

Communication

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Engineering cytochrome P450s for enantioselective cyclopropenation of internal alkynes

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Supporting Information Placeholder

ABSTRACT: We report a biocatalytic platform of engineered cytochrome P450 enzymes to carry out efficient cyclopropene synthesis via carbene transfer to internal alkynes. Directed evolution of a serine-ligated P450 variant, P411-C10, yielded a lineage of engineered P411 enzymes that together accommodate a variety of internal aromatic alkynes as substrates for cyclopropenation with unprecedented efficiencies and stereoselectivities (up to 5760 TTN, and all with >99.9% ee). Using an internal aliphatic alkyne bearing a propargylic ether group, different P411 variants can selectively catalyze cyclopropene formation, carbene insertion into a propargylic C–H bond or [3+2]-cycloaddition. This tunable reaction selectivity further highlights the benefit of using genetically-encoded catalysts to address chemoselectivity challenges.

Cyclopropenes, with *endo*-cyclic double bonds inside a three-membered carbocycle, possess high strain energy, which enables activity in different strain-release transformations for constructing a myriad of useful molecular scaffolds.¹ Carbene transfer to alkynes represents one of the most straightforward approaches to constructing cyclopropenes.^{1a,1b} Small-molecule transition metal complexes based on rhodium, iridium, cobalt and others have been shown to catalyze carbene transfer to terminal alkynes to yield enantio-enriched cyclopropenes.^{2–4} However, enantioselective carbene transfer to internal alkynes still remains largely unexplored. Only two systems with chiral gold/silver⁵ or rhodium⁶ (co-)catalysts have been reported to take internal aromatic alkynes for asymmetric cyclopropene synthesis with good stereoselectivities. These systems require precious metal catalysts in relatively high loading together with complicated ligands and have not been shown to work with internal aliphatic alkynes. We wanted to develop an efficient biocatalytic platform that uses earth-abundant iron to access internal cyclopropenes.

Cytochromes P450 use an iron-heme complex as their catalytic cofactor in their native oxygenase functions.⁷ Recently, directed evolution has significantly expanded the catalytic repertoire of P450 enzymes and other hemoproteins to include non-natural carbene- and nitrene-transfer reactions, as described by our group and others.^{8–10} We recently reported an enzymatic platform of engineered cytochrome P450 enzymes for stereoselective carbene addition to terminal alkynes to forge cyclopropenes and bicyclo[1.1.0]butanes (**Figure 1**).¹¹ We hypothesized that P450 enzymes may achieve even more challenging transformations, such as carbene transfer to internal alkynes for cyclopropene construction. The major difficulty for internal alkyne cyclopropenation lies in the severe steric clash between the linear

π -system and the planar heme cofactor, especially if the reaction involves a concerted carbene-transfer mechanism.¹² Recent mechanistic studies have shown step-wise carbene-transfer processes or even multiple pathways for the same type of reactions with different engineered hemoproteins.¹³ We reasoned that proper engineering of the enzyme active site may direct the desired carbene transfer to proceed through a step-wise pathway, thereby circumventing the steric issue.

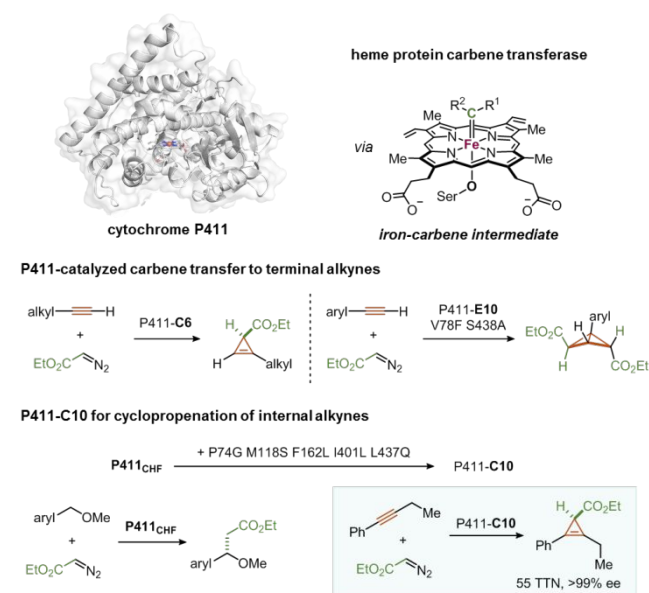


Figure 1. Enzymatic carbene transfer to alkynes for strained carbocycle formation.

We initiated investigation of internal aromatic alkyne cyclopropenation using ethyl diazoacetate (EDA) as the carbene precursor and 1-phenylbutyne (**1a**) as the model alkyne substrate. Screening various hemoprotein variants based on P450s, P411s (P450 with axial ligating residue mutated to serine),¹⁴ cytochromes *c* and globins in the form of whole *Escherichia coli* (*E. coli*) cell catalysts identified a P411 variant, P411-C10, that formed the desired internal cyclopropene (**Figure 1**). P411-C10 belongs to the family of P411_{CHF} (five amino acid substitutions away), which was evolved for a carbene C–H insertion reaction.¹⁵ Surprisingly, the cyclopropene product synthesized by P411-C10 was determined to be a single enantiomer, which suggests the enzyme scaffold binds the alkyne and directs carbene transfer in a well-defined orientation.

For the model reaction with **1a** as the alkyne donor, **C10** in the

form of the whole-cell catalyst exhibited modest activity, with 55 total turnovers (TTN). Directed evolution targeting active-site residues for site-saturation mutagenesis was performed to enhance the overall catalytic efficiency (**Figure 2**). Residue 263, located right above the heme cofactor (in the heme domain), was previously found to play an important role in controlling carbene transfer to phenylacetylene using other P411 variants.¹¹ To our delight, screening the enzyme library made by site-saturation mutagenesis at residue 263 yielded a tryptophan mutation at this site that improved TTN over 11 fold. Sequential mutagenesis targeting sites in the loop regions led to beneficial mutations Q437I, S72F and L436R and afforded the highly efficient variant WIRF, with 2680 TTN towards the desired cyclopropene formation.

P411 **C10**-WIRF's scope of internal alkynes bearing different aromatic rings or carbon chains was then evaluated. For the alkyne

substrates tested (**1b** to **1l**), only cyclopropenes **2c**, **2d**, **2i** and **2j** were synthesized efficiently, and most of the other alkynes with substitutions on the aromatic ring showed poor to moderate reactivities. Thinking that the evolved WIRF variant may have acquired some specificity for the non-substituted aromatic ring or for electron-rich alkynes, we decided to use a less reactive alkyne substrate (compared to **1a**), **1b**, with an electron-deficient *para*-chloro substitution, to further evolve the enzyme (**Figure 2**). A site-saturation library targeting residue 332 afforded mutation S332G, which boosted the total turnover by almost 5 fold. We reasoned that the glycine substitution might help make space in the active site to accommodate substrates with substitutions on the aromatic ring. Mutagenesis of residues close to 332 was investigated, and two additional beneficial mutations, G74A and E70K, yielded the final WIRF_GAK variant with 2320 TTN for substrate **1b**.

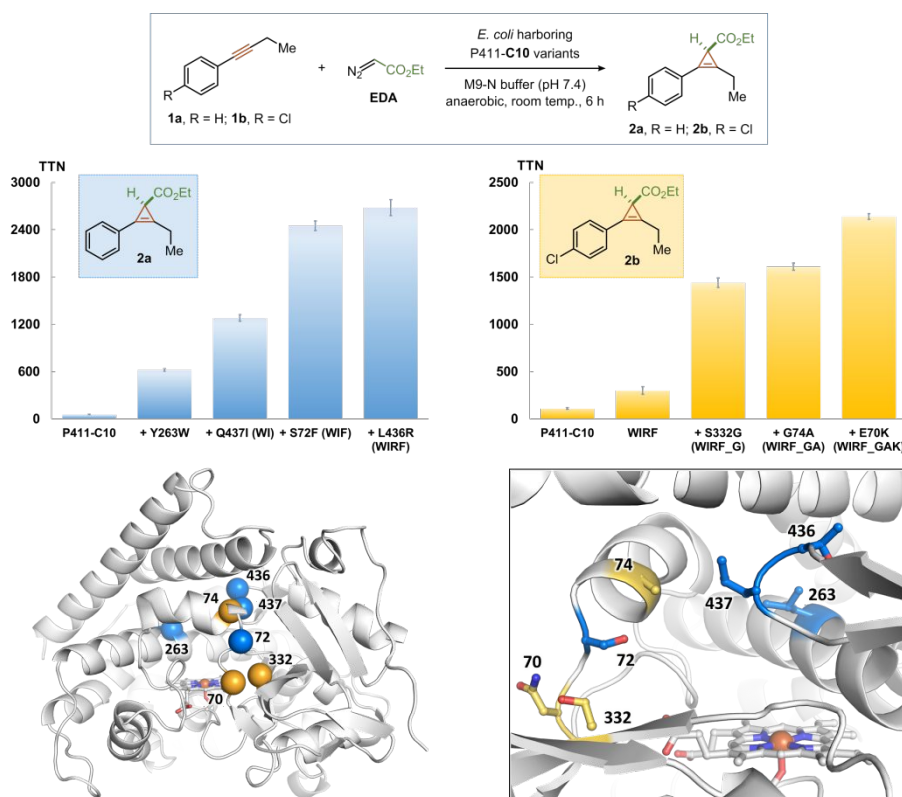


Figure 2. Directed evolution of P411-C10 for internal cyclopropene synthesis. Reaction conditions: 10 mM alkyne, 10 mM EDA, *E. coli* harboring P411-C10 variants ($OD_{600} = 15$ to 60), D-glucose (25 mM), M9-N buffer/EtOH (19:1), anaerobic, 6 h. Product formation was quantified by gas chromatography (GC) and TTNs were determined based on P411 protein concentration. The heme-domain structure of P411-E10 variant (pdb: 5UCW) was used to guide site-saturation mutagenesis; mutation sites are highlighted. See SI for details.

We revisited the substrate scope of this biocatalytic platform using the whole lineage of cyclopropene-forming enzyme variants (from **C10** to WIRF and then to WIRF_GAK) (**Figure 3**). The WIRF variant turned out to be efficient for non-substituted or *ortho*-substituted aromatic alkynes (**1a**, **1c** and **1d**), catalyzing the desired cyclopropene synthesis with 1200 to 2670 total turnovers, while variants from later in the evolution showed impaired activity with these substrates. Although we did not specifically evolve the enzyme for activity on *meta*-substituted aromatic alkynes, variant WIRF_G exhibited improved efficiency for a *meta*-methoxyl alkyne substrate (**1f**), compared to WIRF. For aromatic alkynes bearing *para*-substitutions or *di*-substitutions (**1b** and **1g** to **1l**), the final variant WIRF_GAK catalyzes the desired transformations with unprecedentedly high efficiency compared to all previously reported systems for cyclopropene formation. For instance, an electronically-withdrawing trifluoromethyl-substituted alkyne (**1g**)

was well-accepted by the enzymatic system. It is worth noting that all of the internal cyclopropenes produced enzymatically were determined to be single enantiomers (>99.9% ee for all), which further supports our hypothesis that the engineered enzymes impose a specific binding orientation of the alkyne substrate in the protein active site, allowing for efficient carbene addition to triple bonds with perfect stereocontrol.

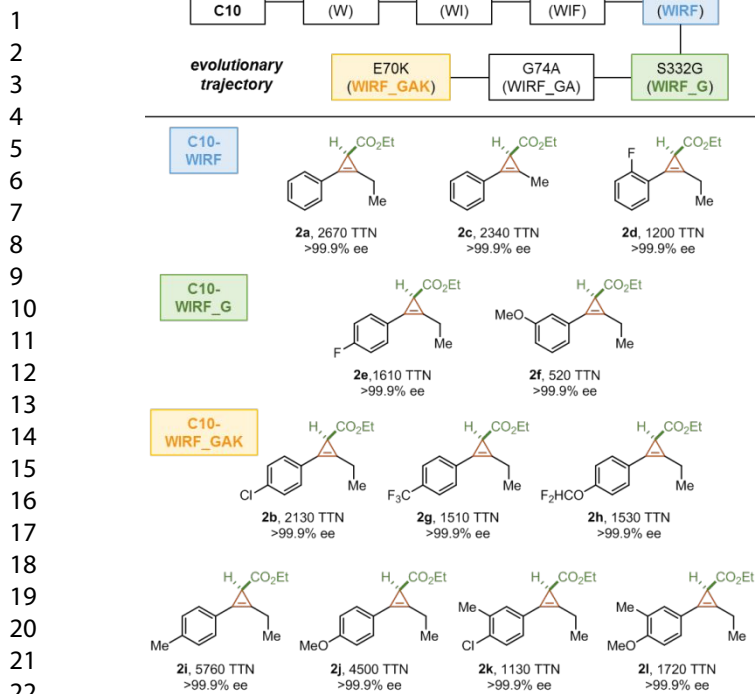


Figure 3. Substrate scope of internal aromatic alkynes for cyclopropane formation. Reactions were performed in quadruplicate under the following conditions: 10 mM alkyne, 10 mM EDA, *E. coli* harboring P411-C10 variants (OD₆₀₀ = 10 to 20), D-glucose (25 mM), M9-N buffer/EtOH (19:1), anaerobic, 16 h. Product formation was quantified by gas chromatography (GC) and TTNs were determined based on protein concentration. See SI for details.

To further demonstrate the utility of this highly stereoselective enzymatic platform, we carried out large-scale preparation of internal cyclopropanes (**Figure 4**). With a simple modification of the reaction conditions using the diazo reagent in excess (2.4 equivalents added in three portions), we obtained high isolated yields of the desired cyclopropane products at mmol scale (90% for **2d** with variant WIRF, and 87% for **2g** with variant WIRF_GAK). Interestingly, the enzyme turnovers of the large-scale reactions are typically higher than those obtained with analytical-scale ones, indicating that the evolved enzymes in whole cells might still retain (partial) activity after the reactions and the turnovers were limited by consumption of the diazo substrate.

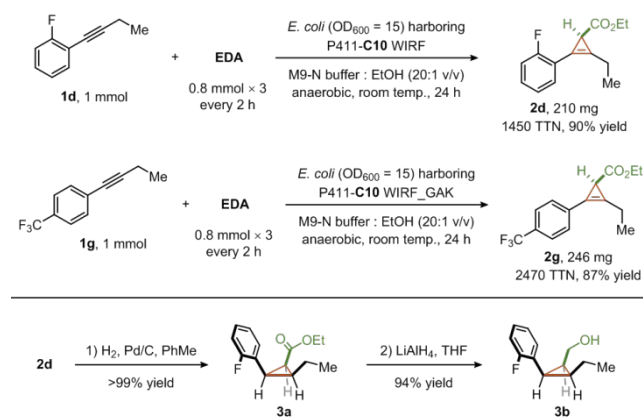


Figure 4. Preparative-scale synthesis of internal cyclopropanes and further derivatization. See SI for details.

Numerous transformations have been developed to furnish diverse molecular structures from versatile cyclopropane building blocks.^{1,2b,2c,6,7} Here, we also derivatized the enzymatically-synthesized cyclopropanes by hydrogenation and ester reduction to afford an all-*cis* cyclopropane product (**Figure 4**), which is otherwise difficult to prepare due to the *cis*-stereochemistry of the three substituents on the cyclopropane ring.

Compared to internal aromatic alkynes described above, internal aliphatic alkynes are typically more challenging targets for enantioselective cyclopropane formation in terms of reactivity and selectivity. As the aryl groups on aromatic alkynes can provide a stabilizing effect through the conjugated system in the carbene transfer process, purely aliphatic alkynes without additional intramolecular effects may suffer from a higher energy barrier for carbene transfer. Additionally, alkyl groups at the two ends of the triple bond are less easy to distinguish than the alkyl and aryl groups on aromatic alkynes. Until now, no systems have been reported for enantioselective cyclopropane synthesis with internal aliphatic alkynes. However, we believed that enzymes can accomplish this, as the enzyme active site is a chiral environment that can recognize minor steric differences for chiral induction.¹⁶

Testing the evolved enzymes for a cyclopropanation reaction with internal aliphatic alkyne **1m** was not fruitful, as only trace activity was observed. However, with the parent enzyme P411-C10 we observed the desired cyclopropane product **2m** (**Figure 5**) with modest activity (43 TTN). This might be because the whole enzyme lineage was evolved for a set of structurally different aromatic alkynes. Further screening of variants in the C10 family identified a triple mutant of C10, C10_VLC, which catalyzed the formation of internal cyclopropane **2m** with improved activity (64 TTN) and perfect stereocontrol (>99% ee). We anticipate that further evolution will lead to more efficient enzymes for internal aliphatic cyclopropane construction, as we have demonstrated for aromatic alkynes.

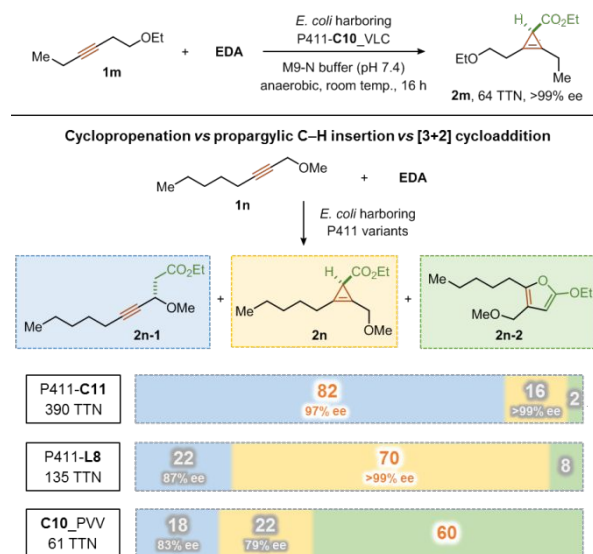


Figure 5. Cyclopropanation of internal aliphatic alkynes and chemoselectivity study with substrate 1n. Reactions were performed in quadruplicate under the following conditions: 10 mM alkyne, 10 mM EDA, *E. coli* harboring P411 variants (OD₆₀₀ = 15 to 20), D-glucose (25 mM), M9-N buffer/EtOH (19:1), anaerobic, 16 h. C10_VLC: C10 T327V Q437L S332C; C11: C10 G74T S118M L162F L401I Q437L; L8: C10 A87P A264S E267D T327P S332A Q437L; C10_PVV: C10 Q437P T327V A87V. See SI for details.

As the parent P411-C10 enzyme was initially engineered for a carbene C–H insertion reaction, we took a deeper look at the chemoselectivity between cyclopropanation and C–H insertion.¹⁵ Internal alkyne substrate **1n**, bearing a propargylic ether group, was found to mainly undergo a carbene insertion reaction into the propargylic C–H bond with high enantioselectivity with catalyst **P411_{CHF}**; a cyclopropene product was also detected as a minor product (see SI for details). However, P411-C10 reversed the chemoselectivity to favor the cyclopropene **2n** as the major product; and a third product observed in low proportion in this latter reaction was confirmed to be a furan derivative, **2n-2**, which may be generated through a [3+2]-cycloaddition.^{11,17} After intensive screening of variants in the families of **P411_{CHF}** and P411-C10, we discovered two related variants, P411-C11 and P411-L8, which could catalyze the C–H insertion reaction and the cyclopropanation reaction with even higher activity and selectivity (compared to **P411_{CHF}** and P411-C10, respectively), as shown in **Figure 5**. And a C10 triple mutant, C10_PVV, was found to flip the chemoselectivity to favor formation of the furan product. These variants are closely related, differing by only a few amino acid substitutions, but gave very different chemoselectivities without any specific enzyme evolution. These results, together with our

ASSOCIATED CONTENT

Supporting Information

Experimental details, and spectral data for all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interests.

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previous demonstration of enzyme-controlled reaction selectivity between C–H insertion and cyclopropanation,¹⁸ highlight how enzyme catalysis can solve chemoselectivity problems in synthetic methodology.

In conclusion, we have developed a versatile biocatalytic platform based on engineered cytochrome P411 enzymes that offers access to an array of structurally diverse internal cyclopropenes through carbene transfer to internal alkynes. This biocatalytic system was evolved rapidly to take internal aromatic alkynes as substrates and furnish the desired cyclopropenes with unprecedentedly high stereoselectivities (>99.9% ee for all). This enzymatic platform is also readily scalable for the production of cyclopropenes in preparative quantities, with even higher efficiencies compared to the analytical-scale reactions. Enantioselective cyclopropanation of internal aliphatic alkynes was also shown to be possible. The versatility and tunability of these biocatalysts has been demonstrated, with chemoselectivity that can be switched among cyclopropanation, carbene C–H insertion and [3+2] cycloaddition. Ongoing studies with this family of P411-C10 variants will help to define the catalytic potential of C10 as a highly promiscuous carbene transferase for non-native transformations.

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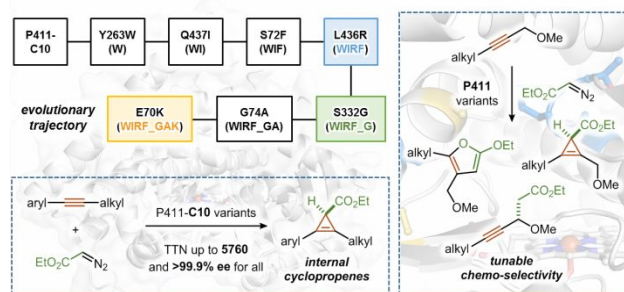
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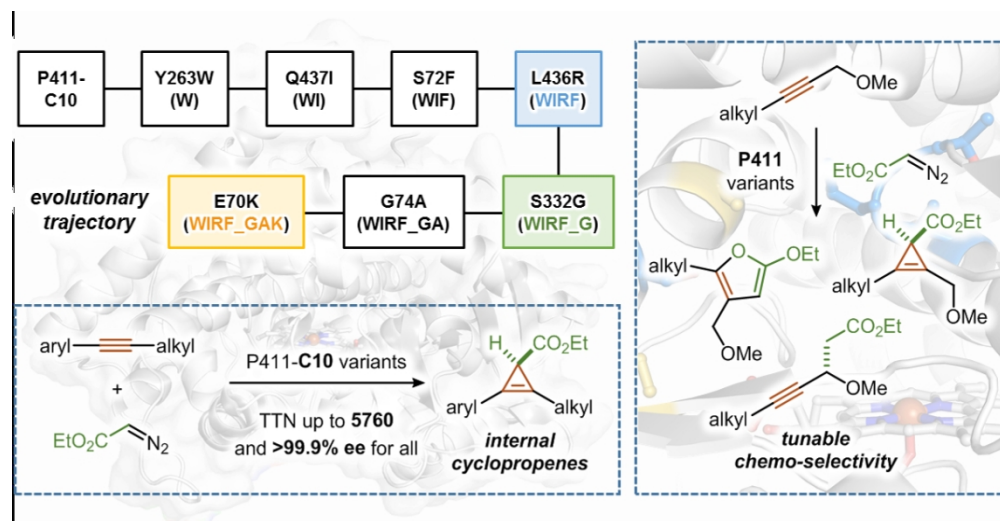
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(18) See refs 10d, 10g and 13 for examples of tunable chemo-selectivities with engineered hemeproteins.

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graphic abstract

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