Directed Evolution

Engineered Biocatalytic Synthesis of β-N-Substituted-α-Amino Acids

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Abstract: Non-canonical amino acids (ncAAs) are useful synthons for the development of new medicines, materials, and probes for bioactivity. Recently, enzyme engineering has been leveraged to produce a suite of highly active enzymes for the synthesis of β-substituted amino acids. However, there are few examples of biocatalytic N-substitution reactions to make α,β-diamino acids. In this study, we used directed evolution to engineer the β-subunit of tryptophan synthase, TrpB, for improved activity with diverse amine nucleophiles. Mechanistic analysis shows that high yields are hindered by product re-entry into the catalytic cycle and subsequent decomposition. Additional equivalents of L-serine can inhibit product reentry through kinetic competition, facilitating preparative scale synthesis. We show β-substitution with a dozen aryl amine nucleophiles, including demonstration on a gram scale. These transformations yield an underexplored class of amino acids that can serve as unique building blocks for chemical biology and medicinal chemistry.

Introduction

Non-canonical amino acids (ncAAs) are useful tool compounds for studying biological phenomena and are components in many pharmaceuticals.[1–3] Altering the side chain structure of α-amino acids can impart new properties on compounds as diverse as small molecule catalysts and peptide therapeutics.[4,5] In particular, bioactive molecules featuring β-N-substitution includes intermediates in biosynthesis,[6] antibiotic analogs of azafuranomycin[7] and aspergillomarasmine A,[8] and protein kinase inhibitors (Figure 1A).[9] Synthesis of ncAAs in high yield and stereocchemical purity is a historical chemical challenge that researchers approached through a variety of methods[10–12] including chiral auxiliaries[13] and asymmetric catalysis.[14,15] Traditional synthetic methods typically rely on protecting group manipulations to attenuate undesired reactions with the amine and carboxylic acid functionalities and must assiduously avoid epimerization of the chiral center.[16,17] More recently, biocatalytic systems have been leveraged to synthesize ncAAs that avoid protecting group manipulations and a variety of C–C bond forming transformations have been developed (vida infra).[18–27] Despite these successes, methods that yield N-containing sidechains have been limited. The potential reversibility of C–N bond formation...
presents a distinct challenge and, if it were overcome, would enable access to new classes of densely functionalized ncAAs.

A variety of enzymes have been identified for the synthesis of ncAAs.[20,26] The β-subunit of the tryptophan synthase complex (TrpB) has proven to be a versatile platform for synthesis of β-substituted amino acids.[26,29] Previous engineering efforts have increased the activity of TrpB when it is expressed independently of its larger complex.[32,30,31] These lineages of standalone TrpB enzymes have served as parents for subsequent evolution to generate a variety of C–C bond forming catalysts that access a range of complex Trp analogs.[26,29] Each of these engineered transformations rests on the same mechanistic principles that were elaborated for the native Trp synthase complexes from *Escherichia coli* (EcTrpS) and *Salmonella typhimurium* (StTrpS).[42–46] Recent studies have confirmed the locations of protons during intermediate steps in the catalytic cycle using a combination of high-resolution X-ray crystallography and NMR spectroscopy, leading to a unified view of the native TrpB mechanism (Figure S1).[42–45] For this study, the key step is a nucleophilic attack into an electrophilic amino acrylate intermediate (Figure 1C). Engineered enzymes catalyze electrophilic aromatic substitution of indoles and rely on the active site to control the chemo- and regioselectivity of the alkylation. Deprotonation of the resultant sigma complex restores aromaticity and serves as a thermodynamic sink for the reaction. Similar principles operate for TrpB variants that act on β-branched γ-Serine (Ser) analogs[46,47] as well as non-indole C-nucleophiles such as oxindoles, azulene, and nitroalkanes.[48–50]

While the C-alkylation reactions of TrpB have been well-behaved, many perplexing and seemingly contradictory features have accumulated while studying the N-alkylation properties of TrpB enzymes. N-substitution was first demonstrated during the investigation of StTrpS. With wild-type protein, reactions with indoline generate a quinonoid adduct that proved sufficiently stable for structural characterization.[45] Mutation at a second-sphere residue from the active site, βD305A, enabled turnover to form 2,3-dihydroxy-γ-tryptophan (DIT) although synthetic utility was not explored.[30] Serendipitously, the TrpB homolog from *Pyrococcus furiosus* (PfTrpB) natively turns over both indoline and indazole.[32] As TrpB evolved to operate with β-branched substrates, it was observed that N-substitution activity decreased, despite the higher nucleophilicity of indolines. Resolving these ambiguities may address longstanding questions about TrpB reactivity and have practical ramifications for synthesis of α,β-diamino acid products. Traditional synthetic methods to access these compounds are well-developed, but typically rely on protecting group manipulations and chiral auxiliaries to control selectivity.[53,54] A biocatalytic route would enable streamlined synthesis and afford the possibility of cascade catalysis. We therefore sought to engineer PfTrpB enzymes for improved N-alkylation and to study what features lead to improved synthetic utility to access these desirable products.

### Results and Discussion

**PfTrpB lineage N-alkylation activity and observations**

We began by surveying a subset of previously engineered PfTrpB variants (reviewed by Almhjell P. et al.[28]) to identify a starting point for directed evolution of N-alkylation activity. Indoline was selected as a model N-alkylation substrate. We tested enzymatic β-substitution with both Ser and γ-Threonine (Thr) using high substrate concentrations to mimic preparative scale conditions (Figure S2). We observed that the evolution for stand-alone activity had no significant effect on reactivity with Ser and indoline (1a). All enzymes were at least 10-fold less reactive with Thr, although activity on this substrate improved steadily through the lineage that led to PfB9. This enzyme has eight mutations, none of which are in the active site, and spectroscopic properties that closely mimic the native PfTrpS complex.[23,55] Previously evolution for improved C-alkylation of Thr led to the variant PfT7E5, but this enzyme was noticeably less efficient at N-alkylation with Ser and Thr.[56] Although PfB9 yields less DIT than Pf0B2, this activity is partially offset because PfB9 expresses at a higher titer than Pf0B2 (Figure S3).[46,52] Further, PfB9 is a well-characterized variant with multiple solved crystal structures, as well as kinetic and spectroscopic data that enable analysis of the differences between C- and N-alkylation.[55] Therefore, we selected PfB9 as the parent for directed evolution for improved N-alkylation activity.

A striking observation while assessing N-alkylation was the relative stability of DIT and β-Me-DIT to reaction conditions. Whereas DIT persisted up to 24 h, the relatively small amount of β-Me-DIT that was formed decomposed overnight (Figure S2B). We hypothesized that the product might be breaking down through a competing β-lyase pathway (Figure 2A). Whereas C-alkylation has a strong driving force that limits elimination of indole, protonation of...
indoline does not break aromaticity and generates a good leaving group for elimination to re-form the amino acrylate E(A–A) intermediate, which is prone to transamination with K82, followed by irreversible hydrolysis yielding pyruvate. To study this lyase reaction, we isolated DIT and resubjected purified product to reaction conditions with P2B9 in the presence and absence of Ser and Thr (Figure 2B, S5). These data showed that DIT rapidly decomposes, but that Ser is an effective competitive inhibitor for the DIT-lyase reaction. We considered the role of Thr competing for the active site with β-MeDIT, however the low yields for this reaction precluded direct analysis. Instead, we measured the effect of Thr on inhibiting the breakdown of DIT and found that Thr does not exert the same kinetic competition that Ser does, which explains why past studies had difficulty accumulating and isolating β-alkylated N-alkylated amino acids.[23,47] Reactions can be driven to modestly higher yields with 7.5 equivalents of amino acid donor (Figure S5), but such conditions are not ideal for practical implementations.

To further understand the challenges associated with N-alkylation, we considered the extent to which the active site of TrpB is predisposed through its history in natural evolution to promote C-alkylation. In the absence of an enzyme, indoline is an intrinsically more potent nucleophile than indole. We assessed the impact of the active site on chemoselectivity by each nucleophile to directly compete for β-substitution. Indole and indoline are sterically similar and nucleophilic attack generates intermediates with similar sp³ hybridization. Nevertheless, P2B9 had a strong preference for C-alkylation of indole over N-alkylation with indoline, at least 250-fold (Figure S6). These results highlight the evolutionary legacy of the TrpB enzymes and the challenges associated with engineering enzymes to engage in new modes of reactivity.

PfTrpB engineering

Information from previous directed evolution campaigns with PfTrpB represents a rich source of potentially activating mutations. We designed a recombination library that simultaneously mutagenized seven positions in or near the active site but restricted the sequence space to 2–4 possible mutations that were activating in other contexts (Table S1, Figure S7). We screened for improved activity with Thr and indoline and found a single point mutation, H165F, that conferred a modest 2-fold increase in activity (Figure S7, S10). We were initially surprised not to observe activating variants containing the L161 V mutation, which was previously shown to create more space for the β-Me group of Thr.[47] We expressed and purified the 2B9-L161 V variant and found that it was indeed a deactivating mutation for β-N-alkylation (Figure S8). This result buoyed our confidence that screening data were accurate and that, contrary to our initial hypothesis, recombination of mutations that are activating for C-alkylation does not enrich in sequences that are proficient at N-alkylation.

At this stage, even concerted effort to conduct preparative scale reactions with Thr and indoline failed to generated product in sufficient yields and we shifted focus to β-substitution with Ser. In this reaction, the 2B9-I165F variant was still activating and had a smaller 1.6-fold effect (Figure 3). For the next round of evolution, we targeted the residue His275, which is a second sphere residue previously found to have a profound effect on the nucleophile specificity of TrpB, including C vs N-alkylation.[57] To capture the effects of the mutation on multiple potential N-nucleophiles, we used substrate-multisplexed screening (SUMS).[57,58] This technique places multiple substrates in direct competition and can read out on both overall catalyst activity and shifts in specificity that are otherwise hidden to single-substrate screening.

We assayed the 2B9-I165F variant with a mixture of anilines, morpholine, indoline, and indazole and adjusted substrate concentrations so that all products were observable (Figure 3A, see Supporting Information for additional details on assay design).

SUMS of the His275 site-saturation library showed a clear trend wherein larger aromatic residues were activating for N-alkylation (Figure S9). A benefit of the SUMS method is that we observed that this increase in activity was general to all nucleophiles present in the mixture, limiting concerns about generating an overly specific catalyst. His275Trp was the most activating mutation, corresponding to a ≈6-fold

Figure 3. Engineering PfTrpB for N-alkylation. A) Substrate mixture and corresponding PfTrpB generated ncAAs. B) PfTrpB engineering results. The left vertical axis gives the total product formed in fold-activity relative to P2B9 (diamond). The right vertical axis corresponds to the relative abundance of each product. PfTrpB (3.2 μM), indoline and indazole (1 mM), aniline (10 mM), N-Et aniline (10 mM), morpholine (10 mM), Ser (100 mM), 10 % DMSO, KPi buffer (200 mM), pH = 8.0, 75 °C, and 30 min. Reactions conducted in triplicate. C) Crystal structure of Pf2B9 with active site residues in gray (PDB : 6AM8) superimposed with the DIT quinonoid (E(Q)) from StTrpS (PDB : 3CEP) in cyan. Atoms are colored so that nitrogen is blue, oxygen is red, and phosphorus is orange.
increase in activity compared to parent (Figure 3B). With two steps of directed evolution, we identified a double variant P2B9-I169F-H275W, hereafter called PfA04, with improved N-alkylation activity.

Characterization of PfA04

To understand how the identified mutations impacted catalysis, we studied the reactivity of indole and DIT with PfA04. We compared progress curves between P2B9 and PfA04 for the β-N-substitution with indole. These data showed that PfA04 is indeed a faster catalyst and reactions go to higher conversion than for P2B9 (Figure 4B). However, DIT persisted for less time, indicating that the protective effect of Ser was diminished for PfA04. We measured Michaelis–Menten parameters for β-N-substitution with indole. For the parent P2B9, the reaction proceeded with a $k_{\text{cat}}$ of 0.5 s$^{-1}$, which is 2-fold less than the $k_{\text{cat}}$ for β-substitution with indole.$^{[55]}$ However, these reactions differ dramatically in their $K_M$, which is at least 2,000-fold higher for indole than for indole. After directed evolution, P2A04 had an apparent 22-fold boost in catalytic efficiency for β-substitution with indole. This increase stems from a 6-fold decrease in $K_M$ for indole and a 3.6-fold increase in $k_{\text{cat}}$ (Figure 4C, Figure S11). To directly observe changes in substrate specificity, we again performed competition experiments between indole and indole and measured the ratios of C- vs N-alkylation products. This experiment showed that P2A04 is still specific for indole, by ≈140-fold (Figure S12). To further probe the mechanism of P2A04, we turned to UV/Vis spectroscopic analysis.

The PLP cofactor is a convenient spectroscopic probe for the examination of mechanistic intermediates in the P2TrpB catalytic cycle. In its resting state, P2TrpB variants display an absorbance peak at 412 nm (Figure S13), which corresponds to the absorbance of the internal aldime E(Ain). Addition of Ser results in β-elimination to form an amino acrylate, E(A–A), that absorbs at 350 nm (Figure S13A). This intermediate can decompose through a hydrolysis pathway, resulting in formation of pyruvate and ammonia (Figure S13C). Previously, directed evolution for improved C-alkylation with attenuated electrophiles or hindered electrophiles was correlated with a decrease in the rate of the hydrolysis reaction.$^{[36,47]}$ We observed the opposite rate effect with P2A04. That is, in the absence of a nucleophile, the rate of hydrolysis increased ≈6-fold to 34 min$^{-1}$.

Upon addition of indole to E(A–A) preformed on P2A04, there is a rapid spectroscopic change and accumulation of a broad absorbance around ≈430 nm and a sharp feature at 465 nm. We attribute the longer wavelength species to the previously characterized E(Q343) and multiple species may be contributing to the shorter bands.$^{[40,59]}$ We measured a progress curve under the same conditions as used for spectroscopic analysis and observed a tight correlation between a decrease in the E(Q343) band over time and an increase in concentration of DIT (Figure 4D). This relationship indicates that E(Q343) is not a thermodynamically stable adduct, otherwise it would increase in population as the concentration of catalysis increases. Instead, we hypothesize that E(Q343) is formed as a steady-state intermediate that accumulates prior to some rate-limiting step. In this scenario, the loss in E(Q343) absorbance is due to the decrease in active site occupancy of indole, which has a relatively high $K_M$ (4.5 mM) and is being consumed in the reaction. This hypothesis is consistent with analysis of P2B9. This parent enzyme has a larger accumulation and persistence of E(Q343), which has a slower rate of catalysis (Figure S14). Together, these data suggest that the increased rate of catalysis for P2A04 is attributed to an increase in the rate of either protonation of E(Q343) to form an external aldime or release of the amino acid. This hypothesis is in line with studies of S1TrpS, which showed that protonation of E(Q343) was exceptionally slow and responsible for the previous intractability of N-alkylation.$^{[45,51,59]}$
**Scope of PfTrpB N-alkylation**

We last explored the synthetic utility of the PfA04 enzyme. Synthetic methods are of highest utility when substrate concentrations are high, which also affords the possibility that products with poor solubility may precipitate from reactions and limit product reentry. Correspondingly, we conducted preparative scale reactions with >100 mM nucleophile and used two equivalents of Ser, which serves both as a substrate for productive catalysis and as a competitive inhibitor for product breakdown.

As PfA04 was highly active with indolines, we conducted reactions with relatively dilute catalyst (0.013 mol %). A large-scale reaction with 140 mM indoline 1a gave 1.18 g of DIT 1b in 82 % isolated yield (Figure 5A). Owing to high-concentration conditions, precipitation was observed within the first hour, minimizing product re-entry into the active site. Reactions with the 5-fluoro analog 2a proceeded smoothly and gave 2b in good yield, 58 %, while the 5-Cl analog 3b gave 3b in 34 % yield. Substrates with electron-donating groups at the 5- and 6-positions (4a and 5a, respectively) afforded product in modest yields (54 % 4b and 31 % 5b) with 0.013 mol % catalyst. Utilizing a racemic mixture of 2-methyl analog 6a gave 6b in a 50 % yield. Surprisingly, $^1$H NMR showed no evidence of diastereoselectivity for this transformation.

The sterically demanding substrates 3-spirocyclopropyl indoline 7a and tetrahydroquinoline 8a reacted less efficiently and reactions used an increased catalyst loading (0.05 mol %) to obtain isolable products, which were low.

![Figure 5. Substrate scope for the engineered TrpB catalyst, PfA04. A) Reaction conditions are provided, and catalyst loading was varied according to the relative efficiencies of the reactions. B) Oxidation of 1b leads to N-isotryptophan, 1c. C) Cascade reaction of PfA04 with the H120N variant of the Trp decarboxylase from Ruminococcus gnavus, RgnTDC_H120N. The decarboxylase intercepts the amino acid 8b and produces high yields of the corresponding amino acid, out-competing depletion of 8b through the β-lyase reaction.](image-url)

Throughout past studies with P/TrpB variants, indazole 9a has been a well-behaved substrate. The variant P/A04 maintains high productivity and we isolated the corresponding product 9b in 86% yield with just 0.025 mol% catalyst. Previous studies, however, have not measured activity with substituted indazoles which we were surprised to find were less-reactive. The 5-hydroxy analog 10a reacted slowly, but we were able to obtain 10b in 22% yield using 0.05 mol% catalyst. As with reactions with indoline, reactions with 10a were limited by product re-entry, as indicated by a decrease in product concentrations over time, even with five equivalents of Ser.

We surveyed a variety of additional classes of N-nucleophiles and found that the active site was highly sensitive to subtle changes in substrate structure. Whereas indoline reacted very efficiently, N-ethyl-aniline reacted poorly, and the product was only detected on analytical scale (Figure S15). Aniline 11a and p-anisidine 12a reacted slowly, but the products did not effectively re-enter the catalytic cycle under the conditions used for preparative scales. Consequently, we were able to isolate products 11b and 12b in high yield. Activity on other heterocycles, such as morpholine, was consistently low, as was propargyl amine. In these cases, isolation of the anticipated products was not pursued due to the low conversion of starting material and difficulties associated with purification of dilute, polar amino acids. Isolation of the metal chelating amino acid 13b from reactions with 8-amino-quinoline 13a was straightforward and gave a modest yield of 42% at 0.05 mol% catalyst. While assaying substituted indolines, we found that the electron-rich 6-hydroxy-indoline 14a reacted efficiently but did not yield an N-alkylation product. Instead, 14b was isolated for which 1H NMR analysis revealed a change in regioselectivity corresponding to C-alkylation at C5. Alkylation of a 6-membered aromatic ring has not been previously reported for TrpB enzymes. Reactions with relatively high catalyst loadings maintain their synthetic tractability due to the high soluble expression of the catalyst, approximately 500 mg purified protein L-1 culture.

We last considered downstream synthetic utility of Pf/A04 and the products described here. We assessed product stability by storing αβ-diamino acids for 3 months at room temperature in NMR-tubes and re-analyzed the products to look for evidence of decomposition. DIT and 9b remained stable after 3–5 months of storage at ambient temperature inferred by 1H NMR. Oxidation of DIT could be promoted with dichlorodiacyanouquinine (DDQ). Reaction with isolated DIT proceeded smoothly to give the N-iso-tryptophan (Figure 5B). We note that the isolation of this material was quite cumbersome, and a small portion of Boc-protected material was isolated to confirm product identity. Last, we considered the utility of P/A04 alkylation transformations in a cascade context. Even reactions with low yield might be useful in a cascade context, where product re-entry into the catalytic cycle can be limited by modification with a downstream enzyme. We demonstrated this scenario by reacting 8a with P/A04 and an engineered Trp decarboxylase, RgTrDC_H120N, and generated the corresponding tryptamine analog 8c in high yield, albeit with a challenging isolation (Figure 5C). In the future, these isolations would benefit from additional synthetic effort for repeated use. Protecting groups could be chosen for complementarity or, as we have shown here, these products can be used immediately in a cascade setting in their unprotected form.

### Conclusion

In this study, we engineered the β-subunit of tryptophan synthase from Pyrococcus furiosus for efficient β-N-substitution. These N-nucleophiles were previously used as inhibitors of TrpB enzymes. We performed directed evolution for increased activity on a mixture of N-nucleophiles resulting in general improvements to catalytic activity. Mechanistic analysis showed how two mutations accelerate the proteolysis and release of the model amino acid DIT. Preparative scale reactions were often driven by the selective precipitation of products, which enabled gram-scale synthesis of DIT. This reaction adds to a growing repertoire of N-alkylation reactions in biocatalysis, which operate on their own to access desirable products and are enabling transformations for complex cascade catalysis.

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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 from the corresponding author upon reasonable request.

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Engineered Biocatalytic Synthesis of \( \beta \)-N-Substituted-\( \alpha \)-Amino Acids

The \( \beta \)-subunit of tryptophan synthase was engineered for efficient \( N \)-alkylation to access densely functionalized non-canonical amino acids. Mechanistic analysis guided preparative scale synthesis, adding a valuable new enzyme to the biocatalytic toolbox.