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Navigating the Unnatural Reaction Space: Directed Evolution of Heme Proteins for Selective Carbene and Nitrene Transfer

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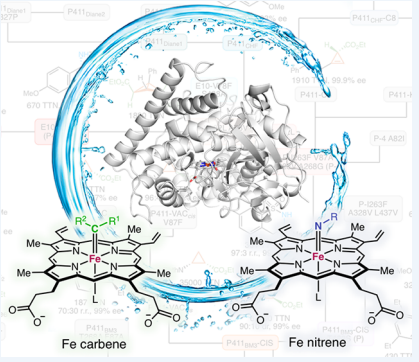
CONSPECTUS: Despite the astonishing diversity of naturally occurring biocatalytic processes, enzymes do not catalyze many of the transformations favored by synthetic chemists. Either nature does not care about the specific products, or if she does, she has adopted a different synthetic strategy. In many cases, the appropriate reagents used by synthetic chemists are not readily accessible to biological systems. Here, we discuss our efforts to expand the catalytic repertoire of enzymes to encompass powerful reactions previously known only in small-molecule catalysis: formation and transfer of reactive carbene and nitrene intermediates leading to a broad range of products, including products with bonds not known in biology. In light of the structural similarity of iron carbene (Fe=C(R^1)(R^2)) and iron nitrene (Fe=NR) to the iron oxo (Fe=O) intermediate involved in cytochrome P450-catalyzed oxidation, we have used synthetic carbene and nitrene precursors that biological systems have not encountered and repurposed P450s to catalyze reactions that are not known in the natural world. The resulting protein catalysts are fully genetically encoded and function in intact microbial cells or cell-free lysates, where their performance can be improved and optimized by directed evolution. By leveraging the catalytic promiscuity of P450 enzymes, we evolved a range of carbene and nitrene transferases exhibiting excellent activity toward these new-to-nature reactions. Since our initial report in 2012, a number of other heme proteins including myoglobins, protoglobins, and cytochromes *c* have also been found and engineered to promote unnatural carbene and nitrene transfer. Due to the altered active-site environments, these heme proteins often displayed complementary activities and selectivities to P450s.



Using wild-type and engineered heme proteins, we and others have described a range of selective carbene transfer reactions, including cyclopropanation, cyclopropenation, Si–H insertion, B–H insertion, and C–H insertion. Similarly, a variety of asymmetric nitrene transfer processes including aziridination, sulfide imidation, C–H amidation, and, most recently, C–H amination have been demonstrated. The scopes of these biocatalytic carbene and nitrene transfer reactions are often complementary to the state-of-the-art processes based on small-molecule transition-metal catalysts, making engineered biocatalysts a valuable addition to the synthetic chemist's toolbox. Moreover, enabled by the exquisite regio- and stereocontrol imposed by the enzyme catalyst, this biocatalytic platform provides an exciting opportunity to address challenging problems in modern synthetic chemistry and selective catalysis, including ones that have eluded synthetic chemists for decades.

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amination to furnish enantioenriched unprotected primary amines.[4]

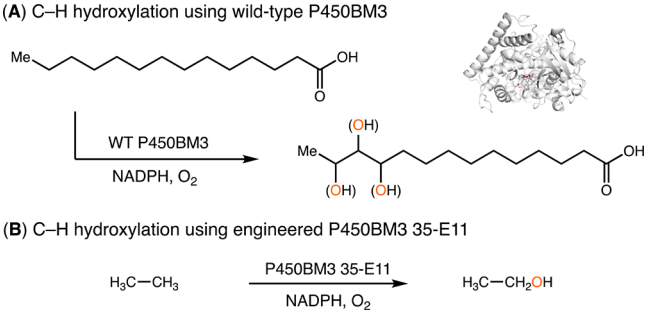
INTRODUCTION

Nature's malleable protein catalysts, enzymes, are capable of facilitating highly demanding transformations with exquisite control.[5−7] A significant fraction of enzymes assemble and exploit metal ions for catalysis. These metalloenzymes catalyze some of the most challenging biological reactions, including photosynthesis,[8] nitrogen fixation,[9] methane oxidation[10] and methylmalonyl-CoA isomerization,[11] with unique reactivity not observed in small-molecule organometallic complexes or enzymes based on purely organic cofactors.

Our laboratory has been fascinated by enzymes of the iron-containing cytochromes P450 (CYPs) family for decades. P450s constitute a subset of hemoproteins using the heme prosthetic group for their native functions.[12,13] Found in all kingdoms of life, P450s catalyze extraordinary oxygenation reactions under exceptionally mild conditions.[14−16] In these P450-catalyzed oxidation reactions, a Fe(IV)═O porphyrin radical cation species (compound I) is the key oxidizing intermediate for oxene transfer reactions.[17] Nature has tamed this intermediate and diversified its reactivity for a vast array of oxidative transformations.

Using P450BM3 from *Bacillus megaterium* (CYP102A1),[18] a self-sufficient P450 naturally fused to its diflavin redox partner, our group[19−23] and others[12,18,24,25] have engineered highly active P450 variants for the oxidation of simple alkanes and other organic substrates (Scheme 1). Starting a decade ago, our

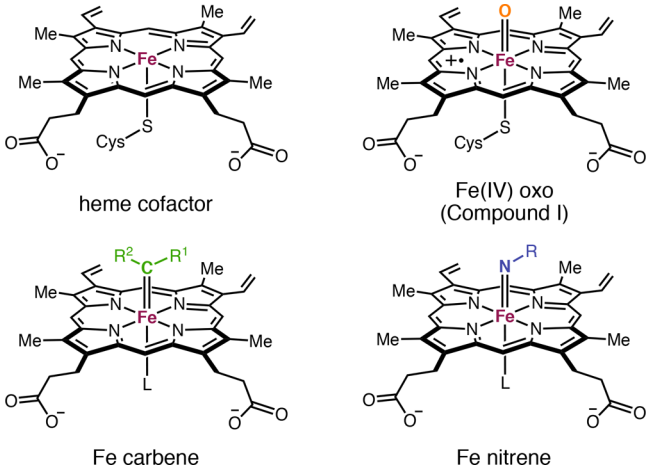
Scheme 1. Native and Engineered Activity: P450BM3-Catalyzed C−H Hydroxylation



group became interested in expanding the already immense catalytic repertoire of P450s to encompass reactions not known in the biological world. Inspiration came from two sources: (1) Evolution does it—new activities arose during P450 evolution, and there was no reason to think that further innovation was not possible using evolutionary engineering methods. (2) Creative chemists had already shown that the heme cofactor and various derivatives can catalyze reactions not known in nature.[26−29] We reasoned that some of these should be available to the heme cofactor embedded in a protein, and that directed evolution could be used to draw out these latent functions.

Of special interest to us were the myriad carbene[30−33] and nitrene[34−36] transfer reactions that synthetic chemists had developed. Metal porphyrin complexes,[26,27] including Fe porphyrins, are privileged catalysts for such transformations. As the vital intermediates in these metalloporphyrin-catalyzed processes, metal carbenes and nitrenes supported by the porphyrin ligand have been extensively investigated. In light of the structural similarity between these synthetic Fe carbenes and Fe nitrenes and the native Fe oxene (compound I), we thought we should be able to generate Fe carbene and nitrene intermediates in P450s by exploiting synthetic precursors not present in nature (Scheme 2). Furthermore, we posited that the

Scheme 2. Structural Similarity of Fe Oxene, Carbene, and Nitrene



selective transfer of these reactive species to organic substrates within the enzyme active site would lead to a similarly broad spectrum of synthetically useful transformations.

In 2012 and 2013, we described repurposing P450s for new-to-nature carbene and nitrene transfer reactions.[1,37] Since then, a plethora of biocatalytic stereoselective C−C, C−N, C−Si, and C−B bond forming processes, including ones that are difficult or impossible to achieve with current small-molecule catalysts, have been developed by us and by other research groups who have joined this exciting journey of discovery. In this Account, we survey the evolution of heme proteins for non-natural carbene and nitrene transfer reactions in our lab. We also present the conceptual advances and strategies that were pivotal to this development.

NON-NATURAL BIOCATALYTIC CARBENE TRANSFER: INITIAL DISCOVERY

At the outset of our efforts to create new-to-nature enzymes, designing an enzyme to catalyze a new reaction, especially one that is unknown in biochemistry, was widely regarded as a formidable challenge. After all, building a new active site requires placement of multiple functional groups in a binding pocket that can orient substrate(s) and stabilize one or more transition states, depending on the reaction. Designing enzymes *de novo* was (and still is) notoriously difficult, and it was not clear how a conservative process of directed evolution, one mutation at a time, could create catalytic novelty.

However, evolution has done it countless times. Less appreciated perhaps by the admirers of enzymes' specificity and selectivity is the fact that many enzymes are also catalytically "promiscuous":[38−40] they are capable of catalyzing, at some level, chemical reactions unrelated to their biological functions. In the history of evolution, this promiscuity has played a central role in the emergence of new biological functions.[41] In recent years, the significance of catalytic promiscuity has been increasingly recognized, and the structural, mechanistic, and evolutionary implications of catalytic promiscuity are being

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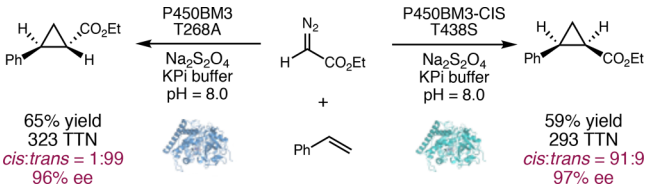
elucidated.[42,43] Inspired by nature's strategy, we sought to harness promiscuity to discover new enzyme chemistries, especially ones that are synthetically useful and challenging to achieve with small-molecule catalysts. In this context, the demonstrable promiscuity of P450s originating from their structural flexibility[44] makes them excellent candidates for discovery of new-to-nature activities.

For our initial study, we focused on enantioselective cyclopropanation of alkenes. An enantioenriched, polysubstituted cyclopropane is a common structural element in a variety of medicinal agents. More importantly, Woo previously showed that Fe porphyrin complexes could catalyze such transformations in a racemic fashion.[45] We discovered that various heme proteins, including myoglobin and cytochrome *c*, exhibited the desired activity, but a large in-house collection of P450BM3 variants accumulated during our research on P450 oxygenation proved instrumental to showing that selectivity and activity were readily tunable by protein engineering. Upon evaluating a panel of P450BM3 variants, we found that several displayed dramatically enhanced cyclopropanation activity relative to hemin in water, as well as promising enantioselectivity and complementary diastereoselectivity (Scheme 3).[1] Specifically, P450BM3 with a single T268A mutation exhibited excellent *trans* selectivity for the cyclopropanation of styrene. In contrast, variant P450BM3-CIS (9-10A-TS F87 V T268A)[46] showed promising *cis* selectivity, which is rarely observed in small-molecule-catalyzed cyclopropanation processes. It also represented a departure from the innate diastereoselectivity of Fe porphyrin-catalyzed cyclopropanation,[47] highlighting the role of the protein secondary coordination sphere in controlling the reaction selectivity.

Scheme 3. P450-Catalyzed Diastereo- and Enantioselective Cyclopropanation of Styrenes (2012)[a]



[a]TTN = total turnover number. Conditions: 30 mM styrene, 10 mM EDA, 0.2 mol % catalyst, and 10 mM $Na_2S_2O_4$.

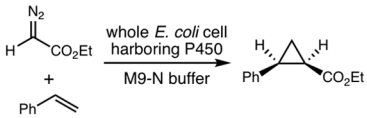
Mutation T268A was found to be generally activating for cyclopropanation in a series of P450BM3 variants, likely by enlarging the active site for carbene transfer. The T268 residue in P450BM3 operates as the essential hydrogen bond donor in the cleavage of the O−O bond in the native O_2 activation and oxygenation reaction.[48] Previous investigation showed that replacing this threonine with an alanine led to a substantial decrease in the native oxygenase activity. The activating effect of T268A in cyclopropanation thus implies fundamental mechanistic dichotomy between the native oxene transfer and the non-native carbene transfer chemistry.[49]

AXIAL SERINE-LIGATED "P411" FOR CARBENE TRANSFER

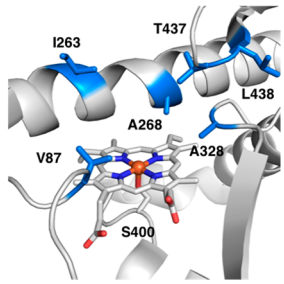
For cyclopropanation, the P450 has to be reduced to its ferrous state to initiate the catalytic cycle. In our early work, P450s were used either as purified protein or in cell-free extract in the presence of exogenous reducing agents such as sodium dithionite ($Na_2S_2O_4$). We reasoned that we would be able to develop an *in vivo* cyclopropanation if the heme enzyme could be readily reduced by endogenous reducing agents such as NAD(P)H. Such whole-cell biocatalysts would greatly simplify the protein engineering process and enable new routes to the sustainable production of valuable compounds.

We replaced the cysteine thiolate ligand in P450BM3 with a weakly donating serine (Scheme 4). The CO-binding assay of this engineered P450 variant revealed a ferrous CO Soret peak at 411 nm instead of 450 nm in the UV−vis spectrum,[50] which is why we refer to the axial-serine-ligated P450 variants as "P411" enzymes. Replacement of this critical Cys residue was known to abolish its monooxygenation activity; in fact, the cysteine thiolate ligand is the *only* amino acid residue that is conserved in all known functional P450s. Electrochemical measurements indicated that the redox potential $E^{\circ\prime}$(Fe(III)/Fe(II)) of P411 shifted to −293 mV, which is well within the range of reduction by NADPH ($E^{\circ\prime}$ = −320 mV). P411BM3-CIS demonstrated dramatically enhanced *in vivo* activity for cyclopropanation. By further increasing the substrate concentration, total turnover numbers of up to 67 800 were achieved in whole *Escherichia coli* cells (Scheme 4). This represented the first highly active biocatalyst for carbene transfer. The advent of P411s turned out to be a key advance for biocatalytic carbene and nitrene transfer reactions. In subsequent studies, we found that serine-ligated P411s were uniquely active for a range of new-to-nature reactions where P450s displayed no or negligible activity.

Scheme 4. Rationally Engineered "P411" Catalyst for *in Vivo* Cyclopropanation (2013)



catalyst	TTN	*cis*:*trans*	ee
P450BM3-CIS	950	22:78	60%
P411BM3-CIS	3700	76:24	96%
P411BM3-CIS[a]	67800	90:10	99%



[a]Reaction carried out with neat substrates (170 mM EDA) for 24 h. All other reactions were carried out with 8.5 mM EDA.

FURTHER DEVELOPMENT OF BIOCATALYTIC CYCLOPROPANATION

Expansion of biocatalytic cyclopropanation followed two directions: (1) We further engineered P450BM3 variants to broaden the substrate scope of cyclopropanation, and (2) we explored other heme proteins for biocatalytic cyclopropanation. In subsequent P450BM3 engineering, we continued to explore the role of the Fe-binding axial ligand. Heme proteins naturally use a range of axial ligands, including cysteine, histidine, and tyrosine. The identity of the axial ligand profoundly influences the catalytic activity of heme enzymes.[51] We found that the C400H variant exhibited the highest activity for *in vivo* cyclopropanation, while the C400Y and C400S variants shared similar kinetic profiles (Scheme 5).[52]

Starting from P450BM3 T268A C400H, iterations of site-saturation mutagenesis (SSM) and screening furnished P450BM3 Hstar (Schemes 6),[52] a highly active catalyst for the cyclopropanation of a wide range of α-substituted acrylamides

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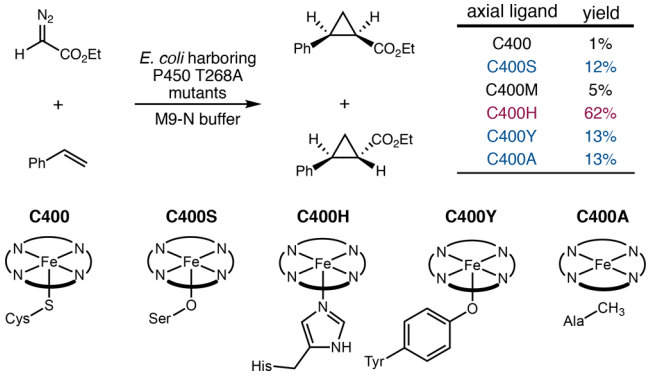
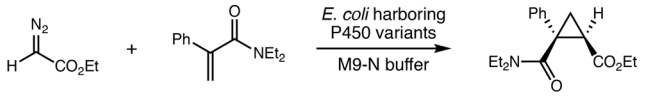
Scheme 5. Axial Ligand Effect in the Biocatalytic Cyclopropanation *in Vivo* and the Development of P450BM3 Hstar (2014)



axial ligand	yield
C400	1%
C400S	12%
C400M	5%
C400H	62%
C400Y	13%
C400A	13%

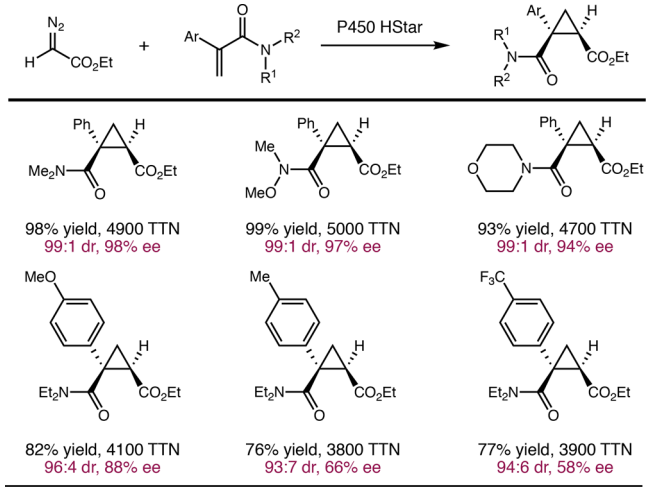
Scheme 6. Development of P450BM3 Hstar



catalyst	yield	TTN	*cis*:*trans*	ee
P450BM3 T268A C400H	81%	-	6:94	42%
P450 HStar (P450BM3 T268A C400H V78M L181V T438W)	92%	-	2:98	92%
P450 HStar H92N H100N	-	20100	4:96	83%

and acrylates (Scheme 7).[53] Subsequent studies on the inactivation mechanism of P450BM3 Hstar revealed that

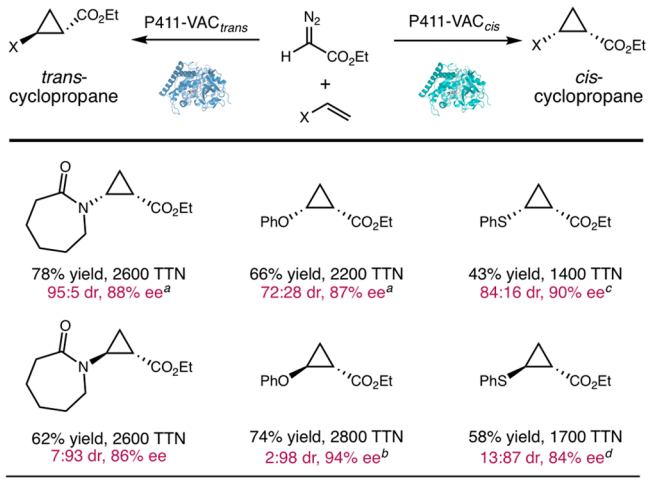
Scheme 7. Substrate Scope of P450BM3 Hstar



undesired modification of nucleophilic amino acid residues took place following reaction with the incipient electrophilic Fe carbene.[54] To reduce inactivation, we performed site-directed mutagenesis of residues prone to modification, which afforded a further improved biocatalyst, P450BM3 Hstar H92N H100N (>20 000 total turnover number, TTN).[54]

Deeper mutational screening of the serine-ligated P411BM3-CIS was also fruitful. Directed evolution of P411BM3-CIS led to a set of complementary catalysts, P411-VAC$_{cis}$ and P411-VAC$_{trans}$, providing access to both the *cis*- and *trans*-diastereomers of heteroatom-substituted cyclopropanes (X = N, O, and S, Scheme 8).[55] Later on, using P411-VAC$_{cis}$ as the template, single mutants P411-VAC$_{cis}$ V87C and P411-VAC$_{cis}$

Scheme 8. Diastereodivergent Biocatalytic Synthesis of Heteroatom-Substituted Cyclopropanes (2018)

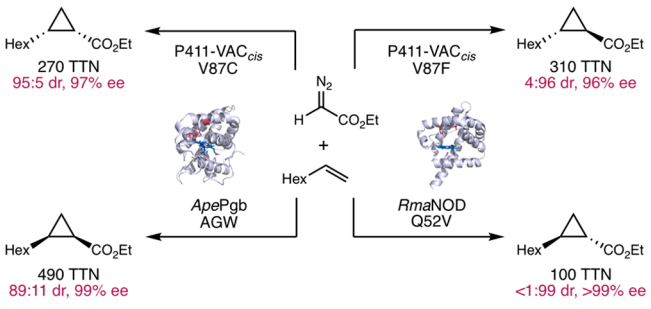


[a]Reaction carried out with P411-VAC$_{cis}$ V87T. [b]Reaction carried out with P411-VAC$_{cis}$ V87I. [c]Reaction carried out with P411-VAC$_{cis}$ A328N. [d]Reaction carried out with P411-VAC$_{cis}$ V87F.

V87F were found to furnish the *cis*- and *trans*-product, respectively, in the cyclopropanation of terminal aliphatic alkenes.[56]

Cognizant of the vast structural diversity of hemoproteins, we surmised that stereoselectivities not found in P450BM3-catalyzed processes could be achieved by exploring other classes of heme proteins with altered active-site environments. Prior to this study, elegant work from the Fasan laboratory demonstrated the utility of engineered myoglobins in carbene transfer.[57] We focused on thermophilic heme proteins due to their superior stability and evolvability.[56] We found that wild-type *Aeropyrum pernix* protoglobin (*Ape*Pgb) and *Rhodothermus marinus* nitric oxide dioxygenase (*Rma*NOD) furnished measurable activity for the cyclopropanation of 1-octene (Scheme 9). Notably,

Scheme 9. Stereodivergent Biocatalytic Synthesis of Disubstituted Cyclopropanes: Access to All Four Stereoisomers (2018)



*Rma*NOD and *Ape*Pgb displayed opposite diastereoselectivity, and evolved variants *Rma*NOD Q52V and *Ape*Pgb W59A Y60G F145W furnished the (1*S*, 2*S*)- or (1*R*, 2*S*)-product with excellent stereocontrol. Combined with P411-VAC$_{cis}$ V87C and P411-VAC$_{cis}$ V87F, we developed a stereodivergent platform to access all four stereoisomers of cyclopropanation products with excellent diastereo- and enantiocontrol.

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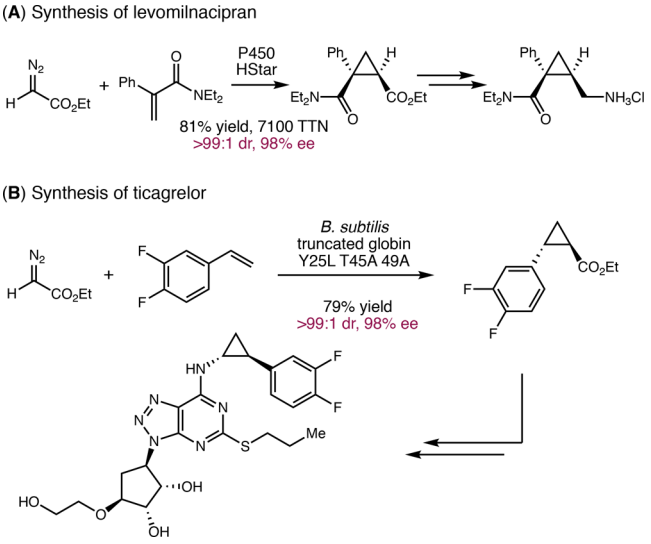
With a series of evolved biocatalysts, we applied these heme proteins in the stereoselective preparation of key cyclopropane intermediates of pharmaceuticals, including levomilnacipran[52] and ticagrelor (Scheme 10).[58] In particular, a truncated globin

Scheme 10. Biocatalytic Stereoselective Synthesis of Cyclopropane Cores of Medicinal Agents (2014 and 2016)



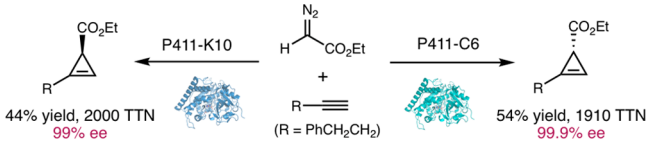
from *Bacillus subtilis*[59] was evolved to provide the desired stereoisomer of the ticagrelor cyclopropyl ester precursor with excellent diastereo- and enantioselectivity.[52] Fasan's group also demonstrated the gram-scale syntheses of tasimelteon and a TRPV1 inhibitor using their evolved myoglobin variants in whole-cell transformations.[60] Collectively, these results showcased the potential utility of non-natural biocatalysis in the manufacturing of value-added pharmaceuticals.

BIOCATALYTIC CYCLOPROPENATION AND BICYCLOBUTANATION

Cyclopropenes are highly strained carbocycles. With strain release, cyclopropenes can be easily converted to a broad spectrum of synthetically useful molecular architectures. Asymmetric catalytic cyclopropenation of alkynes is one of the most widely used methods for the preparation of these motifs in an enantiopure form. However, in contrast to Rh-,[31,61−66] Cu-, Ir-,[67] and Co-based[68] catalysts, no Fe porphyrin complexes were known to catalyze the cyclopropenation process.[47]

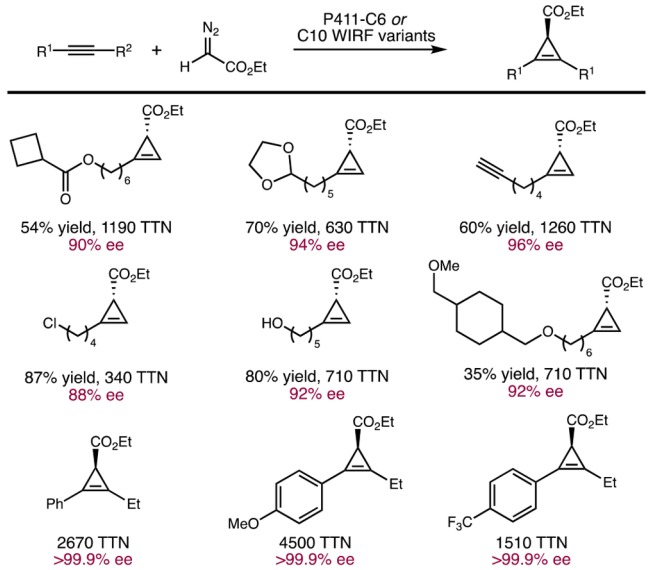
Curious whether engineered P411s could catalyze cyclopropenation, we tested a terminal aliphatic alkyne as the substrate and found that a P411 variant (P-4) previously engineered for nitrene transfer could furnish the desired cyclopropene with modest activity (260 TTN, 91% ee).[69] Using P-4 as the parent, SSM at position 87 was carried out, as residue 87 resides above the heme cofactor, and it is known to play an essential role in controlling the stereochemistry in native oxygenation reactions.[70] The P-4 A87F mutant showed improved activity and enantioselectivity relative to its predecessor (290 TTN, 94% ee). Surprisingly, a single A87W mutation completely reversed the enantiomeric outcome of this cyclopropenation process, affording the opposite enantiomer in excellent enantioselectivity (240 TTN, −94% ee). The complete reversal of stereochemistry (+94% ee → −94% ee) upon the introduction of a single mutation, rarely observed in protein engineering, highlighted the outstanding evolvability of P411 biocatalysts. With the P-4 A87F and A87W mutants, additional rounds of SSM and screening ultimately produced variants P411−K10 and P411−C6. These final variants allowed for the enantiodivergent synthesis of cyclopropenes with excellent enantiocontrol (Scheme 11). Recently, using P411 variants

Scheme 11. Directed Evolution of Enantiodivergent P411 Catalysts for Cyclopropenation (2018)



further evolved from the C−H alkylation lineage,[3] internal alkynes could also be effectively transformed, affording 1,2-disubstituted cyclopropenes in uniformly outstanding enantioselectivity (>99% ee, Scheme 12).[71]

Scheme 12. P411-Catalyzed Cyclopropenation of Terminal Aliphatic Alkynes and Internal Alkynes (2018 and 2020)



When aromatic alkynes were subjected to these enzymatic conditions, the carbene transfer did not stop at the cyclopropene stage. Instead, sequential delivery of the carbenoid fragment to the alkyne substrate afforded the highly strained bicyclobutane as the final product (Scheme 13).[69] This unusual double transfer required the same enzyme to accommodate both the alkyne and the cyclopropene substrate, indicating a distinct promiscuity of evolved P411. Furthermore, the bicyclobutane product formed with complete *exo*, *endo* selectivity. This diastereoselectivity differed from the *endo*, *endo* selectivity observed in the only known intermolecular bicyclobutanation, catalyzed by an osmium porphyrin.[72] Thus, P411-catalyzed intermolecular bicyclobutanation represents a general method to prepare interesting bicyclobutanes using easily available starting materials.

BIOCATALYTIC SI−H AND B−H INSERTION

Silicon is the second most abundant element in Earth's crust. Despite the abundance of silicon in nature, enzymes capable of

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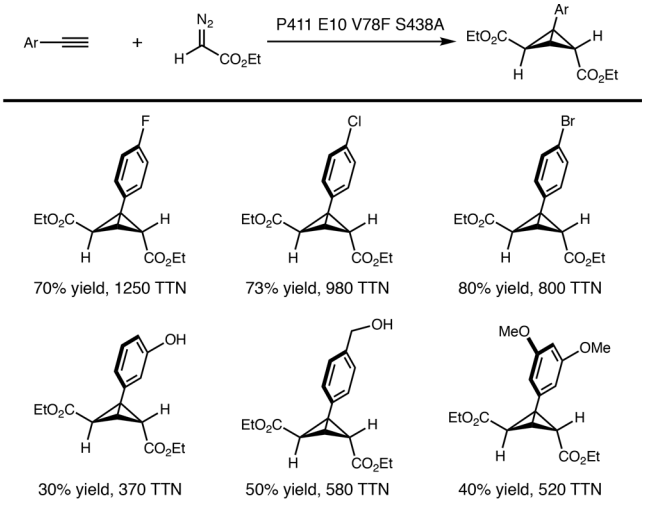
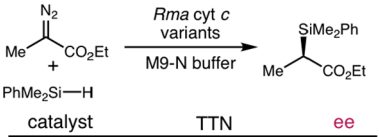
Scheme 13. P411-Catalyzed Bicyclobutanation of Aromatic Alkynes (2018)



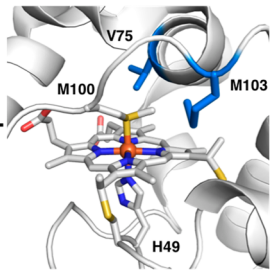
catalyzing the construction of carbon−silicon bonds are not known.[73] Prior art in metal-carbene chemistry demonstrated that the insertion of metal carbenoids into Si−H bonds could lead to the construction of C−Si bonds. We recognized that, by repurposing heme proteins to catalyze the carbenoid insertion into Si−H bonds, we could develop a biocatalytic system to forge Si−C bonds.

We were particularly interested in the Si−H insertion of α,α-disubstituted diazo compounds because the use of such carbenoid precursors would engender a stereogenic center at the α-carbon (Scheme 14). Although P450s were ineffective in

Scheme 14. Directed Evolution of *Rma* cyt *c* for Enantioselective Si−H Insertion (2016)

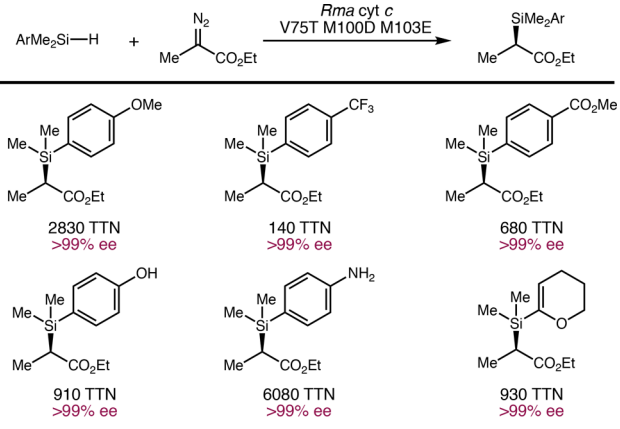


catalyst	TTN	ee
WT *Rma* cyt *c*	44	97%
M100D	549	>99%
V75T M100D	892	>99%
V75T M100D M103E	1520	>99%



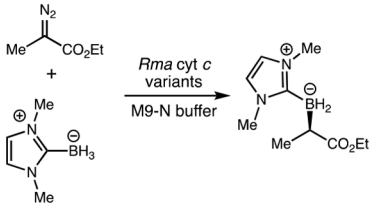
inducing appreciable enantiodifferentiation, we found that *Rhodothermus marinus* cytochrome *c* (*Rma* cyt *c*) afforded the Si−H insertion product with excellent enantioselectivity (97% ee, 44 TTN).[2] Even though *Rma* cyt *c*'s native function is not catalysis, its solvent-exposed heme center apparently accommodates carbenoids bearing two large α-substituents, thus permitting their selective transformation. Further active-site engineering ultimately gave a triple mutant *Rma* cyt *c* V75T M100D M103E ("TDE") that catalyzed the Si−H insertion with 1518 TTN and >99% ee. It tolerated a range of hydrosilane substrates, giving rise to enantioenriched silanes with outstanding enantiocontrol (Scheme 15). More recently, using machine-learning-assisted directed evolution, we engineered an orthogonal set of *Rma*NOD biocatalysts to access either the (*S*)- or the (*R*)-enantiomeric product.[74]

Scheme 15. Engineered *Rma* cyt *c*-Catalyzed Enantioselective Si−H Insertion (2016)



Similar to silicon, biosynthetic machineries to form carbon−boron bonds were also not known. Furthermore, enantiopure organoboron compounds are coveted synthetic intermediates in organic synthesis, since well-established stereospecific transformations of these species allow access to a wide range of medicinally relevant scaffolds. Encouraged by our success in Si−H insertion, we embarked on the development of biocatalysts for enantioselective B−H insertion (Scheme 16).[75] Using *N*-

Scheme 16. Directed Evolution of *Rma* cyt *c* for Enantioselective B−H Insertion (2017)



catalyst	TTN	ee
WT *Rma* cyt *c*	120	70%
M100D	1850	76%
V75R M100D	2300	-
V75R M100D M103T	2490	95%

heterocyclic carbene (NHC)-boranes as our borylation agents due to their stability under aqueous conditions, we quickly found that wild-type *Rma* cyt *c* readily promoted the desired B−H insertion reaction in living *E. coli* cells, furnishing the corresponding enantioenriched organoborane product in 70% ee and 120 TTN. Directed evolution by targeting residues near the heme cofactor furnished *Rma* cyt *c* V75R M100D M103T (BOR^{R1}), which provided the organoboron product in 2490 TTN and 95% ee.

A notable feature of this biocatalytic B−H insertion lies in its compatibility with an exceptionally broad range of α,α-disubstituted diazo compounds (Scheme 17).[75,76] Using iterative SSM and screening, the BOR variants could be evolved quickly for the customized synthesis of diverse enantioenriched organoboron products that are versatile building blocks in organic synthesis. Flexible loop engineering[7] proved particularly fruitful in the directed evolution of *Rma* cyt *c* for borylation reactions. α-Trifluoromethyl-substituted organoboranes[76] and α-boryl lactones[77] could be synthesized with good enantioselectivity using $BOR\text{-}CF_3$[76] and BOR^{LAC},[77] respectively.

Collectively, the successful development of non-natural Si−H and B−H insertion reactions demonstrates a new strategy to unveil and augment the catalytic capability of proteins. Furthermore, the integration of enzymatic Si−H and B−H insertion into metabolic pathways in living cells could

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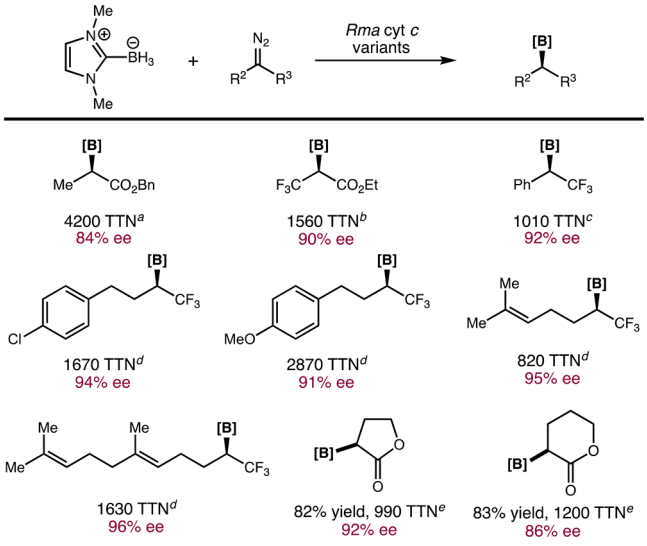
Scheme 17. Engineered *Rma* cyt *c*-Catalyzed Enantioselective B−H Insertion (2017−2019)



[a]Reaction carried out with *Rma* cyt *c* V75R M100D M103D. [b]Reaction carried out with *Rma* cyt *c* V75R M100D M103F. [c]Reaction carried out with *Rma* cyt *c* Y71C V75P M89C M99C M100D. [d]Reaction carried out with *Rma* cyt *c* Y44I V75S M99A M100L M103D (BOR-CF_3). [e]Reaction carried out with *Rma* cyt *c* V75R M99Q M100D T101Y M103Y (BOR^{LAC}). The absolute stereochemistry of these products were not determined.

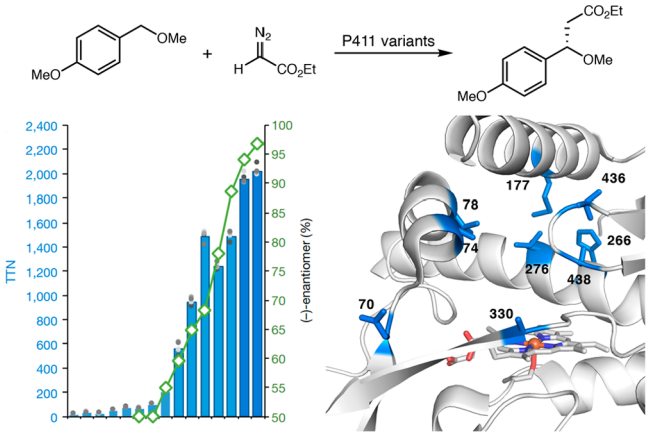
potentially enable the production of unnatural organosilicon and organoboron compounds *in vivo*, thus bringing new opportunities to synthetic biology.

BIOCATALYTIC C−H INSERTION

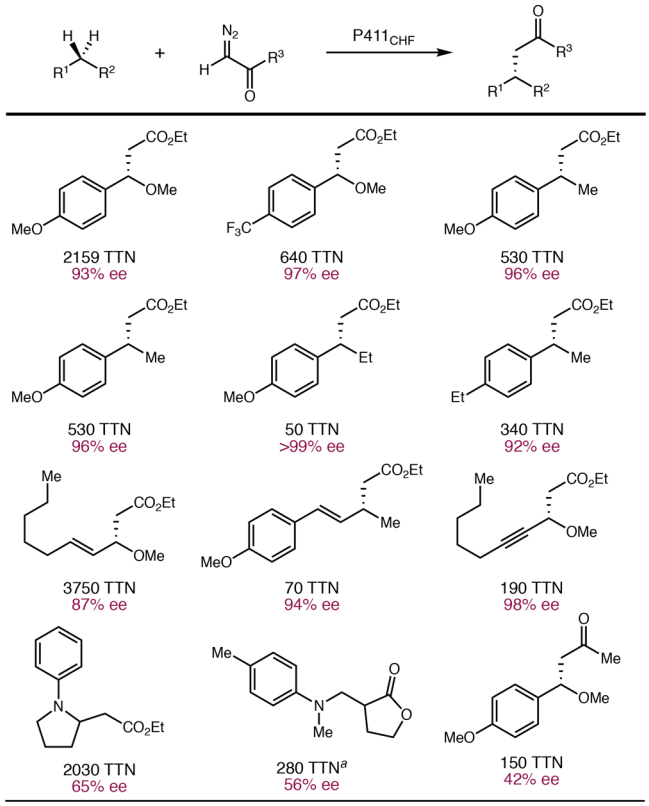
From a reactivity perspective, the insertion of a metal carbenoid into C−H bonds is much more challenging than insertion into Si−H and B−H bonds. Due to the ubiquity of C−H bonds in organic molecules, site- and stereoselective C−H insertion reactions hold the promise to significantly simplify the syntheses of organic compounds. Unlike other late transition metals such as Rh, Cu, Ru, Ag, and Co, Fe catalysts displayed low activities for the catalytic insertion of carbenoids into C−H bonds in previous work.[78,79] Prior to our work, a generally applicable Fe catalyst for the intermolecular C−H insertion was not available. Furthermore, iron-catalyzed enantioselective C−H insertion was recognized as a formidable challenge.

In our heme protein collections of P450s, P411s, cytochromes *c*, and globins, we found only two variants, P-4 A82L and *Rma*NOD Y32G, with measurable activity for the intermolecular C−H insertion reaction (TTN = 13 and 7, respectively), which underscored the challenges this transformation presents.[3] Ultimately, however, directed evolution over 14 rounds of mutagenesis and screening culminated in the highly active variant $P411_{CHF}$ for C−H alkylation (Scheme 18). $P411_{CHF}$ differs from wild-type P450BM3 by 23 amino acids and lacks the FAD domain of the reductase domain. This truncated P411 variant catalyzed the carbene insertion into a variety of C(sp^3)−H bonds, including benzylic, allylic, propargylic, and heteroatom-stabilized ones, with excellent enantioselectivity (Scheme 19). The use of acceptor carbenes for asymmetric C−H insertion is relatively underdeveloped with rhodium catalysts. Thus, these engineered P411s provided a new solution

Scheme 18. Directed Evolution of $P411_{CHF}$ for Enantioselective Intermolecular C−H Insertion (2019)



Scheme 19. $P411_{CHF}$-Catalyzed Enantioselective Intermolecular C−H Insertion (2019)[a]



[a]Variant P411-IY(T327I) was used.

to enantioselective C−H insertion using acceptor carbenes. Furthermore, variants in the $P411_{CHF}$ lineage permitted the stereodivergent C−H insertion with *N*-methyl tetrahydroquinoline, thus allowing for the formal synthesis of both enantiomeric forms of natural product cuspareine.

The biocatalytic enantioselective C−H insertion nicely illustrates the power of directed evolution in solving challenging problems in catalysis. Recently, we were able to engage α-trifluoromethyl- and perfluoroalkyl-substituted diazo compounds[80] in C−H alkylation (Scheme 20).[81] A set of stereocomplementary P411 catalysts was engineered to access either enantiomeric trifluoroethylation product.

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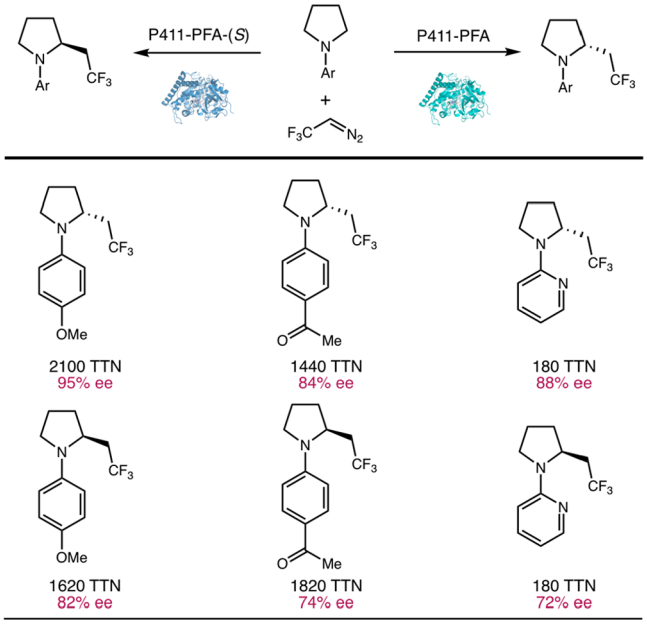
Scheme 20. P411-PFA-Catalyzed Enantioselective Intermolecular C−H Insertion (2019)



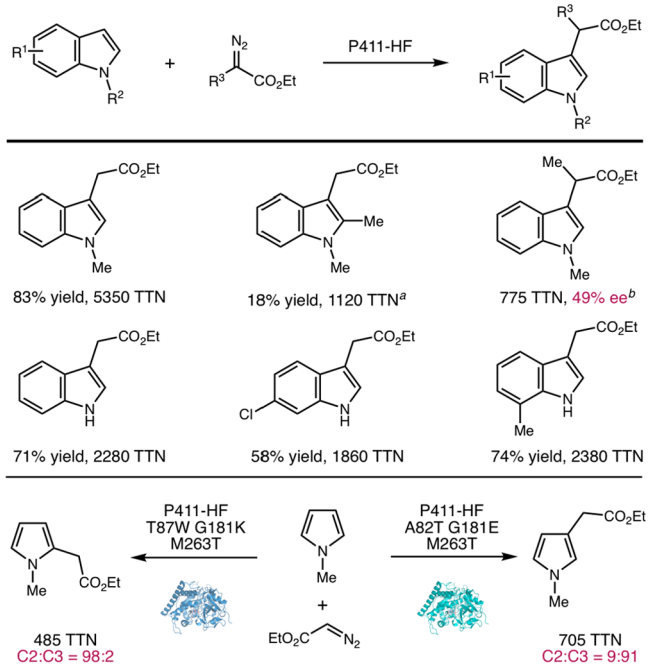
Mutation L401P dramatically enhanced the catalytic activity and the enantioselectivity of P411-PFA (1510 TTN, +88% ee → 4070 TTN, +98% ee). The L401 residue is located next to S400 bound to the heme cofactor. Previous study on another P450 enzyme, P450cam, showed that the leucine-to-proline mutation of the residue next to the heme-binding cysteine disrupted the hydrogen bonding network of the thiolate ligand, thus enhancing the donor strength of the proximal thiolate.[82−84] Our finding showcased the importance of this hydrogen bonding network of the Fe-binding residue in abiological transformations.

Recently, Fasan,[85] Koenigs,[86] and our group[87] all described biocatalytic methods for the C(sp^2)−H functionalization of electron-rich heterocycles including indoles and pyrroles. We found that a range of indole substrates could be alkylated at the 3-position using evolved P411-HF (Scheme 21). When Me-EDA was used as the diazo source, P411 biocatalysts could be evolved to furnish the alkylation product in an enantioselective fashion. This is a rare example of an evolved P411 enzyme showing promising stereocontrol for α,α-disubstituted diazo compounds. Prior to this, engineered *Rma* cyt *c* was the only protein class exhibiting good stereocontrol for these substrates. Moreover, when pyrrole was used as the substrate, the site selectivity could be easily tuned using the P411 catalyst, leading to the C2 or C3 alkylation product with good regiocontrol.

BIOCATALYTIC C−H AMIDATION: INITIAL WORK

Enantiopure amines represent key pharmacophores in small-molecule therapeutics.[88] Consequently, general catalytic methods for stereoselective assembly of amines have long been sought after.[89] In this context, catalytic nitrene transfer, i.e., the delivery of a nitrene fragment (R−N:) to organic substrates, is a powerful technique for the synthesis of valuable amines from readily available alkanes and alkenes. In a pioneering study published in 1985, Dawson and Breslow demonstrated that mammalian microsomal P450s could catalyze C−H amidation with a turnover frequency (TOF) of ca. 2 min^{-1} using iminoiodinanes as the nitrene precursor.[90] Unfortunately, the

Scheme 21. Truncated P411-Catalyzed Selective C−H Functionalization of Indoles and Pyrroles (2019)

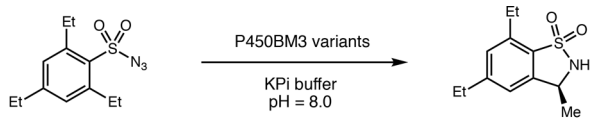


[a]Reaction carried out with P411-HF M263A. [b]Reaction carried out with P411-HF C87A M263E A268G T327P A328Y L437 M.

rapid solvolysis of iminoiodinanes[91] made it challenging to further optimize this system.

Almost 30 years later, concurrent to our investigations into carbene transfer, we revisited this nitrene transfer chemistry and engineered P450BM3 variants to enhance the activity for C−H amidation (Scheme 22).[37] We found sulfonyl azides to be

Scheme 22. P450-Catalyzed Enantioselective C−H Amidation for Sultam Synthesis (2013)



entry	catalyst	TTN	ee
1	P450BM3	2.1	-
2	P450BM3 T268A	15	36%
3	P411BM3	32	20%
4	P411BM3 T268A	120	58%
5	P411BM3-CIS	310	67%
6	P411BM3-CIS T438S	383	73%
7	P411BM3-CIS T438S[a]	430	87%

[a]Reaction carried out using P411 in intact *E. coli* cells.

superior nitrene precursors for biocatalytic amination reactions, in part due to the excellent water stability of organic azides. Similar to what we observed in P450BM3-catalyzed cyclopropanation, the C−H amidation also starts with ferrous heme, and the T268A and C400S mutations were found to be beneficial. Whole-cell biotransformations employing P411-CIS T438S (variant "P") provided the sultam products with improved activity and enantioselectivity compared to the *in vitro* reaction. Parallel to our investigation, in 2014, Fasan and

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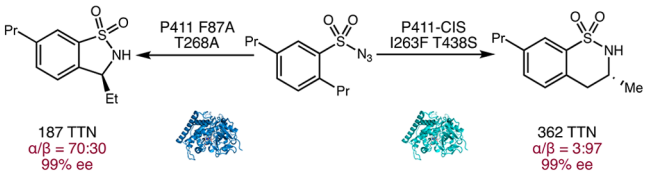
co-workers also accomplished intramolecular C−H amidation using wild-type and engineered P450BM3.[92]

In a subsequent study, we demonstrated that either the six- or the five-membered amidation product could be prepared with excellent site selectivity in an enzyme-controlled fashion (Scheme 23).[93] Specifically, the I263F variant was found to

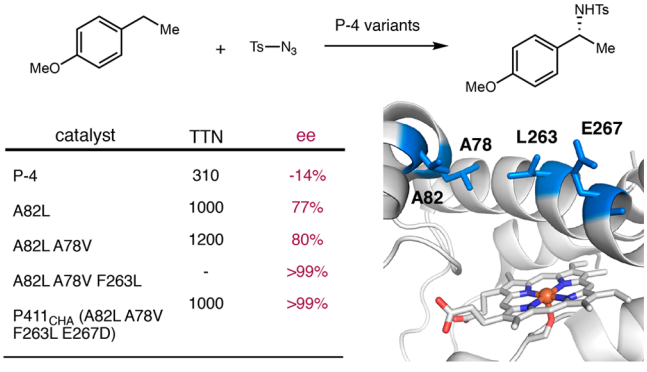
Scheme 23. Enzyme-Controlled, Regiodivergent C−H Amidation (2014)



favor the homobenzylic amidation, whereas the P450BM3 T268A F87A variant was selective for benzylic amidation. This regiodivergent amidation showcased the power of enzymes to override the innate regiochemical preference through the delicate control of substrate binding in the active site. It also underscored the possibility of achieving site selectivities previously inaccessible with existing small-molecule catalysts.

Furthermore, we expanded this biocatalytic enantioselective C−H amidation to intermolecular processes.[94] In contrast to intramolecular nitrene transfer, only the serine-ligated P411 variants could promote the entropically demanding intermolecular amidation. The P-4 A82L variant, a P411 previously developed for sulfide imidation, was critical to this development, showing low levels of promiscuous activity for benzylic C−H amidation (Scheme 24). Introduction of three active-site

Scheme 24. Directed Evolution of Intermolecular C−H Amidase (2017)

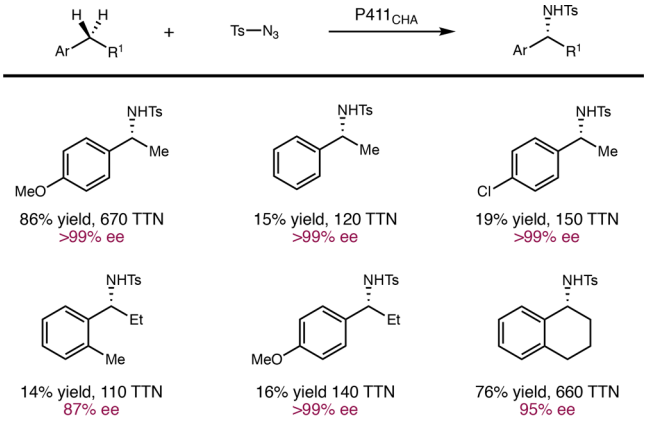


catalyst	TTN	ee
P-4	310	-14%
A82L	1000	77%
A82L A78V	1200	80%
A82L A78V F263L	-	>99%
$P411_{CHA}$ (A82L A78V F263L E267D)	1000	>99%

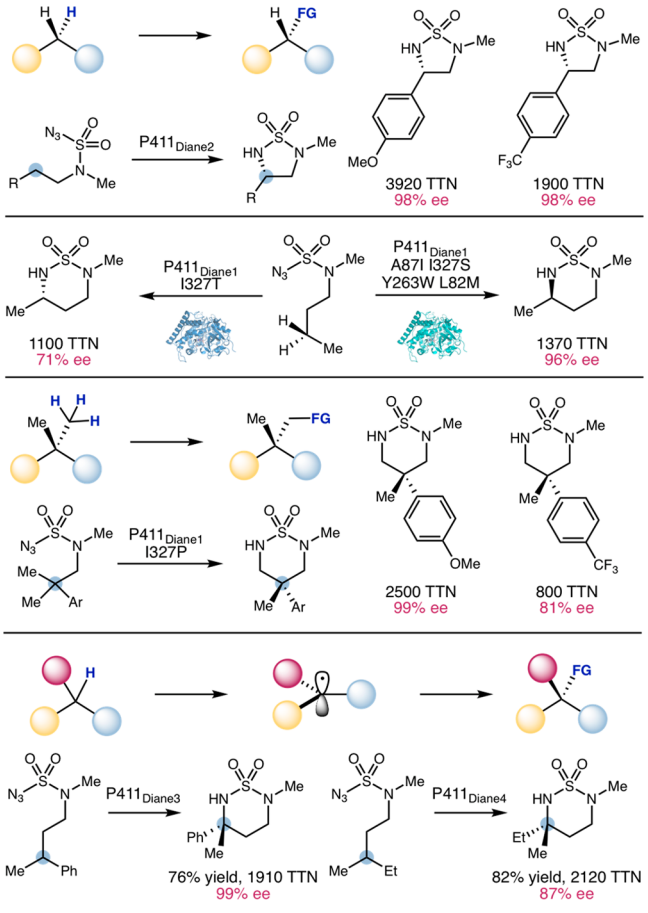
mutations greatly improved catalytic efficiency. The final $P411_{CHA}$ variant effectively transforms diverse benzylic substrates into the corresponding sulfonamides with excellent enantioselectivity (Scheme 25).

More recently, with a significantly expanded library of P411 variants, we were eager to address unmet challenges in the area of enantioselective C−H functionalization using P411 nitrene transferases (Scheme 26). In principle, three classes of enantioselective C(sp^3)−H functionalization reactions could be developed. Starting from a substrate bearing two prochiral secondary C(sp^3)−H bonds, asymmetric C−H functionalization would furnish products with a trisubstituted stereocenter. Desymmetrization of geminal dimethyl-substituted substrates via primary C(sp^3)−H functionalization would generate a

Scheme 25. P411-Catalyzed Intermolecular Benzylic C−H Amidation (2017)



Scheme 26. P411-Catalyzed Asymmetric Amidation of Primary, Secondary, and Tertiary C(sp^3)−H Bonds (2019)



quaternary stereocenter. Furthermore, enantioconvergent functionalization of tertiary C(sp^3)−H bonds would allow for the formation of tetrasubstituted stereocenters. Such enantioconvergent tertiary C−H functionalizations have long eluded synthetic chemists, presumably due to the lack of suitable mechanisms for stereoconvergence as well as effective catalysts to induce asymmetry.[95]

Using the intramolecular C−H amidation of sulfamoyl azide as a model system, we demonstrated that all three modes of enantioinduction could be achieved using P411 catalysts.[96]

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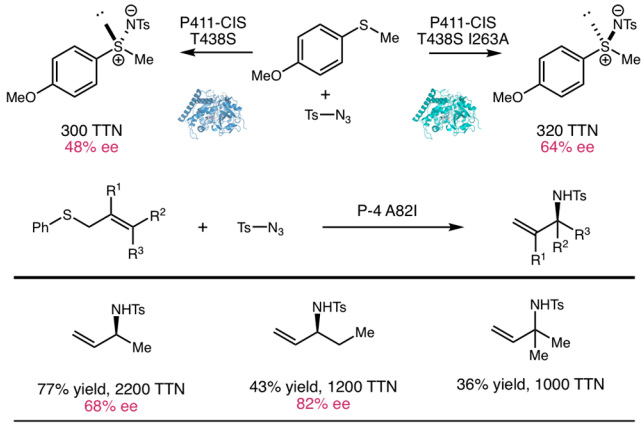
Among all the heme proteins tested, variants from the $P411_{CHF}$ carbene transferase lineage provided the most promising results for the amidation of secondary C(sp^3)−H bonds. Active-site engineering led to $P411_{Diane2}$, a variant capable of transforming a range of benzylic substrates into 1,2-diamines with excellent enantioselectivity. In addition, unactivated secondary C(sp^3)−H bonds were also amidated effectively. By using a set of P411s that only differed by four mutations, both enantiomers could be accessed with good stereocontrol. Furthermore, $P411_{Diane1}$ I327P allowed the primary C(sp^3)−H amidation to occur with excellent activity.

We also developed enantioconvergent tertiary C−H amidations via a putative stereoablative hydrogen atom transfer (HAT)/stereoselective rebound mechanism. With evolved $P411_{Diane3}$ and $P411_{Diane4}$, tetrasubstituted stereocenters possessing a nitrogen group could be efficiently accessed. Notably, this biocatalytic platform could be engineered to construct "methyl−ethyl" stereocenters with excellent enantioselectivity (87% ee). Due to the minimal steric and electronic difference, catalytic asymmetric formation of "methyl−ethyl" stereocenters is a notoriously difficult problem. Our study demonstrated that engineered enzymes could overcome this long-standing obstacle in asymmetric catalysis.

BIOCATALYTIC IMIDATION OF SULFIDES

P450-catalyzed enantioselective oxidation of sulfides is a powerful method for the preparation of optically active sulfoxides. Early in our nitrene transfer study, we wondered whether we could develop an analogous nitrene transfer to sulfides to generate enantiomerically enriched sulfimides, which are key intermediates en route to many sulfoximide-containing agrochemicals and pharmaceuticals (Scheme 27). It was found

Scheme 27. P411-Catalyzed Asymmetric Imidation of Sulfides (2014 and 2016)



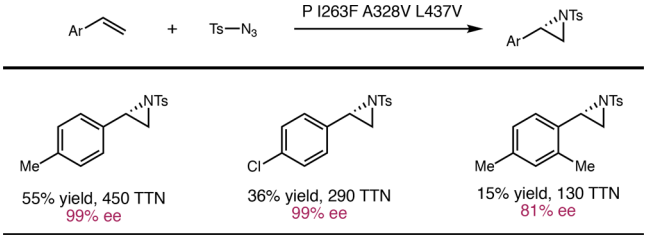
that the P411-CIS T438S variant already showed good activity toward the imidation of sulfides without further engineering (300 TTN, +48% ee).[97] Enantiodivergent imidation could also be achieved using an orthogonal set of P411 biocatalysts. Again, this early example showed that, despite the homochirality of biogenic protein catalysts, either antipode of the product could still be conveniently accessed by engineering the protein.

The spontaneous [2,3]-sigmatropic rearrangement of allylic sulfimides leads to allylic amines. To reprogram this biocatalytic imidation for the asymmetric preparation of amines, we re-engineered our P411 nitrene transferases using phenyl allyl sulfide as the model substrate.[98] Using variant "P" as the template, active-site engineering provided variants P-4 and P-4 A82I for the effective conversion of both the (*E*)- and the (*Z*)-substrates. Further investigation revealed that this sulfide imidation was highly enantioselective. Partial erosion of stereochemical purity of the resulting allylic amine occurred during the [2,3]-sigmatropic rearrangement presumably due to the competing *endo* and *exo* transition states.

BIOCATALYTIC AZIRIDINATION OF OLEFINS

Optically pure aziridines are versatile building blocks to a variety of nitrogen-containing compounds. We demonstrated that P411s could catalyze the asymmetric aziridination of styrene derivatives using tosyl azide as the nitrene source (Scheme 28).[99] Through directed evolution, the competitive azide

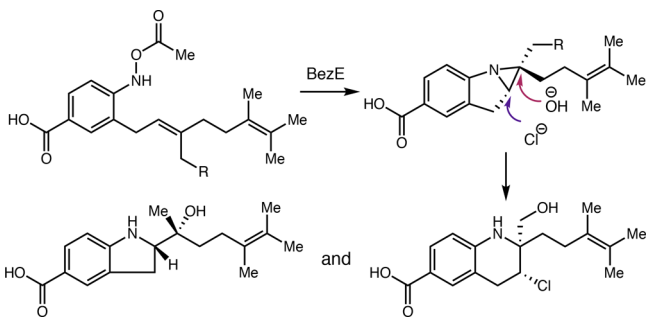
Scheme 28. P411-Catalyzed Asymmetric Aziridination (2015)



reduction could be suppressed. The final variant P I263F A328V L437V allowed for the preparation of a range of enantioenriched aziridines from electron-rich and electroneutral styrenes. Due to their attenuated nucleophilicity, electron-deficient styrenes were less effective substrates under these conditions.

It was long believed that nitrene transfer to olefins has no counterpart in the biological world. However, in 2018, spurred by the report of P411-catalyzed nitrene transfer, the Ohnishi group found that, during the biosynthesis of benzastatin natural products in *Streptomyces* sp. RI18, a cytochrome P450 (BezE) is responsible for the intramolecular aziridination via a nitrene transfer mechanism using *N*-acetoxyaniline as the nitrene surrogate (Scheme 29).[100] We were thrilled that mechanistic insights gained through unnatural reactions in turn informed the discovery of natural enzymes that operate through unconventional pathways in biosynthesis.

Scheme 29. Bez-Catalyzed Nitrene Transfer in the Biosynthesis of Benzastatins (2018)



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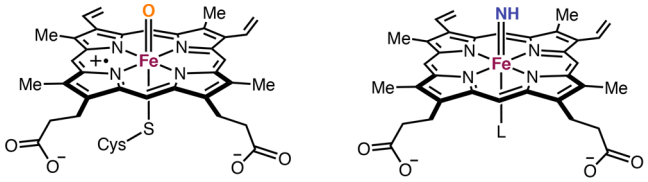
BIOCATALYTIC C−H AMINATION USING EQUIVALENTS OF UNPROTECTED NITRENE

In previously developed biocatalytic nitrene transfer reactions, sulfonyl azides were the privileged nitrene source. Unfortunately, the removal of the *N*-sulfonyl group requires harsh reaction conditions, thus limiting the synthetic utility of nitrene transfer products. To circumvent this problem, we initiated a program to explore alternative nitrene precursors with no protective groups attached to the nitrogen. We were particularly intrigued by *N*-hydroxylamine esters ($RC(O)O-NH_2$) as aminating reagents, because the transformation of these reagents would furnish unprotected primary amines.[101−103] The straightforward synthesis of unprotected, enantioenriched primary amines remains a challenging problem. The successful development of biocatalytic primary amination via a nitrene transfer mechanism would allow for the conversion of unfunctionalized alkanes and alkenes into enantioenriched primary amines, thereby complementing transaminase-catalyzed processes.

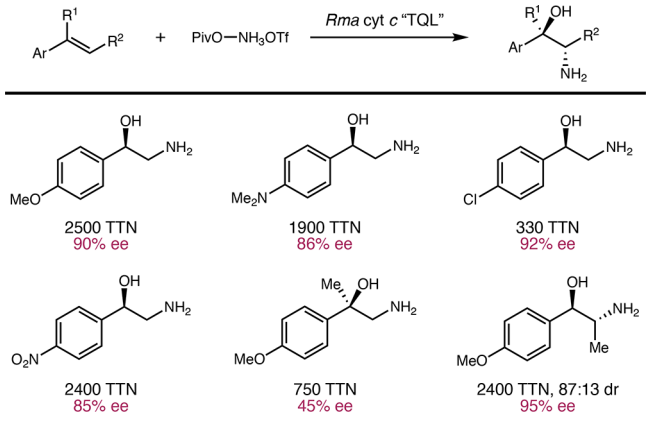
From an enzymologist's perspective, the putative iron nitrene species "Fe═NH" invoked in these amination reactions represents the closest structural mimic of the Fe═O intermediate (compound I) in native P450 catalysis (Scheme 30). Thus, understanding the bonding and reactivity of this presumed "Fe═NH" intermediate is of considerable interest to the bioinorganic community.

Scheme 30. Comparison between Fe═O and Fe═NH



We first targeted the amination of styrene derivatives (Scheme 31). Using wild-type *Rma* cytochrome *c* as the catalyst, we found that the transfer of unprotected nitrene to styrenes afforded aminoalcohols as the final product, presumably due to the rapid ring opening of the unprotected aziridine under the reaction conditions.[104] Starting from *Rma* cyt *c*, active-site engineering

Scheme 31. Engineered *Rma* Cytochrome *c*-Catalyzed Asymmetric Aminohydroxylation (2019)

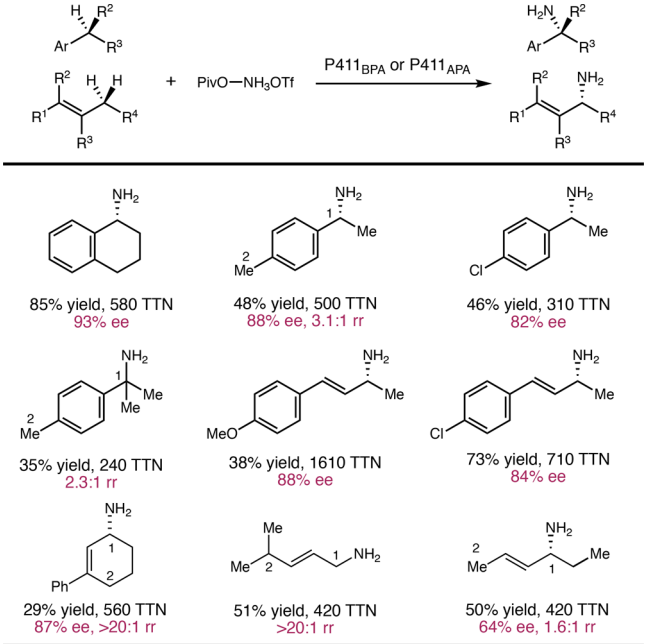


furnished the "TQL" variant, a highly active biocatalyst for the asymmetric aminohydroxylation of diverse styrenes including electron-deficient ones. Further mechanistic study showed that the hydrolysis of the unprotected, enantiopure aziridines is enantiospecific. This finding suggested that enzymatically formed enantioenriched aziridine is a possible precursor to the 1,2-aminoalcohol product.

We next explored C−H amination reactions using unprotected nitrene equivalents. Using a P411 variant from the $P411_{CHA}$ lineage[94] as the parent, we engineered $P411_{BPA}$ for the primary amination of benzylic C−H bonds with high activity and excellent enantiocontrol. Most notably, no small-molecule catalysts are known to promote the primary amination of $C(sp^3)-H$ bonds despite extensive previous efforts dedicated to the study of Fe-catalyzed amination.[101−103] The successful development of this biocatalytic primary amination of $C(sp^3)-H$ bonds illustrates the enormous potential of enzymes to enable desirable reactivities elusive to small-molecule catalysts.

Even more exciting, P411 enzymes were found to catalyze intermolecular allylic C−H amination with excellent enantioselectivity (Scheme 32). Variant $P411_{APA}$ chemoselectively

Scheme 32. P411-Catalyzed Asymmetric Amination of Benzylic and Allylic $C(sp^3)-H$ Bonds (2020)



aminates the allylic C−H bond, leaving the olefin moiety completely untouched. This chemoselectivity is orthogonal to the *Rma* cyt *c* TQL variant previously engineered for olefin aminohydroxylation.[104] With $P411_{BPA}$ and $P411_{APA}$, an array of easily available hydrocarbon starting materials could be converted into value-added primary amines. Further expanding this intermolecular amination activity to unactivated $C(sp^3)-H$ bonds would furnish an unprecedented, enzymatic method for the amination of hydrocarbon feedstocks.

EVOLUTIONARY TRAJECTORY OF P450 CARBENE TRANSFERASES AND NITRENE TRANSFERASES

P450BM3 has been the primary workhorse for the new-to-nature reactions described in this Account. Starting from the wild-type enzyme, we have evolved a family of P450BM3

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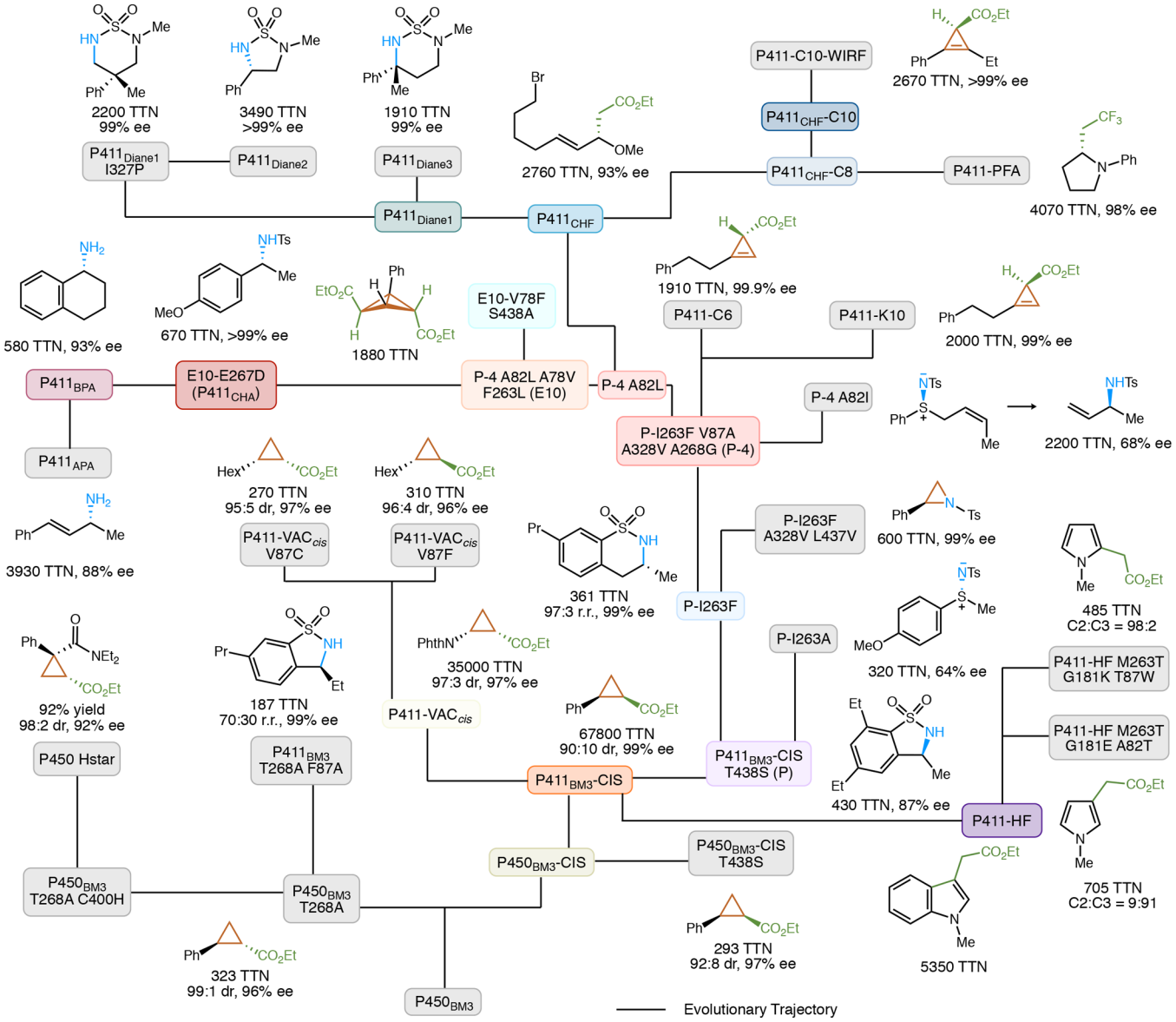

Figure 1. Evolutionary trajectory of P450 carbene and nitrene transferases.

variants to serve as highly active carbene and nitrene transferases. It is interesting to follow the evolutionary trajectories of these different enzymes, delineated in Figure 1. These enzymes are all very closely related: the amino acid substitutions in key P450 variants are summarized in Table 1.

As can be seen from Table 1, except for several early variants, the majority of our evolved P411 carbene and nitrene transferases share the same ancestor, P411-CIS. We often found that a newly engineered carbene transferase acquired promiscuous activity for previously elusive nitrene transfer reactions. Similarly, newly evolved nitrene transferases opened up exciting opportunities for challenging carbene transfer reactions. Thus, there is a great deal of functional overlap in these new enzymes, which provides starting points for evolution and optimization of yet more novel functions.

Active-site evolution by site-saturation mutagenesis and screening has proven fruitful for P450-catalyzed carbene and nitrene transfer. Perhaps this is not surprising, since these activities do not require the same finely tuned amino acid environment evolved for P450BM3's native function. Upon a closer examination of the data in Table 1, it is evident that the following residues are important for tuning the enzyme activity and selectivity: (1) residues 70−87 in the B′ region; (2) residues 177−181 in the F helix; (3) residues 261−269 in the I helix; (4) residues 327−330 in the K/K′ loop; (5) residues 436−438 in the loop; and (6) the iron-binding residue 400. The ability to quickly improve an abiological reaction of interest by iteratively mutating a relatively small set of key residues makes this protein engineering approach very attractive for catalyst development. To help future engineering of P450BM3 biocatalysts for other transformations, we label privileged active-site residues of P450BM3 in Figure 2, whose mutation resulted in substantial improvements in previously developed carbene and nitrene transfer enzymes. Beneficial mutations will of course also lie at more distant residues, as is often found in directed evolution campaigns. Thus, further optimization of a new catalytic activity is possible if the screening capacity is sufficient to capture these rarer events.

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Table 1. Directed Evolution of P450BM3 as Carbene and Nitrene Transferases

entry	variant	type	reaction	mutations relative to WT P450BM3	parent	mutations relative to parent	ref
1	P450BM3 T268A	carbene	cyclopropanation	T268A	P450BM3	T268A	1
2	P450BM3 T268A F87A	nitrene	C−H amidation	F87A, T268A	P450BM3 T268A	F87A	93
3	P450 Hstar	carbene	cyclopropanation	V78M, L181V, T268A, C400H, L437W	P450BM3 T268A	V78M, L181V, C400H, L437W	39, 52, 54
4	P450BM3-CIS (PDB: 4H24)	carbene	cyclopropanation	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, E442K	9-10A-TS[46]	F87V, T268A	1
5	P411BM3-CIS (PDB: 4H23)	carbene	cyclopropanation	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, E442K	P450BM3-CIS	C400S	50
6	P411-VAC$_{cis}$	carbene	cyclopropanation	L75Y, V78A, F87V, P142S, T175I, L181I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, L437F, T438Q, E442K	P411BM3-CIS	L75Y, L181I, L437F, T438Q	55
7	P411-VAC$_{trans}$	carbene	cyclopropanation	V78A, F87L, P142S, T175I, L181R, A184V, S226R, H236Q, E252G, I263G, T268A, A290V, L353V, I366V, C400S, L437F, E442K	P411BM3-CIS	V87L, L181R, I263G, L437F	55
8	"P" (P411-CIS T438S)	nitrene	C−H amidation, sulfide imidation	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K	P411BM3-CIS	T438S	37, 97
9	P I263A	nitrene	sulfide imidation	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263A, T268A A290V, L353V, I366V, C400S, T438S, E442K	"P"	I263A	97
10	P I263F (PDB: 4WG2)	nitrene	C−H amidation	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263F, T268A A290V, L353V, I366V, C400S, T438S, E442K	"P"	I263F	93
11	P I263F A328V L437V	nitrene	aziridination	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263F, T268A, A290V, A328V, L353V, I366V, C400S, L437V, T438S, E442K	P I263F	A328V, L437V	99
12	"P-4"	nitrene	sulfide imidation	V78A, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263F, T268G, A290V, A328V, L353V, I366V, C400S, T438S, E442K	P I263F	V87A, A268G, A328V	98
13	E10 (PDB: 5UCW)	nitrene	C−H amidation	A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263L, T268G, A290V, A328V, L353V, I366V, C400S, T438S, E442K	P-4	A78V, A82L, F263L	94
14	P411$_{CHA}$	nitrene	C−H amidation	A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263L, E267D, T268G, A290V, A328V, L353V, I366V, C400S, T438S, E442K	P-4	A78V, A82L, F263L, E267D	94
15	E10 V78F S438A	carbene	bicyclobutanation	V78F, A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263L, T268G, A290V, A328V, L353V, I366V, C400S, T438A, E442K	E10	V78F, S438A	71
16	P411−K10	carbene	cyclopropenation	S72W, V78S, P142S, T175I, A184V, L188C, S226R, H236Q, E252G, F261G, I263F, T268G, T269L, A290V, T327P, A328V, A330V, L353V, I366V, C400S, T436M, L437F, T438S, E442K	P-4	S72W, A78S, A87F, L188C, F261G, T269L, T327P, A330V, T436M, L437F	71
17	P411−C6	carbene	cyclopropenation	V78W, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, F261M, I263F, T268G, A290V, T327V, A328Y, L353V, I366V, C400S, T436H, L437G, T438S, E442K	P-4	A78W, F261M, T327V, V328Y, T436H, L437G	71
18	P411$_{CHF}$	carbene	C−H alkylation	N70E, A74P, V78L, A82L, F87A, P142S, T175I, M177L, A184V, S226R, H236Q, E252G, I263Y, H266V, T268G, A290V, A328V, A330Y, L353V, I366V, C400S, T436L, E442K ΔFAD	P-4 A82L	N70E, A74P, A78L, M177L, F263Y, H266V, A330Y, T436L, S438T	3
19	P411$_{PFA}$	carbene	C−H alkylation	N70T, A74G, V78L, A82L, F87A, P142S, T175I, A184V, S226T, H236Q, E252G, I263Y, H266V, T268G, A290V, T327V, A328V, A330V, L353V, I366V, C400S, I401P, T436L, L437Q, E442K ΔFAD	P411$_{CHF}$-C8 (P411$_{CHF}$ P74G I401L L437Q)	E70T, L177M, R226T, T327V, Y330V, L401P	81
20	P411$_{Diane2}$	nitrene	C−H amidation	A74G, V78L, A82L, F87A, P142S, T175I, M177L, A184V, S226R, H236Q, E252G, I263W, T268G, A290V, T327P, A328V, L353V, I366V, C400S, T436L, L437F, E442K ΔFAD	P411$_{CHF}$-C2 (P411$_{CHF}$ E70N P74G T327I L437Q)	Y263W, I327P, Q437F	96
21	P411-WIRF	carbene	cyclopropenation	N70E, S72F, A74G, V78L, A82L, F87A, M118S, P142S, F162L, T175I, M177L, A184V, S226R, H236Q, E252G, I263W, H266V, T268G, A290V, A328V, A330Y, L353V, I366V, C400S, I401L, T436R, L437I, E442K ΔFAD	P411$_{CHF}$-C10 (P411$_{CHF}$ P74G M118S F162L I401L L437Q)	S72F, Y263W, L436R, Q437I	71
22	P411$_{BPA}$	nitrene	C−H amination	V78M, A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263M, E267D, T268P, A290V, T327A, A328V, L353V, I366V, C400S, I437F, T438G, E442K	E10 L263M	V78M, E267D, T268P, T327A, I437F, T438G	4

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Table 1. continued

entry	variant	type	reaction	mutations relative to WT P450BM3	parent	mutations relative to parent	ref
23	$P411_{APA}$	nitrene	C−H amination	V78M, A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263M, E267D, T268P, A290V, T327A, A328V, L353V, I366V, N395R, C400S, I437F, T438A, E442K	$P411_{BPA}$ G438S	N395R, S438A	4

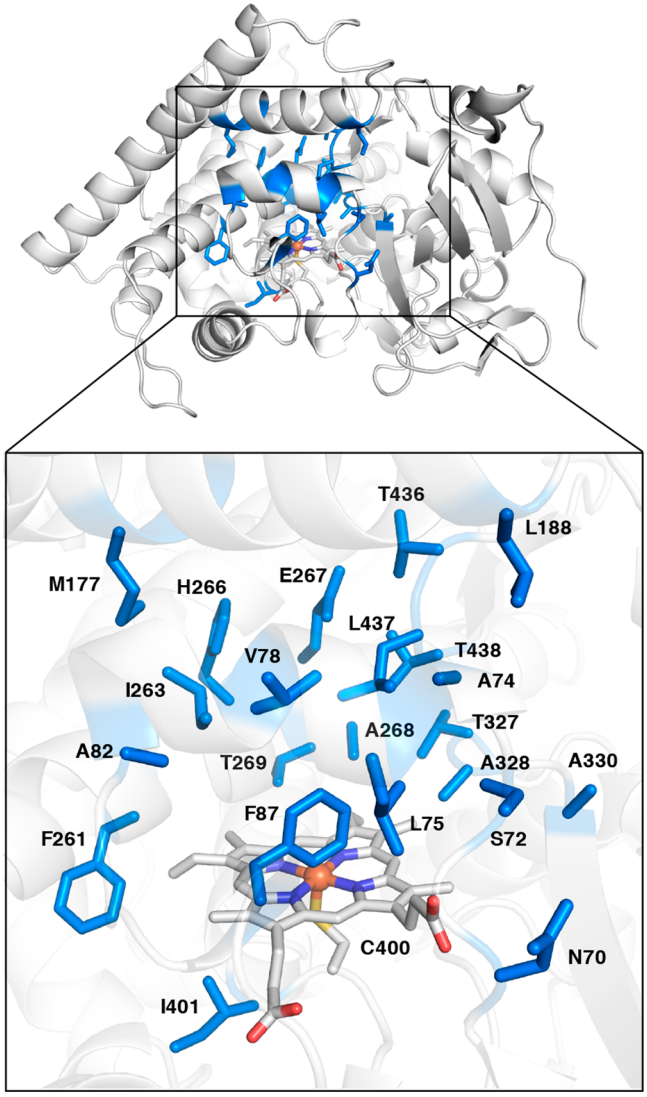

Figure 2. Key active-site residues in P450BM3 for the engineering of carbene and nitrene transferases.

CONCLUDING REMARKS

Over the past eight years, the field of unnatural biocatalysis has witnessed an explosion, and a variety of challenging stereoselective C−C and C−heteroatom bond forming transformations have been realized through the directed evolution of heme proteins. However, from a synthetic chemist's perspective, biocatalytic carbene and nitrene transfer is far from mature. Numerous processes of significant synthetic utility remain to be discovered or optimized. For example, by taking advantage of the precise site- and stereocontrol imposed by the enzyme, exciting new possibilities of selective C−H functionalization await development.

Developing new biocatalytic reactions with no natural counterparts was once considered very difficult. Thus, one might ask why these new-to-nature enzymatic carbene transfer and nitrene transfer activities were achieved so easily. In retrospect, several factors may have played important roles (in addition to the inherent catalytic promiscuity of heme and heme proteins, which enabled improvement using reliable directed evolution approaches). First, the generation of Fe carbene and Fe nitrene is highly exergonic. Unlike the generation of compound I in native oxygenation reactions, the formation of Fe carbene and Fe nitrene is likely kinetically facile and does not require complex interactions with the protein scaffold. Second, the nascent iron carbene and iron nitrene are highly active, allowing for both bimolecular and unimolecular reactions to occur rapidly at ambient temperature. Based on these considerations, we propose that reaction rate (k_{cat}) is more important than substrate binding (K_M) for the initial development of new-to-nature reactions.

Third, despite the highly active nature of iron carbene and nitrene intermediates, their synthetic precursors including diazo compounds and organic azides are relatively stable under physiological conditions, even in living cells. This bioorthogonality limits the spontaneous decay of substrates and ensures the productivity of enzymatic chemistry. Fourth, the excellent evolvability of heme proteins dramatically accelerated engineering campaigns. Last but not least, numerous ideas taken straight from synthetic organic and organometallic chemistry guided our design of new-to-nature biocatalytic processes. Standing on the shoulders of giants, we saw great opportunities at the colliding fields of synthetic chemistry and protein engineering.

We anticipate that the strategies underlying the work detailed in this Account will lead to the development of yet more enzymes for challenging transformations. After all, the astonishing structural and functional diversity of naturally available enzymes, and other proteins, will continue to provide exciting opportunities for catalyst discovery. Using an interdisciplinary approach combining genome mining, enzymology, protein engineering, and synthetic organic chemistry, a broader range of new-to-nature biocatalytic transformations is yet to come.

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Notes

The authors declare no competing financial interest.

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Biographies

Yang Yang received his bachelor's degree in chemistry from Peking University in 2011. He carried out graduate research at MIT under the direction of Prof. Stephen L. Buchwald, studying copper hydride-catalyzed asymmetric hydrofunctionalization of simple olefins. He was an NIH Postdoctoral Fellow in the Arnold lab at Caltech evolving P450 enzymes for asymmetric C−H functionalization. In 2020, he started as an Assistant Professor in the Department of Chemistry and Biochemistry at the University of California, Santa Barbara.

Frances H. Arnold is the Linus Pauling Professor of Chemical Engineering, Bioengineering and Biochemistry at the California Institute of Technology. She received her Ph.D. in chemical engineering from UC Berkeley in 1985. She was awarded the 2018 Nobel Prize in Chemistry for her pioneering work in directed enzyme evolution.

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