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Enantioselective Aminohydroxylation of Styrenyl Olefins Catalyzed by an Engineered Hemoprotein

Inha Cho[a], Christopher K. Prier[b], Zhi-Jun Jia[a], Ruijie K. Zhang[a], Tamás Görbe [c], and Frances H. Arnold*[a]

Abstract: Chiral 1,2-amino alcohols are widely represented in biologically active compounds from neurotransmitters to antivirals. While many synthetic methods have been developed for accessing amino alcohols, the direct aminohydroxylation of alkenes to unprotected, enantioenriched amino alcohols remains a challenge. Using directed evolution, we have engineered a hemoprotein biocatalyst based on a thermostable cytochrome *c* that directly transforms alkenes to amino alcohols with high enantioselectivity (up to 2500 TTN and 90% ee) under anaerobic conditions with *O*-pivaloylhydroxylamine as an aminating reagent. The reaction is proposed to proceed via a reactive iron-nitrogen species generated in the enzyme active site, enabling tuning of the catalyst's activity and selectivity by protein engineering.

The 1,2-amino alcohol motif is a privileged scaffold present in numerous bioactive compounds, drugs, and natural products such as neurotransmitters, β-blockers, anti-malarial and anti-HIV drugs, and antibiotics.[1] Optically active amino alcohols serve other important roles as chiral auxiliaries, ligands, bases, and catalysts in asymmetric reactions.[2] Multiple enzymatic routes have been developed to access chiral amino alcohols from diols, ketones, and esters,[3] or through multistep enzyme cascades from styrenyl olefins.[4] To enable single-step enantioselective synthesis of amino alcohols from olefins,[5] several methods, such as the pioneering Sharpless asymmetric aminohydroxylation of alkenes,[6] have been developed. These chemical methods unfortunately suffer from various drawbacks related to their scope, selectivity, or the use of toxic metals. Furthermore, all of these methods install a nitrogen atom bearing a protecting group, necessitating further steps to reveal the free amine functionality. Aminooxidation can also be achieved by the aziridination of alkenes followed by ring opening, but the majority of asymmetric aziridination reactions also introduce a nitrogen in a protected form.[7]

Amination methods that directly transfer a simple NH unit to olefins have been developed only recently. In 2014, Kürti and coworkers developed rhodium-based catalysts and an aryl hydroxylamine precursor for the synthesis of NH-aziridines.[8] Morandi and coworkers subsequently reported the synthesis of 2-amino-1-arylethanols from styrenes, using iron phthalocyanine (FePc) as the catalyst and *O*-pivaloylhydroxylamine triflic acid (**1**) as the nitrogen source (**Figure 1A**).[9] While further work by these laboratories and others has introduced other hydroxylamine-based reagents for the direct installation of unprotected amines,[10] enantioselective versions have not been reported. Given the prevalence of the amino alcohol motif in medicinal agents, methods that can directly access this structural unit in an enantioselective fashion from abundant olefins are highly desirable.

Our group and others have engineered iron hemoproteins to perform nitrene transfer reactions using sulfonyl azides as nitrene precursors, demonstrating that enzymes have the ability to perform and control the selectivity of challenging non-biological amination processes.[11] Fasan and coworkers also reported enzymatic generation of nitrenes from azidoformates,[12] and Hartwig and coworkers reported the use of sulfonyl azides for nitrene transfer with artificial iridium-containing metalloenzymes.[13] In all of these cases, however, nitrene transfer with engineered hemoproteins produces protected amine products. Furthermore, the use of azide-based reagents may pose safety concerns for large-scale synthesis.

In a remarkable recent report, Tsutsumi and coworkers identified a wild-type cytochrome P450 that performs nitrene transfer in benzastatin biosynthesis through the cleavage of an N-O bond, demonstrating that hydroxylamines can function as nitrene precursors in biological systems (**Figure 1B**).[14] Inspired by nature's chemistry and Morandi's FePc catalyst system, we aimed to develop a hemoprotein catalyst for one-step production of chiral unprotected amino alcohols from olefins, a transformation not known in biology. We thought to discover such a reaction using nitrene source **1** as the precursor to a putative unprotected iron-nitrenoid intermediate **2** (**Figure 1C**). We anticipated that intermediate **2** could react with an olefin, adding a nitrogen from the reagent and an oxygen from water to form a 1,2-amino alcohol product. In this work, we present the directed evolution of an enzyme based on the highly thermostable *Rhodothermus marinus* (*Rma*) cytochrome *c* that generates and transfers the unprotected iron-nitrenoid for the efficient synthesis of chiral amino alcohols from olefins.

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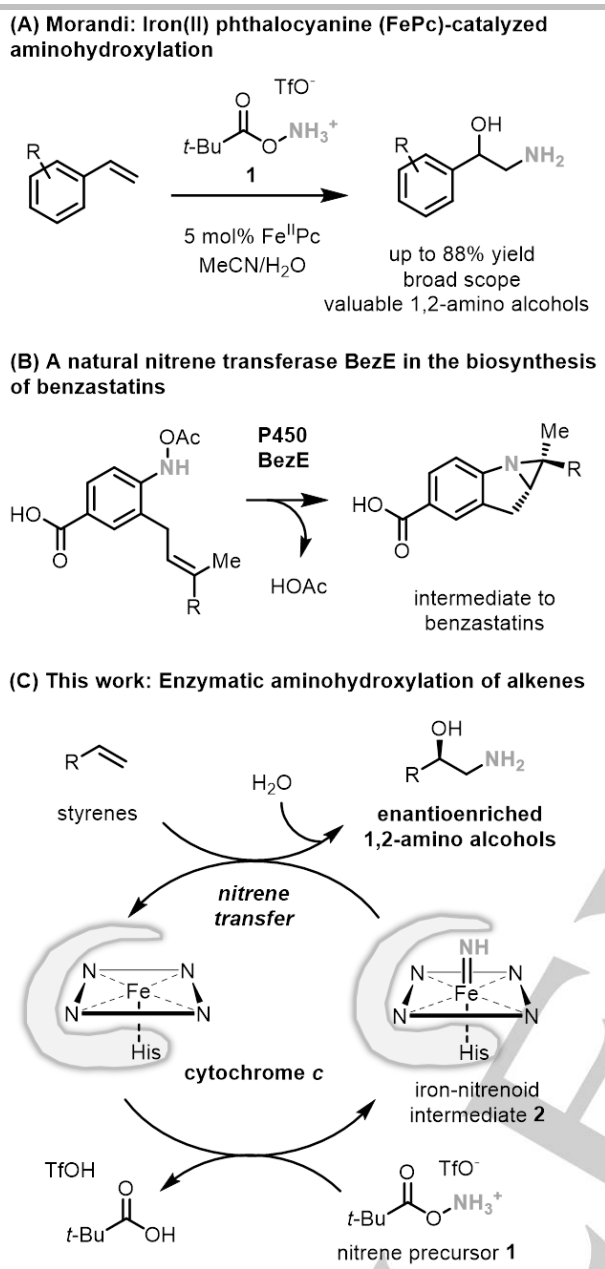

Figure 1. (A) FePc-catalyzed aminohydroxylation of olefins delivers racemic amino alcohol products.[9] (B) The biosynthesis of benzastatins proceeds via BezE-catalyzed nitrene formation from an N-acetoxy species, followed by intramolecular aziridination.[14] (C) Proposed enzymatic, enantioselective aminohydroxylation of alkenes.

Various catalysts were evaluated for aminohydroxylation activity, including cytochrome P450 monooxygenase from *Bacillus megaterium* ($P450_{BM3}$), $P450_{BM3}$ variants having the Cys400 axial ligand replaced with Ser (P411's), the reduced heme cofactor alone, iron phthalocyanine (FePc), protoglobins, and cytochrome *c* variants (**Supplementary Tables 1 and 2**). Whereas FePc catalyzes aminohydroxylation of olefins as reported,[7] the reduced heme cofactor as well as most of the tested hemoproteins showed no or trace activity. The wild-type *Rhodothermus marinus* (*Rma*) cytochrome *c*, an extremely thermostable (T_m = 106 °C) and small (124 aa) protein which coordinates heme using distal methionine and proximal histidine ligands in a small solvent-accessible pocket,[15] converted 4-vinylanisole **(3)** to aminoalcohol **4** with 4% yield under anaerobic conditions and remarkably provided **4** with 32% ee. This result indicated that the configuration at the newly formed stereocenter is controlled by the protein, suggesting that highly enantioselective synthesis of amino alcohols could be achieved through protein engineering.

Rma cytochrome *c* was used as the template for directed evolution to improve activity and enantioselectivity. Starting from the distal ligand M100, the closest residues facing the iron of the heme cofactor were targeted for sequential site-saturation mutagenesis in order to create an active site tailored for the putative iron-nitrenoid intermediate and the olefin substrates. Nearby residues that do not directly face the heme cofactor were also subjected to site-saturation mutagenesis with the hypothesis that these residues could impact substrate binding. For each round of mutagenesis, beneficial mutations were identified by screening for increased product yield and enantioselectivity using reverse phase LC-MS and normal phase HPLC, respectively. Focusing initially on improving product formation, four consecutive rounds of site-saturation mutagenesis at residues closest to the heme yielded *Rma* cytochrome *c* M99V M100S T101P M103G, which produces amino alcohol **4** with 360 TTN, a nine-fold improvement over the wild-type *Rma* cytochrome *c*, and 70% ee (**Figure 2A**). We then selected mutations that provided the greatest improvements in enantioselectivity in subsequent rounds. Two additional site saturation libraries targeting residues T98 and M76 yielded a further 5-fold increase in turnovers (1,800 TTN) and an increase in ee to 85%. The final round of site-saturation mutagenesis and screening introduced the Y44T mutation. This final variant, *Rma* cytochrome *c* TQL, provided the product with 2,500 turnovers and 90% ee under optimal conditions.

The crystal structure of *Rma* wild-type cytochrome *c* (**Figure 2B**) shows the heme iron is coordinated by the distal ligand M100, the heme cofactor, and the axial ligand H49, leaving neither a free coordination site nor space for substrate binding, at least according to the static structure. Nonetheless, the wild type protein catalyzes the reaction with 40 TTN. Mutation of M100 to serine presumably weakens coordination to iron and promotes nitrenoid formation. Residues M99 and M103 in the same loop as M100 were also mutated to smaller residues (99V and 103G), which may increase the flexibility of the distal loop. This may promote alternative conformations of the distal loop, potentially allowing new binding poses for the substrates, as observed previously in computational studies on a *Rma* cytochrome *c* catalyst evolved for carbene Si-H insertion.[16] Residue Y44 is farther from the heme center but is among the residues that surround the active site opening to the aqueous environment. Mutation to threonine at this position may improve substrate access to the active site.

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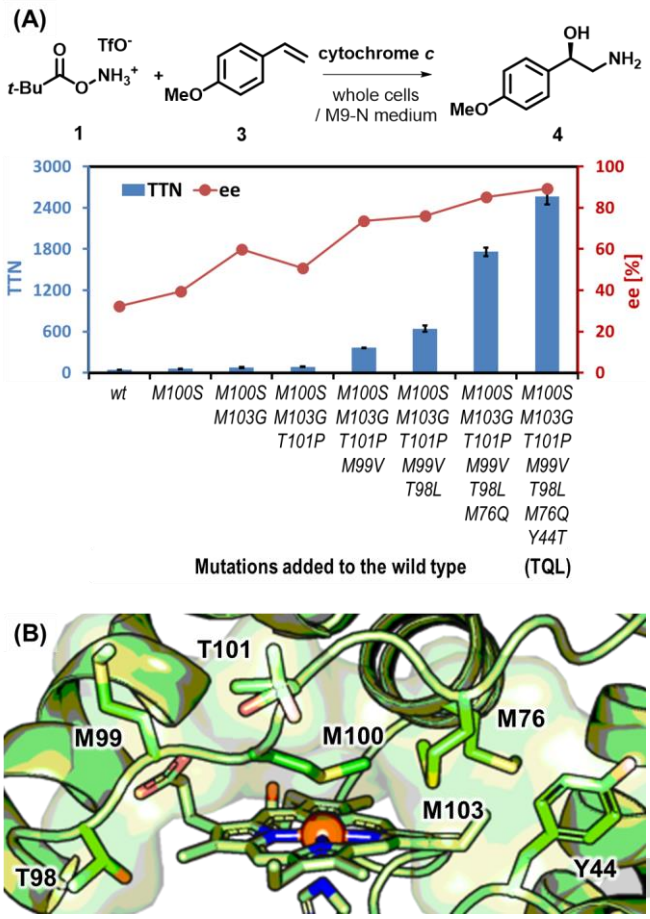
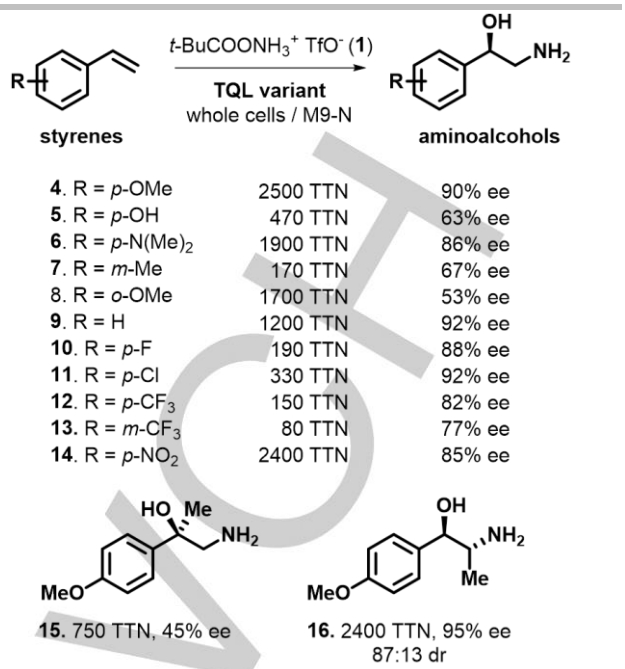


Figure 2. (A) Directed evolution of *Rma* cytochrome *c* from wild type to the TQL variant. (B) Crystal structure of wild-type *Rma* cytochrome *c* (PDB ID: 3CP5),[17] with mutated residues marked.



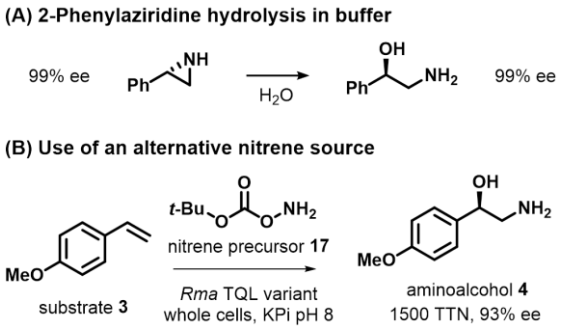
Scheme 1. Substrate scope of aminohydroxylation catalyzed by *Rma* cytochrome *c* TQL variant. Aminoalcohols **4** – **14** are synthesized from substituted styrenes, **15** is synthesized from *p*-isopropenylanisole, and **16** is from *trans*-anethole. Absolute configuration of **4** and **9** is the *R*-enantiomer, and the configuration of other products is assigned by analogy. The major diastereomer of product **16** is *threo*-**16** based on NMR.

Testing a variety of styrenyl olefin substrates, we found that cytochrome *c* TQL tolerates a range of substitutions on the aromatic ring (**Scheme 1**). Styrenes bearing electron-donating as well as -withdrawing substituents at the *para* position are accepted as substrates, undergoing aminohydroxylation with hundreds of turnovers in several examples. *Meta*- and *ortho*-substitutions are also tolerated, although these styrenes are converted with reduced turnovers and enantioselectivities. The TQL variant was also found to be active on α- or β-substituted styrenes to generate amino alcohols **15** and **16**. Interestingly, examples of activity on internal olefins are rare in both the FePc-catalyzed aminohydroxylation[9] and hemoprotein-catalyzed aziridination with sulfonyl azides.[11d] Aminohydroxylation of *trans*-anethole yielded amino alcohol **16**, an analog of the neurotransmitter β-hydroxyamphetamine, with 87:13 dr and 95% ee. Less activated olefins are challenging substrates, with 4-phenyl-1-butene and 1-octene providing only trace amounts of aminohydroxylation products. We next investigated whether aminohydroxylation using TQL could be achieved on a preparative scale. A 2.0-mmol scale aminohydroxylation of olefin **3** using whole cells in phosphate buffer under anaerobic conditions furnished product **4** in 61% (204 mg) isolated yield and 85% ee. Aminohydroxylation of *trans*-anethole was performed on 1.0-mmol scale and yielded 95 mg of product **16** (see Supporting Information for details). The reaction can also be performed with purified cytochrome *c* TQL, proceeding to 1500 TTN and 85% ee over 24 hours.

Our working mechanistic hypothesis for styrenyl olefin aminohydroxylation involves formation of a reactive complex between iron and the nitrogen reagent, which may be either the nitrene **2** or the corresponding nitrene radical.[18] At this point, nitrene transfer to the styrene could proceed to give an aziridine intermediate. To test whether this aziridine is a viable reaction intermediate, we synthesized racemic 2-phenylaziridine and separated the enantiomers via chiral HPLC. Incubating each enantiomer in aqueous buffer either with or without the purified TQL variant resulted in enantiospecific hydrolysis with inversion of configuration at the benzylic center (**Scheme 2A**). These results demonstrate that the aziridine may be a species *en route* to the amino alcohol, with any enantioselectivity conferred by the enzyme in the aziridination step persisting in the product. Alternatively, the putative nitrene or nitrene radical may engage the styrene to produce an iron-bound carbocation intermediate, which could be intercepted by water to provide the amino alcohol without the intermediacy of an aziridine (**Supplementary Figure 1**).[9] The aminohydroxylation of *trans*-anethole supports such a scenario, as the observed major *threo* diastereomer is inconsistent with a mechanism involving stereospecific aziridination and hydrolysis. Further study will be required to delineate the exact reaction pathway and characterize the reactive iron-nitrogen species.

We also performed aminohydroxylation of olefin **3** with a different nitrene precursor, *O*-(*tert*-butoxycarbonyl)-hydroxylamine (**17**). When the reaction was performed using catalyst-harboring *E. coli* whole cells, aminohydroxylation of **3** with reagent **17** yielded amino alcohol **4** with 1,500 TTN with 93% ee (**Scheme 2B**). This demonstrates that hemoproteins can utilize different reagents to access the putative unprotected nitrenoid intermediate. The effect of the nitrene precursor on the

reaction may provide an additional handle for reaction engineering.



Scheme 2. (A) Enantiospecific hydrolysis of 2-phenylaziridine occurs in both the presence and absence of the TQL cytochrome *c* variant, indicating that the aziridine is a viable intermediate *en route* to enantioenriched products. (B) An alternative nitrene source **17** also provides the amino alcohol product **4**.

Wild-type *Rma* cytochrome *c* displayed a limited ability to catalyze the amination of styrenyl olefins with hydroxylamine reagent **1**. Directed evolution improved this promiscuous function, delivering the cytochrome *c* TQL variant, an efficient catalyst for the synthesis of unprotected amino alcohols directly from alkenes. The heme cofactor of TQL is based on the most widely available transition metal in the Earth's crust, iron, and the enzyme exhibits levels of activity and selectivity toward aminohydroxylation that have been difficult to achieve with diverse synthetic methods. As the catalyst is fully genetically encoded, it can be tuned by directed evolution and produced and used in bacteria. This approach also provides a path to taming and utilizing an unusual and synthetically valuable unprotected nitrene equivalent, potentially enabling biocatalytic solutions to further challenges in direct amination chemistry.

Acknowledgments

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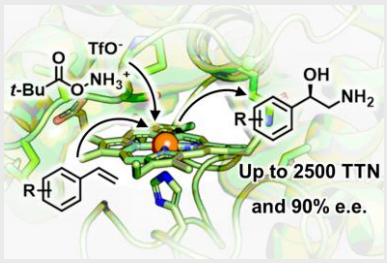
A hemoprotein catalyst was engineered to transform alkenes directly to amino alcohols with high enantioselectivity. Derived by directed evolution from a thermostable cytochrome *c*, the protein catalyst uses *O*-pivaloylhydroxylamine to generate a reactive iron-nitrogen species.



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Page No. – Page No.

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