Supporting Information

Rapid, selective and stable HaloTag-LbADH immobilization directly from crude cell extract for the continuous biocatalytic production of chiral alcohols

Johannes Döbber\textsuperscript{a, b}, Martina Pohl\textsuperscript{a}, Steven V. Ley\textsuperscript{b}, Biagia Musio\textsuperscript{b}\textsuperscript{*}

\textsuperscript{a} IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich, Germany.
\textsuperscript{b} Department of Chemistry, University of Cambridge, Cambridge, CB21EW, UK.

\textsuperscript{*}bm450@cam.ac.uk

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S1. General

All reagents and solvents were obtained from commercial sources and used without further purification. Flash column chromatography was performed using high-purity grade silica gel (Merck grade 9385) with a pore size 60 Å and 230–400 mesh particle size under air pressure. Analytical thin layer chromatography (TLC) was performed using silica gel 60 F254 pre-coated glass backed plates and visualized by ultraviolet radiation (254 nm) and/or potassium permanganate solution as appropriate. 1H NMR spectra were recorded on a 600 MHz Avance 600 BBI Spectrometer as indicated. Chemical shifts are reported in ppm with the resonance resulting from incomplete deuteration of the solvent as the internal standard (CDCl3: 7.26 ppm). 13C NMR spectra were recorded on the same spectrometer with complete proton decoupling. Chemical shifts are reported in ppm with the solvent resonance as the internal standard (13CDCl3: 77.16 ppm, t). 19F NMR spectra were recorded on a 376 MHz Avance III HD Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm with CFCl3 as the external standard (CFCl3: 0.00 ppm). Specific optical rotation was recorded on a Perkin-Elmer Model 343 digital polarimeter, using a Na/Hal lamp set at 589 nm and with a path length of 100 mm. [α]D values were measured using spectroscopy grade solvent at the specified concentration (in g/100 mL) and temperature. If not otherwise stated, chiral HPLC analysis was conducted on an Agilent 1100 Series Chromatography system using mixtures of hexane/2-propanol as eluent on Chiralpak OD-H, ChiralART SA or ChiralART SB columns. Chiral GC analysis was conducted on anAgilent Technologies 6890N system equipped with a β-cyclodextrin column (CP-Chiralsil-Dex CB 25 m, 0.25 mm) and an FID detector.

S2. Construction of expression plasmid, cell cultivation and preparation of lyophilized crude cell extracts

The construction of the expression plasmid pET22b-halotag-lbadh was performed with the Gibson Assembly ® Cloning Kit (NEB). The DNA sequence encoding for the HaloTag and the linker for the connection to the LbADH was synthesized as linear fragments (Eurofins Genomics, Ebersberg, Germany). Specific overhangs for the integration of this sequence into pET22b which was opened by restriction digestion with NdeI were introduced by PCR (Primer 1, 2; see S11). After successful ligation, the DNA sequence encoding for the LbADH was amplified and again, specific overhangs were introduced by PCR for the integration into pET22b-halotag (Primer 3, 4; see S11). pET22b-halotag was linearized by PCR, too (Primer 5, 6; see S11), and ligation was performed according to the instructions given by NEB.

E.coli BL21 (DE3) cells were transformed with pET22b-halotag-lbadh and production of the resulting fusion enzyme was performed in LB medium at 20 °C. For cell disruption, cells were resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM MgCl₂. A detailed protocol about the cell cultivation and disruption is described in Döbber & Pohl 2017. The resulting crude cell extract was frozen at -20 °C and lyophilized (Alpha 1-4 LD plus, Christ, Osterode am Harz, Germany). The lyophilized crude cell extract was stored at -20 °C for further use.

Cloning, cell cultivation and chromatographic purification of the untagged LbADH are described elsewhere.
S3 General batch procedure for the immobilization of HaloTag-LbADH on the HaloLink™ resin.

Immobilization in batch was performed on HaloLink™ Resin (Promega, Madison, WI, USA). Lyophilized crude cell extract (25mg/ml) was dissolved in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM MgCl₂x6H₂O and 0.5 vol% Triton X-100. 200 µl of the HaloLink™ slurry were transferred into a 1.5 ml microreaction tube (Eppendorf, Hamburg, Germany). The resin was washed three times with 1 ml of the mentioned phosphate buffer (see above) and finally suspended in 100 µl buffer. Immobilization was started by adding 100 µl of the crude cell extract solution (25 mg/ml, see above) and the mixture was incubated for 1h at 25 °C and 1200 rpm in a ThermoMixer® (Eppendorf, Hamburg, Germany). Afterwards, the resin was washed three times with 1 ml phosphate buffer (see above) and immobilizates were directly used or stored at 4 °C.

S4. Determination of protein concentration and activity assay

Protein concentrations of free and immobilized proteins were determined with the BC Assay Protein Quantitation Kit (Interchim, Montluçon, France) as described elsewhere.¹ For the characterization of the immobilizates in comparison to the untagged, free LbADH, the activity was determined by following the conversion of benzaldehyde to benzyl alcohol. Assays were performed with 20 mM benzaldehyde in a total volume of 1ml containing 50 mM potassium phosphate, pH 7.0, and 1 mM MgCl₂x6H₂O at 25 °C as well as 1200 rpm in a ThermoMixer® (Eppendorf). 100 µg of immobilized HaloTag-LbADH and 20 µg of free, untagged LbADH, respectively, were used for one assay. NADPH was added either equimolar with respect to benzaldehyde or was used in a concentration of 0.5 mM together with 10 % (v/v) 2-propanol when cosubstrate based cofactor regeneration was applied. Benzaldehyde and benzyl alcohol were detected by HPLC on a Chiralpak IE column using an Agilent 1260 Infinity Quarternary LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1260 Diode Array Detector. The column was operated with a mobile phase consisting of 50 % (v/v) acetonitrile and 50 % (v/v) deionized ultra-pure water with an isocratic flow of 1 ml/min at 20 °C. Benzaldehyde was detected at 250 nm with an approximate retention time of 5.2 min and benzyl alcohol was detected at 215 nm with a retention time of 3.9 min. Toluene was used as an internal standard and was detected at 215 nm with a retention time of 6.3 min. One Unit (U) of specific activity is defined as the amount of enzyme in mg which catalyzes the formation of 1 µmol benzyl alcohol per minute under the described conditions.

S5. General procedure for the HaloTag-LbADH catalyzed reduction of acetophenone 1a in repetitive batch in the presence of different 2-propanol concentrations.

To analyze the activity and stability of immobilized HaloTag-LbADH in the presence of 2-propanol, a repetitive batch was performed. Reactions were performed in 1.5 ml microreaction tubes (Eppendorf, Hamburg, Germany) in a total volume of 1 ml. Reaction tubes were shaken at 1200 rpm and 25 °C in a ThermoMixer® (Eppendorf). Reactions were performed with 30 mM acetophenone 1a, 0.5 mg immobilized HaloTag-LbADH, 0.5 mM NADPH, 50 mM potassium phosphate, pH 7.0, 1 mM MgCl₂x6H₂O and different 2-propanol concentrations (10 vol%, 25 vol%, 50 vol%, 75 vol%, 90 vol%). The reaction solution was incubated for 5 h a day. Then, the reaction was stopped by centrifugation and the supernatant was removed from the immobilized HaloTag-LbADH. Afterwards, immobilized HaloTag-LbADH was washed three times with 1 ml buffer (50 mM potassium phosphate, pH 7.0, 1
mM MgCl$_2$$\cdot$6H$_2$O, 0.5 vol% Triton X-100) and stored at 4 °C until the next cycle was started the next day. Acetophenone 1a and phenylethanol 2a were detected by HPLC on a Chiralpak IE column using an Agilent 1260 Infinity Quaternary LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1260 Diode Array Detector. The column was operated with a mobile phase consisting of 50 % (v/v) acetonitrile and 50 % (v/v) deionized ultra-pure water with an isocratic flow of 1 ml/min at 20 °C. Acetophenone was detected at 250 nm with an approximate retention time of 5.4 min and phenylethanol was detected at 215 nm with a retention time of 4.1 min. Toluene was used as an internal standard and was detected at 215 nm with a retention time of 6.3 min.

![Graph](image1.png)

**Fig. S1.** Effect of 2-propanol on the activity of HaloTag-LbADH. Assay: acetophenone (30 mM), 2-propanol (10 – 90 vol%), NADPH (0.5 mM), Triton X-100 (0.5 vol%), KPi 50 mM (pH 7.0), MgCl$_2$$\cdot$H$_2$O (1 mM). Acetophenone and 1-phenylethanol were detected by HPLC.

![Graph](image2.png)

**Fig. S2.** Repetitive conversion of acetophenone in batch by immobilized HaloTag-LbADH in the presence of 10 vol% 2-propanol. Four consecutive cycles were performed over 4 days and storage in between at 4 °C. Reaction: 0.5 mg/ml immobilized HaloTag-LbADH, 30 mM acetophenone, 10 vol% 2-propanol, 0.5 mM NADPH, 0.5 vol% Triton X-100, 50 mM KPi buffer, pH 7.0, 1 mM MgCl$_2$$\cdot$6H$_2$O, V = 1ml, T = 25 °C. Acetophenone 1a and phenylethanol 2a were detected by HPLC.
**Fig. S3.** Repetitive conversion of acetophenone in batch by immobilized HaloTag-LbADH in the presence of 25 vol% 2-propanol (see Figure 1).

**Fig. S4.** Repetitive conversion of acetophenone in batch by immobilized HaloTag-LbADH in the presence of 50 vol% 2-propanol (see Figure 1).
**Fig. S5.** Repetitive conversion of acetophenone in batch by immobilized HaloTag-LbADH in the presence of 75 vol% 2-propanol (see Figure 1).

**Fig. S6.** Repetitive conversion of acetophenone in batch by immobilized HaloTag-LbADH in the presence of 90 vol% 2-propanol (see Figure 1).

**S6. General procedure for the immobilization of HaloTag-LbADH on the HaloLink™ resin in flow.**

A glass Omnifit® column (Kinesis, Benchmark microbore column 3 MM / 50 MM 2 X F) was loaded with wet HaloLink™ resin (360 mg), allowing particles sedimentation. The reactor was connected to the pump (Syrris Asia Syringe Pumps, equipped with Asia Blue Syringes of 500 µl / 1 ml) by PTFE tubing and end fittings. The resin was washed with Kpi 50 mM pH 7 for 1h (flow rate 30 µl/min). Then, a solution (5 ml) of the cell crude extract (250 mg) in Kpi (50 mM, pH 7) containing MgCl₂·6H₂O (1.0 mM) was pumped continuously through the packed bed. The efflux was monitored in real-time by a UV/Vis detector (Flow-UVTM, Uniqsis Ltd).
General procedure for the HaloTag-LbADH catalyzed reduction of ketones 1a-1p in flow.

A solution of the ketone (50 mM), MgCl₂·6H₂O (1.0 mM) and NADPH (0.5 mM) in Kpi (60% V/V, 50 mM, pH 7), 2-propanol (10% V/V) and THF (30% V/V) was pumped (flow rate 30 µl/min) through the HaloTag-LbADH packed bed reactor, prepared according to the procedure in section S6. The efflux was monitored in real-time by a UV/Vis detector. The collected solution was extracted with pentane and the organic phase was dried over MgSO₄ and concentrated in vacuo. The crude was purified as described for each compound.

To analyze the long-term stability of the HaloTag-LbADH, the conversion of acetophenone 1a in flow in the presence of 2-propanol was investigated. Acetophenone 1a and phenylethanol 2a were detected by HPLC as described in S5.

![Figure 1: Long-term stability of HaloTag-LbADH. A packed-bed reactor (5 cm Omnifit® column, V = 350 µl) containing 4 mg HaloTag-LbADH immobilized on 360 mg of wet HaloLinkTM Resin was prepared.](image)

General procedure for the preparation of (S)-2-phenyloxirane by a two-step chemoenzymatic transformation in flow.

A solution of the ketone 1h (50 mM), MgCl₂·6H₂O (1.0 mM) and NADPH (0.5 mM) in Kpi (60% V/V, 50 mM, pH 7), 2-propanol (10% V/V) and THF (30% V/V) was pumped (flow rate 30 µl/min) through the HaloTag-LbADH packed bed reactor, prepared according to the procedure in section S6. The efflux was mixed with a second stream containing an aqueous solution of NaOH (1 mM, flow rate 30 µl/min). The resulting mixture was passed through a 2-inputs glass microreactor (1.0 ml). The collected solution was extracted with pentane and the organic phase was dried over MgSO₄ and concentrated in vacuo. The crude was analyzed by ¹H-NMR and chiral GC.

General procedure for the preparation of the racemic compounds (±)-2a-p and (±)-5.

Compounds (±)-2a, (±)-2b, (±)-2c, (±)-2d, (±)-2e, (±)-2f, (±)-2k, (±)-2l, (±)-2m, (±)-2o and (±)-5 were obtained from commercial sources and used without further purification as reference for GC and HPLC analyses. Compounds (±)-2g, (±)-2h, (±)-2i, (±)-2j, and (±)-2n were prepared according to the reported procedure.
S10. Spectral characterization of compounds 2a-2p and 5.

(R)-(+-)1-Phenylethanol, 2a. Isolated by flash chromatography (hexane/AcOEt 80/20), 90% yield. The optical purity of 2a was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run 180 °C for 1 min. (R)-isomer, tR 19.910 min; (S)-isomer, tR 20.037 min, >99% ee. [α]_20^25 = +49.3 (c = 0.82 g/100ml, CHCl₃). The configuration was assigned by comparison with the commercial available (R)-(+-)1-phenylethanol. The NMR spectra are in accordance with the reported data.
(R)-(+)-1-(4-Bromophenyl)ethanol, 2b. Isolated by chromatography (hexane/AcOEt 80/20), 94% yield. The optical purity of 2b was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run 180 °C for 1 min. t₁ 22.376 min; t₂ 22.501 min., >99% ee. \([\alpha]_{D}^{20}=+28\) (c = 0.5 g/100ml, CHCl₃). The NMR spectra are in accordance with the reported data. 8
(R)-(+)-1-(4-Chlorophenyl)ethanol, 2c. Isolated by chromatography (hexane/AcOEt 80/20), 94% yield. The optical purity of 2c (ee > 99%) was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run 180 °C
for 1 min. \( t_1 \) 21.650 min; \( t_2 \) 21.797 min., >99% ee. \( [\alpha]_{\text{D}}^{20} = +48 \) (c = 0.715 g/100 ml, CHCl\(_3\)). The NMR spectra are in accordance with the reported data.\(^1\)
(R)-(+)-1-(4-Methoxyphenyl)ethanol, 2d. Isolated by chromatography (hexane/AcOEt 80/20), 23% yield. The optical purity of 2d was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run 180 °C for 1 min. t₁ 21.650 min; t₂ 21.73 min., >99% ee. [α]₂₀° = +28.5 (c = 1.76 g/100ml, CHCl₃). The NMR spectra are in accordance with the reported data.⁹
(R)-(+)-1-(Naphthalene-2-yl)ethanol, 2e. Isolated by cromatography (hexane/AcOEt 80/20), 95% yield. The optical purity of 2e (ee > 99%) was assessed by GC analysis, using the following method: initial temperature 40 °C holded for 13 min, ramp 15 °C/min to 180 °C holded for 10 min, post run...
180 °C for 1 min. $t_1$ 24.534 min; $t_2$ 24.657 min., >99% ee. $[\alpha]^{20}_{D89} = +40$ (c = 0.3 g/100ml, CHCl$_3$). The NMR spectra are in accordance with the reported data.
(R)-(+)\text{-1-}(\text{Tol-4-yl})\text{ethanol, 2f. Isolated by chromatography (hexane/AcOEt 80/20), 70\% yield. The optical purity of 2g was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run 180 °C for 1 min. t}_1 20.42 min; t}_2 20.583 min., 99\% ee. [\alpha]^{20}_D = +54.4 (c = 0.69 g/100ml, CHCl_3). The NMR spectra are in accordance with the reported data.\text{\footnote{1}}
(R)-(+) 4-(1-Hydroxyethyl)benzaldehyde, 2g. Isolated by chromatography (hexane/AcOEt 80/20), 97% yield. The optical purity of 2g (ee > 99%) was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run...
180 °C for 1 min. \( t_1 \) 21.063 min; \( t_2 \) 23.24 min., >99% ee. \([\alpha]_{\text{D}}^{20} = +40 \) (c = 0.3 g/100ml, CHCl\(_3\)). The NMR spectra are in accordance with the reported data.\(^{10}\)
(S)-(+)-2-Bromo-1-phenylethanol, 2h. Isolated by chromatography (hexane/AcOEt 80/20), 98% yield. The optical purity of 2h was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run 180 °C for 1 min. $t_1$ 22.530 min; $t_2$ 22.602 min., >99% ee. $[\alpha]_{589}^20 = +49.4$ (c = 1.1 g/100ml, CHCl$_3$). The NMR spectra are in accordance with the reported data.\(^{11}\)
(S)-(+)-2-Bromo-1-[4-trifluoromethyl]phenyl]ethanol, 2i. Isolated by chromatography (hexane/AcOEt 80/20), 91% yield. The optical purity of 2i was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for 10 min, post run...
180 °C for 1 min. $t_1$ 23.065 min; $t_2$ 23.226 min., >99% ee. $[\alpha]^{20}_{D589} = +35.5$ (c = 0.4 g/100ml, CHCl$_3$). The NMR spectra are in accordance with the reported data.$^{12}$
(S)-(+-)-2-Fluoro-1-phenylethanol, 2j. Isolated by chromatography (hexane/AcOEt 80/20), 99% yield. The optical purity of 2j was assessed by GC analysis, using the following method: initial temperature 40 °C hold for 13 min, ramp 15 °C/min to 180 °C hold for 10 min, post run 180 °C for 1 min. t
20.706 min; $t_2$ 20.815 min., >99% ee. $[\alpha]^{20}_{D,89} = +16.1$ (c = 0.75 g/100ml, CHCl$_3$). The NMR spectra are in accordance with the reported data.\textsuperscript{13}
(R)-(+)-2,2,2-Trifluoro-1-[4-trifluoromethyl]phenyl]ethanol, 2k. Isolated by chromatography (hexane/AcOEt 80/20), 60% yield. The optical purity of 2k was assessed by GC analysis, using the following method: initial temperature 40 °C held for 13 min, ramp 15 °C/min to 180 °C held for
10 min, post run 180 °C for 1 min. $t_1$ 20.770 min; $t_2$ 20.841 min., >99% ee. $[\alpha]^{20}_{589} = +17.8$ (c = 1.2 g/100ml, CHCl₃). The NMR spectra are in accordance with the reported data.¹⁴
(S)-(−)-1-Phenylethane-1,2-diol, 2l. Isolated by chromatography (hexane/AcOEt 80/20), 40% yield. The optical purity of 2l was assessed by HPLC analysis, using a ChiralART SC column, hexane/2-2-
propanol 95/5, flow rate 1.0 ml/min. $t_1$ 20.115 min; $t_2$ 21.322 min., >99% ee. $[\alpha]_\text{D}^{20} = +65.5$ (c = 0.6 g/100ml, CHCl$_3$). The configuration was assigned by comparison with the commercial available (R)-(-)-1-phenylethane-1,2-diol. The NMR spectra are in accordance with the reported data.$^{15}$
(R)-(+)-1-(1-Adamantyl)ethanol, 2m. PS-Tosyl hydrazine (2.39 mmol/g, 0.6 g) was added to a mixture of the reaction crude (obtained according to the general procedure described in S5) and AcOH (5%) in THF (2 mL). This mixture was stirred for 30 min, then filtered and washed with MeOH (8
mL). The compound 2m was obtained with 71 % yields. The % ee of the benzoate derivative, prepared according to the reported procedure, was determined by HPLC analysis, using a ChiralART SA column, hexane/2-2-propanol 97/3, flow rate 0.5 ml/min. t₁ 8.144 min; t₂ 8.673 min., 79% ee. [α]²⁰⁰D = +1.25 (c = 0.98 g/100ml, CHCl₃). The NMR spectra of 2m are in accordance with the reported data.¹⁶
(R)-(-)-Benzyl-3-hydroxybutanoate, 2n. Isolated by chromatography (hexane/AcOEt 80/20), 97% yield. The optical purity of 2n was assessed by HPLC, using a Chiralpak OD-H column, hexane/2-
propanol 98/2, flow rate 0.5 ml/min. t₁ 50.918 min; t₂ 73.901 min, >99% ee. [α]₂⁰° = -30 (c = 0.84 g/100ml, CHCl₃). The NMR spectra are in accordance with the reported data.
(R)-(−)-Phenylbut-3-yn-2-ol, 2o. Isolated by chromatography (hexane/AcOEt 80/20), 99% yield. The optical purity of 2o was assessed by HPLC, using a ChiralART SB column, hexane/2-2-propanol 95/5,
flow rate 0.5 ml/min. t₁ 19.469 min; t₂ 42.1 min., >99% ee. \([\alpha]_{589}^{20} = +32.4\) (c = 0.9 g/100ml, CHCl₃). The NMR spectra are in accordance with the reported data.¹⁸
(S)-2-Phenyl-oxirane, 5. Conversion as determined by GC and $^1$H-NMR, 98% yield. The optical purity of 5 was assessed by GC analysis, using the following method: initial temperature 40 °C hold for 4 min., ramp 15 °C/min to 180 °C hold for 10 min, post run 180 °C for 1 min. (R)-isomer, $t_R$ 7.694 min;
(S)-isomer, $t_R$ 8.109 min., 98% ee. The configuration was assigned by comparison with the commercial available (R)-(+)2-phenyloxirane. The NMR spectra are in accordance with the reported data.\textsuperscript{19}

S11. DNA and protein sequences

Primer sequences

Primer 1: tttaagaaggagatatacatATGAAACATCACCATCACCATCACGCAGAAATTGGTACG

Primer 2: tttattcagcagacgatCGCGGCCGCTTTCGCCGC

Primer 3: cagcgcggaagccgcgcgATGTCTAACCCTTTGGATG

Primer 4: gcttttttagcagccgatcCTATTGAGCAGTGTAGCC

Primer 5: GATCCGGCTGCTAACAAAG

Primer 6: CGCGGCCGCTTTCGCCGC

DNA sequence of halotag-\textit{lbadh}

ATGAAACATCACCATCACCATCACCATCACGCAGAAATTGGTACGGGATTTCCGTTTGACCCGCATTATGTGGAGGTTCTGGGTGAACGCATGCACTACGTGGATGTTGGTCCCGCGCGATGGCACACCGGTGCTGTTTCTGCATGGTAATCCGACCTCCAGCTATGGTACATGGCGCAAAACATATTCCCCCATCATCGCCCCACACACCATCGCTGATTGCCCGGATGCCTCCTAGCGACATCGGACAGCCTTGGGATTTGGGCTACTTCTTCGACGATCACGTACGGTTTATGGACGCCTT
Amino acid sequence of HaloTag-LbADH

2 L. Kulishova, PhD Thesis, Heinrich-Heine University Düsseldorf, 2010
3 http://syrris.com/flow-products/asia-modules/asia-syringe-pump