Tailoring Tryptophan Synthase TrpB for Selective Quaternary Carbon Bond Formation

Markus Dick,[1] Nicholas S. Sarai,[1] Michael W. Martynowycz,[2] Tamir Gonen,[2] and Frances H. Arnold[1,*]

[1]Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, 1200 East California Boulevard, Pasadena, California 91125, United States

[2]Howard Hughes Medical Institute, David Geffen School of Medicine, Departments of Biological Chemistry and Physiology, University of California, Los Angeles, Los Angeles, California 90095, United States

Supporting Information

ABSTRACT: We previously engineered the tryptophan synthase β-subunit (TrpB), which catalyzes the condensation reaction between L-serine and indole to form L-tryptophan, to synthesize a range of modified tryptophans from serine and indole derivatives. In this study, we used directed evolution to engineer TrpB to accept 3-substituted oxindoles and form C–C bonds leading to new quaternary stereocenters. At first, the TrpBs that could use 3-substituted oxindoles preferentially formed N–C bonds by attacking the oxindole N_1 atom. We found, however, that protecting the nitrogen encouraged evolution towards C-alkylation, which persisted even when this protection was removed. After seven rounds of evolution leading to a 400-fold improvement in activity, variant *Pf*$_{quat}$ efficiently alkylates 3-substituted oxindoles to selectively form new stereocenters at the γ-position of the amino acid products. The configuration of the new γ-stereocenter of one of the products was determined from the crystal structure obtained by microcrystal electron diffraction (MicroED). Substrates structurally related to 3-methyloxindole such as lactones and ketones can also be used by the enzyme for quaternary carbon bond formation, where the biocatalyst exhibits excellent regioselectivity for the tertiary carbon atom. Highly thermostable and expressed at > 500 mg/L *E. coli* culture, TrpB *Pf*$_{quat}$ provides an efficient and environmentally-friendly platform for the preparation of noncanonical amino acids bearing quaternary carbons.

Keywords: TrpB, noncanonical amino acid, asymmetric catalysis, quaternary carbon, oxindole, C–C bond formation, directed evolution

Introduction

In order to meet nature's demand for chiral and selective biotransformations, enzymes have evolved to exert a high level of stereo-, regio-, and chemoselective control. Laboratory methods of directed evolution can similarly tailor enzyme selectivity for target-oriented biocatalysis.[1] We have used directed evolution to engineer variants of the β-subunit of tryptophan synthase (TrpB), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, for synthesis of noncanonical amino acids (ncAAs). Natively, TrpB catalyzes formation of a C–C bond between L-serine and indole to make L-tryptophan (Figure 1a).[2] We evolved this enzyme to accept a variety of indole analogues and nitroalkanes with activated sp^3-carbon atoms as nucleophiles (Figure 1b).[3-8] However, these studies revealed limitations including chemo- and stereoselective control we would need to address in order to realize TrpB's broad potential as a biocatalyst for C–C bond formation.

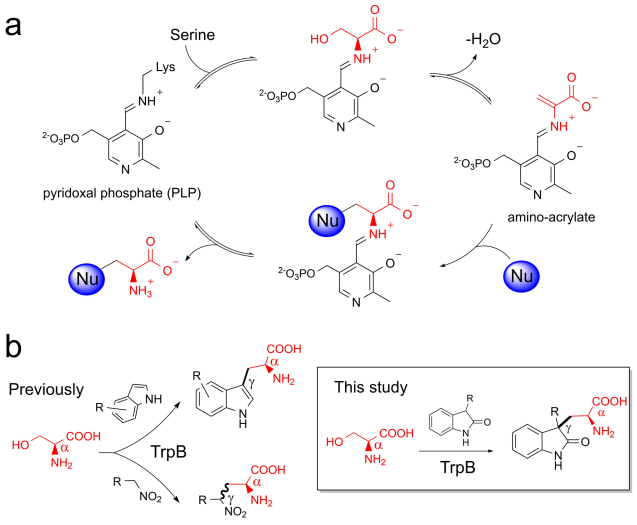

Figure 1. a) Catalytic cycle of amino acid synthesis using TrpB. b) Previous biocatalytic reactions catalyzed by variants of TrpB[5-6, 8] and proposed stereo- and chemo- selective reaction using engineered TrpB.

Our previous efforts focused first on engineering TrpB to accept nucleophiles structurally and electronically similar to indole. We recently found we could branch out to nitroalkanes with some TrpB variants, taking advantage of the known nucleophilicity of these substrates that comes from the low pK_a of the C–H bond adjacent to the electron-withdrawing nitro group.[8,9] We observed, however, that TrpBs do not catalyze C–C bond formation if the substrate is only marginally nucleophilic. Nucleophilic atoms such as nitrogen and sulfur instead serve as competing donors for nucleophilic attack, leading to the formation of C–N and C–S bonds rather than the desired C–C bond in nonnatural substrates.[2, 10]

TrpB is highly stereoselective and can retain the absolute configuration of the C_α of L-serine after product formation (Figure 1b).[3-7]

However, the stereoselectivity at potential new stereogenic centers is unknown. Substrates with nucleophilic sp^3-hybridized carbons have the ability to form a new quaternary carbon at the γ-position. As enzymes that catalyze the formation of quaternary carbon stereocenters are rare, this capability can open new applications for TrpB in asymmetric catalysis. This potential has not yet been explored because indole derivatives with an sp^2-carbon atom lack chirality, and the γ carbon of nitroalkanes easily epimerizes (Figure 1b).[8]

In this study, our goal was to address these limitations by engineering TrpB for unprecedented C–C bond formation with 3-substituted oxindoles. We challenged TrpB with 3-methyloxindole **1**, which has a tertiary, sp^3-carbon atom that could be used to form a quaternary carbon (Figure 1b). In addition, 3-methyloxindole **1** contains two nucleophilic atoms, C_3 and N_1, with which we could probe chemoselectivity for C–C bond formation. Engineering TrpB for activity on 3-methyloxindole **1** would provide biocatalytic access to 3,3-disubstituted oxindoles (Figure 2a), a motif present in a broad range of natural and synthetic biologically active compounds (Figure 2b).[11-12] Various approaches to form 3,3-disubstituted oxindoles have been explored in synthetic chemistry, including C–H activation and chiral catalysts such as phosphoramides or alkaloid-derived urea catalysts.[13-15] These methods may require protection of the nitrogen and/or activation of C_3 and almost always use high chiral catalyst loadings. In contrast, enzymes function under mild reaction conditions and can exert unparalleled stereocontrol. An enzyme with this activity, however, has not been reported.

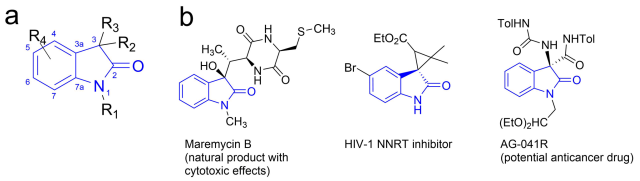

Figure 2. a) Core structure of substituted oxindoles. b) Examples of natural products/bioactive molecules derived from 3,3-disubstituted oxindoles.[13, 16-17]

Results

Screening for activity with 3-methyloxindole

In previous work where TrpB was engineered for the synthesis of tryptophan derivatives, enzymes from the thermophilic organisms *Pyrococcus furiosus* (*Pf*TrpB) and *Thermotoga maritima* (*Tm*TrpB) had sufficient activity with the indole derivatives to enable screening for beneficial mutations.[2-3, 5-7] When we tested the wild-type enzymes and engineered variants from our collection *via* mass spectrometry-based screening with 3-methyloxindole **1** as a nucleophile and serine **2** as the electrophile, several variants showed activity, with up to 28% HPLC yield for the expected mass (SI, Figure 1). However, these enzymes formed the N-alkylated oxindole **3a** (Figure 3a) rather than the desired C-alkylated product **3b**. Certain variants also produced traces of a second, more polar peak, which corresponds to the C-alkylation product **3b** (SI, Figure 2), but because both **3a** and **3b** have the same molecular mass and similar retention times on reverse-phase HPLC, rapid screening for the C-alkylation product **3b** was not possible.

Like nitroalkanes, oxindoles can theoretically tautomerize to activate the C_3 carbon atom by forming an enolate species with an sp^2-hybridized carbon center (Figure 3c). To investigate the equilibrium between the enolate and keto forms, NMR spectra were acquired for 3-methyloxindole **1** and nitromethylbenzene in aqueous solution. Only the nitroalkane displayed a shift in equilibrium towards sp^2-hybridization at C_3; no activated tautomer was observed for the oxindole (SI, Figure 3). This finding indicates that TrpB needs to overcome a high activation barrier to enable nucleophilic attack by the C_3 atom of 3-methyloxindole **1**.

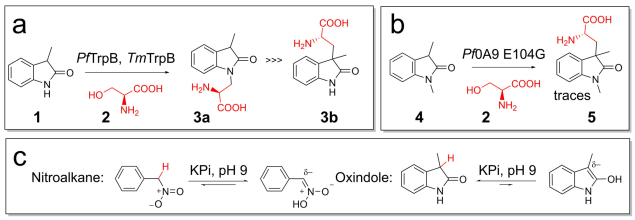

Figure 3. a) N-alkylation reaction using *Pf*TrpB and *Tm*TrpB variants. b) *Pf*0A9 E104G shows trace activity for C-alkylation of 3-substituted oxindoles if the nitrogen of the nucleophile is protected. c) Tautomeric forms of nitromethylbenzene and 3-methyloxindole **1** in aqueous solution under TrpB reaction conditions. While both tautomers of the nitroalkane could be detected in NMR experiments, only the keto form was visible in the NMR spectra of the oxindole, indicating that the equilibrium lies on the left.

Reasoning that protecting the nitrogen could coerce the enzyme to favor C-alkylation, we tested whether the enzyme could accept 1,3-dimethyloxindole **4** (Figure 3b). This reaction would construct 3,3-disubstituted oxindoles with a methylated nitrogen, a common motif in natural products (Figure 2b). Again, a panel of engineered and wild-type TrpB enzymes was evaluated; the highest activity for formation of N-methylated 3,3-dialkylated oxindole **5** was found with *Pf*0A9 E104G, a variant previously evolved for nitrotryptophan synthesis.[5] However, even this best variant showed only trace activity (total turnover number (TTN) < 1).

Engineering TrpB *Pf*0A9 E104G for improved C-alkylation activity

To improve the activity of *Pf*0A9 E104G on the N-protected 1,3-dimethyloxindole **4**, we introduced random mutations by error-prone PCR and tested variants for activity with an LC-MS based screen (SI, Figure 4). One variant was identified with two mutations (W172R and S185T) that gave a 6-fold improvement in product formation compared to parent. Interestingly, W172R and S185T were not beneficial as single mutations, indicating an epistatic effect between the two (SI, Figure 5). To evaluate whether our chemoselectivity modification strategy was effective, we tested this variant for activity on 3-methyloxindole **1**, which lacks protection on the nitrogen. Gratifyingly, the main product was the C-alkylated oxindole (Figure 4a). Thus, a single round of directed evolution was sufficient to switch the chemoselectivity from N- to C-alkylation.

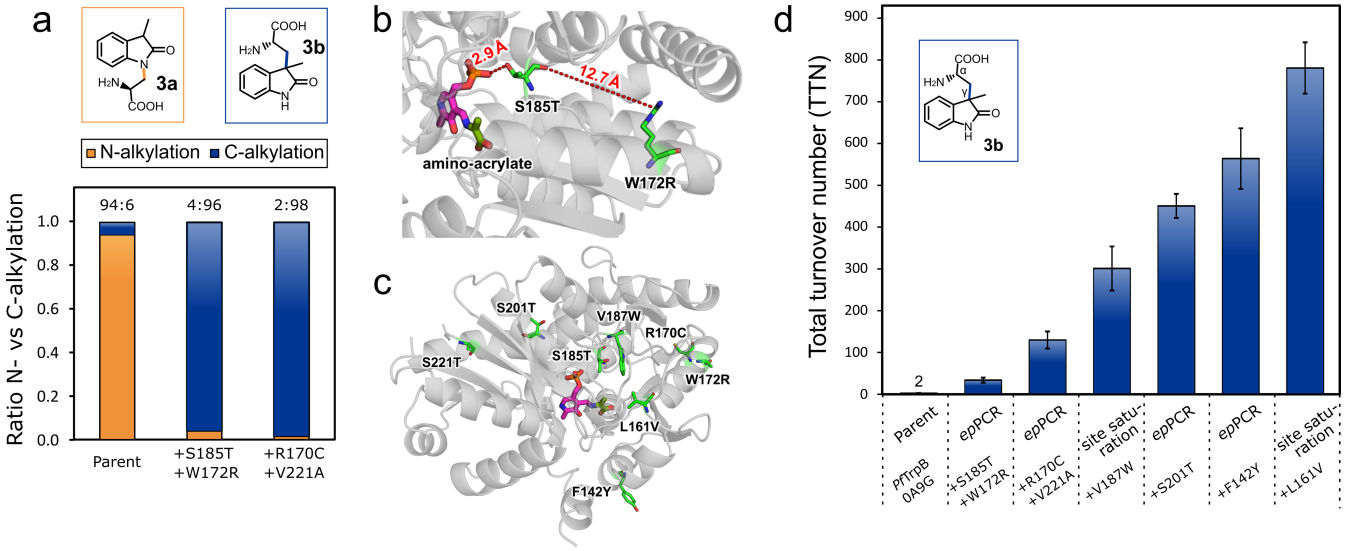

Figure 4. a) Ratio of N- to C-alkylation of 3-methyloxindole **1** using the parent enzyme and the best variants after the first two rounds of directed evolution. b) Distances of R172 and T185 from the active site of TrpB shown on the structure of an evolved variant of TrpB with the PLP-bound amino-acrylate (PDB ID: 5vm5). c) Locations of all beneficial mutations collected after 6 rounds of directed evolution. d) Total turnover number (TTN) for the best variant after each round of directed evolution, determined using 100 mM 3-methyloxindole **1** and serine **2** as substrates and 20 μM of purified enzyme (*ep*PCR = error prone PCR).

Notably, only the S185T mutation is proximal to the active site and can interact directly with the substrate (Figure 4b). By contrast, W172R is 15 Å from the catalytic site and a direct interaction with T185 seems unlikely. One of the benefits of directed evolution is that it explores mutations that cannot be predicted easily.[18-19] It is difficult to explain the effects of these mutations on the activity and chemoselectivity of TrpB, and they would not have been found with targeted mutagenesis. A second round of random mutagenesis uncovered two additional mutations (R170C and V221A) that further doubled activity for oxindoles with and without nitrogen protection (Figure 4a+d).

Since only one of the four mutations in the best variant at this point is close to the active site, we postulated that the binding pocket for the substrate might still be optimized for indole, the natural nucleophile of TrpB, rather than for oxindole. We therefore constructed site-saturation mutagenesis libraries at seven residues that cover the nucleophile-binding site (SI, Figure 6a) and were previously shown to affect the activity of TrpB with nitroalkanes.[8] Screening these libraries on the unprotected 3-methyloxindole **1** identified three beneficial mutations: G104D, I183E and V187W (SI, Figure 6b). A recombination library was constructed; the best variant contained only one of these (V187W), which gave a 3-fold improvement in product formation.

Three further rounds of mutagenesis and screening resulted in final variant Pf_{quat} with three additional mutations (Figure 4c+d); this variant gave 60% HPLC yield with > 99% chemoselectivity. Pf_{quat} expresses at high levels (> 500 mg/L of *Escherichia coli* culture) and can be purified easily by heat treatment at 75 °C. As a result, we were able to synthesize 122 mg (52% isolated yield) of product **3b** from a reaction using 1 mmol substrate **1** and Pf_{quat} in cell lysate from 100 mL of *E. coli* culture.

Substrate scope

We next evaluated the substrate scope of TrpB Pf_{quat}, evolved for activity on **1**. We chose oxindoles with substituents on the aromatic ring and at the 3-position, and other structurally related molecules (Figure 5a). Substrates with alkyl chains at the 3-position from methyl to butyl (products **3b, 9-11**) were accepted, with the longer alkyl groups leading to reduced product formation. Thus, even bulky oxindoles are accepted by the enzyme and the activity uncovered can serve as a starting point for directed evolution. 3-hydroxyl oxindole is a structural motif in maremycin A, B, C and D and represents the core structure of the protease inhibitor TMC-95A.[20] We tested and also found activity for 3-hydroxyl oxindole, leading to product **12**.

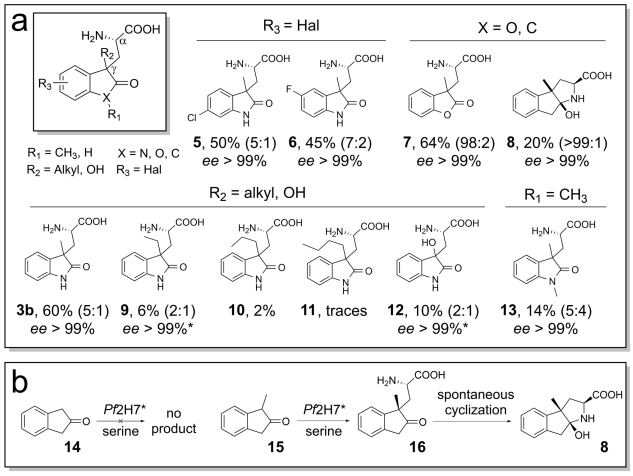

Figure 5. a) Scope of amino acid products using 50 mM oxindole-based nucleophile, 2 eq. of serine **2** and 0.2 mol% of purified TrpB Pf_{quat}. The percentages are the HPLC yields, and the diastereomeric ratio in parentheses refers to the configuration at the γ-position (*S:R*). The *ee*-values represent the enantiomeric purity for both diastereomers. For compounds **9** and **12** (indicated with *), the *ee* of only one diastereomer could be determined. b) Activity of TrpB Pf_{quat} for 2-indanones.

In order to explore the ability of the enzyme to accept nucleophiles beyond oxindoles, we tested a tertiary lactone and a ketone. To our surprise, when a lactone was used, conversion to product **7** was even higher than for product **3b**. Based on this result, we challenged the TrpB variant with two different ketones, 2-indanone **14** and 1-methyl-2-indanone **15** (Figure 5b). Interestingly, while no

activity was observed for substrate **14**, which lacks a tertiary carbon atom at the 1-position, Pf_{quat} converted the 1-methylated ketone **15** to product **16** with 20% HPLC yield and > 95% regioselectivity for the tertiary carbon. Spontaneous nucleophilic attack of the free amino group on the carbonyl carbon leads to the final cyclic product **8**.

A list of substrates not accepted by Pf_{quat} for C–C-bond formation is shown in SI, Figure 7.

Determination of relative configuration

Since the products shown in Figure 5a have two stereocenters at the α- and γ-positions, four stereoisomers are possible in theory. HPLC and NMR analysis of the products confirmed the formation of two diastereomers. Both diastereomers exhibit an *ee* of > 99%, indicating that two of four possible stereoisomers predominate. From previous studies of TrpB in nature and biocatalysis, it is known that the enzyme does not catalyze the formation of D-amino acids at a measurable level. Thus, we inferred by analogy that the α-position remains *S*-configured while the γ-carbon is responsible for the two diastereomers (Figure 5a).

We decided to use MicroED to determine the configuration of the new stereocenter at the γ-position.[21-22] Lyophilized powder of the major diastereomer of the product made from oxindole **3b** with an optical purity > 99% was applied directly onto an EM grid. Needle-shaped nanocrystals were identified, and continuous rotation MicroED[22-23] data were collected. The data from two nanocrystals were merged to increase completeness, and the structure was solved by *ab initio* direct methods to 0.9 Å resolution (Figure 6a). We were surprised to discover that the structure represented two enantiomers in a centric space group, where both stereocenters are either *S*- or *R*-configured. We reasoned that this compound only crystallizes as a racemic mixture under these conditions. Because MicroED is a very sensitive method which allows structural determination using nanocrystals, hardly detectable traces of the *R*-configured amino acid were sufficient for the formation of the racemic crystals.

Based on the overwhelming preference of TrpB to form amino acids with an *S*-configured α-carbon, we deduced that the *S*, *S* stereo centered mirror was the predominant configuration. In order to validate this observation with a second, independent structural determination, we also collected MicroED data from compound **14** (Figure 6b). This structure was solved using *ab initio* direct methods in an acentric space group using data from two nanocrystals, with a single molecule in the asymmetric unit. The structure was solved with the second quaternary center in the *S*-configuration. By analogy to these two structures using NMR data we deduced that the *S*-configuration at the γ-position predominates in all products shown in Figure 5a.

The stereoselectivity of Pf_{quat} is substrate-dependent and decreases when bulkier substituents are present at the α-position or at N_1. Gratifyingly, both the lactone- and the ketone-based products **7** and **8** were produced with excellent diastereoselectivity of more than 95:5.

Discussion

Unlocking new reactivity of TrpB

TrpB has been engineered for the synthesis of diverse L-tryptophan analogues and other noncanonical amino acids. However, the enormous variety of ncAAs found in natural products and useful for biotechnological applications extends far beyond the current scope of TrpB,[24-25] which has mostly been limited to specific sp^2–sp^3 carbon bond formation between serine and indole analogues.

In this study, we extended TrpB's scope to the reaction between serine **2** and a variety of oxindoles. While TrpB variants capable of using 3-methyloxindole **1** form an N–C bond between serine and N_1, directed evolution generated a *Pyrococcus furiosus* TrpB variant exhibiting excellent chemoselectivity for C–C bond formation and stereoselective control at both the α- and γ-position of the product. The desired oxindole-based ncAAs were obtained with > 400-fold improved total turnover numbers while retaining high thermostability and expression level (> 500 mg protein/L *E. coli* culture). This engineering approach demonstrates that the chemoselectivity of TrpB can be switched between different, competing functional groups. The range of biotechnologically relevant ncAAs encompasses those with sp^3 or sp^2 C–C bonds as well as C–N, C–S and C–O bonds.[25] Our strategy provides a proof of principle that the chemoselectivity and stereoselectivity of TrpB may be tailored to expand its portfolio of noncanonical amino acid products.

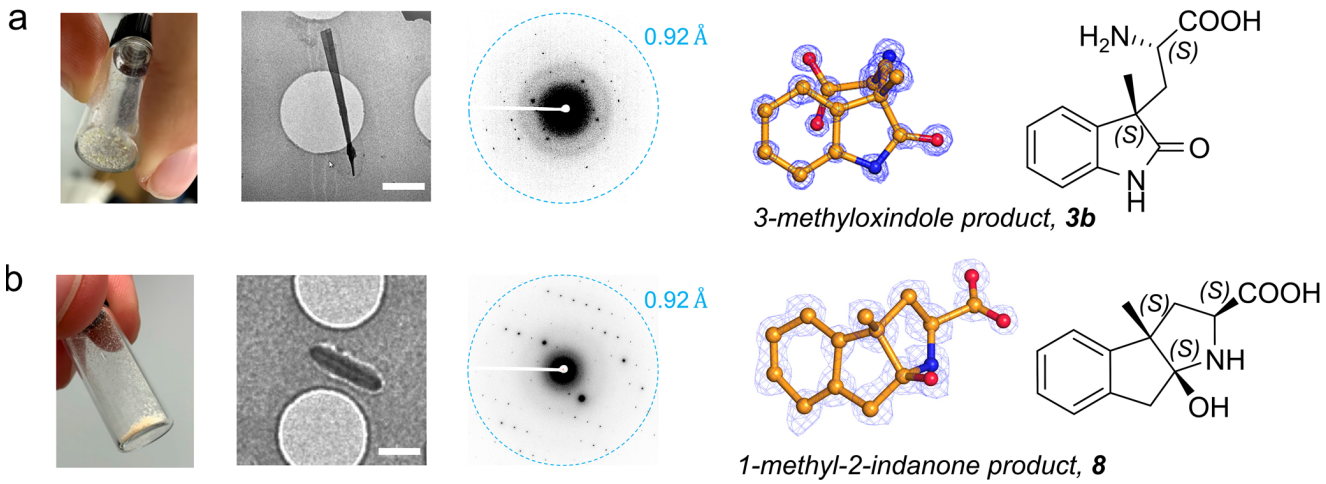

Figure 6. Structures of a) the ncAA product derived from 3-methyloxindole **1**, which was obtained as a resin-like material and b) the cyclized ncAA product derived from 1-methyl-2-indanone **15**, which was obtained as a white/yellow powder. Both products **3b** and **8** were capable of forming nanocrystals on an EM grid. These nanocrystals diffracted to 0.9 Å resolution. Scale bars are 1 µm. $2F_o$-F_c density is contoured at the 2σ level.

Formation of quaternary carbon atoms in organic synthesis and biocatalysis

Quaternary carbon centers are found in many biologically active natural products,[26] but they are notoriously challenging to prepare. This is reflected by the fact that of the 200 top-grossing pharmaceuticals in 2012, none bear quaternary carbons introduced by chemical synthesis.[27] The enzymes that synthesize quaternary carbon-bearing natural products include radical-SAM dependent enzymes, which use a Fe-S-cluster, prenyltransferases that prenylate the 3-position of tryptophan, and cyclases that synthesize natural products including valenic acid or cholesterol.[28-31] Recent developments in synthetic chemistry have led to new strategies for enantioselective synthesis of quaternary carbon centers using chiral catalysts such as palladium complexes.[27] Enzymatic approaches, however, have rarely been addressed. Indeed, the repertoire of C–C bond-forming enzymes employed in biotechnology and biocatalysis is mostly limited to aldolases and ThDP-dependent enzymes.[32] Both of these classes use activated carbonyl compounds as nucleophiles. As such, they are restricted in their substrate scope to aldehydes and ketones and cannot be used to form quaternary carbon centers.

Biocatalytic synthesis of 3,3-disubstituted oxindoles

We reasoned that TrpB should be able to use activated tertiary carbons as nucleophiles to form quaternary carbon centers. Oxindoles were chosen as model substrates because they possess an activated tertiary carbon and 3,3-disubstituted oxindoles represent a structural motif found in many natural products.[13, 16-17] Two previous studies describe enzymatic approaches to the formation of molecules containing the oxindole substructure. Pietruszka *et al.* used laccase-driven oxidation of catechols which undergo a non-enzymatic Michael addition with N-protected oxindoles to form 3,3-disubstituted oxindoles;[33] the enzyme did not directly catalyze arylation of the oxindole. Recently, Black *et al.* reported that 'ene'-reductases can catalyze an abiological, radical cyclization reaction to form 3,3-disubstituted oxindoles from N-protected α-haloamides.[34] The engineered TrpB described here directly functionalizes unprotected oxindoles at C_3.

TrpB for selective quaternary carbon bond formation

The evolved TrpB variant Pf_{quat} is not limited in its nucleophile scope to oxindoles. Reasoning that TrpB might also utilize heterocycles with similar properties to oxindoles, we tested the enzyme on non-oxindole substrates. Replacing the N_1 atom of 3-methyloxindole **1** with carbon or oxygen leads to ketone and lactone structures (Figure 5) that were also converted by TrpB Pf_{quat} to the corresponding products **7** and **8**. These results reveal a broader ability of Pf_{quat} to utilize a diverse suite of carbonyl-containing nucleophiles bearing a tertiary carbon atom at the 1-position. They also demonstrate the exquisite chemo- and regioselectivity of Pf_{quat} for quaternary carbon bond formation.

Highlighting the regioselectivity of Pf_{quat} is its ability to exclusively functionalize the tertiary carbon rather than the secondary carbon of ketone **15**. Ketones and lactones bearing quaternary carbon centers at the α-position are a structural motif found in many natural products with medicinal properties.[27, 35-36] Hence, much effort in organic synthesis has been invested to enable regioselective alkylation of α-substituted ketones to form quaternary carbon centers. Synthesizing such compounds, especially with high enantiomeric purity, can be very challenging, as the substituted product is more sterically hindered and thus kinetically disfavored.[27, 37-38]

Pf_{quat} represents a powerful biocatalytic platform for functionalizing α-carbons of ketones and lactones. Coupled with its ability to synthesize an array of 3,3-disubstituted oxindoles, this genetically-encoded catalyst is capable of providing access to valuable products with diverse properties. These products highlight the ability of TrpB to address chemo- and regioselectivity challenges while expanding the synthetic space available to biocatalysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Additional figures (Figures S1−S7), experimental procedures, crystallographic information (PDF) and NMR spectra (PDF).

AUTHOR INFORMATION

Corresponding Author

*frances@cheme.caltech.edu.

Notes

The authors declare the following competing financial interest: The contents of this paper are the subject of a patent application submitted by Caltech (M.D. inventor).

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) and by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R01GM125887. The Gonen lab is supported by funds from the Howard Hughes Medical Institute. The authors thank David K. Romney, Patrick J. Almhjell, Susanne Bähr, Nicholas J. Porter, Sanel Suljic and Brian M. Stoltz for helpful discussions.

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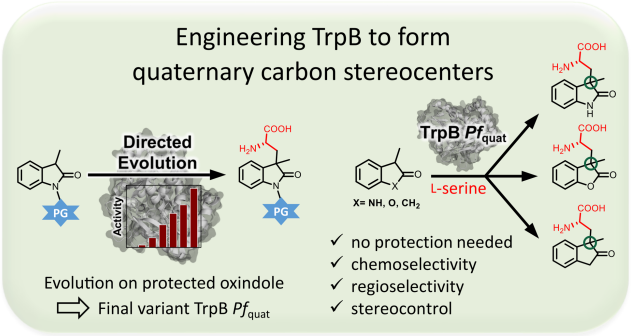
Engineering TrpB to form
quaternary carbon stereocenters
Directed
Evolution
PG
Activity
COOH
PG
Evolution on protected oxindole
Final variant TrpB Pfquat
TrpB Pfquat
X= NH, O, CH2
L-serine
COOH
COOH
COOH
✓ no protection needed
✓ chemoselectivity
✓ regioselectivity
✓ stereocontrol