Supporting Information

Biocatalytic Asymmetric Michael Additions of Nitromethane to α,β-Unsaturated Aldehydes via Enzyme-bound Iminium Ion Intermediates

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1. Materials and Methods

1.1 Materials

Chemicals were purchased from Sigma Aldrich, Across, Merck or Fluka (unless noted otherwise) and were used without further purification. The *α,β*-unsaturated aldehydes **2b-k** were prepared using previously reported methods.[1] Synthesis of racemic reference compounds, required for chiral analysis of enzymatic products, was done according to published protocols.[2]

1.2 General methods

NMR spectra were recorded on a Brucker 500 MHz spectrometer. Enzymatic assays were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). Reverse phase HPLC was carried out using an in-house analytical HPLC equipped with a Shimazu LC-10 AT pump and a Shimazu SPD-M10A diode array detector. Gas chromatography was carried out with a HP 5890 series II gas chromatograph using a chiral GTA column (30 m × 0.25 mm × 0.12 µ; Supelco).

1.3 Screening 4-OT single mutants for enhanced Michael addition activity

A systematic mutagenesis strategy was applied to identify residue positions at which mutations give a marked improvement in the activity of 4-OT for addition of **1** to **2a**. For this, a previously constructed collection of 1040 single 4-OT mutants was used.[3] The proteins were produced in *E. coli* and cell free extracts (CFEs) were prepared according to a reported procedure.[3] Briefly, 1.25 mL of LB medium supplemented with 100 µg/mL ampicillin and 100 µM IPTG was inoculated from a glycerol stock of the corresponding mutant and the culture was grown overnight at 37 °C in a 96-deep well plate. The cells from the overnight cultures were harvested by centrifugation and lysed with 250 µL of Bugbuster (Novagen), supplemented with 25 U/mL benzonase, for 40 min. After centrifugation, the obtained CFE was used for the activity assay. The reaction mixtures (100 µL) consisted of CFE (40% v/v), **1** (25 mM), **2a** (0.25 mM) and ethanol (5% v/v) in 20 mM HEPES buffer (pH 6.5). The reaction was initiated by adding 5 µL from a stock solution of **2a** (5 mM in ethanol). Prior to starting the measurements, the 96-well plates were shaken for 1 min at 500 rpm to ensure proper mixing. The depletion in the absorbance of **2a** was monitored at 292 nm for 3 h with 3 min time intervals.

1.4 Protein expression and purification

The expression and purification of wild-type 4-OT and 4-OT mutants were based on protocols described elsewhere.[4] A sample of each purified protein was analyzed by ESI-MS to confirm that the protein had been processed correctly and the initiating methione had been removed. The purified protein was flash frozen in liquid nitrogen and stored at -80 °C until further use.

1.5 Chiral analysis

The enantiomeric ratio of the enzymatically synthesized compounds **3a**, **3d**, **3i** and **3k** was analyzed directly using HPLC or GC with a chiral stationary phase. The enantiomeric ratio of the compounds **3h** and **3j** was analyzed by chiral HPLC after derivatizing the enzymatic products into a cyclic acetal, prepared according to a literature procedure, and using chemically synthesized racemic cyclic acetal reference compounds for comparison.[3] For compounds **3b**, **3c**, **3e**, **3f** and **3g**, the enantiomeric

ratio was analyzed by chiral HPLC after reducing the aldehyde functionality of the enzymatic product into the corresponding alcohol using $NaBH_4$ according to a reported procedure and using chemically synthesized racemic γ–nitroalcohols as reference compounds.[2a] Analytical conditions for each compound are given below.

1.6. Analytical scale reactions

Activity comparison of wild-type 4-OT (4-OT WT) and the variants 4-OT F50A, 4-OT F50I, 4-OT F50V, and 4-OT P1A for addition of **1** to **2a** was carried out by UV spectroscopic analysis on analytical scale (0.3 mL reaction volume). Purified enzyme (300 µg) was incubated in a 1 mm cuvette with **1** (25 mM) and **2a** (1 mM) in 20 mM HEPES buffer (pH 6.5; 0.3 mL final volume). The reactions were monitored by following the decrease in absorbance at 290 nm, which corresponds to the depletion of substrate **2a**.

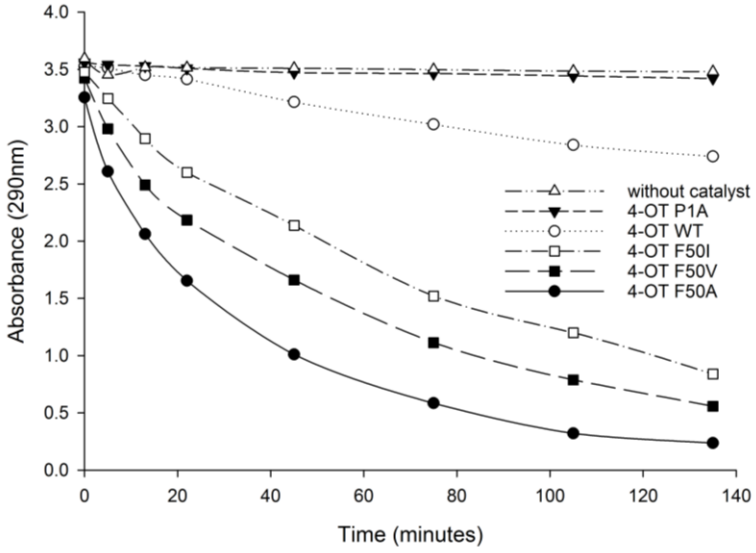


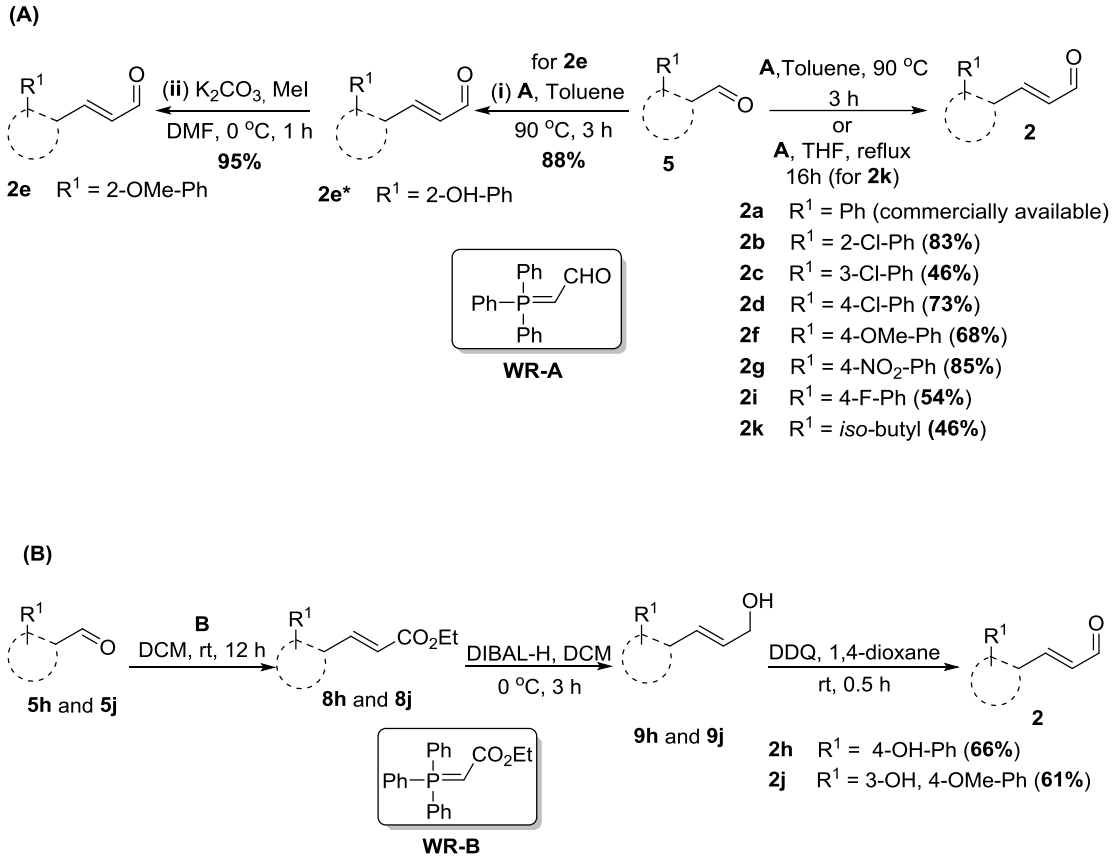
Figure S1. UV traces for monitoring the depletion of **2a** in the presence of purified 4-OT WT, 4-OT F50A, 4-OT F50I, 4-OT F50V and 4-OT P1A. The mutant enzymes F50I, F50V and F50A showed a 6-fold, 8-fold, and 15-fold increase in activity, respectively, based on the initial substrate depletion rates (from 0-13 min). Analysis of the corresponding products showed that the mutant enzymes F50I, F50V and F50A allow the production of (*R*)-**3a** with e.r. values of 99:1, 97:3 and 99:1, respectively.

1.7. Kinetic assay

Kinetic assays were performed at 20 °C in 20 mM HEPES buffer (pH 6.5) by following the decrease in absorbance at 290 nm, which corresponds to the depletion of substrate **2b**. An appropriate amount of purified 4-OT F50A enzyme (20 µg) was incubated in a 1 mm cuvette with **1** (25 mM) and **2b** (varying concentrations ranging from 0.1 to 2 mM) in 20 mM HEPES buffer (pH 6.5; 0.3 mL final volume) at 20 °C. The initial rates (µM/s) were plotted versus the concentrations (µM) of substrate **2b**. SigmaPlot was used to fit the data to Michaelis-Menten kinetics to determine the kinetic parameters. The measurements were done in triplo.

2. Chemical synthesis of *α,β*-unsaturated aldehydes (2b-k)

Compound **2a** is commercially available. Synthesis of compounds **2b-k** was carried out *via* the Wittig reaction and according to reported procedures summarized in Scheme S1.



Scheme S1. Synthesis of *α,β*-unsaturated aldehydes **2b-g**, **2i**, and **2k** (Scheme S1A) and **2h**, **2j** (Scheme S1B) *via* the Wittig reaction. Abbreviation: WR, Wittig reagent.

(a) Synthesis of compounds 2b-g, 2i and 2k (Scheme S1A)

Compounds **2b-g**[1a-h], **2i**[1i] and **2k**[1j-k] were prepared according to literature procedures and their ^{1}H NMR spectra match with earlier reported NMR data.[1a-k]

(b) Synthesis of compound 2e (Scheme S1A)

To a stirred solution of (*E*)-3-(2-hydroxyphenyl)acrylaldehyde (1.0 eq) in 5 mL of dry DMF was added K_2CO_3 (1.5 eq) at 0 °C. Subsequently, MeI (1.5 eq) was added dropwise at the same temperature and the reaction mixture was further incubated for 1 h. After completion of the reaction (monitored by TLC with $KMnO_4$ staining), the reaction was quenched with saturated aqueous ammonium chloride, and the reaction mixture was extracted 3 times with ethyl acetate. The organic layers were combined, dried over Na_2SO_4 and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding cinnamaldehyde derivative **2e**. Yellow solid; yield =

95% (52 mg, starting from 50 mg of **2e***). The ^{1}H NMR spectrum matches with previously reported NMR data.[1f]

(**c**). **Synthesis of compounds 2h** and **2j (Scheme S1B)**

Compounds **2h** and **2j** were prepared according to the route shown in Scheme S1B, which involves the preparation of compounds **8h**, **8j**, **9h** and **9j**.

(**i**) **Synthesis of compounds 8h** and **8j (Scheme S1B)**

The reported literature procedure was slightly modified.[1l-m] 4-OH-benzaldehyde (1.0 eq) was dissolved in 5 mL anhydrous DCM and treated with carbethoxy-methylene triphenylphosphorane (1.2 eq) at room temperature. After the complete addition of all the starting material, the reaction mixture was stirred at room temperature for 12 h. After the complete consumption of the starting material (monitored by TLC with KMnO4 staining), the reaction was quenched with saturated aqueous ammonium chloride, and the reaction mixture extracted three times with DCM. The organic layers were combined, dried over Na_2SO_4 and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding α,*β*-unsaturated ester **8h**. Colorless solid; yield = 79% (248 mg, starting from 200 mg of **5h**). Similarly, compound **8j** was prepared by using the same procedure. Colorless solid; yield = 76% (222 mg, starting from 200 mg of **5j**). The ^{1}H NMR data of compounds **8h**[1l] and **8j**[1m] match with earlier reported NMR data.

(**ii**) **Synthesis of compound 9h** and **9j (Scheme S1B)**

The reported literature procedure was slightly modified.[1n-o] To a stirred solution of ethyl (*E*)-3-(4-hydroxyphenyl)acrylate (1.0 eq) in 5 mL of dry DCM was added diisobutylaluminum hydride (DIBAL-H) (1.5 eq, 1.0 M in cyclohexane) dropwise at 0 ^oC. After the complete addition of all the starting material, the reaction mixture was stirred at 0 ^oC for 3 h followed by 1 h at room temperature. After the completion of the starting material (monitored by TLC with KMnO4 staining), the reaction was quenched with saturated aqueous ammonium chloride, and the reaction mixture was extracted three times with DCM. The organic layers were combined, dried over Na_2SO_4 and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding alcohol **9h**. Yellow solid; yield = 73% (104 mg, starting from 200 mg of **8h**). Similarly, compound **9j** was prepared by using the same procedure. Colorless oil; yield = 61% (100 mg, starting from 200 mg of **8j**). The ^{1}H NMR data of compounds **9h**[1n] and **9j**[1o] match with the reported NMR data.

(**iii**) **Synthesis of compounds 2h** and **2j**

To a stirred solution of (*E*)-4-(3-hydroxyprop-1-en-1-yl)phenol (1.0 eq) in 5 mL of dry 1,4-dioxane was added DDQ (1.2 eq) dropwise at 0 ^oC. After the complete addition of all the starting material, the reaction mixture was stirred at room temperature for 30 min. After the completion of the starting material (monitored by TLC with KMnO4 and DNP staining), the reaction was quenched with saturated aqueous $NaHCO_3$, and the reaction mixture was extracted 3 times with EtOAc. The organic layers were combined, dried over Na_2SO_4 and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an

eluent to give the corresponding cinnamaldehyde derivative **2h**. Yellow solid; yield = 66% (65 mg, starting from 100 mg of **9h**). Similarly, compound **2j** was prepared by using above procedure. Light yellow solid; yield = 61% (60 mg, starting from 100 mg of **9j**). The ^{1}H NMR data of compounds **2h**[1o] and **2j**[1p] match with those previously reported.

(**d**). **^{1}H NMR data of compounds 2b-2k, 2e*, 8h, 8j, 9h** and **9j**

(*E*)-3-(2-chlorophenyl)acrylaldehyde (2b). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.75 (d, *J* = 7.7 Hz, 1H), 7.93 (d, *J* = 16.0 Hz, 1H), 7.65 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.45 (dd, *J* = 8.0, 1.1 Hz, 1H), 7.39 – 7.28 (m, 2H), 6.70 (dd, *J* = 16.0, 7.7 Hz, 1H).

(*E*)-3-(3-chlorophenyl)acrylaldehyde (2c). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.72 (d, *J* = 7.6 Hz, 1H), 7.55 (t, *J* = 1.7 Hz, 1H), 7.47 – 7.35 (m, 4H), 6.71 (dd, *J* = 16.0, 7.6 Hz, 1H).

(*E*)-3-(4-chlorophenyl)acrylaldehyde (**2d**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.71 (d, *J* = 7.6 Hz, 1H), 7.53 – 7.48 (m, 2H), 7.46 – 7.39 (m, 3H), 6.69 (dd, *J* = 16.0, 7.6 Hz, 1H).

(*E*)-3-(2-hydroxyphenyl)acrylaldehyde (**2e***). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.68 (d, *J* = 8.0 Hz, 1H), 7.79 (d, *J* = 16.0 Hz, 1H), 7.50 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.31 (td, *J* = 8.1, 1.6 Hz, 1H), 7.07 – 6.93 (m, 2H), 6.89 (dd, 1H), 6.59 (s, 1H).

(*E*)-3-(2-methoxyphenyl)acrylaldehyde (**2e**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.67 (d, *J* = 7.9 Hz, 1H), 7.82 (d, *J* = 16.1 Hz, 1H), 7.52 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.40 (ddd, *J* = 8.9, 7.5, 1.7 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.93 (d, *J* = 8.3 Hz, 1H), 6.77 (dd, *J* = 16.1, 7.9 Hz, 1H), 3.89 (s, 3H).

(*E*)-3-(4-methoxyphenyl)acrylaldehyde (**2f**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.65 (d, *J* = 7.8 Hz, 1H), 7.54 – 7.50 (m, 2H), 7.43 (d, *J* = 15.8 Hz, 1H), 6.97 – 6.92 (m, 2H), 6.61 (dd, *J* = 15.8, 7.8 Hz, 1H), 3.86 (s, 3H).

(*E*)-3-(4-nitrophenyl)acrylaldehyde (**2g**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.78 (d, *J* = 7.4 Hz, 1H), 8.32 – 8.27 (m, 2H), 7.73 (d, *J* = 8.8 Hz, 2H), 7.53 (d, *J* = 16.1 Hz, 1H), 6.81 (dd, *J* = 16.1, 7.4 Hz, 1H).

(*E*)-3-(4-hydroxyphenyl)acrylaldehyde (**2h**). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.20 (s, 1H), 9.58 (d, *J* = 7.9 Hz, 1H), 7.67 – 7.57 (m, 3H), 6.84 (d, *J* = 8.6 Hz, 2H), 6.66 (dd, *J* = 15.8, 7.9 Hz, 1H).

(*E*)-3-(4-fluorophenyl)acrylaldehyde (**2i**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.69 (d, *J* = 6.6 Hz, 1H), 7.57 (s, 2H), 7.45 (d, *J* = 16.0 Hz, 1H), 7.13 (t, *J* = 7.2 Hz, 2H), 6.65 (dd, *J* = 15.8, 7.1 Hz, 1H).

(*E*)-3-(3-hydroxy-4-methoxyphenyl)acrylaldehyde (**2j**). ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.58 (d, *J* = 7.8 Hz, 1H), 9.30 (s, 1H), 7.59 (d, *J* = 15.8 Hz, 1H), 7.23 – 7.10 (m, 2H), 7.00 (d, *J* = 8.3 Hz, 1H), 6.60 (dd, *J* = 15.8, 7.8 Hz, 1H), 3.83 (s, 3H).

(*E*)-5-methylhex-2-enal (**2k**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.54 (d, *J* = 8.0 Hz, 1H), 7.09 (dd, *J* = 15.3, 10.0 Hz, 1H), 6.08 (dd, *J* = 15.4, 8.0 Hz, 1H), 2.11 (t, *J* = 6.7 Hz, 2H), 1.74 (dh, *J* = 13.3, 6.6 Hz, 1H), 0.93 (d, *J* = 6.7 Hz, 6H).

Ethyl (*E*)-3-(4-hydroxyphenyl)acrylate (**8h**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.64 (d, *J* = 16.0 Hz, 1H), 7.40 (d, *J* = 8.6 Hz, 2H), 7.02 (s, 1H), 6.88 (d, *J* = 8.6 Hz, 2H), 6.29 (d, *J* = 15.9 Hz, 1H), 4.27 (q, *J* = 7.1 Hz, 2H), 1.34 (t, *J* = 7.1 Hz, 3H).

Ethyl (*E*)-3-(3-hydroxy-4-methoxyphenyl)acrylate (**8j**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.58 (d, *J* = 15.9 Hz, 1H), 7.13 (d, *J* = 2.1 Hz, 1H), 7.01 (dd, *J* = 8.3, 2.1 Hz, 1H), 6.82 (d, *J* = 8.3 Hz, 1H), 6.28 (d, *J* = 15.9 Hz, 1H), 5.81 (s, 1H), 4.24 (q, *J* = 7.1 Hz, 2H), 3.90 (s, 3H), 1.32 (t, *J* = 7.1 Hz, 3H).

(*E*)-4-(3-hydroxyprop-1-en-1-yl)phenol (**9h**). ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.48 (s, 1H), 7.22 (d, *J* = 8.5 Hz, 2H), 6.70 (d, *J* = 8.5 Hz, 2H), 6.41 (d, *J* = 16.0 Hz, 1H), 6.12 (dt, *J* = 15.9, 5.4 Hz, 1H), 4.79 (t, *J* = 5.5 Hz, 1H), 4.05 (t, *J* = 4.8 Hz, 2H).

(*E*)-5-(3-hydroxyprop-1-en-1-yl)-2-methoxyphenol (**9j**). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.95 (s, 1H), 6.93 – 6.72 (m, 3H), 6.38 (d, *J* = 15.9 Hz, 1H), 6.19 – 6.04 (m, 1H), 4.78 (t, *J* = 5.4 Hz, 1H), 4.06 (t, *J* = 4.7 Hz, 2H), 3.75 (s, 3H).

3. Biocatalytic synthesis of γ-nitroaldehydes (3a-k) using 4-OT F50A

Reaction mixtures (20 mL) contained nitromethane (**1**, 25 mM), an *α,β*-unsaturated aldehyde (**2a-k**, 3 mM, except for **2g** which was used at 2 mM) and 4-OT F50A enzyme (72 µM, except for **2g** and **2i** for which 36 µM enzyme was used) in HEPES buffer (20 mM, pH 6.5). Stock solutions of **2a-k** were prepared in absolute ethanol. The reaction mixture was incubated at room temperature and the progress of the reaction was monitored by UV-VIS spectrophotometry (**2a-j**) or GC (**2k**). To monitor the progress of the enzyme catalyzed reactions of **2a-j** with **1**, aliquots (80 µL) were taken from the reaction mixture, diluted with 160 µL of 20 mM HEPES buffer (pH 6.5), and analyzed by UV-VIS spectrophotometry. To monitor the progress of the enzyme-catalyzed reaction of **2k** with **1**, aliquots (100 µL) were taken from the reaction mixture, extracted with toluene (100 µL), and analyzed by GC. After the completion of the reactions, each reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under *vacuo*. The products **3a-e** and **3h** were obtained in high purity and did not require further column purification. The crude products of **3f**, **3g**, and **3i-k** were further purified by silica gel column chromatography (petroleum ether/ethyl acetate from 95:5 to 50:50).

(*R*)-4-nitro-3-phenylbutanal (**3a**). Yellow oil; yield = 92% (10.7 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.71 (s, 1H), 7.37 – 7.33 (m, 2H), 7.31 – 7.27 (m, 1H), 7.25 – 7.22 (m, 2H), 4.68 (dd, *J* = 12.5, 7.2 Hz, 1H), 4.62 (dd, *J* = 12.5, 7.5 Hz, 1H), 4.08 (p, *J* = 7.3 Hz, 1H), 3.01 – 2.91 (m, 2H). The enantiomeric ratio (e.r.) of the enzymatic product **3a** was determined by chiral GC (Astec® CHIRALDEX G-TA column; 30 m × 0.25 mm × 0.12 µm) using the following conditions: 10 °C/min from 70 °C to 170 °C, followed by 20 min at 170 °C; flame ionization detection: t_R (major) = 23.9 min, (minor) = 24.3 min. The absolute configuration of enzymatically prepared **3a** was assigned by comparing to previously reported data.[3]

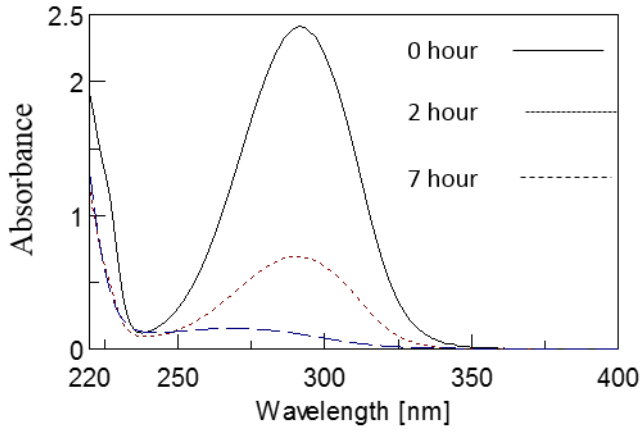


Figure S2. UV spectra monitoring the Michael addition of **1** to **2a** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

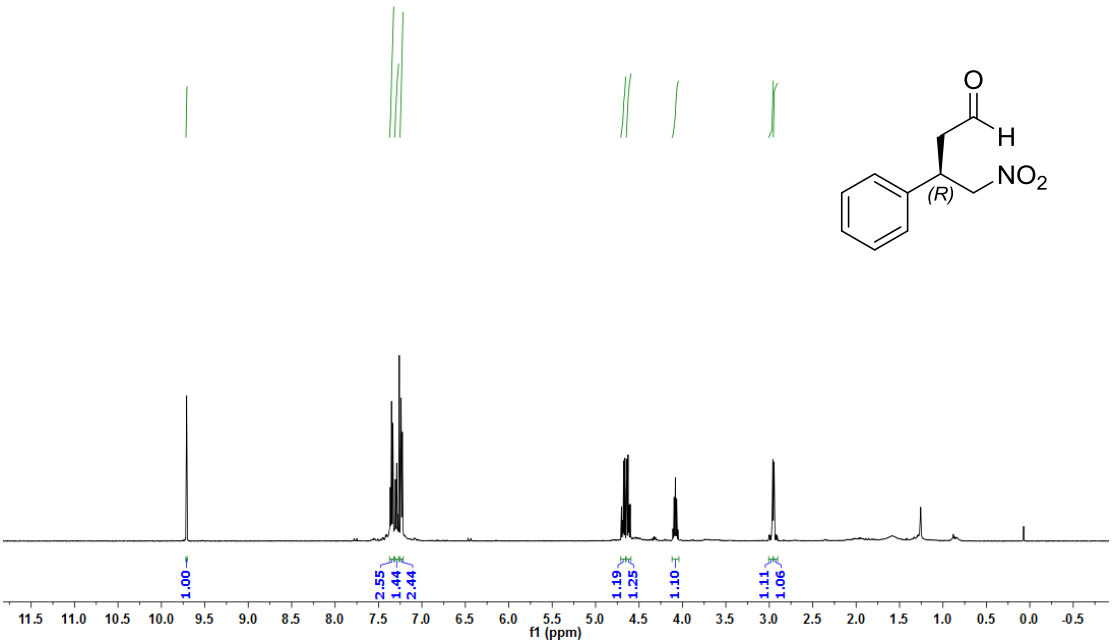


Figure S3. ^{1}H NMR spectrum of enzymatic product **3a**.

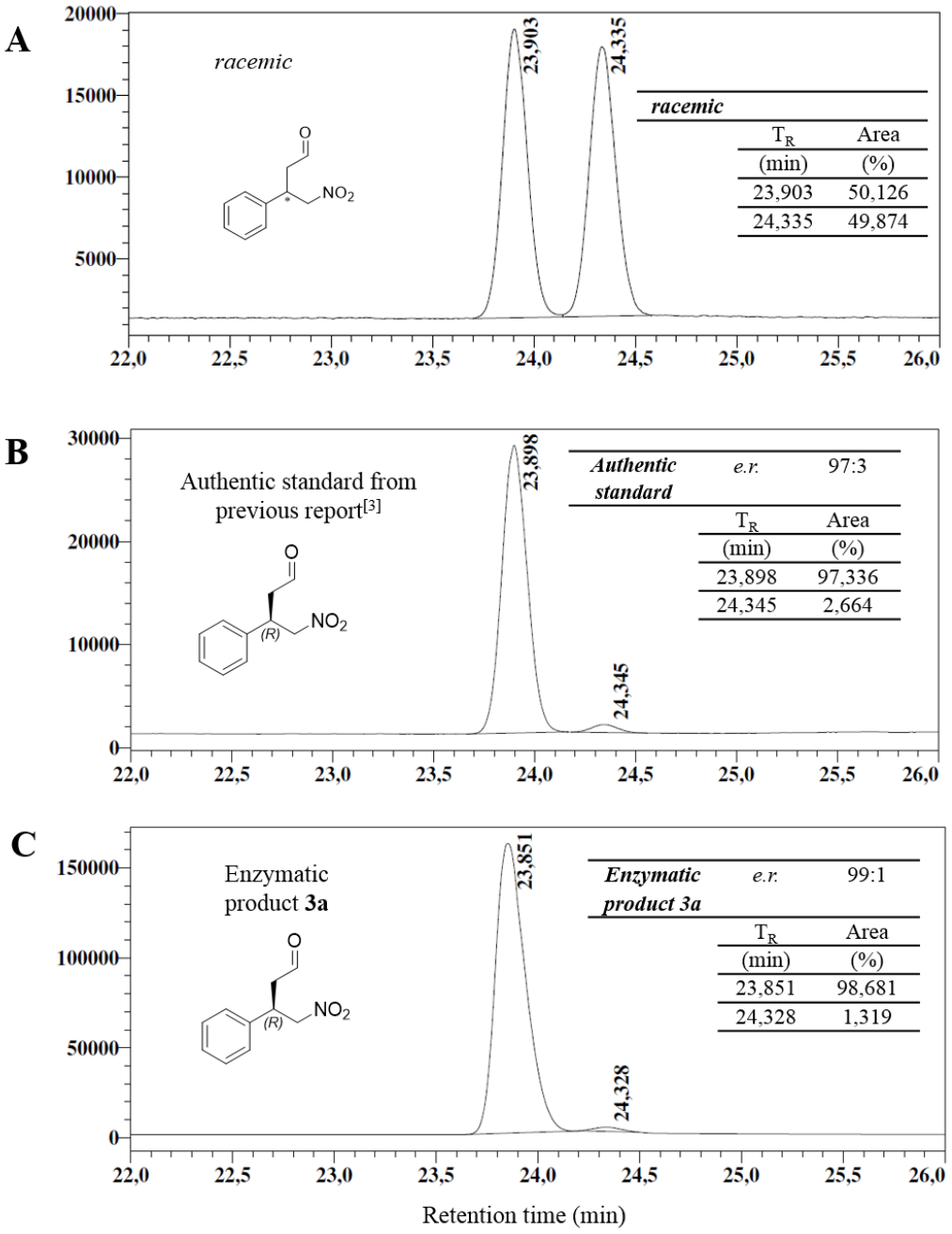


Figure S4. Chiral GC analysis of enzymatic product **3a**.

(*S*)-3-(2-chlorophenyl)-4-nitrobutanal (**3b**). Colorless oil; yield = 90% (12.2 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.74 (t, *J* = 1.1 Hz, 1H), 7.43 – 7.41 (m, 1H), 7.28 – 7.21 (m, 3H), 4.78 (dd, *J* = 12.8, 6.8 Hz, 1H), 4.73 (dd, *J* = 12.8, 6.8 Hz, 1H), 4.55 (p, *J* = 6.9 Hz, 1H), 3.12 – 3.00 (m, 2H). The enantiomeric ratio of the enzymatic product **3b** was determined (after converting the aldehyde functionality into the corresponding alcohol) using reverse phase HPLC on a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 5:95, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R (minor) = 125.9 min, (major) = 134.9 min. The assignment of the absolute configuration of enzymatically prepared **3b** was based on earlier reported chiral HPLC-data.[2a]

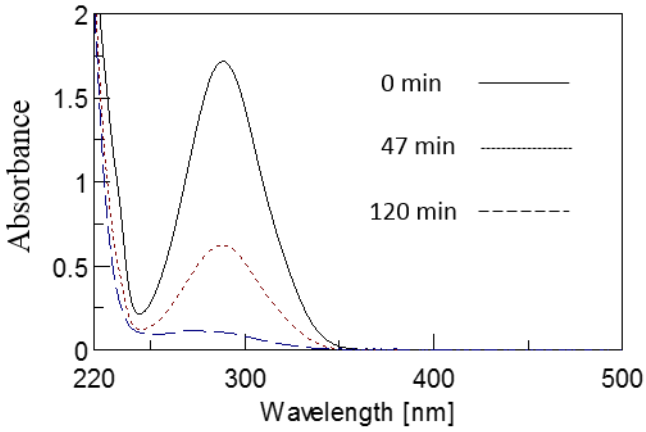


Figure S5. UV spectra monitoring the Michael addition of **1** to **2b** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

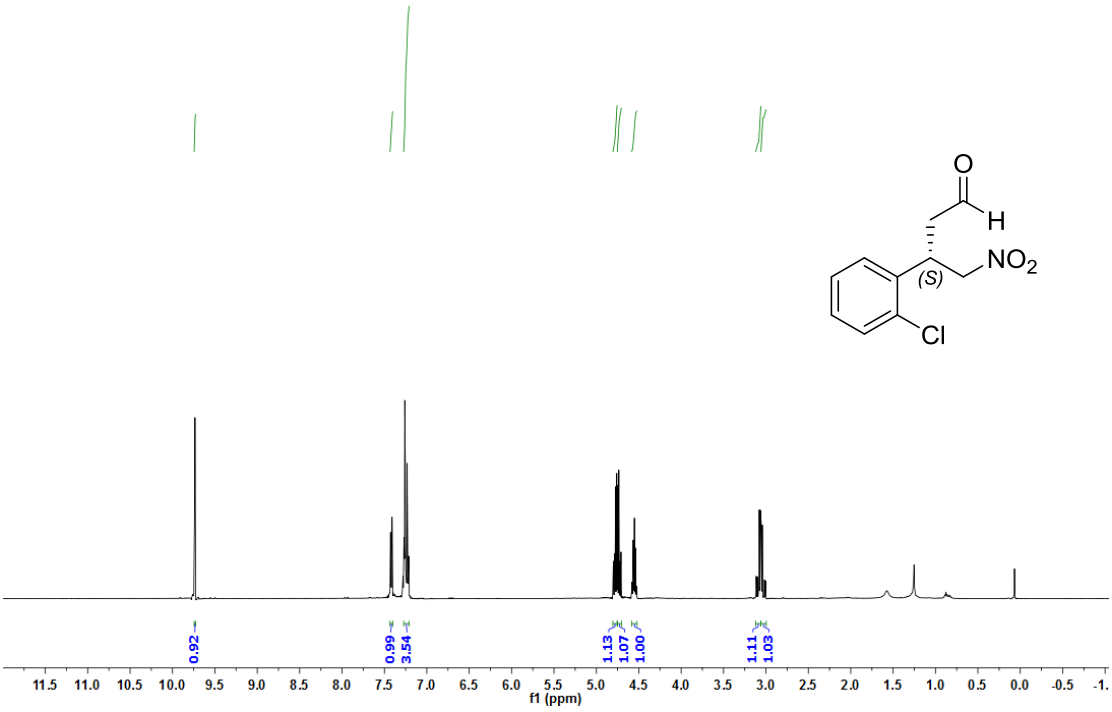


Figure S6. ^{1}H NMR spectrum of enzymatic product **3b**.

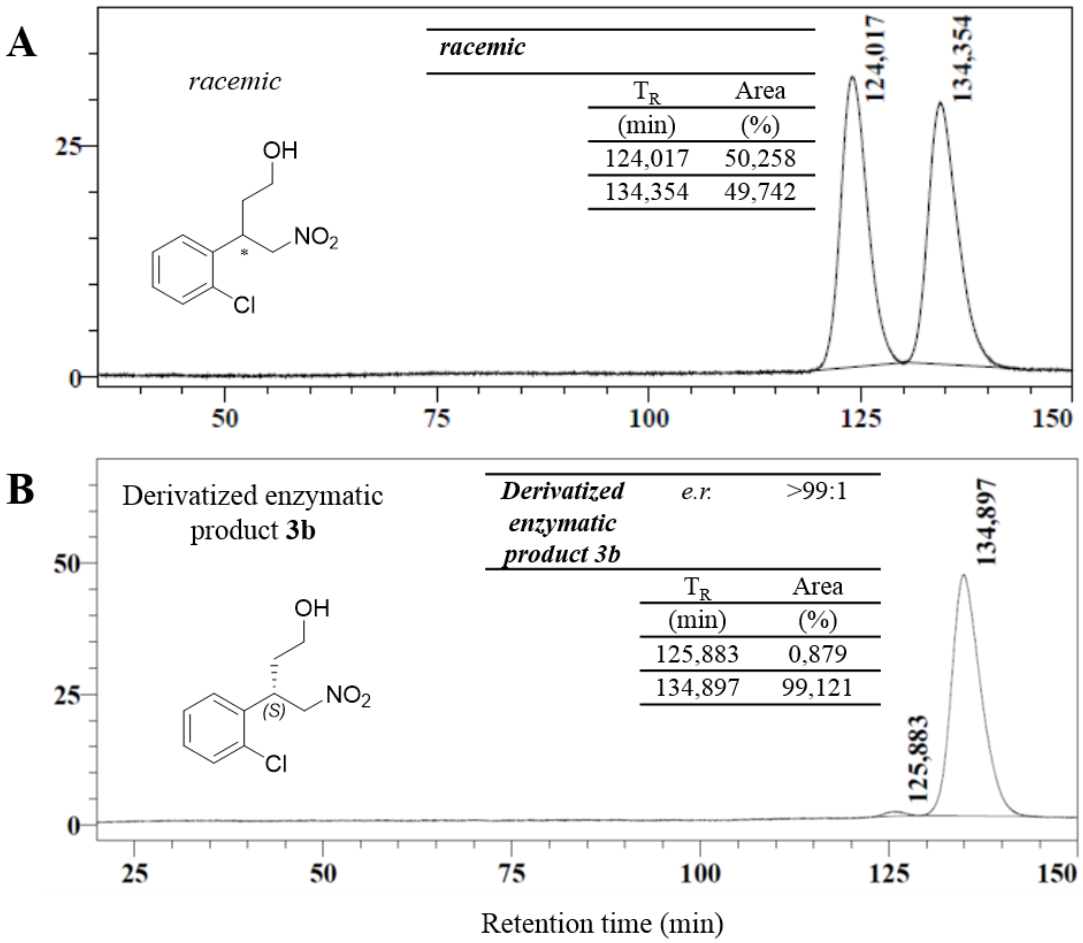


Figure S7. Chiral HPLC analysis of derivatized enzymatic product **3b**.

(*R*)-3-(3-chlorophenyl)-4-nitrobutanal (**3c**). Colorless oil; yield = 93% (12.7 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.71 (s, 1H), 7.29 – 7.27 (m, 2H), 7.23 – 7.22 (m, 1H), 7.14 – 7.12 (m, 1H), 4.68 (dd, *J* = 12.7, 6.9 Hz, 1H), 4.60 (dd, *J* = 12.7, 7.8 Hz, 1H), 4.06 (p, *J* = 7.1 Hz, 1H), 2.96 (dt, *J* = 7.0, 1.1 Hz, 2H). The enantiomeric ratio of the enzymatic product **3c** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 15:85, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R (minor) = 8.5 min, (major) = 9.5 min. The assignment of the absolute configuration of enzymatically prepared **3c** was based on earlier reported chiral HPLC data.[2a]

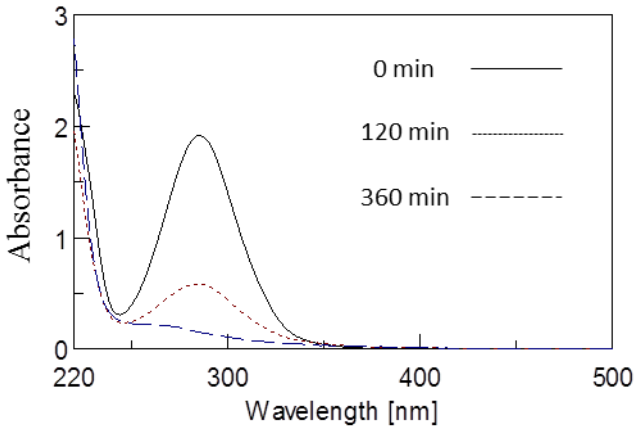


Figure S8. UV spectra monitoring the Michael addition of **1** to **2c** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

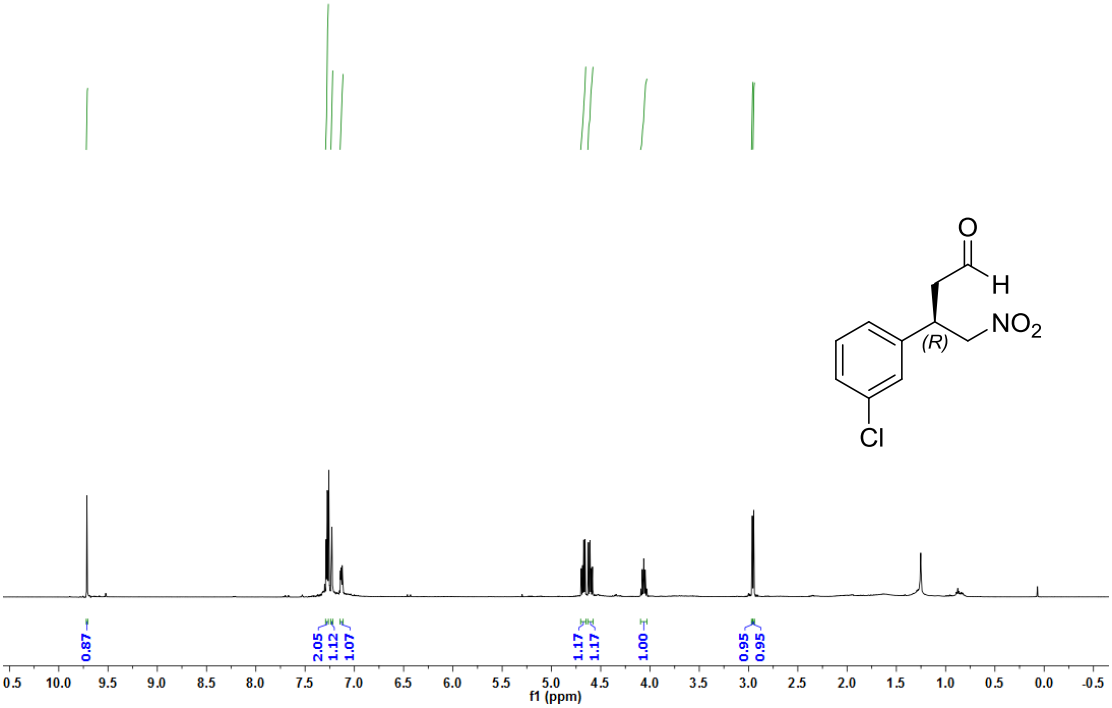


Figure S9. ^{1}H NMR spectrum of enzymatic product **3c**.

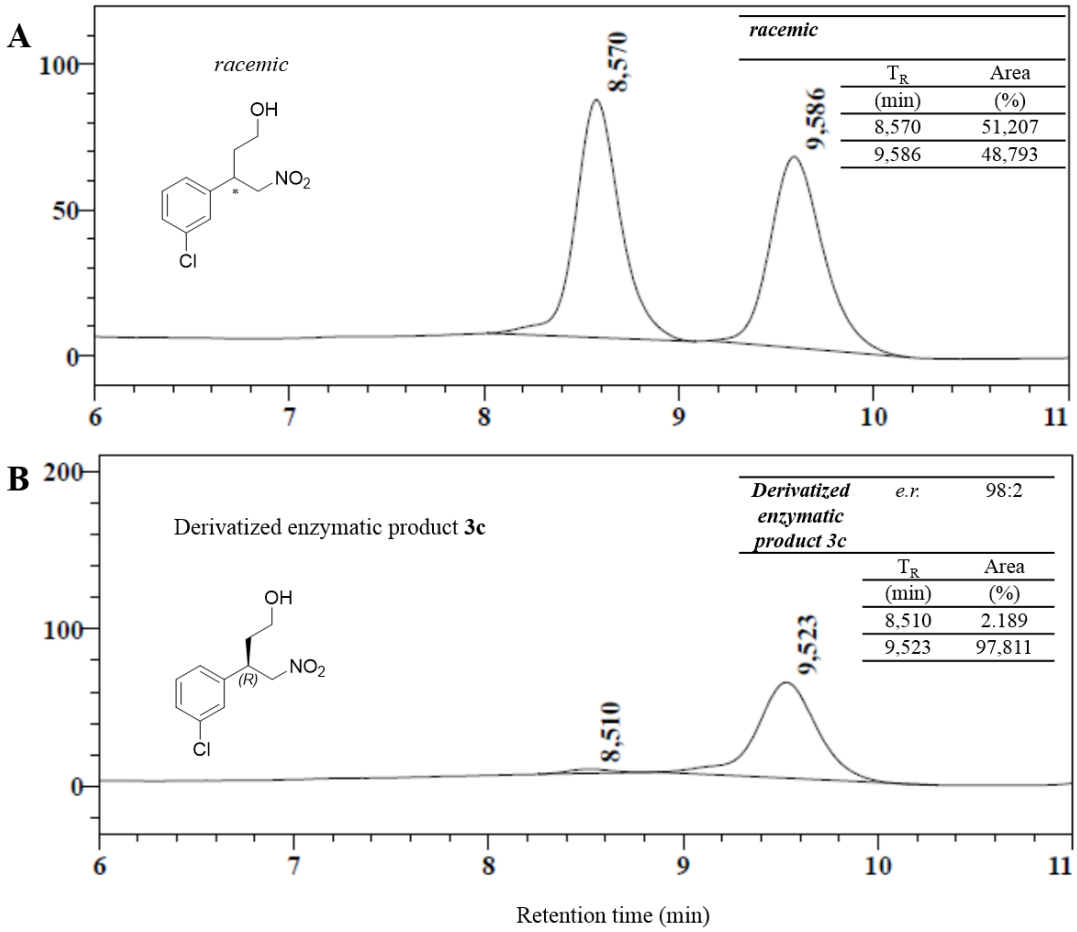


Figure S10. HPLC analysis of derivatized enzymatic product **3c**.

(*R*)-3-(4-chlorophenyl)-4-nitrobutanal (**3d**). Light yellow oil; yield = 93% (12.7 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.71 (s, 1H), 7.34 – 7.31 (m, 2H), 7.19 – 7.16 (m, 2H), 4.67 (dd, *J* = 12.6, 6.9 Hz, 1H), 4.59 (dd, *J* = 12.6, 7.9 Hz, 1H), 4.07 (p, *J* = 7.1 Hz, 1H), 2.95 (dd, *J* = 7.0, 0.9 Hz, 2H). The enantiomeric ratio of the enzymatic product **3d** was determined by reverse phase HPLC using a Chiralpak® AD-RH column (150 mm × 4.6 mm, Daicel) (MeCN/water = 32:68, 25℃) at a flow rate of 0.5 mL/min. UV detection at 220 nm: t_R (major) = 27.6 min, (minor) = 29.6 min. The assignment of the absolute configuration of enzymatically prepared **3d** was based on earlier reported chiral HPLC data.[3]

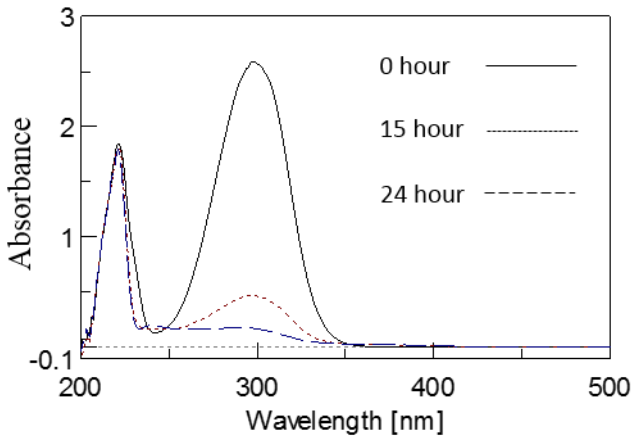


Figure S11. UV spectra monitoring the Michael addition of **1** to **2d** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

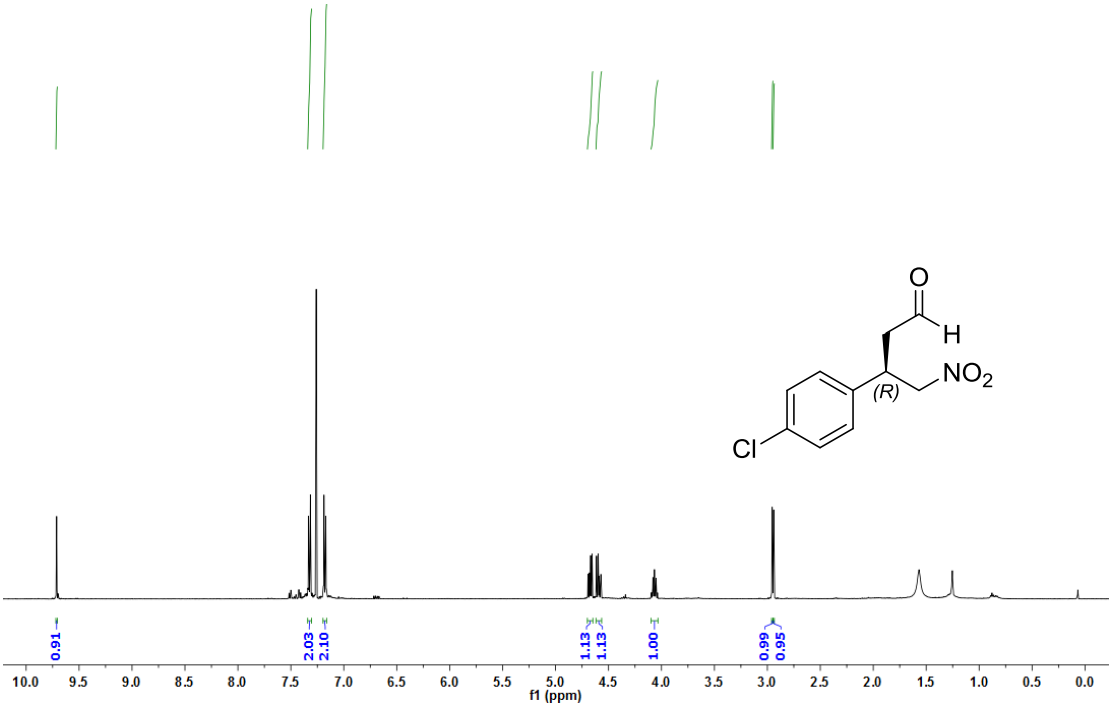


Figure S12. ^{1}H NMR spectrum of enzymatic product **3d**.

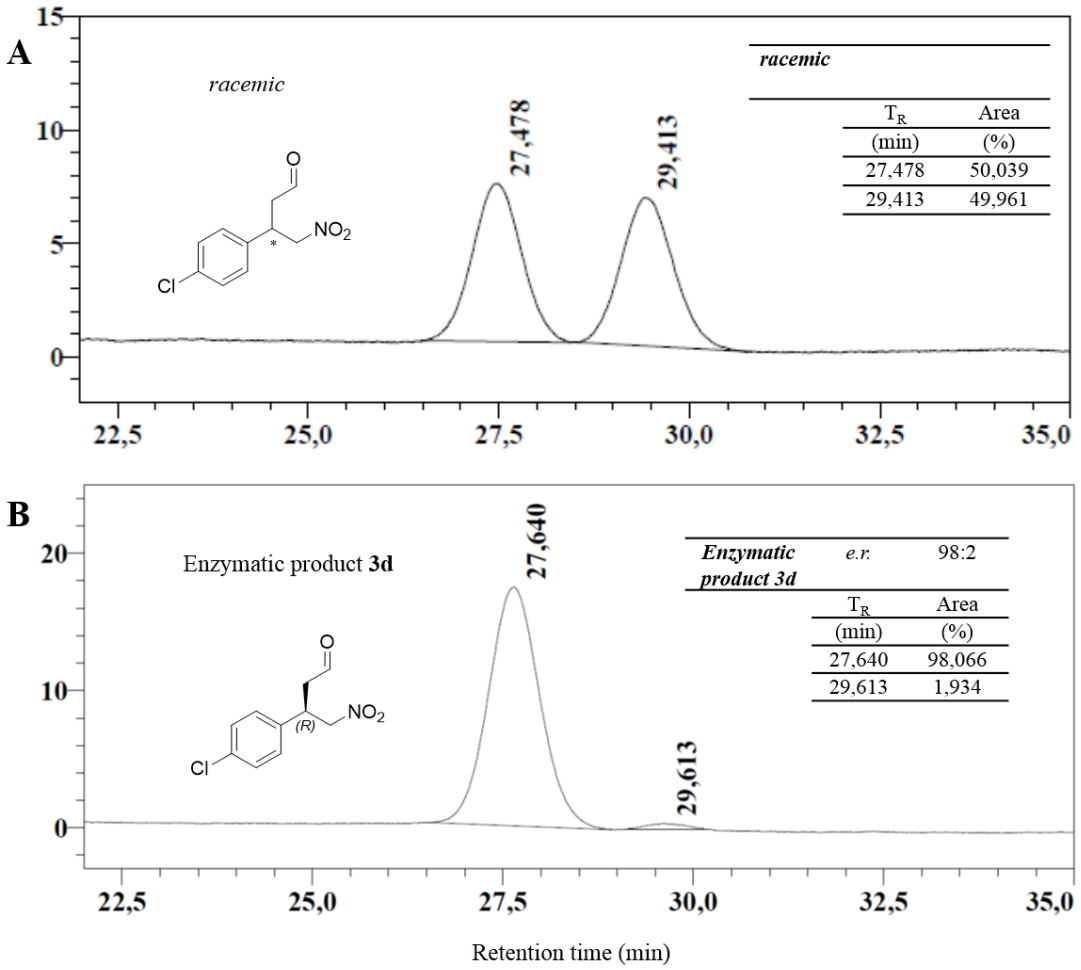


Figure S13. HPLC analysis of the enzymatic product **3d**.

(*S*)-3-(2-methoxyphenyl)-4-nitrobutanal (**3e**). Light yellow oil; yield = 96% (12.8 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.70 (s, 1H), 7.29 – 7.25 (m, 1H), 7.15 (dd, *J* = 7.6, 1.7 Hz, 1H), 6.94 – 6.88 (m, 2H), 4.76 (dd, *J* = 12.5, 7.0 Hz, 1H), 4.71 (dd, *J* = 12.5, 7.2 Hz, 1H), 4.29 (p, *J* = 7.1 Hz, 1H), 3.86 (s, 3H), 3.05 – 2.95 (m, 2H). The enantiomeric ratio of enzymatic product **3e** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® AD-RH column (150 mm × 4.6 mm, Daicel) (MeCN/water = 7.5:92.5, 25℃) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R (minor) = 14.8 min, (major) = 16.2 min. The assignment of the absolute configuration of enzymatically prepared **3e** was based on earlier reported chiral HPLC data.[2a]

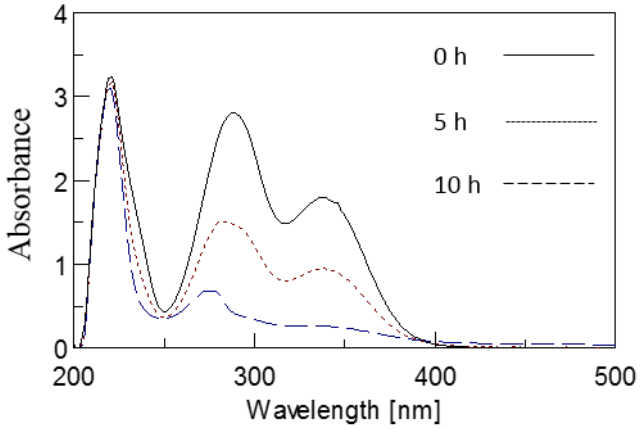


Figure S14. UV spectra monitoring the Michael addition of **1** to **2e** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

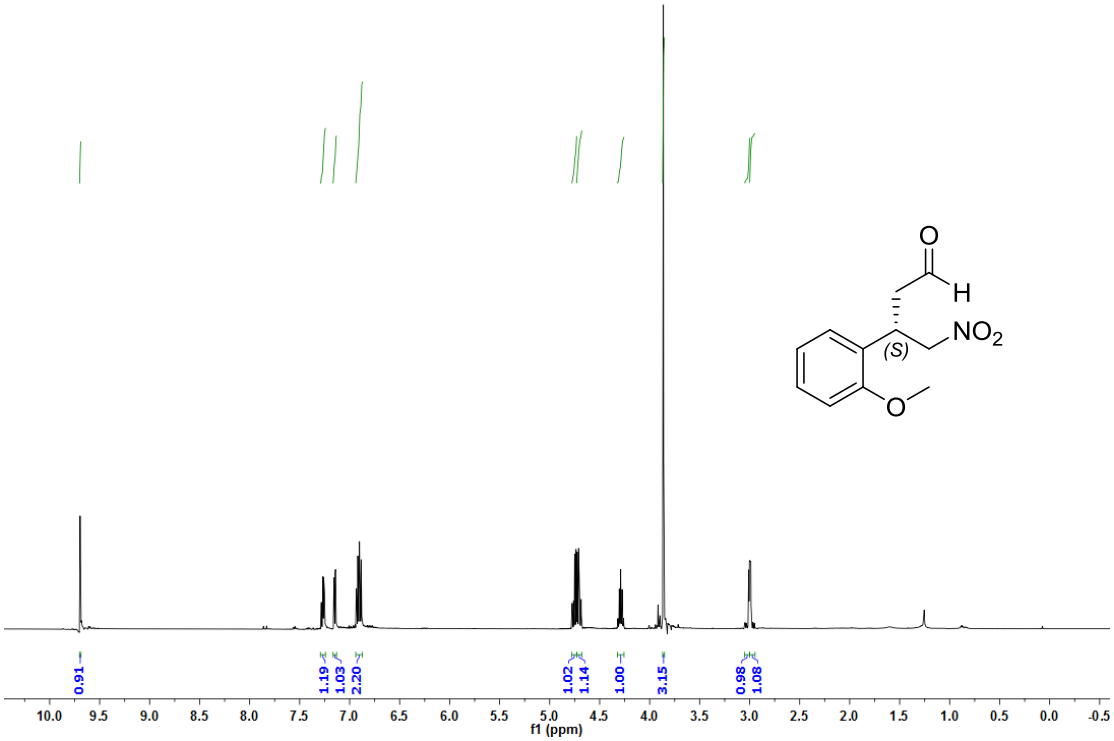


Figure S15. ^{1}H NMR spectrum of enzymatic product **3e**.

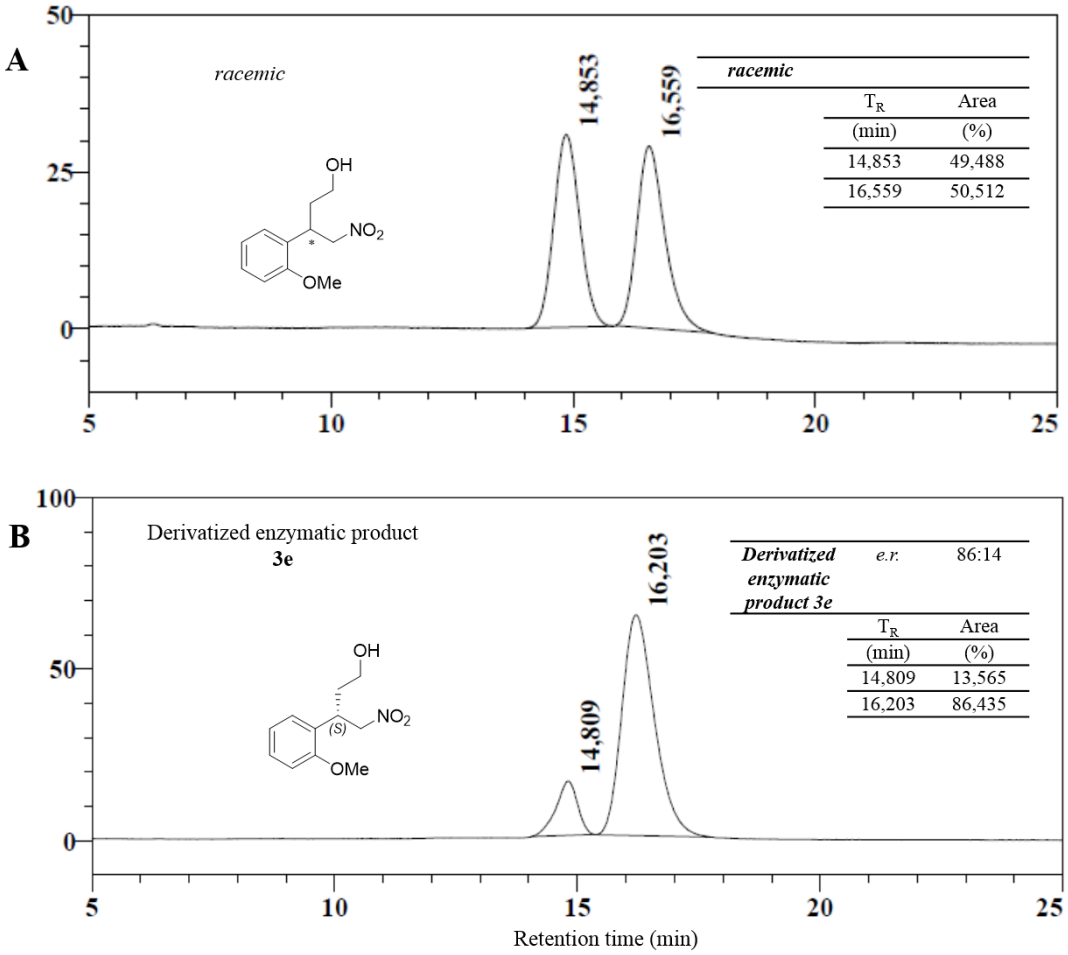


Figure S16. HPLC analysis spectra of derivatized enzymatic product **3e**.

(*R*)-3-(4-methoxyphenyl)-4-nitrobutanal (**3f**). Light yellow oil; yield = 80% (10.7 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.69 (t, *J* = 1.2 Hz, 1H), 7.15 – 7.14 (m, 2H), 6.87 – 6.86 (m, 2H), 4.64 (dd, *J* = 12.4, 7.2 Hz, 1H), 4.57 (dd, *J* = 12.4, 7.7 Hz, 1H), 4.03 (p, *J* = 7.3 Hz, 1H), 3.78 (s, 3H), 2.96 – 2.86 (m, 2H). The enantiomeric ratio of the enzymatic product **3f** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® AD-RH column (150 mm × 4.6 mm, Daicel) (MeCN/water = 15:85, 25℃) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R (major) = 27.3 min, (minor) = 36.3 min. The assignment of the absolute configuration of enzymatically prepared **3f** was based on earlier reported chiral HPLC data.[2a]

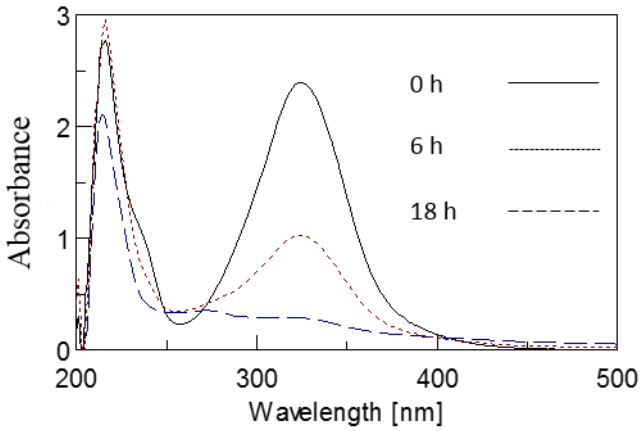


Figure S17. UV spectra monitoring the Michael addition of **1** to **2f** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

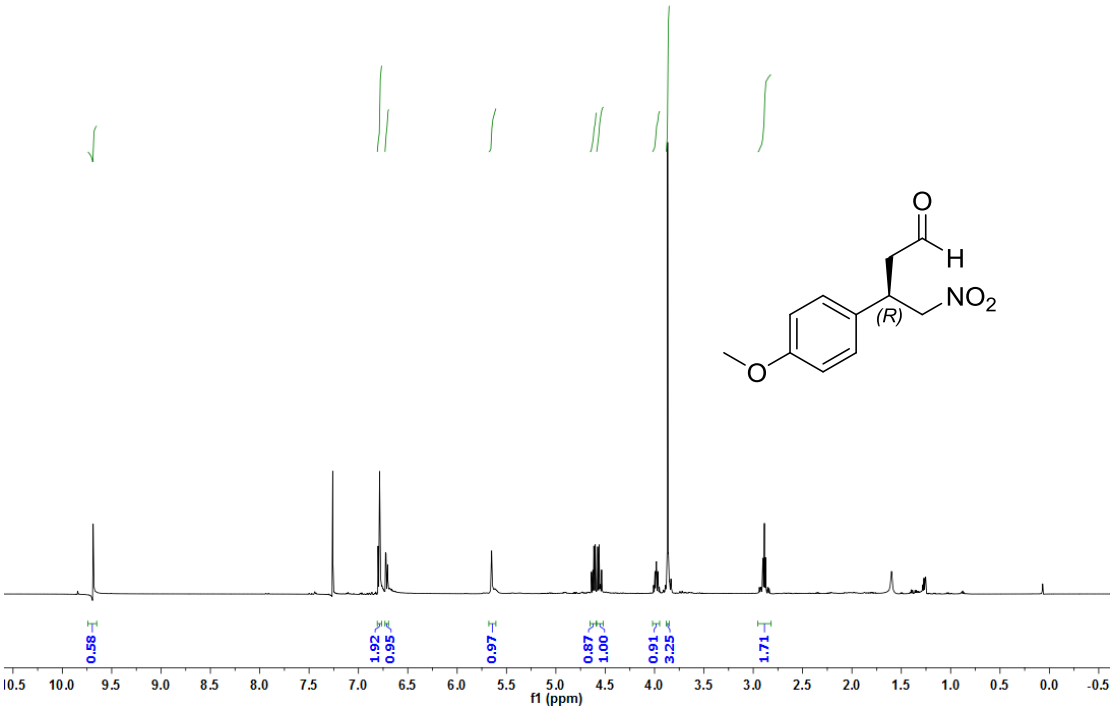


Figure S18. ^{1}H NMR spectrum of enzymatic product **3f**.

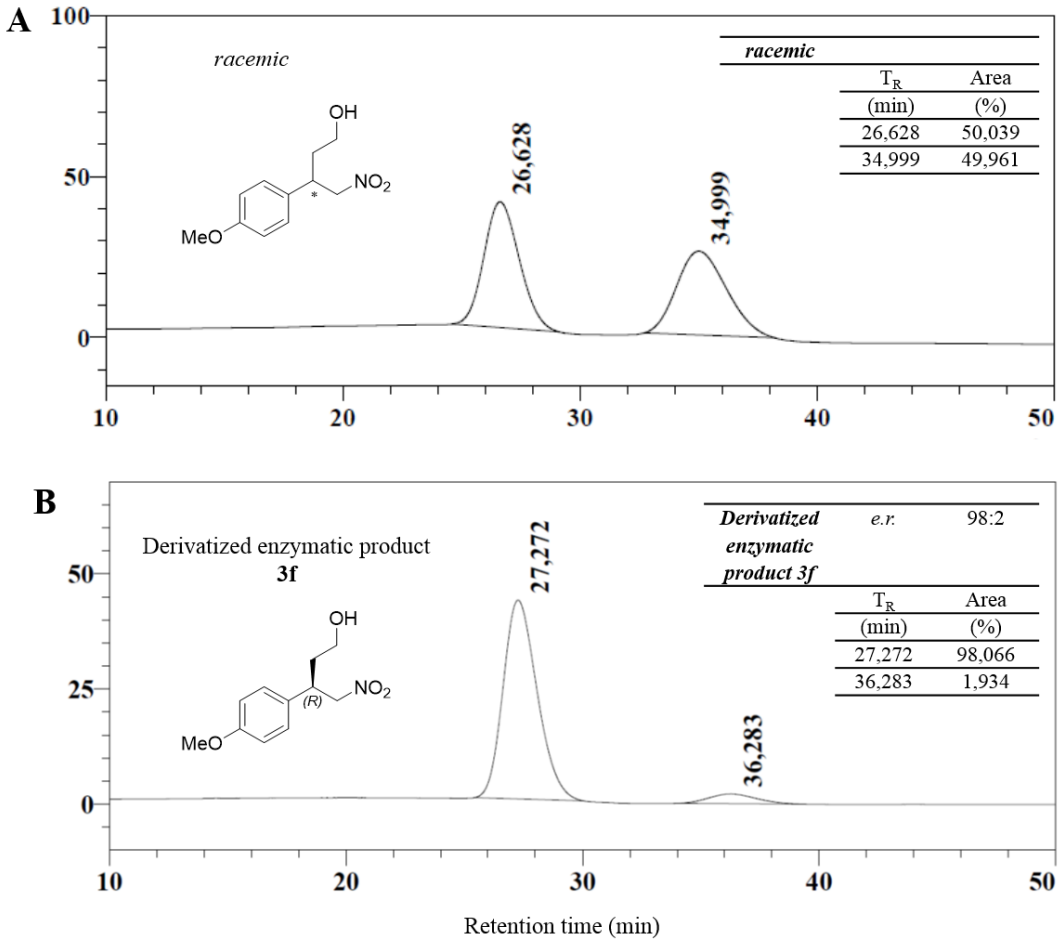


Figure S19. HPLC analysis spectra of derivatized enzymatic product **3f**.

(*R*)-4-nitro-3-(4-nitrophenyl)butanal (**3g**). Light yellow oil; yield = 75% (7.1 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.74 (s, 1H), 8.22 (d, *J* = 8.7 Hz, 2H), 7.45 (d, *J* = 8.7 Hz, 2H), 4.75 (dd, *J* = 12.9, 6.4 Hz, 1H), 4.67 (dd, *J* = 12.9, 8.2 Hz, 1H), 4.24 – 4.19 (m, 1H), 3.04 – 3.02 (m, 2H). The enantiomeric ratio of enzymatic product **3g** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 15:85, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R (major) = 17.4 min, (minor) = 20.1 min. The assignment of the absolute configuration of enzymatically prepared **3g** was based on earlier reported chiral HPLC data.[2a]

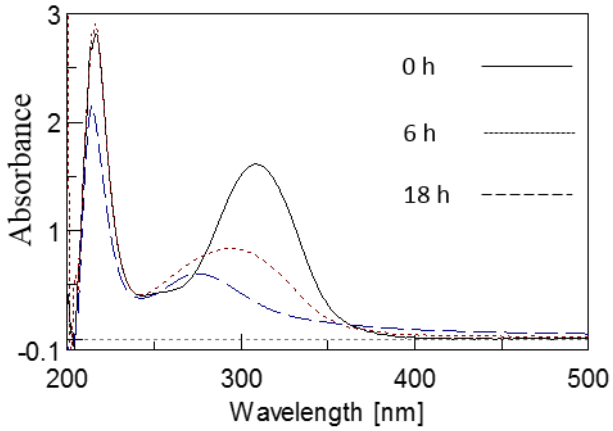


Figure S20. UV spectra monitoring the Michael addition of **1** to **2g** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

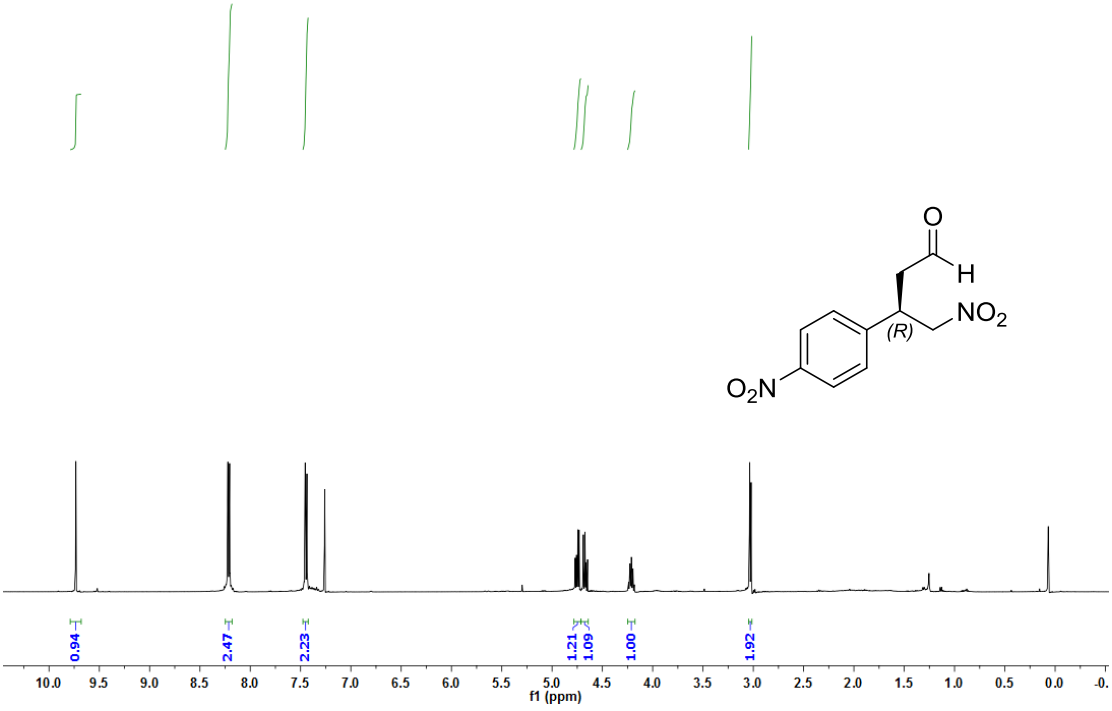


Figure S21. ^{1}H NMR spectrum of enzymatic product **3g**.

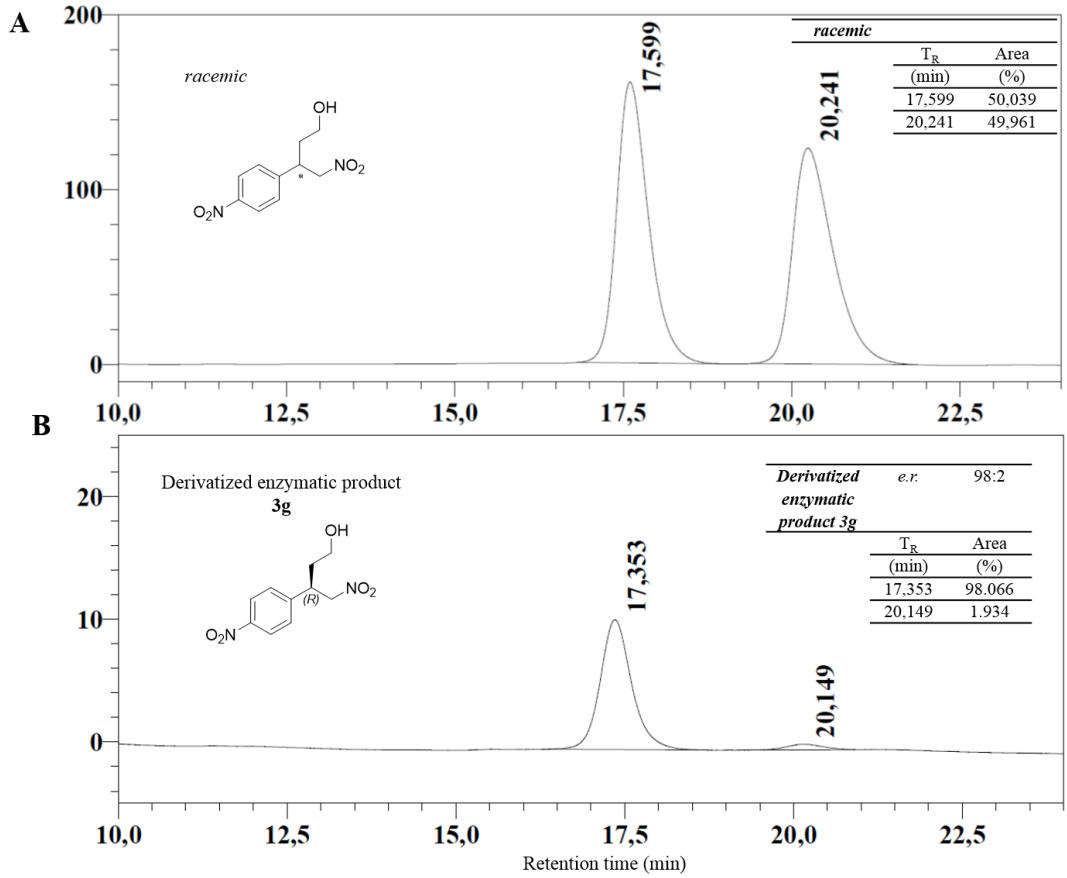


Figure S22. HPLC analysis of derivatized enzymatic product **3g**.

(*R*)-3-(4-hydroxyphenyl)-4-nitrobutanal (**3h**). Light yellow oil; yield = 71% (8.9 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.70 (s, 1H), 7.12 – 7.09 (m, 2H), 6.82 – 6.78 (m, 2H), 4.84 (s, 1H), 4.64 (dd, *J* = 12.4, 7.1 Hz, 1H), 4.57 (dd, *J* = 12.4, 7.7 Hz, 1H), 4.02 (p, *J* = 7.3 Hz, 1H), 2.97 – 2.87 (m, 2H). The enantiomeric ratio of the enzymatic product **3h** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding hemi acetal) using a Chiralpak® AD-RH column (150 mm × 4.6 mm, Daicel) (MeCN/water = 30:70, 25℃) at a flow rate of 0.5 mL/min. UV detection at 210 nm: t_R (major) = 16.8 min, (minor) = 30.6 min. The assignment of the absolute configuration of enzymatically prepared **3h** was based on earlier reported chiral HPLC data.[3]

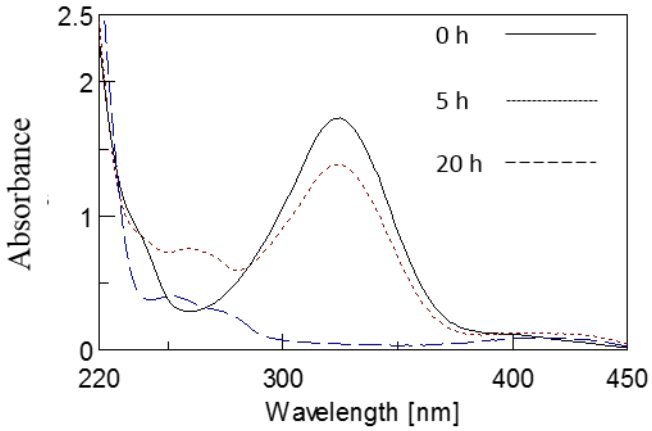


Figure S23. UV spectra monitoring the Michael addition of **1** to **2h** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

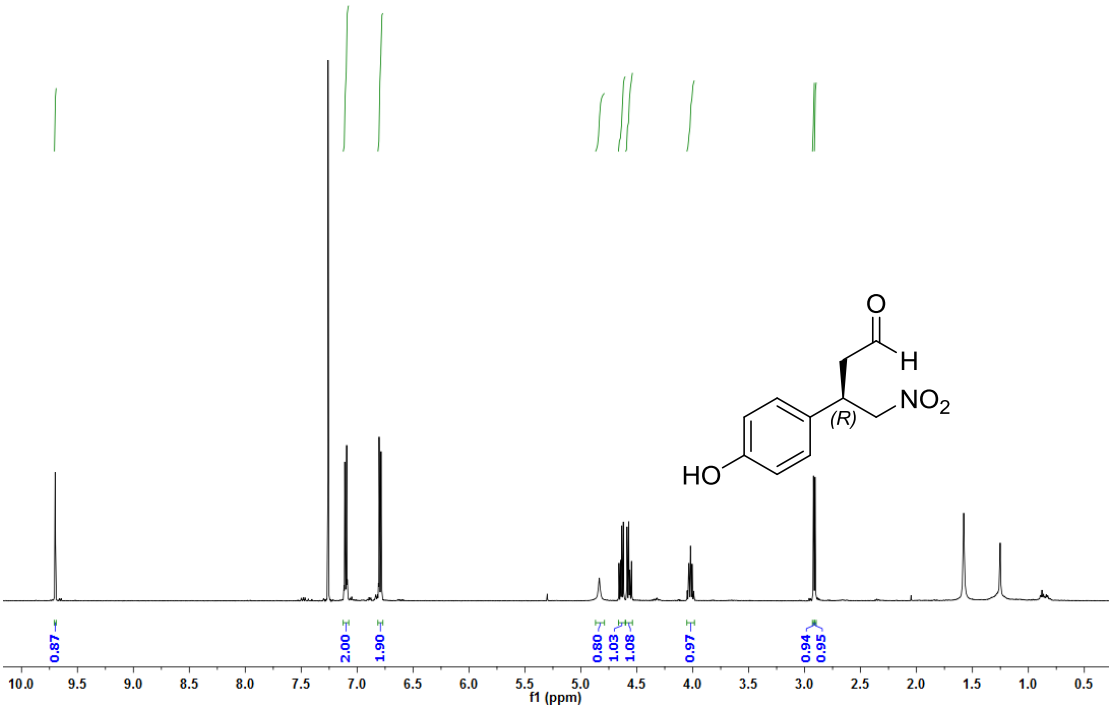


Figure S24. ^{1}H NMR spectrum of enzymatic product **3h**.

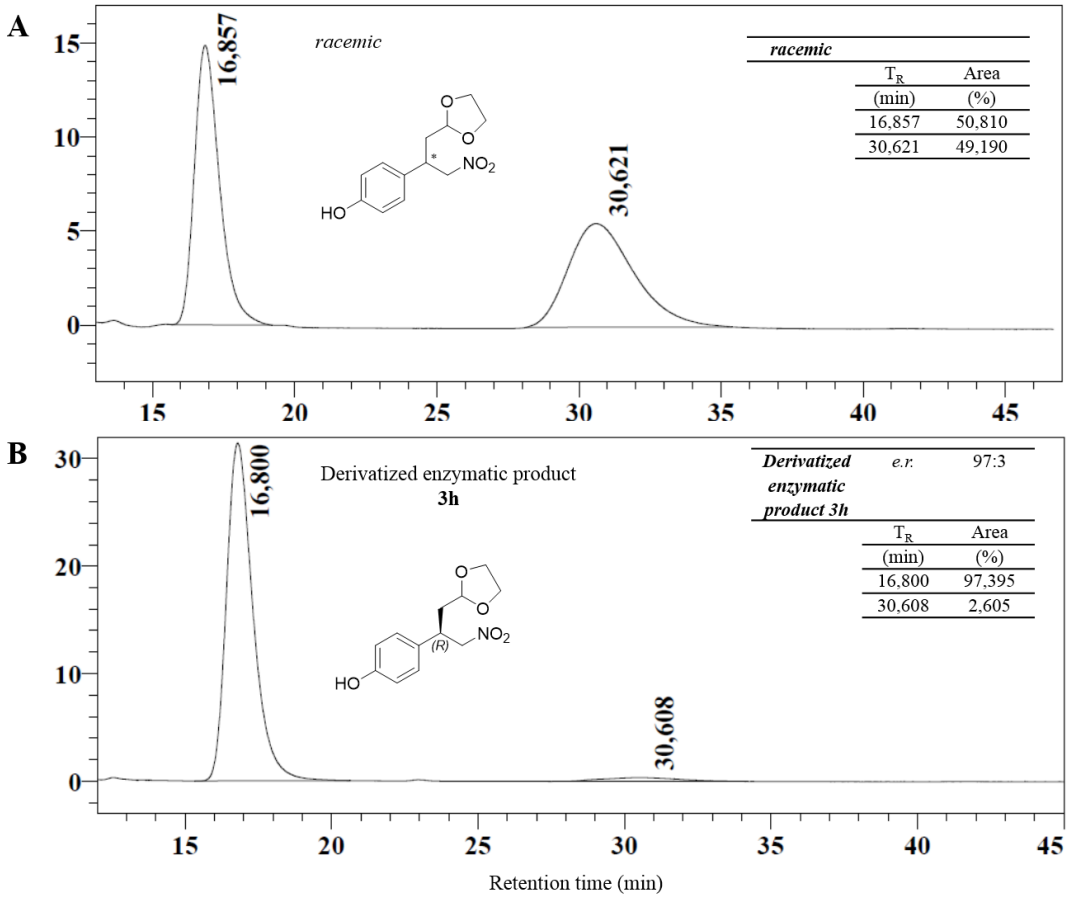


Figure S25. HPLC analysis of derivatized enzymatic product **3h**.

(*R*)-3-(4-fluorophenyl)-4-nitrobutanal (**3i**). Colorless oil; yield = 89% (11.3 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.70 (s, 1H), 7.23 – 7.20 (m, 2H), 7.05 – 7.02 (m, 2H), 4.67 (dd, *J* = 12.6, 6.9 Hz, 1H), 4.59 (dd, *J* = 12.5, 7.9 Hz, 1H), 4.07 (p, *J* = 7.2 Hz, 1H), 2.94 (dd, *J* = 7.1, 1.0 Hz, 2H). The enantiomeric ratio of the enzymatic product **3i** was determined by reverse phase HPLC using a Chiralpak® AD-RH column (150 mm × 4.6 mm, Daicel) (MeCN/water = 15:85, 25℃) at a flow rate of 0.5 mL/min. UV detection at 210 nm: t_R (major) = 48.6 min, (minor) = 51.7 min. The assignment of the absolute configuration of enzymatically prepared **3i** was based on earlier reported chiral HPLC data.[3]

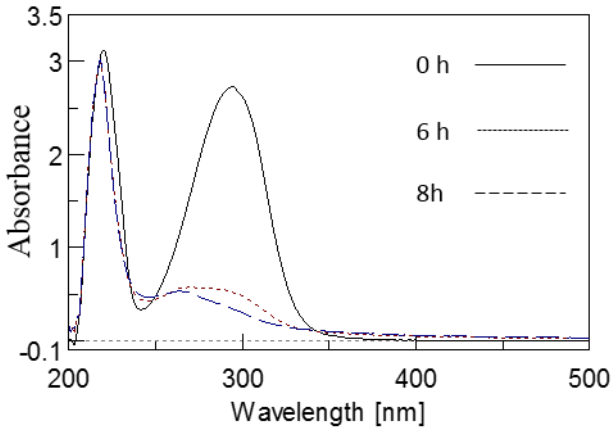


Figure S26. UV spectra monitoring the Michael addition of **1** to **2i** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

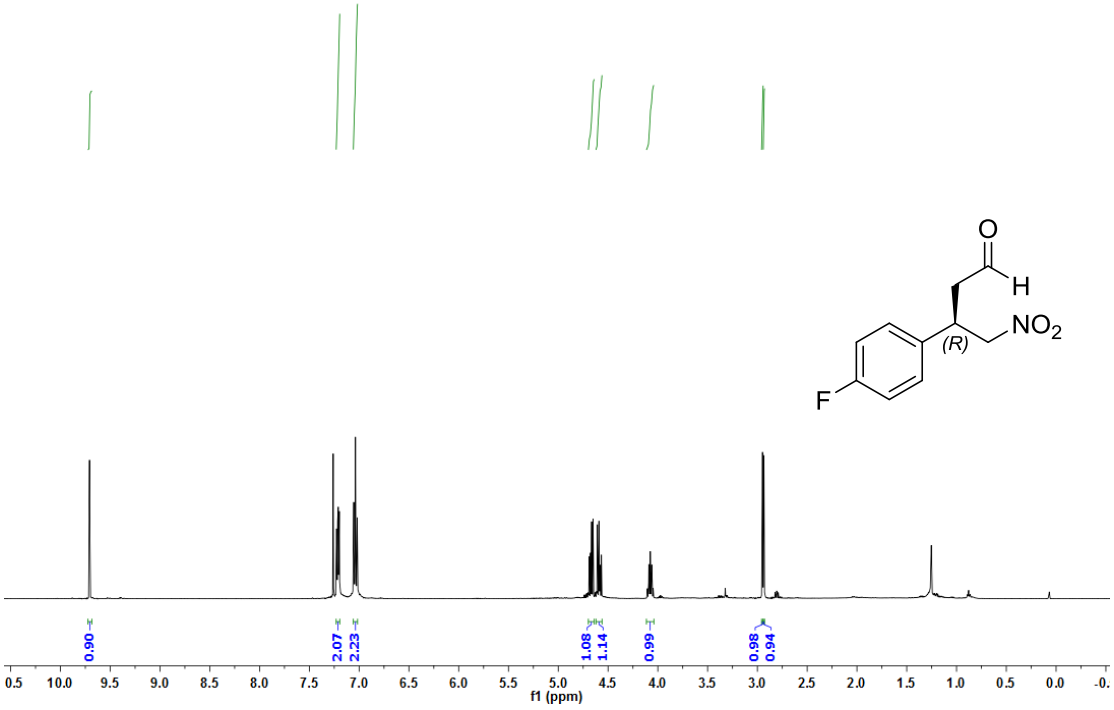


Figure S27. ^{1}H NMR spectrum of enzymatic product **3i**.

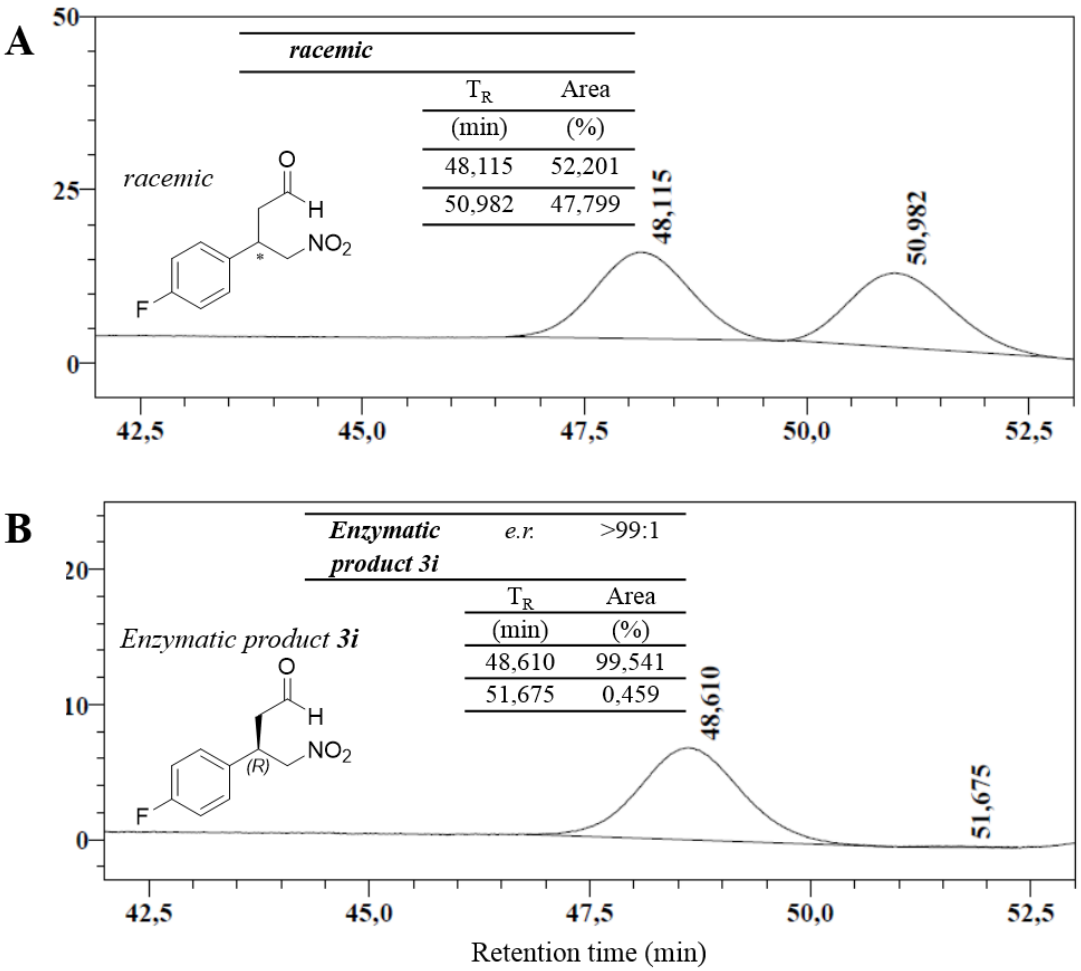


Figure S28. HPLC analysis of enzymatic product **3i**.

(*R*)-3-(3-hydroxy-4-methoxyphenyl)-4-nitrobutanal (**3j**). Colorless oil; yield = 73% (10.5 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.69 (t, *J* = 1.2 Hz, 1H), 6.81 – 6.78 (m, 2H), 6.72 (dd, *J* = 8.2, 2.3 Hz, 1H), 5.63 (s, 1H), 4.62 (dd, *J* = 12.4, 7.2 Hz, 1H), 4.56 (dd, *J* = 12.4, 7.6 Hz, 1H), 3.99 (p, *J* = 7.3 Hz, 1H), 3.87 (s, 3H), 2.94 – 2.84 (m, 2H). The enantiomeric ratio of the enzymatic product **3j** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding cyclic acetal) using a Chiralpak® AD-RH column (150 mm × 4.6 mm, Daicel) (MeCN/water = 30:70, 25°C) at a flow rate of 0.5 mL/min. UV detection at 210 nm: t_R (major) = 21.2 min, (minor) = 30.2 min. The assignment of the absolute configuration of enzymatically prepared **3j** was based on earlier reported chiral HPLC data.[3]

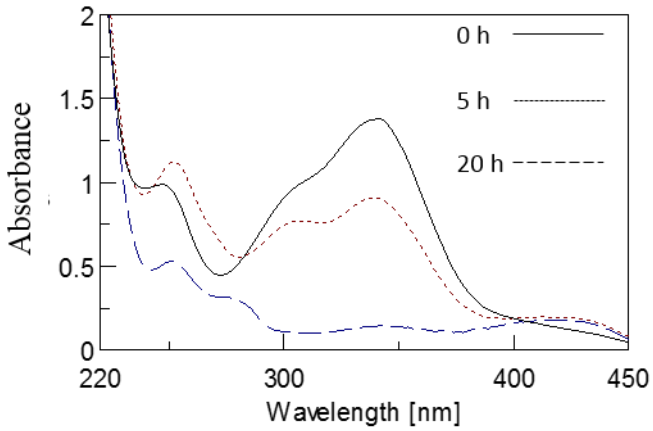


Figure S29. UV spectra monitoring the Michael addition of **1** to **2j** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol)] at pH 6.5.

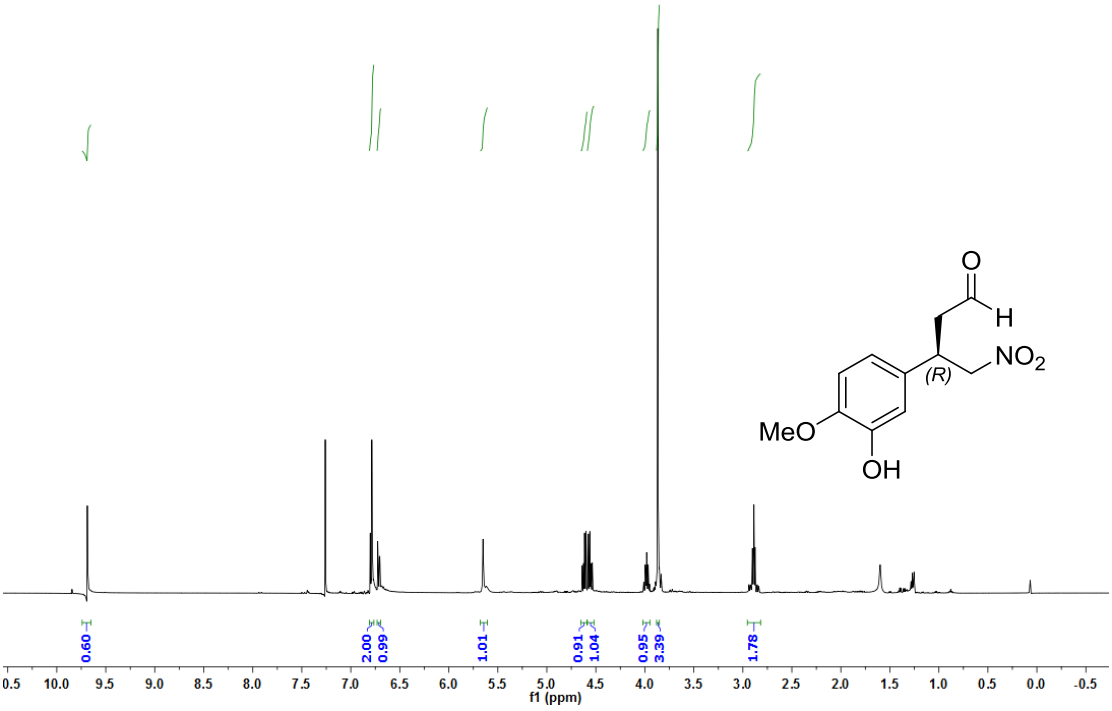


Figure S30. ^{1}H NMR spectrum of enzymatic product **3j**.

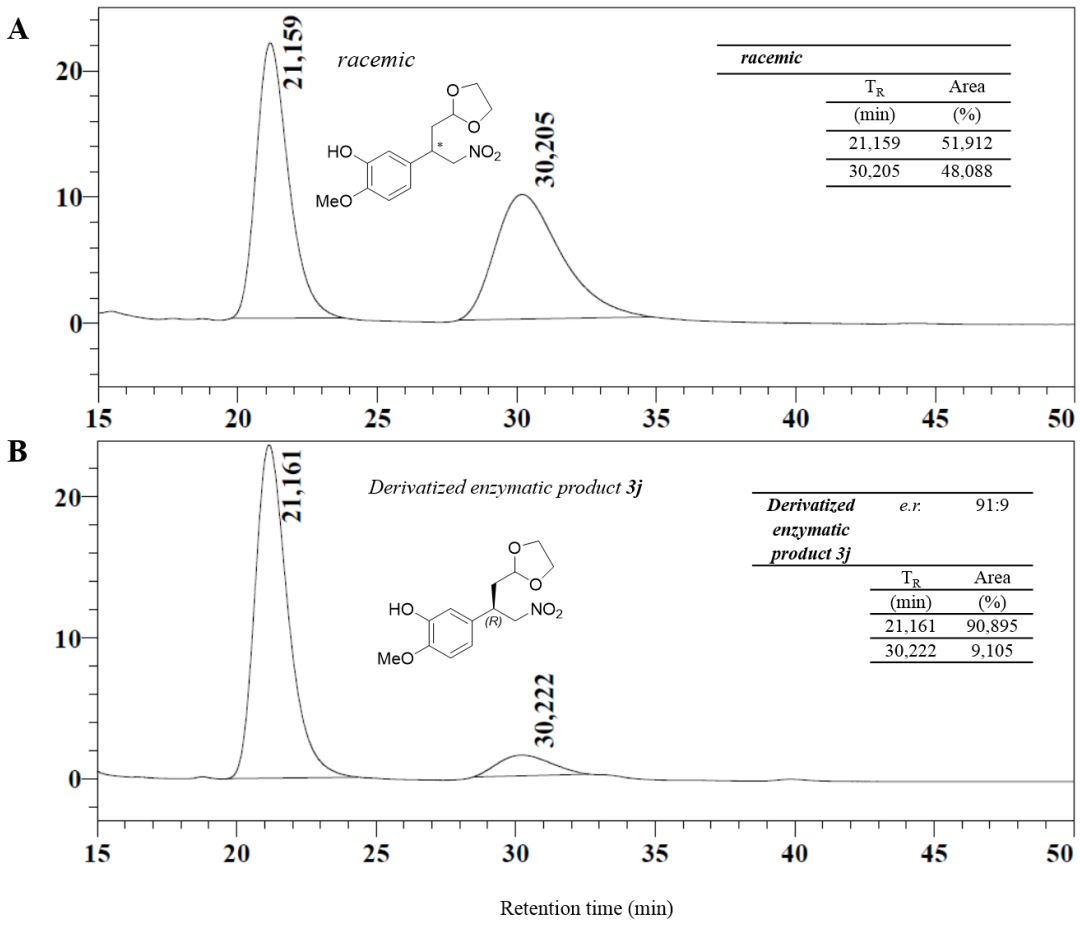


Figure S31. HPLC analysis of derivatized enzymatic product **3j**.

(*S*)-5-methyl-3-(nitromethyl)hexanal (**3k**). Colorless oil; yield = 61% (6.3 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.78 (s, 1H), 4.50 – 4.39 (m, 2H), 2.83 – 2.71 (m, 1H), 2.67 (ddd, *J* = 18.4, 6.9, 0.7 Hz, 1H), 2.57 (ddd, *J* = 18.5, 5.6, 0.9 Hz, 1H), 1.69 – 1.57 (m, 1H), 1.28 (dt, *J* = 7.1, 3.5 Hz, 2H), 0.94 (td, *J* = 15.1, 6.8 Hz, 6H). The enantiomeric ratio of enzymatic product **3k** was determined by chiral GC (Astec® CHIRALDEX G-TA column; 30 m × 0.25 mm × 0.12 µm) under the following conditions: 10 °C/min from 70 °C to 170 °C, followed by 20 min at 170 °C. Flame ionization detection: t_R (major) = 22.3 min, (minor) = 22.6 min. The assignment of the absolute configuration of enzymatically prepared **3k** was based on earlier reported chiral GC data.[3]

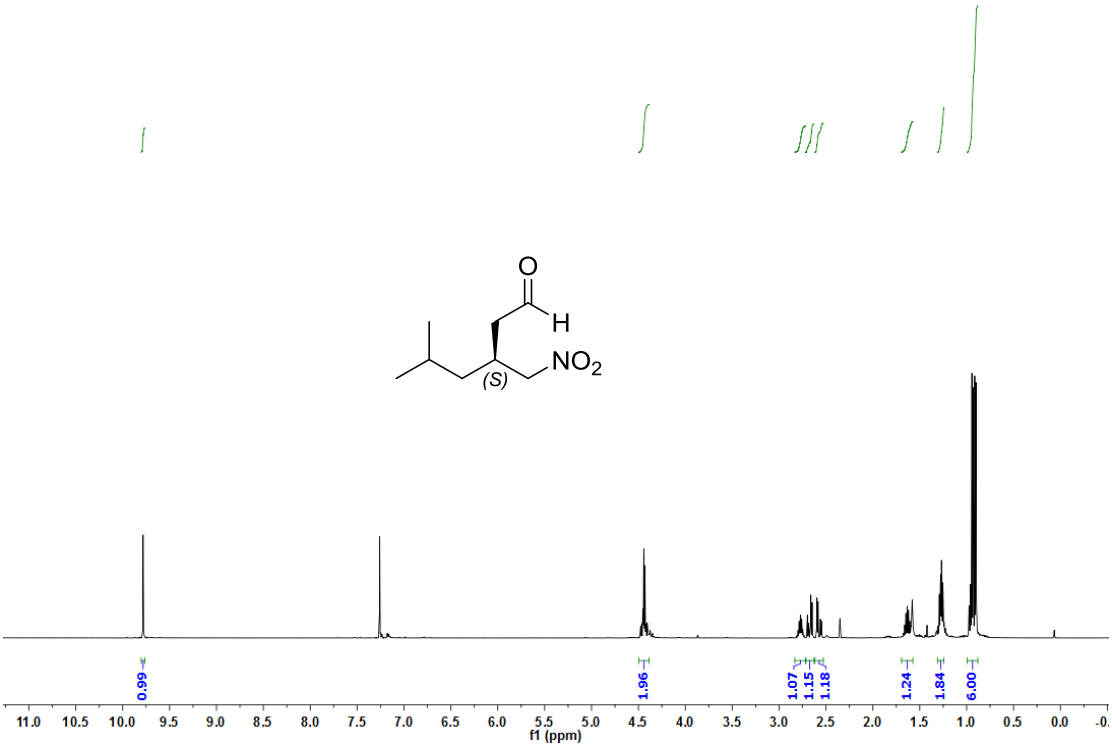


Figure S32. ^{1}H NMR spectrum of enzymatic product **3k**.

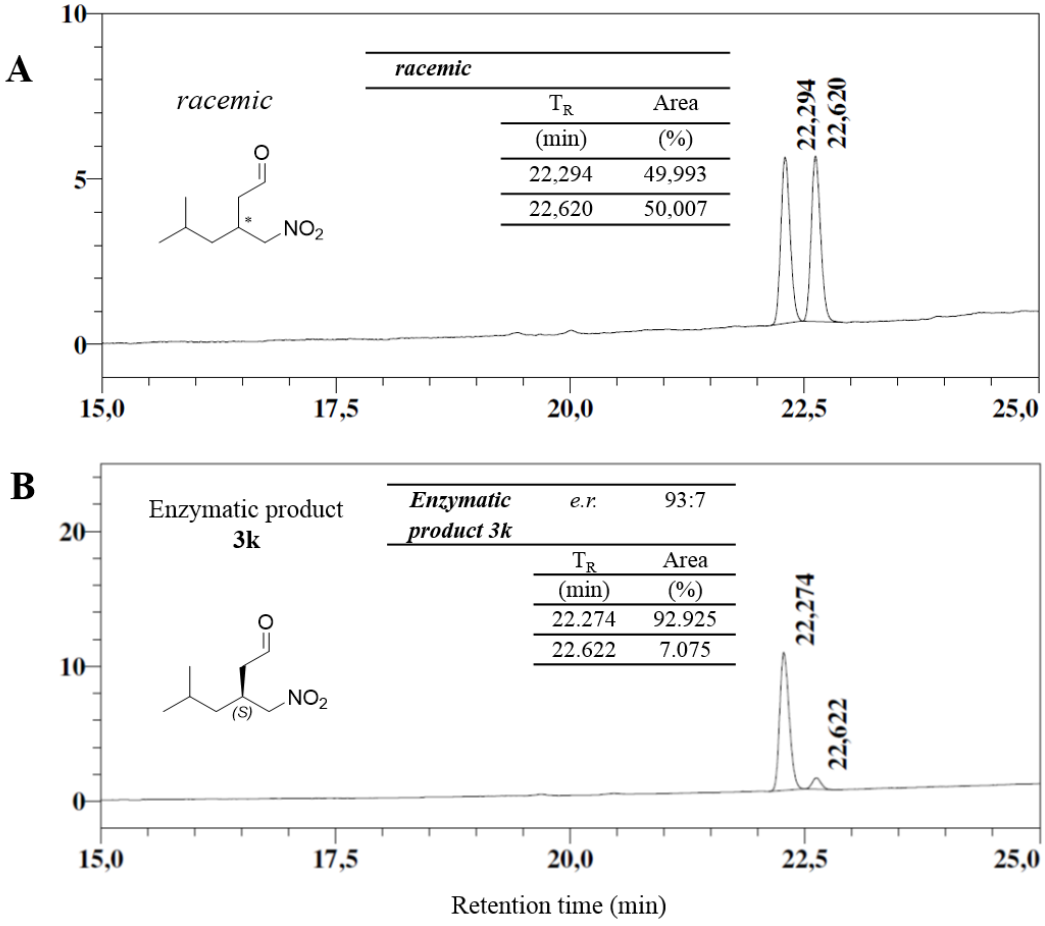


Figure S33. Chiral GC analysis of enzymatic product **3k**.

4. Preparative-scale synthesis of (*R*)-3a

The total volume of the reaction mixture was 50 mL (HEPES buffer, 20 mM, pH 6.5). A stock solution of **2a** (500 mM) in absolute ethanol was prepared. An aliquot of this stock solution (2.5 mL, 25 mM final concentration) was added to HEPES buffer (pH 6.5) containing nitromethane (**1**, 25 mM) and 4-OT F50A (350 µM). The reaction mixture was incubated at room temperature and the progress of the reaction was monitored by UV-VIS spectrophotometry. For this, aliquots (10 µL) were taken from the reaction mixture, diluted with 240 µL of 20 mM HEPES buffer (pH 6.5), and then analyzed by UV-VIS spectrophotometry. After ~50% of the substrate **2a** was consumed, an additional 25 mM of nitromethane **1** was added into the reaction mixture. After the completion of the reaction, the reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under *vacuo* to give crude product **3a** with 96% conversion (^{1}H NMR analysis). The crude product was further purified using flash column chromatography (silica gel, petroleum ether/ethyl acetate from 95:5 to 70:30) to give the pure compound (204.7 mg, 1.1 mmol, 85% yield). The enantiomeric ratio of purified product (*R*)-**3a** was determined using chiral GC as described above.

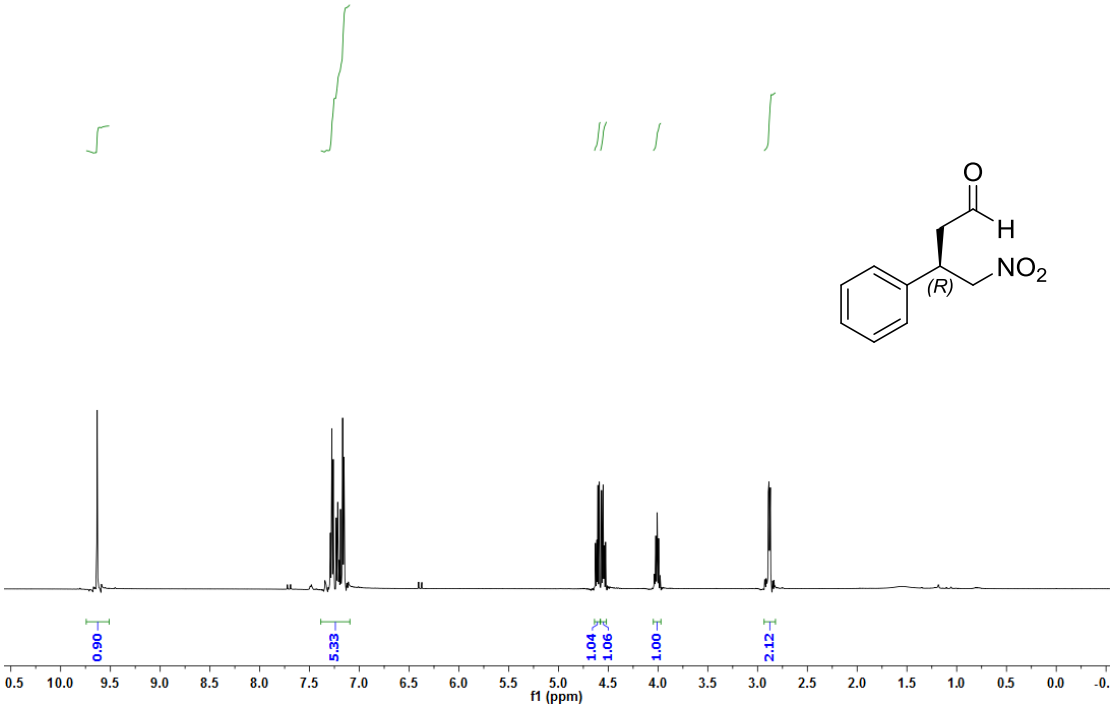


Figure S34. ^{1}H NMR spectrum of enzymatic product **3a**.

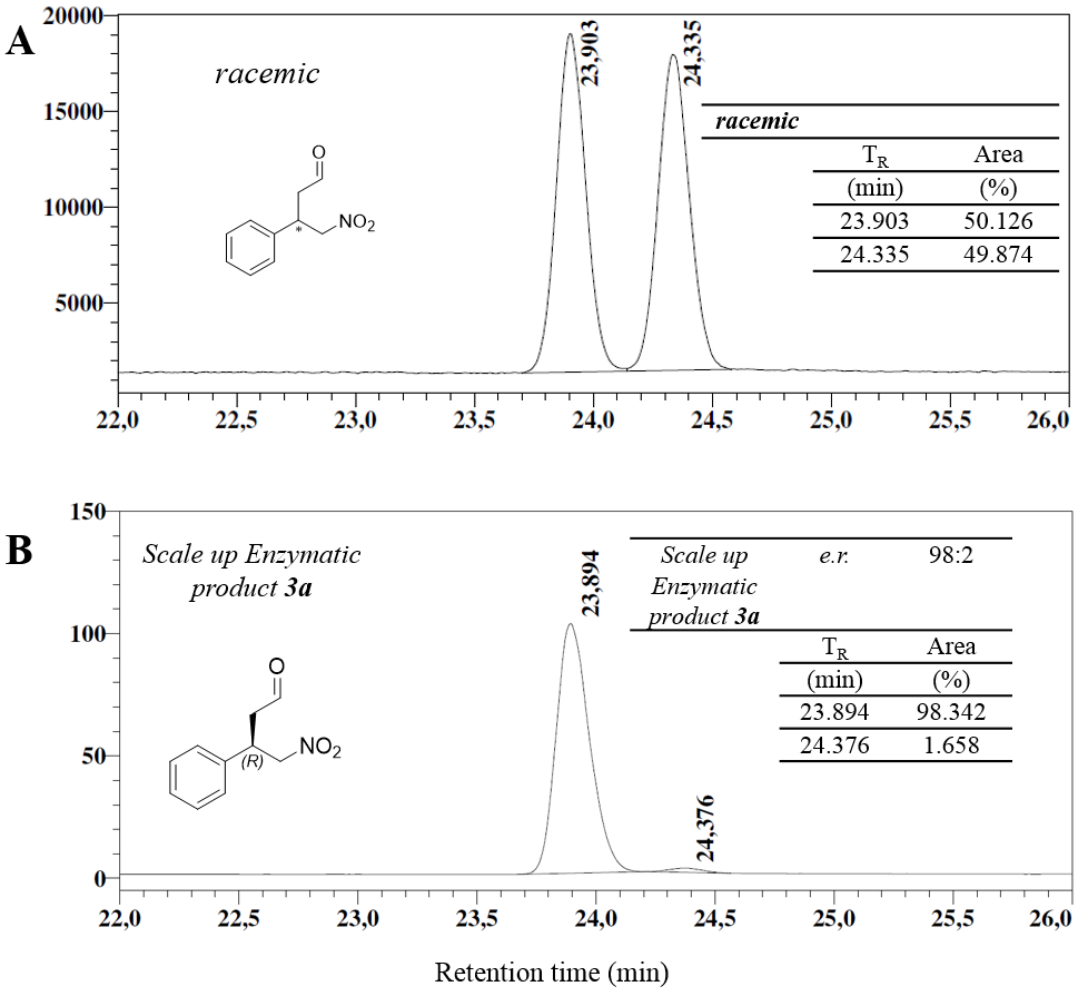


Figure S35. Chiral GC analysis of enzymatic product **3a**.

5. One-pot, four-step enzymatic cascade synthesis of γ-nitrobutyric acids (7a-b)

The reaction mixture (20 mL) contained **5a** (6.36 mg, 3 mM) or **5b** (8.43 mg, 3 mM), 4-OT F50A (219 µM), and ethanol (5% v/v) in HEPES buffer (20 mM, pH 7.3). The reaction was initiated by the addition of **4** (1 mL from a freshly prepared 3 M stock solution, 150 mM final concentration), and the reaction mixture was incubated at room temperature. The progress of the reaction was monitored by GC for **5a** and UV-VIS spectrophotometry for **5b**. After the reaction was completed, the pH of the reaction mixture was adjusted to pH 6.5, and nitromethane (27 µL, final concentration of 25 mM) was added and the progress of the reaction was monitored by GC (consumption of **2a**) or UV-VIS spectrophotometry (consumption of **2b**). After the reaction was finished, the pH of the reaction mixture was adjusted to pH 8.5 followed by the addition of PRO-ALDH(003) (0.5 mg/mL), PRO-NOX(009) (1 mg/mL) and NAD^+ (0.5 mM). After 30 min incubation, the pH was adjusted to pH 5.0, and the reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography

(petroleum ether/ethyl acetate from 95:5 to 50:50) to obtain (*R*)-**7a** (6.7 mg, 53% yield) or (*S*)-**7b** (11.7 mg, 80% yield). The acid functionality of (*R*)-**7a** and (*S*)-**7b** was derivatized to the corresponding methyl ester and ethyl ester, respectively, based on the reported protocol for enantiomeric ratio analysis.[5] For derivatization, the compound **7a** or **7b** (0.1 mmol) was dissolved in dichloromethane (1 mL) and methanol or ethanol (0.2 mL), and cooled down to 0 °C. To this cooled solution, EDC.HCl (0.15 mmol) was added, followed by DMAP (0.01 mmol). After overnight incubation, the reaction mixture was quenched with saturated aq. NH_4Cl and extracted with diethyl ether, and the combined organic layers were dried over anhydrous Na_2SO_4. The dried organic layer was concentrated under *vacuo* and the resulting product dissolved in acetonitrile for chiral HPLC analysis.

(*R*)-4-nitro-3-phenylbutanoic acid (**7a**). Yield = 53% (6.7 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38 – 7.26 (m, 3H), 7.26 – 7.20 (m, 2H), 4.72 (dd, *J* = 12.6, 7.1 Hz, 1H), 4.63 (dd, *J* = 12.6, 7.8 Hz, 1H), 3.97 (p, *J* = 7.4 Hz, 1H), 2.83 (dd, *J* = 7.4, 2.3 Hz, 2H). The NMR data match previously reported NMR data for this compound.[5] The enantiopurity of derivatized **7a** was analyzed by reverse phase HPLC using a chiral column (Chiralpak-ID, 150 mm x 4.6 mm, Daicel®) (MeCN/water 30:70, 25 °C, 1 mL/min flow rate). Detection at 210 nm, retention time (*R*)-**7a**: 32.5 min, (*S*)-**7a**: 34.8 min. The assignment of the absolute configuration was based on earlier reported chiral HPLC data.[5]

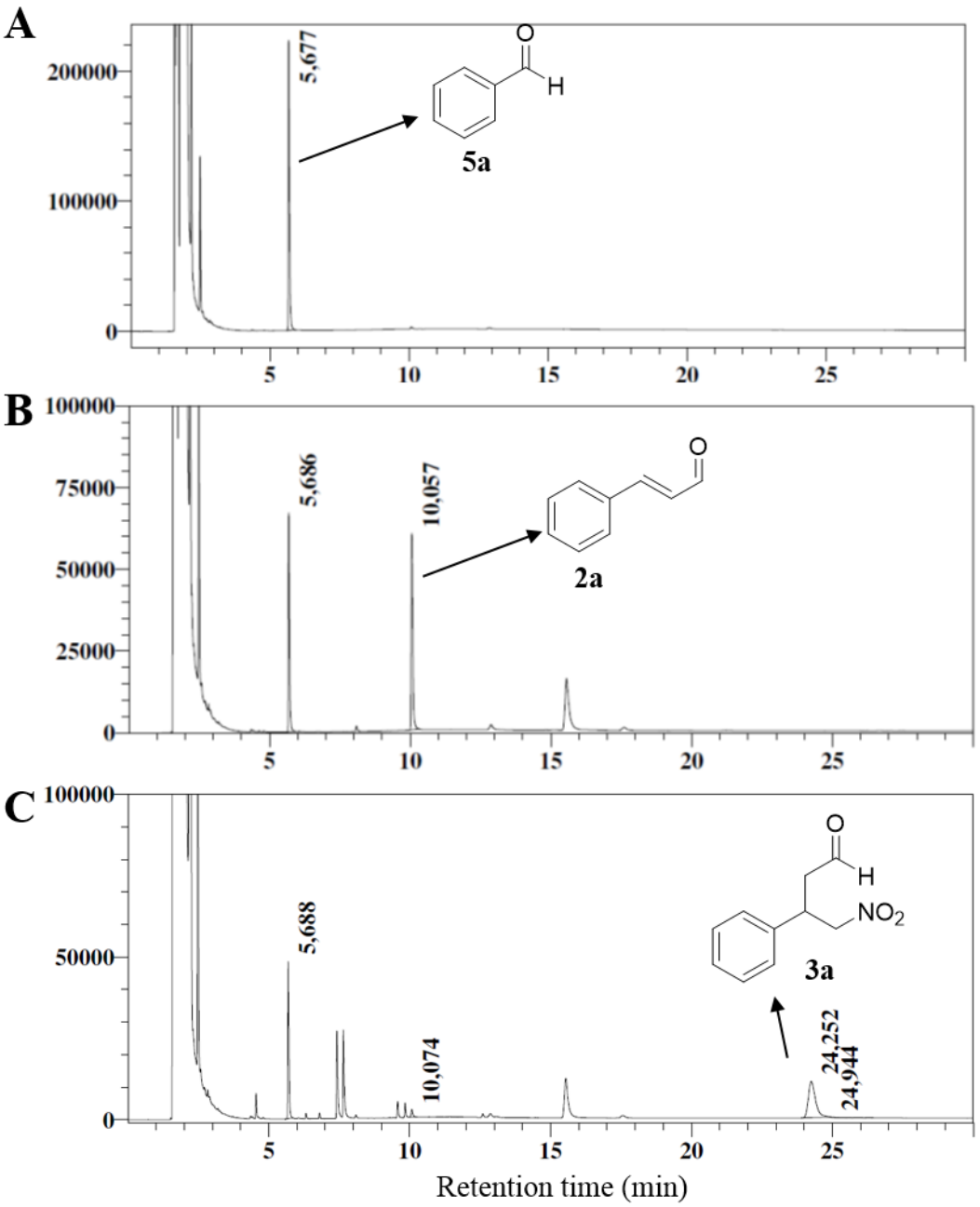


Figure S36. GC spectra monitoring the consumption of **5a** and **2a**, and the formation of **3a** in the one-pot, four-steps cascade synthesis of γ-nitrobutyric acid **7a**. A) 0 h. B) After 7 h, followed by addition of **1**. C) After 12 h.

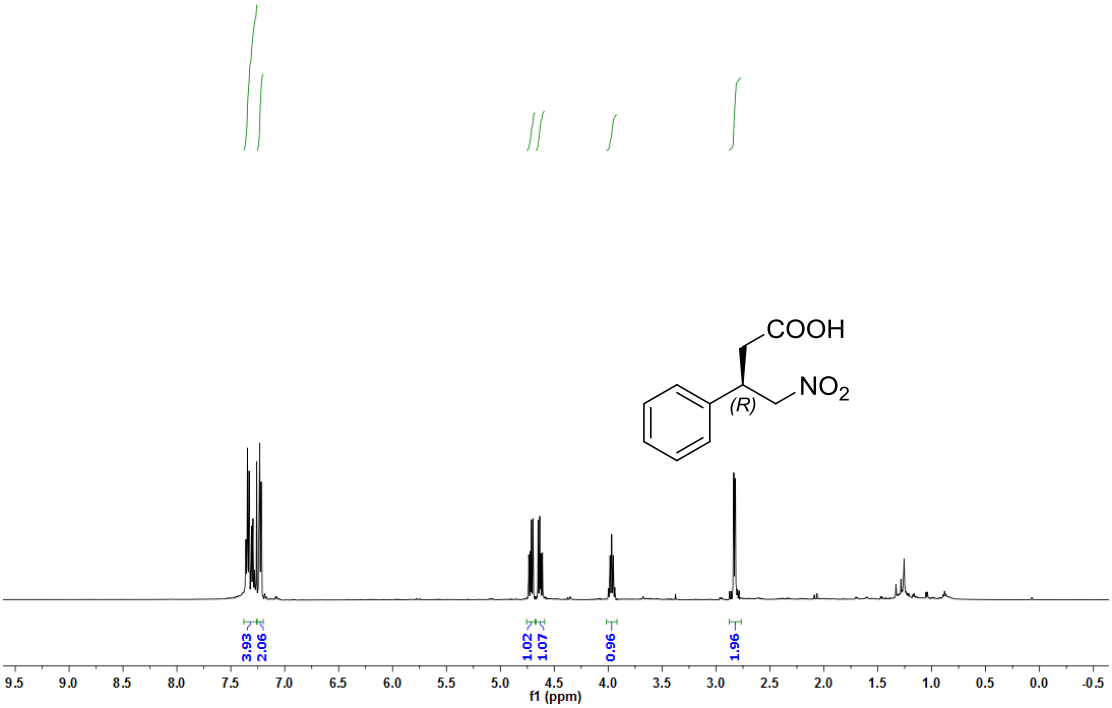


Figure S37. ^{1}H NMR spectrum of **7a** obtained by enzymatic cascade synthesis

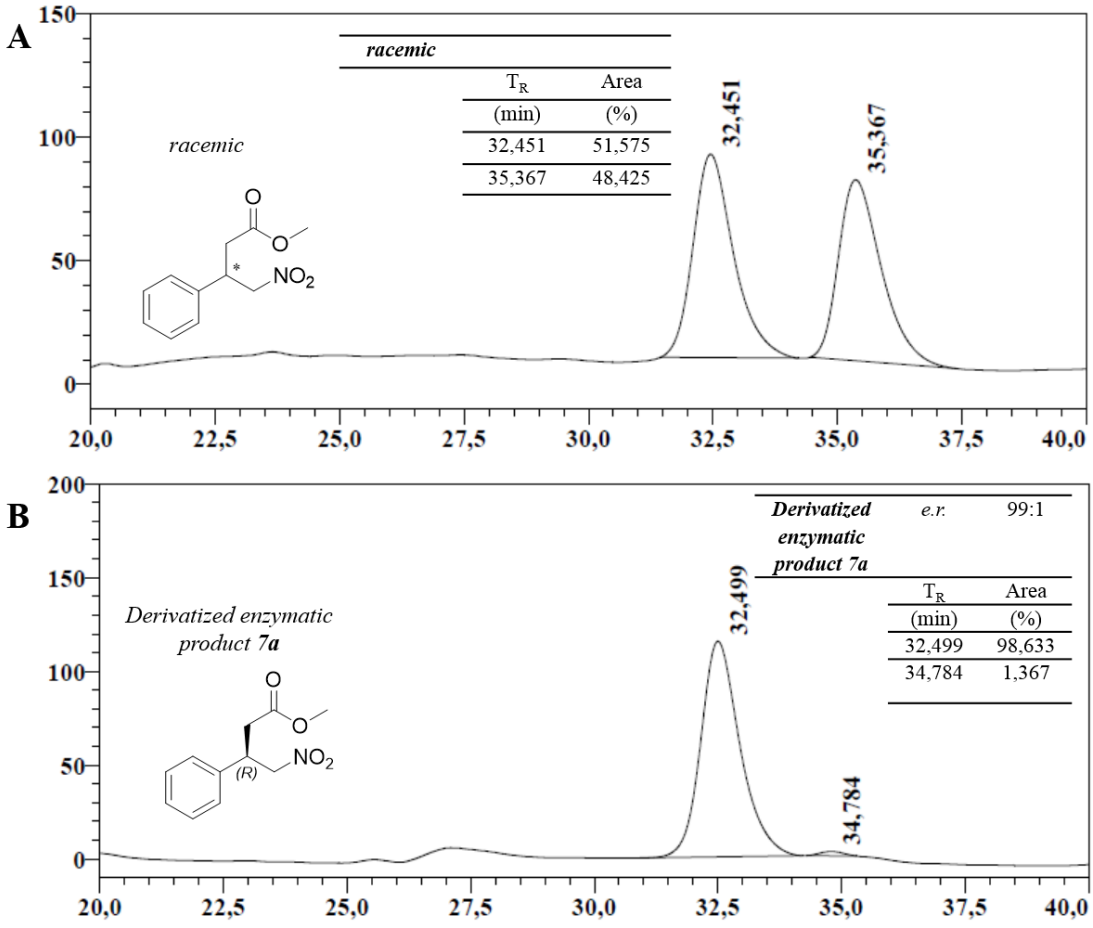


Figure S38. Chiral HPLC analysis of derivatized enzymatic product **7a**.

(*S*)-3-(2-chlorophenyl)-4-nitrobutanoic acid (**7b**). Yield = 80% (11.7 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.45 – 7.39 (m, 1H), 7.29 – 7.21 (m, 3H), 4.84 – 4.72 (m, 2H), 4.45 (p, *J* = 7.0 Hz, 1H), 3.01 – 2.87 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 175.17, 135.21, 133.81, 130.52, 129.31, 128.14, 127.50, 77.32, 36.50, 35.51; HRMS (ESI+): calcd. for $C_{10}H_{10}ClNO_4$, 244.0371; found, 244.0367. The enantiopurity of derivatized **7b** was analyzed by reverse phase HPLC using a chiral column (Chiralpak-ID, 150 mm x 4.6 mm, Daicel®) (MeCN/water 25:75, 25 °C, 1 mL/min flow rate). Detection at 210 nm, retention time: (major) 18.9 min, (minor) 20.3 min. The assignment of the absolute configuration of enzymatically prepared **7b** was based on the unambiguously established absolute configuration of its precursor **3b**.

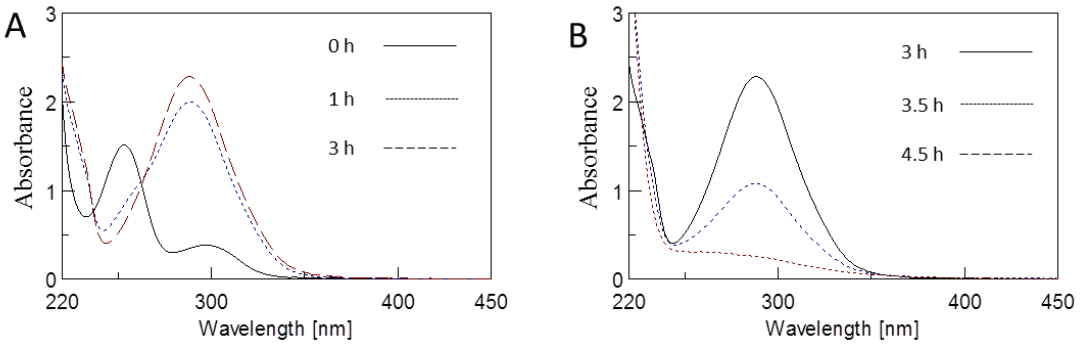


Figure S39. UV spectra monitoring the consumption of **5b** (A) and **2b** (B) in the one-pot four-steps cascade synthesis of γ-nitrobutyric acid **7b**.

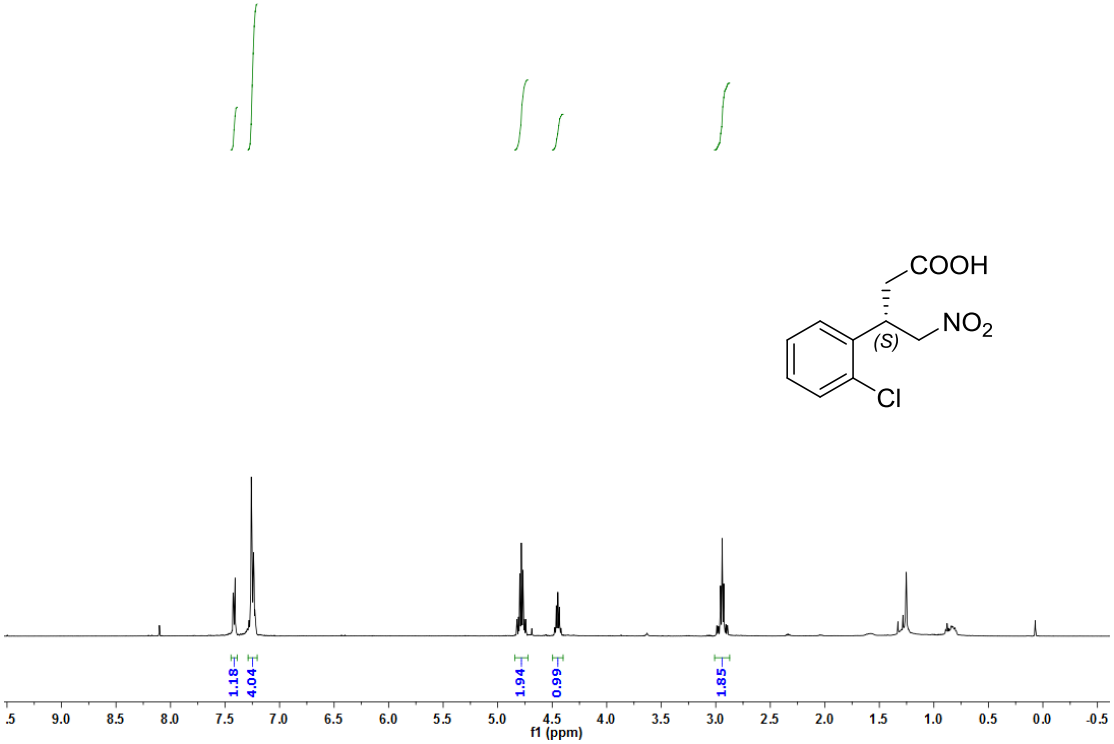


Figure S40. ^{1}H NMR spectrum of **7b** obtained by enzymatic cascade synthesis.

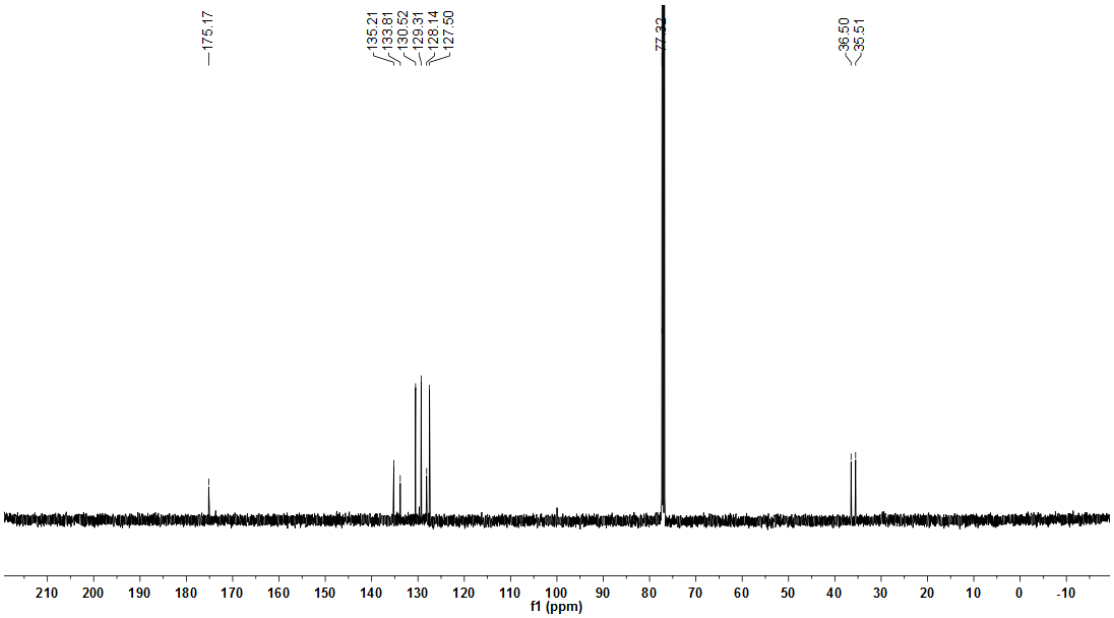


Figure S41. ^{13}C NMR spectrum of **7b** obtained by enzymatic cascade synthesis.

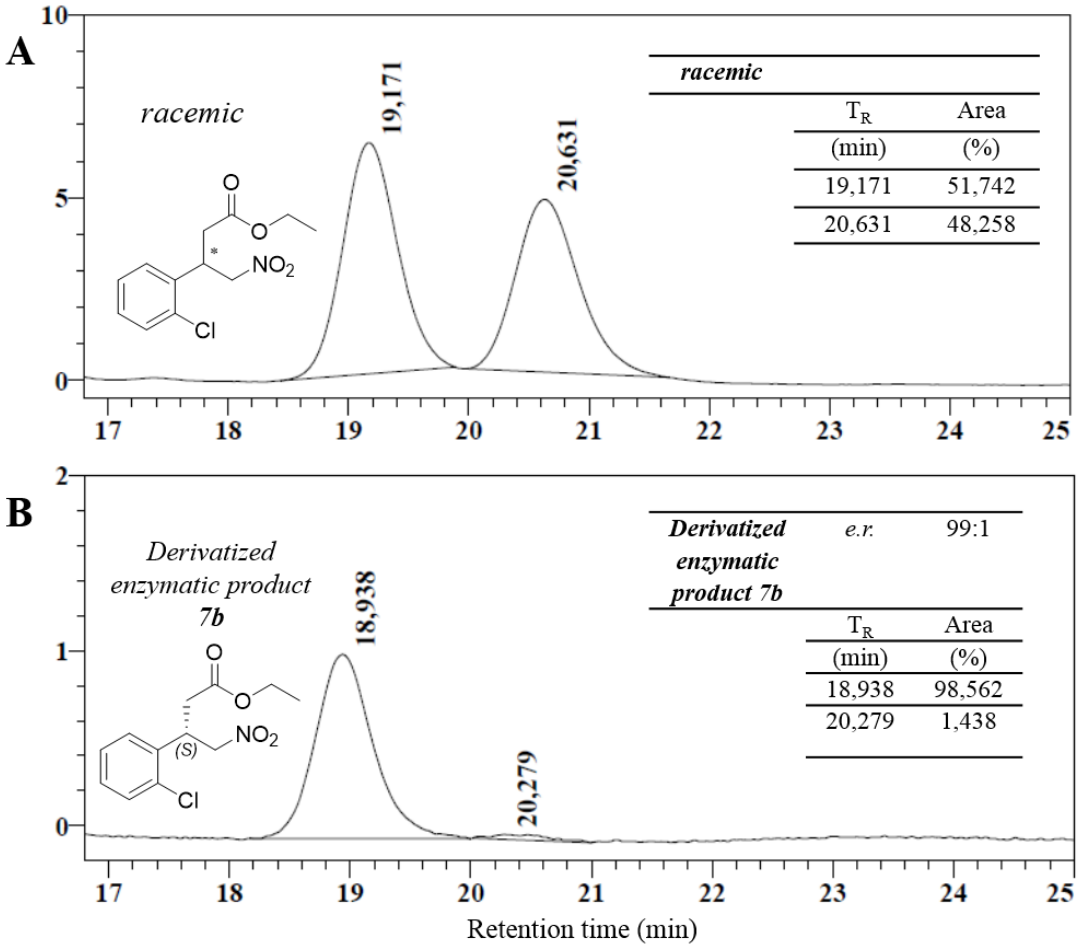


Figure S42. Chiral HPLC analysis of derivatized enzymatic product **7b**.

6. Mass spectral analysis of modified and unmodified 4-OT F50A

An amount of 4-OT F50A (0.5 mg) was incubated with cinnamaldehyde (**2a**, 10 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3) for 1 h at 22 °C (total volume of 1 mL). In a separate control experiment, the same amount of 4-OT F50A was incubated without **2a** under otherwise identical conditions. Subsequently, $NaBH_4$ was added to both samples to give a final concentration of 25 mM, and the samples were incubated at 22 °C for 1 h. The buffer of the two incubation mixtures was exchanged against 20 mM NaH_2PO_4 buffer (pH 7.3) using separate pre-packed PD-10 Sephadex G-25 gelfiltration columns (GE Healthcare, Piscataway, NJ, USA). The two purified 4-OT F50A proteins were analyzed by ESI-MS using the mass spectrometer system described below (Figure S43).

For the peptide mapping studies, 20 µL of each protein solution (0.5 mg/mL) was diluted with 20 µL ammonium bicarbonate (100 mM, pH 8). Then, 10 µL of a 10 ng/µL solution of endoproteinase GluC (Promega) was added and the mixture was incubated for 3 h at 37 °C. Subsequently, the samples were acidified and injected into the LC-MS system, consisting of an Ultimate 3000 UHPLC equipped with a quaternary pump, an autosampler, and a column oven coupled by a HESI-II electrospray source to a Q-Exactive Orbitrap™-based mass spectrometer (all Thermo Scientific, San Jose, CA, USA). For LC separation, a BEH-C18 (1.7 µm particles, 50 × 2.1 mm) column (Waters, Milford, MA, USA) was used, with (A) water and (B) acetonitrile (both containing 0.1% v/v formic acid) as eluents. The following gradient was used: 5% B until 0.5 min, then linear to 90% B in 17.5 min. This composition was held for 2 min, after which a switch back to 5% B was performed in 0.1 min. After 2.9 min of equilibration, the next injection was performed. The LC flow rate was 350 µL/min, the LC column was kept at 60 °C and the injection volume was 10 µL. The HESI-II electrospray source was operated with the parameters recommended by the MS software for the LC flow rate used (spray voltage of 3.5 kV, positive mode); other parameters were sheath gas 40 AU, auxiliary gas 8 AU, cone gas 2 AU; capillary temperature 325 °C; heater temperature 350 °C. The samples were measured in positive mode from m/z 300-2000 at a resolution of 70000 @ m/z 200 and a Top5 data dependant (DDA) scan was performed at a resolution of 17500 at m/z 200. The system was controlled using the software packages Xcalibur 4.1, SII for Xcalibur 1.3 and Q-Exactive Tune 2.9 (all Thermo Scientific). For data processing Peaks studio X (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) was used. The identified peptide fragments are given in Figures S44 and S45. Selected ions of both samples were subjected to MS/MS analysis (Figure S46).

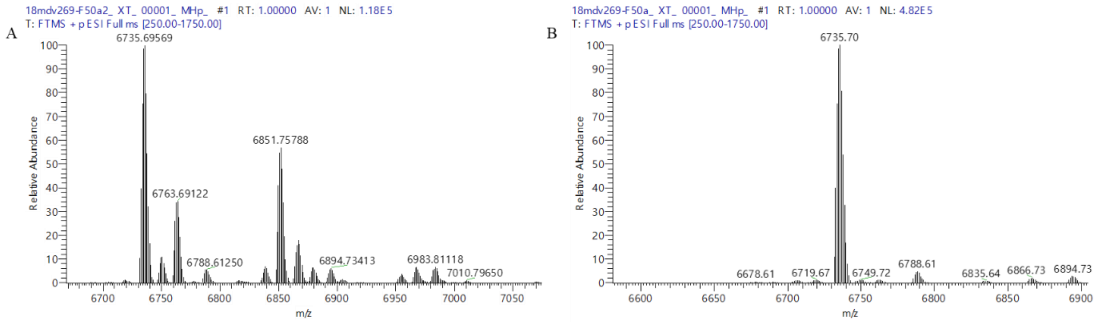


Figure S43. ESI-MS spectra of labeled (A) and unlabeled (B) 4-OT F50A. (A) 4-OT F50A incubated with 10 mM **2a** and afterwards treated with sodium borohydride (expected mass 6851 Da). (B) Unmodified 4-OT F50A treated with sodium borohydride (expected mass 6735 Da).

Peptide List

Peptide	-10lgP	Mass	Length	ppm	m/z	RT	Fraction	Scan	Source File	Intensity Sample 1	#Feature	#Feature Sample 1	Accession	PTM
AISRSLDAPLTSVRVIITE	62.66	2040.1578	19	4.2	681.0627	6.36	1	2145	18mdv27u-label-GluC2-DDA_20181122170307.raw	1.11E9	9	9	us|4-OT-18mdv276|Cguo-4OT	
VSEAISRSLDAPLTSVRVIITE	58.33	2355.3008	22	0.9	1178.6587	6.68	1	2306	18mdv27u-label-GluC2-DDA_20181122170307.raw	9.88E8	8	8	us|4-OT-18mdv276|Cguo-4OT	
M(+15.99) AKGHAGIGGELASKVRR	51.63	1853.0054	18	1.4	464.2593	3.20	1	820	18mdv27u-label-GluC2-DDA_20181122170307.raw	3.55E7	14	14	us|4-OT-18mdv276|Cguo-4OT	Oxidation (M)
MAKGHAGIGGELASKVRR	50.67	1837.0104	18	1.8	460.2607	3.30	1	850	18mdv27u-label-GluC2-DDA_20181122170307.raw	6.36E8	20	20	us|4-OT-18mdv276|Cguo-4OT	
MAKGHAGIGGE	40.24	1026.4917	11	1.0	514.2537	0.90	1	280	18mdv27u-label-GluC2-DDA_20181122170307.raw	6.4E8	31	31	us|4-OT-18mdv276|Cguo-4OT	
M(+15.99)AKGHAGIGGE	34.21	1042.4866	11	1.3	522.2512	0.66	1	184	18mdv27u-label-GluC2-DDA_20181122170307.raw	4.23E7	14	14	us|4-OT-18mdv276|Cguo-4OT	Oxidation (M)
PIAQIHILE	29.59	1032.5968	9	3.5	517.3075	5.69	1	1874	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.97E9	13	13	us|4-OT-18mdv276|Cguo-4OT	
TLIREVSE	28.92	945.5131	8	3.6	473.7655	3.95	1	1148	18mdv27u-label-GluC2-DDA_20181122170307.raw	1.15E8	10	10	us|4-OT-18mdv276|Cguo-4OT	
GRSDEQKE	24.71	947.4308	8	0.1	316.8176	0.33	1	53	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.72E7	24	24	us|4-OT-18mdv276|Cguo-4OT	
PIAQIHILEGRSDE	24.36	1576.8209	14	1.1	526.6148	4.98	1	1561	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.48E6	2	2	us|4-OT-18mdv276|Cguo-4OT	
P(+116.06)IAQIHILE	19.60	1148.6567	9	3.6	575.3377	7.32	1	2578	18mdv27u-label-GluC2-DDA_20181122170307.raw	6.93E8	2	2	us|4-OT-18mdv276|Cguo-4OT	C9H8-nterminus
LASKVRR	18.67	828.5294	7	0.6	415.2722	0.53	1	133	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.41E8	5	5	us|4-OT-18mdv276|Cguo-4OT	
total 12 peptides														

Figure S44. Peptide mapping data of labeled 4-OT F50A digested with protease GluC.

Peptide List

Peptide	-10lgP	Mass	Length	ppm	m/z	RT	Fraction	Scan	Source File	Intensity Sample 2	#Feature	#Feature Sample 2	Accession	PTM
VSEAISRSLDAPLTSVRVIITE	63.12	2355.3008	22	0.5	1178.6582	6.66	3	2352	18mdv27u-unl-GluC-DDA_20181122163511.raw	5.46E9	10	10	us|4-OT-18mdv276|Cguo-4OT	
AISRSLDAPLTSVRVIITE	62.16	2040.1578	19	0.2	1021.0864	6.32	3	2184	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.47E10	19	19	us|4-OT-18mdv276|Cguo-4OT	
MAKGHAGIGGELASKVRR	46.84	1837.0104	18	1.6	460.2606	3.30	3	925	18mdv27u-unl-GluC-DDA_20181122163511.raw	2.06E8	9	9	us|4-OT-18mdv276|Cguo-4OT	
M(+15.99)AKGHAGIGGELASKVRR	43.36	1853.0054	18	0.4	464.2588	3.21	3	891	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.31E7	6	6	us|4-OT-18mdv276|Cguo-4OT	Oxidation (M)
MAKGHAGIGGE	39.29	1026.4917	11	1.0	514.2537	0.91	3	316	18mdv27u-unl-GluC-DDA_20181122163511.raw	2.31E9	31	31	us|4-OT-18mdv276|Cguo-4OT	
M(+15.99)AKGHAGIGGE	33.25	1042.4866	11	3.8	522.2526	0.66	3	205	18mdv27u-unl-GluC-DDA_20181122163511.raw	2.3E8	14	14	us|4-OT-18mdv276|Cguo-4OT	Oxidation (M)
PIAQIHILE	28.69	1032.5968	9	2.6	517.3070	5.66	3	1872	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.74E10	35	35	us|4-OT-18mdv276|Cguo-4OT	
TLIREVSE	28.52	945.5131	8	1.1	473.7643	3.95	3	1230	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.72E8	8	8	us|4-OT-18mdv276|Cguo-4OT	
GRSDEQKE	27.12	947.4308	8	1.1	316.8179	0.47	3	142	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.36E8	25	25	us|4-OT-18mdv276|Cguo-4OT	
PIAQIHILEGRSDE	23.66	1576.8209	14	0.5	526.6145	4.99	3	1640	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.19E6	2	2	us|4-OT-18mdv276|Cguo-4OT	
QKETLIRE	21.47	1015.5662	8	1.0	339.5297	2.86	3	749	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.14E6	2	2	us|4-OT-18mdv276|Cguo-4OT	
LASKVRR	16.69	828.5294	7	0.9	415.2723	0.53	3	157	18mdv27u-unl-GluC-DDA_20181122163511.raw	1.88E8	3	3	us|4-OT-18mdv276|Cguo-4OT	
total 12 peptides														

Figure S45. Peptide mapping of unlabeled 4-OT F50A digested with protease GluC.

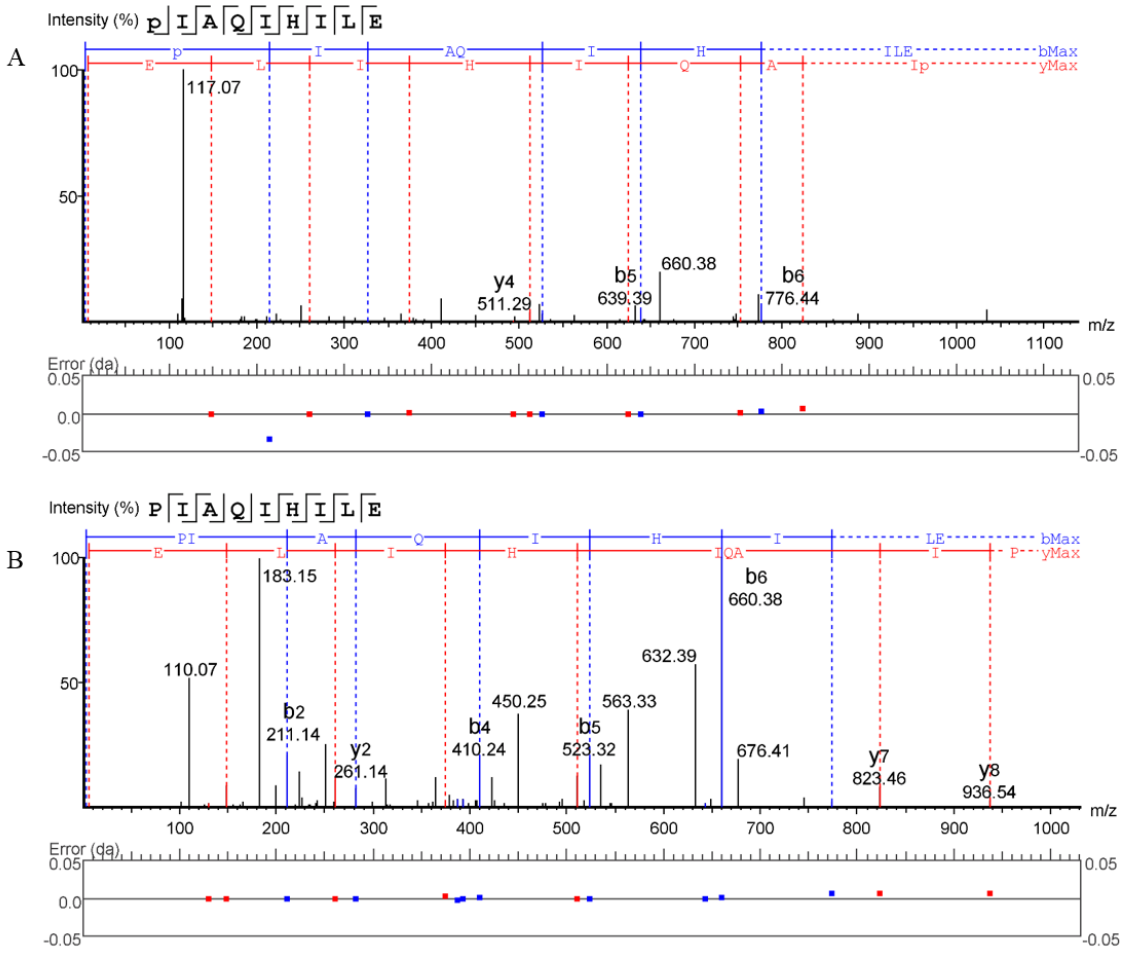


Figure S46. MS/MS analysis of modified and unmodified peptide ions. A) MS/MS spectrum of the ion corresponding to the modified peptide PIAQIHILE obtained from 4-OT F50A treated with **2a** and sodium borohydride. B) MS/MS spectrum of the ion corresponding to the unmodified peptide PIAQIHILE obtained from 4-OT F50A treated with only sodium borohydride. The expected m/z of the b_5 ion (PIAQI) of the modified peptide is 639 Da, whereas that of the unmodified peptide is 523 Da.

7. References

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