Asymmetric Enzymatic Hydration of Unactivated, Aliphatic Alkenes

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Supporting Information

I. Materials and methods

(A) All chemicals and solvents were purchased from commercial suppliers (Acros Organics, Alfa Aesar, Carl Roth GmbH, Macherey-Nagel, Merck, Peqlab, Sigma-Aldrich, VWR) and used without additional purification.

(B) $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance 300 and 500 MHz instrument, in CDCl$_3$ and are referenced to the residual solvent peak. Data for $^1$H NMR are reported in the conventional form: chemical shift ($\delta$ ppm), multiplicity (d = doublet, dd = doublet of doublets, t = triplet, q = quartet, quin = quintet, sext = sextet, m = multiplet), coupling constant (Hz), integration. Data for $^{13}$C NMR are reported in terms of chemical shift ($\delta$ ppm).

(C) Achiral gas chromatography (GC) analyses were conducted with a Shimadzu GC2010 instrument equipped with a flame ionization detector using an Agilent J&W DB-WAX column (30 m x 0.25 mm, 0.25 μm film, part number 122-7032) with hydrogen as carrier gas. Injector temperature: 200°C. Split mode with a split ratio of 5 at 1 μl injection. Detector temperature: 260°C. Depending on the respective alcohol product an individual temperature programme and internal standard was used (table S5). As a final step the oven temperature was increased to 220°C (50 K/min) and held for three minutes.

(D) Chiral GC analysis was performed using derivatized samples. After extraction of biotransformations, 600 µL supernatant was collected in 1.5 mL reaction tubes. A catalytic amount of 2 mg 4-(dimethylamino)-pyridine (DMAP) and 250 µL acetic anhydride were added for derivatization. After one hour at 25 °C and 800 rpm 300 µL water were added to the samples and the organic phase was transferred to a new reaction tube, dried with Na$_2$SO$_4$ and analyzed by chiral GC. Chiral GC analysis of 2-decanol, 2-octanol and 7-bromoheptan-2-ol was conducted with a Shimadzu GC2010 instrument equipped with a flame ionization detector using an Agilent J&W CP-Chirasil-Dex CB column (25 m x 0.25 mm, 0.25 μm film, part number CP7502) with hydrogen as carrier gas. Chiral GC analysis of 1,7-octandiol was conducted with a Shimadzu GC2010 instrument equipped with a flame ionization detector using an Agilent J&W CP-Chirasil-Dex CB column (25 m x 0.25 mm, 0.25 μm film, part number CP7502) with hydrogen as carrier gas. Chiral GC analysis of 1,7-octandiol was conducted with a Shimadzu GC2010 instrument equipped with a flame ionization detector using an Agilent J&W HP-Chirasil-20B column (30 m x 0.32 mm, 0.25 μm film, part number 19091G) with hydrogen as carrier gas. Chiral GC analysis of 2-heptanol, 2-hexanol, 2-pentanol, 4-phenylbutan-2-ol and 2- as well as 3-octanol from the internal alkene hydration was conducted with an Agilent 7820A instrument equipped with a 5977B mass selective detector using an Agilent J&W CP-Chirasil-Dex CB column (25 m x 0.25 mm, 0.25 μm film, part number CP7502) with helium as carrier gas. Injector temperature: 220°C. Split mode with a split ratio of 5 (only for 2-pentanol) and 20 at 1 μl injection. For information regarding internal standards (500 μM) and temperature programs see table S6. As a final step of each GC run the temperature was raised to 200°C (50 K min$^{-1}$) and held for 3 min. Due to background noise, GC analysis of derivatized 2-hexenol, 2-pentenol, 4-phenylbutan-2-ol and 2- as well as 3-octanol from the internal alkene hydration
samples was performed in SIM-mode (2-hexanol: 84.1 m/z; 2-pentanol: 87.1 m/z, 4-phenylbutan-2-ol: 132.1 m/z, 2-octanol: 112.2 m/z, 3-octanol: 112.2 m/z).

(E) Identification of the specificity-determined position 248[1] was accomplished by a systematic analysis of the Hydratase Engineering Database (HyED)[2]. Structure analysis was performed based on the Em-OAH wildtype crystal structure (PDB ID: 4UIR)[3]. Molecular docking and energy minimization were performed using the YASARA software. Docking results and in silico mutations were processed and visualized using PyMOL.

(F) DNA and amino acid sequence of Em-OAH wildtype enzyme

ATGAAATCGGATTTACCCGCAAAATTTGTATAGAATGCTGGAATGCCAGCGGAATATGGCATGGTATTATATCATGAAATAGGATAGCAG
CAGAAAGACCGACCGCTAAATACGCCAGGAATTACCGCGGGTTATAGCGATGAGCTGGAGCTGGAAATCATGGCACTTCTGCTGGAA
AGTCGAAGCGCGTAATACCCATCTGGAATACCCTGGTTAAAGATCTGGACATTCACATTAACACCGAAGGTAGTTGGG
AAAGCAATTAGGTTCATGCTGGTCTGTTGGGTTTATGCACTGTTTATGGATAAAGGGCAATTATATCAAAAAACCA
TGCTGGAATGCACCGGTGATGAAATTCTGGCAGAACTGTGTTATCATCTGGGTATTGAAGATCAACTGGAAAACGTGCAGAAA
AATACCATTGTTCGTACCGCATTCATGCCGTATATTACCTCAA
ATGTTTATGCCTCGTGCCAAAGGTGATCGTCCGCGTGTTGT
GCCGGAAGGTTAAAAACCTGGGTCTGGTTGGTCAGTTTGTGGAAACCAATAATGATGTGGTGTTCACAATGGAAAGCAGCG
TGCGTACCGCAGTATCGCGGTGTTATATAACTCTGAAAGCAGTGAACGATCCGTATAGTG

MNPITSKFDKVLNASSEYGHVNHEPSSEKQRTQNSMPSFSDQIGNYQRNKIGHPVSYDNSKIVYIYSGIAGMSAYFYIR
DHGHPKNTIFPIEOLHIDGEQLDGAENGPTDGYIIRGREGDMTENCYLENLMDQFDIPALEMPAYSVLVDEYLRLNNDNSYXKAR
LINNKGIEKDFSIFGLNMDQTAIRLLCQGSDNLDEYFSFISLKNSFMTFWMFAMENWHELLEELKLYMHFLHAI
DGLNLSLSSLVPFNYQDVTFTPLFRKLFQEGVNHIIIINLTVKLDHHINIEKGNEVIEITQDKEFVIFVPKDYIVVTGTS
MTEDFYFGNYNPIGIIINNTSOGSAWGLWKNNLAKASEIFPKPEFKCSNEISAESWASATLCSKPSALDLKKEYSINVDPK
GTKVTGGI1ITIDSMNLSM5SCTRQPHFQEPQPDFVDLVLYLWYESMKEGNTI1KTMRLCTEGEI1AELC沅HLEGEDQVNSQK
N12IVRTAEPY1DSMMPRAKGDPRVFVFGCNCNLWVLVGQFWETDNVFTMVSSSVESSVTRARRAYKVMLLNMQFVDIPNLQYDI
RRLLAATKTLNDKFPVFGEOILRLKKVLYGTYEFEHVLPGANCEEEHESF1AYNHVNFRE
II. General procedures

(A) QuikChange mutagenesis
Oleate hydratase from Elizabethkingia meningoseptica (C7DLJ6) was cloned into a pET28a(+) vector in former works.\textsuperscript{[2]} Em-OAH mutants were generated by QuikChange mutagenesis via PCR. For primer information, PCR reaction mixture and temperature program see table S2, table S3 and table S4. The resulting PCR products were digested with DpnI, purified over 30 min by MilliQ-dialysis (MF\textsuperscript{TM}-Membrane Filters, 0.025 µm VSWP, Merck Millipore Ltd.) and transformed via electroporation into E. coli strain Dh5α. pDNA was isolated using the “Zyppy\textsuperscript{TM} Plasmid Miniprep Kit” (Zymo Research Corp.) according to the manufacturer’s protocol. 20 µl of pDNA (60-100 ng/µl) and Primer (10 pmol/µl) were sent to GATC Biotech AG for sequencing.

(B) Transformation via electroporation
50 µl electrocompetent cells were thawed on ice, mixed with 100 ng pDNA (retransformation) or 5 µl of purified PCR-constructs (after QuikChange mutagenesis) and transferred to a pre-chilled electroporation cuvette. Electroporation was carried out at 2.5 V, 200 Ω and 25 µF. Cells were regenerated in 2 mL LB-media for 1 h at 37 °C and 180 rpm. 5 µl (retransformation) and 100 µl (after QuikChange mutagenesis), respectively, of cell suspension were streaked on LB agar plates with 30 µg/mL kanamycin and incubated overnight at 37 °C.

(C) Expression of recombinant Em-OAH
Heterologous gene expression of recombinant Em-OAH wildtype and mutants was performed as described before.\textsuperscript{[4]}

(D) Alken biotransformations
Alkene biotransformations were performed at 500 µL-scale in 2 mL glass vials as reaction tubes in a citrate buffer system (50 mM citrate, pH 6.0). Expression cultures were thawed on ice and resuspended in citrate buffer. 50 mg mL\textsuperscript{-1} cell suspension served for whole cell assays applying glucose (100 mM final conc.) and FAD (0.3 mM final conc.). Reactions were started by the addition of substrate (100 mM stock solution in DMSO, 2.5µL stock solution) and decoy molecule (100 mM stock solution in DMSO, 2.5µL stock solution) with a final DMSO concentration of 1% (v/v) and incubated at 25 °C and 180 rpm. Biotransformation samples (triplicates) were extracted with 800 µL MTBE (with addition of 500 µM internal standard). This set-up has been applied for reactions shown in figure S5.

(E) Alken biotransformations with alternative decoy molecules
Biotransformations were performed as described above. Substrate and decoy molecule stock solutions (100 mM) were prepared in DMSO with exception of 6-aminohexanoic acid and 1,6-hexanediol which were solubilized in buffer. This set-up has been applied for reactions shown in figure S7.
(F) Alken biotransformations applying a two-phase system

For alken biotransformations in a two-phase system, expression cultures were directly used without freezing the cell pellets. Biotransformations were performed at the 500 µL-scale in 2 mL glass vials with 100 mg mL⁻¹ cell suspension, glucose (200 mM final conc.) and FAD (0.6 mM final conc.) in a citrate buffer system (50 mM citrate, pH 6.0) with addition of 100 µL bis(2-ethylhexyl) phthalate (BEHP) as organic phase. Alkene substrate (300 mM final conc.) and fatty acid decoy molecule (5 mM final conc.) were added directly to biotransformations without DMSO as cosolvent and reactions were incubated at 25 °C and 180 rpm for seven days. This set-up has been applied for reactions shown in figure 2B and S8 as well as for the substrates 1 and 4 in Fig 2C.

(F) Substrate scope studies

The two-phase system as described above did not work for all the substrates tested in the extended substrate scope studies (see Fig. 2C). To confirm that substrates 2, 3, 5 and 6 were converted by cells containing Em-OAH A248L we used a simple system as described in section D above. Biotransformations were performed at 500 µL-scale in 2 mL glass vials using a citrate buffer system (50 mM citrate, pH 6.0). 100 mg mL⁻¹ cell suspension served as whole cell biocatalyst in combination with glucose (100 mM final conc.) and FAD (0.3 mM final conc.). Reactions were started by the addition of substrate (200 mM stock solution in DMSO, 1% DMSO final conc., 2 mM substrate final conc.) and decoy molecule (20 mM stock solution in citrate buffer, 2 mM decoy molecule final conc) and incubated at 25 °C and 180 rpm for 6 days. Biotransformation samples (triplicates) were extracted with 1000 µL MTBE (with addition of 500 µM internal standard) and analyzed by GC analysis.
III. Supporting figures

Figure S1: Synthesis of racemic and chiral alcohols from unactivated alkenes

Hydration using acids/solid acids/zeolites/clays
(Example for racemic alkene hydration using sulfuric acid)

\[
\begin{align*}
R-\underset{H_2SO_4}{\rightarrow} \quad & \quad [R-\underset{OH}{\rightarrow} \quad \text{racemic}] \\
\end{align*}
\]

Mukaiyama hydration
(not a direct addition of water, this redox-reaction depends on stoichiometric amounts of O\(_2\) and silanes)

\[
\begin{align*}
R-\underset{CoL_2, O_2, \text{Silane}}{\rightarrow} \quad & \quad [R-\underset{OH}{\rightarrow} \quad \text{racemic}] \\
\end{align*}
\]

Oxidation-reduction sequence
(for example the one-pot Wacker oxidation combined with an enantioselective carbonyl reduction using a ketoreductases)

\[
\begin{align*}
R-\underset{\text{Wacker oxidation}}{\rightarrow} \quad & \quad R-\underset{\text{Enantioselective reduction}}{\rightarrow} \quad [R-\underset{OH}{\rightarrow} \quad \text{chiral}] \\
\end{align*}
\]

Resolution of racemic alcohols to generate chiral alcohols
(for example an enzyme-catalyzed resolution using a lipase)

\[
\begin{align*}
R-\underset{\text{OH}}{\rightarrow} \quad + \quad O\underset{R_2, CO_{OR_3}}{\rightarrow} \quad \text{Lipase} \quad \rightarrow \quad R-\underset{\text{OH}}{\rightarrow} \quad + \quad [R-\underset{\text{OH}}{\rightarrow} \quad \text{chiral}] \\
\end{align*}
\]

The asymmetric addition of water across a carbon-carbon double bond is a long-standing challenge in alkene functionalization.\(^{[5,6]}\) Even though the hydration can be readily catalyzed by acids, metal oxides, zeolites and clays,\(^{[7]}\) these catalysts do not offer stereocontrol for this chemical transformation. Currently, the synthesis of chiral alcohols from alkenes depends on two-step oxidation-reduction sequences or resolution of racemic alcohols.\(^{[8,9]}\)
The few reported catalysts for the asymmetric hydration of alkenes show often only moderate stereoselectivity and are limited to substrates with activated carbon-carbon double bonds such as enones and hydroxy-styrenes.\textsuperscript{10,11}
Figure S3: Biocatalysts for the asymmetric addition of water across unactivated alkenes

**LinD-catalyzed isomerization of geraniol to (S)-linalool and dehydration to myrcene**

\[
\text{HO} \quad \xrightarrow{\text{LinD}} \quad \text{HO} \\
\text{Geraniol} \quad \xrightarrow{\text{LinD}} \quad \text{(S)-Linalool} \quad \xrightarrow{\text{LinD}} \quad \text{Myrcene}
\]

**CrtC-catalyzed hydration of lycopene to 1-hydroxy-lycopene and 1,1'-dihydroxy-lycopene**

\[
\text{HO} \quad \xrightarrow{\text{CrtC} + \text{H}_2\text{O}} \quad \text{HO} \\
\text{Lycopene} \quad \xrightarrow{\text{CrtC} + \text{H}_2\text{O}} \quad \text{1-OH-Lycopene} \\
\text{HO} \quad \xrightarrow{\text{CrtC} + \text{H}_2\text{O}} \quad \text{OH} \\
\text{1,1'-OH-Lycopene}
\]

**OAH-catalyzed hydration of oleic acid to (R)-10-hydroxyoctadecanoic acid**

\[
\text{oleic acid} \quad \xrightarrow{\text{OAH} + \text{H}_2\text{O}} \quad \text{OH} \\
\text{(R)-10-hydroxyoctadecanoic acid}
\]

Hydrolyase enzymes (EC 4.2.) catalyze the addition of water to isolated as well as conjugated carbon-carbon double bonds (α,β-unsaturated carbonyls) with the first following the rule of Markovnikov.\textsuperscript{12,13} Interesting examples for the cofactor-independent regio- and stereoselective hydration of unactivated alkenes are the carotenoid-1,2-hydration (CrtC)\textsuperscript{14–18}, the linalool dehydratase isomerase (LinD)\textsuperscript{19–22} and the fatty acid hydratases (e.g. oleic acid hydratase, OAH)\textsuperscript{2,23–26}. 

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Figure S4: Schematic view of the substrate binding pocket of *Em*-OAH wildtype enzyme

In accordance with our “hydratase-decoy molecule system” we divided the V-shaped binding pocket of the oleate hydratase into two theoretical parts (indicated by the dashed line): The substrate 1-decene is bound in the alkyl substrate binding pocket, while the decoy molecule hexanoic acid is accommodated by the carboxy binding pocket. The carbon-carbon double bond of the alkene is located at the bend of the binding pocket near the active site residues E122 and Y241. Binding of the fatty acid decoy molecule is facilitated by positively charged and polar amino acids at the entrance of the substrate binding pocket (indicate as carboxy binding site). The usage of decoy molecules has already been extensively investigated in P450-catalyzed reactions resulting in the hydroxylation of nonnative substrates.\cite{27-29} While the decoy molecules do not participate in the catalysis, they do favor the binding, orientation and stabilization of the substrate close to the active site residues by partially filling the substrate binding pocket.
Figure S5: Relative product formation of 1-decene hydration by Em-OAH wildtype enzyme after three days of incubation applying different hexanoic acid concentrations.

1-Decene hydration by Em-OAH wildtype enzyme was analyzed by varying the concentration of the decoy molecule hexanoic acid. Biotransformations were performed in the 500 µl scale with recombinant E. coli whole cells (50 mg/ml) and addition of glucose (100 mM final conc.) and FAD (0.3 mM final conc.) in a citrate buffer system (50 mM, pH 6). Reactions were started by the addition of substrate (0.5 mM final 1-decene conc.) and decoy molecule (0 mM; 0.1 mM; 0.25 mM; 0.5 mM; 1 mM; 2.5 mM final hexanoic acid conc.) with 1% (v/v) final DMSO concentration. After three days of incubation at 25°C and 180 rpm product formation was analyzed by GC. The result of equimolar hexanoic acid concentrations was set as 100% (0.25 mM 2-decanol).

Best results were obtained with an equimolar amount of the decoy molecule, while 2-decanol formation was strongly decreased at much lower and higher hexanoic acid concentrations (0.2 and 5 equivalents). Chiral GC analysis revealed an excellent stereoselectivity of >99% ee for the S-enantiomer, irrespective of the applied decoy molecule concentration. As described before,\textsuperscript{[4]} nearly no conversion (<5%) was observed without hexanoic acid as a decoy molecule.
Figure S6: Alternative decoy molecules for the hydration of 1-decene by Em-OAH

To further investigate the binding of the decoy molecule in the carboxy binding pocket and the importance of the carboxylic group for substrate recognition, enzyme activation and/or substrate positioning various fatty acids and fatty acid derivatives were tested as alternative decoy molecules. Up to now, hexanoic acid was used as a decoy molecule as it has a carboxylic group, which seems to be essential for substrate recognition. Moreover we assumed, that it has a suitable chain length enabling the simultaneous binding of two molecules within the substrate-binding pocket regarding the hydration of 1-decene. Based on these assumptions, saturated fatty acids of different chain length (C5-C8) were tested as alternative decoy molecules to examine the possibility of steric hindrances within the active site. Moreover, hexanoic acid esters, hexanoamide, hexanal and hexanol were analyzed as decoy molecules to further investigate the importance of the carboxylic group for conversion of alkenes. Finally, the analysis of terminal functionalized hexanoic acid derivatives might reveal information regarding the binding of the decoy molecule in the carboxy substrate binding site.
Figure S7: Relative product formation of 1-decene hydration by Em-OAH applying equimolar concentrations of alternative decoy molecules after two days of incubation.

1-Decene hydration by Em-OAH wildtype enzyme applying alternative decoy molecules was analyzed after two days of incubation and 2-decanol formation with hexanoic acid was set as 100%. Relative product formation without using a decoy molecule is indicated by a dashed line. Biotransformations were performed in the 500 µl scale with recombinant E. coli whole cells (50 mg/ml) and addition of glucose (100 mM final conc.) and FAD (0.3 mM final conc.) in a citrate buffer system (50 mM, pH 6). Reactions were started by the addition of substrate (0.5 mM final 1-decene conc.) and decoy molecule (0.5 mM final conc.) with 1% (v/v) final DMSO concentration. Stock solutions (100 mM) were prepared in DMSO, with exception of 6-aminohexanoic acid and 1,6-hexanediol, which were dissolved in buffer.

Highest 1-decene conversion was observed with heptanoic acid as a decoy molecule (113% relative product formation). The usage of longer and shorter fatty acids, octanoic acid and pentanoic acid, respectively, resulted in a strong decrease in activity with 36% and 18% relative product formation. The results indicate that the usage of heptanoic and hexanoic acid enables the simultaneous binding of two molecules within the substrate binding pocket, while octanoic acid might lead to steric hindrances. However, shorter fatty acids such as pentanoic acid do not seem sufficient for effective enzyme activation and/or alkene stabilization. Hexanoamide did not serve as a decoy molecule for 1-decene hydration as relative conversion rates were comparable to assays without decoy molecule (<5%). This is most probably due to an insufficient hydrogen network at the entrance of the substrate binding pocket, since hexanoamide is only a poor hydrogen bond acceptor. The usage of 1,6-hexanediol as well as 6-hydroxy- and 6-aminohexanoic acid did not result in any significant 2-decanol formation (<5%). This supports the hypothesis that the aliphatic chain of the decoy molecule has to bind in the carboxy substrate binding pocket for efficient catalysis. The polar functionality of the hexanoic acid derivatives might prevent the productive binding within the mostly hydrophobic binding pocket. Moreover, low conversion might result from steric hindrances within the binding pocket. The results indicate that in addition to the carboxylic group the decoy molecule has to be bound in the carboxy substrate binding pocket for effective substrate recognition, enzyme activation and/or alkene positioning. Stereoselectivity of 1-decene hydration with alternative decoy molecules was analyzed by chiral GC. An excellent stereoselectivity of >99% was observed irrespective of the decoy molecule.
Hexanoic acid esters did serve as decoy molecules for 1-decene hydration by Em-OAH whole cells (data not shown). However, as GC analysis revealed the hydrolysis of esters with the result of increasing hexanoic acid concentrations, product formation was not the result of alternative decoy molecule acceptance but of ester hydrolysis due to cellular esterases. Therefore, no information is gained regarding the acceptance of esters for substrate recognition and/or enzyme activation. The characterization of various OAHs indicates that a carboxylic group is essential for hydratase activity, most probably due to substrate recognition by positively charged and polar amino acids at the entrance of the substrate binding pocket.\textsuperscript{[3,30,31]} No activity was observed towards substrates such as methyl and ethyl fatty acid esters lacking the carboxy functionality.\textsuperscript{[25,30,32–34]} About 25% and 12% relative conversion was obtained by using hexanal and 1-hexanol, respectively, as alternative decoy molecules (data not shown). As GC analysis revealed low concentrations of hexanoic acid due to hexanal and 1-hexanol oxidation by cellular enzymes, 2-decanol formation does not seem to be the result of alternative decoy molecule acceptance. Therefore, no information is obtained regarding the acceptance of keto and alcohol functions for substrate recognition by Em-OAH.
**Figure S8:** Chiral GC-analysis for enantioselective hydration of 1-heptene (A), 1-hexene (B) and 1-pentene (C)

Stereoselectivity of alkene hydration by *Em*-OAH wildtype enzyme and generated variants was analyzed by chiral GC-(MS). Figure S9 presents the GC chromatograms of *Em*-OAH variant A248L biotransformations with 1-heptene (A) and *Em*-OAH A248W biotransformations with 1-hexene (B) and 1-pentene (C), respectively. The chromatograms show the product of the enzymatic conversion in green in comparison to the racemic standard in black. 1-Heptene biotransformations were analyzed in SCAN-mode, 1-hexene (84.1 m/z) and 1-pentene (87.1 m/z) biotransformations were evaluated in SIM-mode.
We used the variants *Em*-OAH wild type, *Em*-OAH A248L and *Em*-OAH A248W to screen a set of 23 substrates for alkene hydration activity using pentanoic and hexanoic acid as decoy molecule. Reaction conditions: 75 mg/mL cells, 50 mM citrate buffer pH 6, 100 mM glucose, 0.3 mM FAD, 2 mM decoy molecule, 2 mM Substrate, 1% DMSO, 6 days at 24°C. Comparison with cells containing an empty vector revealed activity for six substrates. The variant A248L in combination with hexanoic acid showed highest conversion. These six substrates have been chosen for further verification, namely: 7-bromo-1-heptene, 7-octen-1-ol, 4-phenyl-1-butene, trans-2-octene, cis-2-octene and 1-octyne. Characterization reactions were performed in quadruplicate and the quantification revealed a standard deviation of <5% of the mean value for each substrate. Comparisons with cells containing an empty vector as negative control did not show any background conversion for characterized substrates (7-bromo-1-heptene, 7-octen-1-ol, 4-phenyl-1-butene, trans-2-octene, cis-2-octene and 1-octyne) except for 4-phenyl-1-butene which shows a low background activity that yields the racemic alcohol as product. Please note that all alkenes were converted to chiral alcohols with very high enantioselectivity which further supports that this activity is based on enzymatic hydration performed by *Em*-OAH A248L.
**Figure S10:** Selectivity in alkene hydration for the expanded substrate scope studies

Chiral GC analysis of the enzymatic, asymmetric hydration of (A) 7-bromo-1-heptene, (B) 4-phenyl-1-butene and (C) 7-octen-1-ol. The top chromatograms show the racemic mixtures and the bottom chromatograms show the products from the enzymatic reaction. The alcohols have been analyzed as acetic ester derivatives. Please note that we do not have single enantiomers to assign the stereocenters. As for all the other substrates (1-decene, 1-octene, 1-heptene, 1-pentane, cis-2-octene and trans-2-octene) (S)-selectivity was confirmed, we suggest that also the alcohols shown in S10 were generated as (S)-enantiomer.
Figure S11: Selectivity for internal alkene hydration

Chiral GC analysis of trans-2-octene and cis-2-octene conversion revealed high regio- as well as enantiocontrol in the enzymatic hydration reaction. Please note that a catalytic, regio- and enantioselective method for alkene hydration has not been available previously. The alcohols were analyzed after derivatization to the corresponding acetic ester derivatives using acetic anhydride. A) Racemic mixtures of 2- and 3-octanol. B) Enzymatic asymmetric hydration of trans-2-octene. C) Enzymatic asymmetric hydration of cis-2-octene. The blue chromatogram corresponds to the control experiment.
IV. Supporting tables

To obtain high conversion, we optimized the substrate concentration for 1-decene and 1-octene hydration. 0.5 mM up to 4 mM final alkene concentration (100 mM stock solution in DMSO) and equimolar concentrations of fatty acid decoy molecule (100 mM stock solution in DMSO) with 1 to 8% (v/v) final DMSO concentration were analyzed. The preliminary experiments were performed in the 2 mL-scale in 2 mL glass vials with recombinant *E. coli* whole cells (100 mg/mL), glucose (200 mM final conc.) and FAD (0.6 mM final conc.) in a citrate buffer system (50 mM citrate, pH 6.0). Reactions were started by addition of substrate and decoy molecule and incubated at 25 °C and 180 rpm. 500 µl of biotransformation samples were extracted with 800 µL MTBE (with addition of 500 µM internal standard) and product formation was analyzed by achiral GC.

Table S1 presents the results of preliminary experiments for preparative scale reactions to optimize the reaction for high conversion. Based on these results, 2 mM 1-decene and 1 mM 1-octene with an equimolar concentration of the decoy molecule were used for preparative scale reactions (250 ml and 500 ml scale).

**Table S1: Preliminary experiments for 1-decen and 1-octen hydration in preparative scale**

| 1-decene [mM] | heptanoic acid [mM] | DMSO (v/v) | 2-decanol [mM] | conversion [%] |
|---------------|---------------------|------------|----------------|----------------|
| 0.5           | 0.5                 | 1 %        | 0.46           | 93             |
| 1             | 1                   | 2 %        | 0.75           | 75             |
| 2             | 2                   | 4 %        | 1.12           | 56             |
| 3             | 3                   | 6 %        | 0.96           | 32             |
| 4             | 4                   | 8 %        | 0.63           | 16             |

| 1-octen [mM]  | hexanoic acid [mM] | DMSO (v/v) | 2-octanol [mM] | conversion [%] |
|---------------|--------------------|------------|----------------|----------------|
| 0.5           | 0.5                | 1 %        | 0.42           | 84             |
| 1             | 1                  | 2 %        | 0.86           | 86             |
| 1.5           | 1.5                | 3 %        | 1.19           | 79             |
| 2.0           | 2.0                | 4 %        | 1.19           | 59             |
| 2.5           | 2.5                | 5 %        | 1.27           | 51             |
**Em-OAH mutants were generated by QuikChange mutagenesis via PCR.** Table S2, table S3 and table S4 present information regarding the degenerated primer, the PCR reaction mixture and the applied temperature program.

**Table S2: Degenerated PCR primer for QuikChange mutagenesis**

| Primer | Sequence 5' --> 3' |
|--------|-------------------|
| OAH1-A248G-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248G-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248V-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248V-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248I-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248I-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248M-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248M-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248F-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248F-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248W-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248W-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248T-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248T-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248C-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248C-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248Y-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248Y-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248N-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248N-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248Q-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248Q-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248D-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248D-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248K-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248K-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248R-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
| OAH1-A248R-rv | GATCATTCAGACCATCAATCTGCAATGCAGAAAAACGATGC |
| OAH1-A248L-fw | GCATCGTTTTCATGTCATGATGGCTGGTCTGAATGATC |
Table S3: PCR reaction mixture

| Volume | Ingredient |
|--------|------------|
| 5 µl   | KOD-Puffer (10x) |
| 4.5 µl | 25 mM MgSO₄ |
| 1 µl   | primer forward (10 µM) |
| 1 µl   | primer reverse (10 µM) |
| 5 µl   | dNTPs (2 mM each dNTP) |
| 0.5 µl | pET28a(+)::Em-OAH (200 ng/µl) |
| 1 µl   | KOD Hot Start polymerase (200 units) |
| ad 50 µl | MilliQ |

Table S4: PCR temperature program

| Temp. | Time |
|-------|------|
| 95 °C | 2 min |
| 95 °C | 0.5 min |
| 50 °C | 0.75 min |
| 70 °C | 3 min |
| 70 °C | 7 min |

20 cycles
Product formation was analyzed by GC-(MS). Depending on the respective alcohol product, different internal standards and temperature programs were used. Table S5 and table S6 present an overview of achiral and chiral GC analysis methods.

Table S5: Temperature programs for biotransformations samples for achiral GC analysis

| Alcohol product       | Internal standard (500 μM) | Heat rate [K/min] | Temp. [°C] | Hold [min] |
|-----------------------|-----------------------------|-------------------|-------------|------------|
| 2-decanol             | 1-octanol                   |                   | 130         | 3          |
|                       |                             | 2                 | 150         | 0          |
| 2-octanol             | 1-decanol                   |                   | 130         | 3          |
|                       |                             | 10                | 180         | 0          |
| 2-heptanol            | 1-pentanol                  |                   | 110         | 5          |
|                       |                             | 15                | 150         | 0          |
| 2-hexanol             | 1-pentanol                  |                   | 90          | 5          |
|                       |                             | 10                | 120         | 0          |
| 2-pentanol            | 3-hexanol                   |                   | 70          | 6          |
|                       |                             | 20                | 120         | 0          |
| 7-bromoheptan-2-ol    | 1-nonanol                   |                   | /           | 50         |
|                       |                             | 15                | 205         | 0          |
|                       |                             | 100               | 310         | 3          |
| Octane-1,7-diol       | 1-nonanol                   |                   | /           | 50         |
|                       |                             | 15                | 205         | 0          |
|                       |                             | 100               | 310         | 3          |
| 4-phenylbutan-2-ol    | 1-nonanol                   |                   | /           | 50         |
|                       |                             | 15                | 205         | 0          |
|                       |                             | 100               | 310         | 3          |
| 3-octanol             | 1-nonanol                   |                   | /           | 50         |
|                       |                             | 15                | 205         | 0          |
|                       |                             | 100               | 310         | 3          |
| 2-octanone            | 1-nonanol                   |                   | /           | 50         |
|                       |                             | 15                | 205         | 0          |
|                       |                             | 100               | 310         | 3          |
| alcohol product | internal standard | heating rate [K/min] | temp. [°C] | hold time [min] |
|----------------|-------------------|----------------------|------------|----------------|
| 2-decanol      | 1-octanol         | /                    | 100        | 1              |
|                |                   | 10                   | 120        | 3.5            |
|                |                   | 10                   | 150        | 0              |
| 2-octanol      | 1-decanol         | /                    | 100        | 0              |
|                |                   | 2                    | 112        | 0              |
| 2-heptanol     | 1-pentanol        | /                    | 65         | 0              |
|                |                   | 5                    | 100        | 0              |
| 2-hexanol      | 1-pentanol        | /                    | 55         | 8              |
|                |                   | 5                    | 65         | 0              |
|                |                   | 10                   | 80         | 0              |
| 2-pentanol     | 3-hexanol         | /                    | 55         | 4              |
|                |                   | 20                   | 85         | 0              |
| 7-bromoheptan-2-ol | 1-nonanol | /                    | 85         | 3              |
|                |                   | 2                    | 110        | 0              |
|                |                   | 20                   | 200        | 5              |
| Octane-1,7-diol | 1-nonanol        | /                    | 60         | 0              |
|                |                   | 0.1                  | 70         | 0              |
|                |                   | 0.2                  | 80         | 0              |
|                |                   | 0.3                  | 90         | 0              |
|                |                   | 0.4                  | 100        | 0              |
|                |                   | 0.5                  | 120        | 0              |
|                |                   | 2.5                  | 200        | 0              |
| 4-phenylbutan-2-ol | 1-nonanol | /                    | 100        | 4              |
|                |                   | 5                    | 145        | 0              |
| 3-octanol / 2-octanol | 1-nonanol | /                    | 64         | 0              |
|                |                   | 0.1                  | 67         | 0              |
V. Preparative scale reactions

General protocol
Preparative scale reaction of 1-decene hydration by Em-OAH wildtype was performed with 70 mg starting material (0.5 mmol; 2 mM 1-decene). 2 mM Heptanoic acid served as decoy molecule at a final DMSO concentration of 4% (v/v). The reaction was carried out in the 250 mL-scale in a 250 mL round-bottom flask using resuspended whole cells (100 mg/mL), glucose (200 mM final conc.) and FAD (0.6 mM final conc.) in a citrate buffer system (50 mM citrate, pH 6.0) by incubating the mixture for six days at 25°C and 150 rpm. The reaction was extracted two times with 200 ml MTBE and the combined organic layers were dried over Na₂SO₄, filtrated and concentrated *in vacuo*. The extract (280 mg) was dissolved in cyclohexane/ethyl acetate and purified by column chromatography (5:1 CH/EA; 30 cm length, 2.5 cm diameter, 28 g silica gel 60). The different fractions were analyzed by thin layer chromatography (2:1 CH/EA; 20 cm length, 1.5 cm diameter, 7.5 g silica gel 60). Again, product fraction were combined and concentrated *in vacuo*. The resulting product extract (51 mg yellowish oil) was dissolved in 20 ml CH/EA (5:1) and four times extracted with the same volume of a saturated NaHCO₃-solution and finally with water. The organic phase was dried over Na₂SO₄, filtrated and concentrated *in vacuo* (35 mg). 10 mg was dissolved in 600 µl deuterated chloroform and analyzed by NMR (¹H, ¹³C). For analysis of stereoselectivity 0.5 µl of the purified product was dissolved in 600 µl MTBE and derivatized with acetic anhydride for chiral GC.

Preparative scale reaction of 1-octene hydration by Em-OAH A248L was performed with 56 mg starting material (0.5 mmol; 1 mM 1-octene). 1 mM Hexanoic acid served as decoy molecule at a final DMSO concentration of 2% (v/v). The reaction was carried out in the 500 mL-scale in a 500 mL round-bottom flask using resuspended whole cells (100 mg/mL), glucose (200 mM final conc.) and FAD (0.6 mM final conc.) in a citrate buffer system (50 mM citrate, pH 6.0) by incubating the mixture for six days at 25°C and 300 rpm. The reaction was extracted three times with 400 ml MTBE and the combined organic layers were dried over Na₂SO₄, filtrated and concentrated *in vacuo*. The extract (532 mg) was directly dissolved in dichloromethane/methanol (98:2) and purified two times by flash chromatography (98:2 DCM/MeOH; 20 cm length, 1 cm diameter, 2.5 g aluminium oxide). The organic phase was concentrated *in vacuo* and the extract (200 mg) was dissolved in CH/EA (10:1) and purified by column chromatography (6:1 CH/EA; 30 cm length, 2 cm diameter, 20 g silica gel 60). The different fractions were analyzed by thin layer chromatography (2:1 CH/EA; pre-coated TLC-sheets ALUGRAM®Xtra SIL G/UV₂₅₄; 0.2 mm, silica gel 60; 2-octanol-Řₕ = 0.55) using a phosphomolybdic acid hydrate staining solution (5 g in 50 ml ethanol). Product fractions were combined (about 100 ml) and extracted one time with 40 ml saturated NaHCO₃ solution and one time...
with water. The organic phase was dried over Na$_2$SO$_4$, filtrated and concentrated in vacuo. The extract (30 mg) was dissolved in n-hexane/EtOAc (10:1) and purified once again by column chromatography (5:1 n-hexane/EtOAc; 20 cm length, 1 cm diameter, 3 g silica gel 60). The result was analyzed by TLC as described above. Product fractions were combined, concentrated in vacuo (15 mg) and 10 mg of the purified product was dissolved in CDCl$_3$ for NMR analysis ($^1$H, $^{13}$C). For analysis of stereoselectivity 0.5 µl of the purified product was dissolved in 600 µl MTBE and derivatized with acetic anhydride for chiral GC.

**(S)-2-Decanol:** The reaction was performed on 0.5 mmol scale. Isolated 35 mg (44 % yield). $^1$H NMR (300 MHz, CDCl$_3$) δ 0.88 (t, $^3$J$_{H-H}$ = 6.6 Hz, 3H), 1.19 (d, $^3$J$_{H-H}$ = 6.2 Hz, 3H), 1.28 – 1.44 (m, 15H), 3.80 (m, 1H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 14.26, 22.82, 23.64, 25.93, 29.42, 29.74, 29.81, 32.03, 39.54, 68.37. NMR data are in accordance with Literature.$^{[35]}$ HREIMS m/z 157.1590 (calcd for C$_{10}$H$_{21}$O$^+$ 157.1592).

**(S)-2-Octanol:** The reaction was performed on 0.5 mmol scale. Isolated 14 mg (22 % yield). $^1$H NMR (300 MHz, CDCl$_3$) δ 0.88 (t, $^3$J$_{H-H}$ = 6.6 Hz, 3H), 1.18 (d, $^3$J$_{H-H}$ = 6.2 Hz, 3H), 1.24 – 1.37 (m, 11H), 3.79 (m, 1H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 14.22, 22.76, 23.62, 25.88, 29.46, 31.96, 39.53, 68.36. NMR data are in accordance with Literature.$^{[35]}$ HREIMS m/z 129.1282 (calcd for C$_8$H$_{17}$O$^+$ 129.1279).
VI. Chemical synthesis of product standards

Synthesis of 7-bromoheptan-2-ol

7-Bromoheptan-2-ol was synthesized according to literature.\textsuperscript{36,37} Under an oxygen atmosphere, a mixture of palladium(II) chloride (99.0 mg, 0.56 mmol, 10 mol%) and copper(II) chloride dihydrate (0.96 g, 5.65 mmol) was dissolved in dimethylformamide (14 mL) and water (2 mL). Then 7-bromohept-1-ene (1.00 g, 5.65 mmol) dissolved in dimethylformamide (3.5 mL) and water (0.5 mL) was slowly added to the mixture under cooling in an ice bath. After stirring the reaction mixture at room temperature for 20 h, hydrochloric acid (3 M, 100 mL) was added followed by an extraction with diethyl ether (3 x 50 mL). The combined organic extract was washed with saturated sodium hydrogen carbonate solution (50 mL) and brine (50 mL), then dried over anhydrous magnesium sulfate. After removing the solvent under reduced pressure the oily crude product was analyzed via NMR spectroscopy and GC/MS. This showed a product composition of unreacted educt and 7-bromoheptan-2-one (32%, 1.81 mmol) in a ratio of 2:1. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \( \delta = 3.41 \, (t, \, J = 7.0 \, Hz, \, 2H, \, CH\textsubscript{2}), \, 2.45 \, (t, \, J = 7.3 \, Hz, \, 2H, \, CH\textsubscript{2}), \, 2.14 \, (s, \, 3H, \, CH\textsubscript{3}), \, 1.87 \, (quin, \, J = 7.2 \, Hz, \, 2H, \, CH\textsubscript{2}), \, 1.60 \, (quin, \, J = 7.6 \, Hz, \, 2H, \, CH\textsubscript{2}), \, 1.44 \, (m, \, 4H, \, CH\textsubscript{2}) \) ppm. \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \( \delta = 208.7, \, 43.4, \, 33.6, \, 32.5, \, 29.9, \, 27.7, \, 22.8 \) ppm. GC/MS m/z = 192.01 (calc.), 192.0 (found).

Without further purification, the crude product containing 7-bromoheptane-2-one (32%, 1.81 mmol) was used in the following reaction step. A solution of sodium borohydrate (0.21 g, 5.43 mmol) in anhydrous ethanol (5 mL) was prepared under nitrogen atmosphere. Then the ketone containing mixture dissolved in ethanol (5 mL) was added under cooling in an ice bath. The reaction mixture was stirred for 18 h at room temperature and quenched by addition of saturated sodium hydrogen carbonate solution (50 mL). After extraction with diethyl ether (3 x 50 mL), the combined organic layers were washed with brine (50 mL), dried over anhydrous magnesium sulfate, filtered, concentrated and purified by column chromatography (10 – 50% ethyl acetate in cyclohexane). The desired compound (0.35 g, 1.80 mmol, 99 %) was obtained with an overall yield of 32% as slightly yellow oil. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \( \delta = 3.80 \, (sext, \, J = 5.7 \, Hz, \, 1H, \, CH), \, 3.42 \, (t, \, J = 6.9 \, Hz, \, 2H, \, CH\textsubscript{2}), \, 1.88 \, (quin, \, J = 7.0 \, Hz, \, 2H, \, CH\textsubscript{2}), \, 1.54 – 1.42 \, (m, \, 6H, \, CH\textsubscript{2}), \, 1.19 \, (d, \, J = 6.2, \, 3H, \, CH\textsubscript{3}) \) ppm. \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \( \delta = 67.9, \, 39.0, \, 33.9, \, 32.7, \, 28.1, \, 24.9, \, 23.6 \) ppm. GC/MS m/z = 194.03 (calc.), 193.9 (found).
Synthesis of octane-1,7-diol

Octane-1,7-diol was synthesized according to literature. A solution of lithium aluminum hydride in dry THF (1 M, 25.0 mL, 25.0 mmol) was cooled to 0°C under nitrogen atmosphere. After adding 7-oxooctanoic acid (0.85 g, 5.37 mmol) dissolved in dry THF (10 mL) over a period of 10 min, the reaction mixture was stirred at room temperature for 4 h. Excess of LiAlH₄ was quenched by adding dH₂O (3 mL) and aqueous sodium hydroxide solution (15%, 1 mL) under ice cooling. The precipitate was filtered off over a pad of celite and washed with ethyl acetate (50 mL), following a washing step of the filtrate with brine (50 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. Octane-1,7-diol (64%, 0.50 g, 3.44 mmol) was obtained as slightly yellow liquid and was used without further purification. ¹H NMR (400 MHz, CDCl₃) δ = 3.79 (sext, J = 6.0 Hz, 1H, CH), 3.64 (t, J = 6.6 Hz, 2H, CH₂), 1.58 (quin, J = 7.1 Hz, 2H, CH₂), 1.48 – 1.30 (m, 8H, CH₂), 1.19 (d, J = 6.2 Hz, 3H, CH₃) ppm. ¹³C NMR (100 MHz, CDCl₃) δ = 68.1, 63.0, 39.2, 32.7, 29.4, 25.7 (2C), 23.5 ppm.
VII. NMR spectra

$^1$H NMR: (S)-2-decanol (product of the preparative scale reaction)

$^{13}$C NMR: (S)-2-decanol (product of the preparative scale reaction)
$^1$H NMR: (S)-2-octanol (product of the preparative scale reaction)

$^{13}$C NMR: (S)-2-octanol (product of the preparative scale reaction)
$^1$H NMR: 7-bromoheptan-2-one (crude mixture)

$^{13}$C NMR: 7-bromoheptan-2-one (crude mixture)
$^1$H NMR: 7-bromoheptan-2-ol (racemic product standard from chemical synthesis)

$^{13}$C NMR: 7-bromoheptan-2-ol (racemic product standard from chemical synthesis)
$^1$H NMR: octane-1,7-diol (racemic product standard from chemical synthesis)

$^{13}$C NMR: octane-1,7-diol (racemic product standard from chemical synthesis)
VIII. High resolution EI-MS spectra

Purified sample

2-Decanol

OH
**Purified sample**

**2-Octanol**

\[ \text{C}_8\text{H}_{17}\text{O}_1 \]
IX. References

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