Photoenzymatic Synthesis of α-Tertiary Amines By Engineered Flavin-Dependent 'Ene'-Reductases

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Supporting Information Placeholder

ABSTRACT: α-tertiary amines are a common motif in pharmaceutically important molecules but are challenging to prepare using asymmetric catalysis. Here, we demonstrate engineered flavin-dependent 'ene'-reductases (EREDs) can catalyze radical additions into oximes to prepare this motif. Two different EREDs were evolved into competent catalysts for this transformation with high levels of stereoselectivity. Mechanistic studies indicate that the oxime contributes to the enzyme templated CT-complex formed between the substrate and cofactor. These products can be further derivatized to prepare a variety of motifs, highlighting the versatility of ERED photoenzymatic catalysis for organic synthesis.

Chiral amines are privileged pharmacophores present in a wide variety of small molecule drugs.[1-3] Owing to their unique pharmacological and agrochemical properties, catalytic methods for their asymmetric synthesis have received considerable synthetic interest. Strategies for preparing α-secondary amines are well developed with frequent application of asymmetric reduction of imines and enamines, as well as biocatalytic transamination and reductive amination.[4-5] In contrast, strategies for preparing enantioenriched α-tertiary amines remain underdeveloped and desired for drug design and natural product synthesis.[6-7] Stereoselective delivery of carbanions or radicals to imines, oximes, and hydrazines derived from ketones is an attractive strategy for preparing α-tertiary amines.[8-10] Small molecule catalysts struggle to control facial delivery of reactive intermediates because of the small steric and electronic differences between the two substituents on the prochiral carbon. Enzymes are ideal for this challenge, however natural biocatalytic methods involving additions to imine congeners are rare.[11-12] We hypothesized that substrate promiscuous enzymatic platforms are competent catalysts for non-natural reaction mechanisms that address this challenge.[13]

Our group has pioneered the use a photoexcitation as a strategy for expanding the synthetic capabilities of enzymes.[14] We identified flavin dependent enzymes as an attractive class due to the wide variety of radical chemistry catalyzed by photoexcited flavin.[15] We recently found that flavin-dependent 'ene'-reductases (EREDs) can catalyze asymmetric hydroalkylations of olefins when irradiated with visible light.[16-18] In these reactions, radical formation occurs via electron transfer from flavin hydroquinone (FMN_{hq}) to the substrate via the intermediacy of an enzyme templated charge-transfer (CT) complex (Figure 1b). In these studies, presence of the alkene was essential for forming reactive charge-transfer complexes, with aliphatic amides failing to provide the same absorption feature (Figure 1b). We hypothesize that a $\pi \rightarrow \sigma^*$ interaction between the alkene and alkyl halide is responsible for the enhanced reactivity of these complexes.[19] Based on this observation, we question whether other types of coupling partners would be reactive. Toward our goal of synthesizing α-tertiary amines, we

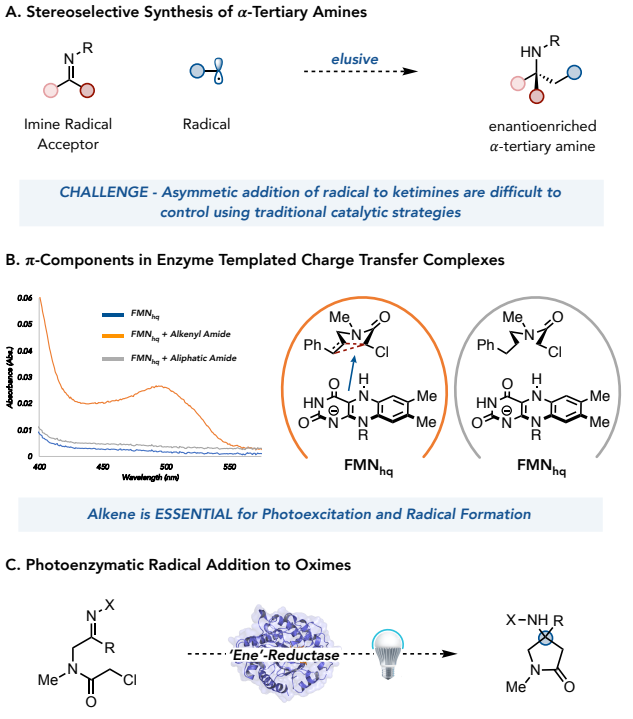


Figure 1. Preparing enantioenriched α-tertiary amines

specifically questioned whether oximes could function electron donors for CT complex formation.[20-23] If effective, we imagined that EREDs could be used for enantioenriched α-tertiary amine synthesis.

We initiated our investigation by exploring the radical cyclization of α-chloroamide **1a** bearing an *O*-benzyloxime (Figure 2) and began by exploring a series of EREDs previously identified as catalysts for photoenzymatic reactions and irradiated the reaction with cyan LEDs. While many EREDs were able to facilitate the desired reaction, none provided both high yield and enantioselectivity. This is stark contrast to our previous studies where effective catalysts could be identified from existing enzyme collections. Seeking to generate selective and efficient catalysts from these less efficacious ones, we elected to conduct protein engineering on two enzymes promising for either yield or enantioselectivity. We selected GluER-T36A, which provides products in 91% yield but with low enantioselectivity (66:34 er) and NCR, which forms product with good enantioselectivity (90:10 er) but low yield (Figure 2).[16, 24]

We began by engineering GluER-T36A for improved enantioselectivity by mutating residues within the protein active site. We tested an existing collection of variants containing active site mutations and found that GluER-G6 (T36A-K317M-Y343F), a variant possessing an active site mutation (Y343F), afforded product with 80:20 er in 95% yield.[19] Building on this catalyst, we conducted site saturation mutagenesis on additional residues lining the protein active site (Y269, W66, Q232, and Y343) and found mutation of the phenylalanine at position 269 to valine (GluER-T36A-K317M-Y343F-F269V) improved the enantioselectivity to 96:4 er, with the product formed in 95% yield (Figure 2).

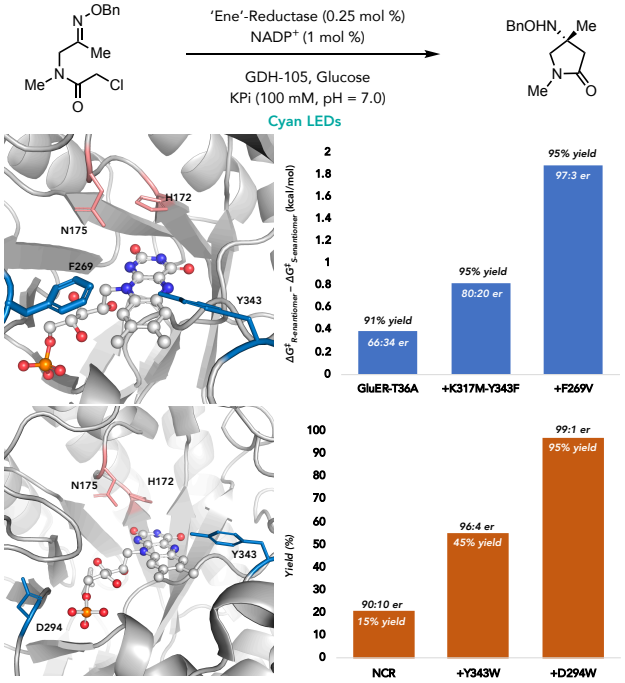
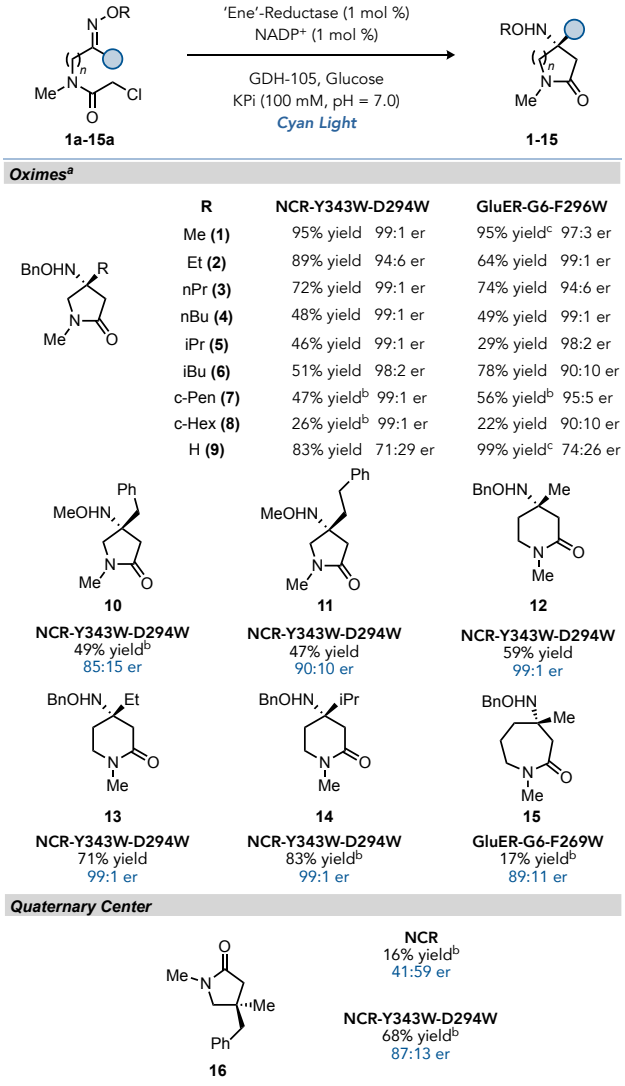


Figure 2. Optimization of Radical Addition to Oximes

Concurrently, NCR was engineered for improved activity. Using a similar approach as was employed with GluER-T36A, we prepared site saturation mutagenesis libraries for residues lining the protein active site (N175, Y177, I231, Q232, R261, T268, F269, N292, Q293, D294, T296, S313, R316, P317, I319, D337, W342, and Y343).[24] In the first-round, mutation of tyrosine 343 to tryptophan (Y343W) increased the yield to 45% with an increase in enantioselectivity. Interestingly, this is the homologous position to that mutated in GluER. A second round of site-saturation mutagenesis revealed conversion of aspartic acid at position 294 to tryptophan (NCR-D294W-Y343W) doubled the activity, providing product in 95% yield with 99:1 er (Figure 2).



[a] Substrate (15 mM), Enzyme (1 mol %), NADP+ (1 mol %), GDH-105 (20% w/w), Glucose (6 equiv.), KPi (100 mM, pH 7.0), 25 °C, 18 h [b] 2 mol% ERED, [c] GluER-G6-F296V

Figure 3. Substrate Scope

With two selective and efficient catalysts in hand, we explored their scope and limitation of these catalysts (Figure 3). We found that NCR-D294W-Y343W

was effective for a variety of substituents on the carbon of the oxime, with all tested examples forming product with outstanding levels of enantioselectivity. Yields decreased with increasing size, most notably with substitution at the α position of the R group as is the case in substrates **6a**, **7a**, and **8a**. This effect is presumably due to poor substrate binding. Interestingly, GluER-G6-F296V was ineffective for these substrates, affording product in low yield. Reexamining the GluER-G6 F269 library with more sterically demanding isopropyl substrate revealed GluER-G6-F269W to be a more reactive catalyst without loss in enantioselectivity. This catalyst was also effective for other substituted oximes, providing product in good yield and promising levels of enantioselectivity. Interestingly, the aldehyde derived oxime **9a** is highly reactive but less enantioselective. Benzylic and homobenzylic substituted oximes proved to be completely unreactive, presumably due to challenges associated with substrate binding. We suspected that this challenge could be addressed by modifying the oxygen substituent of the oxime from benzyl to methyl. We were delighted to find that this change in substrates **10a** and **11a** enhanced the reactivity of these substrates, providing product in promising yields and good selectivity.

Next, we explored the ability to forge larger lactam rings using oximes as coupling partners. We found that NCR-Y343W-D294W is an effective variant for 6-exo-trig cyclizations. Interestingly, changing the substituent on the oxime did not have a detrimental effect on the enantioselectivity of the transformation. Finally, we found that GluER-G6-F269W is capable of catalyzing a 7-exo-trig cyclization on substrate **15a** in low yield but with promising levels of enantioselectivity (Figure 3).

A striking feature of these variants is their ability to control the stereochemical configuration of a fully substituted carbon center. Based on this observation, we questioned whether these catalysts would also be effective for setting quaternary stereocenters. We prepared trisubstituted olefin **16a** and found that the NCR catalyzed the desired reaction with low levels of enantioselectivity and modest yield. In contrast, NCR-Y343W-D294W forms product with significantly enhanced levels of enantioselectivity and promising yield. While further engineering is required to increase the reaction yield, these results highlight the opportunity to control challenging stereocenters using catalytically promiscuous 'ene'-reductases.

Next, we explored the synthetic utility of these hydroxylamine products. The N–O bond of the hydroxylamine can be readily reduced using Pd/C under an atmosphere of hydrogen, cleanly providing the desired α-tertiary amine in good yield. The product can be further reduced to the corresponding pyrrolidine using borane. Finally, the lactam can be hydrolyzed and N–O bond reduced under acidic conditions to afford the corresponding β-amino acid in good yield.

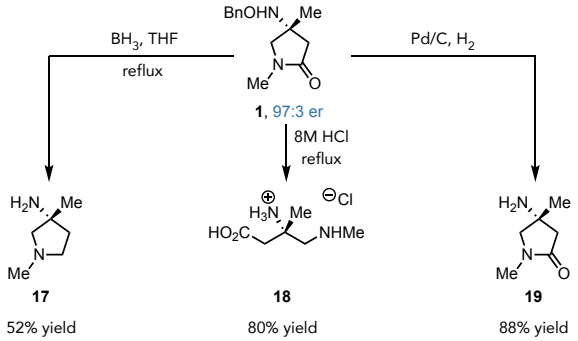


Figure 4. Product Derivatization

Having established engineered EREDs as effective asymmetric catalysts for radical additions into oximes and trisubstituted alkenes, we drew our attention to the biochemical basis for improved reactivity in the engineered variants. Noting the robust improvement in yield and enantioselectivity for both NCR and GluER variants upon mutating the Y343 residue, we looked to the reported crystal structure of NCR for insight into the role of this site.[24] We noted that the Y343 hydroxyl residue forms a polar contact with nicotinamide. We hypothesize this interaction constitutes a binding contact with NADPH that facilitates flavin turnover. However, since EREDs operate under a ping-pong mechanism in which NADPH and substrate alternatively bind,[25] if NADPH binds much more tightly to the enzyme than substrate the nicotinamide cofactor could act as a competitive inhibitor. To evaluate this possibility, we measured the binding constants for NADPH across the evolutionary trajectory of NCR and GluER. We found that wild type NCR has a tight binding constant for NADPH with $K_D \leq 25\ \mu M$. The Y343W mutation has a remarkable impact on NADPH binding, with $K_D = 702\ \mu M$. Addition of the D294W on top of the Y343W mutation seems to improve NADPH binding but not as tightly as the level of wild type enzyme, as the NCR-D294W-Y343W variant has a K_D of 53 μM. While less pronounced, the GluER variants follow the same trend (Figure S11) supporting the role of the observed mutations to ameliorate the inhibitory effect of strong NADPH contacts with the enzyme while still enabling tight enough binding for flavin reduction.

A key mechanistic feature of the coupling with alkenes is formation of a charge transfer (CT) complex between the flavin hydroquinone, α-chloroamide, and alkene. We questioned whether this type of complex was also responsible for coupling with oximes. UV-vis experiments were conducted with reduced GluER-G6-F269V. When substrate is added, a complex with a maximum absorption around 500 nm is formed, indicating the formation of the CT complex. Interestingly, when a structurally related alkane or ketone are tested, which lack the electron rich π-system, no CT complexes are observed. These results suggest that the oxime is required for CT complex formation. We hypothesize that the electron rich nature of oximes helps facilitate the hyperconjugative

interactions between the π-system and σ^*_{C-Cl} required for the complex formation.

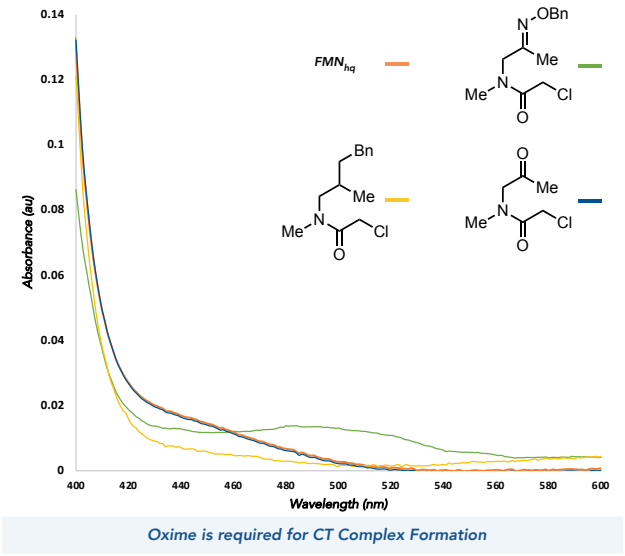


Figure 5. Mechanistic Experiments

In conclusion, we demonstrated that oximes are reactive coupling partners for ERED catalyzed cyclizations enabling the synthesis of enantioenriched α-tertiary amines. While wild-type enzymes were insufficiently selective or reactive, protein engineering was effective in increasing the activity and selectivity of these enzymes. The reaction is found to utilize a similar reaction mechanism to couplings involving alkenes and that protein engineering alters binding of the NADPH cofactor. These engineered catalysts are also effective for reactions to set quaternary stereocenters. Overall, this study highlights the opportunity of novel biocatalytic reaction mechanisms coupled with directed evolution to solve long-standing challenges in chemical synthesis.

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Notes

The authors declare no competing financial interest.

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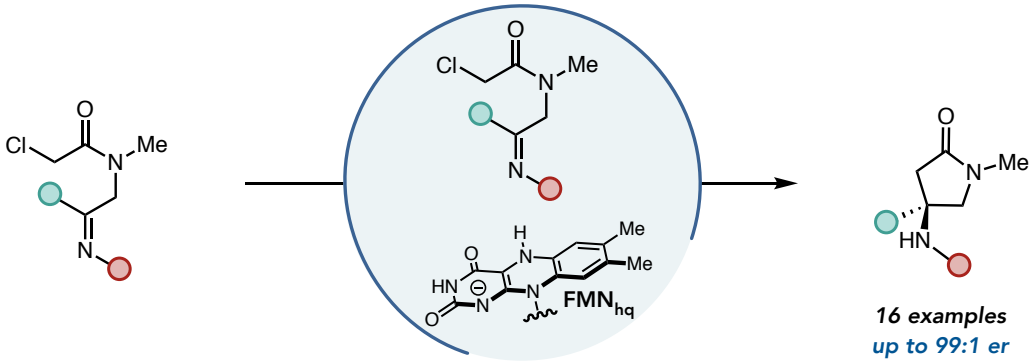

16 examples
up to 99:1 er

Engineered Enzyme-Templated
Charge-Transfer Complex