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## Biocatalysis

# Chiral Alcohols from Alkenes and Water: Directed Evolution of a Styrene Hydratase

Matúš Gajdoš, Jendrik Wagner, Felipe Ospina, Antonia Köhler, Martin K. M. Engqvist, and Stephan C. Hammer\*

**Abstract:** Enantioselective synthesis of chiral alcohols through asymmetric addition of water across an unactivated alkene is a highly sought-after transformation and a big challenge in catalysis. Herein we report the identification and directed evolution of a fatty acid hydratase from *Marinitoga hydrogenitolerans* for the highly enantioselective hydration of styrenes to yield chiral 1-arylethanols. While directed evolution for styrene hydration was performed in the presence of heptanoic acid to mimic fatty acid binding, the engineered enzyme displayed remarkable asymmetric styrene hydration activity in the absence of the small molecule activator. The evolved styrene hydratase provided access to chiral alcohols from simple alkenes and water with high enantioselectivity (>99:1 e.r.) and could be applied on a preparative scale.

The enantioselective addition of water across an alkene is a conceptually simple reaction to synthesize chiral alcohols.<sup>[1,2]</sup> This chemical transformation is highly sought-after as it would offer significant advantages over the well-established enantioselective carbonyl reduction for chiral alcohol synthesis.<sup>[3–5]</sup> In contrast to carbonyl reduction, alkene hydration does not depend on stoichiometric amounts of reducing agents, uses cheap and readily available alkenes and water as the sole reactants, and avoids any byproduct formation (Figure 1). Enantioselective alkene hydration is considered one of the most atom-economical approaches to synthesize chiral alcohols and frequently referred to as a “dream reaction” in organic chemistry.<sup>[1,2,6,7]</sup> The development of catalysts for enantioselective hydration of unacti-

vated alkenes, however, has proven a longstanding challenge in modern catalysis.

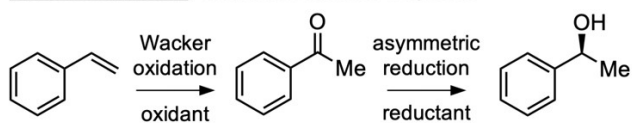
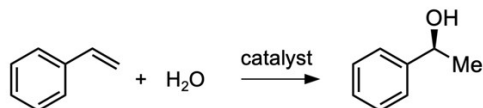
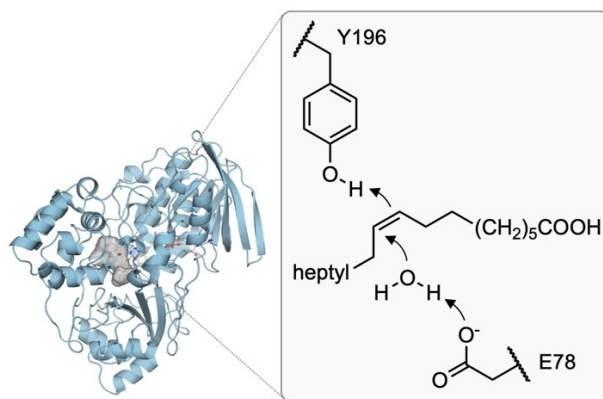
Several impressive multistep reaction sequences have been reported, for example, to convert styrenes into important chiral 1-arylethanols (Figure 1a).<sup>[6–9]</sup> However, these multistep reactions still depend on transition metal catalysts, chiral ligands as well as stoichiometric amounts of redox equivalents and other reagents (Figure S1). To date, not a single synthetic catalyst is available that can, for example, add water to a simple styrene while controlling enantioselectivity in the C–O bond formation (Figure 1a). Please note that a promiscuous enzyme for the enantioselective hydration of 4-hydroxystyrenes was recently reported.<sup>[10,11]</sup> This enzymatic reaction involves a *p*-quinone methide intermediate and the process is thus a conjugate addition to an activated alkene (unsaturated carbonyl compound), a feature that explains the limitation of this reaction to 4-hydroxystyrenes as substrates (Figure S2). In addition, anti-Markovnikov hydration of styrenes to produce 2-arylethanols has recently been reported with a promiscuous monooxygenase<sup>[12]</sup> and through redox hydration using engineered P450s and ADHs in a two-enzyme cascade.<sup>[13,14]</sup>

Several classes of natural enzymes (termed hydratases) are known that add water to unactivated alkenes with outstanding regio- and enantioselectivity.<sup>[15–23]</sup> Alkene hydration by hydratases is proposed to be achieved through precise positioning of the alkene and water substrates in combination with cooperative Brønsted acid-base catalysis, which simultaneously activates the alkene electrophile and the water nucleophile (Figure 1b).<sup>[24,25]</sup> Please note that important details of the mechanism are currently unclear and under discussion.<sup>[26]</sup> Hydratases are not only highly selective, but also highly substrate specific, being mainly limited to their natural substrates, namely carotenoids,<sup>[25,27]</sup> prenylated isoflavones<sup>[15]</sup> and unsaturated fatty acids<sup>[17,28,29]</sup> (Figure S3). This limitation has recently been addressed by rational enzyme engineering. In particular, fatty acid hydratases (FAHs) have been engineered to catalyze enantioselective hydration of fatty acid derivatives such as fatty alcohols<sup>[24,29]</sup> and fatty 1-alkenes<sup>[30]</sup> (Figure S4). In the latter case, hexanoic acid was used as a decoy molecule to activate the FAH for activity on fatty 1-alkenes, potentially by mimicking fatty acid binding.<sup>[31]</sup> Hydratases catalyze a highly desired chemical transformation and promise to solve a longstanding challenge in catalysis, yet this is only true if we are able to engineer enzyme variants that selectively add water to desired alkenes.

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**a** Conversion of alkenes to chiral alcoholsClassical approach: Oxidation reduction sequenceAim: Catalytic enantioselective water addition to styrenes**b** Cooperative Brønsted acid-base catalysis in fatty acid hydratases

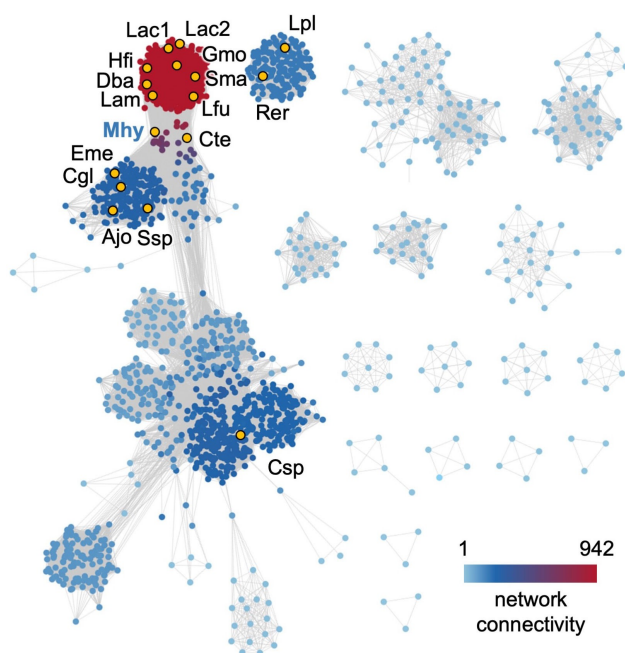
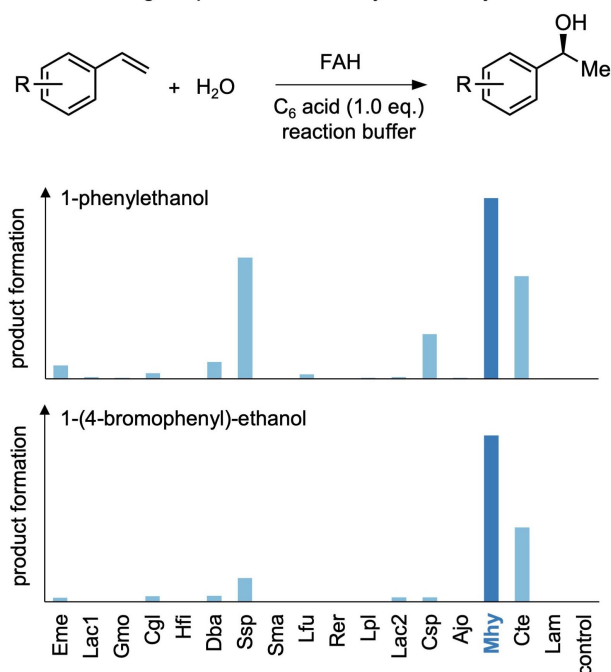
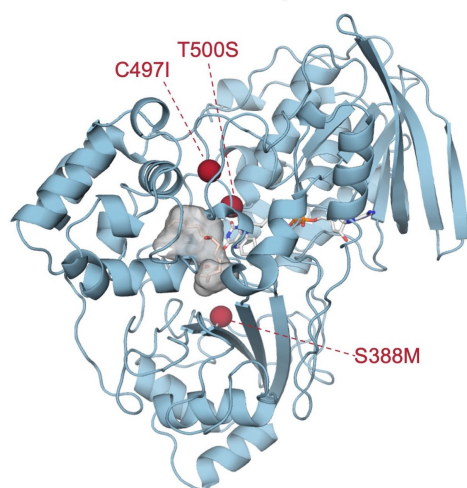
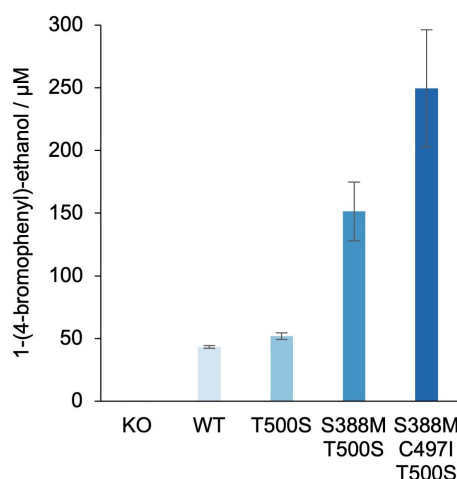
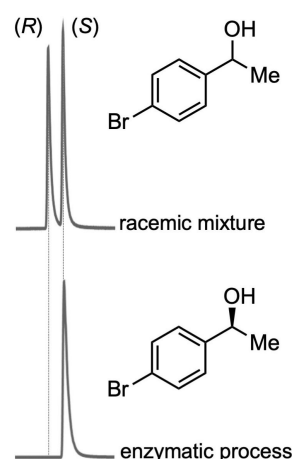
**Figure 1.** Synthesis of chiral alcohols from alkenes. a) A typical approach to access chiral secondary alcohols is the enantioselective reduction of the corresponding ketones using chiral chemocatalysts<sup>[3,4]</sup> or enzymes.<sup>[5]</sup> The ketones can be generated from alkenes by the palladium-catalyzed Wacker oxidation and this oxidation reduction sequence can also be performed in a one-pot process.<sup>[6]</sup> In contrast, the direct enantioselective addition of water across alkenes can generate chiral alcohols with high atom economy. In this desired reaction, water is used as the sole reactant and stoichiometric amounts of reducing agents are avoided.<sup>[1,2,6,7]</sup> b) The proposed mechanism of fatty acid hydratases involves cooperative Brønsted acid-base catalysis to activate the alkene as well as water for asymmetric alkene hydration.<sup>[17]</sup>

Herein we release FAHs from their dependence on fatty-type molecules. We have identified a promiscuous FAH from a thermophilic bacterium and used directed evolution to generate a variant for enantioselective hydration of simple styrenes. The evolved styrene hydratase generates chiral 1-arylethanol with very high enantioselectivity, useful activity and on preparative scale.

To develop a catalyst for enantioselective styrene hydration, we aimed to discover a promiscuous FAH from the natural pool and improve its performance using directed evolution. In a first step we used a Basic Local Alignment Search Tool (BLAST)<sup>[32]</sup> and characterized FAHs<sup>[33]</sup> as input to shed light on the natural diversity of this enzyme class. BLAST searches confirmed that FAHs belong to the Interpro protein family IPR010354, which consists of more than 6000 members. To roughly cover the natural diversity, we decided to generate a sequence similarity network<sup>[34]</sup> and chose an FAH panel from different clusters (Figure 2a). We have selected FAHs mainly based on literature demonstrating functional expression in *E. coli* (see Table S1). In addition, we applied TOME,<sup>[35]</sup> a command line tool that utilize a machine learning model to predict potentially thermostable enzymes from a big panel of homologs. TOME analysis identified three so far unknown FAHs from *Marinitoga hydrogenitolerans* (MhyFAH), *Clostridium tepidiprofundum* (CteFAH), *Lactobacillus amylolyticus* (LamFAH) which complemented the enzyme panel. Cloning of the corresponding synthetic DNA into a pET28a(+) vector and SDS-PAGE analysis after heterologous expression using a standard *E. coli* BL21 strain revealed recombinant protein production of almost all 17 FAHs, including the so far uncharacterized enzymes from the TOME prediction (Figure S5A). In addition, activity analysis using oleic acid as substrate confirmed fatty acid hydratase activity for all produced members of the enzyme panel (Figure S5B).

Having a potentially diverse FAH panel in hand, we started to screen the enzyme library for promiscuous activity using styrene and 4-bromostyrene as model substrates. Reactions were performed with resting whole cells, as previous studies revealed highest activities in whole cell preparations (as compared to cell-free lysates).<sup>[31]</sup> This can be explained by the slow aerobic oxidation of a FAH-bound FADH<sub>2</sub> cofactor after cell lysis and exposure to air.<sup>[17]</sup> FAHs belong to the 10 % of flavoenzymes that are non-oxidoreductases,<sup>[36]</sup> and it has been shown that FAD in its reduced form (FADH<sub>2</sub>) is important for high enzyme activity, potentially by stabilizing a carbocation intermediate after alkene protonation and prior to water attack.<sup>[17]</sup> We performed identification of promiscuous activities in the presence of equimolar concentrations of short chain fatty acids such as hexanoic (C<sub>6</sub>) or heptanoic acid (C<sub>7</sub>). Such molecules mimic fatty acid binding and activate the enzymes for catalysis.<sup>[31]</sup> We found that several FAHs reveal low styrene and 4-bromostyrene hydration activity which was close to the detection limit (Figures 2b and Table S2). What stood out was the catalytic performance of MhyFAH, an FAH from the anaerobe, thermophilic bacterium *Marinitoga hydrogenitolerans* isolated from a black smoker.<sup>[37]</sup> MhyFAH showed the highest level of product formation (as measured by peak area) for both substrates and was therefore chosen for further characterization.

To exclude artifacts from the whole cells and to confirm that styrene hydration occurs in the active site of the hydratase, we initially aimed to prove this promiscuous activity with purified enzyme. However, the purified enzyme was inactive with both styrene substrates, most likely owing to aerobic FADH<sub>2</sub> oxidation. Thus, we continued to confirm promiscuous styrene hydration activity using resting whole cells. We started by comparing the performance of wild-type MhyFAH with MhyFAH-E78A-Y196F, a variant with two “knock-out” mutations in the catalytically relevant coopera-

**a** Sequence similarity network of fatty acid hydratases**b** Screening for promiscuous enzyme activity**c** Structural model of *Mhy*FAH**d** Directed evolution of *Mhy*FAH**e** Enantioselectivity

**Figure 2.** Identification and directed evolution of a promiscuous FAH for asymmetric hydration of styrenes. **a**) Sequence similarity network (SSN) of the oleate hydratase family (IPR010354). A sequence similarity network is useful to visualize a relationship among a large number of protein sequences. Each node is a protein sequence and edges between nodes are only shown if protein sequences share at least a defined level of similarity. Here, an alignment score of 160 has been used that corresponds to 48 % sequence identity. The SSN was generated using the Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST).<sup>[34]</sup> The network connectivity displays the number of edges from one node to other nodes. Consequently, clusters with high network connectivity (red) represent a much higher number of protein sequences than clusters with low network connectivity (light blue). The SSN shows that the more promiscuous enzymes (*Mhy*FAH, *Cte*FAH and *Ssp*FAH) and less promiscuous enzymes (e.g. *Lpl*FAH and *Rer*FAH) occupy different clusters in sequence space. **b**) Promiscuous activity of the chosen enzyme panel was studied using styrene and 4-bromostyrene as substrate. Reactions were performed in the presence of hexanoic acid ( $C_6$  acid, 1 equiv) to mimic fatty acid binding. Product formation was measured as peak area using GC/MS in SIM mode (styrene) as well as HPLC/DAD (4-bromostyrene). **c**) Model of *Mhy*FAH with the active site represented as a grey surface. Amino acids that have been mutated during directed evolution are shown as red spheres. **d**) Directed evolution experiment shown as activity of freshly prepared cell-free extract (lysate conc. 300 mg wcm<sup>-1</sup>, 5 mM 4-bromostyrene, 2 mM heptanoic acid, 24 h, room temperature). Although activities in cell-free extract are lower than in whole cells, these reactions are highly reproducible and this setup has been used to rescreen beneficial mutants. KO represents *Mhy*FAH-E78A-Y196F, a variant with two mutations in the catalytically relevant cooperative acid-base machinery. **e**) Enantioselectivity of the styrene hydratase (*Mhy*FAH S388M-C497L-T500S)-catalyzed reaction.

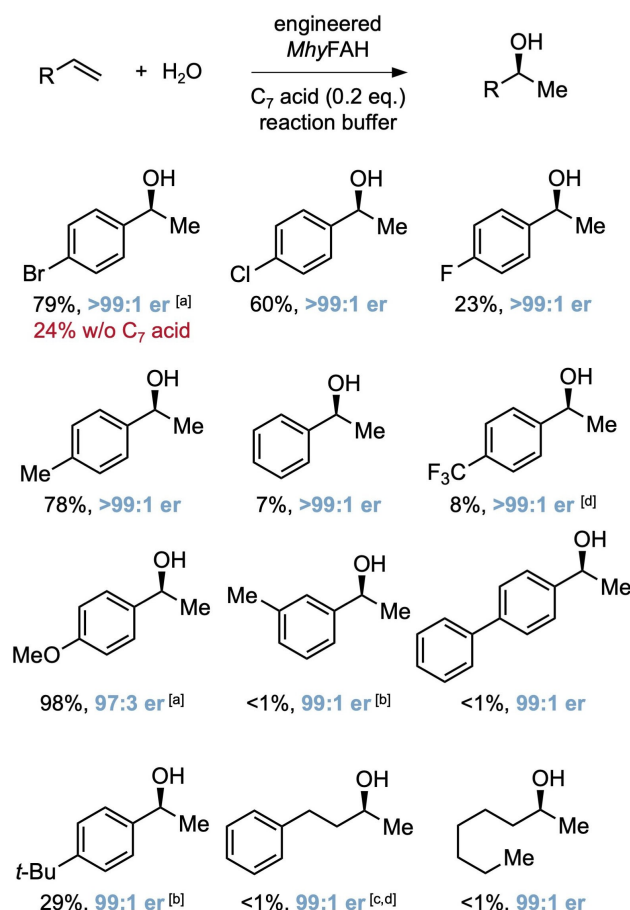


tive acid-base machinery (see Figure 1b). In contrast to the wild-type enzyme, this double mutant was completely inactive for alkene hydration using oleic acid, styrene or 4-bromostyrene as substrate (Figure S6). In addition, chiral GC analysis revealed that the wild-type *MhyFAH* produces 1-arylethanol with outstanding enantioselectivity (e.r. >99.9:0.1, see Figure S7). These results do not only suggest that the promiscuous reaction proceeds in the active site of *MhyFAH* but also represent the first enantioselective hydration of simple styrenes.

Since enantioselective styrene hydration with *MhyFAH* is mainly limited by low activity, we aimed to optimize this enzyme function through directed evolution.<sup>[38]</sup> We applied iterative saturation mutagenesis (ISM)<sup>[39]</sup> in the presence of heptanoic acid (0.4 equiv) using 4-bromostyrene as substrate. Three rounds of ISM, mainly at active site amino acids (Figure 2c and Table S3), generated *MhyFAH* S388M-C497L-T500S (hereafter referred to as styrene hydratase). This triple mutant displays a 5.8-fold increase in activity as measured by product formation (Figure 2d). To our delight, the evolved biocatalyst retained the high stereocontrol in the hydration of 4-bromostyrene (e.r. >99.9:0.1), yielding the chiral alcohol product in practically enantiopure form (Figure 2e). To further confirm the mechanism, we generated a styrene hydratase variant containing two “knock-out” mutations in the proposed cooperative acid-base machinery (E78A and Y196F, see Figure 1b). This mutant did not show 4-bromostyrene hydration activity (Figure S9). In addition, while the wild-type enzyme was inactive after enzyme purification (most-likely due to aerobic FADH<sub>2</sub> oxidation), the styrene hydratase remained partly active in its purified form (Figure S8). The residual activity of the purified enzyme was low, but highly selective to yield the *S* enantiomer (e.r. >99:1, Figure S9), thus, further proving the function of the evolved styrene hydratase. In addition, cell lysis under anaerobic conditions generated cell-free extracts with significantly higher styrene hydration activity as compared to cell-free extracts generated under aerobic conditions (Figure S10). This supports the oxygen-sensitivity of this enzyme and it is in line with the oxygen-sensitivity reported by other fatty acid hydratases (based on aerobic FADH<sub>2</sub> oxidation).<sup>[17,40,41]</sup> The introduced mutations are in the first and second shell of the active site and did not affect expression level of the recombinant protein (Figure S11). It is currently not clear how these mutations increase activity overall. For example, improved enzyme performance could be a result of optimized kinetic parameters or enhanced stability of the triple mutant against FADH<sub>2</sub> oxidation.

Under optimized conditions, enantioselective hydration of 4-bromostyrene was performed with good yield (79%) and excellent enantioselectivity (e.r. >99:1, Figure 3). Strikingly, we found that the styrene hydratase was much less dependent on heptanoic acid activation than the wild-type *MhyFAH*. While heptanoic acid (0.2 equiv) boosted alkene hydration 6.8-fold in the wild-type enzyme, reactions of the styrene hydratase in the absence of heptanoic acid decreased the yield only 3.3-fold, generating the corresponding chiral alcohol with 24% yield (Figure S12). This significant styrene hydratase activity in the absence of

### Substrate scope of the evolved styrene hydratase



**Figure 3.** Substrate scope of the asymmetric styrene hydration reactions. Reaction conditions: *E. coli* whole cells (100 mg w/w mL<sup>-1</sup>), 2 mM alkene and 0.2 equiv heptanoic acid (C<sub>7</sub> acid), 24 h, room temperature. The yields represent mean values from reactions of biological and technical triplicates (*n* = 9). The coefficient of variation (ratio of standard deviation to the mean) in yield determination was within 8%. [a] Reactions were also performed on a preparative 100 mg scale. [b] Reactions were performed with *MhyFAH*-S388M-T500S. [c] The reaction was performed with *MhyFAH*. [d] The absolute configuration of the stereocenter has not been determined.

heptanoic acid supports that directed evolution optimized the enzyme for 4-bromostyrene binding and suggest that further evolution (e.g., of the carboxylic acid binding pocket) might generate enzymes that are completely independent of such small molecule activators.

Next, we studied the substrate scope of the styrene hydratase (Figure 3). We found that various substituted styrenes are converted to the corresponding (*S*)-1-arylethanol with moderate to excellent yield (up to 98%) and very high enantioselectivity (e.r. typically >99:1, Figures 3 and S7, S13). Experiments with styrenes containing electron withdrawing and donating groups revealed an electronic effect on the reaction. While substituents with electron withdrawing groups at 4-position (e.g., 4-F or 4-CF<sub>3</sub>) showed lower yields, activating groups such as 4-Me or 4-OMe revealed highest yields (Figure 3). This electronic effect

suggests that overcoming a transition state with carbocation-like character is part of the rate-determining step in the enzyme-catalyzed reaction. In addition, we have also found activity in the hydration of challenging aliphatic 1-alkenes (4-phenyl-1-butene and 1-octene). These reactions proceeded with low activity but very high enantioselectivity (e.r. 99:1, Figures 3 and S7).

Finally, we aimed to explore scalability of these transformation by performing enantioselective hydration of 4-bromo- and 4-methoxystyrene on preparative 100 mg scale. We performed the reactions with a substrate loading of 2 mM, as experiments with higher substrate concentration (5 mM) generated more product but less yield within 24 h reaction time (Figure S14). The reactions using resting whole cells and 2 mM of substrate yielded chiral 1-arylethanol in useful yields (4-Br: 57 % and 4-OMe: 75 %) and very good selectivity (4-Br: e.r. 99:1 and 4-OMe: e.r. 95:5). These results highlight that highly enantioselective secondary alcohol synthesis can be performed on preparative scale using only unactivated alkenes and water as reactants.

In conclusion, we have reported the identification and directed evolution of a FAH for catalytic enantioselective hydration of styrenes. The reactions proceed with excellent enantioselectivity and often good activity, also on a preparative scale. The synthesis of chiral alcohols by selective addition of water across an alkene is considered a “dream reaction” in synthesis<sup>[1,2,6,7]</sup> due to substrate availability and high atom economy. Asymmetric alkene hydration is proven a long-standing challenge in the field,<sup>[2]</sup> with enantioselective catalysis being currently mainly limited to activated alkenes (e.g.,  $\alpha,\beta$ -unsaturated carbonyl compounds).<sup>[10,42]</sup> Our work shows that directed evolution can tame FAHs not only to accept non-fatty acid substrates, but also to reduce the dependence on the carboxylic acid motif that has been proposed as crucial to the mechanism.<sup>[26]</sup> We believe that this study opens a new avenue for the asymmetric synthesis of many important alcohols from simple alkenes and water.

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

**Keywords:** Alkene Hydration • Biocatalysis • Directed Evolution • Hydratase • Stereoselective Catalysis

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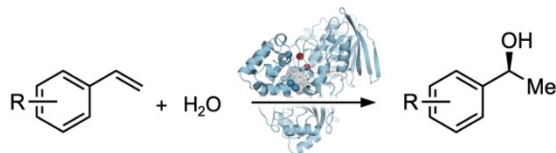
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## Communications

## Biocatalysis

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Chiral Alcohols from Alkenes and Water:  
Directed Evolution of a Styrene Hydratase



- engineered enzyme
- simple starting materials
- enantiomeric ratio > 99:1
- yield up to 98%
- 100 mg scale

The enantioselective addition of water across unactivated alkenes is a much sought-after chemical transformation and a major challenge in catalysis. Now a promiscuous engineered fatty acid

hydratase produces chiral alcohols with high enantioselectivity, also on a preparative scale, using simple alkenes and water as reactants.