Synergistic Photoenzymatic Catalysis Enables Synthesis of *α*-Tertiary Amino Acids Using Threonine Aldolases

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Supporting Information Placeholder

ABSTRACT: *α*-Tertiary amino acids are essential components of drugs and agrochemicals, yet traditional syntheses are step-intensive and provide access to a limited range of structures with varying levels of enantioselectivity. Here, we report the *α*-alkylation of unprotected alanine and glycine by pyridinium salts using pyridoxal (PLP)-dependent threonine aldolases with a Rose Bengal photoredox catalyst. The strategy efficiently prepares various *α*-tertiary amino acids in a single chemical step as a single enantiomer. UV-vis spectroscopy studies reveal a ternary interaction between the pyridinium salt, protein, and photocatalyst, which we hypothesize is responsible for localizing radical formation to the protein active site. This method highlights the opportunity for combining photoredox catalysts with enzymes to reveal new catalytic functions for known enzymes.

Non-canonical *α*-amino acids (ncAAs) and *α*-amino alcohols are essential components of natural products and bioactive molecules (Figure 1A).[1–4] When incorporated into proteins, they can unlock new catalytic functions,[5,6] facilitate mechanistic investigations,[7–9] and enable the facile synthesis of protein conjugates.[10] In therapeutics and agrochemicals, ncAAs provide activity and stability profiles not observed with their naturally occurring conjurers.[11] Beyond proteins and peptides, many chiral small molecule catalysts are derived from amino acids,[12] where non-canonical analogs provide access to regions of chemical space necessary for selective transformations. For all these applications, *α*-secondary amino acids are most common because of their structural similarity to canonical amino acids. Increasingly, *α*-tertiary amino acids are desired because they have enhanced metabolic stability and improved conformational rigidity, leading to improved functions.[13]

Catalytic asymmetric syntheses of *α*-tertiary amino acids are challenging because of the inherent reactivity of the amino acid motif. [14] For *α*-alkylation reactions, the amine and carboxylic acid must be masked to enable enolization, requiring the subsequent removal of the two protecting groups to form the desired ncAA.[15,16] Alternative synthetic strategies, such as nucleophilic addition to *α*-iminoesters, also require protection of both the acid and imine to enable a selective synthesis (Figure 1B)[17–21] Nature avoids these tedious protection and deprotection steps by transiently activating the amino acid for functionalization using 5-pyridoxal phosphate (PLP).[22,23] This cofactor reacts with the amino moiety to form an aldimine, which both protects the amine functional group and acidifies the *α*-protons, while electrostatic and hydrogen bonding interactions with the protein temper the reactivity of the carboxylate. We hypothesized that repurposing native PLP-dependent enzymes might provide an attractive strategy for synthesizing *α*-tertiary amino acids.

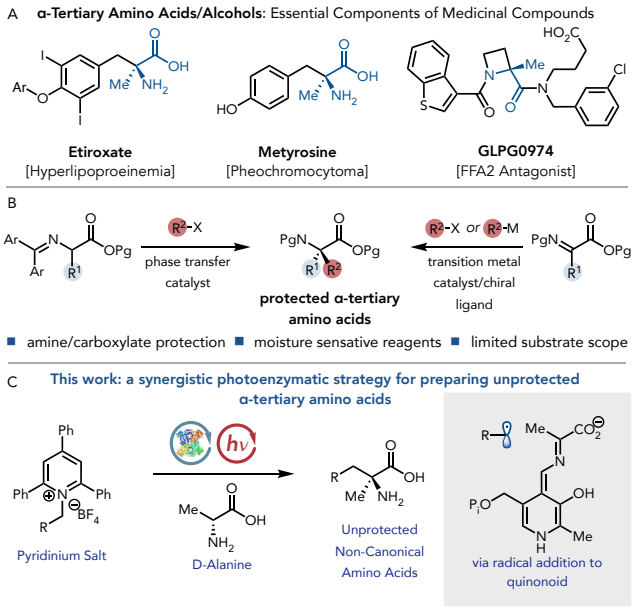
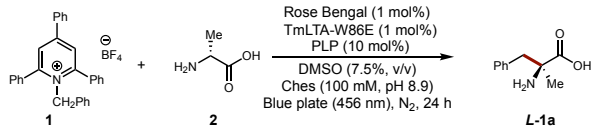


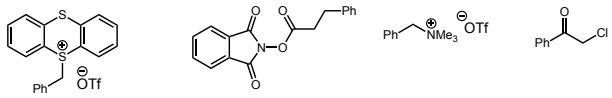
Figure 1. (A) Selective examples of bioactive non-canonical amino acids. (B) Existing methods to access chiral *α*-tertiary amino acids. (C) Our work: *L*-Threonine Aldolase catalyzed *α*-tertiary amino acids synthesis.

Over the past six years, our group has demonstrated that exogenous photoredox catalysts can expand the catalytic function of flavin and nicotinamide-dependent oxidoreductases by generating non-native radicals within their active sites.[24–28]In these studies, we found that electronic activation of radical precursors by the protein and photocatalyst association to the protein was essential for ensuring that radical formation only occurs within the protein active site. Recently, Yang and coworkers demonstrated that this strategy for unlocking new catalytic functions could be used with PLP-dependent tryptophan synthases to deliver *α*-secondary amino acids from alkyl trifluoroborates and serine via a radical Michael addition in good



1 **2** ***L*-1a**

entry	variation	yield (***L*-1a**, %)[b]	er[c]
1	-	90	>99:1
2	with TmLTA	67	>99:1
3	with TeLTA	90	>99:1
4	w/o TmLTA-W86E, 10% free PLP	0	-
5	dark	0	-
6	green kessil	88%	>99:1
7	w/o free PLP	67%	>99:1
8	w/o RB	0	-
9	under O_2 atmosphere	0	-



R-1 n.d. **R-2** n.d. **R-3** n.d. **R-4** n.d.

Figure 2. L-Threonine Aldolase catalyzed model reaction optimization. Reaction conditions: **1** (2.5 umol 1.0 eq, 5.8 mM), D-alanine (12.5 umol, 5 eq, 29 mM), LTA (1 mol% based on **1**) in 100 mM CHES buffer pH 8.9, with 7.5% DMSO (v/v) as cosolvent, final total volume is 430 µL. Reaction mixtures were stirred under anaerobic conditions at room temperature for 24 h. [b] Yield determined via LC-MS relative to an internal standard (Mandelic acid). [c]Enantiomeric ratio (er) determined by chiral HPLC after Boc_2O protection. n.d.: not detected.

yield and good to excellent levels of enantioselectivity.[29] Independently, we questioned whether photoredox catalysts could be used with threonine aldolases to provide a facile synthesis of *α*-tertiary amino acids.

L-threonine aldolases (LTAs) are a large family of pyridoxal 5-phosphate (PLP)-dependent enzymes that are capable of catalyzing synthetically useful aldol reactions between small amino acids (glycine, alanine, serine) and aldehydes to afford *ß*-hydroxyl-*α*-amino acids.[22,23,30] Essential to this reactivity is the formation of the pyridoxal-quinonoid, the nucleophile in the aldol reaction. Given the similarities between the quinonoid and other nucleophilic species involved in catalysis, such as enolates and enamines, we hypothesized that the quinonoid could also serve as a SOMOphile with radical species (Figure 1C). As the protein scaffold blocks one prochiral face of the quinonoid, we expected radical addition to occur with perfect stereoselectivity.[31]

We began our studies using the thermophilic LTA from *Thermotoga maritima* with *D*-alanine as the amino donor.[32] We tested a series of alkyl electrophiles, including thianthrenine salt **R1**, NHPI ester **R2**, tetramethylammonium salt **R3,** *α*-chloroacetophenone **R4**, and Katritzky pyridinium salt **1**, which generate alkyl radicals via single electron reduction.[33] This mechanism of radical initiation was desired because the oxidized state of the photocatalyst could oxidize the PLP-adduct of radical addition.[34,35] When using Rose Bengal as a photoredox catalyst and irradiating with blue LEDs (456 nm), we found the pyridinium salts were the only productive alkyl radical source, furnishing the product with a 65% yield and forming the *S*-product in >99:1 enantiomeric ratio. Unfortunately, the yield was dependent on the preparation of the protein, presumably due to the instability of the protein tetramer. Gront and coworkers found that mutation of tryptophan 86 to glutamic acid (W86E) enhanced the stability of the enzyme by forming a salt bridge with the arginine 120 residue on an adjacent subunit.[32] Introducing this mutation increased the yield to 90% and significantly improved the reproducibility. Other LTAs also demonstrated this non-native reactivity, with the enzyme from *Aeromonas tecta* (TeLTA) exhibiting similar activity and enantioselectivity to TmLTA-W86E (Figure 2, entry 3) (Supplementary Table 1). Control experiments confirmed that light, photocatalyst, and protein are essential for the desired reactivity (Figure 2, entries 5, 7). It is worth mentioning that an additional 10 mol% free PLP was needed to enhance the enzyme activity, likely due to the photodegradation of the quinonoid intermediate as a competing pathway (Figure 2, entry 6, Supplementary Figures 19 and 20).

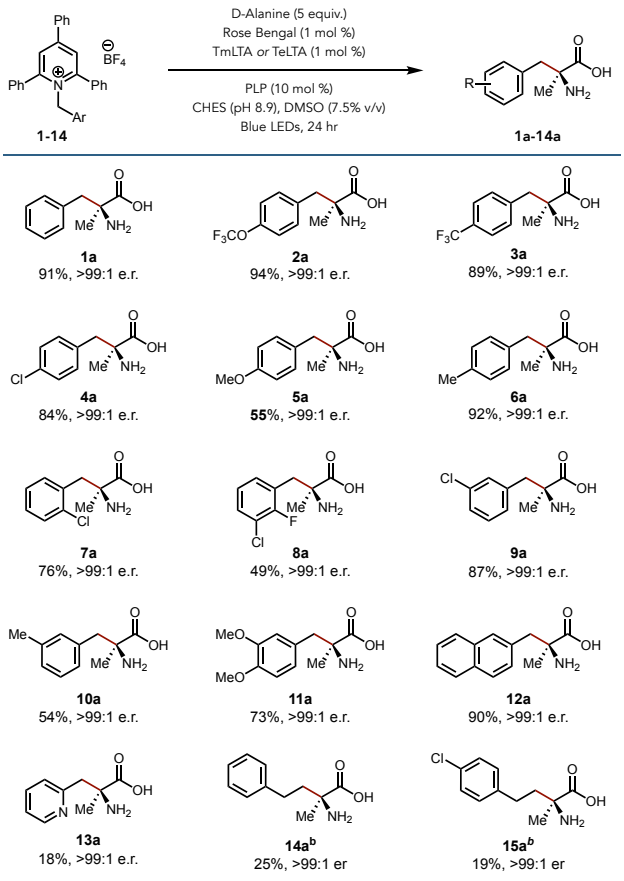


Figure 3. Scope of L-Threonine Aldolase catalyzed non-canonical amino acid synthesis. Reaction conditions: Katritzky salt (2.5 umol 1.0 eq, 5.8 mM), D-Alanine (12.5 umol, 5 eq, 29 mM), TeLTA (1 mol% based on **1**) in 100 mM CHES buffer pH 8.9, with 7.5% DMSO (v/v) as cosolvent, final total volume is 430 µL. Reaction mixtures were stirred under anaerobic conditions at room temperature for 24 h. [a]Yield determined via LC-MS relative to an internal standard (Mandelic acid). Enantiomeric ratio (er) determined by chiral HPLC after Boc_2O protection. [b]Using 5-phenethyl-5H-dibenzo[b,d]thiophen-5-ium trifluoromethanesulfonate instead of Katritzky salt.

With the best conditions identified, we investigated the substrate tolerance of this protocol (Figure 3). Katritzky salts with different *para*-substituents from electron-withdrawing fluoroalkyl groups, such as OCF_3, CF_3, and synthetically useful Cl, were well accepted, giving the desired products in 84-94%

yield with excellent selectivity, with the exclusive formation of the *S*-product (**1a-4a**). Electron-rich substrates provided products in a more modest yield, presumably due to an electronic mismatch between the electron-rich radical and nucleophilic quinonoid (**5a** and **6a**). Sterically demanding *ortho*-substituted arenes and naphthyl pyridinium salts were reactive, affording product in good yield (**7a, 8a, 12a**). *Meta*-position with Cl and methyl led to 37-87% yields in high stereoselectivity (**9a** and **10a**).

This method was effective for preparing the α-tertiary amino acid analog of *L*-DOPA, affording product in 73% yield and 99% enantioselectivity. We also found that a pyridine-derived Katritzky salt was reactive, enabling the facile synthesis of amino acids with electron-deficient *N*-heterocycles and challenging structures to prepare other synthetic methods. Unfortunately, non-benzylic pyridinium salts were unreactive. This limitation was overcome by using sulfonium salts, which afforded a product with moderate yield as a single enantiomer. (**14a** and **15a**). A 2 mmol scale reaction with TeLTA cell-free lysate was conducted with the model substrate **1** and *D*-alanine (Supplementary figures 5 and 6). To ensure the light penetration, we performed the reaction under 525 nm using green Kessil irradiation. The product was isolated in 46% yield, >99:1 e.r., highlighting the potential of our protocol for industrial production

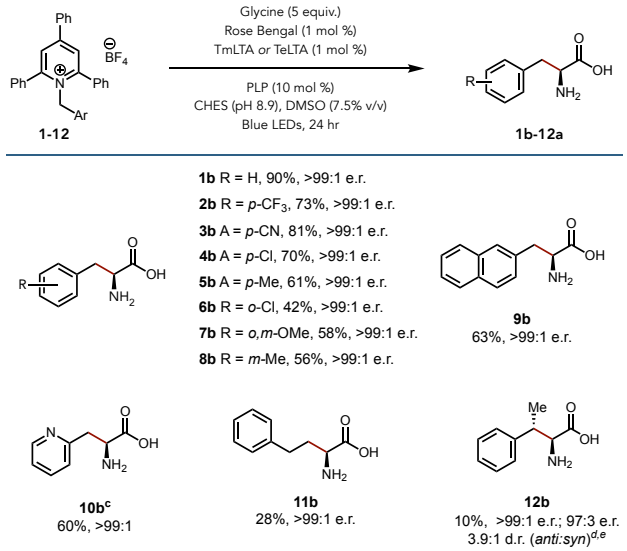


Figure 4. Scope of *L*-Threonine Aldolase catalyzed non-canonical amino acids synthesis. Reaction conditions: Katritzky salt (2.5 umol 1.0 eq, 5.8 mM), glycine (12.5 umol, 5 eq, 29 mM), TeLTA (1 mol% based on **1**) in 100 mM CHES buffer pH 8.9, with 7.5% DMSO (v/v) as cosolvent, final total volume is 430 µL.

Beyond alanine, this biocatalytic system also accepts glycine as a quinonoid precursor. As shown in Figure 4, benzylic Katritzky salts harboring different electronic substituents, such as CF_3 (**2b**), CN (**3b**), Cl (**4b** and **6b**), Me (**5b** and **8b**), MeO (**7b**), and naphthyl (**9b**) were well compatible to afford the desired ncAA products in 42-90% yields and excellent enantioselectivity (>99:1 e.r.). Pyridyl Katritzky salt functioned well with 60% isolated yield, >99:1 e.r. (**10b**). Nonbenzylic sulfonium salt (**11b**), as well as secondary substrate (**12b**) were accommodated to give the corresponding amino acids products with excellent enantioselectivity control, albeit with lower reactivity.

After establishing the synthetic utility of the method, we next conducted experiments to determine the mechanistic nuances of this system. We began by confirming the formation of the quinonoid with glycine and alanine via UV-vis spectroscopy. The quinonoid signal forms immediately upon addition of either amino acid and persists for at least 30 minutes, suggesting this is the steady-state intermediate in these reactions (Supplemental Figure 23). Next, we determined the mechanism of radical initiation. We hypothesized that Rose Bengal's triplet excited state was responsible for reducing the pyridinium salt to form the alkyl radical. Using Stern-Volmer analysis of fluorescence quenching data, we identified a non-linear relationship between pyridinium salt concentration and fluorescence, indicative of a static quenching mechanism (Figure 5A).[36] This observation suggests the cationic pyridinium salt complexes with the dianion Rose Bengal before excitation. Indeed, we observe a red shift in the absorption of Rose Bengal in the presence of **1** (10 nm shift), indicating the formation of a complex. These results indicate that photoinduced electron transfer is responsible for radical formation.

Throughout our studies, we observed negligible formation of bibenzyl due to pyridinium salt dimerization, suggesting that radical formation is controlled and likely occurs near the protein active site. In previous studies, we observed an association between the photocatalysts and oxidoreductases.[24–26] We conducted UV-vis spectroscopy on Rose Bengal with and without TmLTA to determine whether an interaction is present in this system. A red shift in the absorption of Rose Bengal is observed in the presence of TmLTA, indicating that the protein and photocatalyst are associating.[37,38] Fluorescence anisotropy experiments are consistent with a single binding event with K_D = 76 µM for Rose Bengal to the protein (Supplemental Figure 17). Interestingly, when pyridinium salt **1** is added to this mixture, further red shifting of the Rose Bengal absorption is observed with a spectral signature distinct from that of the Rose Bengal/**1** salt complex. Importantly, no change is observed when mixing the pyridinium salt and enzyme, indicating that these two species do not associate with each other without the dye.

Next, we conducted molecular docking to determine possible sites for photocatalyst binding. Previous studies indicate the dianionic Rose Bengal binds to electropositive and hydrophobic regions of proteins.[37,38] Molecular docking using Autodock VINA[39] revealed favorable binding sites in an electropositive region of the protein immediately adjacent to the protein active site. In these binding orientations, the photocatalysts are 6 to 15 angstroms away from the PLP cofactor. With this model in hand, we propose that Rose Bengal templates a ternary complex between the pyridinium salt and enzyme (Figure 5D). Radical initiation events occur more frequently near the quinonoid intermediate, which is formed at high equilibrium concentrations as the photocatalyst preferentially binds adjacent to the protein active site (Supplemental Figure 26). This helps localize the radical formation to the active site, allowing for high-fidelity C-C bond formation over other radical termination pathways. Since only one prochiral face of the PLP intermediate is exposed, the C–C bond is formed with excellent stereocontrol. Once the C–C bond is formed, the resulting radical quinonoid intermediate can be oxidized by the Rose Bengal radical cation to furnish the product external aldimine and Rose Bengal. The product external aldimine can then undergo Schiff base exchange with the active site Lysine 199 to release the product and reform the internal aldimine.

In conclusion, we developed a synergistic photoenzymatic system using *L*-threonine aldolases to prepare unprotected α-tertiary amino acids from pyridinium salts and small amino acids with excellent levels of enantioselectivity. Mechanistic investigations suggested a unique ternary interaction between pyridinium salt, Rose Bengal, and the enzyme that helps localize

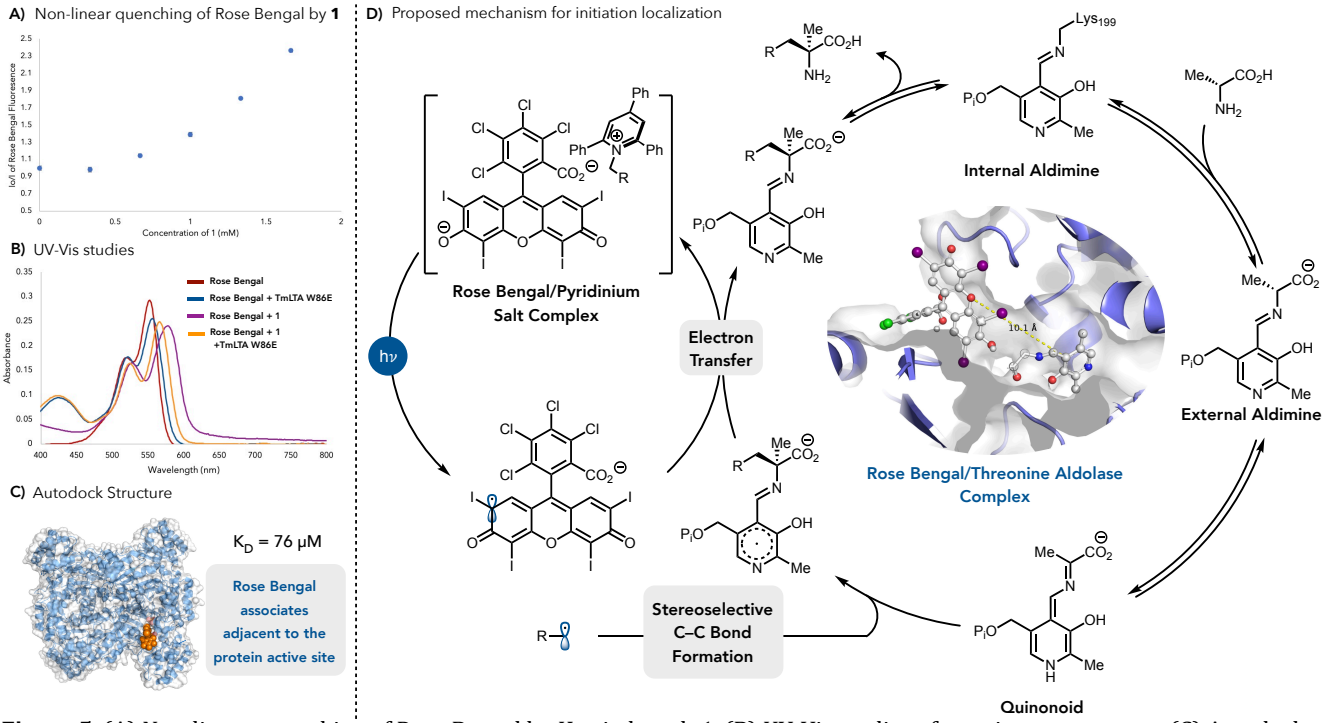


Figure 5. (A) Non-linear quenching of Rose Bengal by Katritzky salt 1. (B) UV-Vis studies of reaction components. (C) Autodock structure of photocatalyst and protein. (D) Proposed reaction mechanism

radical formation to the protein active site. We hypothesize that these interactions can be used with other proteins/photocatalyst combinations to unlock new reactivity.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, NMR spectra, and HPLC traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

The research reported here was supported by the National Institutes of Health National Institute of General Medical Sciences (R21GM146042). The authors would like to acknowledge Venu Vandavasi for assistance running Fluorescence Anisotropy experiments and Eli McAmis for assistance with docking. C.G.P. acknowledges the NSF-GRFP and Eli-Lilly Ted Taylor Fellowship for support. We thank Joshua Turek-Herman for the preliminary experiments.

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