Electronic Supplementary Material (ESI) for Catalysis Science & Technology.


Supporting Information

# Structure of the fungal hydroxylase, CYP505A30, and rational transfer of mutation data from CYP102A1 to alter regioselectivity

Jasmin C. Aschenbrenner,[ab] Ana C. Ebrecht,[a] Carmien Tolmie,[a] Martha S. Smit,[ab] and Diederik J. Opperman*[a]

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## Section A. Material and Methods

### 1. Generation of CYP505A30 mutants

CYP505A30 mutants were generated using the Megaprimer method[1] with the primers stated in Table S1. The mutant CYP505A30_78-328_2 was generated from introducing the 78-328 mutations to CYP505A30_BM3wt. Megaprimer PCR reactions (50 µL) consisted of 1X KOD Hot Start Polymerase buffer, 1.5 mM $MgSO_4$, 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 0.02 $U \cdot mL^{-1}$ KOD Hot Start polymerase (Novagen), 0.4 $ng \cdot mL^{-1}$ template DNA and 0.1 mM of both forward and reverse primers. PCR conditions consisted of an initial denaturation step (95 °C, 2 min), followed by 6 cycles of denaturation at 95 °C (20 s), annealing at 65 °C (10 s) and elongation at 70 °C (15 s) and 28 cycles of denaturation at 95 °C (20 s) and annealing and extension of the megaprimer at 70 °C (3:50 min) with a final extension of 12 min at 70 °C.

**Table S1.** Primers used for introducing mutations via the Megaprimer method.

| Mutation | Construct Name | Forward Primer | Reverse Primer |
|---|---|---|---|
| F93V, I334F | 87-328 | CGATGGCCTCGTCACCGCTCG | GCTCGATTGATTATTGGGAACGTGGGACAGAGGC |
| V88A, I334A | BM3wt | CGAAACGGCGCCAACGATGGCC | GATTGATTATTGGGGCCGTGGGACAGAGGC |
| V84A, V88L, I334V | 1-12G | CTCGCCGAGGCTCGAAACGGCCTGAACGATGGC | GCTCGATTGATTATTGGGACCGTGGGACAGAGGC |

## 2. Data collection and structure determination

**Table S2.** X-ray data collection and refinement parameters.

| Data collection | |
|---|---|
| Beamline | i04 |
| Wavelength (Å) | 0.9795 |
| Space group | $P4_2\ 2_1\ 2$ |
| Cell dimensions a/b/c (Å) | 171.49/171.49/175.65 |
| α/β/γ (°) | 90/90/90 |
| Resolution (Å) | 61.35 - 2.33 (2.37 - 2.33) |
| Unique reflections | 111842 (5522) |
| Completeness (%) | 100.0 (95.9) |
| Mn(I)/sd(I) | 16.2 (0.2) |
| Multiplicity | 13.3 (13.1) |
| $R_{merge}$ | 0.085 (4.010) |
| $R_{pim}$ | 0.024 (1.154) |
| $CC_{1/2}$ | 1.000 (0.296) |
| Molecules in ASU | 4 |
| **Refinement** | |
| $R_{work}/R_{free}$ | 0.2033/0.2339 |
| Number of residues modelled | 1832 |
| B-factors | |
| Amino acids | 89.11 |
| Ligands | 78.51 |
| Waters | 71.64 |
| Rmsd Bond lengths (Å) | 0.0099 |
| Rmsd Bond angles (°) | 1.6466 |
| Ramachandran distribution (%) (favoured/allowed/outliers) | 96.1/3.1/0.8 |
| MolProbity score | 1.86 |
| Poor rotamers (%) | 4.45 |
| Clash score, all atoms | 3.03 |
| PDB ID | 7P6L |

Values in parantheses describe the outer shell.

## Section B. Supplementary Results

### 1. SDS-PAGE analysis of purified CYP505A30 HD

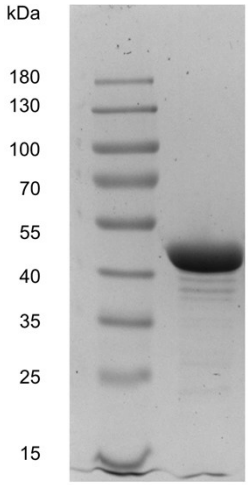


**Figure S1.** SDS-PAGE of CYP505A30 HD (54.7 kDa) after purification with immobilized metal affinity chromatography and size exclusion chromatography.

### 2. SDS-PAGE analysis of the expression levels of CYP505A30 and mutants

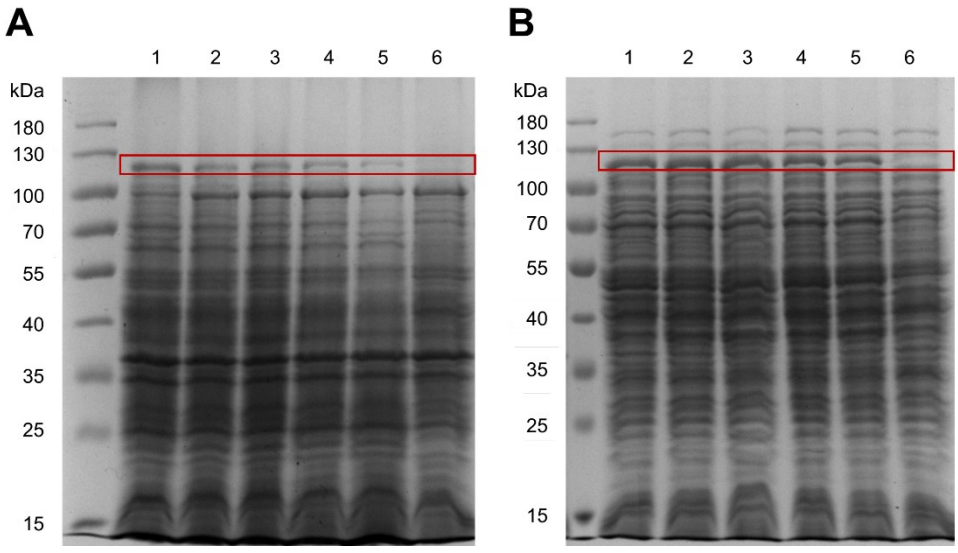


**Figure S2.** SDS-PAGE analysis of the crude (**A**) and cell-free extract (**B**) prepared from *E. coli* BL21-DE3 (Gold) cells transformed with pET22b(+)-plasmids expressing the (**1**) CYP505A30wt, (**2**) CYP505A30_BM3wt, (**3**) CYP505A30_1-12G, (**4**) CYP505A30_87-328 (**5**) CYP505A30_87-328_2 or transformed with (**6**) an empty pET22b(+)-plasmid. The predicted molecular weight of the expressed proteins is approximately 120 kDa.

### 3. Average CYP450 content measured in whole cells, cell-free extract and in biotransformations

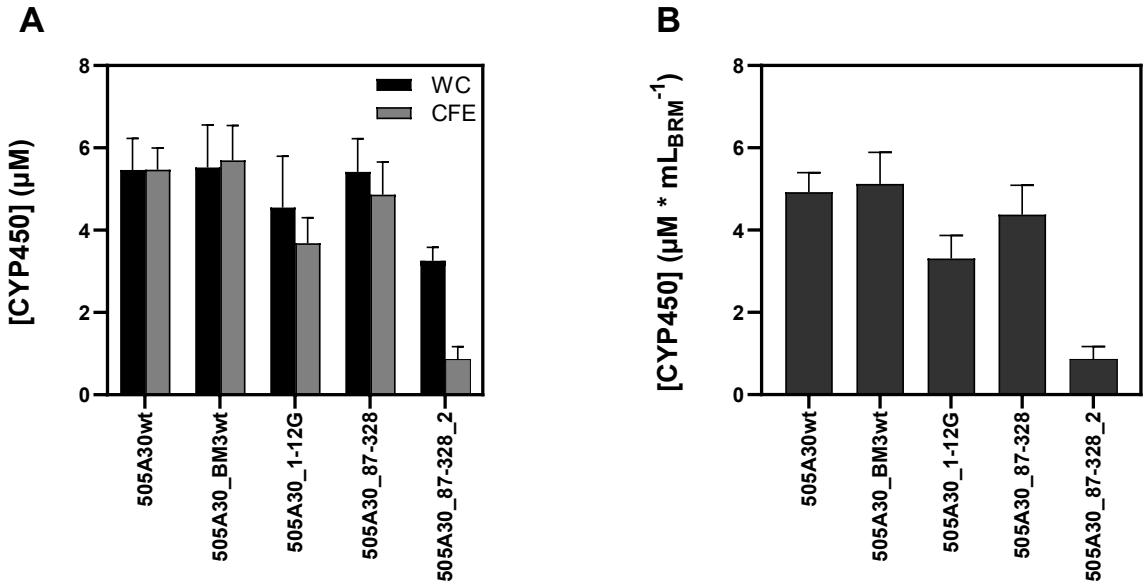


**Figure S3.** (**A**) Comparison of CYP450 content in whole cell (WC) and CFE with a constant wet cell biomass of 1 g in 5 ml buffer measured in duplicates from 5 different experiments. (**B**) Average CYP450 content in CFE BRM.

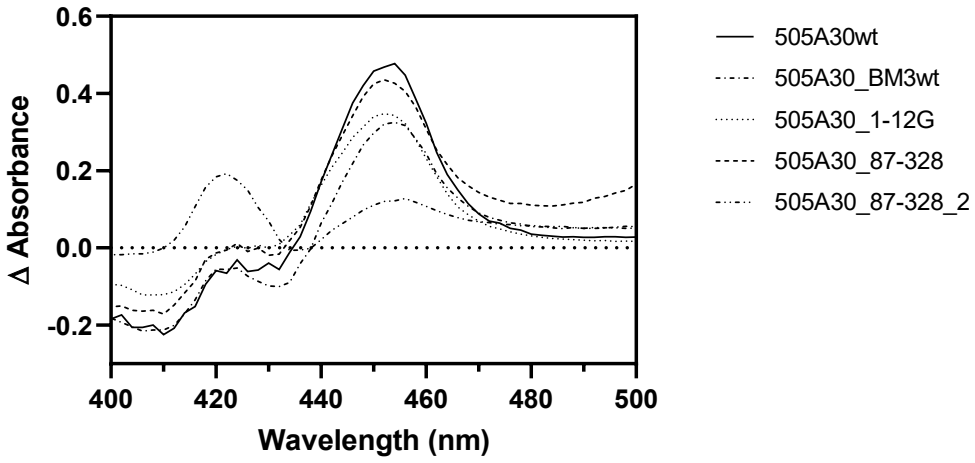


**Figure S4.** Representative examples of CO-difference spectra obtained by CYP505A30wt and mutants in CFE with a constant wet cell biomass of 1 g in 5 ml buffer.

## 4. GC analysis of products from alkane biotransformations

### 4.1. Temperature programs for GC analysis of formed products

**Table S3.** GC programs for the analysis of products formed during biotransformations. The products were analysed on a Finnigan TRACE GC Ultra (Thermo Fisher Scientific). Samples were sillylated for analysis and quantification of diols.

| Analysis | Derivatization | Column | Program | Compound | Retention time [min] |
|---|---|---|---|---|---|
| GC | none | FactorFour VF-5ms column (60 m x 0.32 mm x 0.25 µm, Varian) | 50/2/3/75/0/ 15/190/0/[a] | 4-Octanol<br>3-Octanol<br>2-Octanol<br>2,5-Octanediol<br>3,6-Octanediol<br>2,6-Octanediol<br>2,7-Octanediol<br>1-Undecanol[c] | 8.98<br>9.22<br>9.41<br>13.97<br>14.07<br>14.26<br>14.33<br>16.29 |
| GC | BSTFA | FactorFour VF-5ms column (60 m x 0.32 mm x 0.25 µm, Varian) | 100/1/10/300/5/[b] | 2,5-Octanediol<br>3,6-Octanediol<br>2,6-Octanediol<br>2,7-Octanediol<br>1,8-Octanediol<br>1-Undecanol[c] | 6.14<br>6.24<br>6.50<br>6.72<br>8.51<br>7.76 |
| GC | none | BPX90 (30 m x 0.25 mm x 0.25 µm) | 80/2/15/280/4.66/ [b] | 4-Decanol<br>3-Decanol<br>2-Decanol<br>1-Dodecanol[c]<br>2,7-Decanediol<br>2,8-Decanediol<br>2,9-Decanediol<br>3,8-Decanediol | 3.71<br>3.80<br>3.99<br>6.17<br>8.62<br>8.92<br>9.06<br>8.73 |
| GC | BSTFA | FactorFour VF-5ms column (60 m x 0.32 mm x 0.25 µm, Varian) | 100/1/10/300/5/[b] | 2,7-Decanediol<br>3,8-Decanediol<br>2,8-Decanediol<br>2,9-Decanediol<br>1,10-Decanediol<br>1-Dodecanol[c] | 8.62<br>8.73<br>8.92<br>9.13<br>10.91<br>9.00 |
| GC | none | Astec CHIRALDEX™ G-TA column (30 m x 0.25 mm x 0.12 µm) | 40/20/0.75/70/0/[b] | (*S*)-2-Octanol<br>(*R*)-2-Octanol | 45.4<br>45.7 |

[a] Initial temp (°C)/ time (min)/ slope (°C/min)/ temperature (°C)/ hold time (min)/ slope (°C/min)/ temperature (°C)/ hold time (min), [b] Initial temp (°C)/ time (min)/ slope (°C/min)/ temperature (°C)/ hold time (min), [c] Internal standard

## 4.2. Standard curves

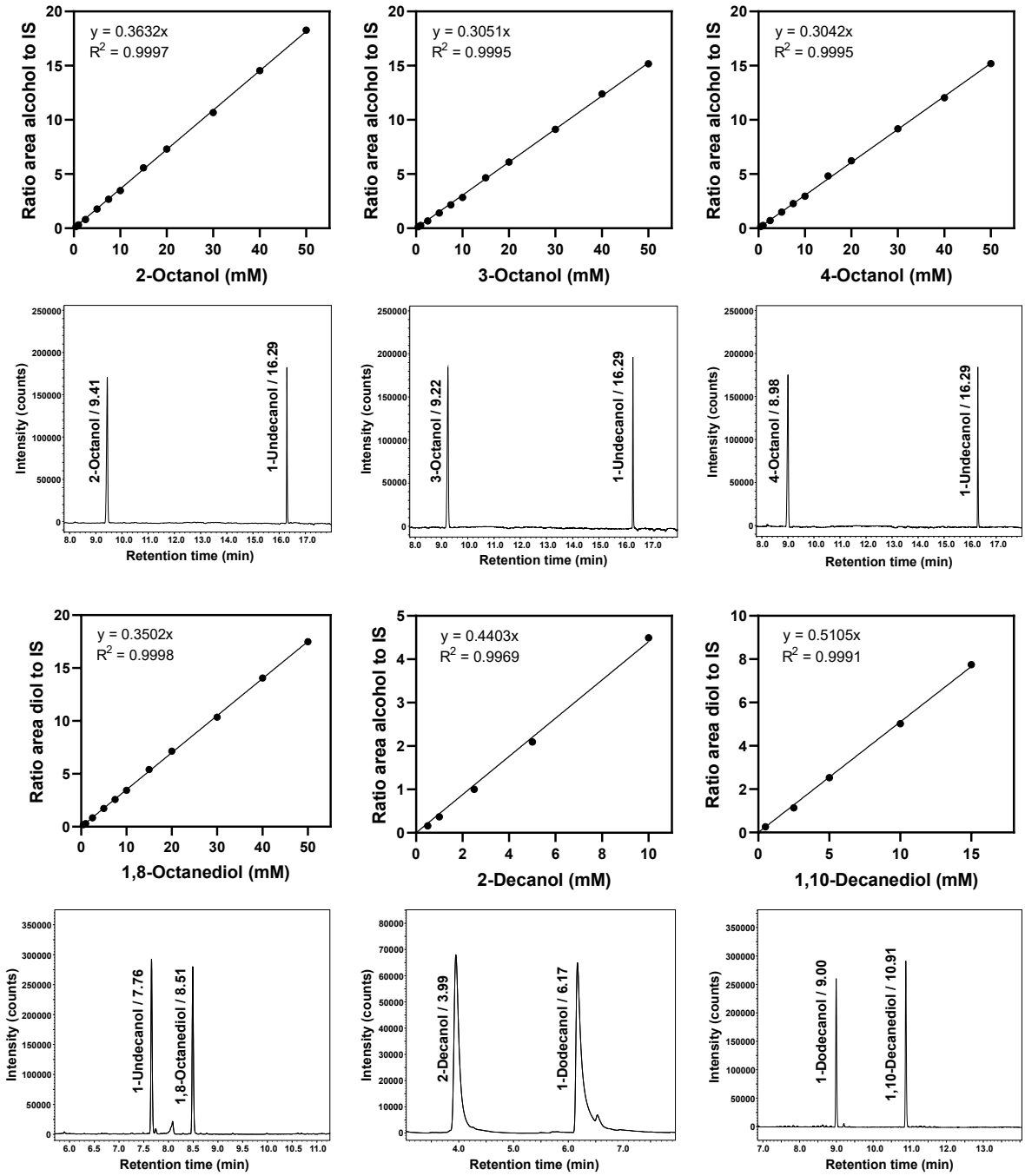


**Figure S5.** Calibration curves and GC chromatograms for the standards. Concentrations of produced alcohols (*n*-octanol and *n*-decanol) were determined from standard curves of 2-, 3- and 4-octanol as well as 2-decanol. Diol concentrations were calculated from 1,8-octanediol and 1,10-decanediol standard curves.

### 4.3. Chromatograms of biotransformations

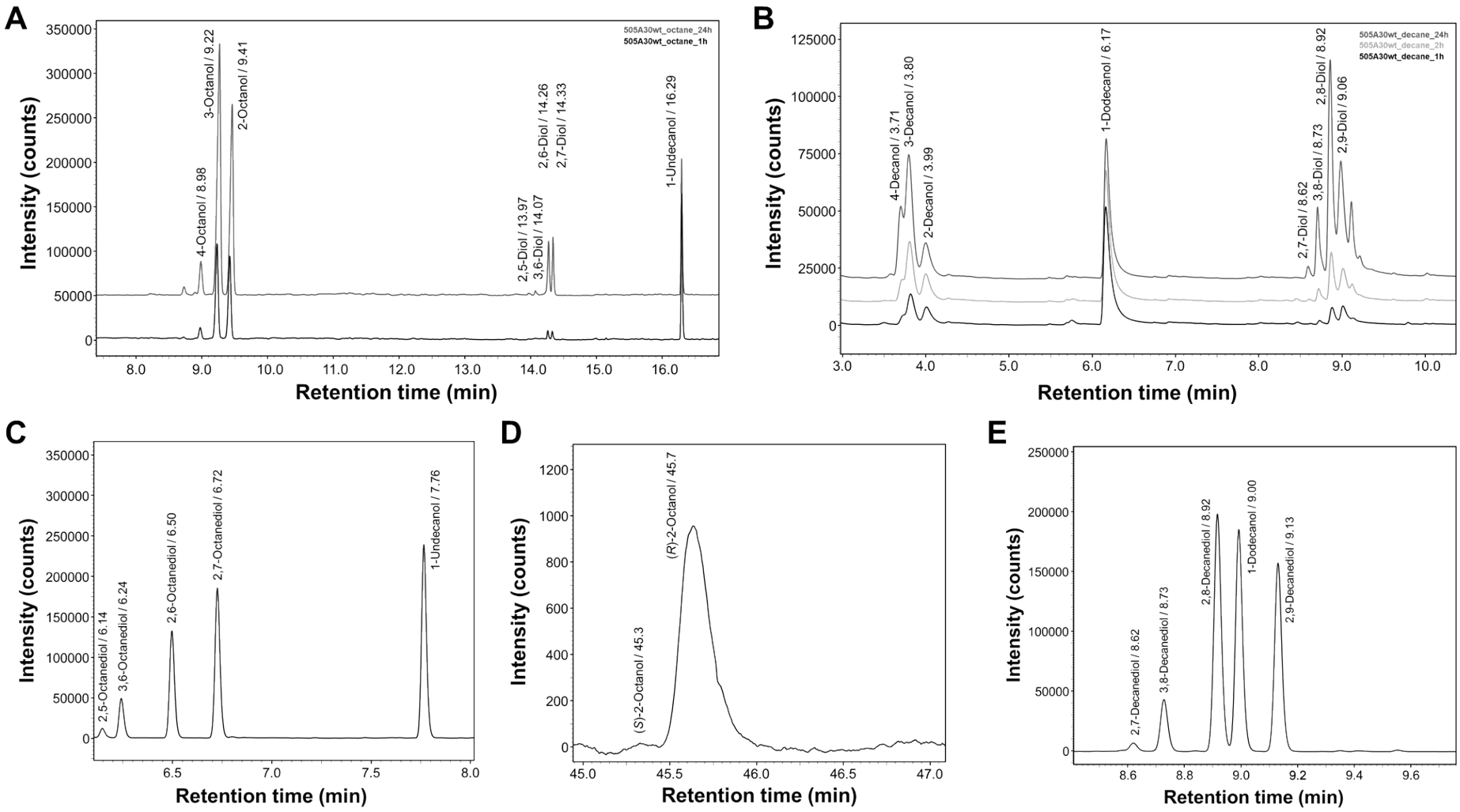


**Figure S6.** Representative examples of GC chromatograms of biotransformations with CYP505A30wt. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated samples for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of 1 h sample of octane biotransformations.

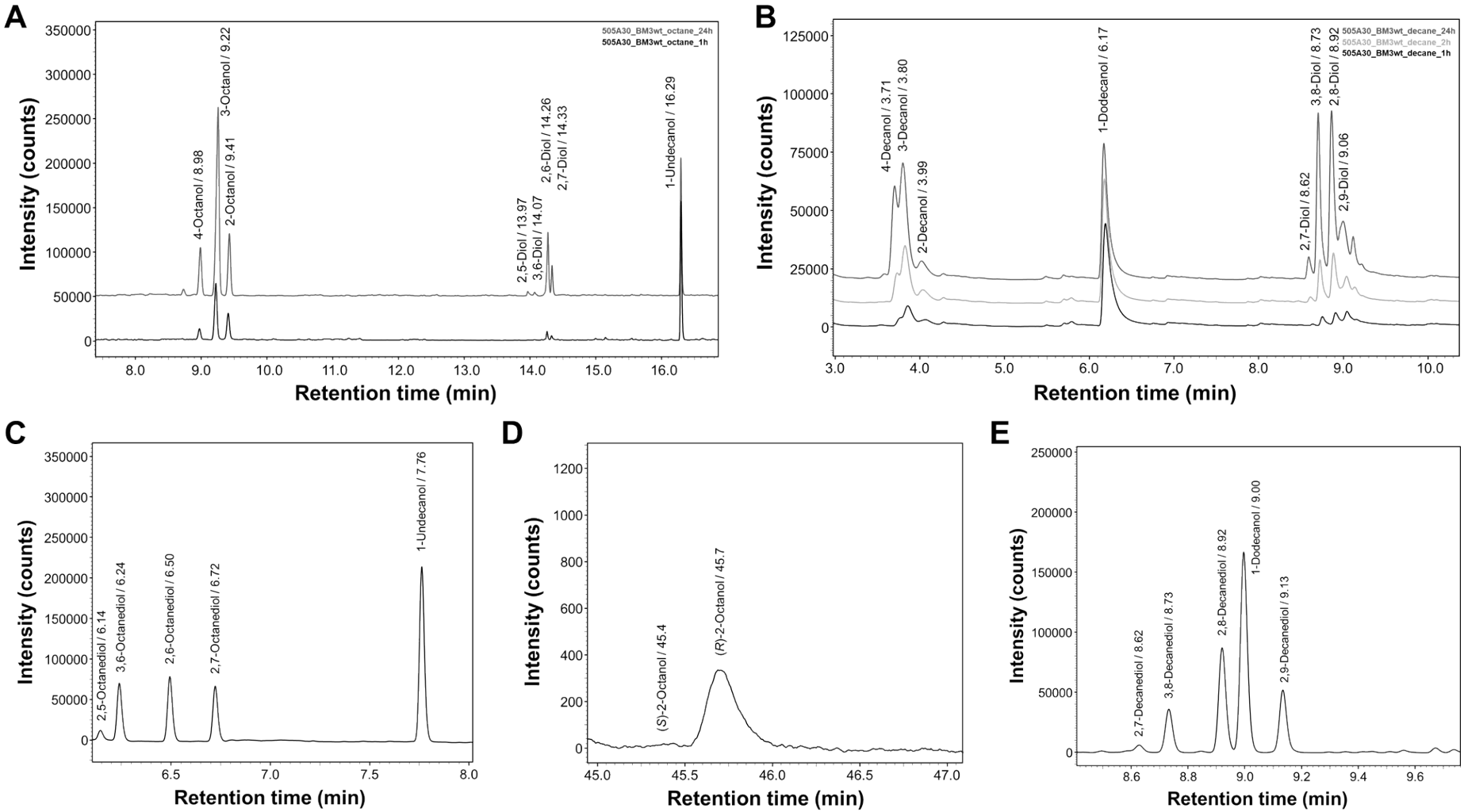


**Figure S7.** Representative examples of GC chromatograms of biotransformations with CYP505A30_BM3wt. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated sample for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of 1 h sample of octane biotransformations.

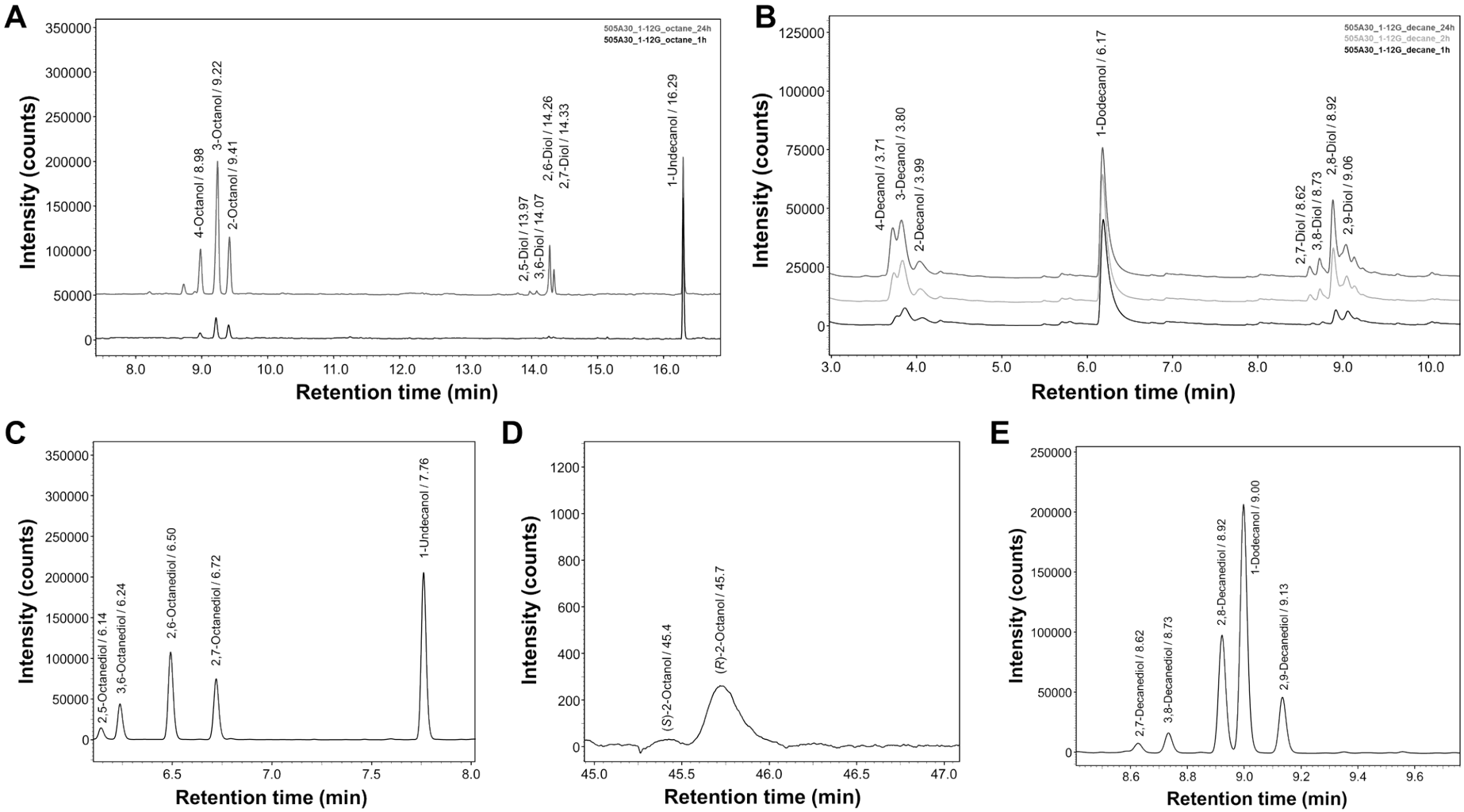


**Figure S8.** Representative examples of GC chromatograms of biotransformations with CYP505A30_1-12G. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated sample for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of 1 h sample of octane biotransformations.

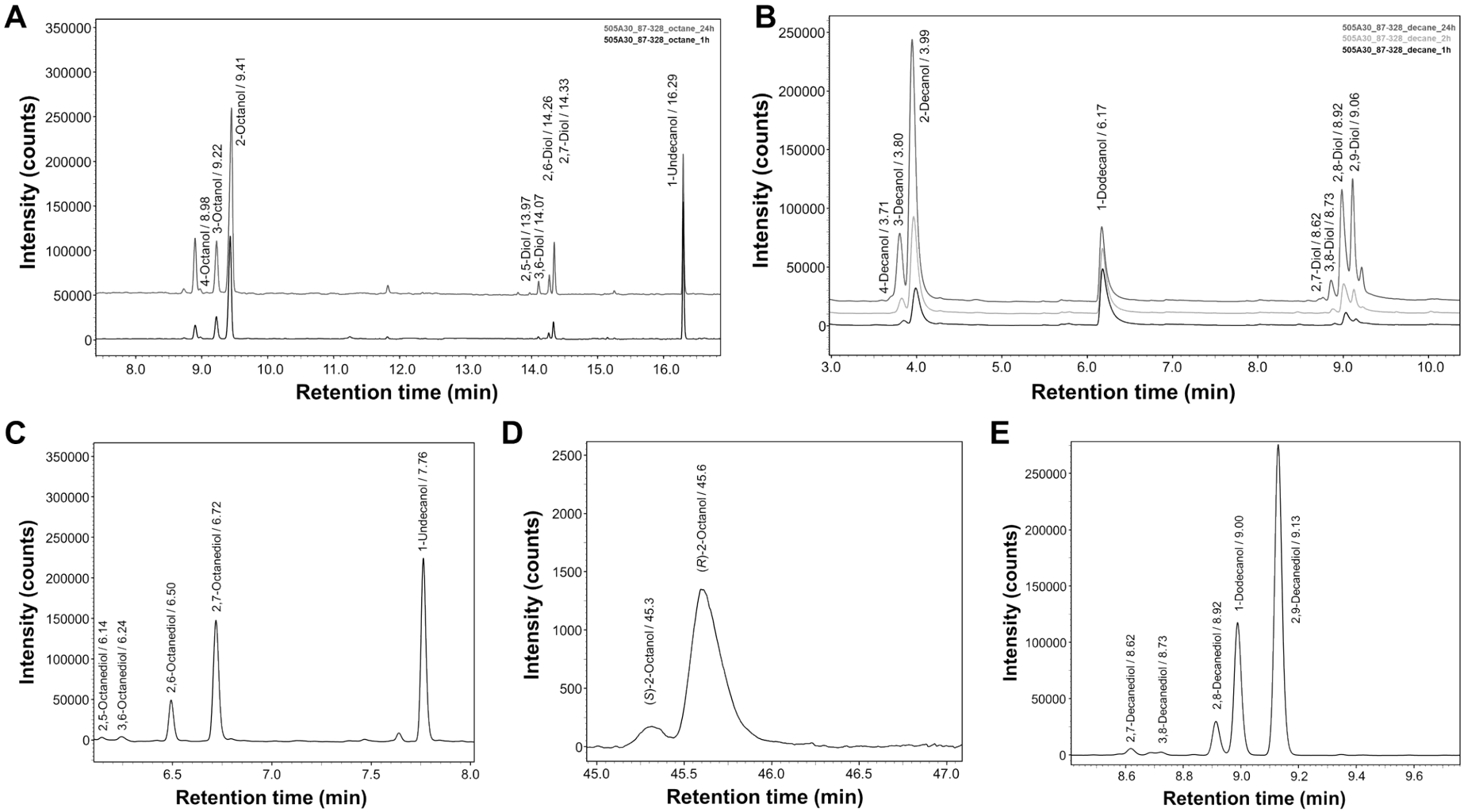


**Figure S9.** Representative examples of GC chromatograms of biotransformations with CYP505A30_87-328. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated sample for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of 1 h sample of octane biotransformations.

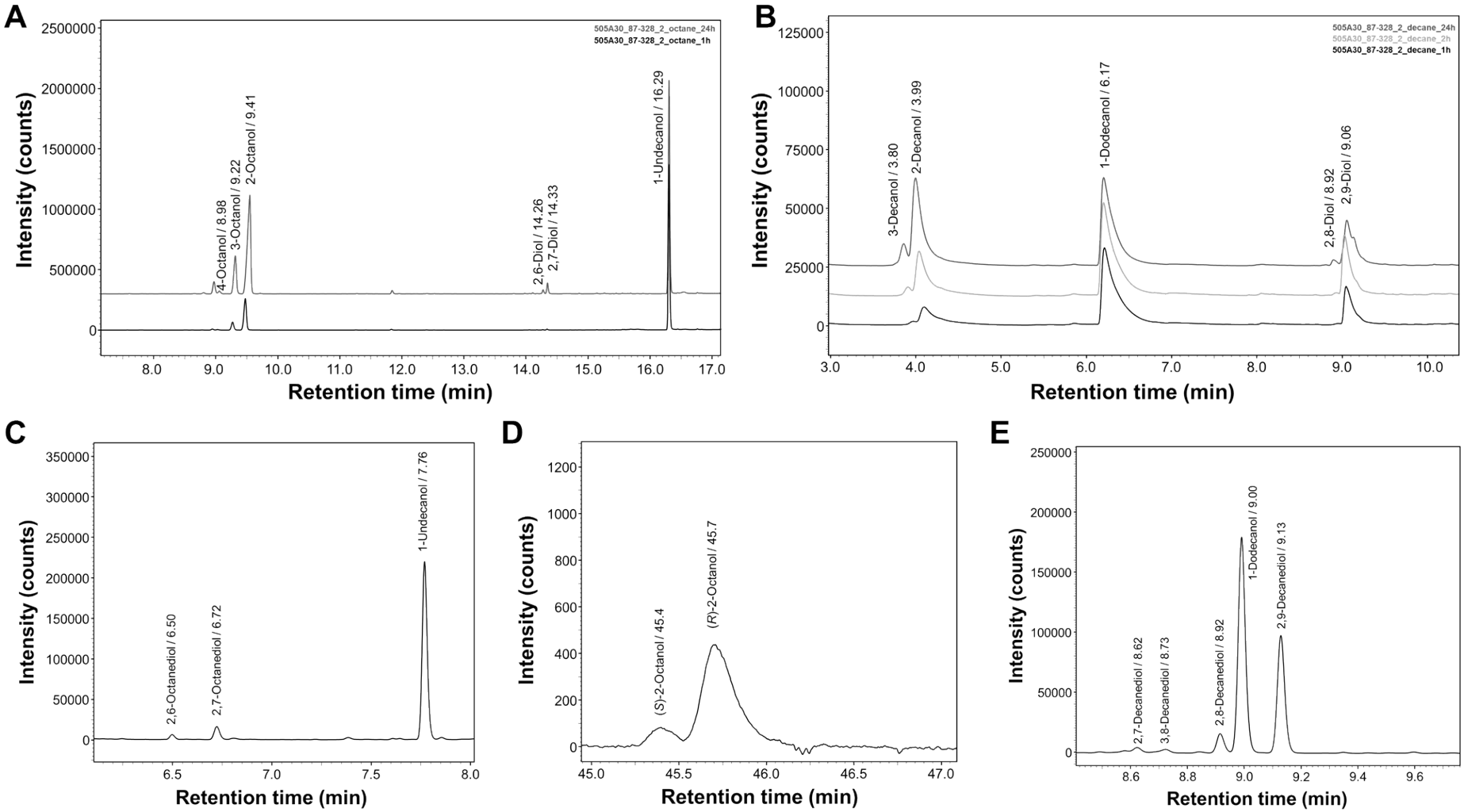


**Figure S10.** Representative examples of GC chromatograms of biotransformations with CYP505A30_87-328_2. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated sample for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of 1 h sample of octane biotransformations.

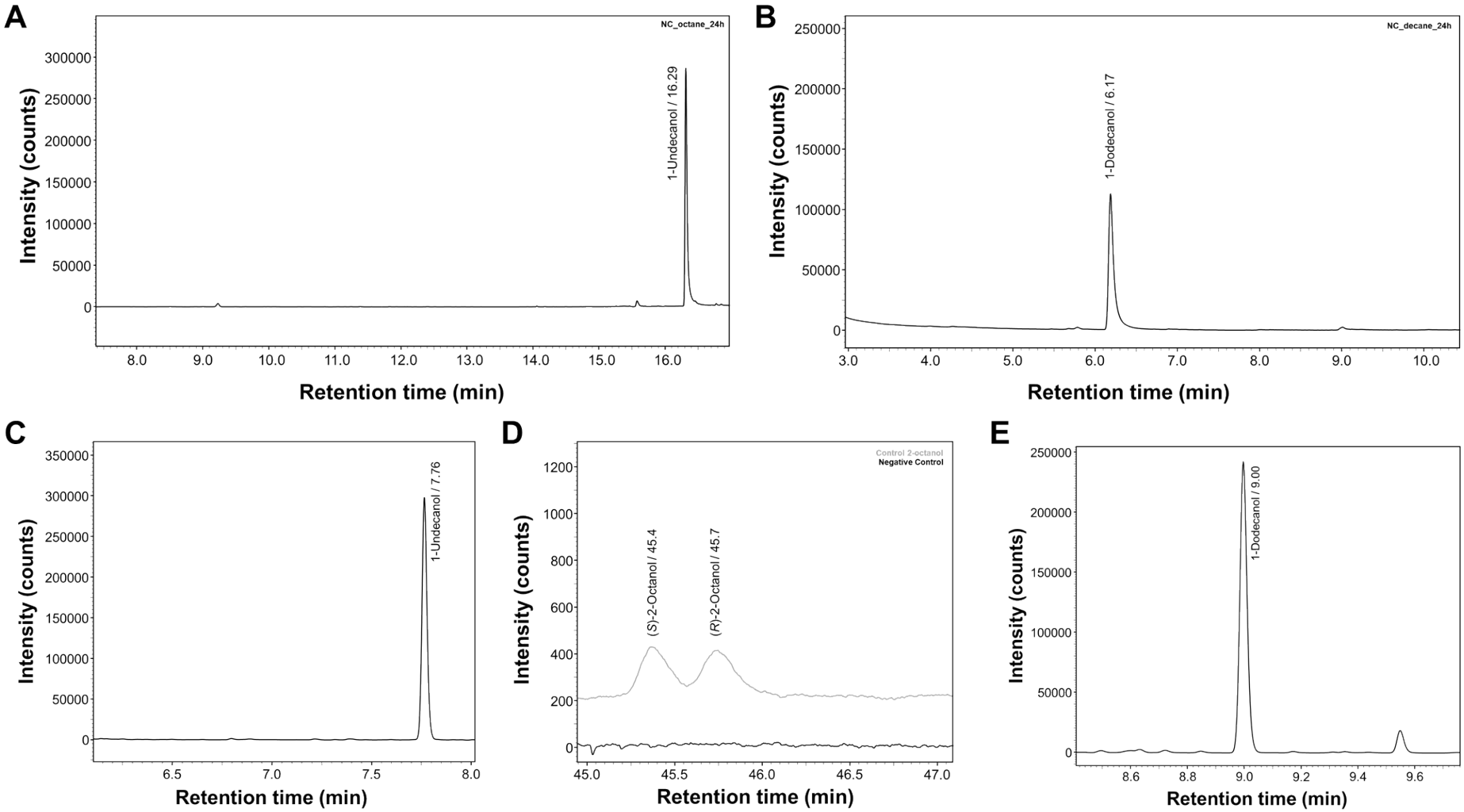


**Figure S11.** Representative examples of GC chromatograms of biotransformations of the negative control. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated sample for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of a racemic 2-octanol sample.

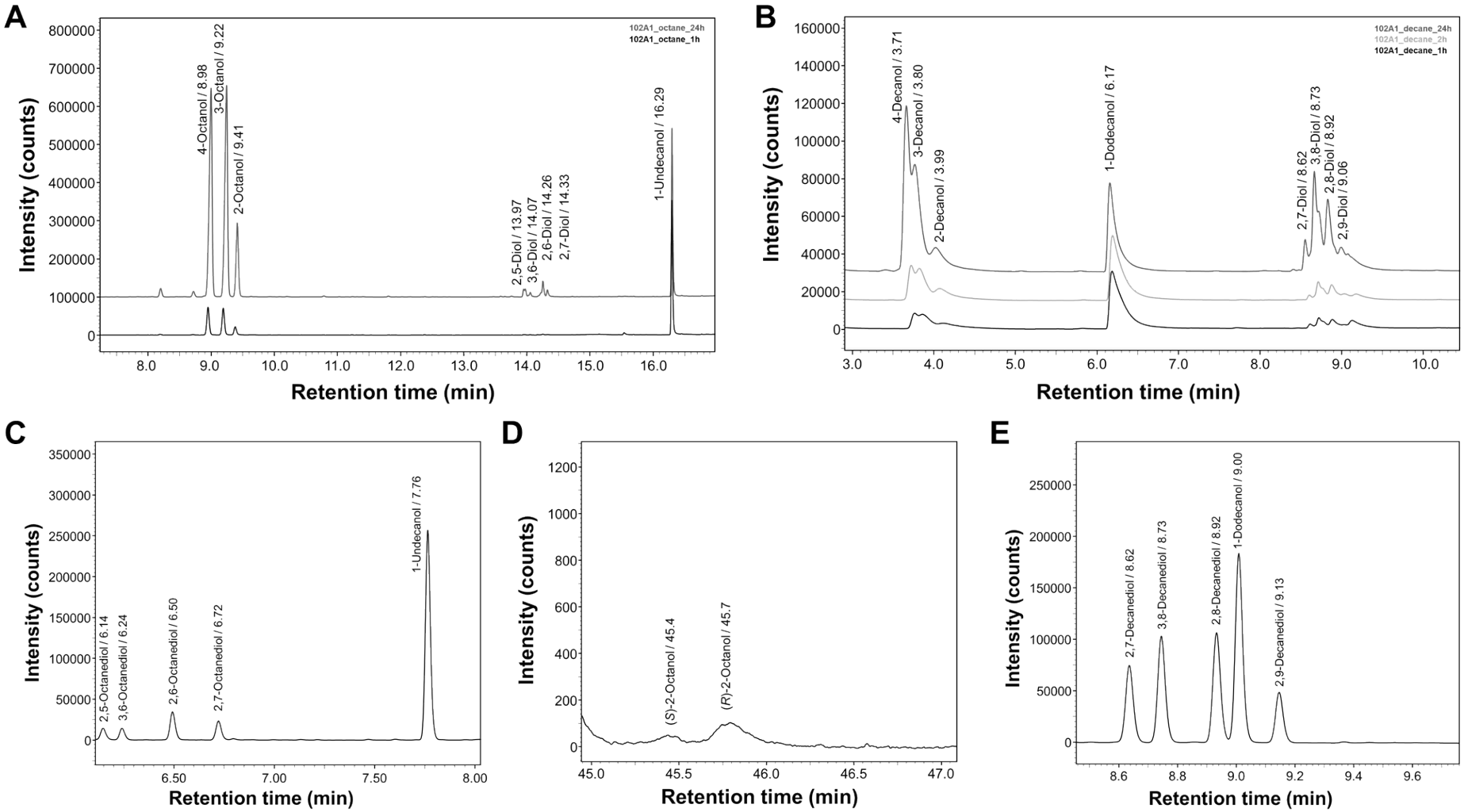


**Figure S12.** Representative examples of GC chromatograms of biotransformations with CYP102A1. GC chromatograms of (A) octane biotransformations at 1 h and 24 h and (B) decane biotransformations at 1 h, 2 h and 24 h. GC chromatograms of silylated sample for diol analysis from octane (C) and decane (E) after 24 h. (D) GC chromatogram of chiral analysis of 1 h sample of octane biotransformations.

### 4.4. Alcohol and diol production during hydroxylation of CYP505A30 and mutants

**Table S4.** Total turnover numbers (TTN) for CYP505A30 (Wt) and mutants in comparison to TTN determined by Pennec *et al.*[2] on CYP102A1 and its variants. Diol formation was not considered in CYP102A1 data[2].

| | Octane | | Decane | | |
|---|---|---|---|---|---|
| Mutation | our work | respective 102A1 variants | our work | respective variants | 102A1 |
| 505A30wt | 7860 ± 1580 | | 2730 ± 480 | | |
| 505A30_BM3wt | 4390 ± 1060 | 2090 | 2300 ± 230 | 1490 | |
| 505A30_1-12G | 4720 ± 1520 | 2630 | 1500 ± 310 | 540 | |
| 505A30_87-328 | 3670 ± 910 | 370 | 3160 ± 960 | 150 | |
| 505A30_87-328_2 | 4660 ± 960 | 370 | 4120 ± 350 | 150 | |

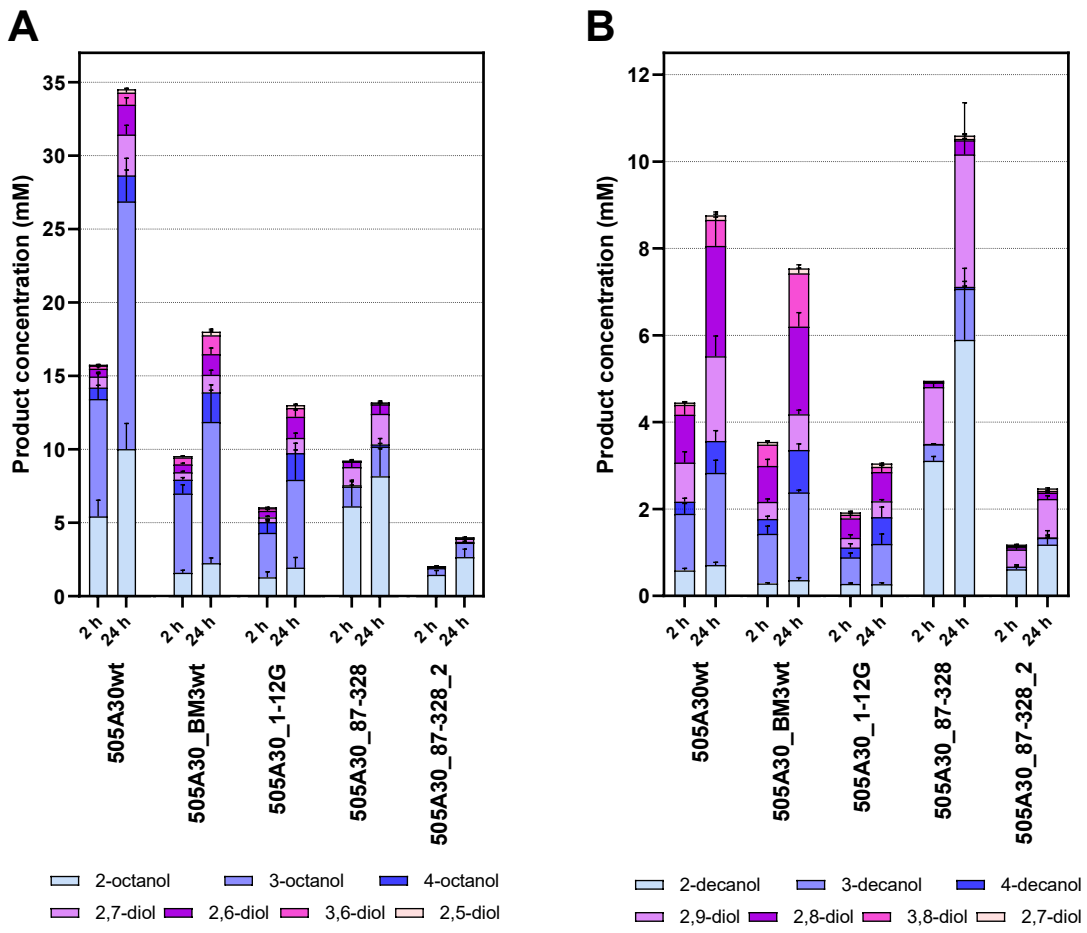


**Figure S13.** Product formation of CYP505A30wt and mutants at 2 h and 24 h of biotransformations of octane (A) and decane (B) based on the average CYP concentration achieved in the BRM.

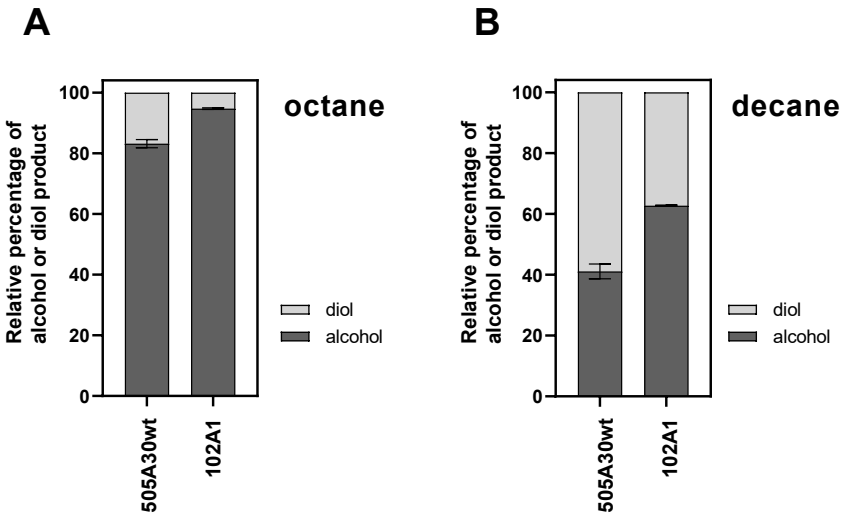


**Figure S14.** Relative percentage of alcohol and diol product formation of CYP505A30wt and CYP102A1 at 24 h of biotransformations of octane (A) and decane (B) with substrate added in excess. [CYP505A30] = 5 µM, [CYP102A1] = 13 µM.

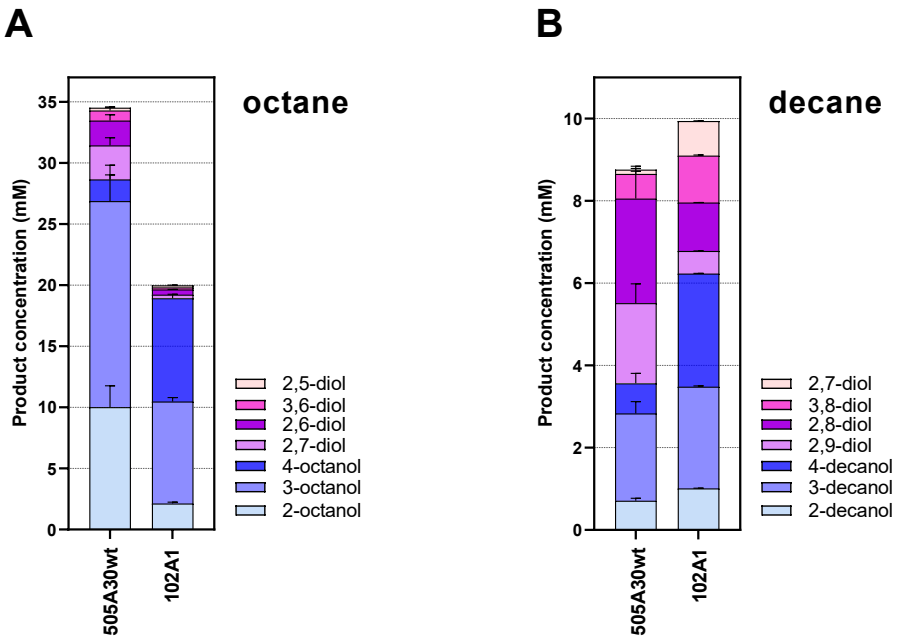


**Figure S15.** Product formation of CYP505A30wt and CYP102A1 at 24 h of biotransformations of octane (A) and decane (B) with substrate added in excess. [CYP505A30] = 5 µM, [CYP102A1] = 13 µM.

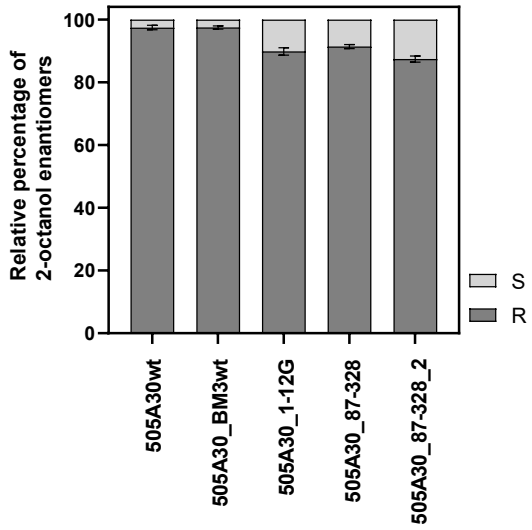


**Figure S16.** Chiral analysis of 2-octanol enantiomers formed by CYP505A30wt and mutants. 1 h data points were analysed and plotted.

## 5. Crystal structure of CYP505A30 HD

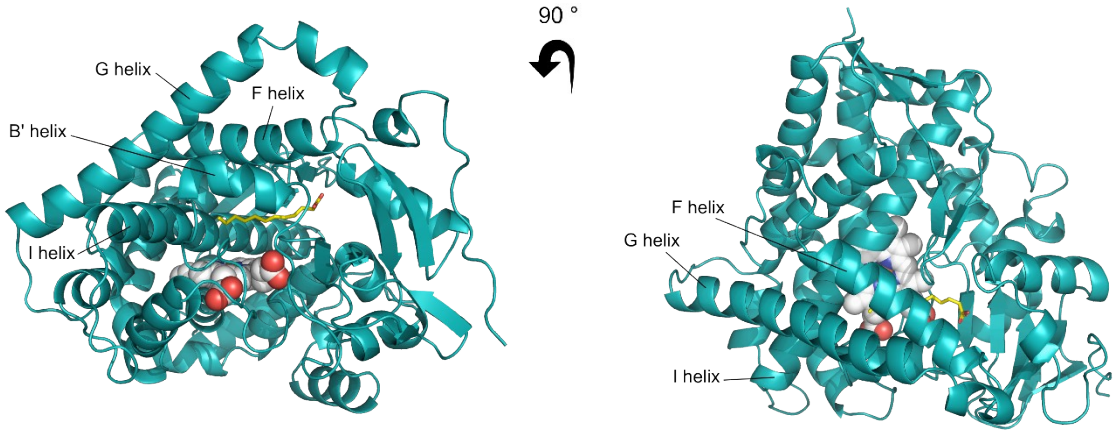


**Figure S17.** Crystal structure of CYP505A30 HD reveals the typical closed fold of CYP450s crystallised with substrate. The haem, shown as spheres, is bound to a conserved cysteine, and the substrate dodecanoic acid is highlighted in yellow.

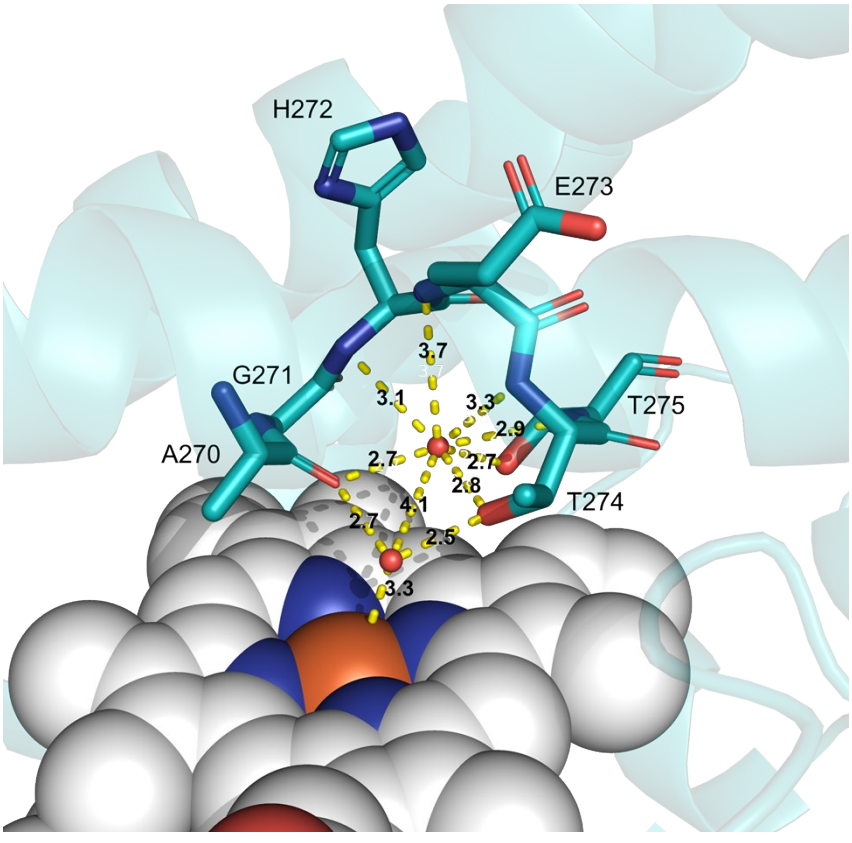


**Figure S18.** I-helix of CYP505A30 HD showing the catalytically important acid-alcohol pair (E273 and T274) and the coordinated water molecules including the hydrogen bonding network.

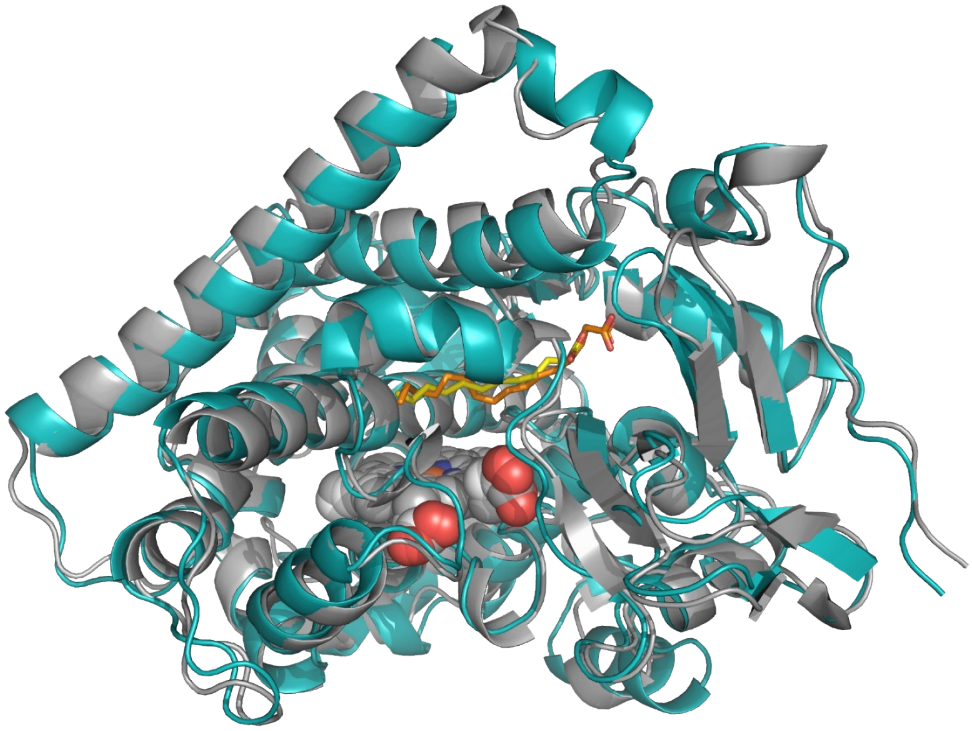

**Figure S19.** Overlay of the crystal structures of CYP505A30 HD (cyan) with dodecanoic acid (yellow) and CYP102A1 (PDB 1fag, grey) with palmitoleic acid (orange) shows a high similarity of the overall structure.

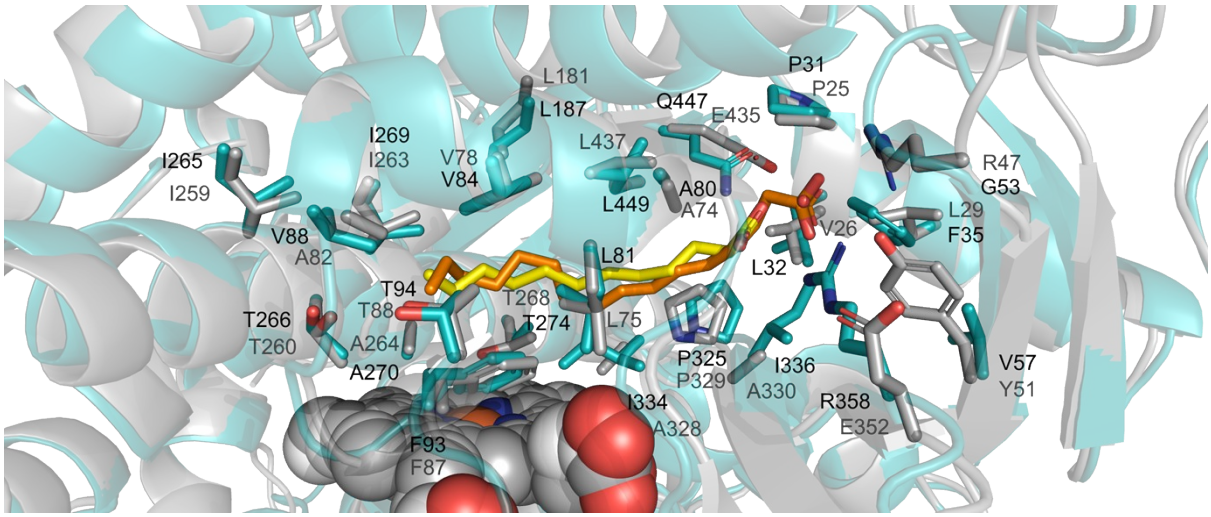


**Figure S20.** Overlay of the active site of CYP505A30 HD (green) with dodecanoic acid (yellow) with the active site of CYP102A1 (PDB 1fag, grey) with palmitoleic acid (orange). 7 residues are located within 4 Å of the substrate in CYP505A30, including F35, F93, I269, I334, I336, R358, L449.

**References**


1 J. Sanchis, L. Fernández, J. D. Carballeira, J. Drone, Y. Gumulya, H. Höbenreich, D. Kahakeaw, S. Kille, R. Lohmer, J. J. P. Peyralans, J. Podtetenieff, S. Prasad, P. Soni, A. Taglieber, S. Wu, F. E. Zilly and M. T. Reetz, *Appl. Microbiol. Biotechnol*., 2008, **81**, 387–397.

2 A. Pennec, C. L. Jacobs, D. J. Opperman and M. S. Smit, *Adv. Synth. Catal*., 2015, **357**, 118–130.