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Enzymatic Construction of Highly Strained Carbocycles

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Abstract

Small carbocycles are structurally rigid and possess high intrinsic energy due to their significant ring strain. These unique features lead to broad applications, but also create challenges for their construction. We report the discovery and engineering of hemeproteins that catalyze the formation of chiral bicyclobutanes, one of the most strained four-membered systems, *via* successive carbene addition to unsaturated carbon–carbon bonds. Enzymes that produce cyclopropenes, putative intermediates to the bicyclobutanes, were also identified. These genetically-encoded proteins are readily optimized by directed evolution, function in *Escherichia coli*, and act on structurally diverse substrates with high efficiency and selectivity, providing an effective route to many chiral strained structures. This biotransformation is easily performed on preparative scale and the resulting strained carbocycles can be derivatized, opening myriad potential applications.

One Sentence Summary

Heme enzymes engineered by directed evolution catalyze the asymmetric formation of highly strained bicyclobutanes and cyclopropenes.

In cyclic organic molecules, ring strain arises from distortions of bond angle and bond length, steric clashes of non-bonded substituents, and other effects (1). The simplest carbocycles, cyclopropanes and cyclobutanes, possess ring strains of 26–28 kcal/mol (2). Introducing carbon–carbon multiple bonds or bridges to these small ring systems induces additional strain as well as structural rigidity. For example, cyclopropenes with an *endo*-cyclic double bond bear a strain of 54 kcal/mol, whereas bicyclo[1.1.0]butanes, folded into puckered structures, distinguish themselves as one of the most strained four-membered systems with around 66 kcal/mol strain (Figure S1) (2). These carbocycles are particularly attractive intermediates in chemical and materials synthesis, since they can undergo strain-release transformations to furnish a myriad of useful scaffolds (3–6). The structural rigidity imparted by strained rings in supramolecular materials can lead to interesting physical properties, such as mechanical stability (7) and high glass-transition temperature (8). The intrinsic energy of these strained structures can also be relieved in response to exogenous force, which leads to radical changes in physical properties (*e.g.* conductivity), a feature highly desirable for stimulus-responsive materials (9, 10).

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High ring strain, however, greatly increases the difficulty of synthesis. A commonly used method for preparing bicyclobutanes starts from dibromo-2-(bromomethyl)cyclopropane substructures and utilizes organolithium reagents for lithium-halogen exchange followed by nucleophilic substitution under rigorously anhydrous and cryogenic conditions (3). An alternative route relies on the double transfer of a carbene to alkynes, but the few examples in the literature are mostly limited to methylene carbene (11–13). Asymmetric bicyclobutane construction is particularly challenging, with multiple chiral centers generated at the same time (14, 15) (Figure S2). Cyclopropene synthesis through enantioselective single carbene addition to alkynes also requires chiral transition metal catalysts based on rhodium (16, 17), iridium (18) and cobalt (19). Development of a sustainable catalytic system that performs with high efficiency and selectivity under ambient conditions would be a significant advance for construction of these useful, highly strained carbocycles.

Enzymes, the catalytic workhorses of biology, are capable of accelerating chemical transformations by orders of magnitude while exhibiting exquisite control over selectivity (20). Although nature synthesizes various cyclopropane-containing products (21), cyclopropene or bicyclobutane fragments are extremely rare (Figure S3) (22, 23). This may be attributed to the lack of biological machinery for synthesizing these motifs and/or the instability of these structures under biological or natural product isolation/purification conditions. Nevertheless, we envisioned that existing enzymes could be repurposed to forge strained carbocycles by taking advantage of their catalytic promiscuity (24, 25) in the presence of non-natural substrates and by using directed evolution to increase the newly-discovered activity and selectivity (26).

In the past several years, we and others have engineered natural hemeproteins to catalyze reactions not known in nature (27–32). We hypothesized that carbene transfer to triple bonds with a heme-dependent enzyme might afford highly strained cyclopropene and bicyclobutane structures and do so enantioselectively. We anticipated several challenges at the outset, especially in bicyclobutane formation, as it involves two sequential carbene additions to the alkyne substrate: 1) the enzyme would need to bind the alkyne in a specific conformation in order to transfer the carbene enantioselectively; 2) the high-energy cyclopropene intermediate generated by the first carbene addition would need to be accepted and stabilized by the protein; 3) compared to methylene carbene used previously, a substituted carbene (*e.g.* with an ester group) might hinder access of the cyclopropene to the iron-carbenoid; and 4) the protein would also be expected to possess precise stereocontrol over the second carbene transfer step regardless of structural differences between the initial alkyne and the cyclopropene intermediate. Despite these challenges, we decided to investigate whether a starting enzyme with this unusual and non-natural activity could be identified, and whether its active site could be engineered to create a suitable environment for substrate binding, intermediate stabilization, and selective product formation.

We first tested whether free heme (± bovine serum albumin (BSA)), which is known to catalyze styrene cyclopropanation (27), could transfer carbene to an alkyne. Reactions using ethyl diazoacetate (EDA) and phenylacetylene (**1a**) as substrates in neutral buffer (M9-N minimal medium, pH 7.4) at room temperature, however, gave no cyclopropene or bicyclobutane product. Next, a panel of hemeproteins including cytochromes P450,

cytochromes P411 (P450 with an axial serine ligand), cytochromes *c* and globins in the form of *E. coli* whole-cell catalysts were tested for the desired transformation under anaerobic conditions (32), but none were fruitful (Figure 1C and Table S1). Interestingly, a P411 variant obtained in a previous cyclopropanation study, P411-**S1** I263W (see Supplemental Materials for sources, sequences and mutations), afforded a furan product (**3b**) with a total turnover number (TTN) of 210. Since other furan analogs have been identified as adducts of carbenes and alkynes (33), we were curious as to how furan **3b** was generated. Preliminary kinetic study of the enzymatic reaction suggested that the enzyme first synthesized an unstable cyclopropene (**3a**), which subsequently rearranged to the furan either spontaneously or with assistance from the enzyme (Figure 1B and S5). This result provided strong evidence that the P411 hemeprotein is capable of transferring a carbene to an alkyne, which is, to our knowledge, an activity not previously reported for any protein or even any iron complex.

To divert the enzymatic reaction to bicyclobutane formation, the enzyme is required to transfer a second carbene to cyclopropene intermediate **3a** before the cyclopropene rearranges to the undesired furan product (Figure 1B). We thus tested P411 variants closely related to P411-**S1** I263W. We reasoned that amino acid residue 263, which resides in the distal pocket, above the heme cofactor, might modulate the rate of this step and that the bulky tryptophan (Trp) side chain at this site may be blocking the second carbene transfer. A P411-**S1** variant with phenylalanine (Phe) instead of Trp at this position (I263F) in fact catalyzed bicyclobutane formation at a very low level (< 5 TTN) (Table S1). Variant '**P4**' with 3 additional mutations relative to P411-**S1** I263F (V87A, A268G and A328V) (28) synthesized the desired bicyclobutane **2a** with 80 TTN and with the formation of furan adduct substantially suppressed (**2a**: **3b** > 50: 1, Figure 1C). Another related P411 variant, **E10** (= **P4** A78V A82L F263L), which was engineered from **P4** for nitrene transfer reactions (29), catalyzed the desired transformation with > 6-fold higher activity (530 TTN, Figure 1E). NMR analysis revealed an *exo*, *endo*-configuration of the enzymatically-produced bicyclobutane **2a**, which is distinct from the only reported achiral *endo*, *endo*-isomer, made using an osmium-porphyrin complex (34, 35). We chose this P411-**E10** variant as the starting template for directed evolution of a more efficient bicyclobutane-constructing enzyme.

Because the side chain of residue 263 influenced formation of the bicyclobutane product, we performed site-saturation mutagenesis (SSM) of variant **E10** at position 263 and screened whole *E. coli* cells expressing the mutated proteins for improved production of bicyclobutane **2a**. The enzyme having leucine at this position (263L) was the most active; other amino acid residues either lowered the reactivity towards bicyclobutane formation and/or delivered more furan product. In parallel, two additional residues in **E10**, V78 and S438, were also targeted by SSM. Aromatic residues were found to be activating at 78, with a phenylalanine or tyrosine mutation giving 1.5 – 2-fold improvement over **E10**. This beneficial mutational effect may stem from a **π-π** stacking interaction between the side chain and the alkyne substrate or the cyclopropene intermediate. A single S438A mutation on a loop residing above the heme also significantly increased the activity, giving >2.5-fold increase in turnover. Finally, recombination of V78F/Y and S438A mutations led to the

discovery of even more powerful biocatalysts for bicyclobutane formation (*e.g.* 1880 TTN with **E10** V78F S438A, Figure 1E and S9).

With the evolved **E10** V78F S438A variant in hand, we next assayed the bacterial catalyst against a panel of aromatic alkyne coupling partners. Biotransformations with 10 different substrates were performed on 0.1 – 0.2 mmol scale. These preparative-scale reactions proceeded smoothly to furnish the corresponding bicyclobutanes with up to 1760 TTN and 80% yield (Figure 2A). Additionally, three alkynes, **1k**, **1l** and **1m**, were transformed in mmol scale, and bicyclobutanes were isolated in hundred-milligram quantities, demonstrating that the biocatalytic transformation is readily scalable. Among the 13 different substrates, the engineered P411 hemeprotein did not exhibit strong preference toward specific electronic or steric features. Electron-deficient halides (**2b**–**2d**), which can be used as pre-functionalities for further transformations as well as electron-rich alkyl or alkoxyl groups (**2e**–**2h** and **2k**) at *meta*- or *para*-position of the phenyl group were accepted by the enzyme. Even heterocyclic substrates such as thiophene (**2j**) served as suitable alkyne partners, albeit with lower reactivity.

Free functionalities, including alcohols (**2i** and **2m**) and a second alkyne (**2l**), are well-preserved, providing an additional opportunity for derivatization of these products. A terminal alkyne allows copper-catalyzed click chemistry, through which bicyclobutane **2l** can be modified with a simple sulfonyl azide (**4a**) or even decorated with biologically relevant fragments, such as a phenylalanine derivative (**4b**). An unprotected hydroxyl group could also offer the possibility of linkage to useful structures. Additionally, in order to probe the enantiopurity of bicyclobutane products, we derivatized **2l** and **2m** with *L*-azido-phenylalanine and (*R*)-Mosher's acid, respectively. The diastereomeric excess of these derivatized products would inform us the enantiomeric ratio of the bicyclobutanes. In fact, we observed only one diastereomer of derivatized bicyclobutanes **4b** and **4c** by NMR. Furthermore, the dicarboxylic esters on the bicyclobutane structure can be reduced easily with a mild reducing reagent, LiBH4, to give diol product **4d** with the strained ring structure preserved. The diol product **4d** allowed for the unequivocal confirmation of the bicyclobutane structure and determination of the absolute configuration through X-ray crystallography.

We next asked whether the enzyme could stop at the cyclopropene product if less reactive aliphatic alkynes are used. To this end, we examined enzyme variants from the P411-**S1** lineage for cyclopropene formation, using phenylbutyne (**5a**) and EDA as starting reagents. Encouragingly, **P4** catalyzed the desired transformation with 260 TTN and 95.5: 4.5 *er*. Further evolution was performed on **P4** to improve its catalytic efficiency. We first targeted position 87, known for its importance to substrate recognition in P450-catalyzed oxidations (36). A87F (290 TTN, 3.0: 97.0 *er*) and A87W (240 TTN, 97.1: 2.9 *er*) were found to exert the opposite enantio-preference, suggesting that residue 87 also controls substrate orientation for non-native carbene chemistry. Using **P4** A87F and **P4** A87W as parents, single- and double-site-saturation mutagenesis were conducted sequentially to improve both reactivity and selectivity (Figure 3A, S11 and S13). The final **K10** and **C6** variants performed with >10-fold higher reactivity compared to the initial **P4** variant and with excellent stereocontrol (99.55: 0.45 *er* and 99.95: 0.05 *er*, respectively).

To evaluate the substrate range of the evolved P411 variants for cyclopropene construction, we focused on P411-**C6** and examined structurally diverse aliphatic alkynes. Enzymatic reactions with 12 alkynes in preparative scale (up to 5.0 mmol scale) afforded the desired cyclopropenes with TTNs ranging from hundreds to thousands and good to excellent stereoselectivities (Figure 3B and 3C). Alkynes with a linear carbon chain (**5b**) or cyclic fragments (**5g**, **5h** and **5j**) all served as good substrates. Different functional groups, including ether (**5f**, **5i** and **5l**), ester (**5d**), acetal (**5e**), chloride (**5k**), and free hydroxyl (**5m**), were well-tolerated. Further optimization of reaction conditions with slow addition of EDA, for example, would likely improve the isolated yields, as we demonstrated for cyclopropene **6h** (66% yield, Figure 3B; and 94% yield, Figure 3C).

Cyclopropenes are used as synthetic building blocks (4, 37), bio-orthogonal imaging precursors (38), and monomers in polymer synthesis (39). Our ability to construct these motifs using bacteria at scale allows us to further explore their potential utility in diverse fields. Here we present two simple transformations of cyclopropenes to build a multi-substituted cyclopropane **7a** and a fused ring system, bicyclo[4.1.0]heptene **7b** (Figure 3C), both of which are substructures common in pharmaceutical candidates and bioactive natural products (21).

In conclusion, we have developed a biocatalytic platform for the construction of highly strained bicyclobutanes and cyclopropenes through directed evolution of a serine-ligated cytochrome P450 (P411) enzyme. That the protein could be quickly adapted to produce these highly strained structures (2–6 mutational steps) highlights the evolvability of the P411 scaffold and its potential to direct the construction of complex motifs. The protein enabled the desired transformations through activation of iron-carbenoid for carbene addition to alkynes, stabilization of the reactive cyclopropene intermediate (in bicyclobutane formation), and precise stereocontrol of the carbene transfer processes. Biotransformations with the evolved enzymes have a surprisingly broad substrate scope with high reactivity and selectivity, providing a route to more than 25 products in preparative scale. This biocatalytic system grants facile access to versatile molecular architectures rarely seen in nature, expanding the set of chemical structures available to biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Facility for analytical support, and the Brian Stoltz lab for use of their polarimeter and chiral gas chromatography. A provisional patent application has been filed through the California Institute of Technology based on the results presented here. Crystallographic coordinates and structure factors have been deposited with the Cambridge Crystallographic Data Centre (https://www.ccdc.cam.ac.uk/) under reference number 1815089 for compound **4d**.

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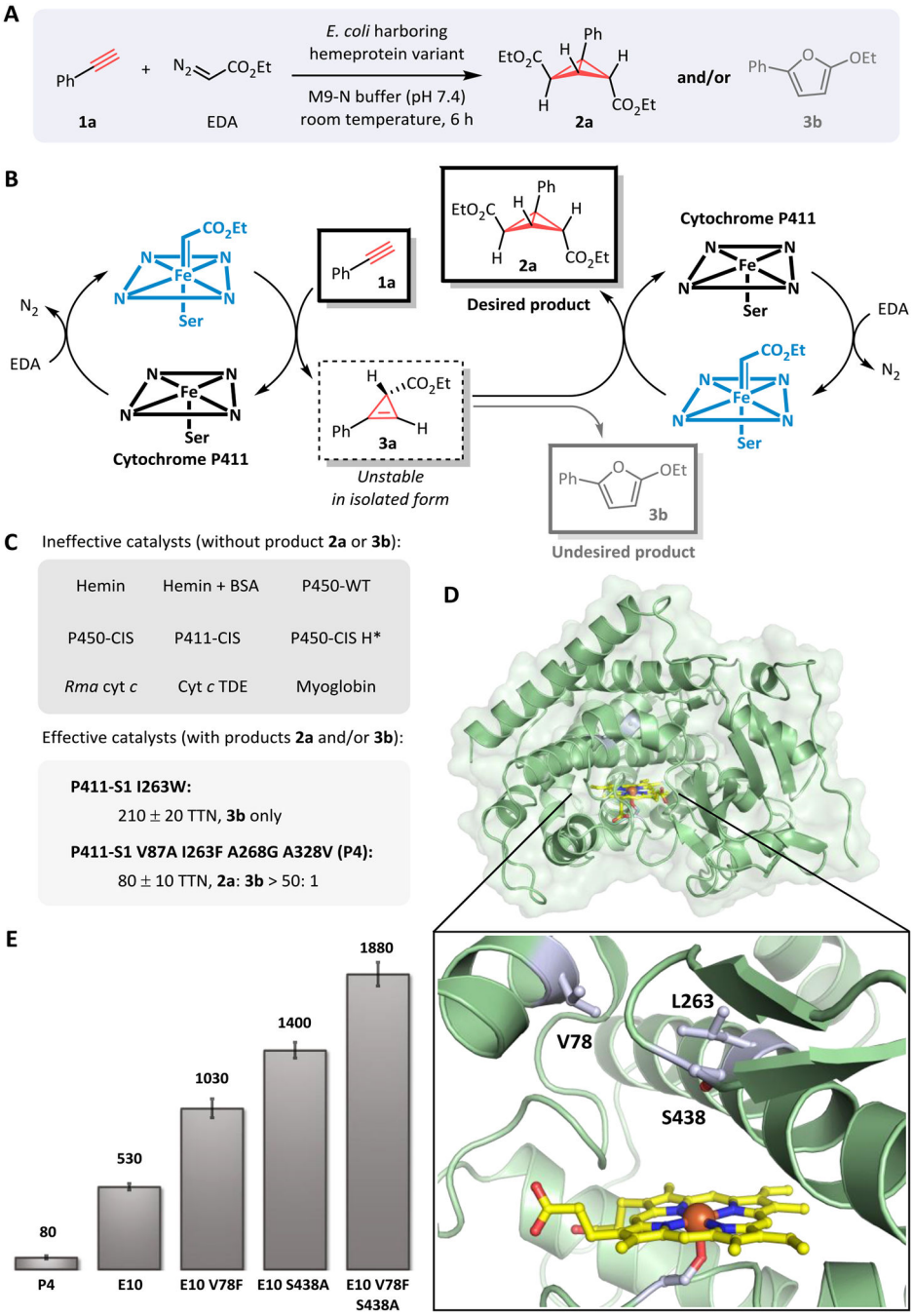

Fig. 1.

(A) Overall reaction of carbene transfer to an alkyne catalyzed by an engineered hemeprotein. (B) Proposed catalytic cycle of carbene transfer to phenylacetylene to form cyclopropene and bicyclobutane structures. (C) Screening of hemin and hemeprotein catalysts for bicyclobutane formation (BSA = bovine serum albumin; for sources, sequences, and mutations in *Bacillus megaterium* P411-**S1** and other proteins, see Supplementary Materials). **(D) X-ray crystal structure of P411-E10 (PDB ID: 5UCW) and view of its distal heme region**. The heme axial ligand is S400, and amino acid residues

V78, L263 and S438 are shown as gray sticks. **(E) Directed evolution of P411-E10 for bicyclobutane formation** (using phenylacetylene and EDA as substrates; numbers refer to total turnovers to product (TTN) measured). Experiments were performed on analytical scale using suspensions of *E. coli* expressing P411-**E10** variants (OD600 = 10–30), 10 mM phenylacetylene, 10 mM EDA, 5 vol% EtOH, M9-N buffer (pH 7.4) at room temperature under anaerobic conditions for 6 h. Reactions performed in quadruplicate. Here TTN refers to the total desired product, as quantified by gas chromatography (GC), divided by total hemeprotein. (Note: because bicyclobutane formation requires two carbene transfers, the number of carbene transfers the hemeprotein catalyzes is 2 x TTN in these reactions.) Further details on reaction conditions and data analysis are provided in the Supplementary Materials.

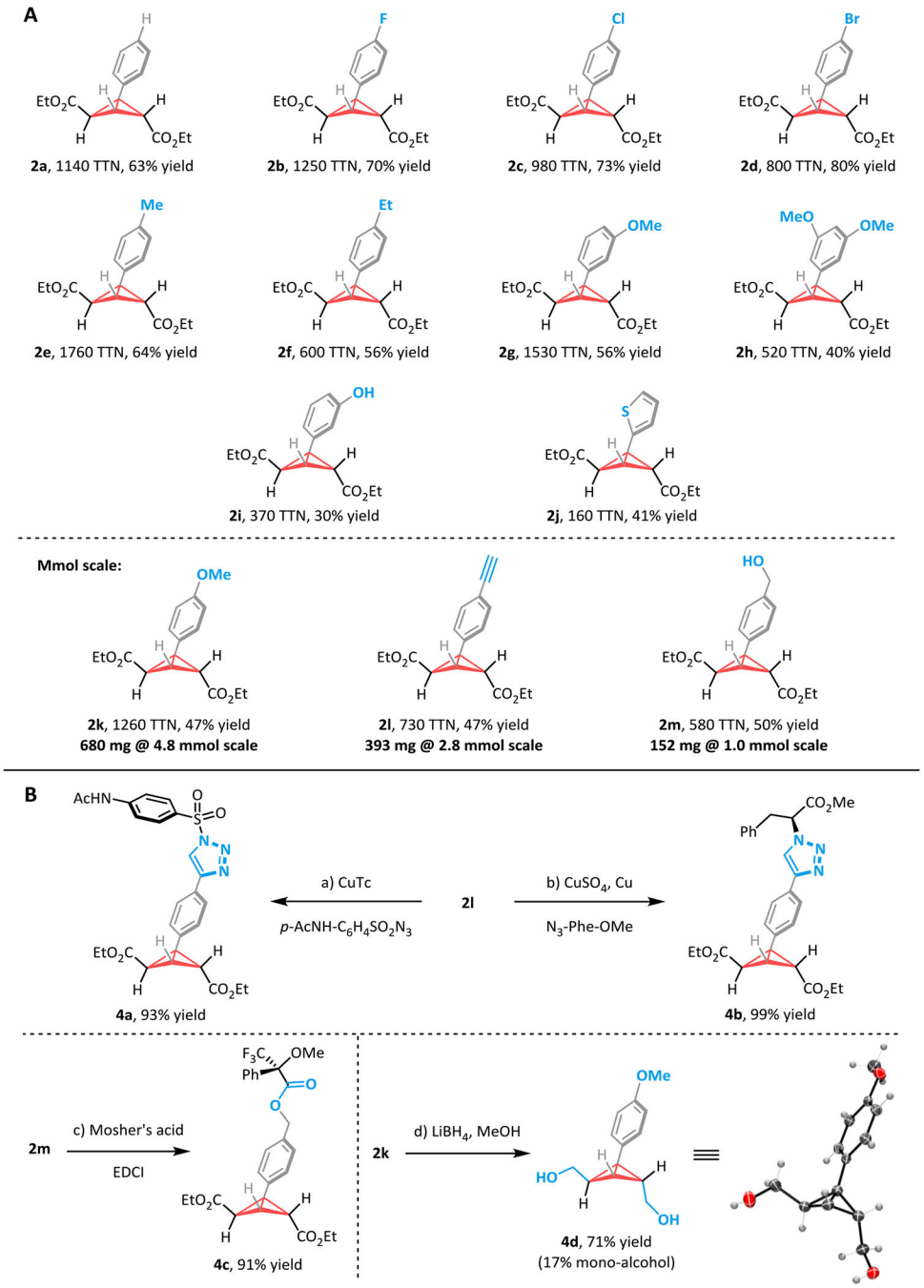

Fig. 2.

(A) Scope of P411-E10 V78F S438A-catalyzed bicyclobutane formation. Standard conditions of preparative-scale reactions (0.1 – 0.2 mmol scale, unless otherwise indicated): suspension of *E. coli* (OD600=15 – 20) expressing P411 **E10**-V78F S438A, 1.0 equiv aromatic alkyne, 2.0 – 4.0 equiv EDA, 10 – 15 mM *D*-glucose, 1 – 5 vol% EtOH, M9-N buffer (pH 7.4) at room temperature under anaerobic conditions for 12 hours. Isolated yields. TTN determined based on isolated yields. **(B) Derivatization of bicyclobutane products**. a) and b) copper-catalyzed click cyclization of **2l** with azide substrates; c) esterification of **2m**

with Mosher’s acid; d) reduction of **2k** to diol with LiBH4. Further details on reaction conditions and data analysis are provided in the Supplementary Materials.

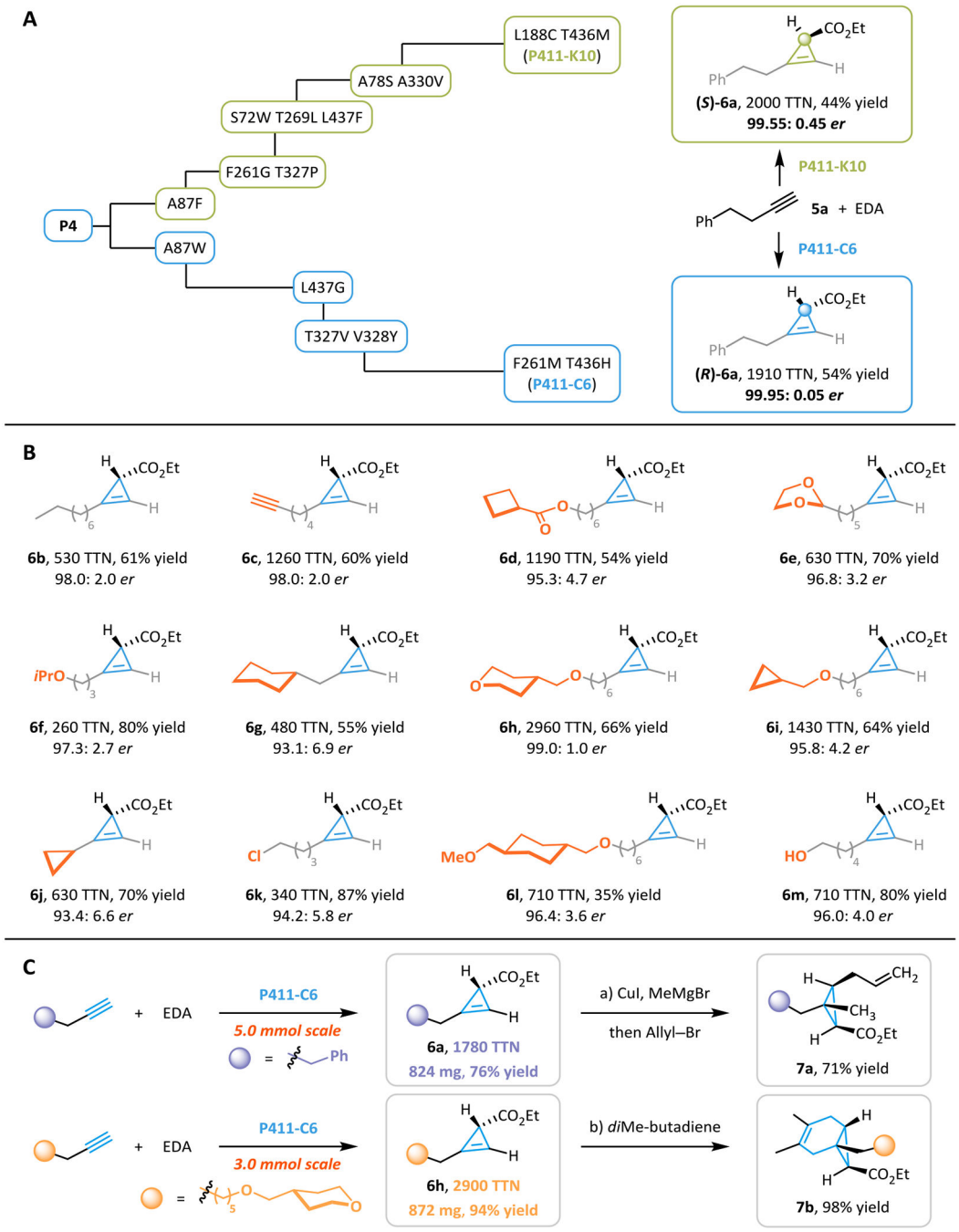

Fig. 3.

(A) Evolutionary trajectory of P411-P4 variants for stereodivergent cyclopropenation of aliphatic alkynes. (B) Scope of P411-C6-catalyzed cyclopropene formation. Conditions of preparative-scale reactions (0.08 – 0.4 mmol scale): suspension of *E. coli* expressing P411-**C6** or **K10** (OD600 = 10 – 32), 10 – 150 mM alkyne, 1.0 – 4.0 equiv EDA (6.0 equiv for **5m**), 10 – 15 mM *D*-glucose, 1 – 5 vol% EtOH, M9-N buffer (pH 7.4) at room temperature under anaerobic conditions for 12 hours. Isolated yields. TTN determined based on isolated yields and enantiomeric ratio (*er*) determined by chiral HPLC. **(C) Enzymatic**

cyclopropenation at mmol scale and derivatization of corresponding products. a) copper-catalyzed addition to cyclopropene **6a** for synthesizing a multi-substituted cyclopropane; b) Diels-Alder reaction of cyclopropene **6h** with 1,3-*di*Me-butadiene to form a fused ring system. Further details on reaction conditions and data analysis are provided in the Supplementary Materials.