SUPPORTING INFORMATION

Self-Immobilizing Fusion Enzymes for Compartmentalized Biocatalysis

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Experimental Procedures / Materials

Cloning of Plasmids

The cloning of the expression plasmids for the His-tagged SC[1] and the GDH-TEV-His, containing a c-terminal TEV-cleavage site between GDH and the His-Tag[2] was previously described. Unless otherwise stated, the genetic construction was performed using the Gibson assembly[3] utilizing PCR-products and synthetic DNA-fragments with 30 bp homologous overlaps. After the assembly, the reaction mixtures were treated with DpnI to remove any remaining vector from prior PCR reactions and then transformed into *E. coli* DH5α cells. All plasmids were purified using ZR Plasmid Miniprep–Classic (Zymo Research, Germany) according to the manufacturer's instructions and sequence verified by commercial sequencing (LGC genomics, Germany).

The previously reported Gateway entry plasmids of Gre2p[2] and GDH[2] were subjected to a LR-reaction with the plasmid pDESTn15 to yield the final expression plasmid generating protein fusions with a c-terminal SBP-Tag (Streptavidin-binding-peptide-tag),[4] Gre2p-SBP or GDH-SBP respectively. For N-terminal protein fusions of the LbADH with SBP the previously reported entry vector[1] was subjected to a LR-reaction with pDESTn16 to yield the pEXPn16_LbADH-SBP plasmid. To generate the expression vector for Gre2p-HOB-His a synthetic DNA fragment (Geneart) of the Gre2p with a HOB M20S[5] connected via a GGGS-linker sequence and containing a c-terminal His-tag and was recombined with a linearized pET22b vector backbone. The expression vector for Gre2p-ST-His was generated via a Gibson assembly of a synthetic DNA fragment encoding for Gre2p, a flexible GGGGS linker, the ST and a c-terminal His-Tag with a linearized pEXP-backbone.

Expression and Purification of Proteins

For heterologous protein expression, *E. coli* BL21(DE3) was transformed with the corresponding expression vector using electroporation. The freshly transformed *E. coli* cells harboring the different plasmids were selected overnight on LB/agar plates containing 100 µg/ml ampicillin at 37°C. Liquid cultures of 20 ml LB medium containing ampicillin were started from the LB/agar plates overnight cultures. The 20 ml cultures were incubated for 14-18h at 37 °C, 180 rpm in a 150 ml shaking flask and then transferred in 2 L LB-medium containing ampicillin in a 4 L shaking flasks and incubated at 37 °C, 180 rpm until the OD_{600} reached a value of 0.6. The temperature was then lowered to 25 °C and IPTG was added to a final concentration of 0.1 mM for additional 16 hours. The cells were harvested by centrifugation (10000xg, 10 min) and resuspended in 30 mL buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM $MgCl_2$, 10 mM Imidazole, pH 8.0). After disruption using ultrasonication, the cell lysate was obtained after centrifugation (45000xg, 1 h), filtered through a 0.45 µm Durapore PVDF membrane (Steriflip, Millipore) and loaded on a HisTrap FF (5 mL) Ni-NTA column (GE Healthcare, Germany) mounted on an Äkta Pure liquid chromatography system (GE Healthcare, Germany). The column was washed with 50 mL buffer A and the 6xHis-tagged proteins were eluted with 100% buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, pH 8.0). The c-terminal His-Tag of the GDH was removed via TEV-protease-cleavage as previously described.[6] As expected this resulted in an increased GDH activity of 8 (µmol*min)/mg (GDH) in contrast to 3 (µmol*min)/mg (GDH-His). For purification of SBP tagged proteins, a 5 ml Strep-Tactin® Superflow® cartridge (iba-lifescience, Germany) was used according to manufacturer's instructions. Subsequently, the buffer was exchanged for the SBP tagged enzymes to 100 mM Triethanolamine pH 7.5, 1 mM $MgCl_2$ (TEA-Mg) by Vivaspin 10 000 MWCO (GE Healthcare). Gre2p-HOB was stored in a 150 mM PBS-buffer pH 7.3 and the Gre2p-ST in 100 mM KP_i pH 7.5.

Samples of the recombinant, purified proteins were typically analyzed by standard discontinuous SDS-polyacrylamide Laemmli-midi-gels. The bands were visualized by Coomassie staining and were compared to the Page Ruler™ prestained protein ladder (Thermo scientific). The concentrations were determined by UV-Vis spectroscopy, using the theoretical molar extinction coefficients at 280 nm, as calculated by the Geneious version 8.0.5 software.[7]

Functionalization of magnetic microbeads (MB)

Magnetic beads (MB) used for immobilization of the SBP-tagged cells were Dynabeads M-280 Streptavidin (MB-STV) from Thermo Fisher Scientific. MBs containing a chlorohexyl ligand on their surface (MB-CH) were prepared from Dynabeads M-280 Streptavidin beads by incubation with the Biotin-PEG-Chlorohexyl conjugate (HaloTag® PEG-Biotin Ligand, Promega) dissolved in 100 mM KP_i pH 7.5 to an final concentration of 2 nmol Biotin-Chlorohexyl per mg bead. The reaction mixture was incubated for at least 1h, 30 °C at a tube rotator and the MBs were subsequently washed with TEA-Mg supplemented with 0.01% (v/v) Tween20 (TEA-T-Mg). MBs containing the SC on the surface (MB-SC) were generated by covalent immobilization of the SC protein (Figure S1) via its lysine residues onto Dynabeads Epoxy (Thermo Fisher Scientific), following the manufacturer's instructions. The accessibility of the ligands on the functionalized particles was tested as previously described.[1]

Enzyme immobilization on magnetic microbeads

The functionalized magnetic microbeads were mixed with 1 $nmol_{protein}/mg_{MB}$ of the corresponding purified SBP-, HOB- or ST-tagged enzyme for 30 min, 30 °C at a tube rotator. The MBs were subsequently washed three times with TEA-T-Mg. The covalent immobilization of LbADH-SBP, Gre2p-SBP and LbADH-SBP via their lysine residues onto Dynabeads Epoxy (MB-Epoxy) was performed following the manufacturer's instructions. To this end, the enzyme buffer was previously exchanged to 100 mM KP_i pH 7.5, 1 mM $MgCl_2$ by Vivaspin 10 000 MWCO (GE Healthcare).

Crude extract immobilization at magnetic microbeads

The plamsids for direct functionalization with crude extract pEXPn15_Gre2p-SBP, pEXPn15_GDH-SBP, pEXPn16_LbADH-SBP, pET_Grep-HOB and pEXP_Gre2p-ST were transformed into *E. coli* BL21(DE3) using electroporation. The freshly transformed *E. coli* cells harboring the different plasmids were selected overnight on LB/agar plates containing 100 µg/ml ampicillin at 37°C. Liquid cultures (0.5 L LB medium containing ampicillin) were started in 2 L shaking flasks at 37 °C and 180 rpm directly from the LB/agar plates overnight cultures. When the OD_{600} had reached a value of approx. 0.6, the expression of the fusion proteins was induced by addition of IPTG to a final concentration of 100 µM. The cells were grown at 25 °C for 16-20h hours, collected via a brief centrifugation and resuspended in 10 ml 100 mM KPi pH 7.5, 1mM $MgCl_2$. After disruption by ultrasonication, the crude cell extract was obtained by centrifugation (45000xg, 10 min). 1.5 ml of the supernatant was used for direct functionalization of 2 mg functionalized beads. For the competitive binding assay 500 µl of LbADH-SBP, Gre2p-HOB and Gre2p-ST crude extract were mixed

and incubated with 2 mg MBs. The microbeads were subsequently washed three times with TEA-T-Mg and 1.5 mg MBs were used for the activity tests and 0.5 mg MBs for the determination of the binding capacity by gel analysis.

Determination of enzyme activity

The specific activities of free enzymes were obtained by preincubation of 5 µM LbADH-SBP, Gre2p-SBP and GDH-SBP diluted in 100 mM TEA-T-Mg for 22h at 30°C, 500 rpm. If not stated otherwise, all enzyme assays were performed with a reaction mixture containing 10 mM substrate nitrodiketone (NDK) **1**[2] in TEA-Mg supplemented with 100 mM Glucose, 1 mM $NADP^+$ and an excess of 5 µM GDH as NRE. The GDH containing reaction mixture was preincubated for at least 30 min at 30°C, before the KRED enzymes were added. The LbADH biotransformation was tested with the GDH-driven NADPH regeneration system or using 5% (v/v) 2-propanol for cofactor regeneration without preincubation. Time dependent samples were taken manually and analyzed by chiral HPLC. For the determination of the average specific activity of free and immobilized enzymes the reaction mixtures contained 5 mM instead of 10 mM NDK **1** and were additionally supplemented with 0.01% (v/v) Tween20, in order to allow a comparison with the reactor experiments. Reactions were started by adding 0.5 µM KRED, 10 µl crude extract or 50 µg KRED-functionalized MB to the reaction mixture and incubation for 20 minutes at 30°C, 1000 rpm in a 2 ml reaction cup. Additional reaction mixture samples of GDH-SBP@MB-Epoxy analyzed after 60 minutes of incubation showed no activity. In order to measure the GDH activity, 0.5 µM GDH or 50 µg GDH-SBP@MB-STV were incubated together with an excess of 5 µM LbADH-SBP using the same conditions as described for the KREDs above.

For HPLC analyses, 50 µL of the crude reactions mixture were extracted with 150 µL ethyl acetate, centrifuged to enable fast phase separation and 50-100 µl of the organic phase were transferred into an HPLC vial and evaporated (Eppendorf Concentrator plus). Conversion, enantiomeric- and diastereomeric excess values were calculated based on the ratios of HPLC signals detected at 210 nm, as previously described.[2]

Determination of MB loading capacity

Prior to the SDS-gel analysis of the protein binding capacity of MB-STV and MB-CH, the non-covalent immobilization of SBP and Biotin-PEG-CH with STV was disrupted by heating 125 µg enzyme functionalized beads in 1 % (v/v) SDS at 95°C, for 10 minutes. The supernatant obtained from 100 µg denatured MB-samples was purified by magnetic separation of the beads and then analyzed by standard discontinuous SDS-polyacrylamide

Laemmli-midi-gels. The bands were visualized by Coomassie staining and were compared with the Page RulerTM prestained protein ladder plus (Thermo scientific) as well as with calibration samples which contained predefined amounts of the corresponding purified protein (0.25, 0.5, 1 and 2 µg). The comparative greyscale analysis was performed with the ImageJ 1.48v software.[8]

Microfluidic Experiments

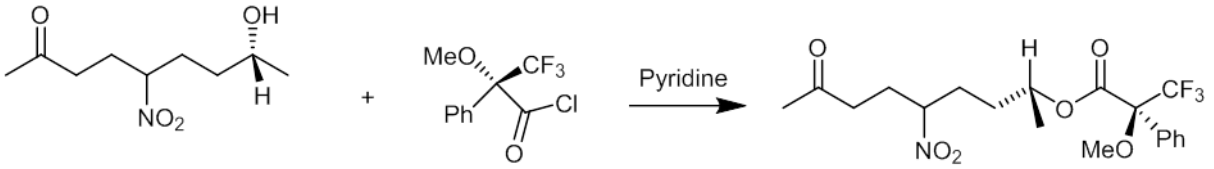
The enzyme funtionalized MBs were loaded into the individual compartments of a four straight channel PMMA chip (microfluidic chipshop, Jena, Germany) through a Mini luer to pippete adapter and a corresponding loaded pipette tip using a negative flowrate of -50 µl/min. The dimensions of one channel are 58.5 × 1.0 × 0.2 mm, which correspond to a reactor volume of 11.7 µl. The magnetic/catalytic zone, which had Nd magnets arranged underneath, had a volume of ~10 µl, which was reduced to 6.8–7.2 µl when 4.5 mg beads were loaded (10 mg correspond to ~6–7 × 10^8 beads with a diameter of 2.7µm). Successful loading of the reactor was monitored by visual inspection. Filled channels were connected with a short PTFE tubing (internal diameter 0.5 mm) using MiniLuer plugs (microfluidic chipshop). The same tubing and plugs were used to connect the inlet of the assembled chip with a CETONI neMESYS syringe pump holding the cofactor/substrate solution and the outlet with the CETONI Compact Positioning System rotAXYS. The HT200 temperature-controlled chipholder (ibidi GmbH, Germany) was set to hold 30°C. The chipholder was modified with Nd permanent magnets, positioned beneath the channels of the chip. The syringe pump was filled with 5 mL cofactor/substrate solution containing 5 mM NDK **1** in TEA-T-Mg, 1 mM $NADP^+$ supplemented with 0.01 % sodium azid to avoid fouling. For cofactor regeneration, the reaction buffer was either complemented with (A) 5% (v/v) 2-propanol, (B) 100 mM glucose, or (C) 100 mM glucose and 10 µM GDH for the setups lacking immobilized NRE. A flow rate of 1 µL/min was used. The chip outflow was automatically fractionated by the rotAXYS system in a 96-well plate which contained 50 µl 7 M NaClO to stop all enzymatic reactions. The samples were subsequently analyzed by chiral HPLC.

Chiral HPLC analysis

The synthesis and the characterization of NDK **1** as well as the analysis of biocatalytic reaction products by chiral HPLC was performed as previously described.[2] In brief, the dried ethyl acetate extractions from the crude reaction mixtures (described above) were resuspended in 100 µl of the mobile phase (90% n-heptan, 10% 2-propanol) and 10-30 µl of the solution were injected into the HPLC instrument (Agilent 1260

series HPLC equipped with a Diode Array Detector (210 nm) on a Lux 3µ Cellulose-1 (150x2.00 mm) chiral column (Phenomenex)). Identical running conditions were used for the analysis of the hydroxy ketones **2** for method A (chromatography solvent 90% n-heptan/ 10% 2-propanol, 10 min isocratic, column oven temperature of 10 °C and a flowrate of 0.5 ml/min) and for diol **3** method B (chromatography solvent 98% n-heptan/ 2% 2-propanol, 20 min isocratic, column oven temperature of 45 °C and a flowrate of 1.0 ml/min).

Preparation of the *S*-MTPA-nitroketone esters of *R*-isomer



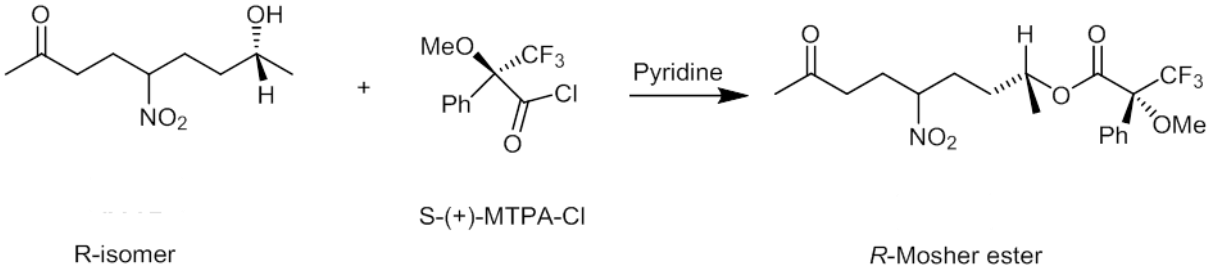
R-isomer R-(+)-MTPA-Cl *S*-Mosher ester

To confirm the absolute configuration of hydroxyketones **2** (see Figure S3), Mosher ester analysis was carried out. To a stirred solution of R-isomer (30 mg, 0.148 mmol) and dry pyridine (37µl, 0.458 mmol, 3.1 equiv.) in dry dichloromethane (5 ml) at room temperature, R-(-)-MTPA-Cl (3**R**, 57 µl, 0.296 mmol, 2.0 equiv.) was added. The reaction progress was monitored by thin-layer chromatography (TLC) on silica gel (1:1::Hex:EtOAc). After complete consumption of the starting material, the reaction mixture was quenched by the addition of water (10 ml) and dichloromethane (10 ml). The aqueous layer was extracted with two additional portions of dichloromethane (10 ml), and the combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo*. The crude product mixture was purified by silica-gel chromatography (eluting with hexanes/ethyl acetate (40:1) to give the S-MTPA-nitroketone ester (54 mg, 87%) as colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$): d 1.33 (m, 3H), 1.53 (m, 1H), 1.59 (m, 2H), 1.81 (m, 1H), 1.96 (m, 2H), 2.12 (d, 3H, *J* = 6.1 Hz), 2.41 (m, 2H) 3.55 (d, 3H, *J* = 7.1 Hz), 4.37 (m, 1H), 5.13 (m, 1H), 7.40 (m, 3H), 7.52 (m, 2H).

^{13}C NMR (500 MHz, $CDCl_3$): d 19.7, 27.1, 29.0, 29.4, 31.5, 38.8, 55.4, 72.3, 86.9, 87.3, 127.0, 128.4, 129.7, 132.3, 166.0, 206.2.

Preparation of the *R*-MTPA-nitroketone esters of *R*-isomer

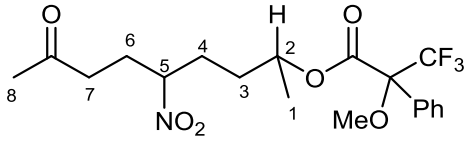


The *R*-MTPA-nitroketone ester of the *R*-isomer was prepared similar as describe above for the *S*-MTPA-nitroketone ester using S-(+)-MTPA-Cl (3S) to obtain a colorless oil (48 mg, 77%).

^{1}H NMR (500 MHz, $CDCl_3$): d 1.25 (m, 3H), 1.54-1.78 (m, 3H), 1.90-2.07 (m, 3H), 2.12 (s, 3H), 2.44 (m, 2H) 3.51 (d, 3H, *J* = 5.4 Hz), 4.47 (m, 1H), 5.13 (m, 1H), 7.40 (m, 3H), 7.51 (m, 2H).

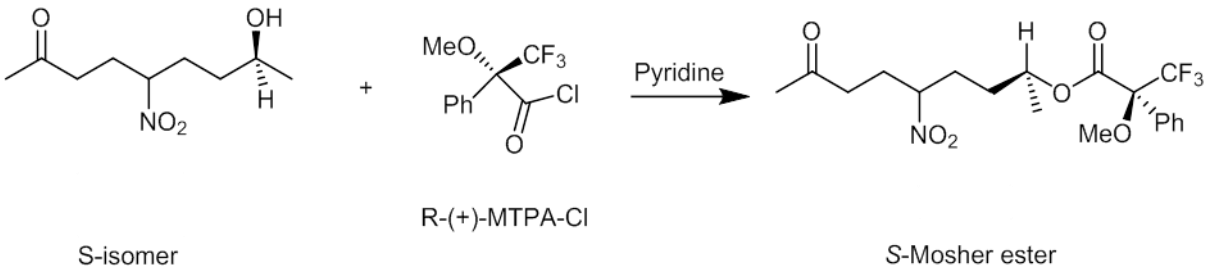
^{13}C NMR (500 MHz, $CDCl_3$): d 19.3, 27.2, 29.3, 29.9, 31.6, 38.7, 55.3, 72.6, 87.1, 127.3, 128.5, 129.7, 132.0, 166.1, 206.2.

Table S1 Δd^{SR} ($=d_S - d_R$) data for the *S*- and *R*-MTPA-nitroketone esters. For analysis and assignment of the stereochemical configuration, see Table S3.



Protons	δ *S*-ester (ppm)	δ *R*-ester (ppm)	$\Delta\delta^{SR}$ ($=\delta_S - \delta_R$) ppm	$\Delta\delta^{SR}$ ($=\delta_S - \delta_R$) Hz (500 MHz)
1-CH_3	1.34	1.25	+0.09	+45
2-CH	5.13	5.13	0.00	0
3-CH2(A)	1.53	1.58	-o.05	-25
3-CH2(B)	1.83	1.94	-0.11	-55
4-CH2	1.60	1.73	-0.13	-65
5-CH	4.36	4.47	-0.11	-55
6-CH2	1.97	2.03	-0.06	-30
7-CH2	2.41	2.44	-0.03	-15
8-CH3	2.11	2.12	-0.01	-5
OCH3	3.55	3.51	+0.04	+20
Ar	7.41	7.40	+0.01	+5
Ar	7.52	7.51	+0.01	+5

Preparation of the *S*-MTPA-nitroketone esters of *S*-isomer



R-(+)-MTPA-Cl

S-isomer

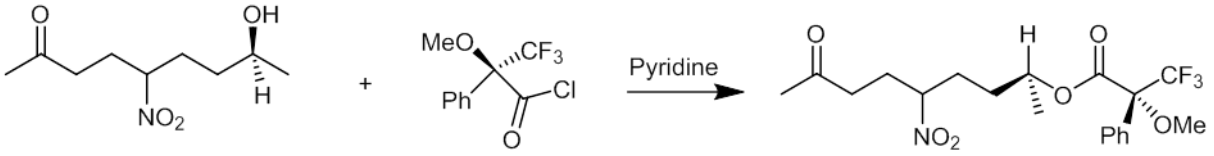
S-Mosher ester

To a stirred solution of S-isomer (30 mg, 0.148 mmol) and dry pyridine (37µl, 0.458 mmol, 3.1 equiv.) in dry dichloromethane (5 ml) at room temperature, R-(-)-MTPA-Cl (3**R**, 57 µl, 0.296 mmol, 2.0 equiv.) was added. The reaction progress was monitored by thin-layer chromatography (TLC) on silica gel (1:1::Hex:EtOAc). After complete consumption of the starting material, the reaction mixture was quenched by the addition of water (10 ml) and dichloromethane (10 ml). The aqueous layer was extracted with two additional portions of dichloromethane (10 ml), and the combined organic layers were dried (Na_2SO_4), filtered and concentrated in *vacuo*. The crude product mixture was purified by silica-gel chromatography (eluting with hexanes/ethyl acetate (40:1) to give the S-MTPA-nitroketone ester (56 mg, 90%) as colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$): d 1.25 (d, *J* = 6.3 Hz, 3H), 1.56 (m, 1H), 1.72 (m, 2H), 1.95 (m, 1H), 2.02 (m, 2H), 2.11 (s, 3H), 2.44 (m, 2H) 3.49 (s, 3H), 4.44 (m, 1H), 5.09 (m, 1H), 7.40 (m, 3H), 7.49 (m, 2H).

^{13}C NMR (500 MHz, $CDCl_3$): d 19.3, 27.3, 29.6, 29.9, 31.7, 38.7, 55.3, 72.9, 87.4, 127.4, 128.5, 129.7, 132.0, 166.0, 206.2.

Preparation of the *R*-MTPA-nitroketone esters of *S*-isomer



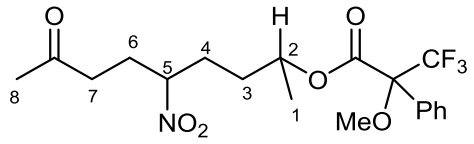
S-isomer S-(+)-MTPA-Cl *R*-Mosher ester

In an entirely analogous fashion, the *R*-MTPA-nitroketone ester was prepared using S-(+)-MTPA-Cl (**3S**) to get colorless oil (52 mg, 85%)

^{1}H NMR (500 MHz, $CDCl_3$):d 1.32 (d, *J* = 6.3 Hz, 3H), 1.52 (m, 2H), 1.63(m, 1H), 1.79 (m, 1H), 1.93 (m, 2H), 2.10 (s, 3H), 2.39 (m, 2H) 3.55 (s, 3H), 4.34 (m, 1H), 5.07 (m, 1H), 7.39 (m, 3H), 7.51 (m, 2H).

^{13}C NMR (500 MHz, $CDCl_3$):d 19.8, 27.2, 29.4, 29.9, 31.8, 38.7, 55.5, 72.9, 87.3, 127.0, 128.4, 129.6, 132.5, 166.0, 206.2.

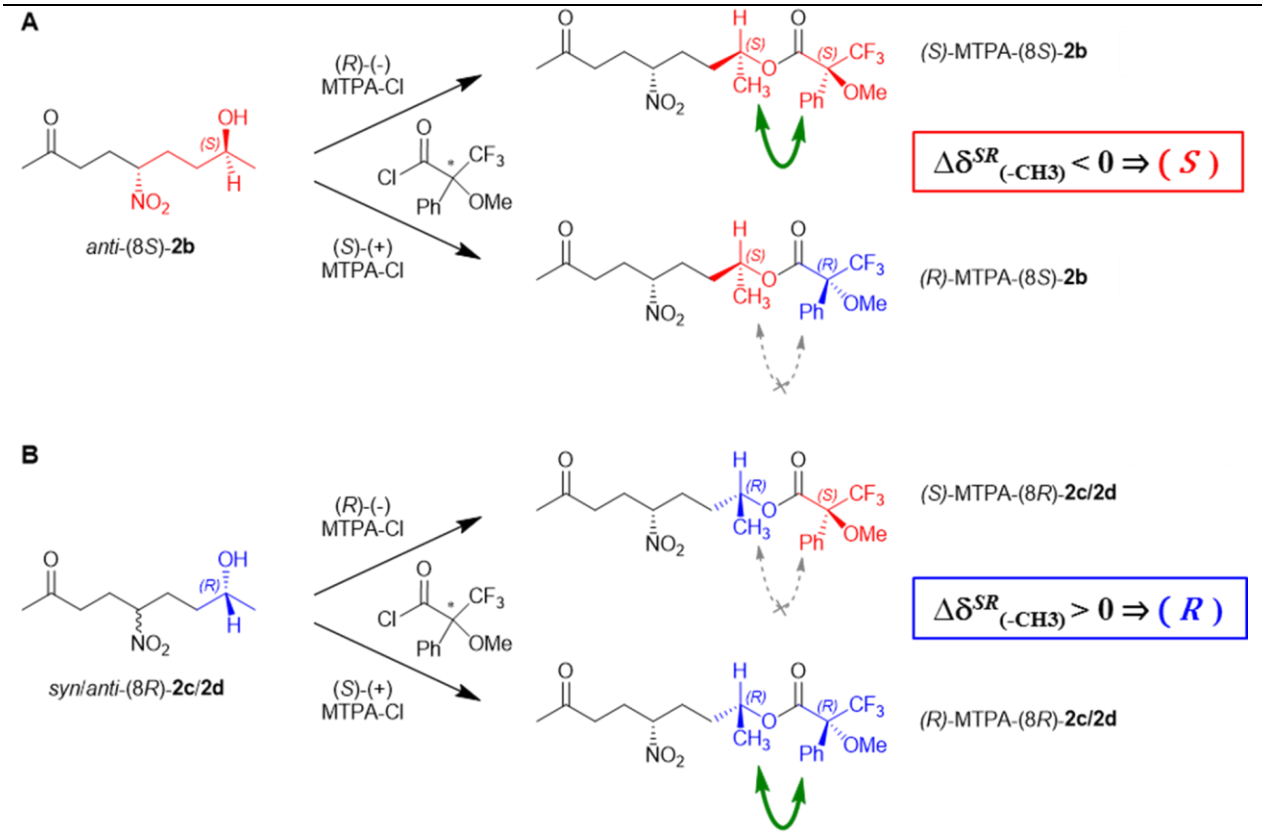
Table S2 Δd^{SR} ($=d_S - d_R$) data for the *S*- and *R*-MTPA-nitroketone esters. For analysis and assignment of the stereochemical configuration, see Table S3.



Protons	d *S*-ester (ppm)	d *R*-ester (ppm)	Δd^{SR} ($=d_S - d_R$) ppm	Δd^{SR} ($=d_S - d_R$) Hz (500 MHz)
1-CH_3	1.25	1.32	-0.07	-35
2-CH	5.09	5.07	+0.02	+10
3-CH2(A)	1.56	1.63	-o.07	-35
3-CH2(B)	1.95	1.79	+0.16	+80
4-CH2	1.72	1.52	+0.20	+100
5-CH	4.44	4.34	+0.10	+50
6-CH2	2.02	1.93	+0.09	+45
7-CH2	2.44	2.39	+0.05	+25
8-CH3	2.11	2.10	+0.01	+5
OCH3	3.49	3.55	-0.06	-30
Ar	7.40	7.39	+0.01	+5
Ar	7.49	7.51	-0.02	-10

Table S3

Mosher-ester analysis[9] for the determination of the absolute configuration of stereoisomeric hydroxyketones **2b** and **2c/2d.** Synthesis and differential chemical shift of the carbinol-methylene of (*S*)-MTPA-**2b** and (*R*)-MTPA-**2b** derived from hydroxyketone *anti*-(8*S*)-**2b (A),** and from (*S*)-MTPA)-**2c/2d** and (*R*)-MTPA)-**2c/2d** derived from *syn/anti*-(8*R*)-**2c/2d (B).** For synthesis and NMR data, see Tables S1, S2. The observed anisotropic shielding towards the carbinol-methylene induced by the phenyl group of one of each two MTPA-esters is indicated with a green arrow (in **A** top, in **B** bottom). The δ / ppm and Δδ / ppm values are summarized in (**C**); [$\Delta\delta^{SR}_{-CH3} = \delta_{(S)\text{-MTPA-}2} - \delta_{(R)\text{-MTPA-}2}$] ≙ differential chemical shift (Δδ / ppm) of carbinol methylene of (*S*)-MTPA-**2** and from (*R*)-MTPA-**2.**



C # 	Substance	$\delta_{(\text{-CH3})}$ / ppm	$\Delta\delta^{SR}$ (=δ_S - δ_R) / ppm (Hz)	
1	(*S*)-MTPA-Ester-**2b**	1.25	-0.07 (-35)	$\Delta\delta^{SR}_{(\text{-CH3})} < 0$
2	(*R*)-MTPA-Ester-**2b**	1.32		⇒ ***2b*:** ***(8S)-OH***
3	(*S*)-MTPA-Ester-**2c/2d**	1.34	+0.09 (+45)	$\Delta\delta^{SR}_{(\text{-CH3})} > 0$
4	(*R*)-MTPA-Ester-**2c/2d**	1.25		⇒ ***2c/2d*: *(8R)-OH***

Figures

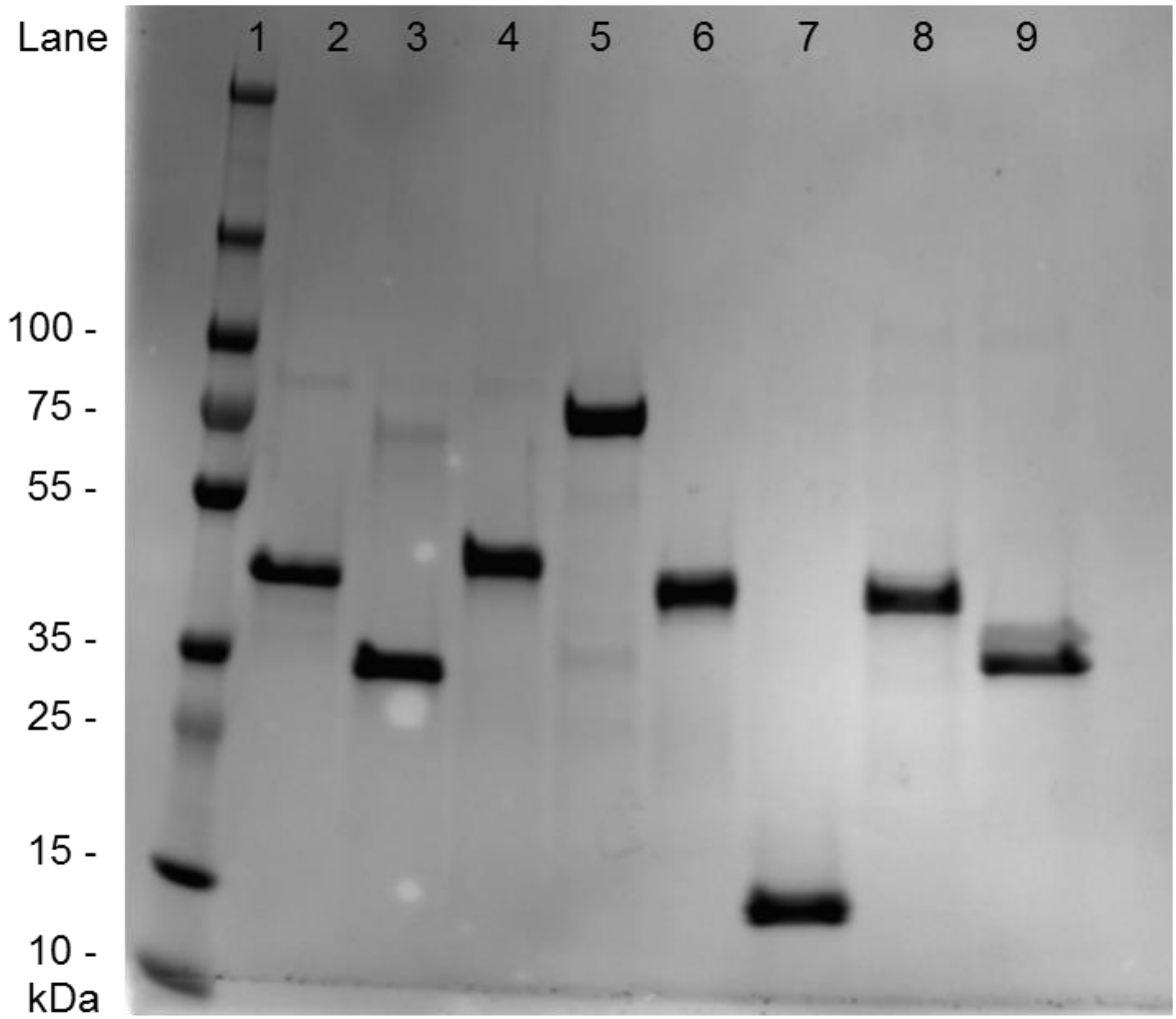


Figure S1 Characterization of the enzymes used in this study using a 4-15% Coomassie stained SDS-PAGE (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad Laboratories, Germany). Lane 1: PageRuler Prestained Protein Ladder Plus (Thermo Scientific); lane 2: LbADH-SBP (32.4 kDa), lane 3: GDH-SBP (36.6 kDa) lane 4: Gre2p-SBP (46.6 kDa), lane 5: Gre2p-HOB (72.7 kDa); lane 6: Gre2p-ST (40.8 kDa); lane 7: SC (15.4 kDa); lane 8: GDH-TEV-His: 32.9 kDa; lane 9: GDH 28.5 kDa.

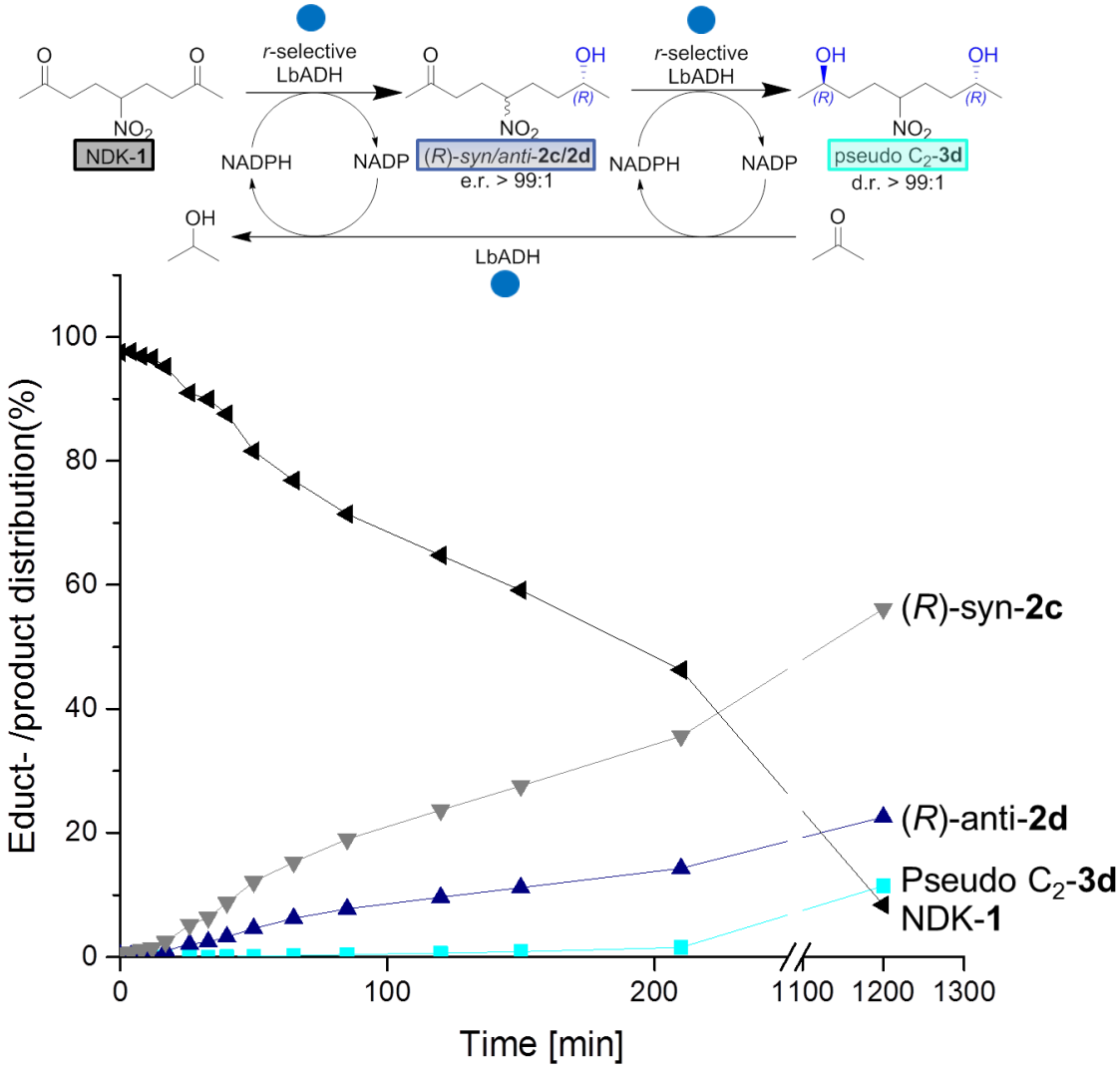


Figure S2 Time dependent (*R*)-selective conversion of NDK-**1** by the LbADH-SBP to (*r*)-syn/anti-**2c/d** hydroxyketone and further reduction to pseudo C_2-diol **3d**. The LbADH-SBP was supplied with 2-propanol as a cosubstrate for the cofactor regeneration of NADPH. The reaction schemes are shown above the corresponding product formation graph. A reaction mixture containing 0.5 µM KRED and 10 mM NDK-**1** was incubated at 30°C, 1000 rpm. Samples were taken after defined time intervals and analyzed by chiral HPLC.

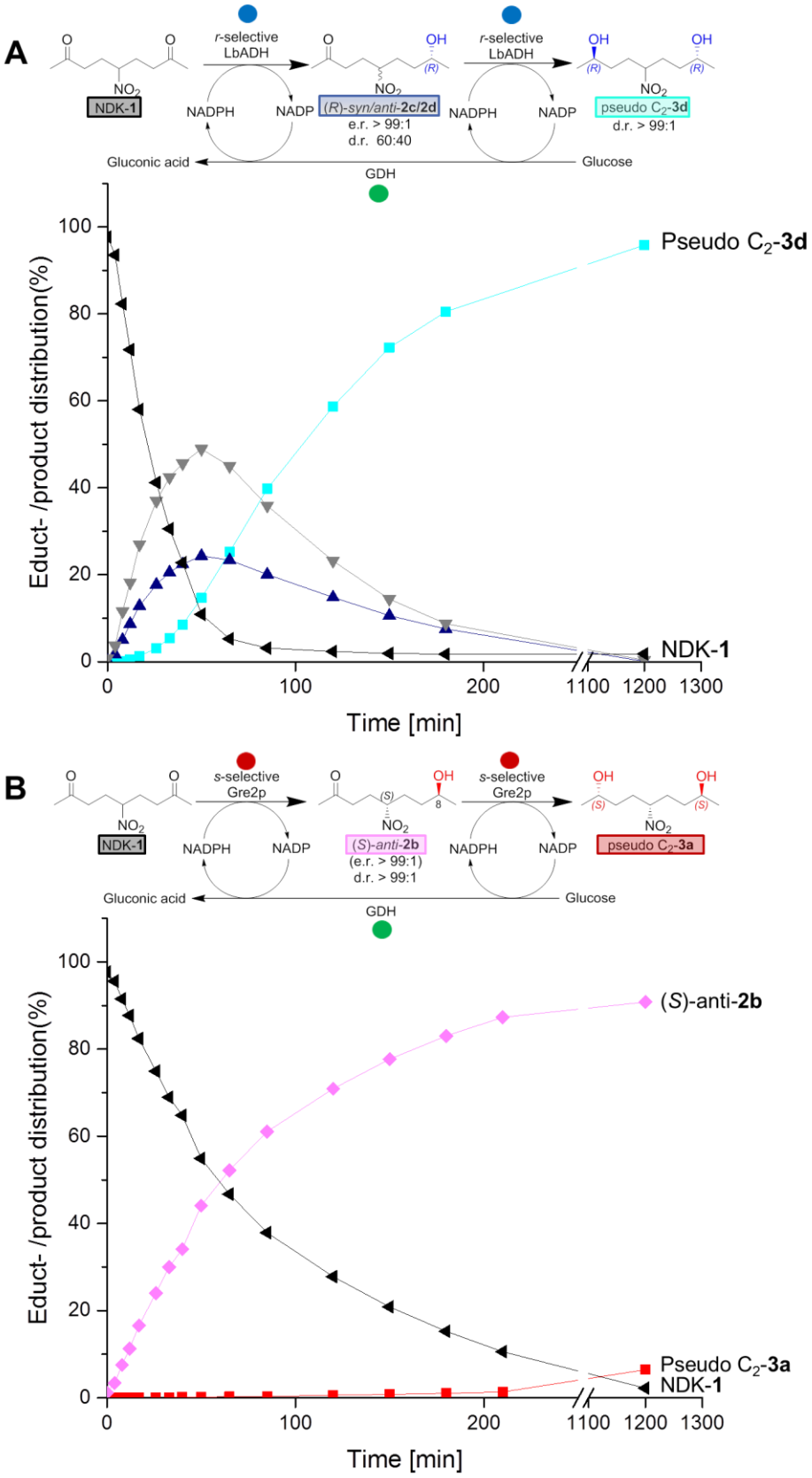


Figure S3 Time dependent stereoselective conversion of NDK-**1** by (**A**) LbADH-SBP to produce (*r*)-syn/anti-**2c/d** hydroxyketones and further reduction to pseudo C_2-diol **3d** and (**B**) Gre2p-SBP to generate (*S*)-anti-**2b** hydroxyketone, which is slowly further

reduced to pseudo C_2-diol **3a**. The KREDs were supplied with reduced cofactor by GDH catalyzed in situ NADP(H)-regeneration. The reaction schemes are shown above the corresponding product formation graph. A reaction mixture containing 0.5 µM KRED and 10 mM NDK-**1** was incubated at 30°C, 1000 rpm and as NRE 5 µM GDH with 100 mM glucose. Samples were taken after defined time intervals and analyzed by chiral HPLC. The absolute configuration of the hydroxyketone products **2b/c/d** was verified by Mosher ester analysis (Tables S1-S3)

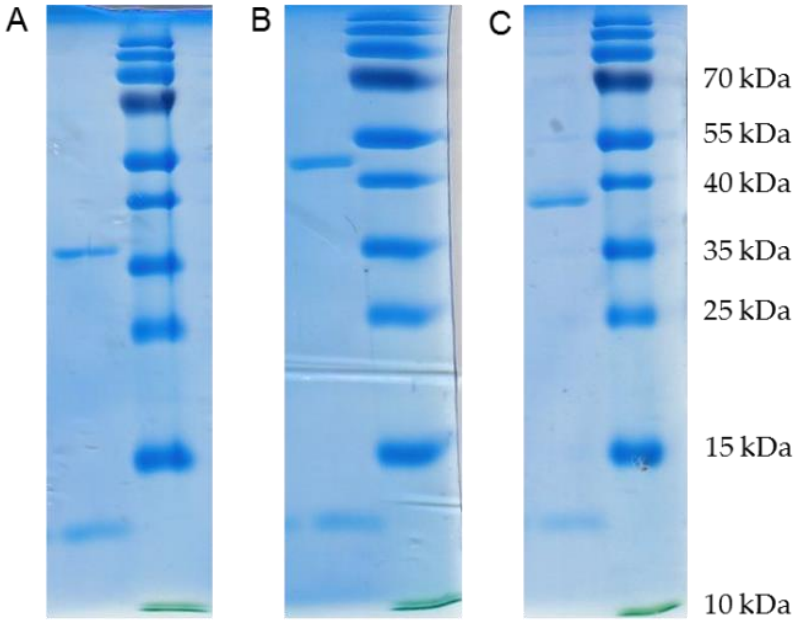


Figure S4: Coomassie stained SDS-PAGE gel of streptavidin functionalized magnetic beads coated with the stereoselective ketoreductases (**A**) LbADH-SBP (33 kDa), (**B**) Gre2p-SBP (46 kDa), and (**C**) GDH-SBP (37 kDa) after cooking at 95°C. The band at ~12 kDa shows the STV-monomers, which are not covalently linked to the bead-surface.

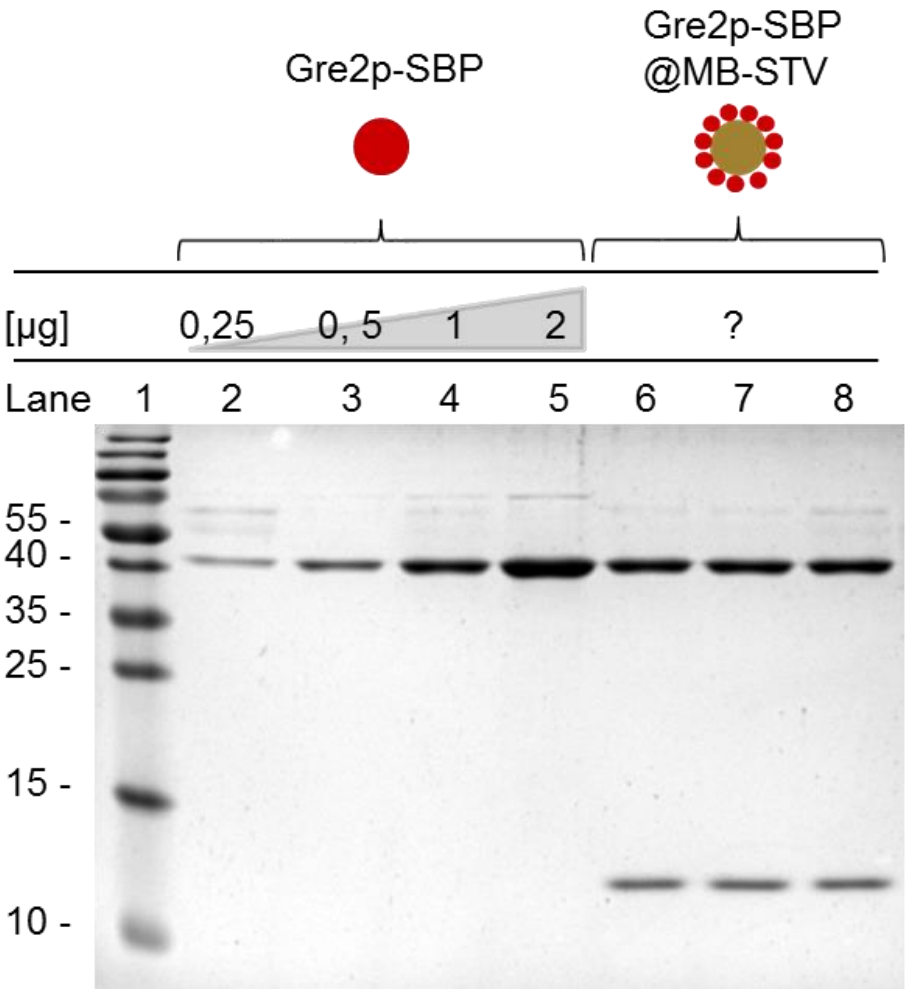


Figure S5 Representative 12% Coomassie stained SDS-PAGE for the characterization of the binding capacity of Gre2p-SBP at magnetic beads by comparative greyscale analysis. Lane 1: PageRuler Prestained Protein Ladder (Thermo Scientific); lane 2-5: Gre2p-SBP (46.6 kDa) in variabel amounts ranging from 0.25 µg to 2 µg; lane 6-8: Supernatant obtained from denatures 100 µg of Gre2p-SBP@STV-MB. The band at ~12 kDa shows the STV-monomers, which are not covalently linked to the bead-surface.

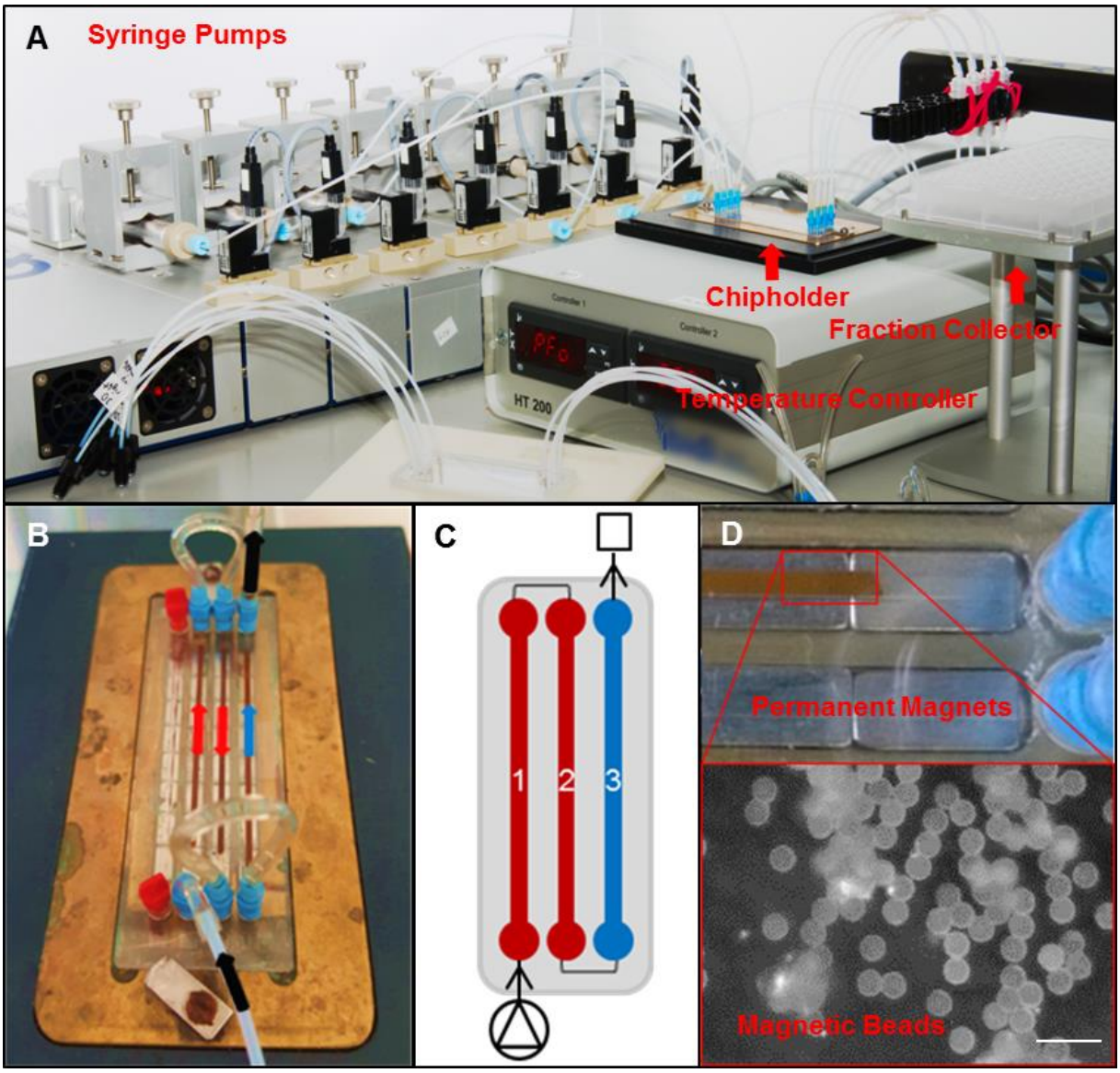


Figure S6 Photograph of the fluidic setup used in this study. (**A**) The syringe pumps holding the cofactor/substrate solution were connected with the four straight channel PMMA chip by conventional PTFE tubing. The reactor-outflow was automatically fractionated into a 96-well plate by the rotAXYS Positioning System, which was modified for parallel sampling of up to 4 samples. Photograph (**B**) and schematic representation (**C**) of the compartmentalized reactor from Figure 2C. Note that the two red modules produce only the (*S*)-configured hydroxyketone **2b**, which is further (*R*)-selectively converted in the sequentially coupled blue module to *meso*-anti-diol **3c**. The inlet and outlet for the reaction mixture are indicated by black arrows. The compartments can be interconnected by conventional PTFE-tubing, which leads to up to four possible compartments per chip. (**D**) The chips were embedded in a temperature controlled chipholder with Nd magnets arranged underneath to hold the magnetic beads in the

reactor. The magnetic zone holds a volume of ~10 µl, which is reduced by to about 6.8-7.2 µl when 4.5 mg beads are loaded. The inset shows a microscope image of the MBs (scalebar 5 µm).

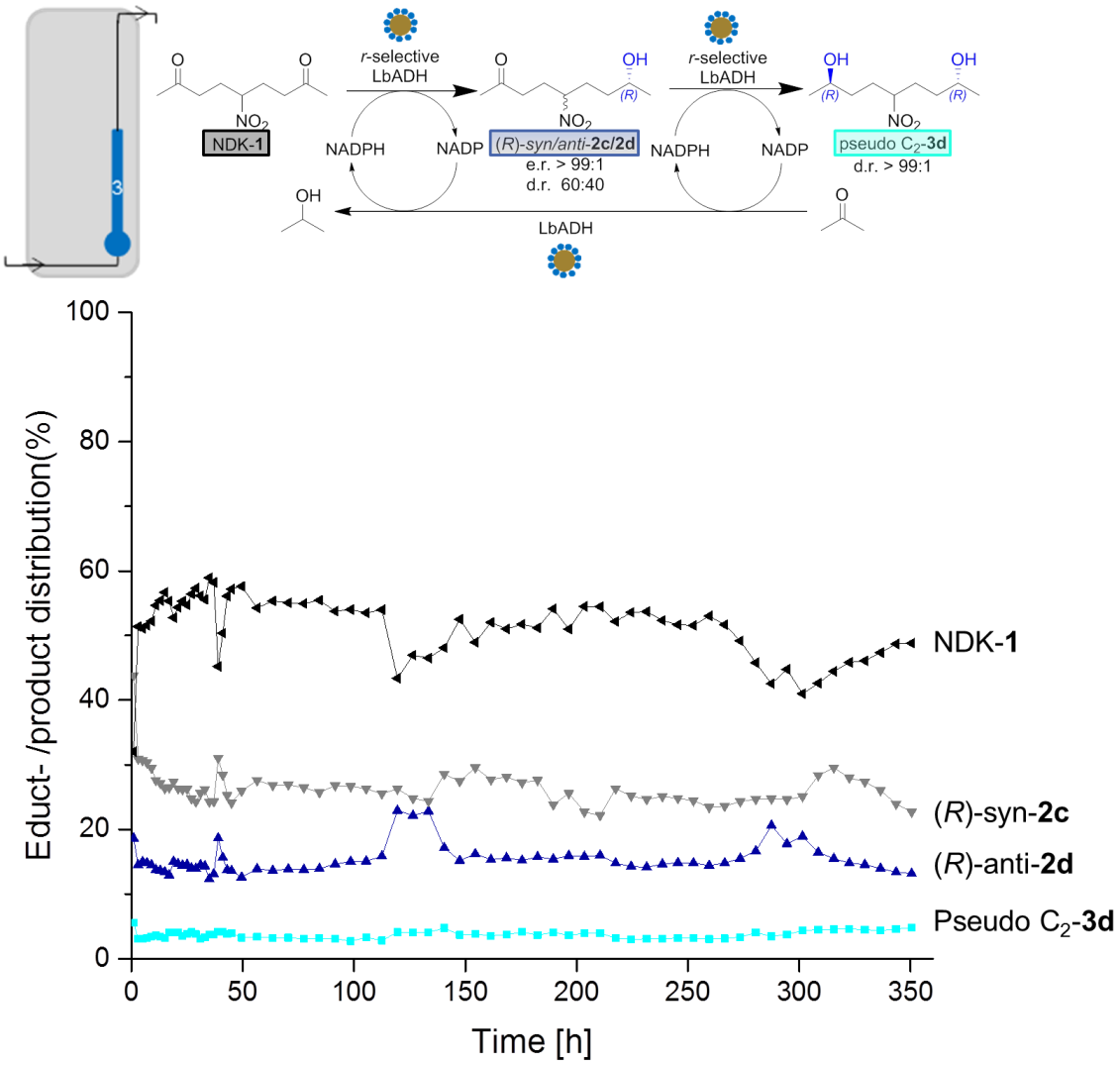


Figure S7 Educt- and product distribution in the outflow of a module half filled with LbADH-SBP@MB-STV analyzed by chiral HPLC. The LbADH-SBP converts NDK-**1** with (*R*)-selectivity to produce (*r*)-syn/anti-**2c/d** hydroxyketone which are further reduced to pseudo C_2-diol **3d**. The LbADH-SBP was supplied with 2-propanol as a cosubstrate for the cofactor regeneration of NADPH. Chip configurations and reaction schemes are shown above the corresponding product formation graphs. The module continuously generated (*R*)-products over 350 h.

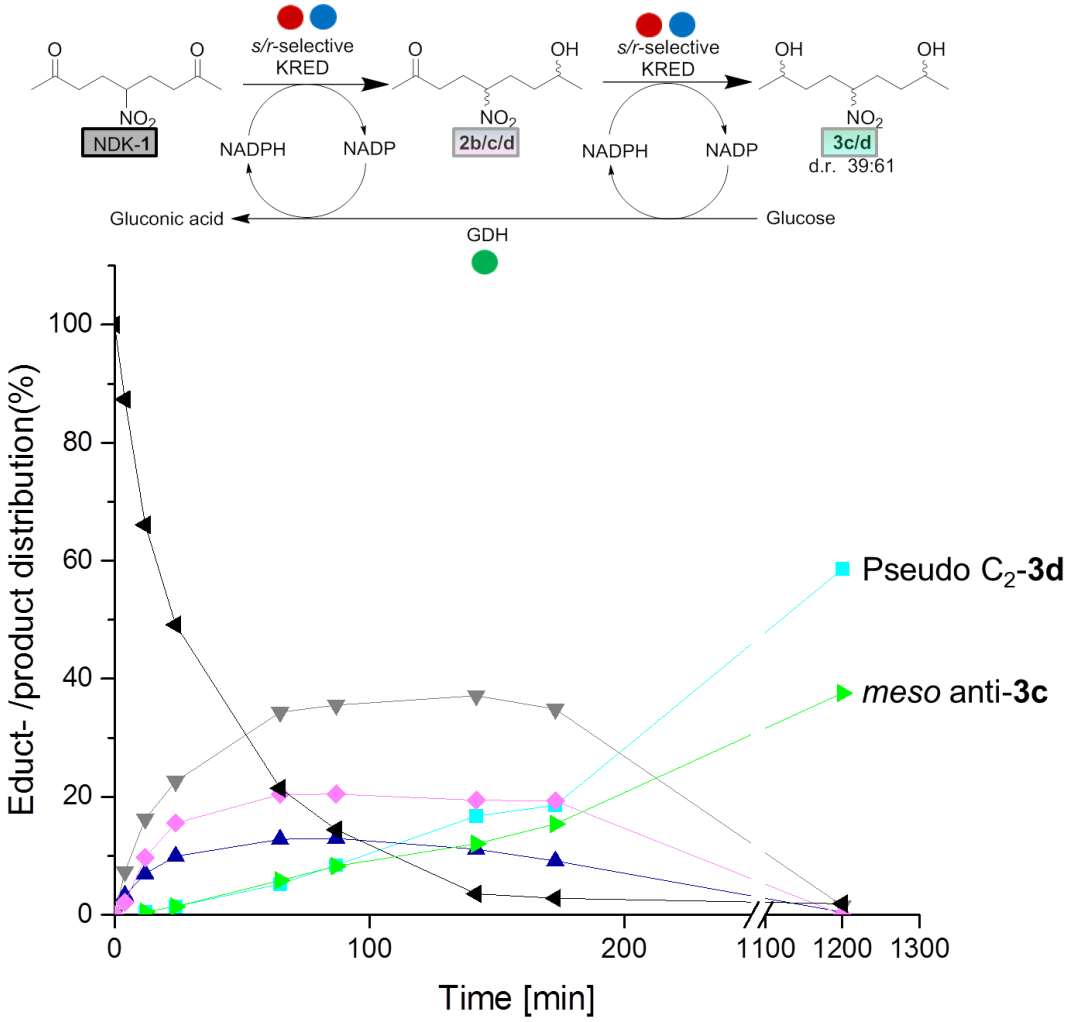


Figure S8 Time dependent one-pot conversion of NDK-**1** conducted with an identically enzyme ratio as used in the sequential packed-bed reactor shown in Figure 2C (26% Gre2p-SBP, 3% LbADH-SBP, 71% GDH-SBP). The reaction mixture containing 0.5 µM KRED (0.45 µM Gre2p-SBP and 0.05 µM LbADH-SBP) and 1.23 µM GDH-SBP was incubated with 5 mM NDK-**1** at 30°C, 1000 rpm. Samples were removed after defined time intervals and analyzed by chiral HPLC. Note that the one-pot reaction leads to unselective formation of hydroxyketones **2b/c/d**, which are further reduced to mainly pseudo C_2-diol **3d** along with *meso*-anti-diol **3c** (d.r. 39:61).

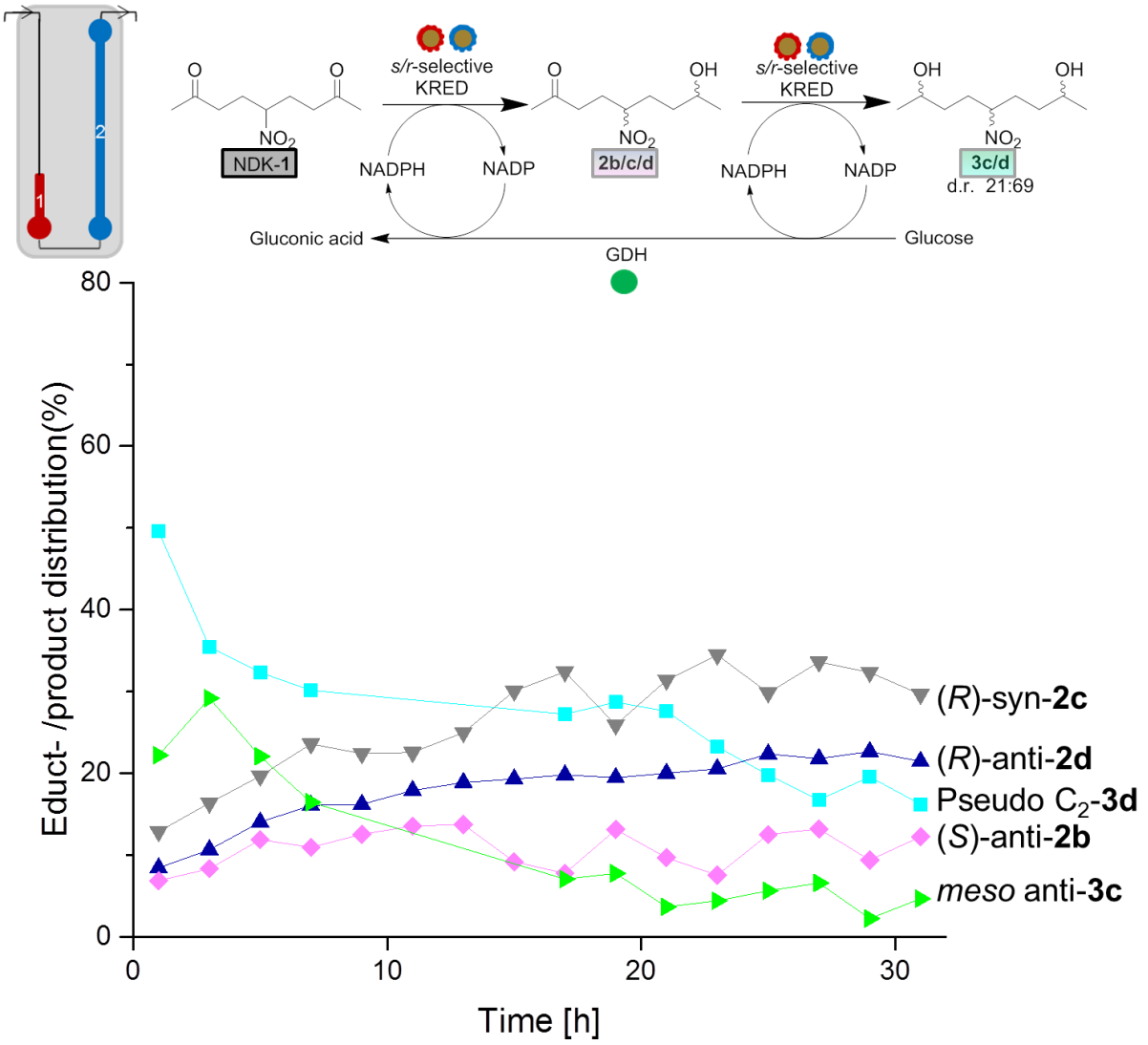


Figure S9 Represetative data obtained from differently configured microreactors containing variable relative amounts of bead-immobilized enzymes (Fig. 3, main text). The stereoselective reduction of NDK **1** was done in a two-compartment microfluidic packed-bed reactor with 10 µM GDH as soluble NRE. The first compartment contained 1 mg of Gre2p-SBP@MB-STV (illustrated by the red color) and the second 4 mg of LbADH-SBP@MB-STV (blue), corresponding with a relative amount of bead-immobilized enzymes of 1:4. Chip configurations and reaction schemes are shown above the corresponding product formation graphs. Note that the blue module exclusively generates (*R*)-configured hydroxyketones **2c/d** and diol **3d** products, whereas the red module produces only the (*S*)-configured hydroxyketone **2b**. In the coupled reaction where NDK-1 is sequentially reduced by the red and the blue module, *meso*-anti-diol **3c** is formed with an inital d.r. of 21:69 (d.r. after 3±1h: 45:5).

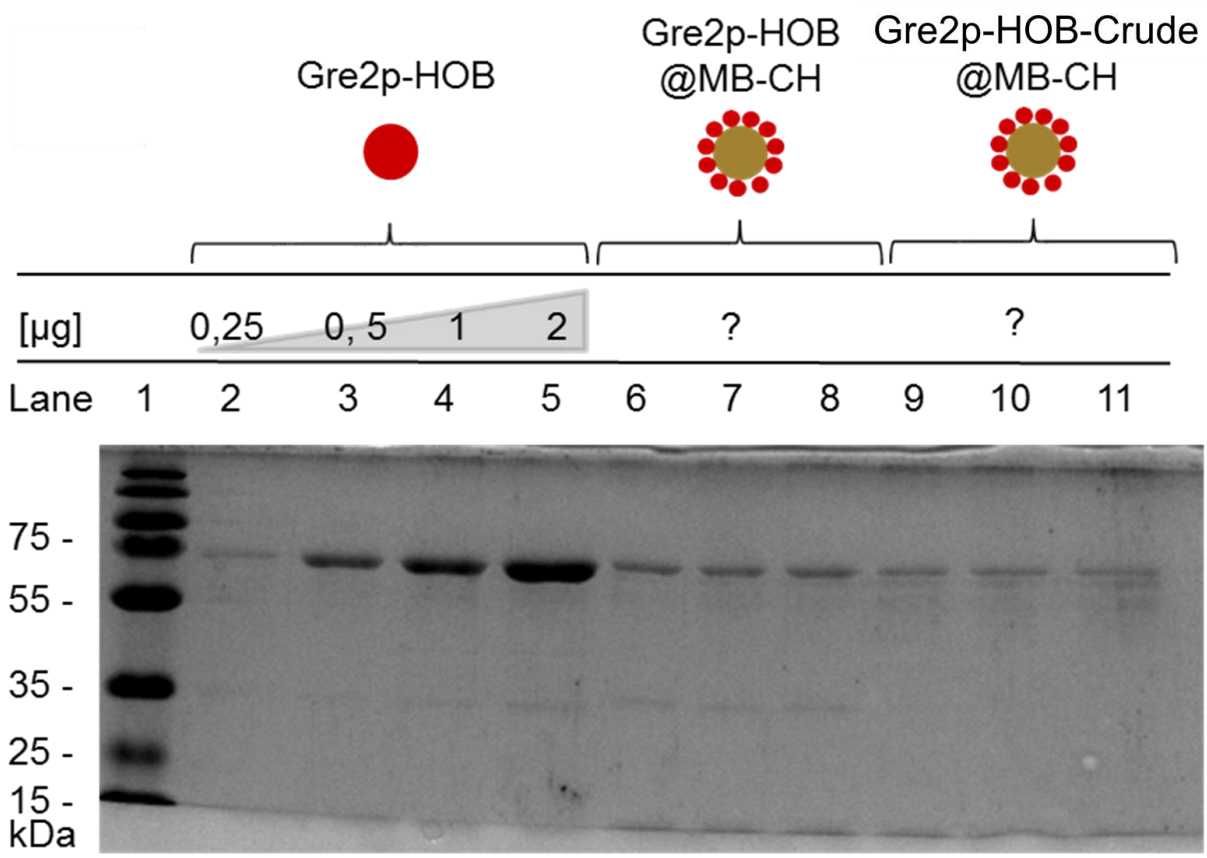


Figure S10 Representative 16% Coomassie stained SDS-PAGE for the characterization of the binding capacity of Gre2p-HOB and Gre2p-HOB-crude at CH functionalized magnetic beads by comparative greyscale analysis. Lane 1: PageRuler Prestained Protein Ladder plus (Thermo Scientific); lane 2-5: Gre2p-HOB in different amounts ranging from 0.25 µg to 2 µg (72.7 kDa); lane 6-8: 100 µg of Gre2p-HOB@MB-CH and lane 9-11: 100 µg of Gre2p-HOB-Crude@MB-CH.

Table S4 Remaining specific activities of free enzymes after 22h preincubation at 30°C.

	MW	Homomeric subunits	Specific activty[a]		
	[$g_{protein}$ $mol_{subunit}^{-1}$]		[µ$mol_{substrate}$ min^{-1} $mg_{protein}^{-1}$]	[µ$mol_{substrate}$ min^{-1} µ$mol_{subunit}^{-1}$]	Remaining activity [%]
GDH-SBP	36598	4	0.9 ± 0.1	33 ± 4	100 ± 22
LbADH-SBP	32338		13.7 ± 1	443 ± 32	104 ± 10
Gre2p-SBP	46640	1	1.1 ± 0.1	51 ± 5	50 ± 17

[a] Activities were determined compared to the freshly prepared enzymes (Table 1, main manuscript) using chiral HPLC. Data represent the mean of triplicate analyzes ± 1 SD.

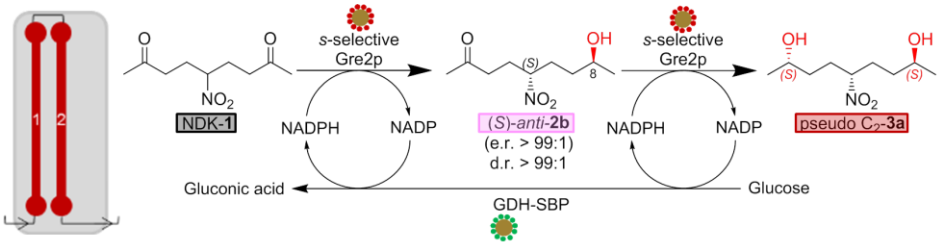
Table S5 Biocatalytic activities of self-immobilizing enzymes.

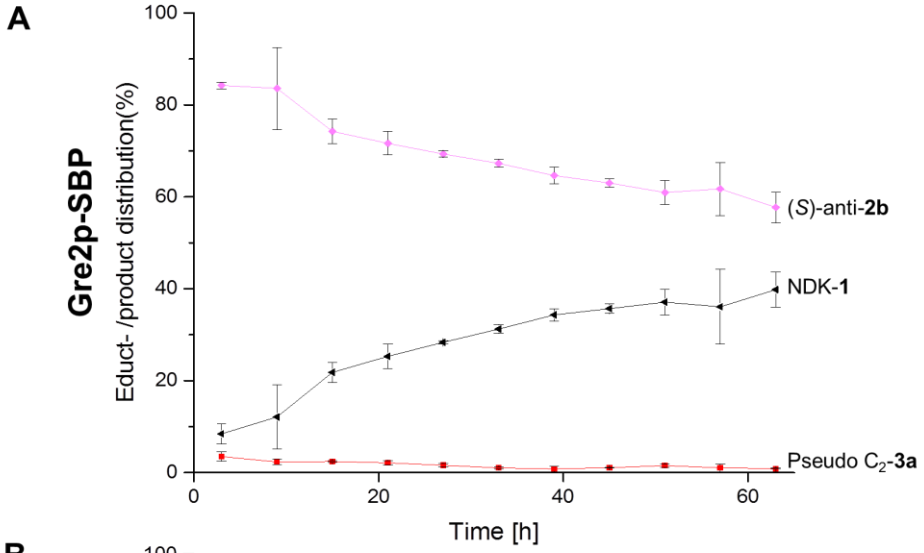
	LbADH-SBP-Crude	Gre2p-HOB-Crude	Gre2p-ST-Crude	MB-STV	Products [%][c]	
					R[a]	*S*[b]
1	x	-	-	-	>99	<1
2	-	x	-	-	<1	>99
3	-	-	x	-	<1	>99
4	x	x	x	x	>99	<1

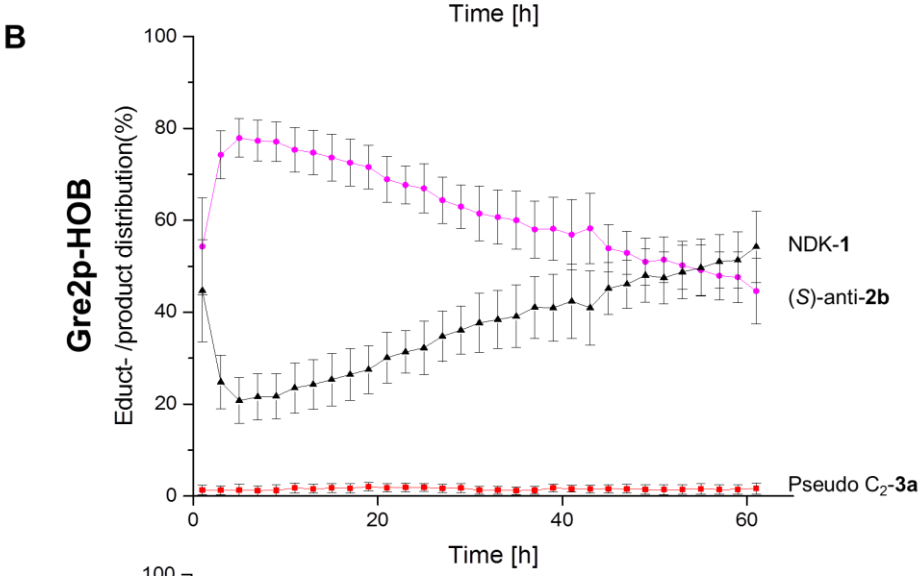
[a] Overall amount of *R*-configured hydroxyketones **2c**/**2d** and diol **3d** as determined by chiral HPLC analysis.

[b] Only the *S*-configured hydroxyketone **2b** was produced under the given reaction conditions.

[c] Data represent the mean of triplicate analyzes.


1
2
O
O
NO2
NDK-1
s-selective
Gre2p
NADPH
NADP
O
OH
(S)
8
NO2
(S)-anti-2b
(e.r. > 99:1)
d.r. > 99:1
s-selective
Gre2p
NADPH
NADP
OH
OH
(S)
(S)
NO2
pseudo C2-3a
Gluconic acid
Glucose
GDH-SBP


A
Gre2p-SBP
Educt- /product distribution(%)
Time [h]
(S)-anti-2b
NDK-1
Pseudo C2-3a


B
Gre2p-HOB
Educt- /product distribution(%)
Time [h]
NDK-1
(S)-anti-2b
Pseudo C2-3a

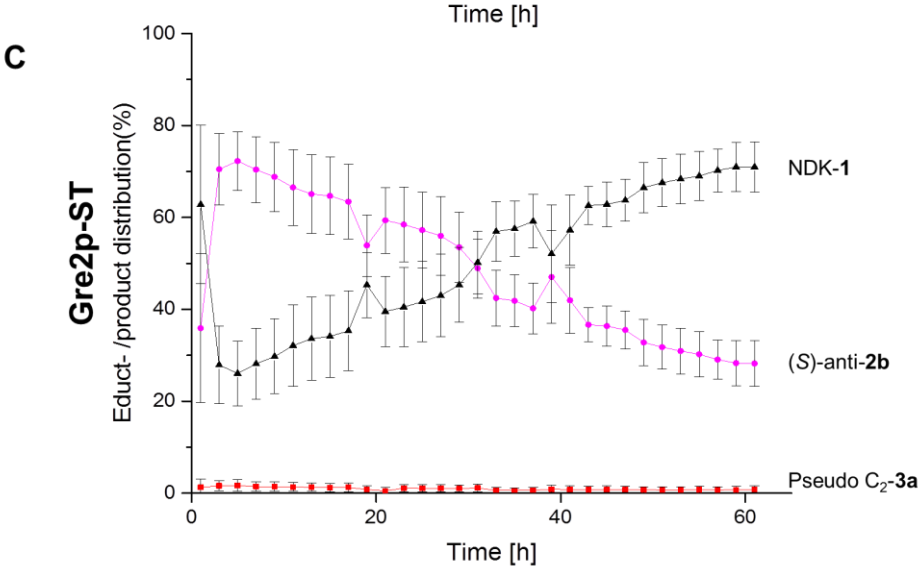

C
Gre2p-ST
Educt- /product distribution(%)
Time [h]
NDK-1
(S)-anti-2b
Pseudo C2-3a

Figure S11 Direct comparison of two interconnected compartments, each harboring 3 mg GDH-SBP@MB-STV mixed with either 1.5 mg (**A**) Gre2p-SBP@MB-SBP, (**B**) Gre2p-HOB@MB-CH, or (**C**) Gre2p-ST@MB-SC. Chip configurations and reaction schemes are shown above the product formation graphs. The curves show the amounts of educt/products determined in the outflow of the reactor by chiral HPLC. Note that the red module produces only the (S)-configured hydroxyketone **2b**. Data represent the mean of at least duplicate analyzes, error bars indicate standard error of the mean. Also note that, regardless of the covalent nature of HOB- and SC-based immobilization, the enzymatic activity of Gre2p decreases over time, similar as observed for the non-covalent SBP tag.

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