Supporting Information

Induced axial chirality in biocatalytic asymmetric ketone reduction

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Contents

Biology

Reagents

E. coli strain BL21-Gold(DE3) was obtained from Stratagene. Restriction enzyme *Dpn*I, Taq DNA ligase and 100 mM stock solutions of dATP, dTTP, dGTP and dCTP were purchased from New England Biolabs. T5 exonuclease was provided by Epicenter. $NADP^+$ was obtained from Calbiochem. Glucose dehydrogenase 105 was purchased from Codexis. Alcohol dehydrogenases were purchased from X-Zyme (Johnson Matthey Catalysts) or kindly gifted from evocatal (Düsseldorf/Germany). KOD hot start polymerase, dNTPs for PCR, $MgSO_4$ and DNA vector pRSFDuet-1 were obtained from Novagen. Phusion Polymerase was provided by Finnzymes. Oligonucleotides were purchased from Invitrogen in standard, desalted form and used without further purification (**Table S4**). DNAse I, and lysozyme were obtained from AppliChem. LB medium, in a form of premixed powder, and kanamycin were obtained from Roth. IPTG and PEG-4000 were obtained from Fermentas. TB medium contained yeast extract (24g/L), peptone (12g/L), glycerol (4 mL/L), KH_2PO_4 (0.017 M) and K_2HPO_4 (0.072 M).

Screening of commercial ADHs for asymmetric reduction of ketones 1a-f

Alcohol dehydrogenases (ADHs) from two different commercial kits ("Alcohol Dehydrogenase Kit" from Johnson Matthey Catalysts and "Alcohol Dehydrogenases Screening Kit" from evocatal) were screened for asymmetrical reduction of compounds **1a**-**f**. Reactions were carried out in 1.5 mL tubes. Briefly, 5-15 mg of each lyophilized ADH were dissolved in 500 µL reaction mixture containing Glucose (100 mM), cofactor NAD^+ or $NADP^+$ (1 mM), specified by the supplier depending on the ADH, Glucose Dehydrogenase (GDH 105, Codexis; 0.04 Units), ketone (5 mM) dissolved in acetonitrile and phosphate buffer (pH 7.0, 100 mM). Reaction was carried out at 30°C for 16 h in thermomixer with shaking (700 r.p.m.). After incubation time, the reaction media was extracted with ethyl acetate (500 µL) and organic layer submitted to GC analysis.

Construction of pRSF-TbSADH* plasmid

To express TbSADH protein, *adhB* gene from *Thermoanaerobacter ethanolicus* (that encodes for TeSADH protein) was subcloned into pRSFDuet-1 vector. The backbone of PRSFDuet-1 plasmid was amplified by PCR using primers pRSF-*Nco*I (ggt ata tct cct tat taa agt taa aca aaa tta ttt cta cag g) and pRSF-*Avr*II (taa cct agg ctg ctg cca ccg ctg agc aat aac) as previously described.[1] TeSADH gene was amplified from template pADHB1M1-kan[2] (plasmid harboring the *adh*B gene from *T. ethanolicus* E39 used previously in our laboratory[3]) by PCR using primers pRSF-TbSADH*-Fw (ttt tgt tta act tta ata agg aga tat acc atg aaa ggt ttt gca atg ctc agt atc gg) and pRSF-TbSADH*-rv (gtt att gct cag cgg tgg cag cag cct agg tta gca taa aaa tgg act aag tcc cc). The oligonucleotides contained at 5´ end a complementary sequence to vector pRSFDuet-1 (complementary regions are underlined). PCR conditions contained 5 µL of 10 X KOD hot star polymerase buffer, 5 µL dNTPs (2 mM each), 1.25 µL of the appropriate forward and reverse primers (100 ng each), 2 µL $MgSO_4$ (25 mM), 1 µL template DNA (50 ng) and 0.5 µL of KOD polymerase in a final volume of 50 µL. Products from both PCRs were digested with *Dpn*I at 37°C for 3 h for removing of template DNA, and purified with a QIAquick PCR purification spin column (Qiagen). The two purified DNA fragments were ligated by *in vitro* isothermal assembly.[4] Briefly, 2.5 µL of purified TeSADH insert (125 ng) and 2.5 µL of purified backbone vector (125 ng) were mixed with 15 µL of assembly master mixture prepared as previously described[1] and incubated 50°C for 1h. 3 µL of the reaction was used to transform electro into *E. coli* BL-21 Gold (DE3) electro competent cells. Plasmid from 5 transformants selected on LB agar plates containing kan (50 µL/mL) was isolated and sequenced for confirming the TeSADH gene sequence (1064 nucleotides from ATG methionine initiation codon to final ATT stop codon). The sequence of the 5 plasmids

[1] R. Agudo, G.-D. Roiban, M. T. Reetz, *ChemBioChem* **2012**, *13*, 1465-1473.
[2] D. S. Burdette, F. Secundo, R. S. Phillips, J. Dong, R. A. Scott, J. G. Zeikus, *Biochem. J.* **1997**, *326*, 717-724.
[3] F. Schulz, F, Leca, F. Hollmann, M. T. Reetz, *Beilstein J. Org. Chem*. **2005**, 1-10.
[4] D. G. Gibson, L. Young, R.-Y. Chuang, J. C. Venter, C. A. Hutchison, H. O. Smith, *Nat. Methods* **2009**, *6*, 343-345.

revealed that they contained 1 silence mutation (nucleotide position C141G) and 4 missense mutations (nucleotide positions T272C, C557A, C938G and A974G that encode for Trp91Arg, Ala186Asp, Pro313Arg and Glu325Arg amino acid changes, respectively) regarding original TeSADH sequence.[5] However, recent sequencing results indicate that the original sequence of *T. ethanolicus* 39E has indeed C, G and G nucleotides in 272, 938 and 974 positions, respectively,[6] sharing exactly the same sequence as ADH from *Thermoanaerobacter brokii.*[6b] Therefore, for the current work we have used TbSADH protein with A186D substitution, referred as TbSADH*.

In order to clarify if A186D substitution affects the activity of the enzyme using our substrates, we compared the activity of the two enzymes: TbSADH Wt (after reversion of mutation C557A; kindly provided by Dr. Zhi-Gang Zhang) and TbSADH* using compound **1a** as substrate. We performed the same experiment using TbSADH W110A and TbSADH* W110A enzymes. The results show no differences neither in activity, stereospecificty, or thermostability between TbSADH and TbSADH* backgrounds. Furthermore, since this mutation also appeared in the original sequence of plasmid pADHB1M1-kan we store in our laboratory, we did not perform any additional mutagenesis experiment for reverting this residue, therefore we used TbSADH* as template for subsequent library creation.

The plasmid created was termed pRSF-TbSADH* (**Sequence 1**).

TbSADH* protein expression and activity tests

Plasmid pRSF-TbSADH* was transformed in both *E. coli* BL-21 Gold (DE3) and BOU730 (a bioengineered *E. coli* strain that express GDH upon IPTG induction from *Bacillus megaterium*)[1] electro competent cells. One single transformant of each was inoculated in 5 mL of LB with kan (50 µg/mL). After 5 h of incubation at 37°C with shaking, this preculture was transferred to 100 mL of TB with kan (50 µg/mL). Cultures were grown at 30°C until O.D. of 0.7-0.9 at 600 nm was reached, then IPTG was added to a final concentration of 0.2 mM and the cultures were let to grow at 30°C with vigorous agitation. After 20 h, cells were collected by dividing the culture in 1 mL aliquots. Aliquots were centrifuged at 10.000 r.p.m for 6 min at room temperature, supernatant discarded and pellets stored at -20°C until their further use.

One aliquot of *E. coli* BL-21 Gold (DE3) and BOU730 cells expressing TbSADH* were suspended in 300 µL of phosphate buffer (pH 7, 100 mM), sonicated (5 second pulse, power 40%) and centrifuged at 10.000 r.p.m. for 10 min. Supernatants were recovered, heated at 75°C for 15 min to achieve the precipitation of non-thermostable proteins from *E. coli* and centrifuged again for elimination of the cellular debris.

Aliquots taken before and after heating treatment were analyzed by Tris glycine SDS-PAGE. The TbSADH* were ≥95% pure after heat treatment, as judged by SDS-PAGE electrophoresis and staining with Page Blue Protein Staining Solution (Fermentas) (**Figure S1**). TbSADH* concentrations were determined measuring the absorbance of heated samples at 280 nm and using 30.940 M^{-1} cm^{-1} as extinction coefficient value of TbSADH*, previously used for determination of TbSADH protein concentration.[7]

Different experimental conditions were tested in order to improve conversion and eantioselectivity of the product. We have performed different experiments varying the pH of phosphate buffer, concentration of isopropanol, concentration of cofactor and temperature.

For TbSADH* expressed into *E. coli* BL-21 Gold cells, one aliquot of proper pellet was resuspended in lysis buffer [phosphate buffer (pH 6 to 10, 100 mM), lysozyme (14 mg/mL) and DNAse I (6U/mL)] and incubated at 37°C during 20 min at 700 r.p.m. in a thermomixer. After this time, tubes were heated in the incubator at 75°C for 15 min more and then centrifuged at 10.000 r.p.m at 4°C for 15 min to pull down the cellular debris. Supernatant (c.a. 500 µL) was transferred to a new 1.5 mL tube, containing 500 µL reaction buffer (phosphate buffer (pH 6 to 10, 100 mM), isopropanol (2% to 30% final concentration v/v), $NADP^+$ or NAD^+ (10 to 500 µM final concentration) and compound **1a** (5 mM final

[5] D. S. Burdette, C. Vieille, J. G. Zeikus, *Biochem. J*. **1996**, *316*, 115-122.
[6] a) K. I. Ziegelmann-Fjeld, M. M. Musa, R. S. Phillips, J. G. Zeikus, C. Vieille, *Protein Eng. Des. Sel*. **2007**, *2*, 47-55. b) M. M. Mussa, R. S. Phillips, Catal. *Sci. Technol*. **2011**, *1*, 1311-1323.
[7] R. Mishra, L. Olofsson, M. Karlsson, U. Carlsonn, I. A. Nicholls, P. Hammarström, *Cell. Mol. Life Sci*. **2008**, *65*, 827-839.

concentration)]. The sample was incubated at 25 to 60°C for 16 h at 700 r.p.m. in a thermomixer. After incubation time, 700 µL of reaction media was extracted with ethyl acetate (700 µL) and organic layer submitted to GC analysis. For TbSADH* expressed in BOU730 cells, the procedure to determine the optimal reaction conditions was the same as described above except the samples were not heated at 75°C for 15 min and glucose was added to reaction buffer (100 mM final concentration) instead isopropanol.
Control experiments using either no transformed BOU730 or BL-21 Gold (DE3) cells were carried out upon the same conditions explained above. No conversion of **1a** was observed in both cases.
Based on the data obtained during these preliminary assays, further experiments were performed using heat-treated TbSADH* expressed into *E. coli* BL-21 Gold cells with reaction buffer containing phosphate buffer pH 8.0, (100 mM), isopropanol (5-10% final concentration v/v), and $NADP^+$ (25-50 µM final concentration), and incubated at 37°C.

TbSADH* library creation by QC PCR

TbSADH* libraries were created by QuikChange PCR[8] using pRSF-TbSADH* vector as template. PCR reactions contained 5 µL of 10 X KOD hot star polymerase buffer, 5 µL dNTPs (2 mM each), 1.25 µL of the appropriate forward and reverse degenerate primers (100 ng each), 2 µL $MgSO_4$ (25 mM), 1 µL template DNA (c.a. 50 ng) and 0.5 µL of KOD polymerase. PCR method used was the same for all libraries created: the PCR reaction started at 95°C (3 min), continued with 25 cycles of 95°C (I min), 53°C (1 min) and 68°C (10 min), and finished with 15 min at 68°C. PCR products were digested with *Dpn*I at 37°C for 3 h to eliminate the template DNA and then they were dialyzed on Millipore MF-membrane filters (0.05 µm) against distilled water for 20 min. 5 µL of the dialyzed plasmid were used to transform 50 µL of electro competent BL-21 Gold (DE3) cells. Transformation mixture was spread on LB agar plates containing kan (50 µL/mL).
Codon degeneracy evaluation of each library was carried out by scraping colonies from the corresponding plates (≥ 100 colonies) with the help of 3 mL of LB medium as previously described.[1] The plasmid pools were extracted by miniprep and submitted for sequencing.

Prescreening of TbSADH* libraries for active mutants for asymmetric ketones reduction

Individual *E. coli* BL-21 Gold (DE3) colonies harboring mutated plasmids were randomly picked and inoculated in 96 deep well plates sealed with gas permeable seals containing 800 µL LB with kan (50 µg/mL). The three first wells of a plate were used for culturing control colonies (i.e. colonies harboring pRSF-TbSADH* plasmid). Cultures were grown during 5 h at 37°C with gentle agitation. Glycerol stock solutions in sterile 96 well microtiter plates were prepared at this step and kept at -80°C. Aliquots of 100 µL were transferred from the growth culture to a new 96 deep-well plates sealed with gas permeable seals containing 900 µL of TB with kan (50 µg/mL) and IPTG (0.2 mM), and then, cultures were allowed to grow overnight at 30°C with vigorous agitation for protein overexpression. After incubation, plates were centrifuged at 4000 r.p.m. at room temperature for 20 min for harvesting cells. After discarding supernatant, pellets were resuspended in 500 µL of lysis buffer [phosphate buffer (pH 8.0, 100 mM), lysozyme (14 mg/ml) and DNAse I (6U/mL)] and incubated during 45 min at 37°C with vigorous agitation. After this time, plates were heated in the incubator for additional 15 min at 60°C and then centrifuged at 4000 r.p.m at 4°C for 20 min to pull down the cellular debris. 250 µL of supernatant were transferred to a 96 deep-well plate containing 250 µL of reaction buffer [phosphate buffer (pH 8.0, 100 mM), isopropanol (5% final concentration v/v), $NADP^+$ (25 µM final concentration) and ketone (2.5 mM final concentration)]. The reaction was carried out at 37°C with vigorous agitation. After 16 h of incubation, reaction mixture was extracted with ethyl acetate (400 µL) and subjected to chiral GC analysis. Plasmid DNA from clones showing ee values higher than 80% were extracted and sequenced.

[8] H. H. Hogrefe, J. Cline, G. L. Youngblood, R. M. Allen, *BioTechniques* **2002**, *33*, 1158–1165.

Initially, plates were heated at 75°C for 15 min. However, we realized there were no active mutants in library I86. Previously, it has been described that mutant TbSADH I86A is less thermostable than Wild type protein.[9] Therefore we decided to repeat the screening, by heating the I86 library plate at 55°C after the pellets resuspension in lysis buffer. Using these milder conditions, hits in this particular library emerged. SDS-PAGE analysis of those hits found in this library, confirmed that after heating treatment at 60°C, there was the same amount of TbSADH* as before (Data not shown). Thus, we decided to perform the screening and further enzymatic reactions heating samples at 60°C.

Saturation mutagenesis of position I86

QuikChange method was used for saturation mutagenesis at residue I86 to generate those mutants that did not emerge as hits after I86 library screening (i.e. I86 amino acid residue substituted either by R, N, D, C, Q, H, L, K, F, P, S, W, Y or V). PCR reactions contained 5 µL of 10 X KOD hot star polymerase buffer, 5 µL dNTPs (2 mM each), 1.25 µL of the appropriate forward and reverse degenerate primers (100 ng each), 2 µL $MgSO_4$ (25 mM), 1 µL template pRSF-TbSADH* (c.a. 50 ng) and 0.5 µL of KOD polymerase. PCR method used was the same for all libraries created: the PCR reaction started at 95°C (3 min), continued with 25 cycles of 95°C (1 min), 53°C (1 min) and 68°C (10 min), and finished with 15 min at 68°C. PCR products were digested with *Dpn*I at 37°C for 3 h to eliminate the template DNA and then they were dialyzed on Millipore MF-membrane filters (0.05 µm) against distilled water for 20 min. Dialyzed plasmid (5 µL) was used to transform 50 µL of electro competent BL-21 Gold (DE3) cells. The presence of all mutations was confirmed by sequencing.
One single colony of BL-21 Gold (DE3) harboring the proper mutant TbSADH* was inoculated in 5 mL of LB with kan (50 µg/mL), and this cell culture was overgrown, induced, pelleted and collected in 1 mL aliquots for further use as described above. No differences in the TbSADH* expression level among all mutants was observed as judged by SDS-PAGE analysis (Data not shown).

Medium scale procedure to prepare alcohol (*S*)-2a

Medium scale synthesis reactions were performed both using promising hits from plate screening to exclude false positives and mutants in position I86 produced by QuikChange. A single colony from *E. coli* BL-21 Gold (DE3) containing pRSF-TbSADH* with desired mutation was inoculated in 4 mL of LB with kan (50 µg/mL). After 5 h of incubation at 37°C with shaking, this preculture was transferred to 50 mL of TB with kan (50 µg/mL). Culture was grown at 30°C until O.D. of 0.7-0.9 at 600 nm was reached, then IPTG was added to a final concentration of 0.2 mM and the culture was allowed to grow at 30°C overnight with vigorous agitation. Expression level of TbSADH* was checked by SDS-PAGE electrophoresis before further analysis. We confirmed by SDS-PAGE that the TbSADH* expression was similar in all mutants tested, and the incubation at 60 °C did not decrease the amount of TbSADH* produced (Data not shown). Routinely, 1.2±0.2 mg/mL of TbSADH* were obtained from 50 mL cultures. Cells were pelleted by centrifugation (20 min, 4000 r.p.m. at room temperature) and resuspended in 4 mL of lysis buffer [phosphate buffer (pH 8.0, 100 mM), 50 mg of lysozyme and 30 µL of DNAse I (stock solution at 3000 U/mL)] and incubated during 45 min at 37°C with vigorous agitation. After this time, suspensions were incubated 15 min at 60°C. Cellular debris was pelleted by centrifugation (4000 r.p.m. 20 min at 4°C) and the supernatant (c.a. 4 mL) was recovered and diluted to 5mL with reaction buffer [phosphate buffer (100 mM, pH 8.0), isopropanol (10% final concentration v/v), $NADP^+$ (50 µM final concentration)]. Reaction was started by addition of 30 mg of compound **1a**, at 37°C with vigorous agitation. After 2 h, additional 30 mg of compound **1a** were added (63 mM final concentration) and after 20 h of incubation, 700 µL of reaction mixture was extracted with ethyl acetate (700 µL) and subjected to GC analysis.

[9] M. M. Musa, N. Lott, M. Laivenieks, L. Watanabe, C. Vieille, R. S. Phillips, *ChemCatChem* **2009**, *1*, 89-93.

Up-scaling procedure of alcohol (*S*)-2a

For scaling up asymmetric reduction reaction, 20 mL of LB with kan (50 µg/mL) were inoculated with a single colony from *E. coli* BL-21 Gold (DE3) expressing TbSADH* W110T mutant, and incubated for 6 h at 37°C with vigorous shaking. Full volume was added to 500 mL of TB containing kan (50 µg/mL). Culture was grown at 30°C until O.D. of 0.7-0.9 at 600 nm was reached, then IPTG was added to a final concentration of 0.2 mM for protein expression induction and the culture was allowed to grow at 30°C overnight with vigorous agitation. Cells were pelleted by centrifugation (20 min, 4000 r.p.m. at room temperature) and pellet was resuspended in 30 mL of lysis buffer [phosphate buffer (pH 8.0, 100 mM), 400 mg of lysozyme and 400 µL of DNAse I (stock solution at 3000 U/mL)] and incubated during 45 min at 37°C with vigorous agitation. After this time, suspensions were incubated 15 min at 60°C. Cellular debris was pelleted by centrifugation (4000 r.p.m. 20 min at 4°C) and supernatant (c.a. 30 mL, 600 mg TbSADH*) was recovered and diluted to 50 mL with reaction buffer [phosphate buffer (100 mM, pH 8.0), isopropanol (10% final concentration v/v), $NADP^+$ (50 µM final concentration)]. Reaction was started by addition of 225 mg of compound **1a**, and was carried out at 37°C with vigorous agitation. After 2 h, additional 225 mg of compound **1a** were added, and after 2 additional hours, 180 mg more of compound **1a** were added (67 mM final concentration, 630 mg in total, 3.33 mmol) and allowed to react for 20 h. The reaction mixture was extracted with ethyl acetate (4 x 100 mL), organic phase was dried with Na_2SO_4, evaporated and the residue subjected to column chromatography to give (*S*)-**2a** as a colorless liquid (534 mg, 84%). (R_f=0.43 ethyl acetate/petroleum ether=1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.89 (s, 1H), 3.87 (m, 1H), 2.75-2.66 (m, 1H), 2.46-2.37 (m, 1H), 2.15-2.05 (m, 2H), 1.95-1.83 (m, 2H), 1.70 (br s, 1H), 1.54-1.40 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=143.12, 98.80, 68.89, 35.46, 34.46, 31.70, 27.49 ppm; HRMS (APCI+): calcd for $C_7H_{12}BrO$ [*M*+*H*]$^+$: 191.0066, 193.0046; found: 191.0066, 193.0046. GC analyses of the reaction crude extract indicated a final conversion >99% and 99% ee (*S*).

Up-scaling procedure of alcohol (*R*)-2a

Up-scaling of (*R*)-**2a** alcohol from ketone **1a** [67 mM, 500 mg (added at once), 2.64 mmol] was performed as described for the alcohol (*S*)-**2a** but using *E. coli* BL-21 Gold (DE3) expressing the mutant TbSADH* I86A. Purification on column chromatography afforded (*R*)-**2a** as a colorless liquid (400 mg, 81%). GC analyses of the reaction crude extract indicated a final conversion >99% and 97% ee (*R*).

Small scale reactions using 1b-f, 3, 5a-b and acetophenone as substrates

We performed the reduction of compounds **1b-f**, **3** and **5a-b** to the corresponding alcohols using hits specifically evolved for the reaction of **1a**. Single colonies from glycerol stocks in *E. coli* BL-21 Gold (DE3) of the corresponding mutants were isolated and grown in LB with kan (50 µg/mL) during 5 h at 37°C. This pre-inoculum was transferred to 100 mL of TB with kan (50 µg/mL) and the cell culture was induced, grown, and divided in 1 mL aliquots as described above for “TbSADH* protein expression and activity tests” section. Expression levels of different mutants were checked by SDS-PAGE electrophoresis as described above (1 mg/mL of TbSADH*). Aliquots were treated as described above, heating them at 60°C. Ketone reduction reactions were carried out in 1 mL reaction buffer [phosphate buffer (pH 8.0, 100 mM), isopropanol (10% final concentration v/v), $NADP^+$ (50 µM final concentration) and the proper ketone substrate **1b-f**, **3**, or **5a-b** (1 mg, 5-8 mM final concentration)]. Reactions were carried out at 37°C with vigorous agitation. After 16 h of incubation, products were extracted with ethyl acetate and subjected further to GC analysis.

Mutants in position I86 produced by QuikChange were also tested based on this protocol using **1a** (5 mM final concentration) as starting material.

Reactions using acetophenone as starting material were carried out as described above. Determination of the absolute configuration was made after comparison with authentic commercial standards, results being summarized in Table S5.

Substrate 1a modeling in the active site of TbSADH

3D structure of 4-(bromomethylene)cyclohexanone (**1a**) was generated with MAESTRO modeling software (Schrödinger). Using as receptor protein the TbSADH complexed with (*S*)-2-butanol (PDB 1BXZ),[10a] we generated with Glide application (MAESTRO software) 20 poses of substrate **1a** for replacing the (*S*)-2-butanol molecule in the active site of TbSADH.

For modeling substrate **1a** in the catalytic site of TbSADH, we performed a similar procedure as previously described by Ziegelmann *et al.*[6a] The TbSADH-(*S*)-2-butanol complex was superimposed with the TbSADH-$NADP^+$ complex (PDB 1YKF)[10b] using PyMOL. After alignment, the entire 1BXZ complex, except for the (*S*)-2-butanol, was removed. Then we merged the lowest energy conformation pose of **1a** generated before with reactive oxygen closest to C-OH from (*S*)-2-butanol. The result was a single TbSADH model containing $NADP^+$, Zn and both substrates. $NADP^+$s nicotinamide ring was rotated 90° to allocate both substrates in the catalytic site as previously described.[6a] We fitted manually **1a** substrate in PyMOL to superimpose its C-O group with C-OH from (*S*)-2-butanol. Finally, (*S*)-2-butanol was removed from the final model TbSADH-$NADP^+$-Zn-**1a** to generate the image that appears in Figure 1from main text. This is not meant to be a systematic theoretical treatment; rather, it serves as a rough guide for choosing CAST sites.

[10] a) C. Li, J. Heatwole, S., soelaiman, M. Shoham, *Proeins Struct. Funct. Gent*. **1999**, *37*, 619-627; b) Y. Korkhin, A. J. Kalb(Gilboa), M. Peretz, O. Bogin, Y. Burstein, F. Frolow, *J. Mol. Biol*. **1998**, *278*, 967-981.

Table S1. Reduction of model ketones 1a-f with commercial alcohol dehydrogenases.[a][b]

Entry	ADH[c]	Cofactor[d]	Substrate											
			1a→2a		1b→2b		1c→2c		1d→2d		1e→2e		1f→2f	
			Conv. (%)	%*ee*	Conv. (%)	%*ee*	Conv. (%)	%*ee*	Conv. (%)	%*ee*	Conv. (%)	%*ee*	Conv. (%)	%*ee*
1	A1	$NADP^+$	39	46(*S*)	≥ 99	47(*S*)	≥ 99	97(*S*)	≥ 99	60(*S*)	≥ 99	27(*S*)	≥ 99	39(*R*)
2	A2	$NADP^+$	≤ 20	n.d.[e]	≥ 99	23(*S*)	≥ 99	96(*S*)	≥ 99	58(*S*)	≥ 99	27(*S*)	≥ 99	38(*R*)
3	A3	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	≤ 20	n.d.[e]	≤ 20	n.d.[e]	≤ 20	n.d.[e]	26	73(*R*)
4	A4	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	41	84(*R*)	≤ 20	n.d.[e]	35	85(*R*)	83	96(*R*)
5	A5	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	32	55(*R*)	≤ 20	n.d.[e]	≤ 20	n.d.[e]	31	85(*R*)
6	A6	$NADP^+$	≥ 99	83(*S*)	≥ 99	42(*S*)	90	78(*S*)	≥ 99	60(*S*)	≥ 99	28(*S*)	≥ 99	39(*R*)
7	A7	NAD^+	≥ 99	64(*R*)	≥ 99	1(*S*)	≥ 99	97(*R*)	≥ 99	98(*R*)	≥ 99	99(*R*)	≥ 99	96(*R*)
8	A8	NAD^+	≥ 99	96(*R*)	≥ 99	62(*R*)	87	98(*R*)	≥ 99	95(*R*)	≥ 99	≥ 99(*R*)	≥ 99	99(*R*)
9	A9	NAD^+	≥ 99	81(*S*)	≥ 99	66(*S*)	95	42(*S*)	≥ 99	80(*S*)	≥ 99	84(*S*)	≥ 99	68(*S*)
10	A10	NAD^+	≥ 99	96(*R*)	≥ 99	73(*R*)	≥ 99	98(*R*)	≥ 99	95(*R*)	≥ 99	≥ 99(*R*)	≥ 99	99(*R*)
11	A11	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	95	56(*R*)	≤ 20	n.d.[e]	≤ 20	n.d.[e]	59	90(*R*)
12	A12	NAD^+	≥ 99	71(*S*)	≥ 99	38(*S*)	93	34(*S*)	≥ 99	79(*S*)	≥ 99	83(*S*)	≥ 99	35(*S*)
13	A13	NAD^+	≥ 99	95(*R*)	≥ 99	67(*R*)	97	98(*R*)	≥ 99	95(*R*)	≥ 99	≥ 99(*R*)	≥ 99	99(*R*)
14	A14	NAD^+	≥ 99	74(*R*)	≥ 99	27(*R*)	96	98(*R*)	≥ 99	97(*R*)	≥ 99	99(*R*)	≥ 99	96(*R*)
15	A15	NAD^+	54	91(*R*)	60	68(*R*)	81	96(*R*)	29	92(*R*)	82	98(*R*)	≥ 99	≥ 99(*R*)
16	A16	$NADP^+$	≥ 99	83(*S*)	≥ 95	42(*S*)	89	93(*S*)	≥ 95	66(*S*)	≥ 99	27(*S*)	≥ 99	39(*R*)
17	A17	NAD^+	50	52(*S*)	34	10(*R*)	52	40(*R*)	26	58(*S*)	38	42(*S*)	≤ 20	n.d.[e]
18	A18	NAD^+	≤ 20	n.d.[e]	≥ 99	2(*R*)	97	97(*R*)	≥ 99	97(*R*)	≥ 99	99(*R*)	≥ 99	96(*R*)
19	A19	NAD^+	29	48(*R*)	≤ 20	n.d.[e]	34	53(*R*)	≤ 20	n.d.[e]	50	75(*R*)	67	93(*R*)
20	A20	NAD^+	≥ 99	83(*S*)	≥ 99	65(*S*)	95	58(*S*)	≥ 95	80(*S*)	≥ 99	85(*S*)	≥ 99	73(*S*)
21	A21	NAD^+	≥ 99	93(*R*)	≥ 99	74(*R*)	95	96(*R*)	≥ 99	96(*R*)	99	96(*R*)	≥ 99	≥ 99(*R*)
22	A22	NAD^+	41	73(*R*)	≤ 20	n.d.[e]	60	93(*R*)	≤ 20	n.d.[e]	27	90(*R*)	≥ 99	99(*R*)
23	A23	$NADP^+$	≥ 99	81(*S*)	53	35(*S*)	95	91(*S*)	≥ 99	60(*S*)	56	6(*S*)	≥ 99	40(*R*)
24	A24	$NADP^+$	≥ 99	87(*S*)	≥ 99	51(*S*)	90	94(*S*)	≥ 99	61(*S*)	57	25(*S*)	≥ 99	38(*R*)
25	A25	NAD^+	≥ 99	61(*R*)	≥ 99	0	88	93(*R*)	≥ 99	97(*R*)	≥ 99	95(*R*)	≥ 99	96(*R*)
26	A26	NAD^+	≤ 20	n.d.[e]	≥ 99	60(*S*)	85	61(*S*)	≥ 99	80(*S*)	≥ 99	84(*S*)	≥ 99	72(*S*)
27	A27	$NADP^+$	≤ 20	n.d.[e]	≥ 99	48(*S*)	90	95(*S*)	≥ 99	61(*S*)	≥ 99	28(*S*)	≥ 99	39(*R*)
28	A28	$NADP^+$	≤ 20	n.d.[e]	≥ 99	44(*S*)	93	95(*S*)	≥ 99	61(*S*)	≥ 99	25(*S*)	≥ 99	39(*R*)
29	A29	NAD^+	41	80(*R*)	≤ 20	n.d.[e]	67	87(*R*)	≤ 20	n.d.[e]	61	96(*R*)	≥ 99	99(*R*)
30	A30	NAD^+	≥ 99	87(*R*)	≥ 99	72(*R*)	88	84(*R*)	≥ 99	91(*R*)	≥ 99	96(*R*)	≥ 99	98(*R*)
31	A31	NAD^+	≥ 99	82(*S*)	≥ 99	66(*S*)	93	63(*S*)	≥ 99	81(*S*)	≥ 99	85(*S*)	≥ 99	73(*S*)
32	A32	$NADP^+$	≥ 99	81(*S*)	≥ 99	42(*S*)	99	96(*S*)	≥ 99	59(*S*)	≥ 99	27(*S*)	≥ 99	40(*R*)
33	A33	NAD^+	≥ 99	65(*R*)	≥ 99	11(*R*)	94	96(*R*)	≥ 99	98(*R*)	≥ 99	98(*R*)	≥ 99	95(*R*)

34	A34	$NADP^+$	≥ 99	82(*S*)	≥ 99	46(*S*)	97	96(*S*)	≥ 99	59(*S*)	≥ 99	27(*S*)	≥ 99	40(*R*)
35	A35	$NADP^+$	≥ 99	84(*S*)	≥ 99	48(*S*)	93	95(*S*)	≥ 99	60(*S*)	≥ 99	28(*S*)	≥ 99	40(*R*)
36	1.1.010	NAD^+	≥ 99	29(*R*)	95	43(*R*)	91	91(*R*)	≥ 99	71(*R*)	≥ 99	58(*R*)	≥ 99	34(*R*)
37	1.1.020	NAD^+	≥ 99	91(*R*)	90	86(*R*)	96	89(*R*)	≥ 99	95(*R*)	≥ 99	97(*R*)	≥ 99	97(*R*)
38	1.1.030	NAD^+	≥ 99	83(*R*)	≥ 99	5(*R*)	94	95(*R*)	≥ 99	96(*R*)	≥ 99	96(*R*)	≥ 99	93(*R*)
39	1.1.040	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	60	92(*R*)	81	68(*R*)	64	44(*R*)	83	60(*R*)
40	1.1.130	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	≤ 20	n.d.[e]	59	72(*R*)	≤ 20	n.d.[e]	≤ 20	n.d.[e]
41	1.1.140	NAD^+	≤ 20	n.d.[e]	≤ 20	n.d.[e]	≤ 20	n.d.[e]	36	41(*R*)	53	30(*R*)	≤ 20	n.d.[e]
42	1.1.190	$NADP^+$	≥ 99	72(*S*)	93	24(*R*)	57	39(*R*)	61	38(*R*)	95	31(*R*)	≥ 99	47(*R*)
43	1.1.200	NAD^+	≥ 99	2(*S*)	≥ 99	1(*S*)	94	18(*S*)	≥ 99	53(*S*)	≥ 99	54(*S*)	≥ 99	22(*S*)
44	1.1.210	NAD^+	≥ 99	11(*R*)	≥ 99	0(*R*)	≥ 99	97(*R*)	≥ 99	94(*R*)	≥ 99	98(*R*)	≥ 99	92(*R*)
45	1.1.250	$NADP^+$	≥ 99	25(*S*)	≥ 99	15(*S*)	84	35(*S*)	≥ 99	32(*S*)	≥ 99	35(*S*)	≥ 99	4(*R*)
46	1.1.260	$NADP^+$	≥ 99	51(*R*)	≥ 99	50(*R*)	91	94(*R*)	≥ 99	97(*R*)	≥ 99	97(*R*)	≥ 99	92(*R*)
47	1.1.270	$NADP^+$	≥ 99	15(*S*)	≥ 99	3(*S*)	88	85(*S*)	≥ 99	29(*S*)	≥ 99	10(*S*)	≥ 99	54(S)

[a] 5-10 mg of each lyophilized ADH were dissolved in 500 µL of reaction mixture containing glucose (100 mM), cofactor NAD^+ or $NADP^+$ (1 mM), Glucose Dehydrogenase (GDH 105, Codexis, 0.04 Units),**1a-f** (5 mM) dissolved in acetonitrile and phosphate buffer (pH 7.0, 100 mM). Reaction performed at 30°C with shacking during 16 h.
[b] Average of at least 2 independent measurements. Deviation in the values obtained did not excess the 15% of the average ee value shown.
[c] Alcohol dehydrogenases from entries 1 to 35 were purchased from X-Zyme. Alcohol dehydrogenases from entries 36 to 47 were gifted by evocatal.
[d] Cofactor used upon recommendation of the supplier.
[e] Value not determined due to low conversion.

Table S2. Reduction of model ketone 1a with TbSADH* mutants obtained by saturation mutagenesis at position I86.[a,b]

Entry	Mutation	Conversion[c]	%*ee*
1	TbSADH*	≥99%	65(*R*)
2	I86A	≥99%	97(*S*)
3	**I86R**	**≥99%**	**64(*S*)**
4	**I86N**	**≥99%**	**97(*S*)**
5	**I86D**	**≥99%**	**98(*S*)**
6	**I86C**	**≥99%**	**98(*S*)**
7	**I86Q**	**≥99%**	**88(*S*)**
8	I86E	≥99%	95(*S*)
9	I86G	≥99%	98(*S*)
10	**I86H**	**≥99%**	**97(*S*)**
11	**I86L**	**≥99%**	**61(*S*)**
12	**I86K**	**75%**	**86(*S*)**
13	I86M	≥99%	92(*S*)
14	**I86F**	**23%**	**94(*S*)**
15	**I86P**	**≥99%**	**99(*S*)**
16	**I86S**	**≥99%**	**95(*S*)**
17	I86T	≥99%	92(*S*)
18	**I86W**	**<5%**	**n.d.**[d]
19	**I86Y**	**71%**	**98(*S*)**
20	**I86V**	**93%**	**20(*S*)**

[a] Conversion and % *ee* values shown are obtained from average of two independent experiments. Deviation in the values obtained did not excess the 3% of the average ee value shown.

[b] Entries in bold are those mutants obtained by QuikChange. Rests of them are the same that appear in Table 2 from main text. They have been included here only for comparison.

[c] Compound **2a** was the major product when using **1a** as substrate. (≤5% side products in all cases).

[e] Value not determined due to low conversion.

Table S3. Performance of best TbSADH* mutants specifically evolved for substrate 1a as catalysts in the asymmetric reduction of ketones 3 and 5a-b.[a]

Entry	Mutation	Substrate					
		3→4		5a→6a		5b→6b	
		Conv. (%)	*ee*%	Conv. (%)	*ee*%	Conv. (%)	*ee*%
1	TbSADH*	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
2	A85V	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
3	I86A	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
4	I86G	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
5	I86E	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
6	I86M	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
7	I86T	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]
8	W110A	≥ 99	≥ 99	65	55	58	50
9	W110E	≥ 99	≥ 99	≤ 5	n.d.[b]	≤ 5	n.d.[b]
10	W110M	≥ 99	≥ 99	≤ 5	n.d.[b]	≤ 5	n.d.[b]
11	W110T	≥ 99	≥ 99	13	79	18	62
12	C295E	≤ 5	n.d.[b]	≤ 5	n.d.[b]	≤ 5	n.d.[b]

[a] Conversion and % *ee* values shown are obtained from average of two independent experiments. Deviation in the values obtained did not excess the 3% of the average ee value shown.
[b] Value not determined due to low conversion.

Table S4. Primers used for cloning and saturation mutagenesis in the present work.

Name	Sequence (5'→3')
pRSF-TbSADH*-Fw	ttttgtttaactttaataaggagatataccatgaaaggttttgcaatgctcagtatcgg
pRSF-*Nco*I	ggtatatctccttattaaagttaaacaaaattatttctacagg
pRSF-*Avr*II	taacctaggctgctgccaccgctgagcaataac
pRSF-TbSADH*-rv	gttattgctcagcggtggcagcagcctaggttagcataaaaatggactaagtcccc
S39-RNG-Fw	ggccccttgcactrnggacattcataccg
S39-RNG-rv	cggtatgaatgtccnyagtgcaaggggcc
A85-RNG-Fw	gcgttgttgtgccarngattacccctgattggtg
A85-RNG-rv	caccaatcaggggtaatcnytggcacaacaacgc
I86-RNG-Fw	cgttgttgtgccagctrngacccctgattggcgg
I86-RNG-rv	ccgccaatcaggggtcnyagctggcacaacaacg
W110-RNG-Fw	ggaatgctggcaggcrngaaattttcgaatgtaaaag
W110-RNG-rv	cttttacattcgaaaatttcnygcctgccagcattcc
Y267-RNG-Fw	ccatcgctaatgtaaatrngtttggcgaaggagag
Y267-RNG-rv	ctctccttcgccaaacnyatttacattagcgatgg
C295-RNG-Fw	ctataaaaggcgggctarngcccggtggacgtctaag
C295-RNG-rv	cttagacgtccaccgggcnytagcccgccttttatag
I86V-Fw	cgttgttgtgccagctgtgacccctgattggcgg
I86V-rv	ccgccaatcaggggtcacagctggcacaacaacg
I86N-Fw	cgttgttgtgccagctaacacccctgattggcgg
I86N-rv	ccgccaatcaggggtgttagctggcacaacaacg
I86D-Fw	cgttgttgtgccagctgacacccctgattggcgg
I86D-rv	ccgccaatcaggggtcagagctggcacaacaacg
I86C-Fw	cgttgttgtgccagcttgcacccctgattggcgg
I86C-rv	ccgccaatcaggggtgcaagctggcacaacaacg
I86Q-Fw	cgttgttgtgccagctcagacccctgattggcgg
I86Q-rv	ccgccaatcaggggtctgagctggcacaacaacg
I86H-Fw	cgttgttgtgccagctcacacccctgattggcgg
I86H-rv	ccgccaatcaggggtgtgagctggcacaacaacg
I86L-Fw	cgttgttgtgccagctctgacccctgattggcgg
I86L-rv	ccgccaatcaggggtcagagctggcacaacaacg
I86F-Fw	cgttgttgtgccagctttcacccctgattggcgg
I86F-rv	ccgccaatcaggggtgaaagctggcacaacaacg
I86P-Fw	cgttgttgtgccagctccaacccctgattggcgg
I86P-rv	ccgccaatcaggggttggagctggcacaacaacg
I86S-Fw	cgttgttgtgccagcttcgacccctgattggcgg
I86S-rv	ccgccaatcaggggtcgaagctggcacaacaacg
I86W-Fw	cgttgttgtgccagcttggacccctgattggcgg
I86W-rv	ccgccaatcaggggtccaagctggcacaacaacg
I86Y-Fw	cgttgttgtgccagcttacacccctgattggcgg
I86Y-rv	ccgccaatcaggggtgtaagctggcacaacaacg
I86K-Fw	cgttgttgtgccagctaagacccctgattggcgg
I86K-rv	ccgccaatcaggggtcttagctggcacaacaacg
I86R-Fw	cgttgttgtgccagctaagacccctgattggcgg
I86R-rv	ccgccaatcaggggtcttagctggcacaacaacg

Table S5. Reduction of acetophenone with TbSADH* mutants produced in this study.

Entry	Mutation	**1a→2a**[a]		**acetophenone → 1-phenylethanol**[b]	
		Conv. (%)	**%*ee***	**Conv. (%)**	**%*ee***
1	WT	≥99	65(*R*)	<5	n.d.[c]
2	I86A	≥99	97(*S*)	97	99(*R*)
3	I86R	≥99	64(*S*)	9	90(*R*)
4	I86N	≥99	97(*S*)	98	99(*R*)
5	I86D	≥99	98(*S*)	97	99(*R*)
6	I86C	≥99	98(*S*)	98	99(*R*)
7	I86Q	≥99	88(*S*)	93	99(*R*)
8	I86E	≥99	95(*S*)	97	99(*R*)
9	I86G	≥99	98(*S*)	97	99(*R*)
10	I86H	≥99	97(*S*)	<5	n.d.[c]
11	I86L	≥99	61(*S*)	93	99(*R*)
12	I86K	75	86(*S*)	<5	n.d.[c]
13	I86M	≥99	92(*S*)	97	99(*R*)
14	I86F	23	94(*S*)	<5	n.d.[c]
15	I86P	≥99	99(*S*)	98	99(*R*)
16	I86S	≥99	95(*S*)	98	99(*R*)
17	I86T	≥99	92(*S*)	98	99(*R*)
18	I86W	<5	n.d.[d]	<5	n.d.[c]
19	I86Y	71	98(*S*)	<5	n.d.[c]
20	I86V	93	20(*S*)	<5	n.d.[c]
21	W110A	≥99	82(*R*)	5	93(*S*)
22	W110E	≥99	91(*R*)	65	97(*S*)
23	W110M	≥99	97(*R*)	73	98(*S*)
24	W110T	≥99	97(*R*)	10	98(*S*)
25	C295E	16	84(*R*)	<5	n.d.[c]
26	A85V	≥95	95(*R*)	<5	n.d.[c]

[a] Values for **1a→2a** reaction are the same that appear in Table 2 from main text and Table S2. They have been included here only for comparison.

[b] 1-phenlyethanol was the major product when using acetophenone as substrate, (≤2% side products in all cases).

[c] Value not determined due to low conversion.

Sequence 1. Plasmid pRSF-TbSADH* sequence. TbSADH* coding sequence is written in bold letters. The additional TAA stop codon at the 3´end of TbSADH* coding sequence, introduced by PCR amplification using primer pRSF-TbSADH*-rv, is underlined.

```
   1 ggggaattgt gagcggataa caattcccct gtagaaataa ttttgtttaa ctttaataag
  61 gagatatacc atgaaaggtt ttgcaatgct cagtatcggt aaagttggct ggattgagaa
 121 ggaaaagcct gctcctggcc catttgatgc tattgtaaga cctctagctg tggccccttg
 181 cacttcggac attcataccg tttttgaagg cgccattggc gaaagacata acatgatact
 241 cggtcacgaa gctgtaggtg aagtagttga agtaggtagt gaggtaaaag attttaaacc
 301 tggtgatcgc gttgttgtgc cagctattac ccctgattgg cggacctctg aagtacaaag
 361 aggatatcac cagcactccg gtggaatgct ggcaggctgg aaattttcga atgtaaaaga
 421 tggtgttttt ggtgaatttt ttcatgtgaa tgatgctgat atgaatttag cacatctgcc
 481 taaagaaatt ccattggaag ctgcagttat gattcccgat atgatgacca ctggttttca
 541 cggagctgaa ctggcagata tagaattagg tgcgacggta gcagttttgg gtattggccc
 601 agtaggtctt atggcagtcg ctggtgacaa attgcgtgga gccggaagaa ttattgccgt
 661 aggcagtaga ccagtttgtg tagatgctgc aaaatactat ggagctactg atattgtaaa
 721 ctataaagat ggtcctatcg aaagtcagat tatgaatcta actgaaggca aaggtgtcga
 781 tgctgccatc atcgctggag gaaatgctga cattatggct acagcagtta agattgttaa
 841 acctggtggc accatcgcta atgtaaatta ttttggcgaa ggagaggttt tgcctgttcc
 901 tcgtcttgaa tggggttgcg gcatggctca taaaactata aaaggcgggc tatgccccgg
 961 tggacgtcta agaatggaaa gactgattga ccttgttttt tataagcgtg tcgatccttc
1021 taagctcgtc actcacgttt tccggggatt tgacaatatt gaaaaagcct ttatgttgat
1081 gaaagacaaa ccaaaagacc taatcaaacc tgttgtaata ttagcataat aacctaggct
1141 gctgccaccg ctgagcaata actagcataa ccccttgggg cctctaaacg ggtcttgagg
1201 ggtttttttgc tgaaacctca ggcatttgag aagcacacgg tcacactgct tccggtagtc
1261 aataaaccgg taaaccagca atagacataa gcggctattt aacgaccctg ccctgaaccg
1321 acgacaagct gacgaccggg tctccgcaag tggcactttt cggggaaatg tgcgcggaac
1381 ccctatttgt ttatttttct aaatacattc aaatatgtat ccgctcatga attaattctt
1441 agaaaaactc atcgagcatc aaatgaaact gcaatttatt catatcagga ttatcaatac
1501 catatttttg aaaaagccgt ttctgtaatg aaggagaaaa ctcaccgagg cagttccata
1561 ggatggcaag atcctggtat cggtctgcga ttccgactcg tccaacatca atacaaccta
1621 ttaatttccc ctcgtcaaaa ataaggttat caagtgagaa atcaccatga gtgacgactg
1681 aatccggtga gaatggcaaa agtttatgca tttctttcca gacttgttca acaggccagc
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1861 gcaaccggcg caggaacact gccagcgcat caacaatatt ttcacctgaa tcaggatatt
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1981 caggagtacg gataaaatgc ttgatggtcg gaagaggcat aaattccgtc agccagttta
2041 gtctgaccat ctcatctgta acatcattgg caacgctacc tttgccatgt ttcagaaaca
2101 actctggcgc atcgggcttc ccatacaatc gatagattgt cgcacctgat tgcccgacat
2161 tatcgcgagc ccatttatac ccatataaat cagcatccat gttggaattt aatcgcggcc
2221 tagagcaaga cgtttcccgt tgaatatggc tcatactctt cctttttcaa tattattgaa
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2761 gtcgttcgct ccaagctggg ctgtgtgcac gaaccccccg ttcagcccga ccgctgcgcc
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2881 gcagccattg gtaactgatt tagaggactt tgtcttgaag ttatgcacct gttaaggcta
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3661 cagcatcgca gtgggaacga tgccctcatt cagcatttgc atggtttgtt gaaaaccgga
3721 catggcactc cagtcgcctt cccgttccgc tatcggctga atttgattgc gagtgagata
3781 tttatgccag ccagccagac gcagacgcgc cgagacagaa cttaatgggc ccgctaacag
3841 cgcgatttgc tggtgaccca atgcgaccag atgctccacg cccagtcgcg taccgtcttc
3901 atgggagaaa ataatactgt tgatgggtgt ctggtcagag acatcaagaa ataacgccgg
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4021 gatcagccca ctgacgcgtt gcgcgagaag attgtgcacc gccgctttac aggcttcgac
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4201 cagcaacgac tgtttgcccg ccagttgttg tgccacgcgg ttgggaatgt aattcagctc
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4321 cacgcgggaa acggtctgat aagagacacc ggcatactct gcgacatcgt ataacgttac
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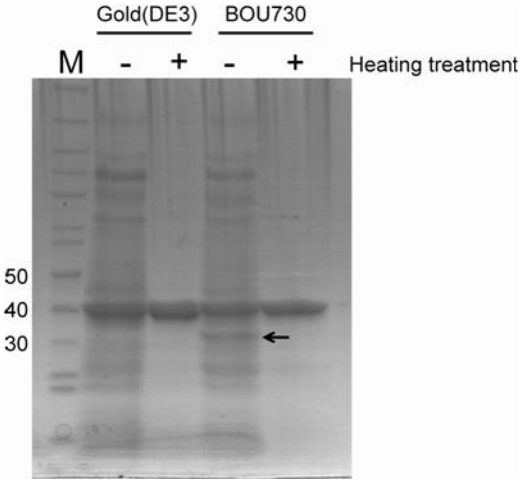


Figure S1. SDS-PAGE analysis of TbSADH* overexpressed either in *E. coli* Gold (DE3) cells or in *E. coli* BOU730 strain in the presence (lines +), or in the absence (lines -), of heating treatment (15 min. at 75°C). Expected molecular mass of TbSADH* is 37.6 kDa. Protein expression levels are similar in both strains. Black arrow indicates overexpression of GDH in BOU730 cells. Note this band disappears after heating treatment, whereas intensity of TbSADH* band is similar either in the absence or in the presence of heating treatment. Molecular standards (line M) of 30, 40 and 50 kDa are indicated.

Chemistry

General remarks

Syntheses of compounds **1a**-**c**,[11] and **1e**[11] as well as the preparation of compounds **4**[12] and **5b**[13] are described previously. All other starting compounds and reagents including dry solvents were purchased from Acros, Sigma-Aldrich and Alfa and used without further purification. NMR spectra were recorded on a Bruker Avance 300 or DRX 400 (^{1}H: 300 MHz or 400 MHz, ^{13}C: 75 MHz or 101 MHz) spectrometer using TMS as internal standard (d=0) unless otherwise noted. High resolution EI mass spectra were measured on a Finnigan MAT 95S spectrometer. High-resolution mass spectra recorded in ESI and APCI mode were performed on a ThermoScientific LTQ-FT spectrometer. Conversion and enantiomeric composition were determined by achiral and chiral gas chromatography as described. Alternatively, product **2c** enantiomeric excess could be measured by HPLC. Analytical thin layer chromatography was performed on Merck silica gel 60 F254q while for column chromatography Merck silica gel 60 (230–400 mesh ASTM) was used. Reactions that required inert atmosphere (nitrogen or argon) were carried out using standard Schlenk techniques. GC Analyses were performed on a HP 6890 series device.

General method for the synthesis of racemic standards

Preparation of **2a-f** and **6a-b**: To a methanol solution (1 mL) of corresponding ketone (1.0 Eg), $NaBH_4$ (2.0 Eg) was added and the reactions were allowed to stir at r.t. until the TLC or GC showed completion (normally within a few minutes). Reactions crudes were subsequently analyzed by GC or HPLC.
Preparation of *rac*-**(4)**: Racemic mixture was prepared according to the experimental procedure described in reference 12.

Absolute configuration determination

Initially, the absolute configuration of (*S*)-**2d** was assigned by comparison with an authentic sample as prepared by Walborsky et al.[14] The absolute configuration of **2a** was determined after its conversion into derivative **2d** by Pd-catalyzed carbonylation and optical rotation comparison with an authentic sample as reported by Walborsky.[14] Determination of the absolute configuration of **2b**, **2c** was made after performing couplings reactions starting from bromoalcohol **2a** as described below.
Determination of the absolute configuration of **2e-f** was made after performing carbonylation reactions starting from bromoalcohol **2a** as described below.

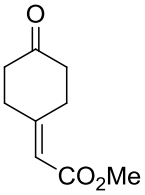
Compounds preparation

General procedure for carbonylation reactions providing compounds 1d, 1f and 2d-f

In a two necked round bottomed flask 4-(bromomethylene)cyclohexanone **1a** (1 Eg) respectively 4-(bromomethylene)cyclohexanol **2a** (1 Eg) and DIPEA (1.2-1.5 Eg) were added to a mixture of dry alcohol (MeOH, EtOH or *i*Pr-OH): dry tetrahydrofuran (3:1, 30 mL) and the solution flushed with argon for 15 min. After this time $Pd(PPh_3)_4$ (0.03% mol) and PPh_3 (0.03% mol) were added and solution flushed for 5 additional min with argon. A balloon filled with CO was then flushed to the solution and a second one was attached to the installation equipped with condenser. Mixtures were refluxed and normally after approximately 24 h GC indicated the total conversion of the starting material and product formation. The reaction crudes were rotaevaporated and their residues loaded on chromatographic columns to afford pure **1d, 1f** and **2d-f** in good yields.

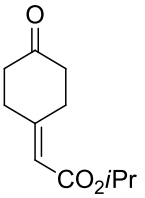
[11] Z.-G. Zhang, G.-D. Roiban, J. P. Acevedo, I. Polyak, M. T. Reetz, A New Type of Stereoselectivity in Baeyer-Villiger Reactions: Access to *E*- and *Z*-Olefins, *Adv. Synth. Catal*., in press.
[12] K. Okuma, Y. Kamahori, K. Tsubakihara, K. Yoshihara, Y. Tanaka, K. Shioji, *J. Org. Chem.* **2002**, *67*, 7355-7360.
[13] G. A. Molander, Y. Le Huerou, G. A. Brown, *J. Org. Chem.* **2001**, *66*, 4511-4516.
[14] H. M. Walborsky, K. Gawronska, J. K. Gawronski, *J. Am. Chem. Soc.* **1987**, *109*, 6719-6726.

Preparation of compound 1d



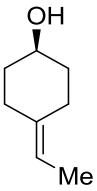
Using the general carbonylation procedure, 4-(bromomethylene)cyclohexanone **1a** (301 mg, 1.59 mmol) and methanol afforded after chromatographic column separation compound **1d** as a white solid (210 mg, 78%). (R_f=0.50 ethyl acetate/petroleum ether=1:2); ^{1}H NMR (300 MHz, $CDCl_3$ δ=5.85 (s, 1H), 3.71 (s, 3H), 3.20 (t, ^3J=6.7 Hz, 2H), 2.66 (t, ^3J=6.6 Hz, 2H), 2.49 ppm (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=210.11, 166.55, 157.15, 115.76, 51.11, 39.57, 38.98, 33.85, 26.83 ppm; MS (70 eV, EI): *m*/*z* (%): 168 $[M]^+$ (100); HRMS (APCI–): calcd for $C_9H_{11}O_3$ $[M\text{-}H]^-$: 167.0714; found: 167.0710.

Preparation of compound 1f



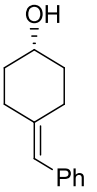
Using the general carbonylation procedure, 4-(bromomethylene)cyclohexanone **1a** (246 mg, 1.30 mmol) and isopropanol afforded after chromatographic column purification compound **1f** as a pale yellow solid (176 mg, 69%). (R_f=0.20 ethyl acetate/petroleum ether=1:6); ^{1}H NMR (300 MHz, $CDCl_3$) δ=5.83 (s, 1H), 5.13-5.01 (br s, 1H), 3.22 (m, 2H), 2.66 (m, 2H), 2.50 (m, 4H), 1.27 (m, 7H), 0.92 (m, 1H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ = 210.27, 165.68, 156.28, 116.68, 67.16, 39.60, 39.01, 33.85, 26.76, 21.93 (2C) ppm; HRMS (EI): calcd for $C_{11}H_{16}O_3$ $[M]^-$: 196.1093; found: 196.1093.

Preparation of (*R*)-4-ethylidenecyclohexanol 2b



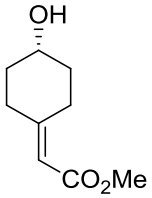
(*R*)-4-(Bromomethylene)cyclohexanol **2a** (75 mg, 0.39 mmol) and $Sn(CH_3)_4$ (272 µL, 1.96 mmol) were added in a 20 mL Schlenk flask containing dry toluene (7 mL), under Ar atmosphere. Over the solution $PdCl_2(PPh_3)_2$ (13.7 mg, 0.05% mol) and PPh_3 (4 mg, 0.01% mol) were further added and the Schlenk tube was sealed with a glass stopper and secured. The mixture was then heated at 100ºC for 16 h. After this time the reaction mixture was carefully cooled down, evaporated and the residue subjected to column chromatography to afford compound (*R*)-**2b** as a viscous colorless liquid (32 mg, 65%). (R_f=0.60 ethyl acetate/petroleum ether=1:1); ^{1}H NMR (300 MHz, CD_3OD) δ=5.03 (q, ^3J=6.7 Hz, 1H), 3.58 (tt, ^3J = 9.4, 3.8 Hz, 1H), 2.51–2.32 (m, 1H), 2.20–2.01 (m, 1H), 2.00–1.80 (m, 1H), 1.81–1.63 (m, 3H), 1.43 (d, ^3J=6.7 Hz, 3H), 1.29–1.08 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ=139.17, 116.99, 70.60, 37.31, 36.25, 34.58, 25.50, 12.96; HRMS (EI): calcd for $C_8H_{14}O$ $[M]^-$: 126.1039; found: 126.1048.

Preparation of (*S*)-4-benzylidenecyclohexanol 2c



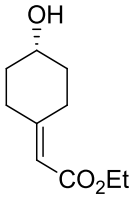
(*S*)-4-(Bromomethylene)cyclohexanol **2a** (83 mg, 0.43 mmol) and phenylboronic acid (1.2 Eg) were added in a 50 mL two necked round bottomed flask. Dioxane/H_2O (4:1, 40 mL) was further added and solution degassed with argon for 15 min, followed by the addition of K_2CO_3 (79 mg, 0.57 mmol). After addition of $Pd(PPh_3)_4$ (13.2 mg, 0.02% mol) and PPh_3 (3 mg, 0.02% mol), the solution was heated to 90ºC and refluxed for 24 h when GC showed reaction completion. The mixture was rotaevaporated and the residue subjected to column chromatography to afford compound (*S*)-**2c** as a viscous colorless liquid (54 mg, 66%). (R_f=0.55 ethyl acetate/petroleum ether=1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ=7.27 (m, 5H), 6.31 (s, 1H), 4.00-3.84 (m, 1H), 2.83-2.76 (m, 1H), 2.52-2.41 (m, 1H); 2.35-1.92 (m, 5H), 1.68-1.40 (m, 2H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ=140.93, 138.23, 128.99 (2C), 128.21 (2C), 126.19, 123.31, 69.66, 36.41, 35.70, 34.02, 25.78 ppm; HRMS (EI): calcd for $C_{13}H_{16}O$ [*M*]$^-$: 188.1195; found: 188.1207.

Preparation of (*S*)-methyl 2-(4-hydroxycyclohexylidene)acetate 2d



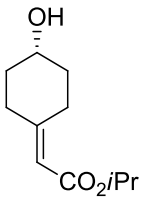
Using the general carbonylation procedure, (*S*)-4-(bromomethylene)cyclohexanol **2a** (90 mg, 0.47 mmol) and methanol afforded after chromatographic column purification compound (*S*)-**2d** as a pale yellow viscous liquid (66 mg, 82%). (R_f=0.34 ethyl acetate/petroleum ether=1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ=5.65 (s, 1H), 3.97-3.89 (m, 1H), 3.68 (s, 3H), 3.38-3.28 (m, 1H), 2.54-2.37 (m, 2H), 2.21-2.12 (m, 1H), 2.01-1.90 (m, 2H), 1.66-1.49 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 167.17, 161.33, 113.71, 68.49, 51.04, 35.88, 35.29, 34.13, 25.75 ppm; HRMS (APCI+): calcd for $C_9H_{14}O_3H_1$ [*M*+*H*]$^+$: 171.1016; found: 171.1016.

Preparation of (*S*)-ethyl 2-(4-hydroxycyclohexylidene)acetate 2e



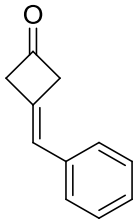
Using the general carbonylation procedure, (*S*)-4-(bromomethylene)cyclohexanol **2a** (74 mg, 0.38 mmol) and ethanol afforded after chromatographic column purification compound (*S*)-**2e** as a pale yellow liquid (63 mg, 88%). (R_f=0.44 ethyl acetate/petroleum ether=1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ=5.64 (s, 1H), 4.14 (q, ^3J=7.1 Hz, 2H), 3.93 (m, 1H), 3.41-3.27 (m, 1H), 2.53-2.36 (m, 2H), 2.24-2.12 (m, 1H), 1.98-1.92 (m, 2H), 1.64-1.49 (m, 3H), 1.27 (t, ^3J=7.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=166.77, 160.92, 114.16, 68.54, 59.76, 35.89, 35.29, 34.14, 25.73, 14.43 ppm; HRMS (APCI+): calcd for $C_{10}H_{17}O_3$ [*M*+*H*]$^+$: 185.1172; found: 185.1172.

Preparation of (*S*)-isopropyl 2-(4-hydroxycyclohexylidene)acetate 2f



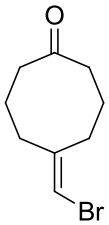
Using the general carbonylation procedure, (*S*)-4-(bromomethylene)cyclohexanol **2a** (80 mg, 0.41 mmol) and isopropanol, afforded after chromatographic column purification compound (*S*)-**2f** as a colorless viscous liquid (74 mg, 89%). (R_f=0.35 ethyl acetate/petroleum ether=1:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ=5.61 (s, 1H), 5.02 (hept, ^3J=6.3 Hz, 1H), 3.92 (m, 1H), 3.37-3.31 (m, 1H), 2.52-2.37 (m, 2H), 2.20-2.10 (m, 1H), 2.00-1.90 (m, 2H), 1.74 (br s, 1H, OH), 1.63-1.53 (m, 2H), 1.24 (dd, ^3J=6.2 Hz, ^4J=1.1 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=166.30, 160.48, 114.66, 68.59, 66.94, 35.87, 35.28, 34.14, 25.70, 22.08 (2C); HRMS (EI): calcd for $C_{11}H_{18}O_3$ [*M*]$^-$: 198.1250; found: 198.1254.

Preparation of 3-benzylidenecyclobutanone 3



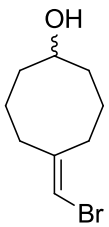
3-Phenylmethylene-cyclobutanol[12] (300 mg, 2.51 mmol) was added in a Schlenk flask under argon together with dry CH_2Cl_2 (2 mL). Dess-Martin reagent (12.55 mL, 3.76 mmol, 0.3 M in CH_2Cl_2) was added and the solution stirred until TLC showed reaction completion (approximately 1h). Reaction mixture was then washed with aqueous satd. $NaHCO_3$ solution (3 x 15 mL) followed by $Na_2S_2O_3$ solution (3 x 15 mL). The organic phase was further dried over anhydrous $MgSO_4$, concentrated, and the residue loaded immediately on a chromatographic column. The collected fractions were washed with satd. $NaHCO_3$ solution, and then evaporated to afford 3-benzylidenecyclobutanone **3** (229 mg, 58%) as a viscous liquid which precipitated immediately after being stored to -80ºC. (R_f=0.58 ethyl acetate/petroleum ether=1:6); ^{1}H NMR (300 MHz, $CDCl_3$) δ=7.35–7.09 (m, 5H), 6.59–6.48 (m, 1H), 3.81 (d, ^2J = 10.3 Hz, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=205.48, 137.03, 129.04, 128.78 (2C), 127.42 (2C), 127.20, 124.45, 56.55, 54.52; HRMS (EI): calcd for $C_{11}H_{10}O$ [*M*]$^-$: 158.0726; found: 158.0743.

Preparation of 5-(bromomethylene)cyclooctanone 5a



Compound **5a** was prepared using a similar procedure like for compound **5b**:[13] Ph_3P=CHBr (1.74 g, 3.99 mmol) was dissolved in toluene (20 mL) and KO*t*-Bu (384 mg, 3.4 mmol) was added. The solution was stirred under Ar at 45°C for 40 min. Cyclooctane-1,5-dione[13] (400 mg, 2.84 mmol) was dissolved in dry toluene (15 mL) in a 100 mL Schlenk flask, under argon atmosphere, and the solution of Ph_3P=CHBr in toluene (20 mL) was added. The reaction was warmed to 95°C for 50 min. Then the mixture was allowed to cool to room temperature. Saturated aqueous NaCl (30 mL) was added and the phases were separated. The aqueous phase was extracted with toluene (3 x 30 mL) and the combined organic phases were dried over anhydrous $MgSO_4$ and then filtered. Solvent was then evaporated under reduced pressure and the residue purified on column chromatography to afford 5-(bromomethylene)cyclooctanone **5a** as a viscous liquid (310 mg, 50%). (R_f=0.30 diethyl ether/petroleum ether=1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ=5.93 (s, 1H), 2.42-1.95 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=214.77, 142.61, 106.63, 43.78, 39.90, 37.44, 31.87, 23.84, 23.11; HRMS (EI): calcd for $C_9H_{13}BrO$ [*M*]$^-$: 216.0144; found: 216.0133.

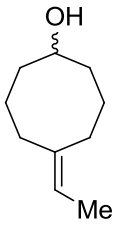
Preparation of *rac*-5-(bromomethylene)cyclooctanol 6a



Compound **5a** was added to a NMR tube containing deuterated methanol (CD_3OD). To the solution $NaBH_4$ was added and then the mixture allowed stirring for 2-3 h. GC showed reaction completion after this time. This sample was used later as control for the GC analyses. NMR showed complete formation of *rac*-**6a**. ^{1}H NMR (300 MHz, CD_3OD) δ=5.92 (q, ^4J=1.0 Hz, 1H), 3.62 (m, 1H), 2.38-2.29

(m, 1H), 2.19–1.97 (m, 3H), 1.82–1.37 (m, 8H). ^{13}C NMR (75 MHz, CD_3OD) δ=147.39, 103.54, 71.84, 38.03, 36.52, 35.65, 32.65, 24.08, 22.95; HRMS (APCI+): calcd for $C_9H_{16}BrO$ $[M+H]^+$: 219.0379, 221.0358; found: 219.0376, 221.0355.

Preparation of *rac*-5-ethylidenecyclooctanol 6b



Compound **5b** was added to a NMR tube containing deuterated methanol (CD_3OD). To the solution $NaBH_4$ was added and then the mixture allowed stirring for 2-3 h. GC showed reaction completion after this time. This sample was used later as control for the GC analyses. NMR showed complete formation of **6b**. ^{1}H NMR (300 MHz, CD_3OD) δ=5.16 (q, ^3J=7.1 Hz, 1H), 3.69 (tt, ^3J=7.6 Hz, ^4J=3.8 Hz, 1H), 2.28–2.12 (m, 1H), 2.09–1.84 (m, 3H), 1.81–1.29 (m, 12H); ^{13}C NMR (75 MHz, Methanol-d4) δ=141.57, 121.60, 72.06, 39.52, 36.76, 35.81, 29.72, 24.61, 24.20, 13.50; HRMS (APCI+): calcd for $C_{11}H_{19}O$ $[M+H]^+$: 155.1430; found: 155.1428.

Determination of conversion and enantiomeric excess (ee)

GC analyses

Compound	Achiral Analysis		Chiral Analysis	
	Conditions	**Retention time (min)**	**Conditions**	**Retention time (min)**
1a→2a	column: 15 m DB-Wax, inner diameter of 0.25 mm; pressure: 0.4 bar H_2; injector: 230°C; oven: temperature gradient: 80–260°C with 6°C/min and then holding 260°C for 10 min, FID detector: 350°C;	12.2	column 25 m Hydrodex, ß-TBDAc, inner diameter of 0.25 mm; pressure: 0.8 bar H_2; injector: 230°C; oven: isothermic at 110°C FID detector: 320°C;	(*R*)-**2a**: 34.8-35.6 (*S*)-**2a**: 36.6-38.2
1b→2b	column: 15 m DB-Wax, inner diameter of 0.25 mm; pressure: 0.4 bar H_2; injector: 230°C; oven: temperature gradient: 60–260°C with 6°C/min and then holding 260°C for 10 min, FID detector: 350°C.	8.0	column 30 m BGB-176, inner diameter of 0.25 mm; pressure: 0.6 bar H_2; injector: 230°C; oven: isothermic at 90°C FID detector: 350°C.	(*R*)-**2a**: 18.6-18.9 (*S*)-**2a**: 19.6-20.1
1d→2d	column: 15 m DB-Wax, inner diameter of 0.25 mm; pressure: 0.4 bar H_2; injector: 230°C; oven: temperature gradient: 80–260°C with 6°C/min and then holding 260°C for 10 min, FID detector: 350°C.	15.8	column 25 m Hydrodex, ß-TBDAc, inner diameter of 0.25 mm; pressure: 0.4 bar H_2; injector: 230°C; oven: isothermic at 150°C FID detector: 350°C.	(*S*)-**9**: 22.6 (*R*)-**9**: 23.1
1e→2e		16.5	column 30 m BGB-174, inner diameter of 0.25 mm; pressure: 0.8 bar H_2; injector: 220°C; oven: isothermic at 140°C FID detector: 350°C.	(*S*)-**6**: 41.9 (*R*)-**6**: 42.6
1f→2f		16.3	column 25 m Hydrodex, ß-TBDAc, inner diameter of 0.25 mm; pressure: 0.8 bar H_2; injector: 230°C; oven: isothermic at 130°C FID detector: 350°C.	(*S*)-**6**: 43.8 (*R*)-**6**: 44.3
3→4	column: 30 m DB-1, inner diameter of 0.25 mm; pressure: 0.5 bar H_2; injector: 220°C; oven: temperature gradient: 60–340°C with 6°C/min and then holding 340°C for 5 min, FID detector: 320°C.	18.0	column 25 m Hydrodex, ß-TBDAc, inner diameter of 0.25 mm; pressure: 0.6 bar H_2; injector: 220°C; oven: isothermic at 140°C FID detector: 320°C.	*(enantiomer 1)*-**4**: 33.2 *(enantiomer 2)*-**4**: 34.1
5a→6a		18.7	column 25 m Ivadex, inner diameter of 0.25 mm; pressure: 0.5 bar H_2; injector: 220°C; oven: temperature gradient: 100–125°C with 0.2°C/min, 125-220°C with 12ºC/min then holding 220°C for 5 min, FID detector: 320°C.	*(enantiomer 1)*-**6a**: 94.0 *(enantiomer 2)*-**6a**: 96.0
5b→6b		14.1	column 25 m Lipodex G, inner diameter of 0.25 mm; pressure: 0.7 bar H_2; injector: 220°C; oven: temperature gradient: 40–100°C with 0.2°C/min, 100-220°C with 12ºC/min then holding 220°C for 5 min, FID detector: 320°C.	*(enantiomer 1)*-**6b**: 205.0 *(enantiomer 2)*-**6b**: 210.0

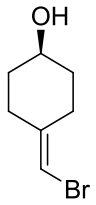
HPLC analyses of 1c and 2c

In the case of alcohol **2c**, HPLC was used to confirm the enantiomeric excess, using as control a racemic sample. Same method was used for chiral and achiral analyses.

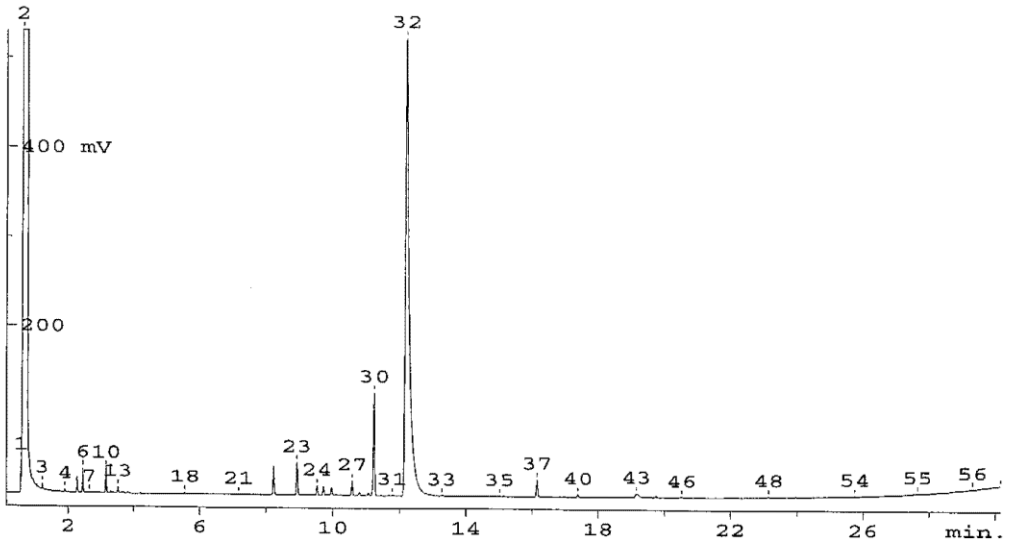
Compound	**Chiral and Achiral Analyses**	
	Conditions	**Retention time (min)**
1c→**2c**	150 mm Kromasil Amycoat RP, 4.6 min i.d., Acetonitrile/water = 50:50. flow: 1.0 mL/min, 16.2 MPa, 298 K; Detection : UV, 220 nm.	**1c**: 7.1 (*S*)-**2c**: 4.6 (*R*)-**2c**: 6.0

GC chromatograms

(*R*)-4-(bromomethylene)cyclohexanol (2a) resulting from biocatalytic asymmetric ketone reduction of 1a with TbSADH*



Achiral chromatogram



********** R513S683.DAT ********** 10-MAY-12 11:13:46

Sample : 12/90977
Cust.ID: AGU-AA-228-02
S.code : in EE

measured: 9-MAY-12 10:17:18
processed: 10-MAY-12 11:13:42
by: CHROM3
with:

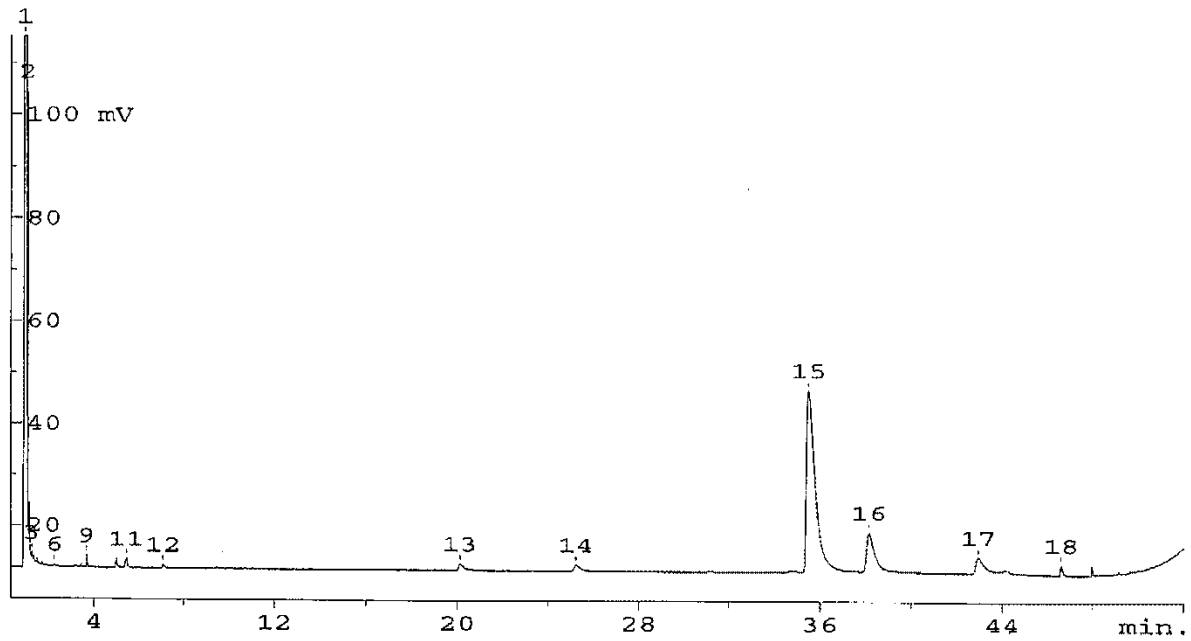
achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min
Zuordnung nach GC/MS a14031 zu ZHN-ZA-068-46; 12/90537

No.	min.	area-%
22	8.23	1.57
23	8.93	1.87
24	9.53	0.57
25	9.72	0.51
26	9.96	0.50
27	10.58	0.88
28	10.80	0.17
30	11.23	6.49
32	12.19	84.3
37	16.16	1.15
40	17.39	0.16
43	19.17	0.70

%- threshold: 0.15 %
30 peaks out of 42 (total area percentage = 1.16 %) are below threshold.
16 peaks had been suppressed.

Instrument : HP6890 / MPS2L , 513
Column : 15m DB-Wax 0.25/0.15df G/492
Detector : FID
Temperature : 230/80 6/min 260 10min iso/350
Gas : 0.4 bar H2
Sample size : 1 uL
Recorder : 1mV

Chiral chromatogram




```
**********     R528S756.DAT     ****************** 11-MAY-12 14:10:39

Sample :  12/90977                        measured:  11-MAY-12 12:39:28
Cust.ID:  AGU-AA-228-02                   processed: 11-MAY-12 14:10:31
S.code :  in EE                           by:        CHROM3
                                          with:

chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-136-01; 11/90008

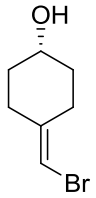
  No.   min.      area-%

   15  35.49      82.6
   16  38.16      17.4

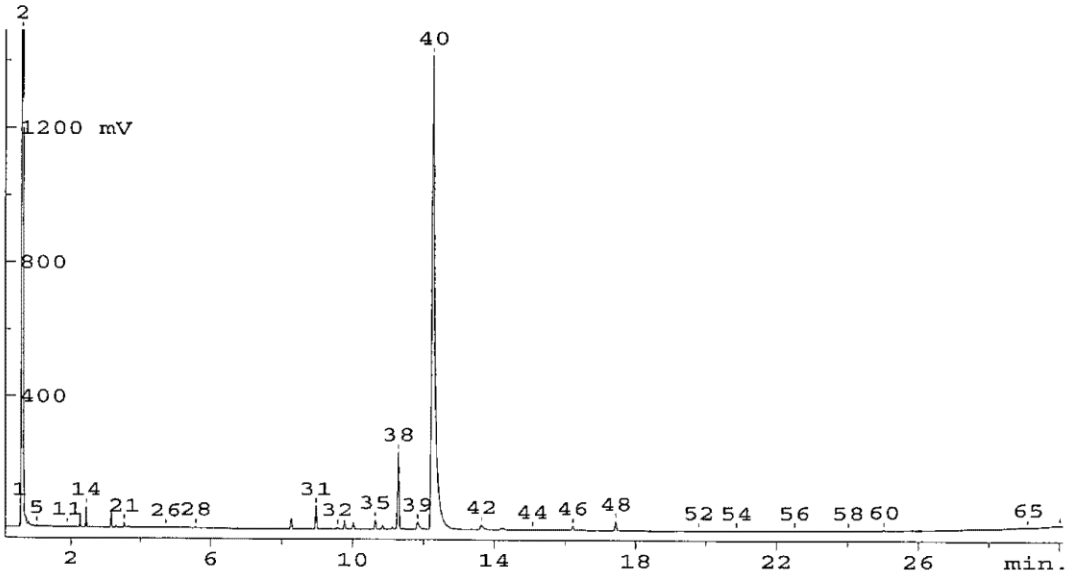
  22 peaks had been suppressed.

 Instrument      : AT 6890 N; 528
 Column          : 25m Hydrodex-y TBDAc 0.25/?df G/580
 Detector        : FID
 Temperature     : 230/110 iso spaeter ausgeheizt/320
 Gas             : 0.8 bar H2
 Sample size     : 1 uL
 Recorder        : Kipp & Zonen 1 mV
```

(*S*)-4-(bromomethylene)cyclohexanol (2a) resulting from biocatalytic asymmetric ketone reduction of 1a with mutant I86A



Achiral chromatogram



********** R513S607.DAT ****************** 24-APR-12 11:50:49

Sample : 12/90882
Cust.ID: AGU-AA-212-01
S.code : in EE

measured: 24-APR-12 09:41:55
processed: 24-APR-12 11:50:25
by: CHROM3
with:

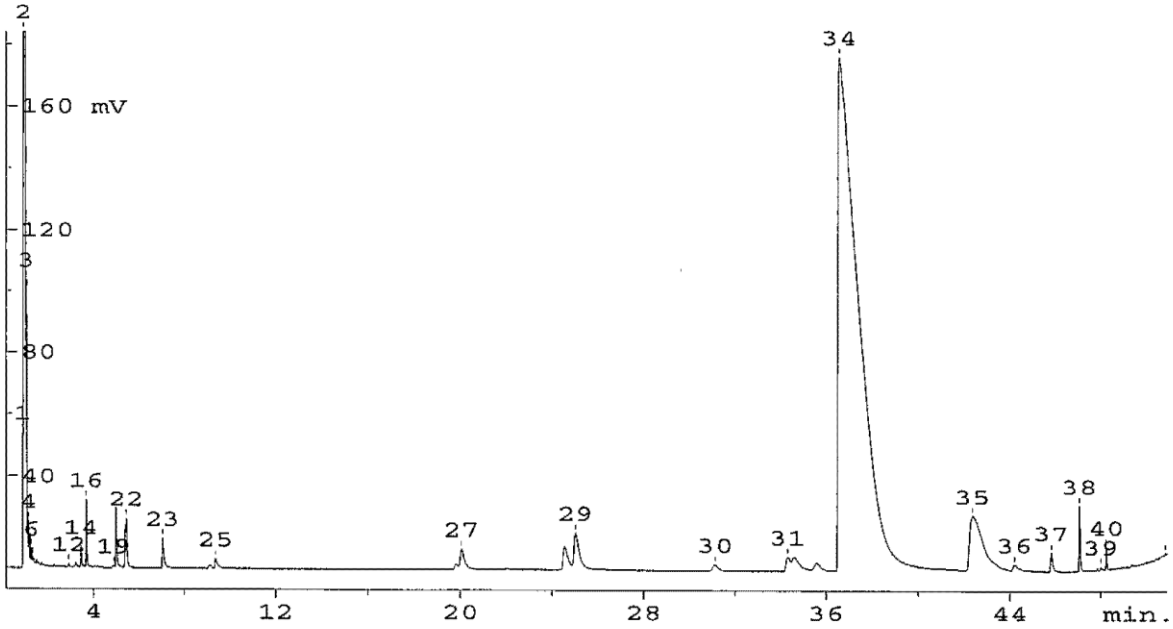
achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min

No.	min.	area-%
29	8.28	0.70
31	8.98	1.59
32	9.58	0.12
33	9.78	0.60
34	10.02	0.53
35	10.64	0.67
36	10.85	0.28
37	11.12	0.16
38	11.29	6.38
39	11.83	1.13
40	12.26	84.8
42	13.64	0.79
43	14.22	0.34
46	16.22	0.39
48	17.44	0.78

%- threshold: 0.10 %
29 peaks out of 44 (total area percentage = 0.74 %) are below threshold.
24 peaks had been suppressed.

Instrument : HP6890 / MPS2L , 513
Column : 15m DB-Wax 0.25/0.15df G/492
Detector : FID
Temperature : 230/80 6/min 260 10min iso/350
Gas : 0.4 bar H2
Sample size : 1 uL
Recorder : 1mV

Chiral chromatogram




```
**********     R528S721.DAT     ****************** 25-APR-12 07:45:08

Sample :   12/90882                      measured:  24-APR-12 13:42:05
Cust.ID:   AGU-AA-212-01                 processed: 25-APR-12 07:45:03
S.code :   in EE                         by:        CHROM3
                                         with:

chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-136-01, 11/90008

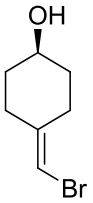
  No.    min.      area-%

   33  35.58       0.44
   34  36.56      99.6

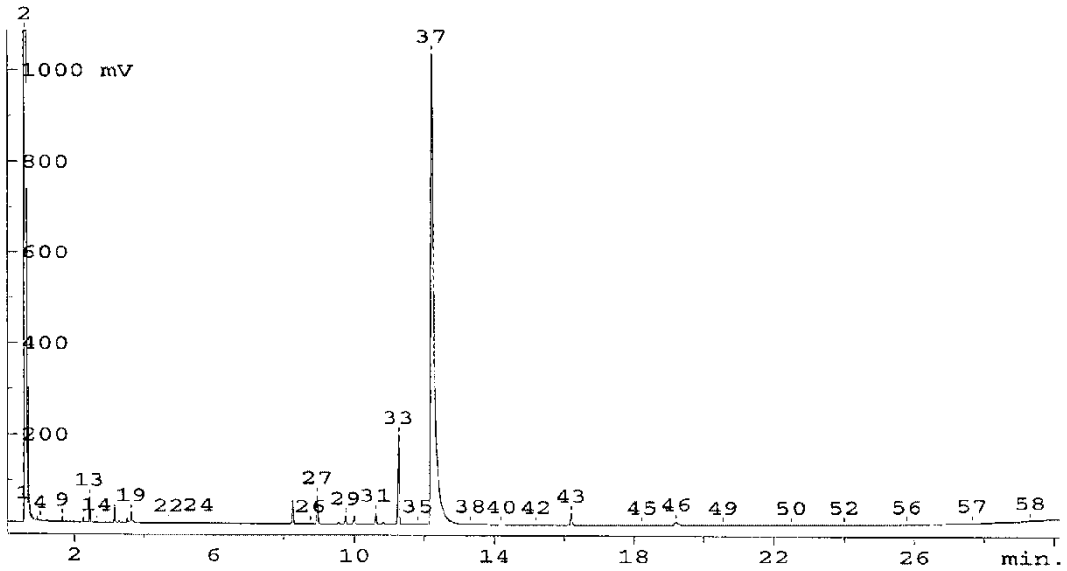
  49 peaks had been suppressed.

 Instrument      : AT 6890 N; 528
 Column          : 25m Hydrodex-y TBDAc 0.25/?df G/580
 Detector        : FID
 Temperature     : 230/110 iso spaeter ausgeheizt/320
 Gas             : 0.8 bar H2
 Sample size     : 1 uL
 Recorder        : Kipp & Zonen 1 mV
```

(*R*)-4-(bromomethylene)cyclohexanol (2a) resulting from biocatalytic asymmetric ketone reduction of 1a with mutant W110T



Achiral chromatogram



********** R513S613.DAT ****************** 25-APR-12 11:00:14

Sample : 12/90883
Cust.ID: AGU-AA-215-17
S.code : in EE

measured: 25-APR-12 07:51:41
processed: 25-APR-12 10:59:48
by: CHROM3
with:

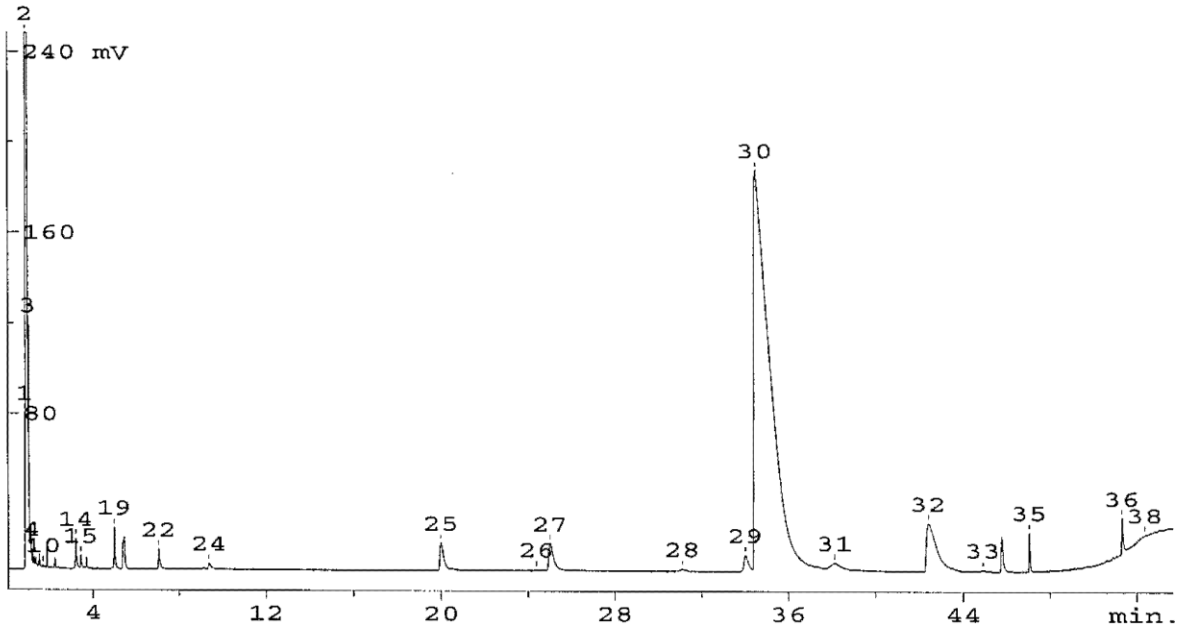
achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min

No.	min.	area-%
25	8.26	1.55
27	8.96	1.89
28	9.56	0.13
29	9.76	0.57
30	10.01	0.57
31	10.62	0.83
32	10.83	0.13
33	11.27	6.59
35	11.81	0.11
37	12.23	85.2
38	13.31	0.14
39	13.62	0.12
43	16.20	0.91
46	19.21	0.70

%- threshold: 0.10 %
25 peaks out of 39 (total area percentage = 0.56 %) are below threshold.
28 peaks had been suppressed.

Instrument : HP6890 / MPS2L , 513
Column : 15m DB-Wax 0.25/0.15df G/492
Detector : FID
Temperature : 230/80 6/min 260 10min iso/350
Gas : 0.4 bar H2
Sample size : 1 uL
Recorder : 1mV

Chiral chromatogram



********** R528S727.DAT ********** 26-APR-12 08:55:31

Sample : 12/90883
Cust.ID: AGU-AA-215-17
S.code : in EE

measured: 25-APR-12 14:01:28
processed: 26-APR-12 08:55:10
by: CHROM3
with:

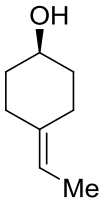
chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-136-01; 11/90008

No.	min.	area-%
30	34.50	98.3
31	38.14	1.68

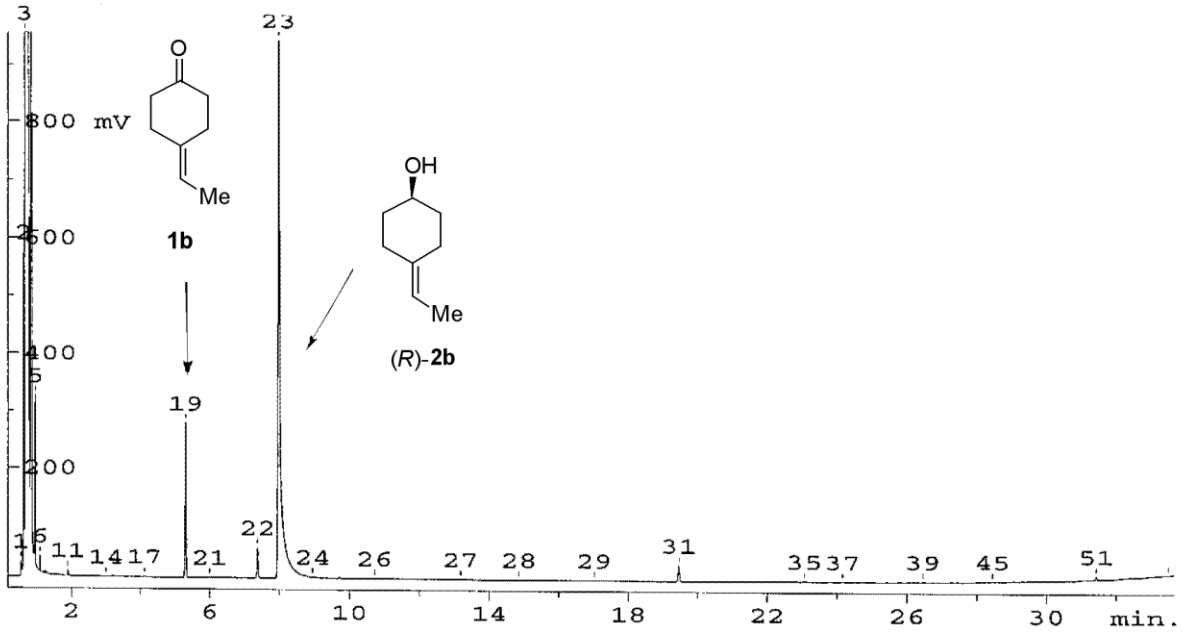
42 peaks had been suppressed.

Instrument : AT 6890 N; 528
Column : 25m Hydrodex-y TBDAc 0.25/?df G/580
Detector : FID
Temperature : 230/110 iso spaeter ausgeheizt/320
Gas : 0.8 bar H2
Sample size : 1 uL
Recorder : Kipp & Zonen 1 mV

(*R*)-4-ethylidenecyclohexanol (2b) resulting from biocatalytic asymmetric ketone reduction of 1b with mutant A85V



Achiral chromatogram




```
**********     R513S693.DAT     ****************** 10-MAY-12 08:09:59

Sample :  12/90980                        measured:   9-MAY-12 19:37:19
Cust.ID:  AGU-AB-235-06                   processed: 10-MAY-12 08:09:55
S.code :  in EE                           by:        CHROM3
                                          with:

achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min
Zuordnung nach GC/MS a14067 zu ZHN-ZA-068-36; 12/90536

  No.    min.      area-%

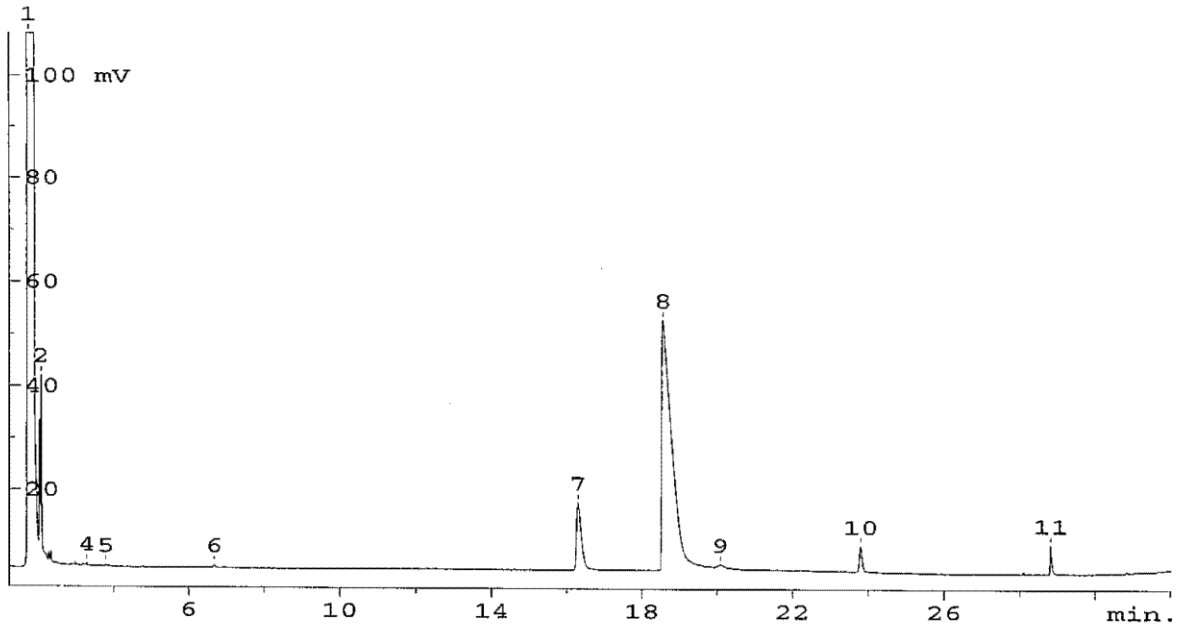
   17    4.12       0.11
   19    5.31      10.2
   22    7.38       2.23
t  23    7.99      84.6
   25    9.72       0.19
   31   19.46       1.56
   51   31.44       0.32

  %- threshold:   0.10 %
  29 peaks out of   36 (total area percentage =   0.75 %) are below threshold.
  21 peaks had been suppressed.

t = area corrected by tangent method

 Instrument      : HP6890 / MPS2L , 513
 Column          : 15m DB-Wax 0.25/0.15df G/492
 Detector        : FID
 Temperature     : 230/60 6/min 260 10min iso/350
 Gas             : 0.4 bar H2
 Sample size     : 1 uL
 Recorder        : 1mV
```

Chiral chromatogram



********** R512S449.DAT ********** 11-MAY-12 07:49:56

Sample : 12/90980
Cust.ID: AGU-AB-235-06
S.code : in EE

measured: 10-MAY-12 18:44:50
processed: 11-MAY-12 07:49:35
by: CHROM3
with:

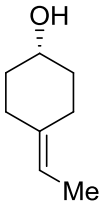
chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-136-03; 11/90011

No.	min.	area-%
8	18.57	98.2
9	20.10	1.81

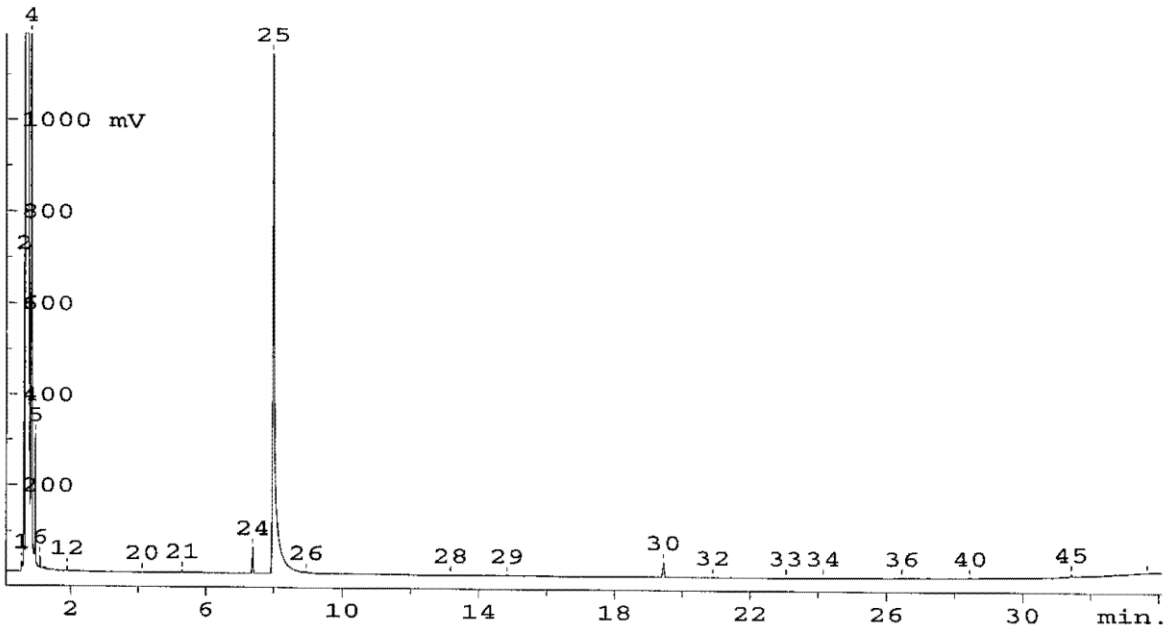
13 peaks had been suppressed.

Instrument : AT 6890N; 512
Column : 30m BGB-176/SE-52 0.25/0.15df G/508
Detector : FID
Temperature : 230/90 iso spaeter ausgeheizt/350
Gas : 0.6 bar H2
Sample size : 1 uL
Recorder : Kipp & Zonen 1 mV

(*S*)-4-ethylidenecyclohexanol (2b) resulting from biocatalytic asymmetric ketone reduction of 1b with mutant I86E



Achiral chromatogram




```
**********    R513S697.DAT    ****************** 10-MAY-12 08:16:01

Sample :  12/90981                      measured:   9-MAY-12 22:58:43
Cust.ID:  AGU-AB-235-21                 processed: 10-MAY-12 08:15:55
S.code :  in EE                         by:        CHROM3
                                        with:

achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min
Zuordnung nach GC/MS a14067 zu ZHN-ZA-068-36; 12/90536

  No.    min.     area-%

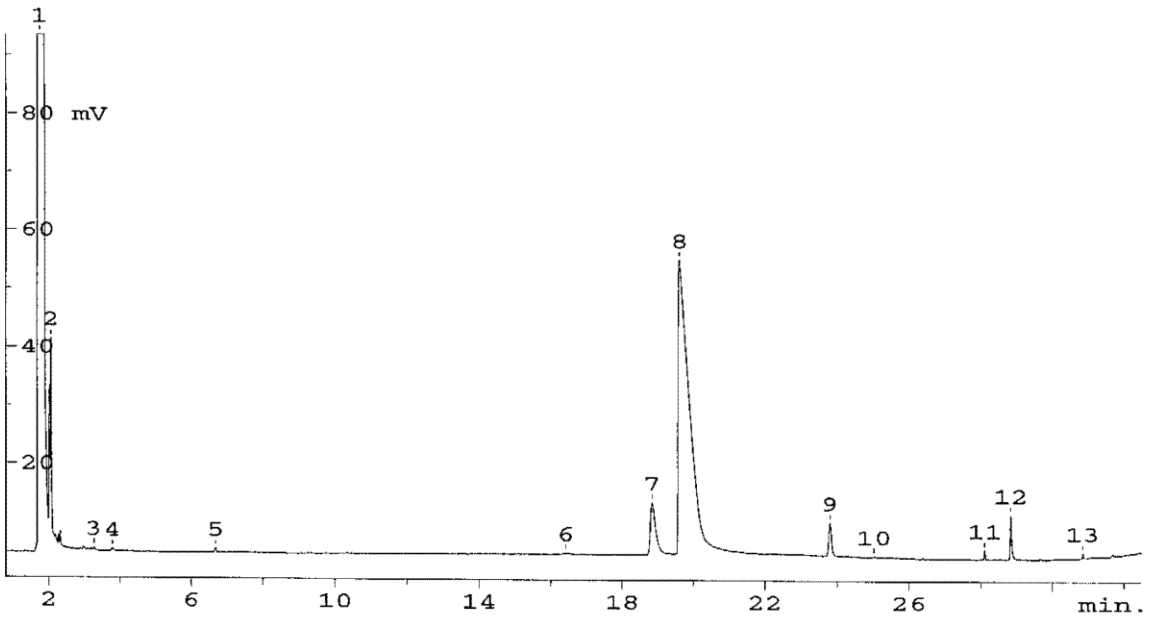
   20    4.12      0.11
   21    5.31      0.17
   24    7.39      2.24
t  25    8.00     94.6
   27    9.72      0.19
   30   19.45      1.72
   45   31.44      0.39

  %- threshold:   0.08 %
  20 peaks out of   27 (total area percentage =   0.60 %) are below threshold.
  24 peaks had been suppressed.

t = area corrected by tangent method

 Instrument      : HP6890 / MPS2L , 513
 Column          : 15m DB-Wax 0.25/0.15df G/492
 Detector        : FID
 Temperature     : 230/60 6/min 260 10min iso/350
 Gas             : 0.4 bar H2
 Sample size     : 1 uL
 Recorder        : 1mV
```

Chiral chromatogram




```
**********    R512S453.DAT    ****************** 11-MAY-12 08:00:11

Sample :  12/90981                      measured:  10-MAY-12 21:32:27
Cust.ID:  AGU-AB-235-21                 processed: 11-MAY-12 08:00:06
S.code :  in EE                         by:        CHROM3
                                        with:

chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-136-03; 11/90011

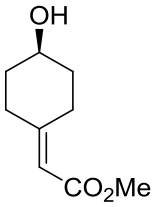
  No.    min.      area-%

    7  18.85       7.82
    8  19.61      92.2

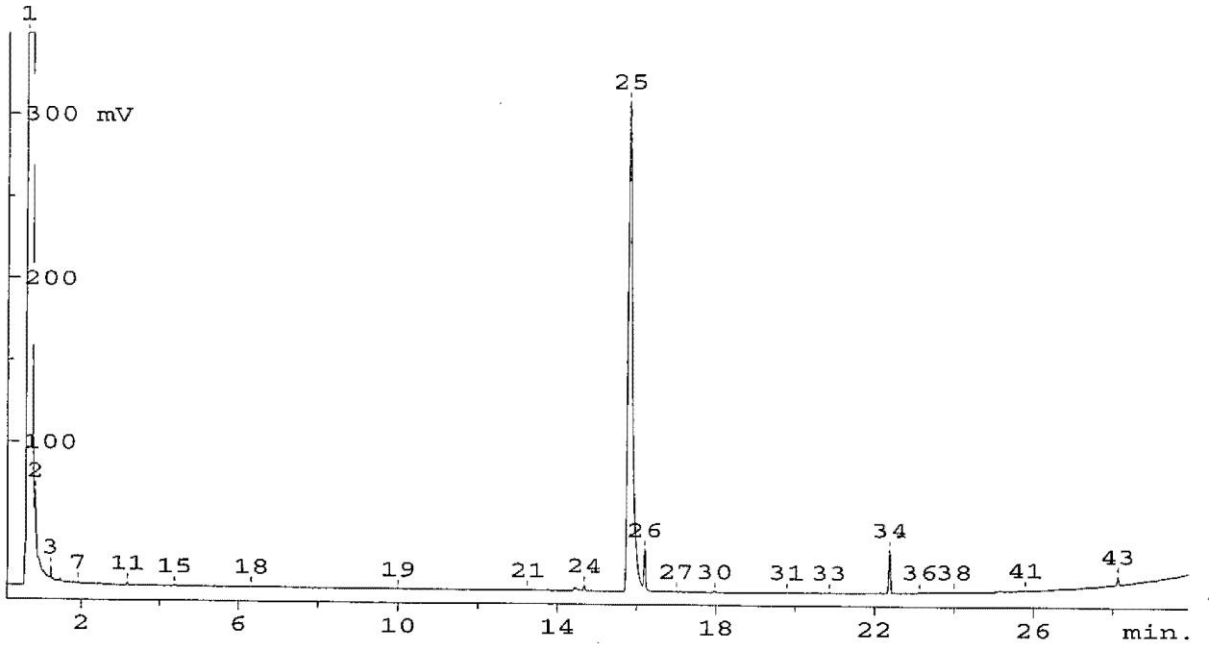
  15 peaks had been suppressed.

 Instrument      : AT 6890N; 512
 Column          : 30m BGB-176/SE-52 0.25/0.15df G/508
 Detector        : FID
 Temperature     : 230/90 iso spaeter ausgeheizt/350
 Gas             : 0.6 bar H2
 Sample size     : 1 uL
 Recorder        : Kipp & Zonen 1 mV
```

(*R*)-methyl 2-(4-hydroxycyclohexylidene)acetate (2d) resulting from biocatalytic asymmetric ketone reduction of 1d with mutant W110T



Achiral chromatogram



********** R513S635.DAT ***************** 8-MAY-12 07:58:18

Sample : 12/90985
Cust.ID: AGU-AB-235-52
S.code : in EE

measured: 7-MAY-12 21:59:39
processed: 8-MAY-12 07:58:14
by: CHROM3
with:

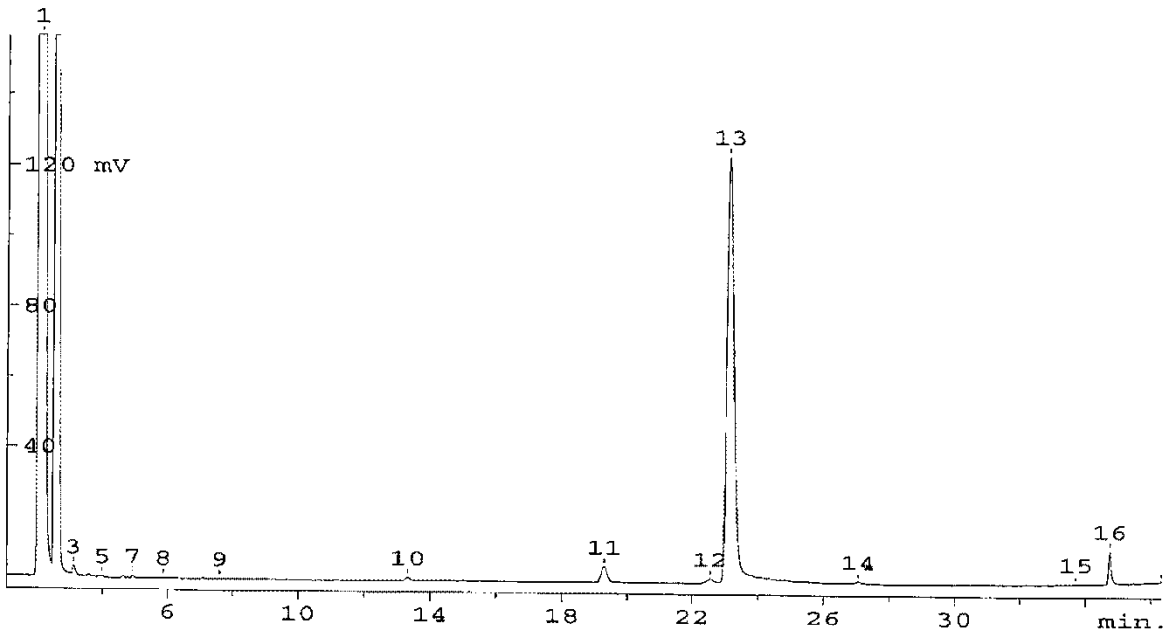
achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min
Zuordnung nach GC/MS a13827 zu AGU-AA-035-09; 11/90047

No.	min.	area-%
23	14.45	0.47
24	14.68	0.42
25	15.80	91.1
26	16.21	3.38
30	17.97	0.10
34	22.38	2.99
40	25.17	0.10
41	25.79	0.12
43	28.13	0.56

%- threshold: 0.10 %
22 peaks out of 31 (total area percentage = 0.80 %) are below threshold.
23 peaks had been suppressed.

Instrument : HP6890 / MPS2L , 513
Column : 15m DB-Wax 0.25/0.15df G/492
Detector : FID
Temperature : 230/80 6/min 260 10min iso/350
Gas : 0.4 bar H2
Sample size : 1 uL
Recorder : 1mV

Chiral chromatogram



********** R512S419.DAT ********** 9-MAY-12 11:01:08

Sample : 12/90985
Cust.ID: AGU-AB-235-52
S.code : in EE

measured: 8-MAY-12 23:09:24
processed: 9-MAY-12 11:01:03
by: CHROM3
with:

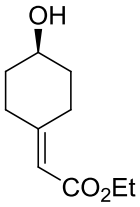
chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-067-01; 11/90045

No.	min.	area-%
12	22.55	0.93
13	23.11	99.1

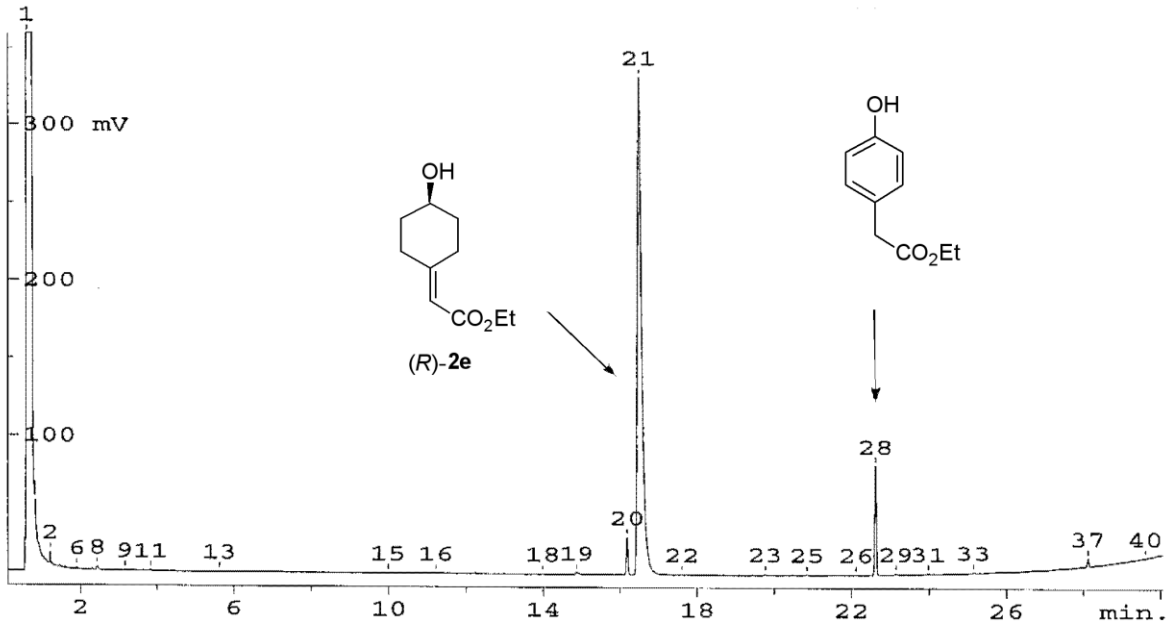
18 peaks had been suppressed.

Instrument : AT 6890N; 512
Column : 25m Hydrodex-b TBDAc 0.25/?df G/589
Detector : FID
Temperature : 230/150 iso spaeter ausgeheizt/350
Gas : 0.4 bar H2
Sample size : 1 uL
Recorder : Kipp & Zonen 1 mV

(*R*)-ethyl-2-(4-hydroxycyclohexylidene)acetate (2e) resulting from biocatalytic asymmetric ketone reduction of 1e with mutant W110T



Achiral chromatogram




```
**********    R513S651.DAT    ******************  8-MAY-12 11:21:05

Sample :  12/90988                      measured:   8-MAY-12 10:05:05
Cust.ID:  AGU-AB-235-53                 processed:  8-MAY-12 11:20:59
S.code :  in EE                         by:        CHROM3
                                        with:

"achirale Uebersichtsanalyse, Auswertung ohne Loesemittelbereich sup bis 3.5min"
"Zuordnung nach GC/MS a14032 zu ZHN-ZA-068-22; 12/90535"

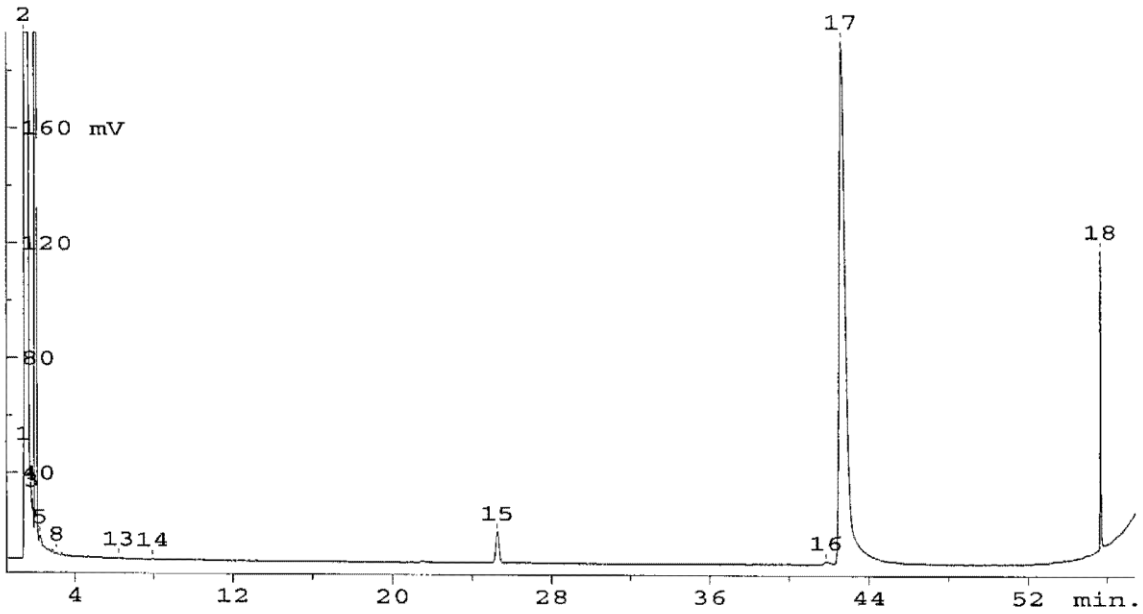
  No.   min.     area-%

   19  14.88      0.26
   20  16.18      2.45
   21  16.49     88.8
   28  22.62      7.13
   33  25.15      0.10
   37  28.12      0.52

  %- threshold:   0.10 %
  21 peaks out of   27 (total area percentage =   0.73 %) are below threshold.
  19 peaks had been suppressed.

 Instrument      : HP6890 / MPS2L , 513
 Column          : 15m DB-Wax 0.25/0.15df G/492
 Detector        : FID
 Temperature     : 230/80 6/min 260 10min iso/350
 Gas             : 0.4 bar H2
 Sample size     : 1 uL
 Recorder        : 1mV
```

Chiral chromatogram



********** R525S504.DAT ********** 9-MAY-12 08:30:47

Sample : 12/90988
Cust.ID: AGU-AB-235-53
S.code : in EE

measured: 9-MAY-12 01:56:48
processed: 9-MAY-12 08:30:25
by: CHROM3
with:

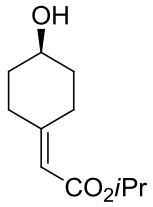
chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-179-01; 11/90196

No.	min.	area-%
16	41.86	0.47
17	42.62	99.5

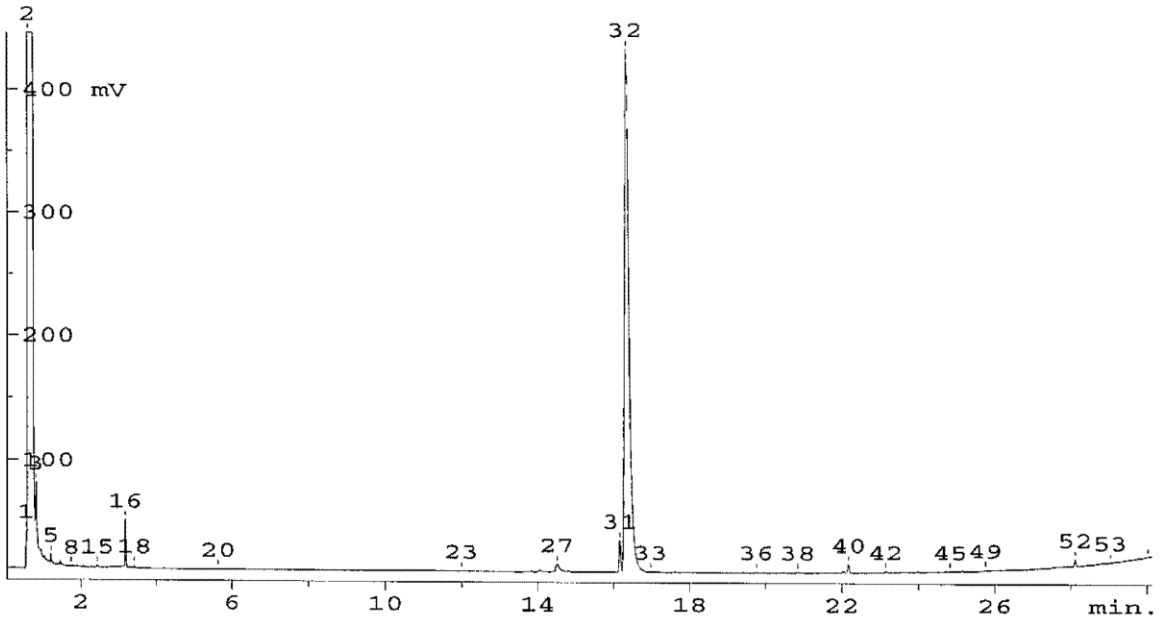
24 peaks had been suppressed.

Instrument	: AT6890N + MPS2L ; 525
Column	: 30m BGB-174/BGB-1701 0.25/0.25df;G/650
Detector	: FID
Temperature	: 220/140 iso spaeter ausgeheizt/350
Gas	: 0.8 bar H2
Sample size	: 1 uL
Recorder	: Kipp & Zonen 1mV

(*R*)-isopropyl 2-(4-hydroxycyclohexylidene)acetate (2f) resulting from biocatalytic asymmetric ketone reduction of 1f with mutant W110T



Achiral chromatogram




```
**********    R513S665.DAT    ******************  9-MAY-12 08:03:12


Sample :  12/90991                        measured:   8-MAY-12 20:40:40
Cust.ID:  AGU-AB-235-54                   processed:  9-MAY-12 08:03:04
S.code :  in EE                           by:         CHROM3
                                          with:

achirale Uebersichtsanalyse, Auswertung chne Loesemittelbereich sup bis 3.5min
Zuordnung nach GC/MS a14034 zu ROI-RA-222-01; 12/90527

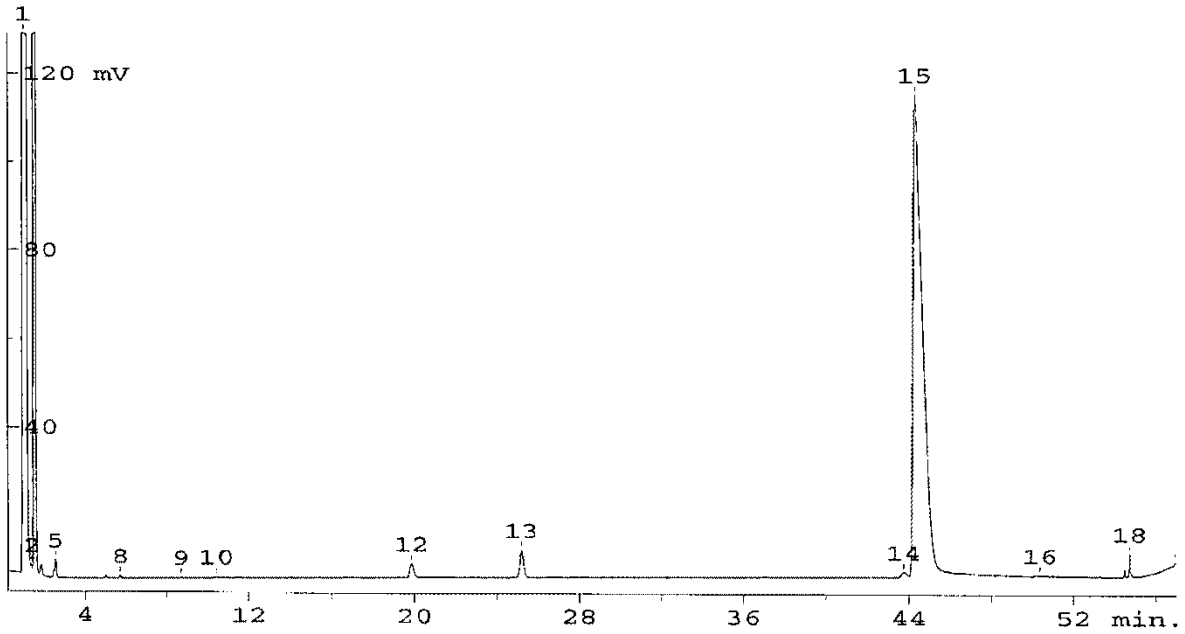
  No.   min.     area-%

   26  14.05      0.17
   27  14.51      1.28
   28  14.81      0.11
   31  16.17      2.05
   32  16.33     94.2
   33  16.98      0.11
   40  22.18      0.54
   51  27.65      0.25
   52  28.12      0.41

  %- threshold:   0.10 %
  25 peaks out of   34 (total area percentage =   0.87 %) are below threshold.
  26 peaks had been suppressed.

 Instrument      : HP6890 / MPS2L , 513
 Column          : 15m DB-Wax 0.25/0.15df G/492
 Detector        : FID
 Temperature     : 230/80 6/min 260 10min iso/350
 Gas             : 0.4 bar H2
 Sample size     : 1 uL
 Recorder        : 1mV
```

Chiral chromatogram




```
**********     R512S436.DAT     ****************** 10-MAY-12 08:57:52

Sample :  12/90991                        measured:  10-MAY-12 01:37:39
Cust.ID:  AGU-AB-235-54                   processed: 10-MAY-12 08:51:30
S.code :  in EE                           by:        CHROM3
                                          with:

chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-222-01; 12/90527

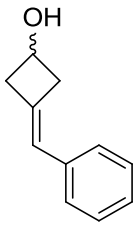
  No.    min.      area-%

   14  43.76       0.61
   15  44.33      99.4

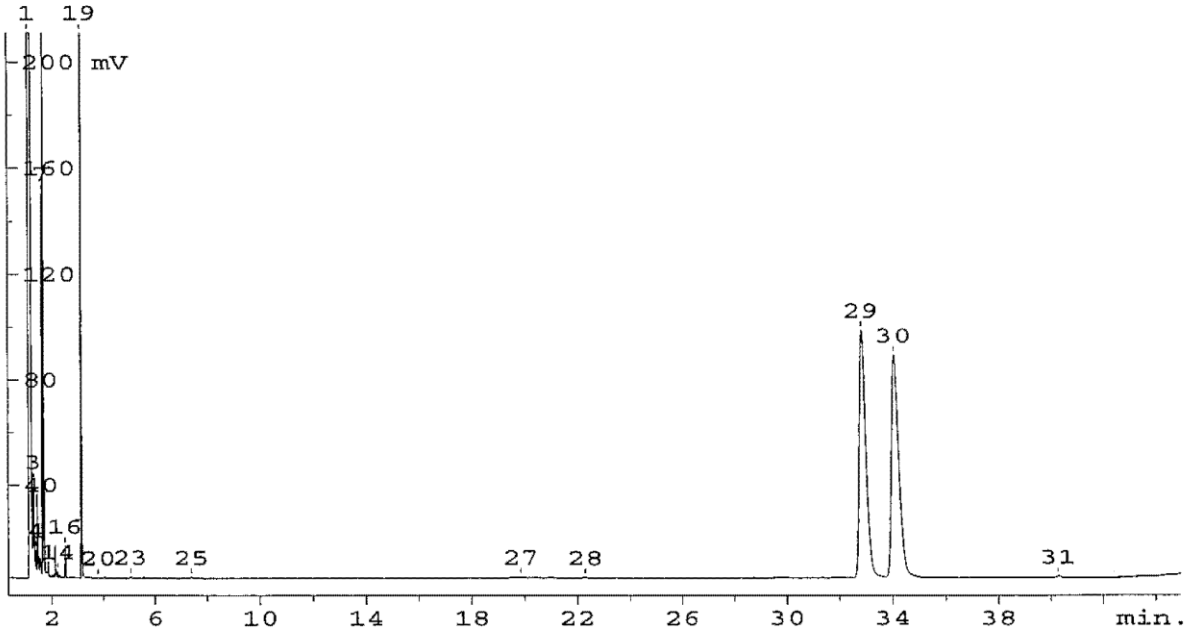
  25 peaks had been suppressed.

 Instrument      : AT 6890N; 512
 Column          : 25m Hydrodex-b TBDAc 0.25/?df G/589
 Detector        : FID
 Temperature     : 230/130 iso spaeter ausgeheizt/350
 Gas             : 0.8 bar H2
 Sample size     : 1 uL
 Recorder        : Kipp & Zonen 1 mV
```

Racemic 3-benzylidenecyclobutanol *rac*-(4) prepared chemically



Chiral chromatogram




```
**********    R509S996.DAT    ****************** 11-JUN-12 14:36:43


Sample :  12/91062                      measured:  11-JUN-12 12:00:28
Cust.ID:  ROI-RA-282-04                 processed: 11-JUN-12 14:36:35
S.code :  in EE                         by:        CHROM3
                                        with:

chirale Messung
Racemat

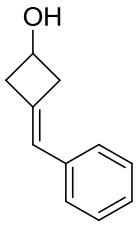
  No.   min.     area-%

   29  32.83     49.7
   30  34.05     50.3

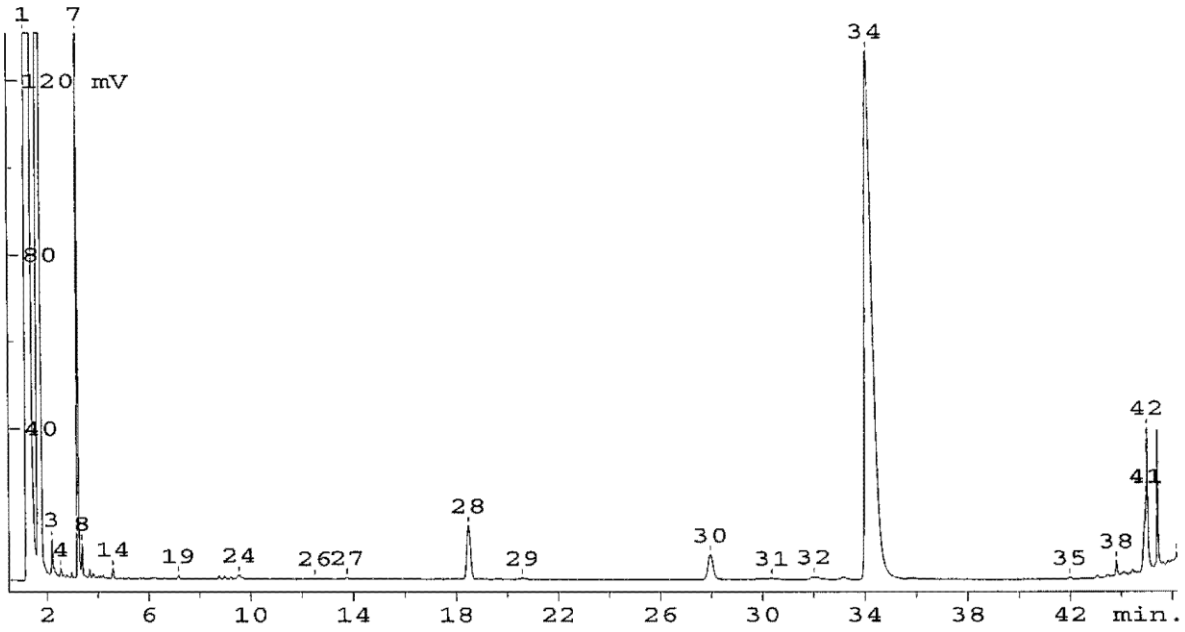
  42 peaks had been suppressed.

 Instrument      : HP 6890  509
 Column          : 25m Hydrodex-b TBDAc 0.25/?df G/625
 Detector        : FID
 Temperature     : 220/140 iso spaeter ausgeheizt/320
 Gas             : 0.6 BAR H2
 Sample size     : 0.5 uL
 Recorder        : 0.5 CM/MIN  1mV
```

Enantiomeric pure 3-benzylidenecyclobutanol 4 resulting from biocatalytic asymmetric ketone reduction of 3 with mutant W110T



Chiral chromatogram




```
**********     R509S999.DAT     ****************** 14-JUN-12 11:06:32

Sample :  12/91107                        measured:  11-JUN-12 16:01:10
Cust.ID:  AGU-AB-253-09                   processed: 14-JUN-12 11:05:21
S.code :  in EE                           by:        CHROM3
                                          with:

chirale Messung, ee-Verhaeltnis
Zuordnung nach Racemat ROI-RA-282-01

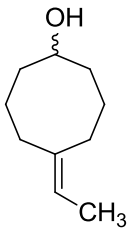
  No.   min.     area-%

   33  33.17      0.36
   34  34.12     99.6

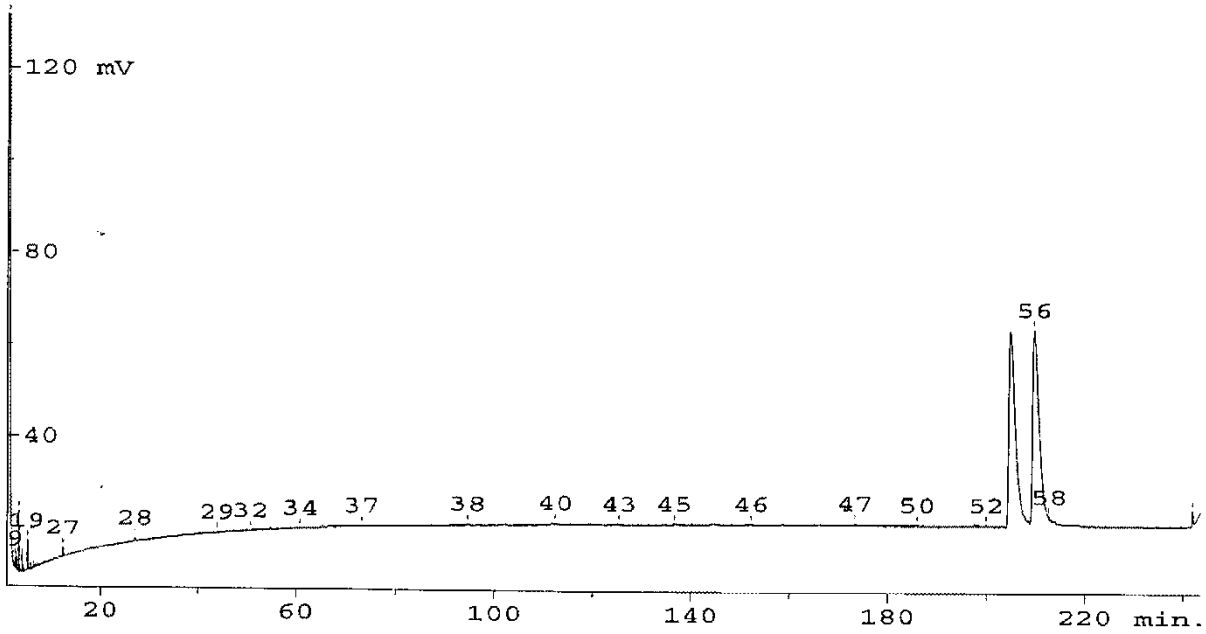
  70 peaks had been suppressed.

 Instrument     : HP 6890  509
 Column         : 25m Hydrodex-b TBDAc 0.25/?df G/625
 Detector       : FID
 Temperature    : 220/140 iso spaeter ausgeheizt/320
 Gas            : 0.6 BAR H2
 Sample size    : 2 uL
 Recorder       : 0.5 CM/MIN  1mV
```

Racemic 5-ethylidenecyclooctanol *rac*-(6b) prepared chemically



Chiral chromatogram




```
**********     R500S873.DAT     ****************** 13-SEP-12 15:20:10

Sample :  12/91344                        measured:  13-SEP-12 11:02:00
Cust.ID:  ROI-RB-322-01                   processed: 13-SEP-12 15:17:46
S.code :  in EE                           by:        CHROM3
                                          with:

chirale Messung
Racemat-Probe

Gr.  No.-no.   min. -min.      area-%

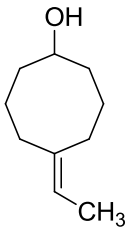
  1   54- 55 204.59-204.63     49.1
  2   56- 58 209.45-212.60     50.9

       Group  1:     49.06%     of all peaks
       Group  2:     50.94%     of all peaks

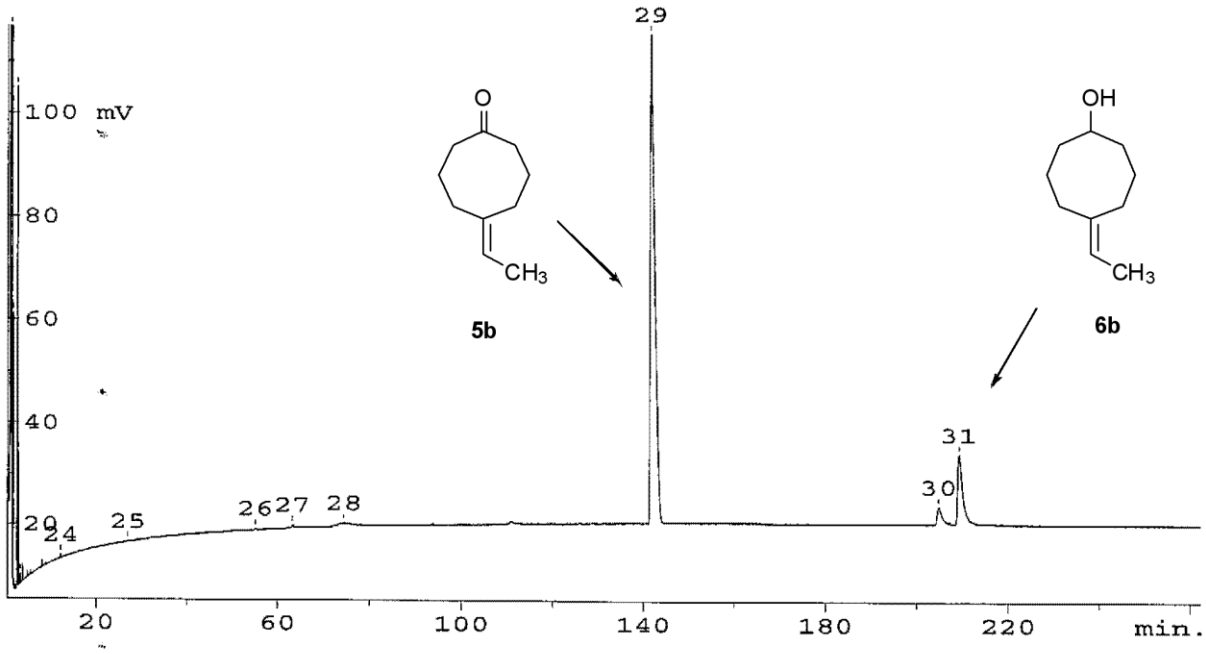
  76 peaks had been suppressed.

 Instrument      : HP 6890, 500
 Column          : 25m Lipodex G 0.25/?df G/602
 Detector        : FID
 Temperature     : 220/40 0.2/min 220 5min iso/320
 Gas             : 0.7 BAR H2
 Sample size     : 0.5 uL
 Recorder        : 0.5 CM/MIN  1mV
```

High enantiomeric excess 5-ethylidenecyclooctanol *rac*-(6b) resulting from biocatalytic asymmetric ketone reduction of 5b with mutant W110T



Chiral chromatogram



********** R500S876.DAT ********** 14-SEP-12 12:13:33

Sample : 12/91442
Cust.ID: AGU-AB-310-02
S.code : in EE

measured: 14-SEP-12 07:43:09
processed: 14-SEP-12 12:13:01
by: CHROM3
with:

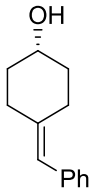
No.	min.	area-%
30	204.75	19.1
31	209.15	80.9

29 peaks had been suppressed.

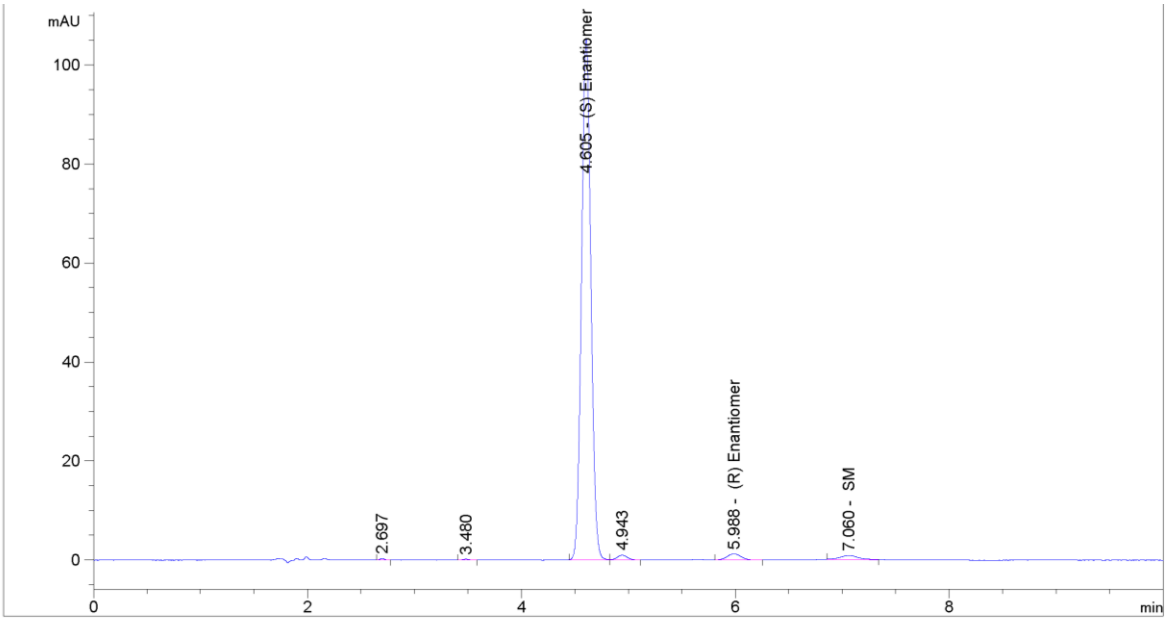
Instrument : HP 6890, 500
Column : 25m Lipodex G 0.25/?df G/602
Detector : FID
Temperature : 220/40 0.2/min 100 12/min 220 5min iso/320
Gas : 0.7 BAR H2
Sample size : 3 uL
Recorder : 0.5 CM/MIN 1mV

HPLC Chromatograms

(*S*)-4-benzylidenecyclohexanol 2c resulting from biocatalytic asymmetric ketone reduction of 1c with enzyme A1 from X-Zyme kit (entry 1 from Table S1)



Chiral chromatogram



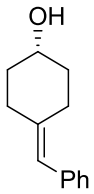

```
=====================================================================
                      Area Percent Report
=====================================================================

Sorted By             :      Signal
Calib. Data Modified  :      8/29/2012 2:25:41 PM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

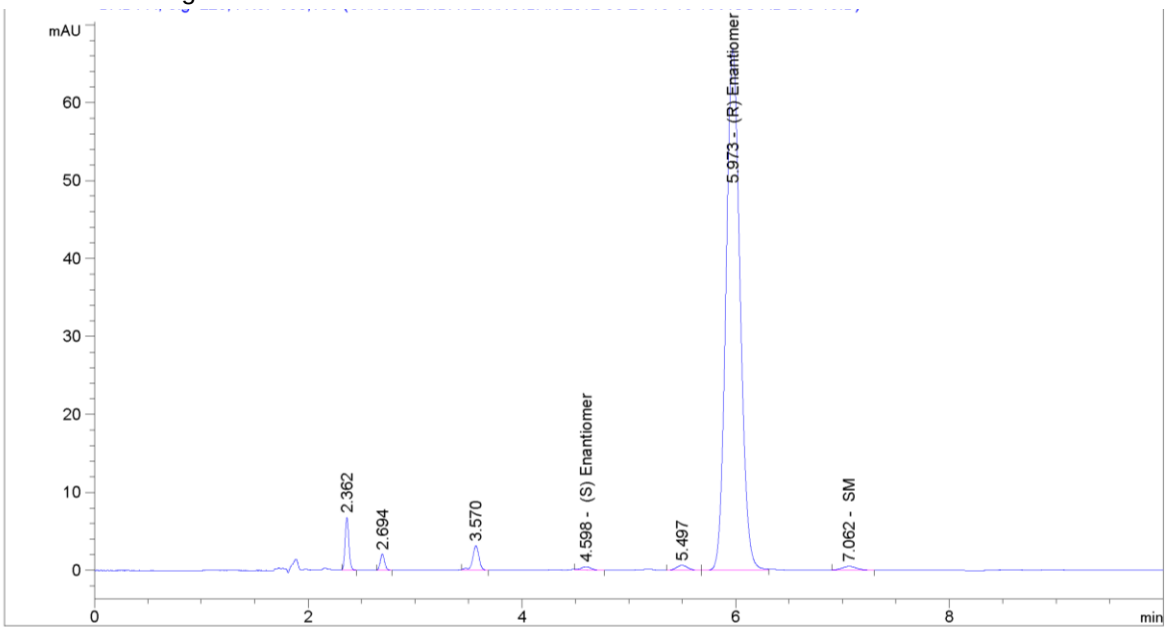

Signal 1: DAD1 A, Sig=220,4 Ref=380,100

Peak RetTime  Type   Width     Area      Area     Name
  #   [min]          [min]   [mAU*s]       %
----|-------|------|-------|----------|--------|---------------------
   1   2.697 BB      0.0458 9.65336e-1   0.1441 ?
   2   3.480 BB      0.0692 8.25498e-1   0.1232 ?
   3   4.605 BV      0.0950  640.68414  95.6156 1. Enantiomer
   4   4.943 VB      0.1005    6.24667   0.9323 ?
   5   5.988 BB      0.1387   10.98311   1.6391 2. Enantiomer
```

(*R*)-4-benzylidenecyclohexanol 2c resulting from biocatalytic asymmetric ketone reduction of 1c with mutant W110M



Chiral chromatogram



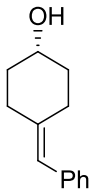

```
=====================================================================
                      Area Percent Report
=====================================================================

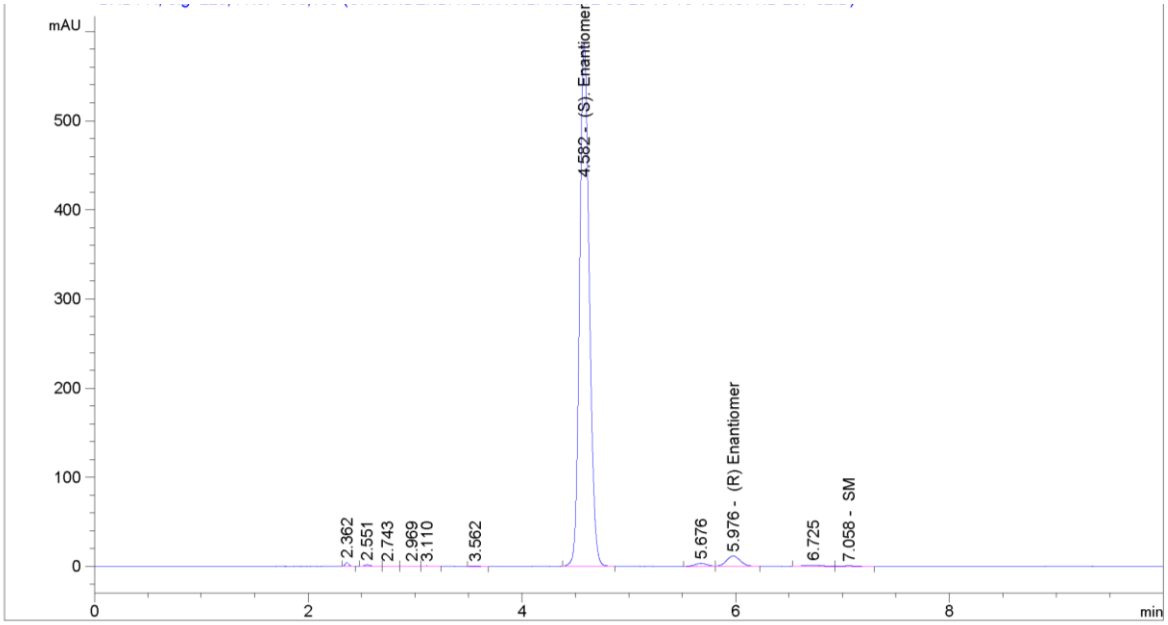
Sorted By             :      Signal
Calib. Data Modified  :      8/29/2012 2:25:41 PM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=220,4 Ref=380,100

Peak RetTime  Type   Width     Area      Area    Name
  #   [min]          [min]   [mAU*s]       %
----|-------|------|-------|----------|--------|--------------------
   1   2.362 BB      0.0384   16.39238   2.4889 ?
   2   2.694 BB      0.0445    5.88224   0.8931 ?
   3   3.570 BB      0.0641   12.98606   1.9717 ?
   4   4.598 BB      0.0997    2.68150   0.4071 1. Enantiomer
   5   5.497 BV      0.1139    4.70003   0.7136 ?
   6   5.973 VB      0.1425  611.42328  92.8356 2. Enantiomer
```

(*S*)-4-benzylidenecyclohexanol 2c chemically prepared from (*S*)-4-(bromo-methylidene)cyclohexanol 2a






```
=====================================================================
                      Area Percent Report
=====================================================================

Sorted By             :      Signal
Calib. Data Modified  :      8/29/2012 2:25:41 PM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=220,4 Ref=380,100

Peak RetTime  Type   Width     Area      Area    Name
  #   [min]          [min]   [mAU*s]       %
----|-------|------|-------|----------|--------|--------------------
   1   2.362 BB      0.0383    9.90413   0.2566 ?
   2   2.551 BB      0.0673    7.22225   0.1871 ?
   3   2.743 BB      0.0682    1.84652   0.0478 ?
   4   2.969 BV      0.1121    3.50893   0.0909 ?
   5   3.110 VB      0.0765    2.35867   0.0611 ?
   6   3.562 BB      0.0681    1.34739   0.0349 ?
   7   4.582 BB      0.0971 3678.45728  95.2847 1. Enantiomer
   8   5.676 BV      0.1284   27.31462   0.7075 ?
   9   5.976 VB      0.1414  104.82336   2.7153 2. Enantiomer
```