Enantioselective biocatalytic formal α-amination of hexanoic acid to L-norleucine

Supporting Information

1. General

All chemicals, catalase (from bovine liver) and lysozyme (from chicken egg) were obtained from Sigma Aldrich (Steinheim, Germany) unless otherwise stated. Hydrogen peroxide was used from a commercial 30% solution.

2. Access to enzymes

P450 peroxygenase from *Clostridium acetobutylicum* ($P450_{CLA}$) and 2-hydroxyisocaproate dehydrogenases from *Lactobacillus confusus* (L-Hic-DH) and *Lactobacillus casei* (D-Hic-DH) were obtained as reported previously.[1] Internal plasmid numbers are pEG306 for $P450_{CLA}$, pEG220 for L-Hic-DH and pEG221 for D-Hic-DH.

$P450_{CLA}$ was used as purified protein (Figure S1).

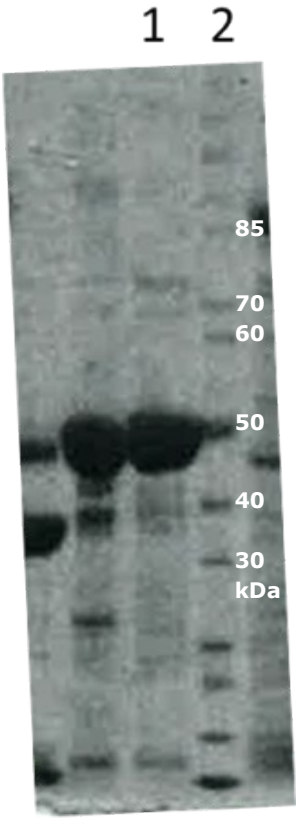


Figure S1. SDS-PAGE from His-tag purification of $P450_{CLA}$. Lane 1: His-Tag purified $P450_{CLA}$ (~48 kDa). Lane 2: PAGE ruler unstained protein ladder from Fermentas.

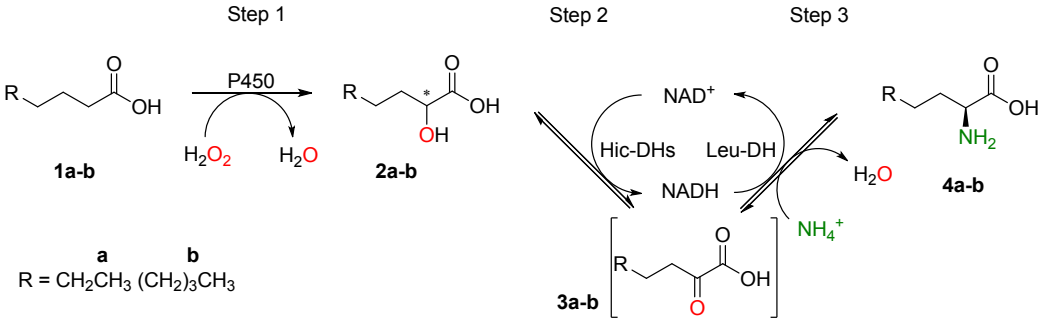
Both Hic-DHs were used as freeze-dried whole-cell preparations. For this, cells were separated from expression media by centrifugation (4000 x g, 30 min, 4 °C). The obtained cell pellet was

resuspended and homogenized in 10 mM potassium phosphate buffer (pH 7.0), shock frozen in liquid N_2 and freeze-dried overnight. The obtained dried powder was stored at 4 °C until use in biotransformations.

Leucine dehydrogenase from *Bacillus cereus* (L-Leu-DH; Uniprot reference number: Q81G11) was cloned as codon optimized synthetic gene into the pEamTA expression vector (*NdeI* and *EcoRI* restriction sites). The plasmid was transformed into *E. coli* BL21(DE3) cells. A 50 mL pre-culture was grown overnight (37 °C, 120 rpm) in TB medium containing 100 mg L^{-1} ampicillin. The main culture (50 mL TB media containing 100 mg L^{-1} ampicillin) was induced adding cells to a starting optical density (OD) of 0.1. Cells were grown at 28 °C and 120 rpm until an OD value of 1.0. Then 0.25 mM IPTG (final concentration) was added to induce expression of L-Leu-DH and cells were incubated for additional 16 h (28 °C, 120 rpm). Cells were harvested by centrifugation (4000 x g, 4 °C, 15 min) and the obtained pellets washed once with ddH_2O. The washed pellet was resuspended in 15 mL KPi buffer (50 mM; pH 7.75) followed by disruption of cells by sonication (4 min at 65 % amplitude; pulse for 2 sec with pausing of 4 sec). Cell debris were removed by centrifugation (24000 x g, 1 h, 4 °C). The supernatant was collected and frozen in liquid N_2 and stored at -20 °C until further use.

3. Individual steps for formal amination of fatty acids to α-amino acids

All enzymatic steps and compounds are referenced herein as numbered in reaction scheme S1.



Scheme S1. General reaction scheme for biocatalytic formal α-amination of fatty acids to amino acids in three-step one-pot cascade.

3.1. Catalytic activity of $P450_{CLA}$ in hydroxylation of 1a-b (step 1)

Characterization of purified $P450_{CLA}$ in the hydroxylation of fatty acids (**1a-b**) was performed by monitoring the amount of products **2a-b** formed upon one-time addition of sub-stoichiometric quantities of H_2O_2, as indicated in Table 1.

Reaction conditions

Reactions were performed in triplicate in Tris-HCl (pH 7.5, 100 mM) using 1 µM $P450_{CLA}$ and 5% (*v*/*v*) EtOH (co-solvent) at RT and 170 rpm shaking in closed glass vials (reaction time as indicated in Table 1, main text). Reactions were quenched by addition of HCl before product extraction, which leads to immediate decolorization, indicative of destruction/loss of heme functionality and thus protein deactivation. Hydroxy acids were extracted and derivatized for GC analysis following a protocol previously reported.[2]

3.2. Catalytic activity of Hic-DHs in oxidation of 2a-b (step 2)

Commercial standards of *rac*-**2a-b** were used to test the activity of both Hic-DHs. To that end, both enzyme preparations were employed in the oxidative mode (step 2, scheme S1), and the progress of the reaction was monitored by following change in absorbance at 340 nm on the spectrophotometer. Oxidation of 2-hydroxy acid substrates was successful as indicated by increasing UV absorption, corresponding to (concomitant) formation of NADH (Figure S2).

Reaction conditions

Reaction mixtures contained 1 or 10 mM *rac*-**2a-b**, 1% (*v*/*v*) DMSO as co-solvent, 10 mM NAD^+ and 0.25 mg of L- and D-Hic-DH (freeze-dried whole-cell preparations) in a final volume of 1 mL buffer (100 mM Tris-HCl, pH 8.5).

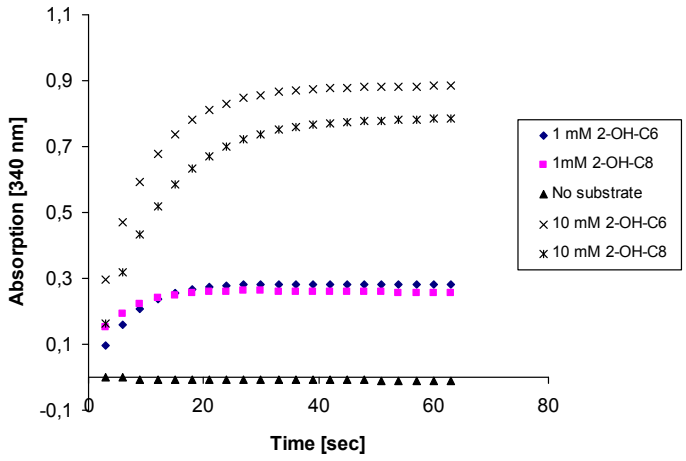


Figure S2. Oxidation of *rac*-2-hydroxyhexanoic (2-OH-C6 = **2a**) and 2-hydroxyoctanoic acid (2-OH-C8 = **2b**) by L-/D-Hic-DH. Following NADH concentrations were calculated after 1 min reaction time with 1 mM **2a**: 45 µM; 10 mM **2a**: 142 µM; 1 mM **2b**: 41 µM; 10 mM **2b**: 126 µM.

3.3. Catalytic activity of L-Leu-DH (step 3)

The activity of L-Leu-DH was tested in the oxidative deamination direction. Deamination of L-norleucine (**4a**) and L-leucine (both commercial standards) was performed using cell-free lysates containing L-Leu-DH from *Bacillus cereus*.

Reaction conditions

Reaction mixtures contained 10 mM substrate, 10 mM NAD^+ and 5 µL Leu-DH preparation in a final volume of 1 mL buffer (Tris-HCl, pH 8.5, 100 mM). Oxidation of α-amino acids to α-ketoacids was successful as indicated by increasing UV absorption at 340 nm, corresponding to (concomitant) formation of NADH (Figure S3).

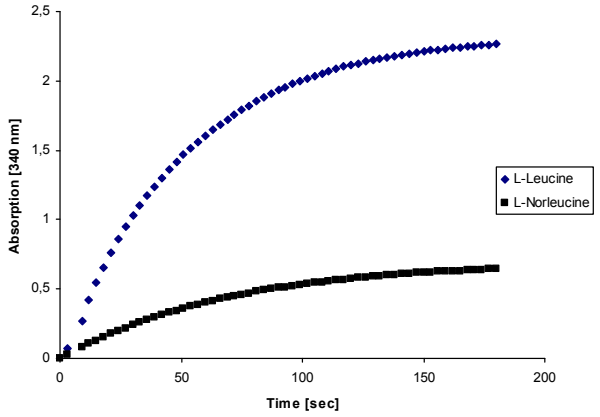


Figure S3. Formation of NADH monitored at 340 nm in the oxidative deamination of L-leucine and L-norleucine (**4a**) using L-Leu-DH (corresponds to a volumetric activity of 14.5 U mL^{-1}). Incubation of NAD^+ with norleucine during 20 min without enzyme did not lead to any detectable increase in absorbance.

4. Bi-enzymatic one-pot conversion of 2-hydroxy fatty acid 2a to amino acid 4a

The compatibility of steps 2 and 3 (Scheme S1) was tested on D-/L-hydroxy hexanoic acid **2a** at pH 8.5.

Reaction conditions

Reaction mixtures contained 10 mM *rac*-**2a**, 5% (*v/v*) EtOH as co-solvent, 10 mM NAD^+, $(NH_4)_2SO_4$ (varying concentration), 0.01 mg each of L- and D-Hic-DH (freeze-dried whole cells), 0.07 U mL^{-1} Leu-DH (cell-free lysate) in a final volume of 1 mL buffer (Tris-HCl, pH 8.5, 100 mM).

Amount of formed norleucine was quantified by GC (section 5.3.2) and data showed that the lowest concentration of ammonium salt (100 mM) yielded highest conversion (Figure S4).

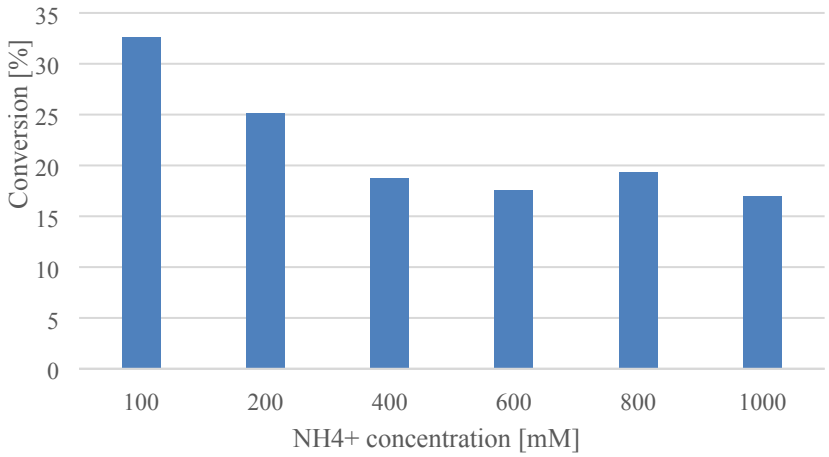


Figure S4. Bi-enzymatic one-pot amination of *rac*-**2a** into **4a** at various ammonium salt concentrations.

5 One-pot cascade set-up for conversion of fatty acids to α-amino acids

5.1. Conversion of fatty acids 1a-b to amino acids 4a-b

Hydroxylation of fatty acid substrates (**1a-b**) was performed using 3 µM $P450_{CLA}$, 10 mM substrate and 2.5% (*v/v*) EtOH (co-solvent) in a final volume of 1 mL buffer (Tris-HCl, pH 7.5, 100 mM). H_2O_2 (1.6 mM) was supplemented every 30 min (36.8 mM total). After 24 h, the hydroxylation reaction (step 1) was stopped by adding 1200 U catalase (1 mg) and 20 µL of $(NH_4)_2SO_4$ (2.5 M stock) and the pH was adjusted to 8.5 using 1 M NaOH. For steps 2 and 3, L- and D-Hic-DHs (0.25 mg each), 10 mM NAD^+ and 0.07 U mL^{-1} Leu-DH (cell-free lysate) were added to the reaction mixture. After 24 h reaction time, 2 µL of the reaction volume were spotted on a TLC plate along with a commercial standard of L-norleucine as reference material (positive control). After elution of products with acetic acid/H_2O/n-butanol as mobile phase (1:1:3), the plate was stained with ninhydrin and heated gently for color development. Only the sample from the cascade with **1a** gave a distinct red spot after ninhydrin staining, indicating formation of a free amine functionality. Under these conditions, substrate **1b** did not display product (amino acid) formation. Following control reactions were performed with **1a** to confirm overall success of the one-pot cascade, leaving out one of the following components at a time: 1) $P450_{CLA}$; 2) L- and D-Hic-DH; 3) L-Leu-DH; 4) fatty acid substrate; 5) H_2O_2; 6) $(NH_4)_2SO_4$; 7) NAD^+. The

reaction samples were analyzed by TLC and formation of **4a** could not be detected in any of the samples.

5.2. Derivatization and GC-MS analysis of cascade product 4a

The reaction sample indicating formation of **4a** (positive ninhydrin staining on TLC plate) was freeze-dried in liquid nitrogen and lyophilized overnight. Freeze-dried residues were resuspended in 300 µL MeOH followed by addition of DMAP (5% in 700 µL MeOH) and 150 µL ethyl chloroformate. The solution was stirred at 700 rpm for 50 min at 50 °C. MeOH was removed by evaporation (dry air flow) and 700 µL HCl (2%) were added. The solution was extracted three times with 600 µL EtOAc and the organic phases were dried over anhydrous Na_2SO_4 prior to injection into GC-MS. In addition, 4 mg of L-norleucine (commercial standard) was treated in the same way and served as reference. The successful production of norleucine from hexanoic acid employing the three step one-pot cascade was confirmed by GC-MS (Figure S5).

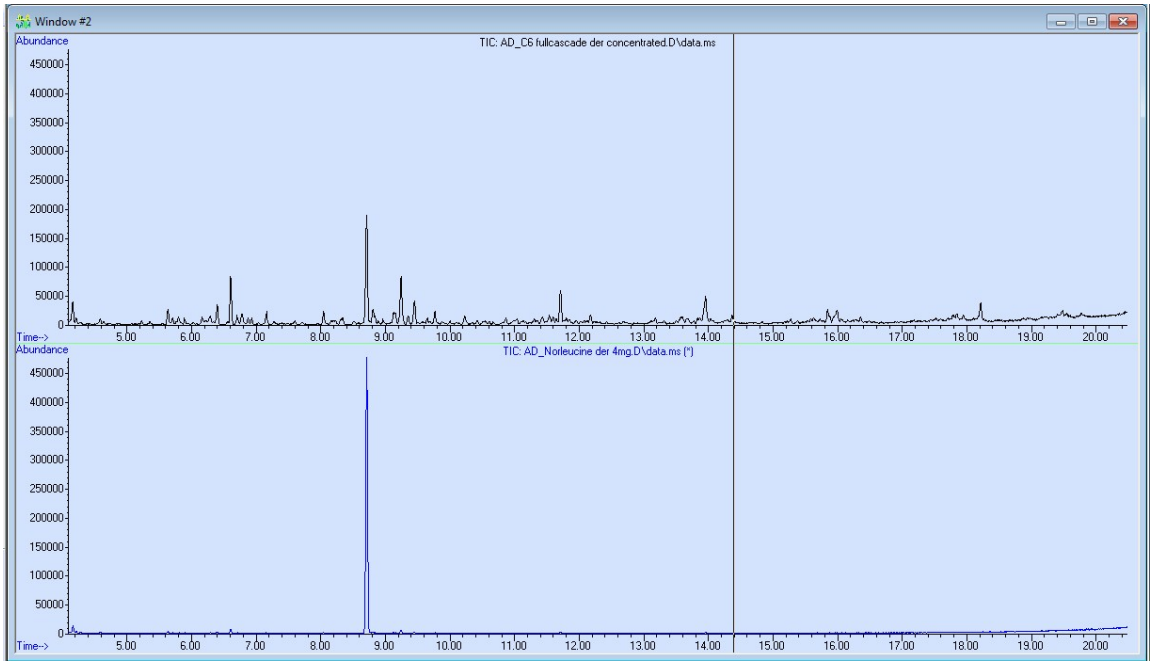


Figure S5. GC-MS chromatogram after one-pot three-step cascade conversion of 10 mM **1a** according to 5.1 and derivatization (upper chromatogram; black line). Lower chromatogram (blue line) shows derivatized sample of 4 mg L-**4a** (commercial standard; retention time 8.75 min). Additional peaks in the reaction sample arise from components originating from the cells which got derivatized during the work-up.

5.3. Conversion of 1a to final product 4a in cascade set-up

5.3.1. Conversion of 1a to 4a

The reaction was performed in 1 mL Tris-HCl buffer (pH 7.5, 100 mM) containing **1a** (1-5 mM), 3 µM $P450_{CLA}$ and 5% (*v*/*v*) EtOH (co-solvent), and 0.8 mM H_2O_2 (from 320 mM stock) was supplemented every 30 min (total 20 mM). The pH was adjusted to 8.5 after completion of first step by adding 5 M NaOH in 1 µL increment until the desired pH was reached, followed by addition of 0.07 U mL^{-1} Leu-DH (cell-free lysate), 0.01 mg mL^{-1} L- and D-HIC-DHs (freeze-dried whole cells), 50 mM $(NH_4)_2SO_4$ and 10 mM NAD^+. Samples were analyzed on GC-FID as described below (section 5.3.2) for determination of conversion. For determination of enantiopurity, an HPLC protocol was developed (section 5.3.3).

5.3.2. GC protocol for determination of conversion

For quantification of norleucine (**4a**), a derivatization protocol was established and a calibration curve was prepared using commercial analytical standard (Figure S6). A defined concentration of norleucine (0-10 mM) was supplemented into a final volume of 1 mL of buffer (Tris-HCl, pH 8.5, 100 mM). Samples were freeze dried, resuspended in 300 µL MeOH followed by addition of 700 µL of a DMAP solution (5% in MeOH) and 150 µL of ethyl chloroformate. The solution was stirred at 700 rpm and 50 °C for 1 h. MeOH was removed by dry air flow followed by addition of 700 µL of 2% HCl. Extraction of products was performed three times with 600 µL EtOAc containing 0.1% (*v*/*v*) 1-decanol as internal standard. The organic phases were dried over Na_2SO_4 prior to injection on GC-FID.

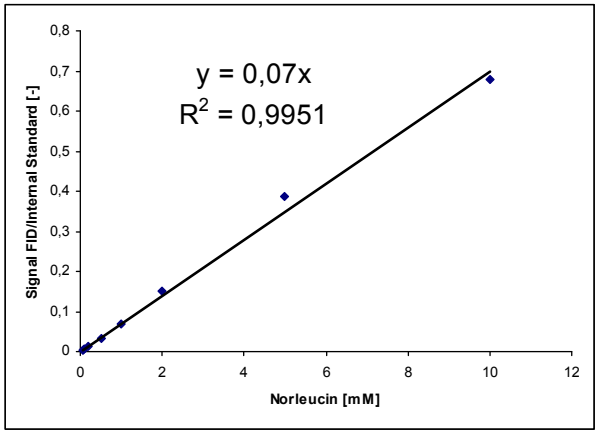
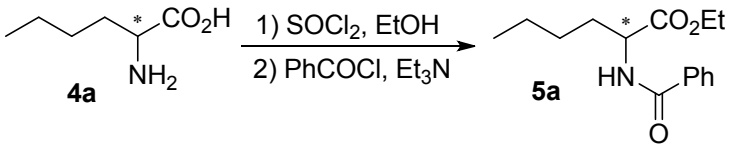


Figure S6. Calibration curve for **4a** after derivatization of standards and GC-FID analysis.

5.3.3. HPLC protocol for determination of enantiopurity of 4a

The protocol was developed using commercial standard of *rac*-norleucine (**4a**). Amino acid **4a** was derivatized according to scheme S2. The procedure does not affect the absolute configuration of the chiral center.



Scheme S2. Derivatization of **4a** to **5a** for analysis on chiral HPLC

A solution of *rac*-**4a** (0.1 mmol) in absolute EtOH (1 mL) was cooled to 0 °C. Neat $SOCl_2$ (51 µL, 0.7 mmol) was added dropwise and the solution allowed to warm-up to RT before heating to reflux for 10 h. The mixture was cooled and solvents removed in vacuo to give a white crystalline solid, which was dissolved in CH_2Cl_2 (0.5 mL). To this solution, Et_3N (28 µL, 0.2 mmol) was added followed by benzoyl chloride (11.6 µL, 0.1 mmol). After stirring for 1 h, the resulting mixture was purified by preparative TLC (4:1 cyclohexane/ethylacetate) to yield *rac*-**5a**. HPLC measurement proceeded on a chiral column (Chiralcel OD-H, 1.0 mL/min, 96:4 *n*-heptane:*i*-PrOH, 30 ºC, 215 nm) and yielded following retention times (see Figures S7-8): D-**4a** 7.79 min, L-**4a** 13.43 min. The product from the reductive amination catalyzed by L-Leu-DH was treated in a similar fashion and the resulting chromatogram yielded a single peak for L-**4a**.

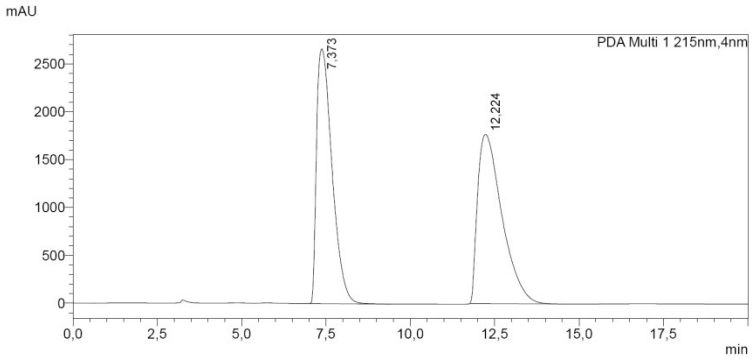


Figure S7. HPLC chromatogram from derivatized authentic sample of *rac*-norleucine (**4a**)

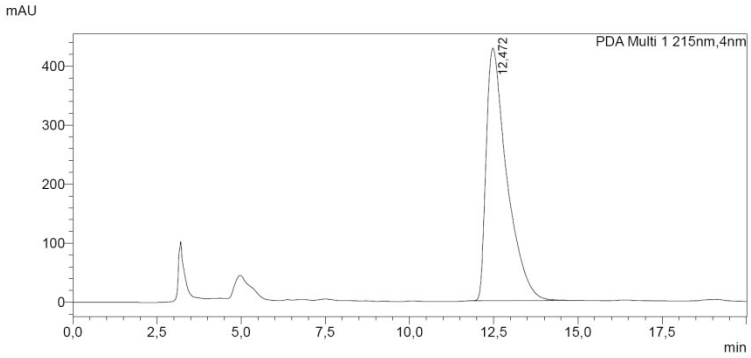


Figure S8. HPLC chromatogram from derivatized norleucine (**4a**) obtained by L-leu-DH.

6. Analytical methods

GC-MS measurements were 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 He as carrier gas. Injector temperature: 250 °C; injection volume: 1 µL; flow rate: 0.7 mL min^{-1}; temperature program 1: 100 °C, hold 0.5 min, 10 °C min^{-1} to 300 °C; EI mode, energy 70 eV, MS Source: 230 °C, MS Quadrupole: 150 °C.

GC measurements were performed on an Agilent Technologies 7890 A GC system equipped with a FID-detector and a 7693A Injector in combination with a 7693 Series Autosampler and using a HP-5 column (30 m x 320 µm, 0.25 µm film, J&W Scientific, Agilent Technologies) and He as carrier gas. Injector temperature 300 °C; injection volume: 1 µL; flow rate: 36.388 cm sec^{-1}, 2.0 mL min^{-1}; split ratio 30:1; temperature program: 100 °C, hold 5 min, 10 °C min^{-1} to 320 °C, hold 0 min.

HPLC measurements were performed on a Shimadzu LC-20AD HPLC system with a DGU-20A5 degasser, a SIL-20AC autosampler, SPD-M20A diode array detector and a CTO-20AC column oven, equipped with a Chiralcel OD-H column (25 × 0.46 cm).

References

[1] a) 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; b) E. Busto, N. Richter, B. Grischek and W. Kroutil, *Chem. Eur. J.* **2014**, *20*, 11225-11228.

[2] A. Dennig, M. Kuhn, S. Tassoti, A. Thiessenhusen, S. Gilch, T. Bulter, T. Haas, M. Hall and K. Faber, *Angew. Chem. Int. Ed.* **2015**, *54*, 8819-8822.