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REVIEW

Tryptophan Synthase: Biocatalyst Extraordinaire

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Abstract Tryptophan synthase (TrpS) has emerged as a paragon of noncanonical amino acid (ncAA) synthesis and is an ideal biocatalyst for synthetic and biological applications. TrpS catalyzes an irreversible, C–C bond forming reaction between indole and serine (Ser) to make L-tryptophan (Trp); native TrpS complexes possess fairly broad specificity for indole analogs, but are difficult to engineer to extend substrate scope or to confer other useful properties due to allosteric constraints and their heterodimeric structure. Directed evolution freed the catalytically relevant TrpS β-subunit (TrpB) from allosteric regulation by its TrpA partner and has enabled dramatic expansion of the enzyme's substrate scope. This review examines the long and storied career of TrpS from the perspective of its application in ncAA synthesis and biocatalytic cascades.

1. Introduction

Noncanonical amino acids (ncAAs) enable researchers to interact with and modify life at the molecular level and are a vital tool for many modern biological studies. Defined as amino acids that are not genetically encoded, ncAAs bear chemical motifs not found in the 20 canonical amino acids and can alter the characteristics of molecules that incorporate them. Though often referred to as unnatural amino acids, many ncAAs do occur naturally as post-translationally modified peptide residues or as intermediates in biosynthesis of secondary metabolites.[1] Nature thus demonstrates that ncAAs can serve as handles to manipulate biochemical properties. Furthermore, substituting canonical amino acids with ncAAs imbues molecules with different functionalities while minimally perturbing structure.[2] As such, ncAAs are seeing growing applications in research, where they are useful as biophysical probes,[2,3] are introduced into polypeptides to create improved or entirely new functions,[4,5] and are incorporated into bioactive small molecules and peptide therapeutics.[6–8]

A barrier to realizing the potential of ncAAs is that they are challenging to synthesize owing to laborious protection and deprotection sequences necessary to prevent epimerization of the chiral center or undesired reactivity with the amine and carboxylate groups.[9] Simpler, more effective, and more direct routes to ncAAs are necessary to better harness their potential applications. A promising approach for ncAA synthesis is to use enzymes, which can perform transformations with exquisite precision in the presence of multiple reactive centers without the need for protecting groups. With carefully tuned active sites, enzymes can overcome regio- and stereoselectivity challenges by directing substrates and reactive intermediates during a catalytic cycle. Another major advantage of enzymes is that they can be combined in one-pot biocatalytic cascades to access value-added products from simple and inexpensive starting materials.[10] However, enzymes from biosynthetic pathways to naturally occurring ncAAs may not be practical to engineer or scale up if, for example, they catalyze reversible reactions, express poorly in recombinant hosts, are allosterically regulated, or have limited substrate scopes.[11] Nevertheless, directed evolution has empowered biocatalysis to be well poised to contribute to ncAA synthesis, and examples of new, engineered ncAA synthases and enzyme cascades are emerging.[11]

Tryptophan synthase (TrpS) is a premier example of an enzyme that can be used for scalable ncAA synthesis. TrpS possesses covetable qualities for an ncAA synthase: it forms a C–C bond between readily available starting materials to make L-tryptophan (Trp) and closely related derivatives in a single enzymatic step.[12] TrpS exists as a heterodimeric complex comprised of two α- and β-subunits (TrpA and TrpB, respectively) that work together to transform indole glycerol phosphate (IGP) and L-serine (Ser) into Trp (**Scheme 1**). The TrpA subunit is responsible for the cleavage of IGP into indole and glyceraldehyde, and does not directly participate in the C–C bond forming step. In fact, TrpA can be bypassed entirely by providing indole analogs to the enzyme complex, where they are transformed by the TrpB subunit into Trp analogs. Although TrpS can be used to synthesize a variety of Trp-based ncAAs, directed evolution of the catalytically relevant TrpB subunit to create a stand-alone enzyme dramatically simplified engineering efforts and allowed for a systematic expansion of accessible ncAA products.[13]

This review provides an overview of how TrpS and its laboratory-evolved TrpB progeny have been used to produce ncAAs. We also give examples of how this enzyme has been incorporated in biocatalytic cascades to access D-amino acids and tryptamine products.

1.1. Properties of tryptophan synthase (TrpS)

Tryptophan synthase is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that has captured the interest of enzymologists and bioengineers for over half a century. The study of TrpS dates back to when the burgeoning field of molecular biology had barely taken its first steps. Discovered in the 1940s, TrpS has served as a model enzyme for a wide range of investigations, from proving gene-protein collinearity[14] to studying the evolution and nature of allostery,[15] conceptualizing and understanding vectorial catalysis and substrate

channeling,[16] and, most relevant to this review, synthesis of ncAAs.

TrpS is found in all domains of life as an αββα heterodimeric complex that catalyzes the formation of Trp from IGP and Ser (**Scheme 1**). The α-subunit (TrpA) and β-subunit (TrpB) experience mutual allosteric activation, the evolutionary history and nature of which are still an active area of research.[17,18] The two subunits interact with one another through rigid-body motion of the TrpB communication (COMM) domain and a monovalent cation (MVC) binding site within TrpB. When IGP binds TrpA, it initiates a conformational change activating TrpB to promote formation of the (PLP)-bound amino-acrylate derived from Ser. The TrpB subunit then reciprocally stimulates TrpA to induce retro-aldol cleavage of IGP, releasing indole.[19] Once released, indole diffuses along a 25-Å long tunnel to the β-subunit where it can immediately participate in a PLP-mediated β-addition reaction, releasing water and Trp.

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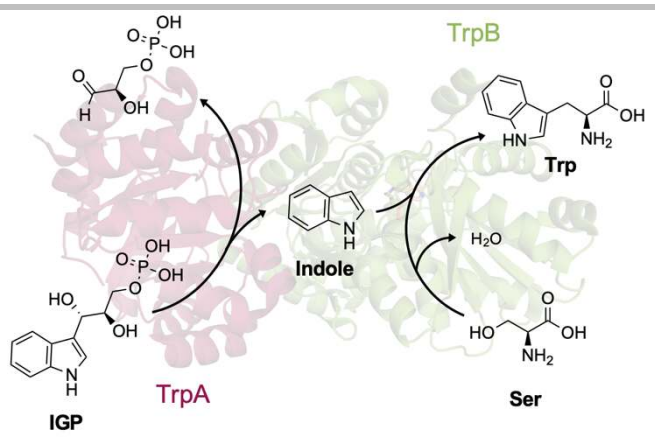
Frances Arnold is the Linus Pauling Professor of Chemical Engineering, Bioengineering and Biochemistry at the California Institute of Technology. She was awarded the 2018 Nobel Prize in Chemistry for her pioneering work in directed enzyme evolution.



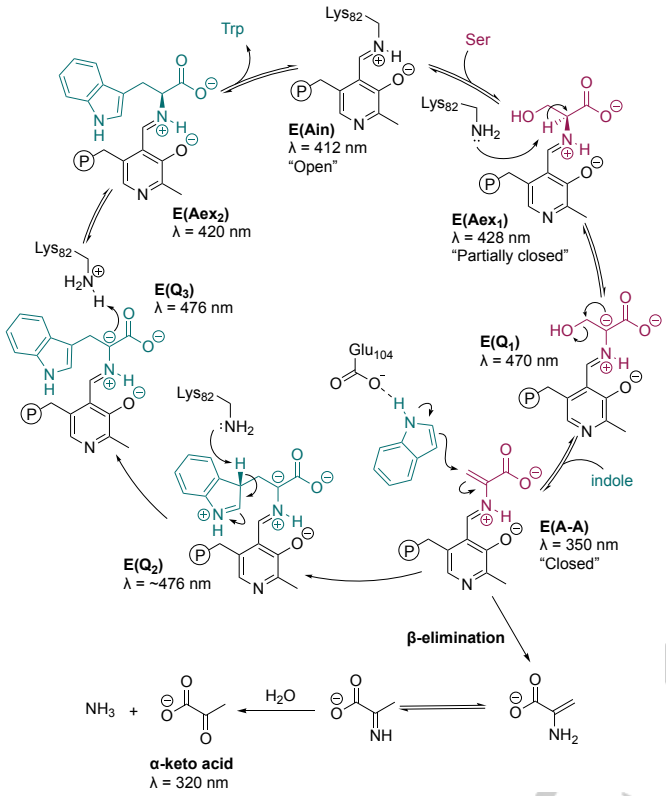
Scheme 1. Native transformation catalyzed by TrpS. TrpA (left, pink) performs a retro-aldol cleavage on indole glycerol phosphate (IGP) releasing indole and glyceraldehyde phosphate. Indole diffuses to the TrpB subunit (right, green) which catalyzes a PLP-mediated β-substitution reaction between indole and L-serine (Ser), releasing water and L-tryptophan (Trp). (PDB:5E0K)

The TrpB PLP cofactor absorbs in the UV-vis region, and each reactive intermediate possesses a characteristic spectral trace, allowing observation of the catalytic cycle via UV-vis spectroscopy. In the TrpB resting state, PLP is covalently bound to the ε-nitrogen of a lysine residue (K82, *Pyrococcus furiosus* TrpB, *Pf*TrpB, numbering) through a protonated Schiff-base linkage referred to as the internal aldimine, **E(Ain)** (λ_{max}= 412 nm).[20] In the first stage of the catalytic cycle (**Scheme 2**), Ser enters the active site and replaces the lysine via transimination to form an external aldimine intermediate, **E(Aex$_1$)** (λ_{max}= 428 nm). This step is concomitant with a rigid-body conformational change in the TrpB COMM domain, with the enzyme adopting a 'partially closed' state.

PLP-dependent enzyme specificity is largely dependent on alignment of the bond to be broken with the π molecular orbital system of PLP.[21–23] TrpB promotes Cα deprotonation by using a hydrogen bonding network formed with the Ser carboxylate that locks the C–H bond periplanar to the PLP π system.[23] The free K82 residue deprotonates the Cα of Ser, ablating the chiral center and forming a carbanion that is delocalized by the PLP cofactor to form a quinonoid intermediate, **E(Q$_1$)** (λ_{max}= 470 nm).[24] Subsequent elimination of the hydroxyl group forms the electrophilic amino-acrylate species, **E(A-A)** (λ_{max}= 350 nm), which is poised for attack by the indole nucleophile. During this step, the COMM domain assumes a 'fully closed' conformation that is stabilized by TrpA.[17,25] If no indole is present, a kinetically competing transimination reaction with the active site lysine can occur, releasing dehydroalanine that hydrolyzes to form ammonia and pyruvate (**β-elimination pathway**).[26] If indole is present, it arrives in the active site of TrpB and is positioned by the catalytic glutamate (E104, *Pf*TrpB numbering) for nucleophilic attack. The catalytic glutamate is important for controlling the regioselectivitiy of the reaction; mutagenesis reveals its crucial role to effect C–C bond formation at C_3 over a C–N bond at N_1.[27]

The beginning of the second stage of the TrpB catalytic cycle is marked by irreversible nucleophilic attack by indole on the **E(A-A)** to form a second quinonoid intermediate, **E(Q$_2$)** (λ_{max}= ~476 nm). The (S)-indolenene species is quickly deprotonated, restoring aromaticity, to reach a third and final quinonoid intermediate, **E(Q$_3$)** (λ_{max}= 476 nm).[28] Cα is then re-protonated

by K82 stereospecifically to form the Trp-bound external aldimine, **E(Aex$_2$)** (λ_{max}= 420 nm), re-establishing the chiral center and completing Trp formation.[29,30] Trp release from the enzyme via transimination by K82 returns PLP to the **E(Ain)** resting state and completes the catalytic cycle.[30]



Scheme 2. Catalytic cycle of TrpB.

2. Synthesis of noncanonical Trp derivatives

Tryptophan derivatives (**Scheme 3**) are a subclass of ncAAs that have been used extensively as probes for chemical biology. Trp itself is a major source of UV absorption and fluorescence in proteins, and its spectral properties, which are highly influenced by the surrounding environment, have been leveraged to study protein dynamics, folding, and ligand binding.[31] Substitutions on the indole moiety, such as in 4-cyanoTrp and 5-hydroxyTrp, as well as Trp isosteres like azaTrps (**1**, **2, 3**, **4**) can enhance or alter these spectroscopic properties to exhibit higher quantum yields or shift excitation/emission spectra.[32–34] Decorations and substitutions on the indole side chain bestow many other useful biochemical properties: fluorinated Trps are used in ^{19}F NMR studies,[35] selenophene and thienyl functional groups are used for phasing crystallographic structures,[36] and halides can allow for site-specific modification through palladium-catalyzed coupling reactions with alkenes and alkynes.[37] Like many other ncAAs, Trp derivatives are biosynthetic precursors to compounds that exhibit diverse pharmacological activities, including anticancer, antibiotic, immunosuppressant, and phytotoxic properties.[38,39]

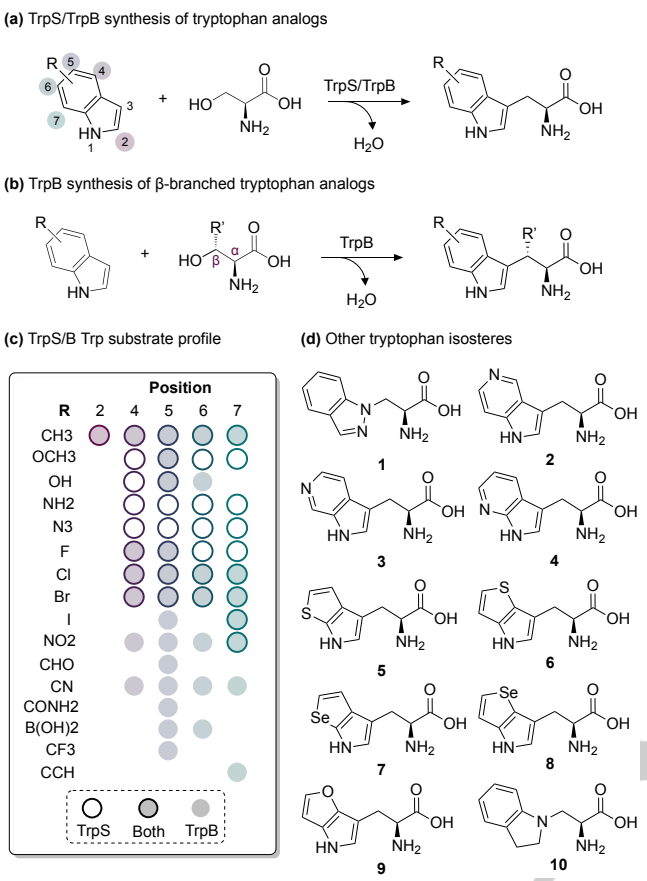
Shortly after the discovery of TrpS, researchers began using substituted methylindoles (2-, 4-, 5-, 6-, 7-CH$_3$-indole) to gain insights into the enzyme's mechanism and pathway regulation.[29,40,41] The synthetic utility of TrpS, however, was first realized in 1974 when Wilcox synthesized a series of Trp derivatives (5-F-, 6-F-, 5-OH-, 5-MeO-, 6-MeO-, 2-CH$_3$-, 5-CH$_3$-, 7-CH$_3$-Trp; **1**, **4**) from Ser and indole analogs using TrpS from *Escherichia coli* (*Ec*TrpS).[42] During the following decades, various wildtype TrpS homologs were shown to have activity on a number of other decorated indoles and indole isosteres: Saito et al. demonstrated the synthesis of azido-substituted Trps (4-, 5-, 6-, 7-N$_3$-Trp) using a TrpS from *Neurospora crassa*;[43] the Phillips group applied TrpS from *Salmonella typhimurium* for the synthesis of chloroTrps (4-, 5-, 6-, 7-Cl-Trp),[44] sulfur, selenium, and oxygen-containing Trp isosteres (**5, 6, 7, 8, 9**),[45–47] as well as azaTrps (**1**, **2, 3**, **4**);[48] Goss and colleagues prepared an exceptionally diverse set of substituted Trp analogs including methyl- (2-, 4-, 5-, 6-, 7-CH$_3$-Trp),[49] amino- (4-, 6-, 7-NH$_2$-Trp),[50] halo- (4-, 5-, 6-, 7-F; 4-, 5-, 6-, 7-Cl; 5-, 6-, 7-Br; 7-I-Trp),[49–52] and nitroTrp (7-NO$_2$-Trp)[52] using TrpS from *E. coli* and *Salmonella enterica*. TrpS has also been found to catalyze a C–N bond forming reaction with indoline to form dihydroisotryptophan (**10**).[27,53,54]

Despite this ability to produce desirable compounds, TrpS still has limitations that restrict practical and widespread use. Even under optimized conditions, ncAA yields with TrpS catalysts are typically under 50%.[49,52] Insolubility of indole compounds in water also limits the substrate concentrations. Although this was partially remedied by addition of co-solvents, the TrpS homologs used lacked solvent tolerance, limiting the effectiveness of this solution.[44] Furthermore, extensive engineering of the TrpB subunit for improved activity or expanded substrate scope was impeded by the need for the TrpA subunit, which does not directly participate in the coupling of indole and Ser but nonetheless increases metabolic load on host cells. Unfortunately, without co-expression and allosteric activation from their corresponding TrpAs, native TrpBs lose most of their activity, rendering them all but useless.[30,55,56]

2.1 Engineering a stand-alone TrpS β-subunit (TrpB)

In 2015, Andrew Buller's team engineered the wild-type TrpB subunit from the hyperthermophilic archaeon *Pyrococcus furiosus* (*Pf*TrpB^{WT}) using directed evolution to recapitulate allosteric activation without TrpA.[56] Using a TrpB derived from a hyperthermophilic organism enabled implementation of a heat-lysis pre-treatment, which selects against enzyme variants that have acquired destabilizing mutations, therefore maintaining enzyme stability over multiple rounds of evolution. Thermostability also simplifies enzyme purification from the mesophilic *E. coli* host and allows screening at elevated temperatures (55–75 °C) that increase indole solubility and thus enable higher substrate loading. Only three rounds of directed evolution and six mutations were needed to increase the catalytic efficiency of *Pf*TrpB^{WT} 83-fold, resulting in a stand-alone variant, *Pf*TrpB^{0B2}, that was even more active than the native TrpS complex. These mutations were found to accelerate catalysis through the same mechanism as TrpA effector binding, by altering the energetics of the numerous transition states of TrpB.[57] When tested against a panel of diverse indole derivatives (2-CH$_3$-, 4-F-, 5-F-, 5-Br-, 6-OH-, 2-aza-, 7-aza-indole; indazole), *Pf*TrpB^{0B2} retained the substrate range of *Pf*TrpS, and in almost all cases initial reaction rates were comparable to or better than the wild-type complex. This

engineered TrpB activation laid the foundation for expansion of the enzyme's substrate scope by directed evolution.



Scheme 3. (a) TrpS- and TrpB-catalyzed synthesis of Trp analogs. (b) TrpB-catalyzed synthesis of β-branched Trp analogs. (c) TrpS/TrpB substrate profile: modified indoles that any TrpS or TrpB variants have been demonstrated to accept. Position represents carbon where substitution, R, occurs on indole moiety. The profile is not to be interpreted as a TrpS/TrpB selectivity profile and gaps in activity may be due to lack of testing (not all indole derivatives are readily available). (d) Other tryptophan isosteres: TrpS and TrpB catalyze the synthesis of a number of Trp analogs bearing heteroatom substitutions.

2.2 Engineering stand-alone TrpB for indole-derived nucleophiles

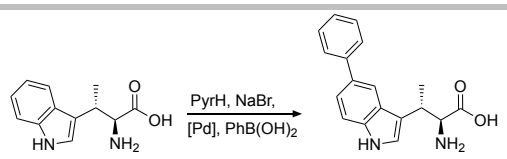
Enzyme homologs are valuable assets that often display divergent activities with non-natural substrates. Javier Murciano-Calles and co-workers investigated homologs of *Pf*TrpB for activity on 5-substituted indoles.[58] They recombined activating mutations discovered by Buller et al. into the TrpB derived from the hyperthermophilic bacterium *Thermotoga maritima* (*Tm*TrpB, 64% sequence identity to *Pf*TrpB) and found a variant with broadly improved activity toward 5-substituted indoles (5-CH_3-, 5-OCH_3-, 5-Cl-, 5-Br-, 5-NO_2-, 5-CHO-, 5-CN-, 5-$B(OH)_2$-Trp) compared to previous catalysts.

With a set of stand-alone TrpB enzymes that were straightforward to express and engineer in hand, David Romney and his team aimed to broaden the platform's substrate scope to include challenging indoles on which TrpS had previously shown poor activity.[56,58] The resultant panel of evolved TrpB enzymes accepted indoles bearing different substitution patterns and functional moieties such as halogen (4-F-; 6-, 7-Cl-; 4-, 6-, 7-Br-; 5-, 7-I-; 5,6-Cl_2-, 5-Br-7-F-, 5-Cl-7-I-Trp), nitro (4-, 5-, 6-, 7-NO_2-Trp), cyano (4-, 5-, 6-, 7-CN-Trp), carboxamide (5-$CONH_2$-Trp), boronate (5-, 6-$B(OH)_2$-Trp), and trifluoromethyl groups (5-CF_3-Trp) with most isolated yields ranging from 70-99%.[13] Christina Boville and colleagues continued engineering one of Romney's variants, *Tm*TrpB^{2F3}, to improve activity for 4-cyanoTrp, a useful blue fluorescent ncAA with high quantum yield and lifetime.[59] This transformation demonstrated a marked improvement over the best synthetic route, which was a palladium-catalyzed cyanation reaction that achieved a maximal yield of 10%.[32] Cells from one liter of *E. coli* shake flask culture expressing the final variant, TmTrpB^{9D8*}, could synthesize 4-cyanoTrp on a larger scale (800 mg, 49% yield). Notably, laboratory-evolved *Tm*TrpB^{9D8*} was discovered to function better at lower temperatures (such as 37 °C), providing for future possible *in vivo* applications.[59]

2.3 β-Branched Trps

Beta-branched amino acids are found in many useful bioactive natural products and pharmaceuticals; however the presence of two adjacent chiral centers makes them particularly challenging to synthesize.[60,61] Buller's team engineered their stand-alone TrpB to accept L-threonine (Thr) as the electrophile to produce β-methylTrp (**Scheme 3b**).[62] Unlike in earlier works, where modified electrophiles ultimately produced the same amino-acrylate as Ser,[63,64] the use of Thr generated an entirely new β-substituted amino-acrylate-like species (amino-crotonate) that diastereoselectively formed a second chiral center upon C–C bond formation. This is remarkable: Thr is a universal and abundant metabolite that TrpS naturally discriminates against. Buller discovered that native TrpS actually binds Thr efficiently, but binding results in decreased affinity for indole and disrupts the allosteric signaling that regulates the catalytic cycle. These effects translate to a >82,000 fold-preference for Ser over Thr in the native enzyme complex when both substrates are present.[65] However, in the absence of Ser competition, *Pf*TrpS—and more importantly *Pf*TrpB^{WT}—showed trace activity with indole and Thr, providing the foothold necessary to apply directed evolution. Starting from an evolutionary intermediate from their previous campaign that had better activity with Thr than the wild-type enzyme, *Pf*TrpB^{4D11}, two rounds of evolution accumulating three new mutations resulted in *Pf*TrpB^{2B9}, which exhibited a >6,000-fold boost in activity for β-methylTrp formation relative to wild-type *Pf*TrpB.[62]

Shortly after this work appeared, the Micklefield group published an engineered *St*TrpS bearing one mutation, L166V, that could also catalyze the formation of β-branched Trps (β-methyl- ; β-methyl-2-, 4-, 6-, 7-CH_3-; β-methyl-4-, 7-F-; β-methyl-7-Cl-; β-methyl-7-OMe-Trp) from Thr and indole analogs.[66] Similar to *Pf*TrpS, the enzyme struggled with 5-substituted indoles. Instead of applying further evolution to increase the substrate scope, the authors took a different approach, using *St*TrpS^{L166V} to synthesize β-methylTrp (**11**), which they then derivatized chemoenzymatically. The flavin-dependent Trp-5-halogenase PyrH was used with $MgCl_2$ or NaBr to create halogenated 5-substituted Trps (5-Br-, 5-Cl-Trp) which could undergo a palladium-catalyzed cross coupling reaction with phenylboronic acid in the same pot to create 5-phenyl-β-methylTrp (**Scheme 4**, **12**).



Scheme 4. One-pot C_5-arylation of β-methylTrp (**11**). Flavin-dependent Trp-halogenase PyrH brominates C_5, which then participates in a palladium-catalyzed Suzuki cross-coupling to install the aryl group (**12**).[66]

Christina Boville and team engineered stand-alone *Pf*TrpB^{2B9} to accept β-branched Ser analogs with longer alkyl chains such as β-ethyl- and β-propylSer.[67] Bulkier alkyl chains at the β-position were thought to hinder nucleophilic attack, allowing the competing β-elimination to unproductively consume the electrophile. Initial activity with *Pf*TrpB^{2B9} on β-ethylSer and indole was too low for high-throughput screening, so the authors mutated an active-site residue presumed to clash sterically with the alkyl β-substitution. Investigating the very same residue mutated by Micklefield et al.,[66] Boville and colleagues discovered that both valine (Val) and alanine (Ala) improved activity. While Ala was slightly less beneficial than Val, the authors rationalized that Ala may provide more room in the active site for electrophiles with longer β-alkyl substituents. This boosted activity enough to enable Boville's team to use a high-throughput UV-based screen with libraries generated by random mutagenesis for three more rounds of evolution. The final variant, *Pf*TrpB^{7E6}, was assayed against combinations of electrophiles (Thr, β-ethyl-, β-propylSer) and nucleophiles (indole; 2-, 4-, 5-, 6-, 7-CH_3-; 4-, 5-F-; 5-Cl-; 7-aza-indole) to determine its substrate scope and generality. Although *Pf*TrpB^{7E6} was only evolved on β-ethylSer, the mutations substantially improved activity for Thr and β-propylSer as well. Notably, *Pf*TrpB^{7E6} required only one equivalent of Thr to achieve a 3.5-fold greater yield for β-methylTrp than the parent *Pf*TrpB^{2B9} did with ten equivalents of Thr. X-ray crystallography, measurement of the deamination rates and UV-spectrophotometric evidence supported the hypothesis that evolution stabilized the closed conformation of the enzyme and generated a more persistent amino-acrylate that was less prone to unproductive β-elimination .[67]

2.4 Engineering stand-alone TrpB for non-indole-derived nucleophiles

TrpB proved its mettle as a noncanonical Trp synthase, but it remained to be seen whether the enzyme could become a more generalized ncAA synthase. The observation of activity with thiol- and nitrogen-based nucleophiles provided precedent for the possibility that TrpB can accept molecules that are not explicitly indole-like to create C–N,[27,54,56,68,69] C–S,[70] and C–Se[70] bonds (**Figure 1**). In principle, any sufficiently activated nucleophile that fits in the active site could react with the amino-acrylate. Carbon-based nucleophiles would be attractive synthons for TrpB, allowing for enzymatic C–C bond formation to make a broad panel of ncAAs. One major challenge, however, is that strong carbon-based nucleophiles, which are normally accessed via deprotonation of weakly-acidic C–H bonds, are highly disfavored in water because of their high basicity (pK_a > 7). Nevertheless, enzymes are known to exert profound effects on substrates to lower activation barriers, making the endeavor at least worth investigation. To our surprise and satisfaction, we discovered TrpB can react with a number of carbon nucleophiles to form novel ncAAs.

Nitroalkanes readily tautomerize to form a nucleophilic carbon alpha to the nitro group and have been used in the past as substrates for C–C bond formation reactions with electrophile-activating enzymes.[71–73] They have also been shown to react with chemically formed amino-acrylates to synthesize a wide range of amino acids, albeit under harsh conditions and with no enantioselectivity.[74–77] This led David Romney to hypothesize that nitroalkanes could act as nucleophiles in the TrpB β-elimination reaction.[78] This was indeed the case, and many of the pre-existing TrpB variants they tested displayed at least some activity with (nitromethyl)benzene (**Scheme 5**, **a**) and the more sterically unwieldy nitrocyclohexane (**Scheme 5, b**). At the standard screening temperature of 75 °C, (nitromethyl)benzene was found to decompose, leading the authors to reduce the reaction temperature to 50 °C. The variant with the best activity on both substrates was subjected to several rounds of site-saturation mutagenesis (SSM) targeting the active site to ultimately produce two specialized variants that both exhibited a maximum of 2,700 turnovers with their respective substrates. Because nucleophilicity is dependent on pH, the authors investigated the effect of pH and discovered that higher pH (pH 9.0) did not necessarily improve the initial reaction rate but did result in higher total turnovers and consequently higher yield. The authors probed the substrate scopes of the two enzymes with a panel of nitrocyclohexane and (nitromethyl)benzene derivatives (**Scheme 5, boxed**). Unfortunately, the carbon alpha to the nitro group is still readily deprotonated after product formation, resulting in stereoablation of the products with newly formed chiral centers. Nevertheless, the authors suggested that the platform could be engineered for α-nitro-substituted substrates to synthesize ncAAs with multiple chiral centers.

Biologically active compounds are replete with all-carbon quaternary centers whose regio- and stereoselective formation is a challenge for both organic synthesis and biocatalysis. In a recent study, Markus Dick and Nicholas Sarai engineered TrpB for selective quaternary bond formation with 3-substituted oxindoles.[79] 3,3-Disubstituted oxindoles are a common motif in natural and synthetic bioactive compounds. Similar to the tautomerization of nitroalkanes to form a nucleophilic carbon, oxindoles exist in an equilibrium between keto and enol tautomers, the latter of which is nucleophilic. In contrast to the nitroalkanes, whose tautomerization in water could be readily observed by NMR, the equivalent tautomer could not be observed for oxindoles, which suggested that there could be a significant activation barrier for TrpB to overcome in order for nucleophilic attack to occur. 3-Methyloxindole (**17**) was initially tested due to its abundance in synthetic and natural compounds. However, the methyl group appeared to sterically hinder nucleophilic attack from C_3, and the initial TrpB variants that were tested primarily formed the N-alkylation product (**Scheme 6**, **18**). A few variants, however, also formed the desired C_3-alkylation product, providing a foothold for directed evolution. To prevent N-alkylation, Dick and Sarai chose to use 1,3-dimethyloxindole (1-CH_3-**17**) as the model substrate to begin evolution. Impressively, a single generation was enough to switch the chemoselectivity almost entirely from N- to C_3-alkylation, allowing the authors to continue with their original substrate, 3-methyloxindole, for the remainder of the evolution. After three more rounds of mutagenesis and screening,

they obtained variant *Pf*TrpB^{quat} that exhibited >99% chemoselectivity for C_3-alkylation and a 52% yield (122 mg) from 1 mmol of 3-methyloxindole using only 100 mL of *E. coli* cell culture. They determined that the enzyme was *S,S*-stereoselective with 3-methyloxindole, though stereoselectivity decreased with bulkier substitutions at C_3. The enzyme could also tolerate ketone and lactone structures, demonstrating its ability to create quaternary centers with a diverse suite of carbonyl-containing nucleophiles bearing a tertiary carbon (**Scheme 6, solid black box and dashed pink box**).

The ability of TrpB to accept non-indole substrates such as nitroalkanes and oxindoles inspired other members of our lab to explore more molecules with known nucleophilic character such as the cyclic aromatic hydrocarbon azulene (**Scheme 7**, **29**). Until this point carbon nucleophiles accepted by TrpS and TrpB possessed heteroatoms that could stabilize the accumulation of charge during nucleophilic attack. Although azulene has no heteroatoms, it experiences a permanent dipole readily apparent in its resonance structure, which is a cycloheptatrienyl cation (tropylium) fused to a cyclopentadienyl anion (Cp^-). We hypothesized that the electron accumulation of the Cp^- stabilized by the tropylium system could promote nucleophilic attack by azulene on the amino-acrylate to form the ncAA β-(1-azulenyl)-L-alanine (**Scheme 7**, AzAla, **30**).[80] AzAla is a blue Trp isostere whose fluorescent properties have been leveraged for spectroscopic studies,[81–83] but whose use was limited due to its difficult, time-sensitive, multi-step synthesis.[84]

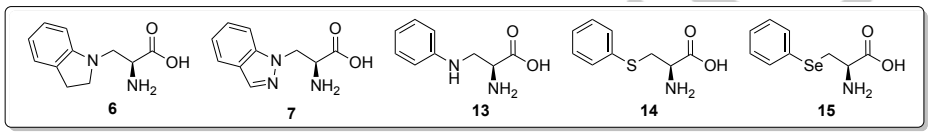
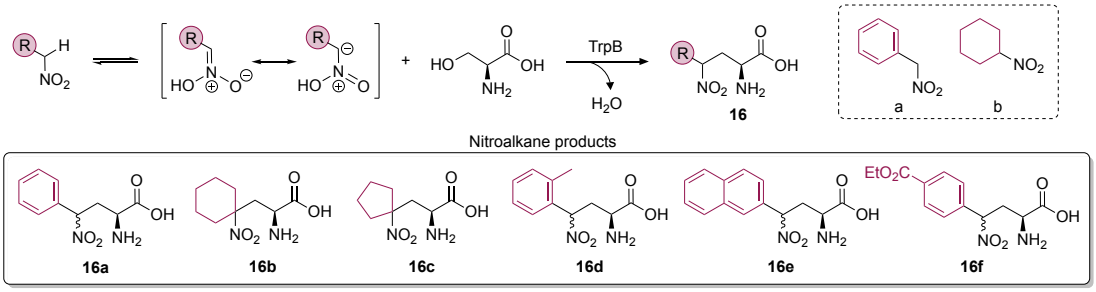
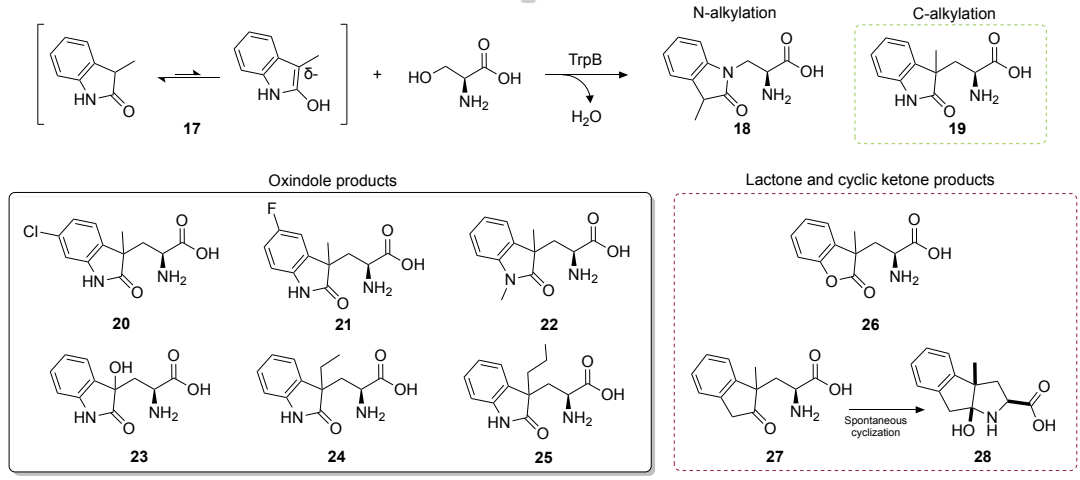


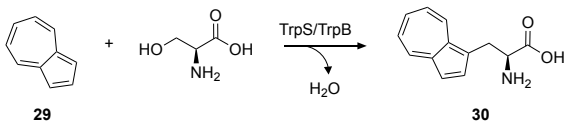
Figure 1. Products of non-carbon and non-indole nucleophiles produced by TrpS and TrpB. [27,54,56,68–70]



Scheme 5. Reaction scheme and product scope (boxed) of engineered TrpB with nitroalkanes as nucleophiles for ncAA synthesis. Nitroalkanes readily tautomerize in water to form a nucleophilic carbon species that reacts with the amino-acrylate intermediate in TrpB to form a new C–C bond. Model substrates **a** and **b** (dashed box) were used for directed evolution of TrpB.[78]



Scheme 6. Reaction scheme and product scope (boxed) of oxindoles as nucleophiles for TrpB-catalyzed ncAA synthesis. Initially, TrpB variants catalyzed primarily N-alkylation of **17** to form **18**. With directed evolution, the regio- and chemoselectivity were switched to favor the desired C–C product (**19,** green dashed box). The final evolved variant accepted oxindoles with aryl (**20, 21**), N-methyl (**22**), and C_3 substitutions (**23, 24, 25**, black box). It also catalyzed the formation of lactone (**26**) and cyclic ketone (**27** & **28**) products (pink dashed box), which suggests that evolution could be applied to expand the scope of ncAA synthesis to encompass other nucleophiles.[79]



Scheme 7. TrpB-catalyzed synthesis of β-(1-azulenyl)-L-alanine (AzAla) (**30**) from azulene (**29**) and serine.[80]

Azulene was tested against a diverse panel of engineered TrpB variants, and nearly every enzyme demonstrated significant activity for the reaction, the exception being variants bearing a glycine mutation at the highly conserved catalytic glutamate (E104G, *Pf*TrpB numbering). We reasoned that the catalytic glutamate may be important for stabilizing the tropylium cation to facilitate the nucleophilic attack from Cp- (**Figure 2**). This hypothesis was supported by examining azulene and indole activity with *Pf*TrpB and *Tm*TrpB variants with and without the glutamate to glycine mutation. While the mutation only attenuated Trp formation and decreased regioselectivity, it completely abolished AzAla formation, demonstrating a critical role for this residue in the non-natural reaction.

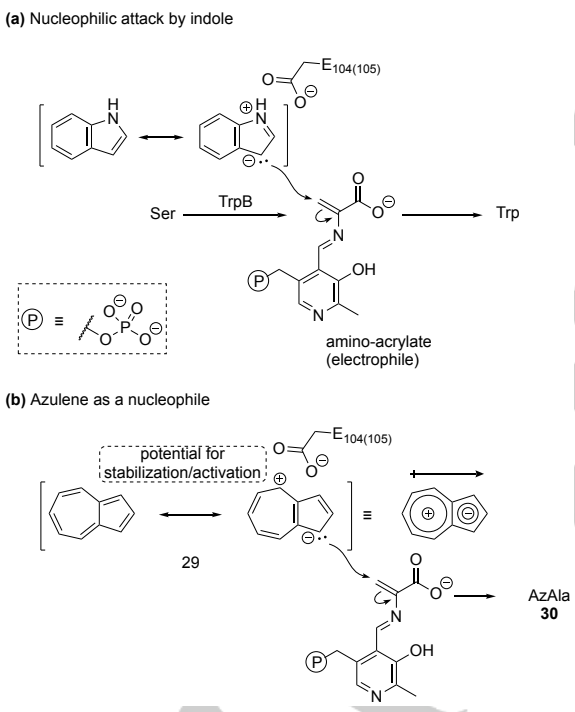


Figure 2. Comparison of proposed mechanism of nucleophilic activation in the TrpB active site for native substrate indole (a) and azulene (**29**) (b). Figure adapted from ref. [80]. Copyright: 2020, ChemBioChem.

Azulene was found to sublime readily at higher temperatures, making its containment at 75 °C difficult. Although many TrpB variants were good candidates for evolution, *Tm*TrpB^{9D8*} was chosen due to its good activity at the lower temperatures used to mitigate substrate loss. It took only one round of evolution and two mutations (W286R and F184S) to improve the turnover rate of *Tm*TrpB^{9D8*} three-fold (from 4.6 to 14.0 turnovers per minute). The final variant, *Tm*TrpB^{Azul}, was used to synthesize AzAla on gram scale (965 mg, 57% isolated yield).[80]

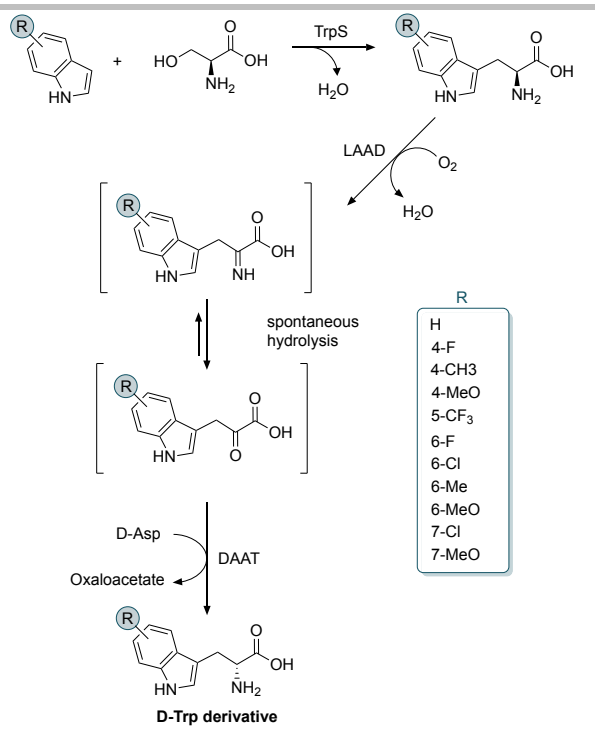
3. Biocatalytic cascades

Designing and optimizing enzymatic cascades can be a laborious process, and to date many do not best their synthetic rivals. Nonetheless, their continued development is paramount for realizing the potential of biocatalysis as a sustainable route to many of the world's chemicals. The ever-expanding catalog of engineered biocatalysts and advances in metabolic engineering have transformed the once pipe dream of whole-cell biocatalytic cascades into an attainable reality. In this realm, stereo- and regioselective enzymes that can be engineered easily and expressed heterologously reign supreme. It is thus not surprising that TrpS and TrpB—which are simple to use and boast large scopes of biologically relevant products—have already been used in a number of cascades. Some of the *in vivo* implementations have required only the host's native TrpS, which speaks to the latent potential of this remarkable complex. Others have made use of the simplicity provided by the stand-alone TrpB platform. In the following section, we highlight notable applications of TrpS and TrpB in biocatalytic cascades and discuss their biotechnological relevance.

3.1 D-Amino acids

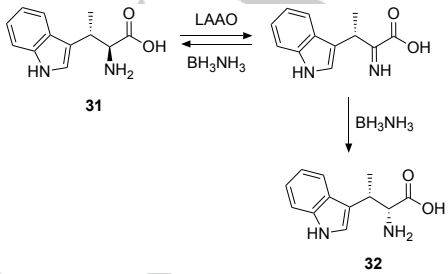
Although L-amino acids comprise an overwhelming majority of amino acids in natural and synthetic compounds, their mirror counterparts are still found in many bioactive molecules and are important targets for enantiopure synthesis. D-amino acids face similar synthetic challenges to L-amino acids but lack their diverse abundance of synthases, making direct biocatalytic access difficult. Unfortunately, TrpS's strict retention of stereoselectivity for making the L-amino acid hinders its ability to be repurposed as a D-amino acid synthase. Nevertheless, because TrpS still represents a simple way to make Trp derivatives, numerous groups have combined TrpS with downstream enzymes to access various D-Trps.

Parmeggiani and colleagues coupled TrpS from *S. enterica* (*Se*TrpS) with an L-amino acid deaminase (LAAD) followed by an engineered D-alanine aminotransferase (DAAT) to synthesize D-Trp derivatives in a one-pot, two-step transformation (**Scheme 8**).[85] LAAD and DAAT were found to possess promiscuous activity for L-Ser, putting Ser in direct competition with L-Trp for stereoinversion and lowering the overall enantiomeric excess (ee) of the product. To circumvent this, the cascade was converted into a one-pot telescopic system whereby the stereoinversion biocatalysts were introduced after the TrpS-mediated synthesis was complete. This proved to be an effective strategy to synthesize numerous D-Trp derivatives at gram scale with high yields and high ee.



Scheme 8. Biocatalytic cascade for synthesis of D-Trp derivatives. In the first reaction, a substituted indole is transformed into its respective Trp derivative by TrpS. In the second reaction, an L-amino acid deaminase (LAAD) deaminates the Trp to form an imine intermediate, which then spontaneously hydrolyzes to the α-keto acid. A D-alanine aminotransferase (DAAT) transaminates the α-keto acid with D-Asp, forming oxaloacetate and the D-Trp. Adapted with permission from ref. [85]. Copyright: 2019, ACS Catalysis.

In their preparation of β-branched Trps using TrpS, the Micklefield group used an L-amino acid oxidase (LAAO) and excess ammonia borane to produce the enantiopure D-configured epimer of β-methylTrp (**Scheme 9**, (*2R,3S*)-β-methylTrp, **32**).[66] Ammonia borane nonspecifically reduces the imine product formed by the LAAO to form both the L- and D-amino acids; LAAO re-oxidizes the L-isomer to the imine while the D-isomer accumulates. The synthesis was performed in two steps in separate pots and resulted in an overall yield of 66%. While the authors only demonstrated this proof of concept with β-methylTrp (**31**), the LAAO they used exhibits a broad substrate scope, and it is likely the method could be used to make other derivatized D-β-branched Trps.[86]



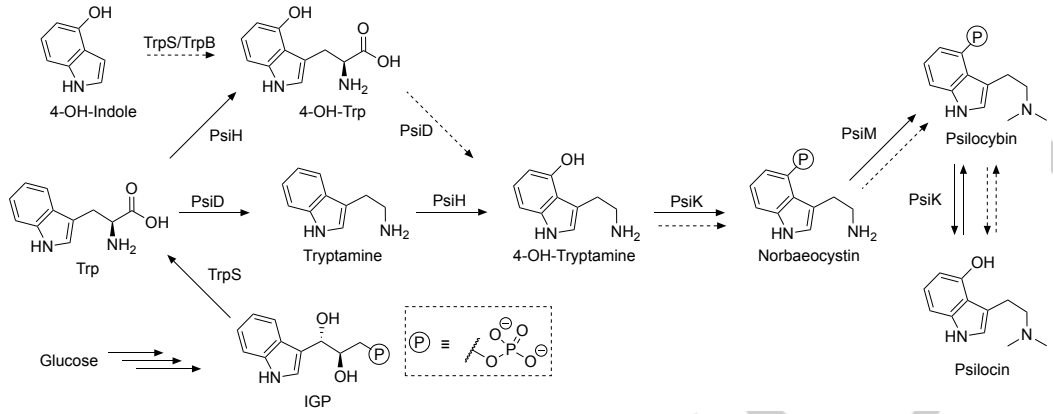
Scheme 9. One-pot chemoenzymatic approach for the stereoinversion of β-methylTrp (**31**) to form D-β-methylTrp (**32**).[66]

3.2 Tryptamine products

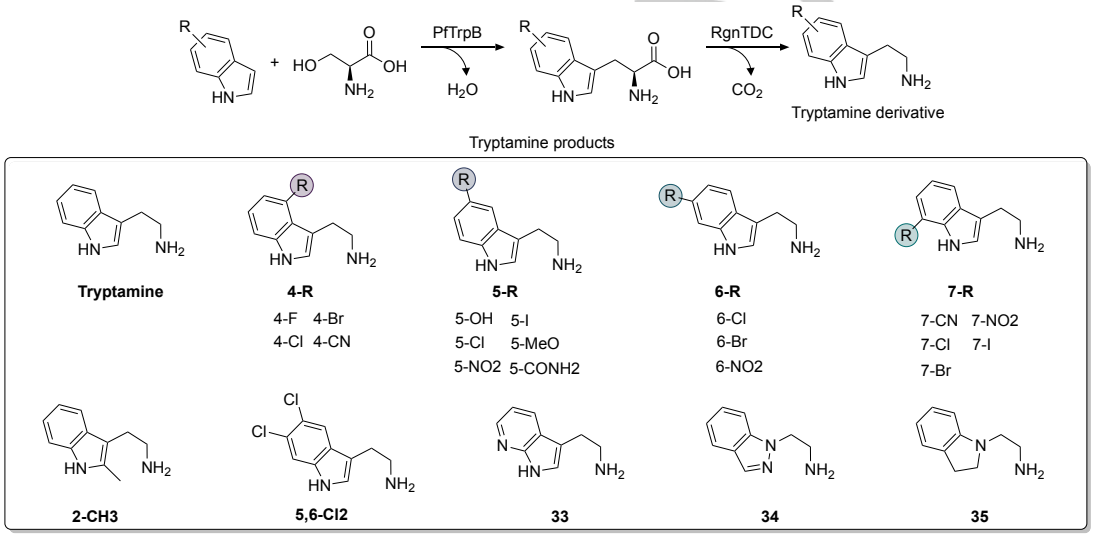
In addition to its essential role as a proteinogenic amino acid, Trp is a precursor to numerous primary and secondary metabolites across all domains of life.[87] Tryptamines are one such class of Trp-derived molecules that possess a wide range of bioactive properties. Reflecting their importance, significant efforts to develop synthetic approaches for tryptamines have resulted in several effective methodologies.[88–91] However, there is still room for biocatalysis to improve upon the cost, sustainability, and level of oversight needed for their synthesis. Recently, there has been interest in the study of psychoactive natural products like psilocybin as treatments for psychological and neurological afflictions. Psilocybin is a hallucinogenic tryptamine that is an effective treatment option for patients with anxiety,[92] substance addiction,[93,94] and depression,[95,96] and it is possible that the molecule will be approved as a pharmaceutical drug. Unfortunately, psilocybin is produced only in very small and inconsistent amounts by the mushroom *Psilocybe cubensis,* making commercial extraction impractical.[97]

Recent elucidation of the natural biosynthetic pathway by the Hoffmeister group revealed that TrpS lies upstream of only four enzymes, PsiH, PsiD, PsiK, and PsiM (which provide monooxygenase, decarboxylase, kinase, and methytransferase activities, respectively) to reach psilocybin (**Scheme 10, black arrows**).[98] The relative simplicity of the pathway coupled with the product's newfound pharmacological relevance encouraged them to investigate whether the cascade could be expressed in a model host organism to provide a scalable biosynthetic route. Indeed, the proteins expressed in *E. coli* allowed Fricke et al. to validate the putative activities of each enzyme and demonstrate an *in vitro* biosynthetic cascade of psilocybin.[99] In characterizing the enzymes, they discovered, perhaps unsurprisingly, that *P. cubensis* TrpB (*Pc*TrpB) could accept 4-hydroxyindole to produce 4-hydroxyTrp. Fortuitously, PsiD could accept 4-hydroxyTrp, which obviated the need for PsiH and further simplified the *in vitro* synthesis to a four-enzyme cascade that starts from 4-hydroxyindole (**Scheme 10, dashed arrows**).

Since then, there have been several instantiations of the cascade in different hosts. The first *in vivo* attempt ported the four enzymes downstream of TrpS into *Aspergillus nidulans,* accomplishing a modest 110 mg/L titer and more importantly establishing precedence for further *in vivo* applications.[100] Adams et al. transferred the cascade without PsiH into *E. coli*, instead exploiting the promiscuity of *Ec*TrpS to synthesize 4-hyroxyTrp from 4-hydroxyindole that was provided exogenously.[101] Attempts at *Ec*TrpS overexpression did not improve titer, with native levels of expression sufficient for the pathway's flux. Scale up led to 1.16 g/L of psilocybin after 72 hours, a ten-fold enhancement over the previous method. Most recently, Milne et al. transferred the entire pathway into *Saccharomyces cerevisiae* for the *de novo* biosynthetic production of psilocybin.[102] They chose to use the natural functionality of TrpS to engineer a biosynthetic route to psilocybin rather than exploit the enzyme's promiscuity for relatively costly 4-hydroxyindole. A fed-batch fermentation process yielded 627 mg/L of psilocybin and 580 mg/L of psilocin, the dephosphorylated bioactive form of psilocybin, after 200 hours. However, their metabolically engineered pathway takes about three-fold longer to reach approximately the same titer as the *in vivo* pathway starting from 4-hydroxyindole put forth by Adams et al.[101]



Scheme 10. Enzymatic synthesis of psilocybin. Black arrows represent natural pathway and pathway implemented by Milne et al. in *S. cerevisiae*.[102] Dashed arrows represent synthetic pathway that exploit promiscuity of TrpS to synthesize 4-hydroxyTrp from 4-hydroxindole.[99,101]



Scheme 11. One-pot, two-step synthesis of tryptamine derivatives with TrpB and and *Ruminococcus gnavus* Trp decarboxylase (*Rgn*TDC). Top: reaction scheme, bottom boxed: product scope.

Psilocybin is one of the most well-known examples of a tryptamine, perhaps second only to the neurotransmitter serotonin. However, tryptamines are an abundant motif among alkaloid natural products, and substitutions around the aromatic indole ring have profound effects on their bioactive properties. In Nature, substitutions are installed after Trp biosynthesis by specific enzymes, not unlike the natural psilocybin pathway. Harnessing these enzymes for biocatalysis to produce tryptamine derivatives, however, presents the arduous task of identifying and expressing separate tailoring enzymes for different Trp modifications.[103]

The modular and convergent nature of TrpB to combine substituted indoles with Ser offers a simpler and more general method to access Trp analogs. Buller and colleagues hypothesized that coupling a stand-alone TrpB with a promiscuous Trp decarboxylase would create a simple and streamlined route to diverse tryptamines.[104] No Trp decarboxylases that accept a broad range of substrates had been reported, so they tested a variant from the gut microbe *Ruminococcus gnavus* (*Rgn*TDC), whose active site appeared to be large enough to accommodate substituted indoles. *Rgn*TDC possessed relatively high promiscuous activity suitable for immediate biocatalytic application with TrpB. Because the two enzymes operate at dramatically different optimal temperatures (75 vs. 37 °C), *Pf*TrpB^{2B9} was combined with *Rgn*TDC in a one-pot, two-step reaction to produce a range of tryptamine derivatives with isolated yields ranging from 12-99% (**Scheme 11**). The ability to access a large number of products by combining TrpB and *Rgn*TDC is a testament to the versatility and power that generalist enzymes bring to biocatalysis.

5. Summary and outlook

TrpS is a remarkable enzyme and ideal ncAA synthase. Its long history as a model enzyme led to substantial knowledge about its allosteric regulation and catalytic mechanism that granted enzyme engineers the ability to re-imagine its function. In

this review, we have covered major applications of TrpS, from humble beginnings preparing simple Trp analogs to becoming a powerful platform of TrpB enzymes that produce entirely new-to-nature ncAAs, as well as its use in biocatalytic cascades to make new Trp-derived products. The no-frills TrpB platform dramatically simplifies enzyme engineering efforts, enabling the rapid expansion and exploration of the synthase's substrate scope. Recent success in evolving TrpB to catalyze C–C bonds with new, non-indole nucleophiles bodes well for the platform's further expansion into ncAA space. We envision that the discovery of highly stable and evolvable TrpB variants that function well at moderate temperatures will promote future applications in cascades, replacing multi-step syntheses that currently must accommodate different temperature optima. Mesophilic TrpB variants may also be used for *in vivo* synthesis to improve intracellular delivery of the ncAA (indole passes through cellular membranes more readily than a charged amino acid), reducing the amount of product that must be supplied exogenously, which could improve incorporation rates and reduce costs. *In vivo* synthesis and incorporation of ncAAs into proteins or secondary metabolites might also be used for robust biocontainment.[101,105,106]

Although many substituted indoles are commercially available, one of the roadblocks for biocatalytic synthesis of Trp derivatives by TrpS and TrpB is the high price and limited availability of these substrates. This issue is highlighted by Milne et al., who used PsiH to install a hydroxyl group on Trp rather than provide expensive 4-hydroxyindole to TrpS directly.[102] However, as we reach beyond the domain of naturally occurring chemical motifs, it is more difficult to find enzymes like PsiH that can make desired Trp modifications. In this non-natural space the TrpB platform truly shines for ncAA synthesis; it is simpler to engineer TrpB to accept new derivatives than it is to discover and engineer whole new enzymes that modify Trp. Therefore, advances in indole analog synthesis will continue to make TrpB a desirable route to Trp analogs. We expect that further applications of TrpS and TrpB are on the horizon and demonstrations like those from Parmeggiani et al.[85] and McDonald et al.[104] will inspire others to use this biocatalyst extraordinaire.

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Conflict of Interest

FHA is a co-founder of Aralez Bio (San Leandro, CA), which uses TrpB variants to produce ncAAs.

Keywords: biocatalysis • amino acids • enzyme • biotransformation • protein engineering

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REVIEW

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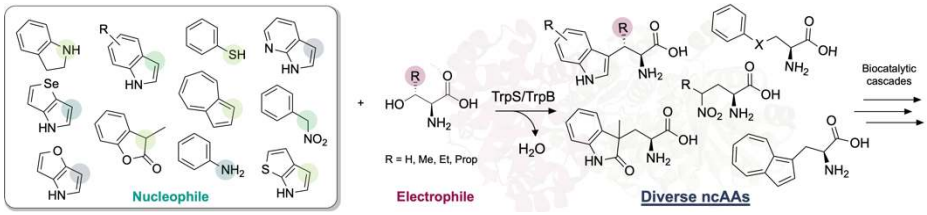

Biocatalyst extraordinaire! Tryptophan synthase (TrpS) natively catalyzes the formation of tryptophan but also possesses remarkable promiscuous activity for synthesizing a wide range of noncanonical amino acids (ncAAs). This review details the history of TrpS as a ncAA synthase, from the characterization of its naturally broad substrate scope and engineering efforts to expand the range of its non-natural chemistry to applications in biocatalytic cascades to synthesize diverse natural and xenobiotic compounds.

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