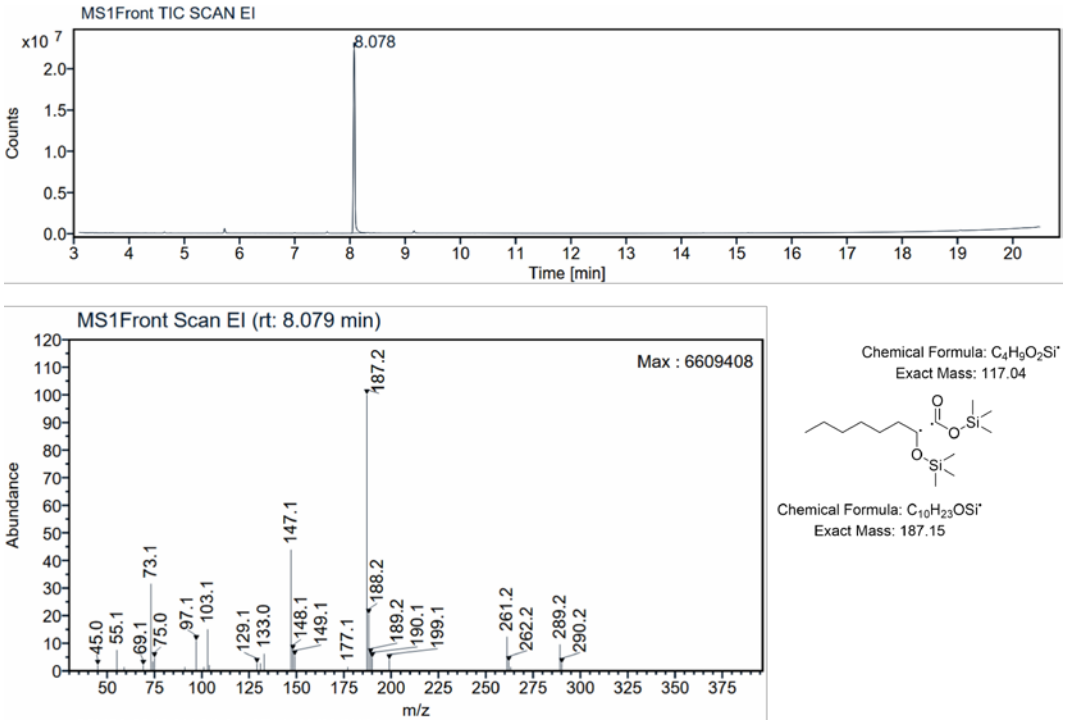

*Figure S8: GC-MS chromatogram and MS-spectrum for **2a** derivatised with BSTFA-TMCS. Method GC-03.*

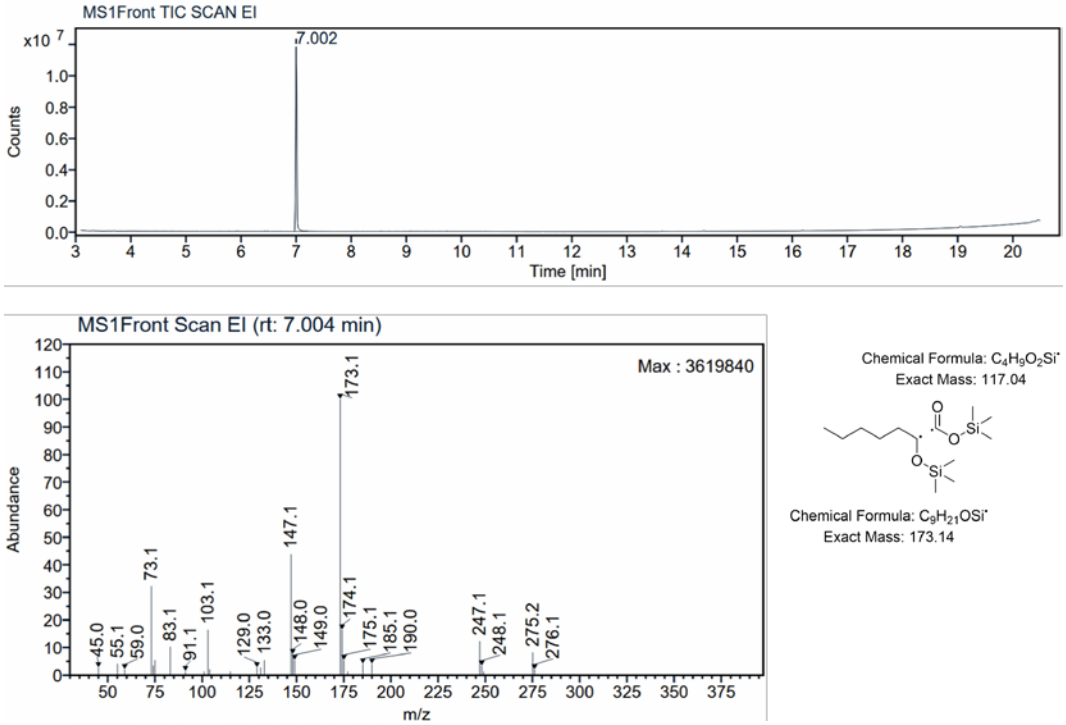

*Figure S9: GC-MS chromatogram and MS-spectrum for **2b** derivatised with BSTFA-TMCS. Method GC-03.*

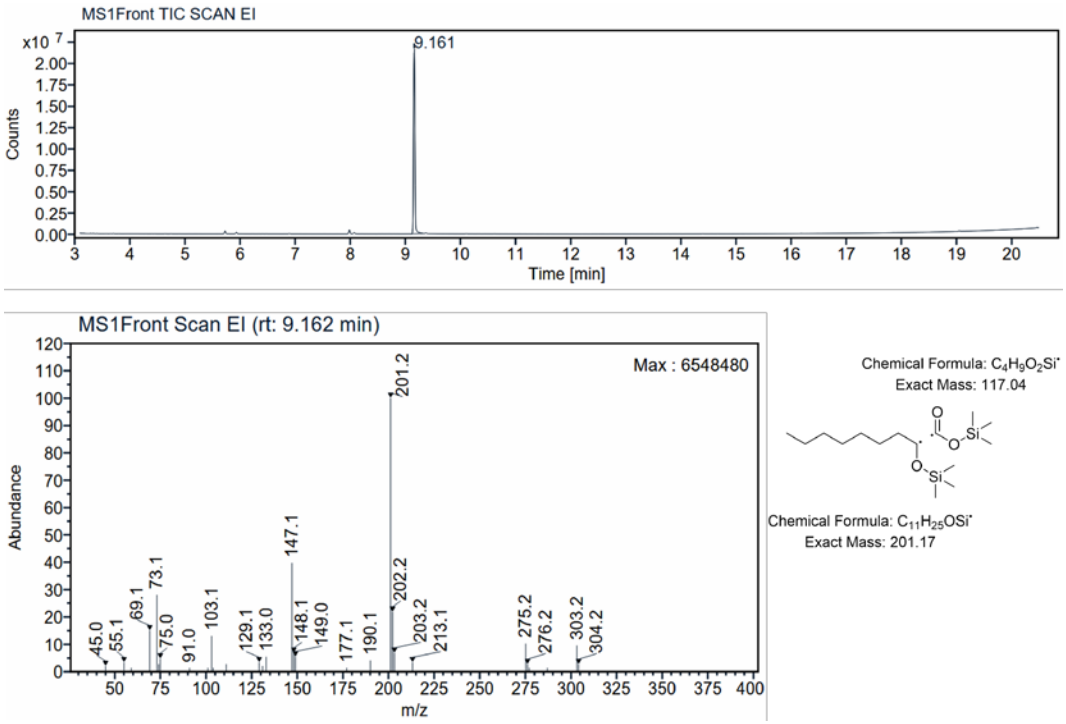

*Figure S10: GC-MS chromatogram and MS-spectrum for **2c** derivatised with BSTFA-TMCS. Method GC-03.*

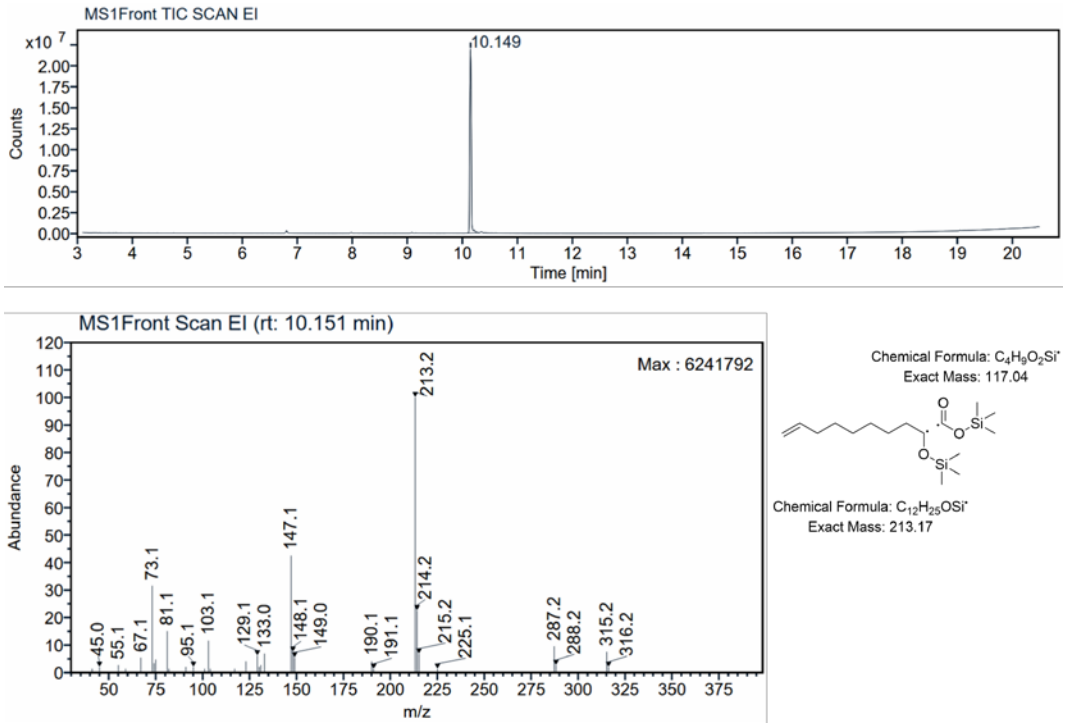

*Figure S11: GC-MS chromatogram and MS-spectrum for **2d** derivatised with BSTFA-TMCS. Method GC-03.*

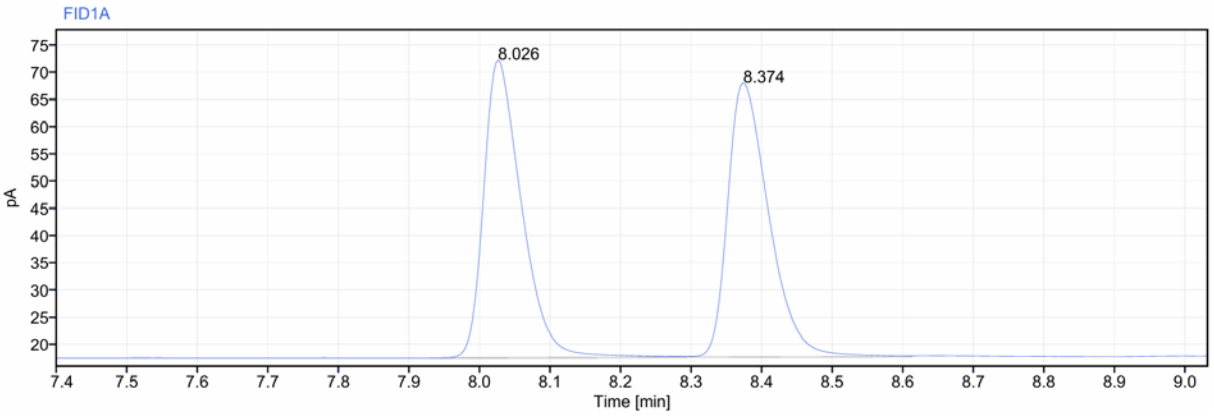

*Figure S12: GC-FID chromatogram of rac-**2a** derivatised with MeOH/ethyl chloroformate. (R)-**2a** (8.026 min), (S)-**2a** (8.374 min). Method GC-02.*

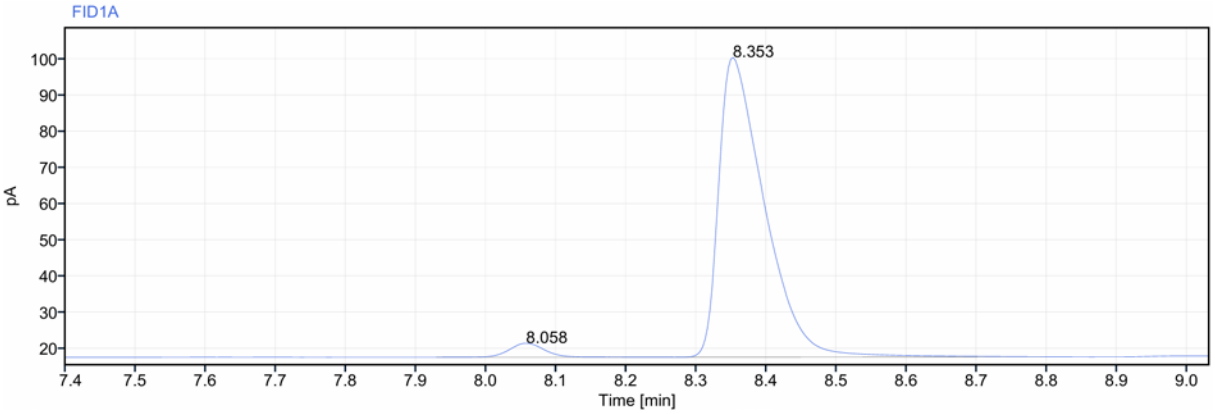

*Figure S13: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2a** formed in the α-hydroxylation of **1a** by $PO_{SPα}$ variant V3-P04. (R)-**2a** (8.058 min), (S)-**2a** (8.353 min). Method GC-02.*

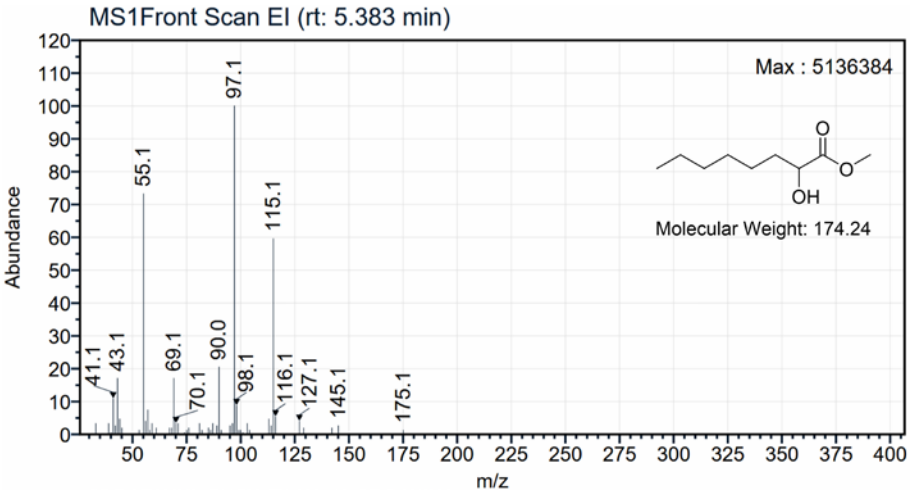

*Figure S14: MS-Spectrum of **2a** derivatised with MeOH/ethyl chloroformate.*

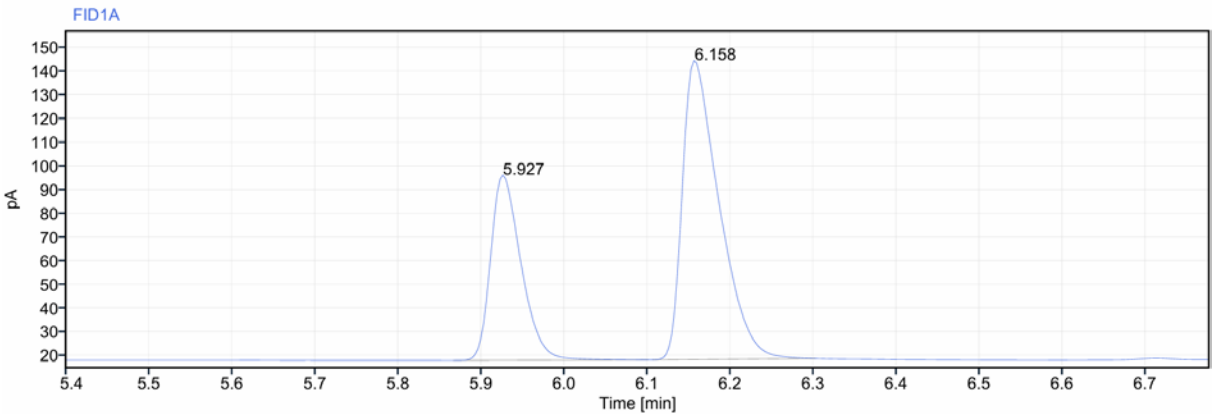

*Figure S15: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2b** formed in the α-hydroxylation of **1b** by PO_{CLA}. (R)-**2b** (5.927 min), (S)-**2b** (6.158 min). Method GC-02.*

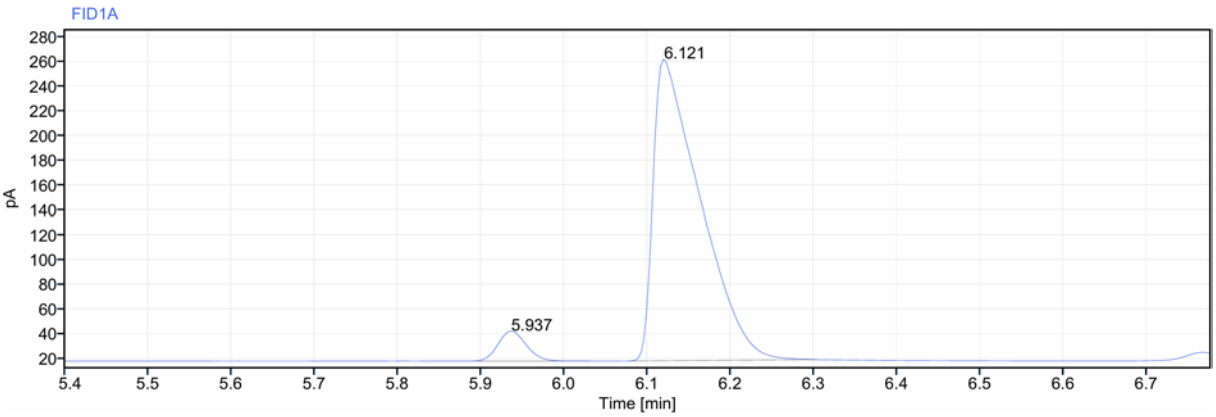

*Figure S16: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2b** formed in the α-hydroxylation of **1b** by $PO_{SPα}$ variant V3-P04. (R)-**2b** (5.937 min), (S)-**2b** (6.121 min). Method GC-02.*

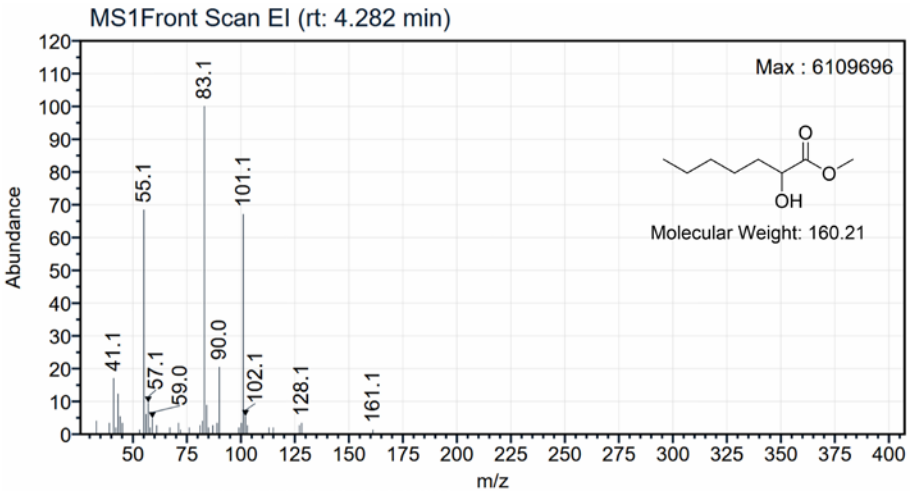

*Figure S17: MS-Spectrum of **2b** derivatised with MeOH/ethyl chloroformate.*

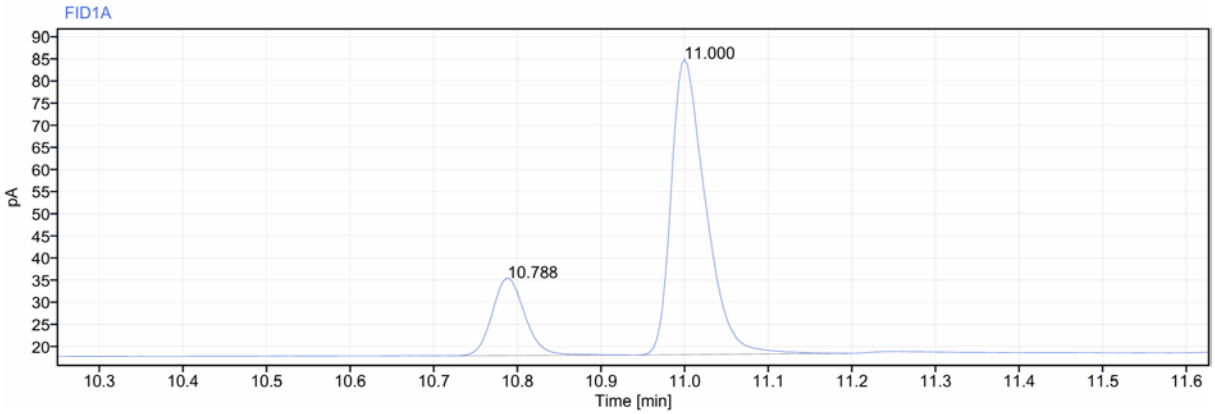

*Figure S18: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2c** formed in the α-hydroxylation of **1c** by PO_{CLA}. (R)-**2c** (10.788 min), (S)-**2c** (11.000 min). Method GC-02.*

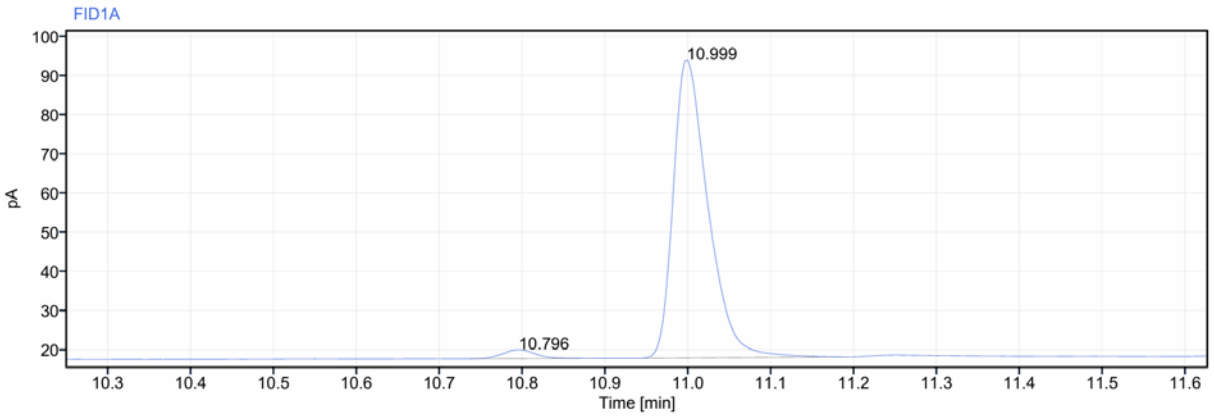

*Figure S19: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2c** formed in the α-hydroxylation of **1c** by $PO_{SPα}$ variant V3-P04. (R)-**2c** (10.796 min), (S)-**2c** (10.999 min). Method GC-02.*

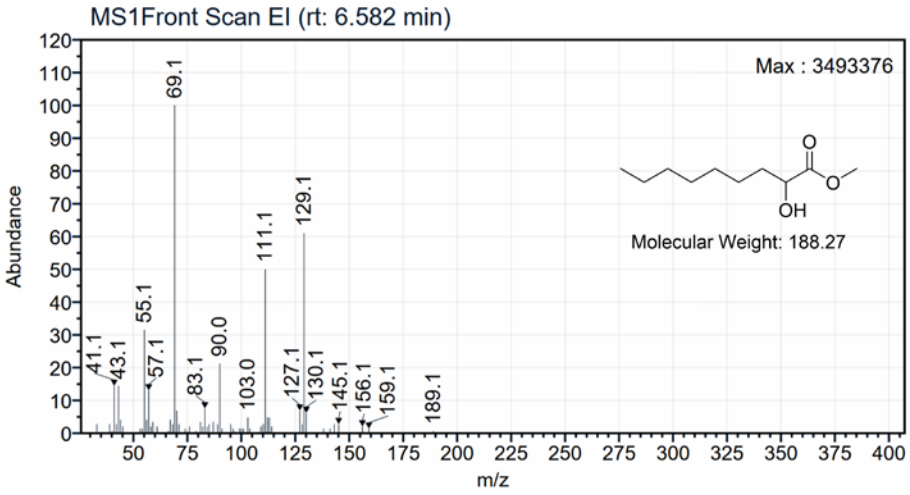

*Figure S20: MS-Spectrum of **2c** derivatised with MeOH/ethyl chloroformate.*

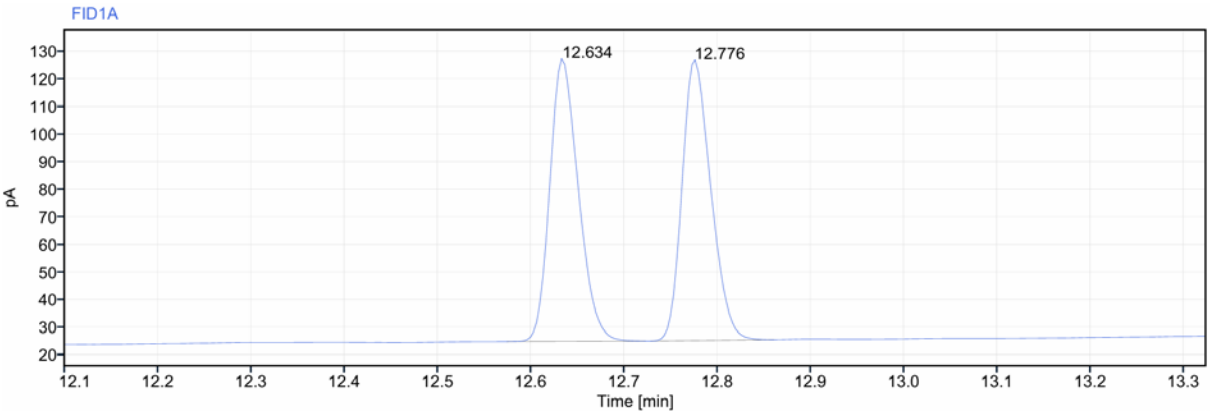

*Figure S21: GC-FID chromatogram of rac-2-hydroxydecanoic acid (**2e**) derivatised with MeOH/ethyl chloroformate. (R)-**2e** (12.634 min), (S)-**2e** (12.776 min). Method GC-02. Retention times of **2d** and **2e** were found to be identical.*

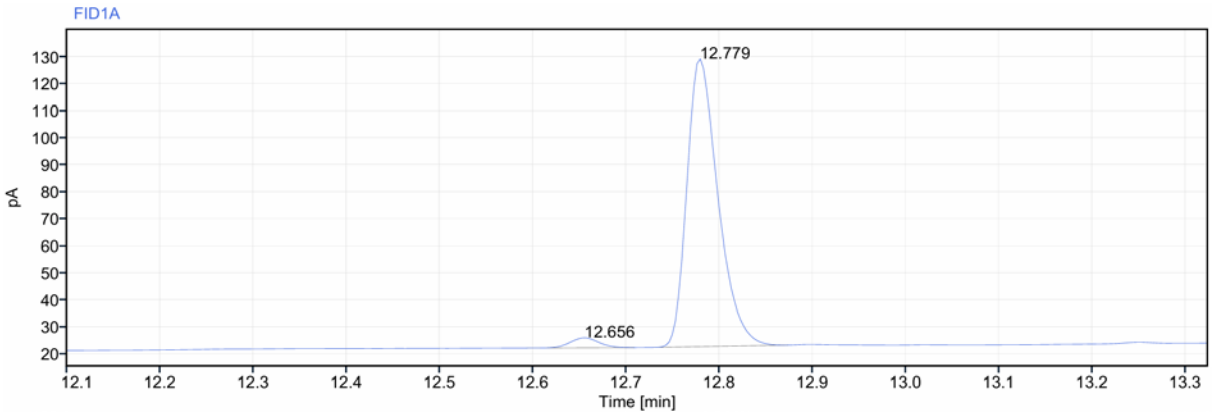

*Figure S22: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2d** formed in the α-hydroxylation of **1d** by $PO_{SPα}$ variant V3-P4. (R)-**2d** (12.656 min), (S)-**2d** (12.779 min). Method GC-02.*

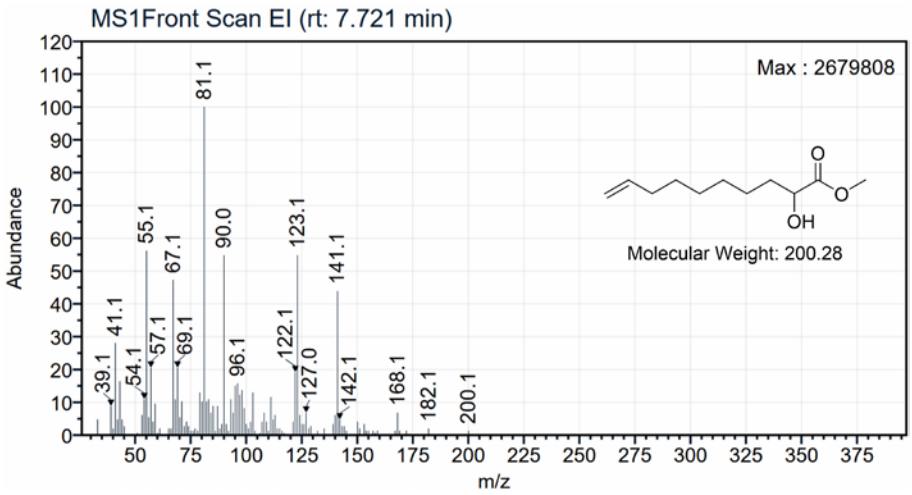

*Figure S23: MS-Spectrum of **2d** derivatised with MeOH/ethyl chloroformate.*

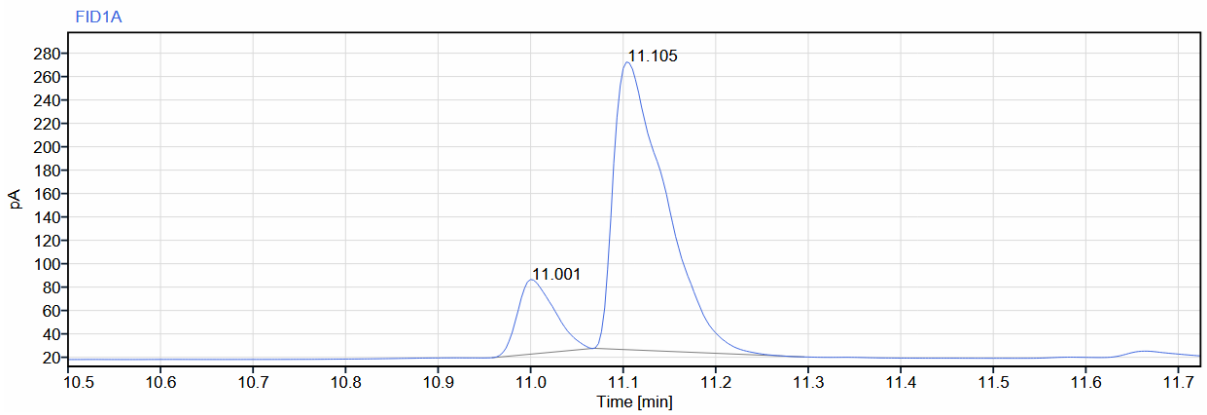

*Figure S24: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2d** formed in the α-hydroxylation of **1d** by PO_{CLA}. Method GC-02. The shift in retention times and change in peak shape compared to Figure S22 is due to the use of an older, more spent column.*

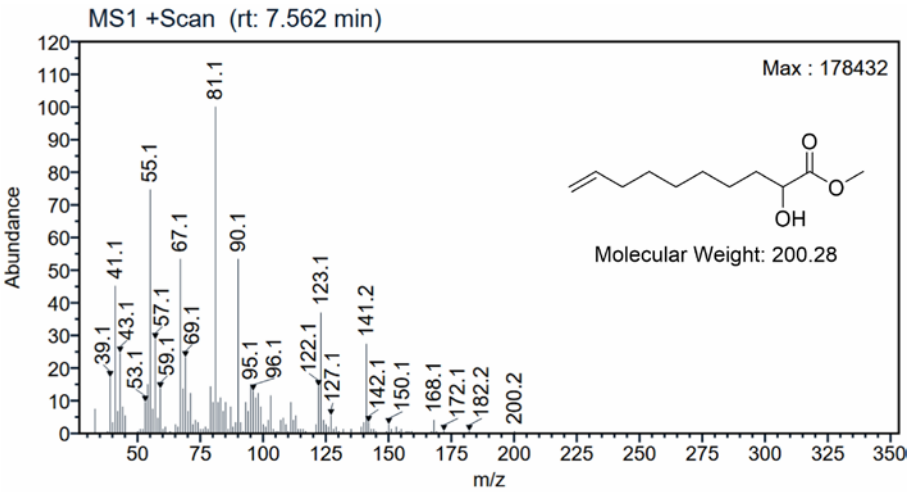

*Figure S25: MS-Spectrum of **2d** formed in the α-hydroxylation of **1d** by PO_{CLA} and derivatised with MeOH/ethyl chloroformate.*

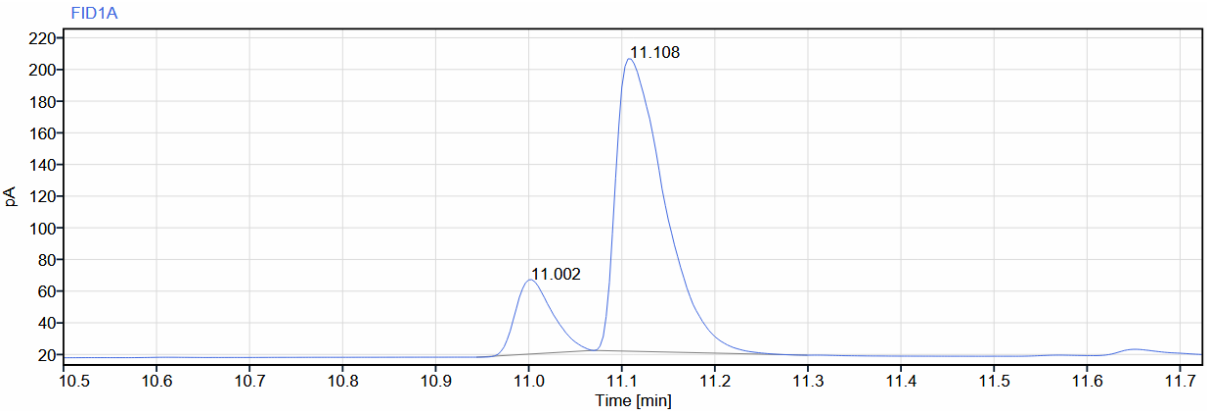

*Figure S26: GC-FID chromatogram of MeOH/ethyl chloroformate-derivatised **2e** formed in the hydrogenation of **2d**. Method GC-02. The shift in retention times and change in peak shape compared to Figure S21 is due to the use of an older, more spent column.*

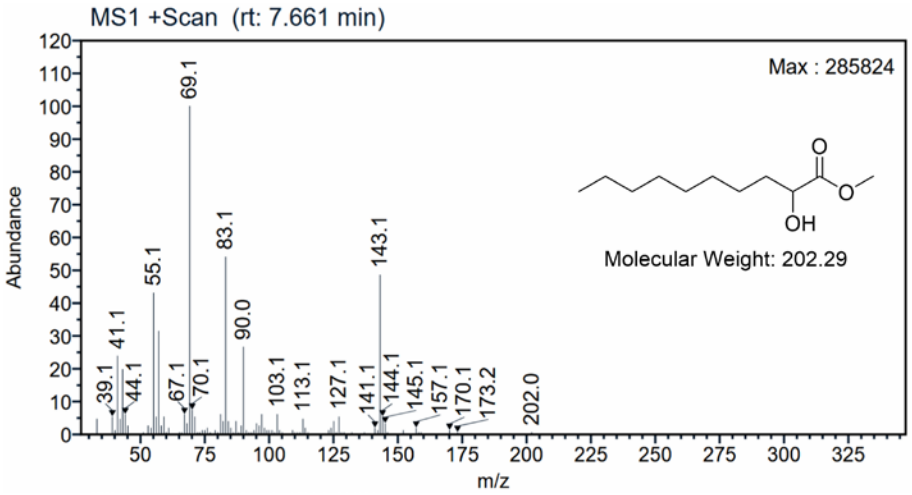

*Figure S27: MS-Spectrum of **2e** formed in the hydrogenation of **2d** and derivatised with MeOH/ethyl chloroformate.*

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

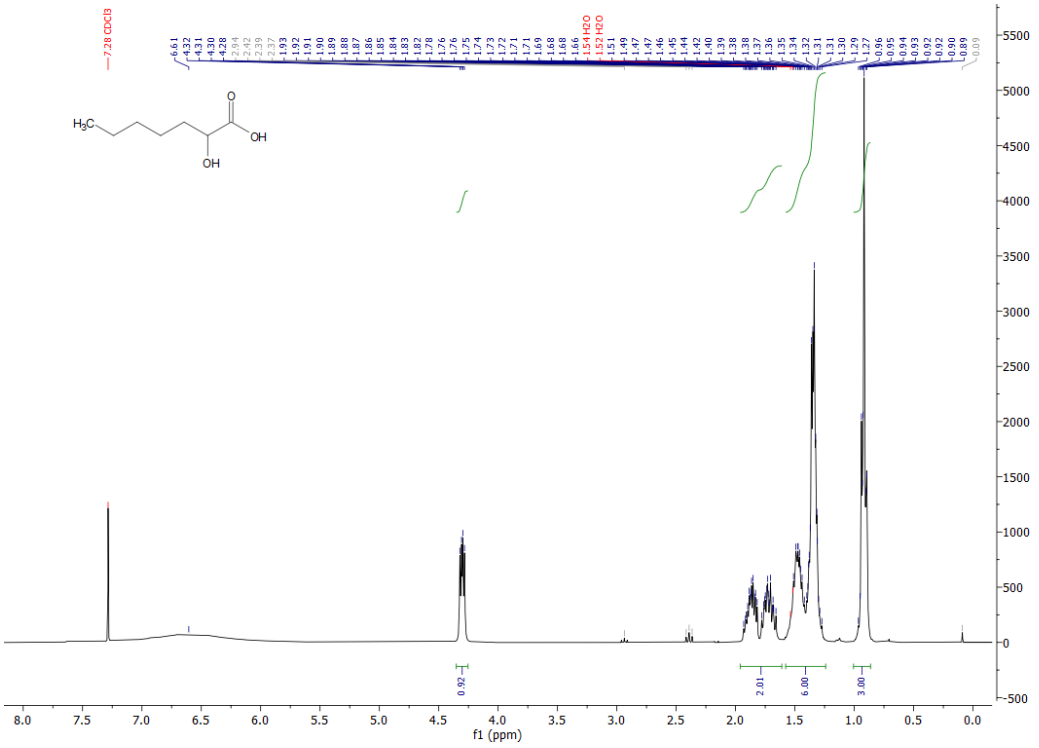

*Figure S28: ^{1}H NMR (300 MHz, $CDCl_3$) of **2b** isolated from the preparative-scale experiment conducted with PO_{CLA}.*

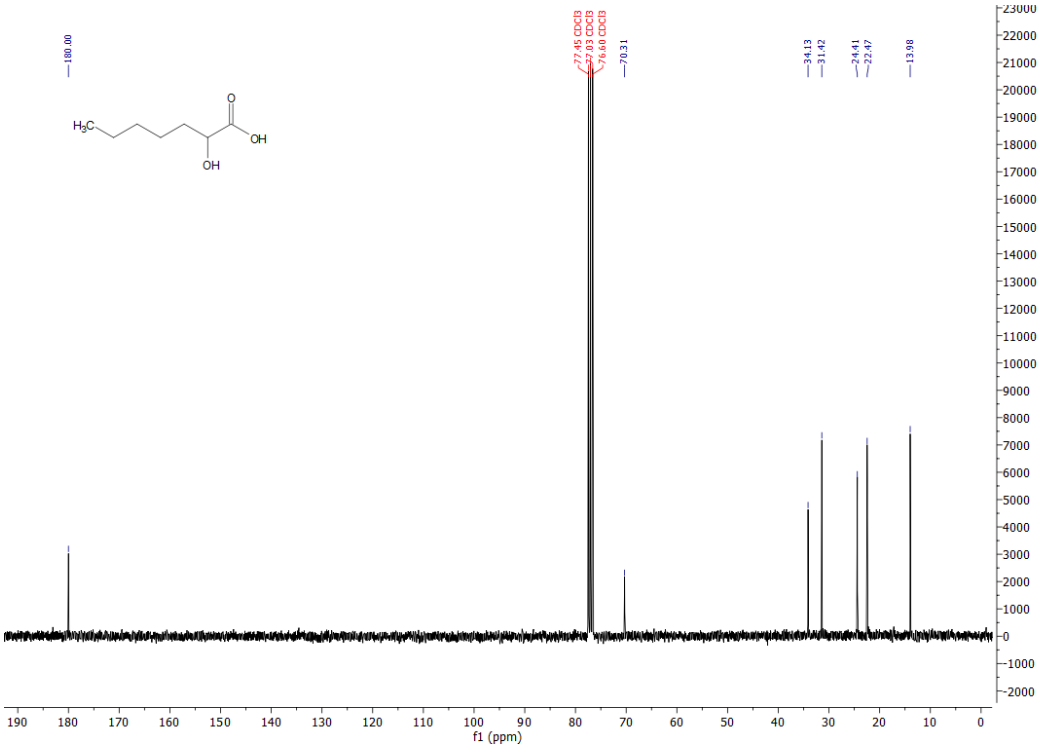

*Figure S29: ^{13}C NMR (75 MHz, $CDCl_3$) of **2b** isolated from the preparative-scale experiment conducted with PO_{CLA}.*

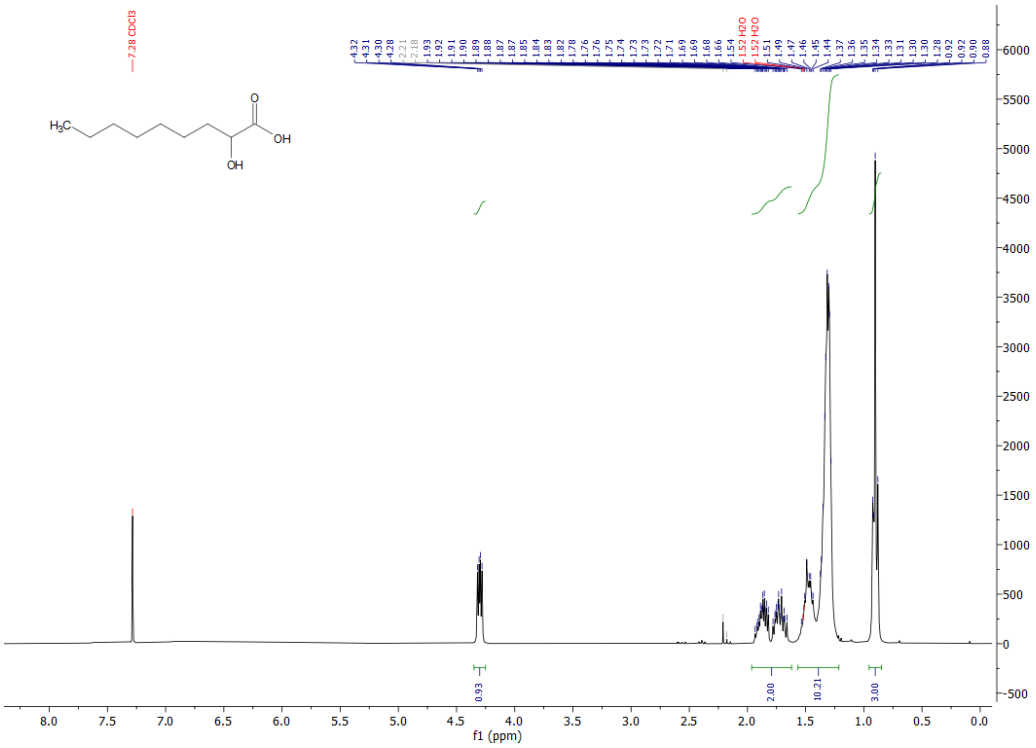

*Figure S30: ^{1}H NMR (300 MHz, $CDCl_3$) of **2c** isolated from the preparative-scale experiment conducted with PO_{CLA}.*

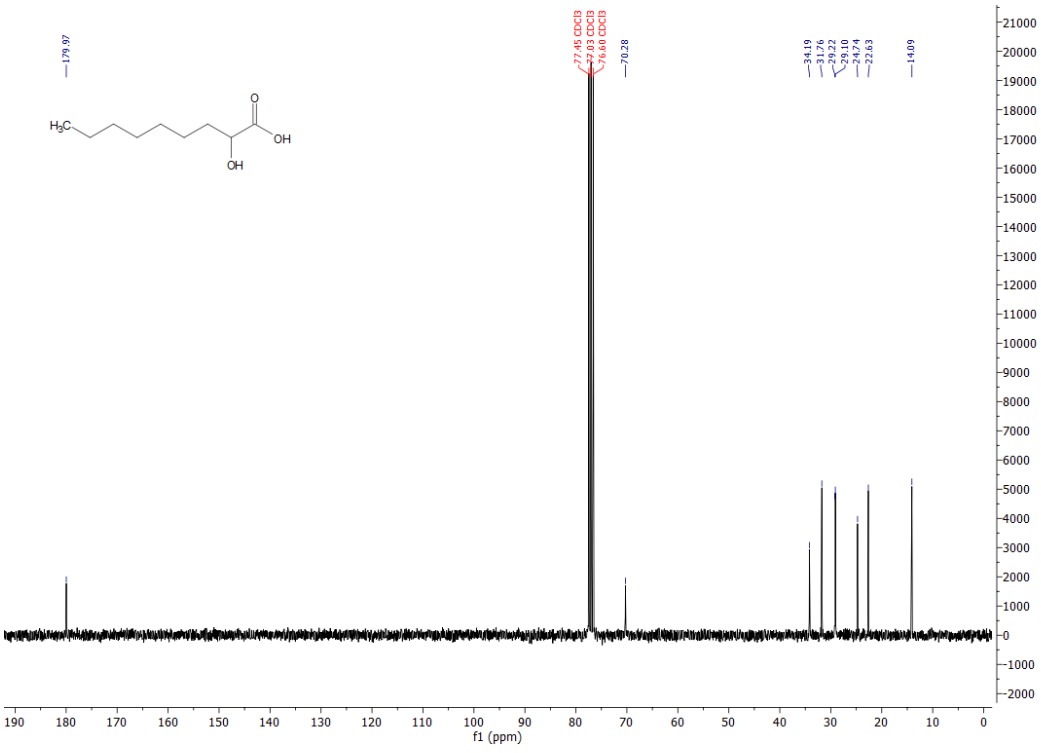

*Figure S31: ^{13}C NMR (75 MHz, $CDCl_3$) of **2c** isolated from the preparative-scale experiment conducted with PO_{CLA}.*

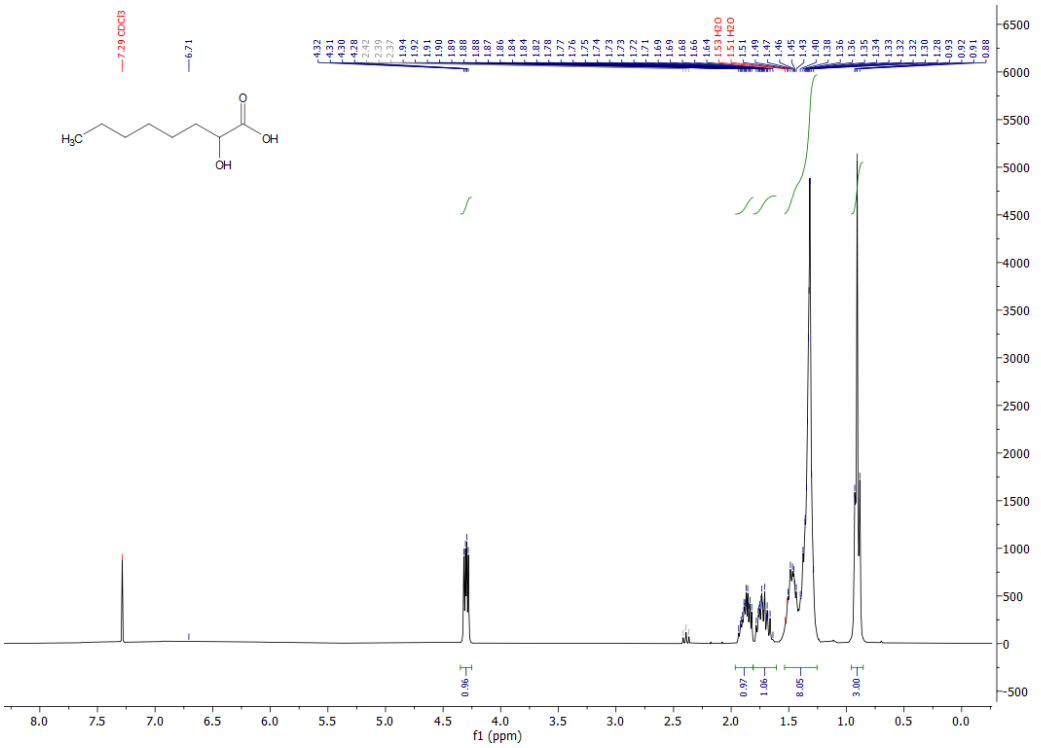

*Figure S32: ^{1}H NMR (300 MHz, $CDCl_3$) of **2a** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

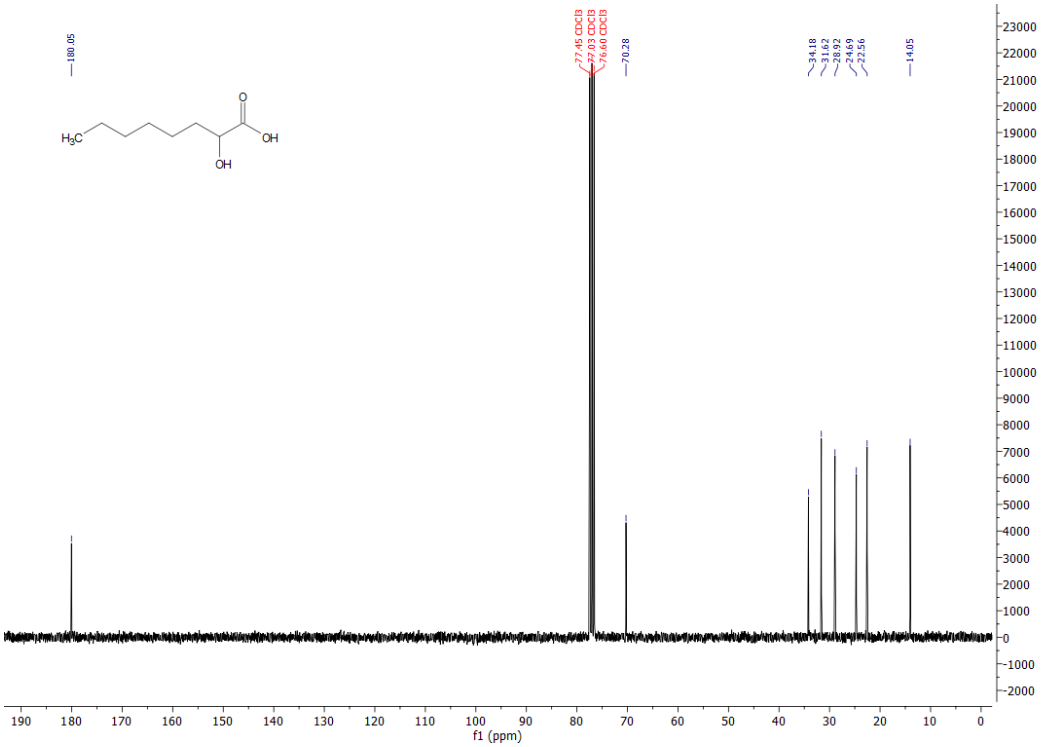

*Figure S33: ^{13}C NMR (75 MHz, $CDCl_3$) of **2a** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

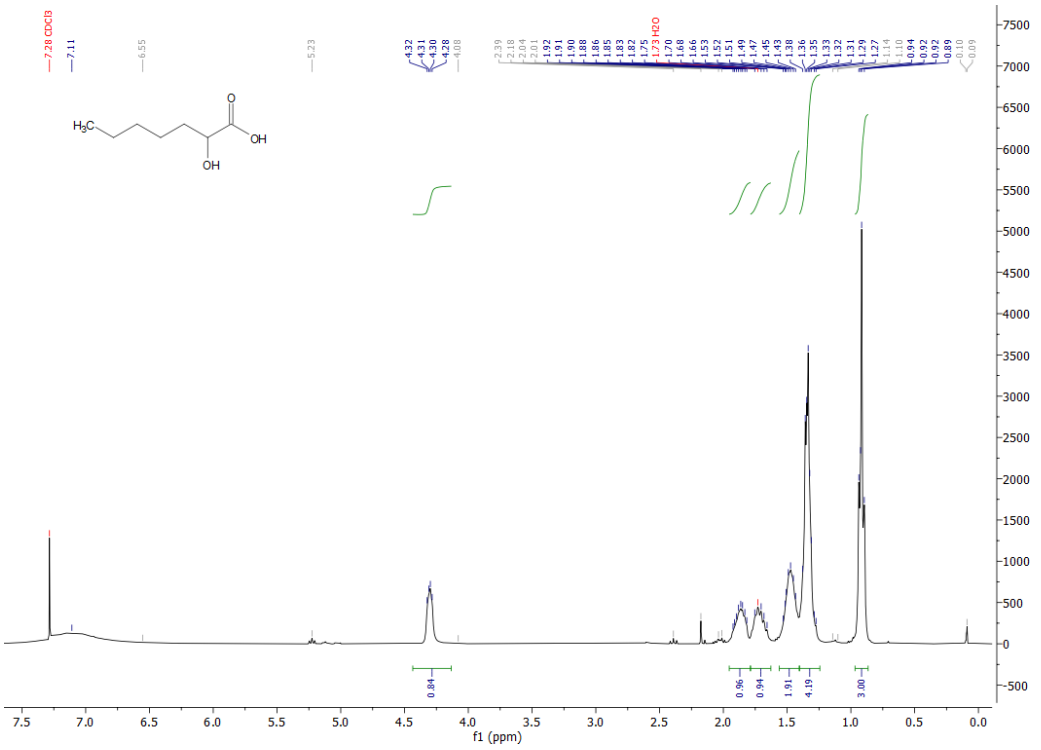

*Figure S34: ^{1}H NMR (300 MHz, $CDCl_3$) of **2b** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

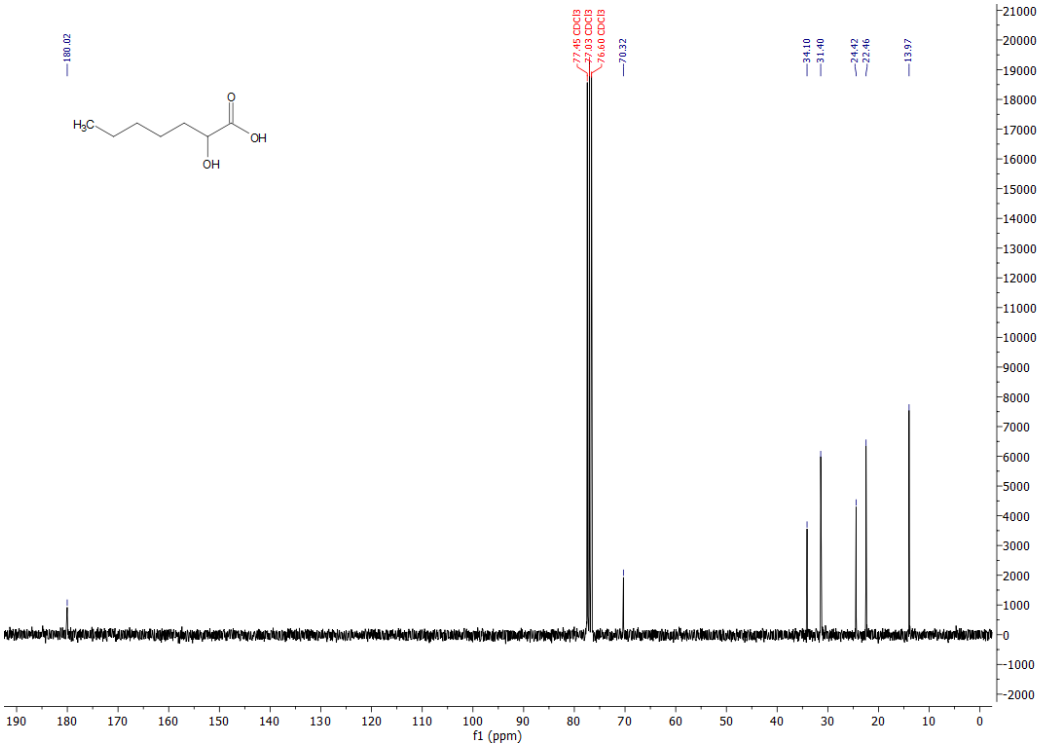

*Figure S35: ^{13}C NMR (75 MHz, $CDCl_3$) of **2b** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

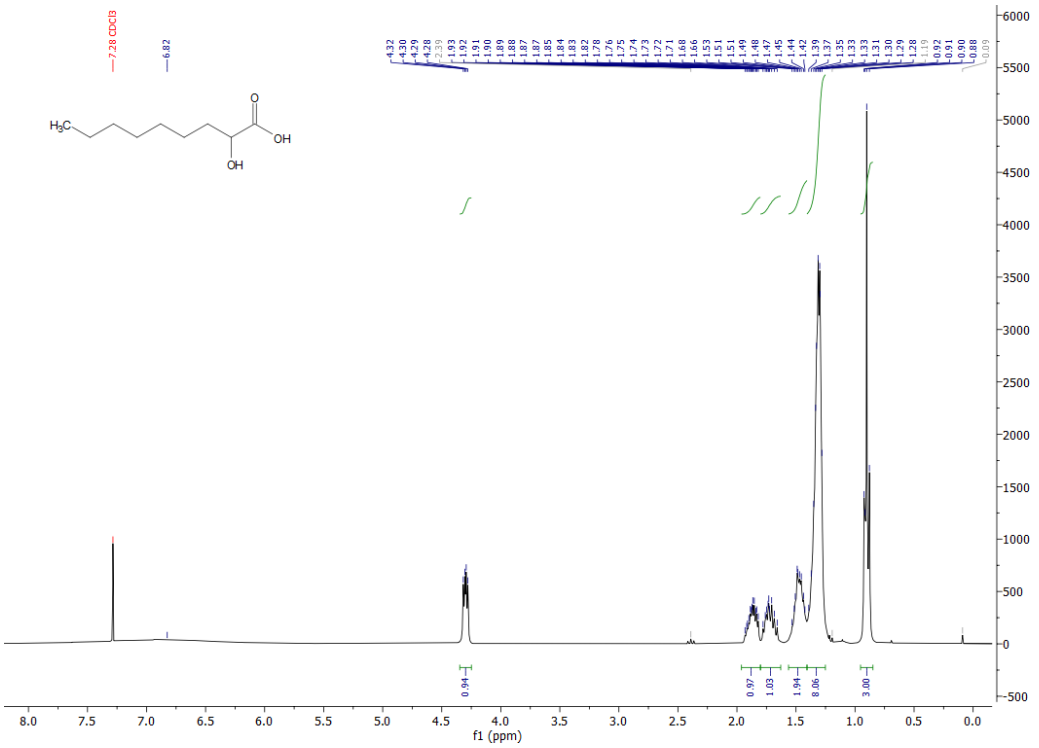

*Figure S36: ^{1}H NMR (300 MHz, $CDCl_3$) of **2c** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

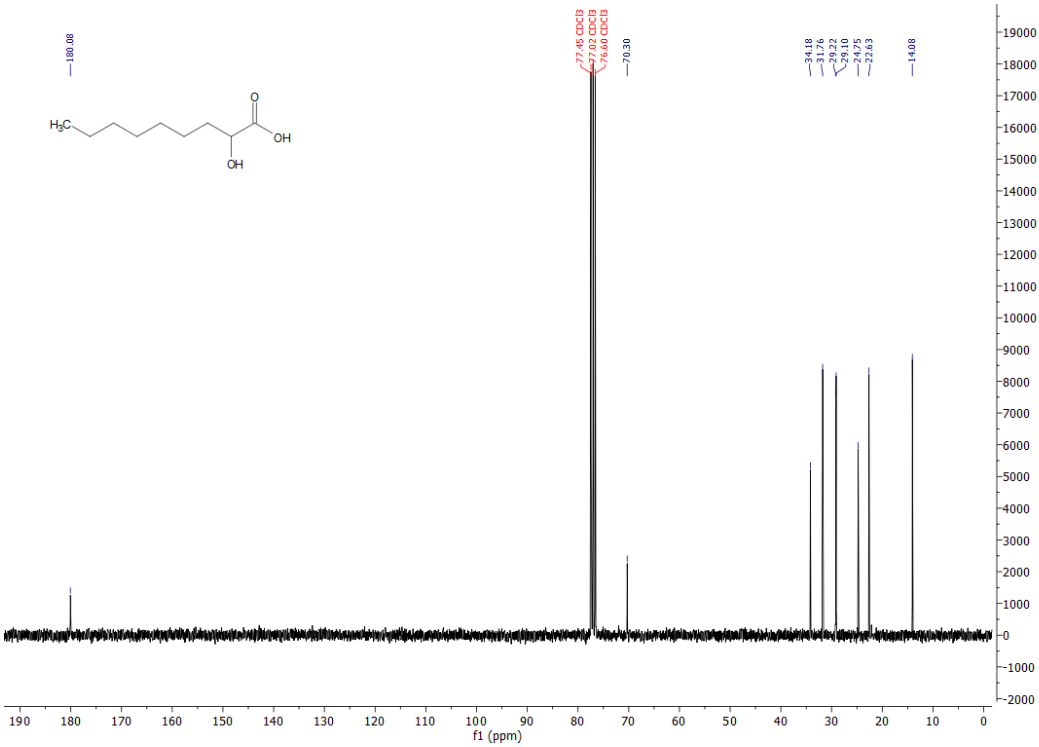

*Figure S37: ^{13}C NMR (75 MHz, $CDCl_3$) of **2c** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

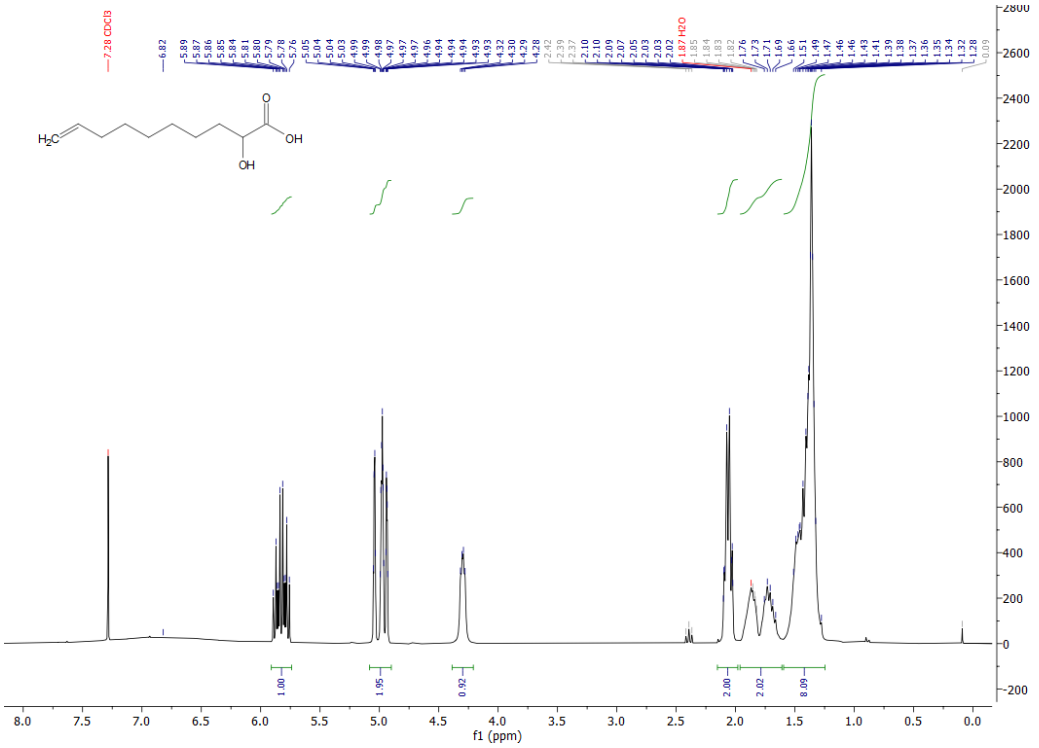

*Figure S38: ^{1}H NMR (300 MHz, $CDCl_3$) of **2d** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

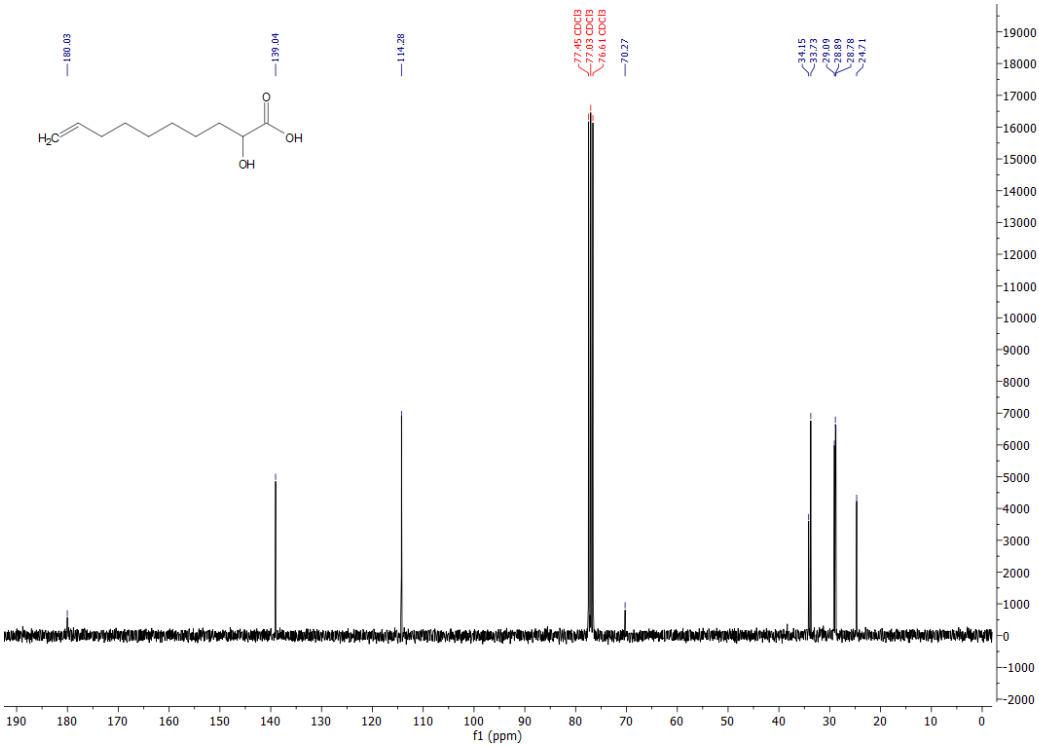

*Figure S39: ^{13}C NMR (75 MHz, $CDCl_3$) of **2d** isolated from the preparative-scale experiment conducted with $PO_{SPα}$ V3-P04.*

Literature

1. F. P. Guengerich, M. V. Martin, C. D. Sohl and Q. Cheng, *Nature Protocols*, 2009, **4**, 1245-1251.
2. R. A. Friesner, J. L. Banks, R. B. Murphy, T. A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky, E. H. Knoll, M. Shelley, J. K. Perry, D. E. Shaw, P. Francis and P. S. Shenkin, *J. Med. Chem.*, 2004, **47**, 1739-1749.
3. M. M. Aboelnga, *RSC Adv.*, 2022, **12**, 15543-15554.