

Engineering Catalytically Self-Sufficient P450s

Hans Renata*



Cite This: *Biochemistry* 2023, 62, 253–261



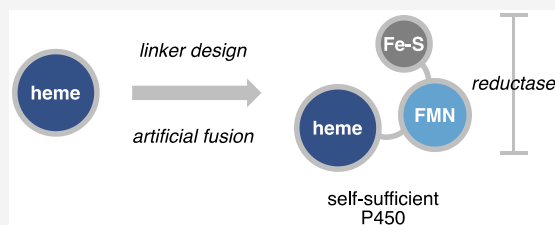
Read Online

ACCESS |

Metrics & More

Article Recommendations

ABSTRACT: The P450 superfamily comprises some of the most powerful and versatile enzymes for the site-selective oxidation of small molecules. One of the main drawbacks for the applications of the P450s in biotechnology is that the majority of these enzymes is multicomponent in nature and requires the presence of suitable redox partners to support their functions. Nevertheless, the discovery of several self-sufficient P450s, namely those from Classes VII and VIII, has served as an inspiration for fusion approaches to generate chimeric P450 systems that are self-sufficient. In this Perspective, we highlight the domain organizations of the Class VII and Class VIII P450 systems, summarize recent case studies in the engineering of catalytically self-sufficient P450s based on these systems, and outline outstanding challenges in the field, along with several emerging technologies as potential solutions.



Cytochromes P450 is a superfamily of heme-dependent enzymes that perform a wide array of oxidative transformations.¹ Members of the superfamily share the same overall fold and are defined by the presence of a heme prosthetic group that is axially ligated by a conserved Cys residue. The name of the superfamily is derived from the signature spectroscopic absorption maximum at 450 nm that can be obtained upon complexation of the ferrous form of the enzyme with CO.² P450s commonly act as monooxygenases, which are defined by the transfer of just one of the oxygen atoms from molecular oxygen to the substrate. In addition to their key roles in xenobiotic degradation and drug metabolism in humans,³ these enzymes also serve as key catalysts in the biosynthesis of secondary metabolites.⁴ Increasingly, some of these enzymes have also been widely investigated for biotechnological applications,⁵ including the production of alcohols as biofuels and fine pharmaceuticals.

The unique reaction of the P450s is derived from their ability to generate a highly reactive oxoiron(IV)porphyrin π cation radical (also known as “Compound I”, Figure 1).⁶ During the generation of this species, two electrons are delivered to the heme center in a sequential fashion, supplied by NAD(P)H through the intermediacy of redox protein partners. Due to the need for redox partners, most P450s are referred to as “multicomponent P450s” though several P450s, such as P450BM3⁷ and P450RhF,⁸ contain natural fusion to their reductase partners.⁹ These “self-sufficient” P450s have been shown to have superior catalytic activity and coupling efficiency—a term that is described to measure the efficiency NAD(P)H usage and commonly defined by the molar ratio of formed product(s) to consumed NAD(P)H—by virtue of the proximity to their redox partners. It is worth noting that the aforementioned parameters may be intimately linked as uncoupling can lead to the buildup of reactive oxygen species

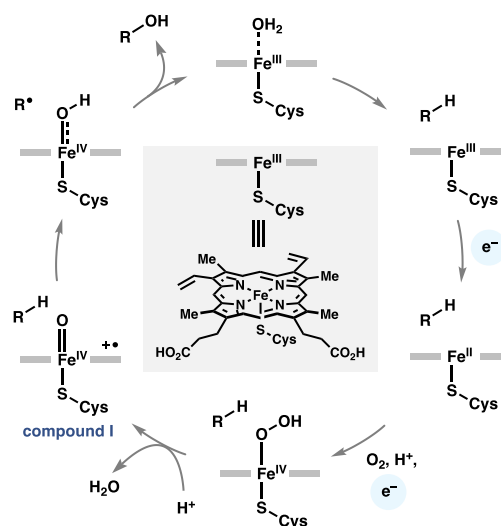


Figure 1. P450 catalytic cycle featuring the generation of compound I as the active oxidizing species.

such as superoxide anion and hydrogen peroxide, which affect the activity of the enzyme. The multicomponent nature of many P450s also presents a significant challenge for their biochemical characterization: the cognate redox partners are not always

Special Issue: Protein Engineering

Received: June 10, 2022

Revised: August 16, 2022

Published: August 31, 2022



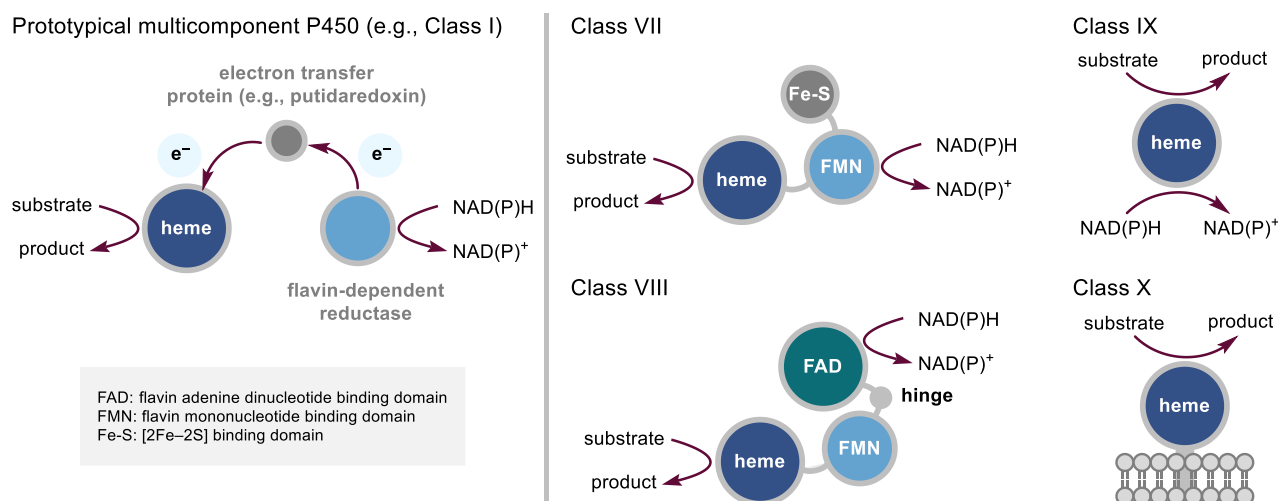


Figure 2. Domain organizations of Classes VII–X P450s and their comparison to a prototypical multicomponent P450 system.

encoded in the biosynthetic gene cluster of the P450 in context and, thus, may be difficult to find.¹⁰ Nevertheless, many bacterial P450s are known to be permissive with regards to their redox partners, and there are several surrogate redox partners¹¹ that are widely used for P450 characterization, such as the spinach ferredoxin (Fdx)/ferredoxin reductase (Fdr) system, the bovine adrenodoxin (Adx)/adrenodoxin reductase (Adr) system, and the putidaredoxin (Pdx, also known as CamB)/putidaredoxin reductase (Pdr, also known as CamA) system from *P. putida*. One of the key technical disadvantages of this approach is the need to independently express these redox partners, which significantly complicates application in enzymatic synthesis and biotechnology, especially since the relative expressions of these partners need to be modulated to achieve optimal reconstitution. As a workaround, the discovery of self-sufficient P450s has served as an inspiration to develop artificial P450 chimeras with the same properties. This approach is especially attractive since several self-sufficient P450s, such as P450BM3, are known to possess superior catalytic activity and coupling efficiency relative to their multicomponent counterparts. Thus, this approach may offer a viable solution to address some of the key bottlenecks in the synthetic applications of the P450s, provided that the catalytic properties are not impaired during the chimera generation. This Perspective highlights recent case studies in protein engineering approaches to generate self-sufficient P450s. We will first provide a brief primer on the different P450 Classes and the organization of their heme and reductase domains, focusing on several self-sufficient P450s that have been well-studied. Next, recent case studies that draw inspiration from self-sufficient P450s to generate artificial chimeras will be covered. Finally, we conclude by discussing current outstanding challenges in the area and several recent developments in protein engineering that might merit incorporation in the creation of self-sufficient P450s.

P450 Classes. The P450s were initially categorized into just two different Classes, namely the cytosolic Class I P450s and the membrane-bound eukaryotic Class II P450s.¹² Subsequent discoveries have led to the expansion of the Class system, and most recently, a ten-Class system has been put forth, defined by the identity of the redox partners, domain organization, and localization. In general, Classes I–VI are multicomponent systems, and Classes VII–X are self-sufficient enzymes whose domains are expressed from a single gene to produce a single

polypeptide (Figure 2). For the purpose of this review, we will focus on Classes VII–X, and the readers are referred to several excellent reviews^{10,12} for more thorough discussions on Classes I–VI.

Class VII. The bacterial Class VII P450 system consists of an N-terminal P450 domain fused to a phthalate dioxygenase reductase (PDR) domain.¹³ The latter is made up of an NAD(P)H-dependent FMN-binding domain and a [2Fe-2S]-containing Fdx. During the catalytic cycle, the FMN domain transfers electrons from NAD(P)H to the electron acceptor Fdx domain. CYP116B2 from *Rhodococcus* sp. (P450RhF) is the first characterized member of the Class.⁸ Initial work by Turner and co-workers identified this P450 through unbiased PCR amplification with degenerate primers, characterized its novel domain organization, and established initial heterologous expression system in *E. coli*. In this study, the Turner lab also demonstrated that recombinant P450RhF is able to catalyze the *O*-dealkylation of 7-ethoxycoumarin, though a later study showed that this reaction proceeded with low coupling efficiency (10–15%) and turnover.^{8c} Since this discovery, several other Class members have been identified.¹⁴ As we will see in later sections, the reductase domain of P450RhF (RhFRed) has also proven to be a versatile partner in the construction of catalytically self-sufficient P450 chimeras.

Class VIII. Class VIII P450s contain a similar domain organization to Class VII but utilize a different set of redox partners. Instead of a PDR domain, the redox partner of Class VIII consists of a eukaryotic-like diflavin cytochrome P450 reductase (CPR). This CPR domain comprises an FMN-binding domain that links the N-terminal heme domain and the C-terminal FAD-binding domain. The latter uses NAD(P)H as an electron donor to initiate an electron transfer relay process to the FMN domain and subsequently to the heme domain. The first characterized member of the family, CYP102A1 from *Bacillus megaterium*, also known as P450BM3,⁷ is one of the most catalytically active P450s and has been one of the most well-studied members of the superfamily from the vantage point of both mechanistic enzymology and biotechnological application. Wild-type P450BM3 is responsible for the hydroxylation of long-chain fatty acids at their subterminal positions with very high coupling efficiency (*ca.* 90% or higher). Seminal work from the Arnold laboratory showed that this P450 can be engineered for the hydroxylation of short-chain alkanes¹⁵ (e.g., *n*-octane, *n*-

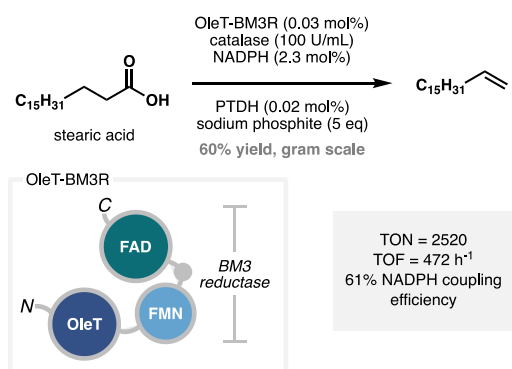
propane, ethane) with native-like efficiency. Concurrently, this enzyme has also been engineered¹⁶ for the site-selective hydroxylation of a variety of small molecules, including terpenes, alkaloids, and drug-like substances, culminating in several applications in the chemoenzymatic synthesis of complex natural products.¹⁷

Class IX and Class X. The Class IX system is made up exclusively of nitric oxide reductases, which catalyze the reduction of NO to N₂O.¹⁸ Unlike the other Classes, members of this Class do not require any redox partner domain and are capable of using NADH directly as reductant. Similar to Class IX, Class X P450s also function in the absence of any redox partners. Members of this Class are typically membrane bound and also belong to the CYP74 family.¹⁹

Recent Examples of Engineering Catalytically Self-Sufficient P450s. The discovery of naturally self-sufficient P450s has served as an inspiration and template for the development of artificial self-sufficient systems from multi-component P450s that bypass the need for individual expressions of the reductase partners. To date, this development has almost exclusively relied on covalently linking the P450 in context with the reductase domains of naturally self-sufficient P450s through artificial fusion. In this section, we highlight recent examples from the literature that showcase the viability and biocatalytic utility of this approach.

OleT. The P450 OleT_{JE} catalyzes the conversion of saturated fatty acids to terminal olefins using H₂O₂ as the terminal oxidant.²⁰ Interestingly, OleT_{JE} is capable of using O₂ as the oxidant and NAD(P)H as the electron donor in the presence of redox partners. To bypass the need for separate redox partner expression and simplify the protein production and reaction system, Lu et al. investigated the generation of self-sufficient OleT_{JE} chimeras by fusing the P450 to the reductase domains of P450RhF and P450BM3.²¹ Between the two, the fusion protein containing P450BM3 reductase (OleT-BM3R, Scheme 1) was

Scheme 1. Oxidative Decarboxylation of Stearic Acid with OleT-BM3R Chimera

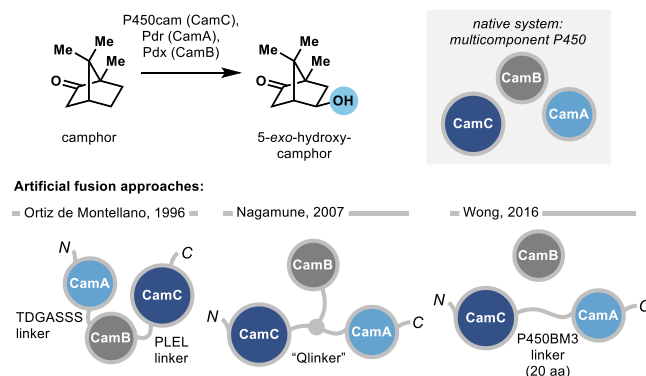


found to be superior. Using stearic acid as a model substrate, the reaction proceeded with 2520 turnover number (TON), 472 h⁻¹ turnover frequency, and 61% NADPH coupling efficiency. These numbers correspond to a 7.3-fold improvement in turnover frequency and a 2.1-fold improvement in NADPH coupling efficiency relative to the nonfused counterpart. Furthermore, this coupling efficiency represents a marked improvement over a previous reconstitution effort with the CamA/CamB reductase system by Faber and co-workers.^{20c} The reaction was coupled to a phosphite dehydrogenase-based

regeneration system for large scale application and substrate scope examination, resulting in the production of more than ten terminal alkenes with moderate to high yields. Further comparison of the OleT-BM3R system with the previously established combination of OleT and Pdx/Pdr showed marked improvements in product titer (1.4 g L⁻¹ vs 0.93 g L⁻¹) and volumetric productivity (117 mg L⁻¹ h⁻¹ vs 42.5 mg L⁻¹ h⁻¹). The various head-to-head comparisons performed in this report highlight the advantages of developing a self-sufficient variant of OleT_{JE}. Notably, 1-heptadecene could be produced from stearic acid using the cell-free lysate system with 60% yield on gram scale, further demonstrating the scalability of the biotransformation.

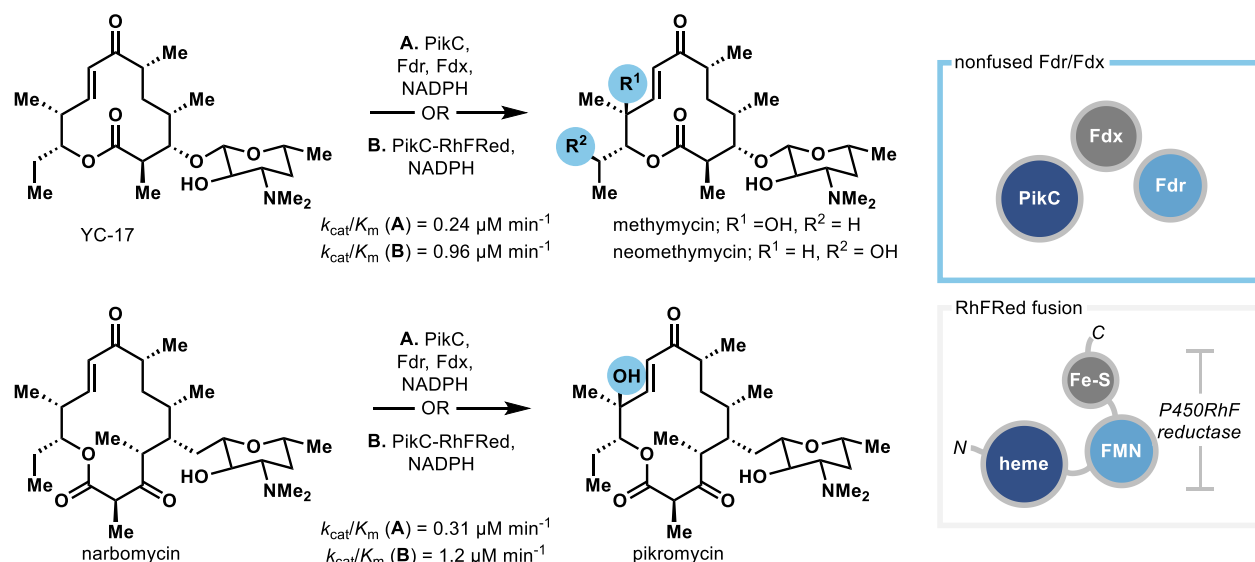
P450cam. Cytochrome P450cam, also known as CYP101, serves to convert camphor to 5-*exo*-hydroxycamphor in *P. putida*.²² This enzyme was the first P450 to be structurally characterized through X-ray diffraction,²³ which facilitates extensive mechanistic studies on the superfamily. As a Class I P450, the catalytic activity of P450cam is supported by the flavoprotein putidaredoxin reductase (Pdr/CamA) and the [2Fe-2S]-containing putidaredoxin (Pdx/CamB). In the catalytic cycle, Pdx shuttles electrons from Pdr to P450cam with NADH acting as the electron source. Following compound I generation in the monooxygenase protein, hydrogen atom abstraction from camphor and radical rebound affords 5-*exo*-hydroxycamphor. In 1996, Ortiz de Montellano and co-workers reported the generation of a Pdr-Pdx-P450cam triple fusion system (Scheme 2).²⁴ Four constructs were tested in total,

Scheme 2. Comparison of Several Self-Sufficient or Partially Sufficient P450cam Fusions for the Production of 5-*exo*-Hydroxycamphor



achieved by varying the location of the reductases (i.e., N-terminal vs C-terminal), as well as the length and the sequence of the linkers. Among these four, Pdr-Pdx-P450cam, containing Pdx that is flanked by Pdr at its N-terminus and P450cam at its C-terminus, was observed to exhibit the highest catalytic activity as measured by the rate of O₂ consumption. Comparison of this triple fusion system with the native system in which Pdr, Pdx, and P450cam were mixed in a 1:1:1 ratio showed that the two systems perform comparably, although the O₂ consumption rate of the native system is faster at higher enzyme concentrations. It should also be noted that the product formation rate of the triple fusion system is markedly lower than the optimally reconstituted native system which consists of a 1:8:20 ratio of P450cam:Pdr:Pdx. Complementary to this work, Nagamune designed a branched fusion strategy to covalently link together P450cam, Pdr, and Pdx, achieved by first fusing P450cam and

Scheme 3. Comparison of PikC Reconstitution with Spinach Fdr/Fdx and Fusion with RhFRed in the Hydroxylation of YC-17 and Narbomycin



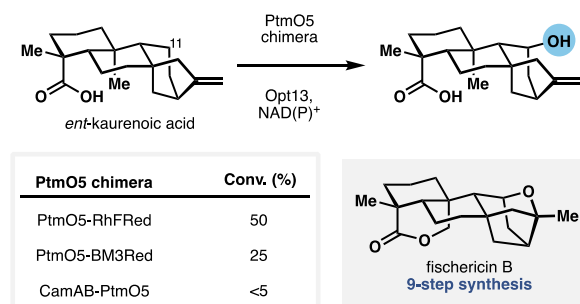
Pdr, followed by cross-linking the chimera with Pdx through the action of a transglutaminase.²⁵ This construct proved to be ca. 10-fold more efficient than de Montellano's system and provided almost perfect coupling efficiency (99%). More recently, Johnson and Wong investigated an alternative fusion approach comprising a partial fusion of Pdr to the C-terminus of P450cam through a linker peptide, which was then reconstituted with free Pdx.²⁶ By coexpressing a ferrochelatase from *E. coli*, optimizing the expression conditions, and varying the identity of the polypeptide linker, the authors were able to obtain up to 85% heme incorporation. The partial fusion system provided similar *in vivo* camphor oxidation efficiency to the native system and >90% coupling efficiency. This approach provides an alternative strategy for the construction of self-sufficient P450s with the Pdx/Pdr reductase system, though its generality remains to be shown.

Artificial Fusion with RhFRed. Two seminal studies by Misawa and co-workers hinted at the generality of using the reductase from P450RhF (RhFRed) to support P450 activity in a fusion arrangement.²⁷ Specifically, 5-*exo*-hydroxycamphor, protocatechuate, and 1-octanol were successfully produced from camphor, 4-hydroxybenzoate, and *n*-octane with recombinant *E. coli* cells that express P450cam-RhFRed, P450Bzo-RhFRed, and P450Balk-RhFRed, respectively. To further verify the viability of this fusion approach, the Sherman laboratory performed a series of *in vitro* studies using purified PikC-RhFRed and benchmarked this chimera against a multicomponent system supported by the spinach Fdr/Fdx reductase (Scheme 3).²⁸ PikC is involved in the biosynthesis of pikromycin, methymycin, and neomethymycin by catalyzing the final C–H oxidation step on the macrolactones narbomycin and YC-17.²⁹ Spectral binding titrations suggested that the fusion arrangement has minimal impact on substrate binding affinity. Michaelis–Menten analyses further showed that the catalytic efficiency of the reactions with both YC-17 and narbomycin was improved by approximately 4-fold in the fusion arrangement. These studies have spurred further investigation on RhFRed as a viable reductase partner in artificial fusion systems with various P450s. To date, this strategy has been the most well-explored for the engineering of self-sufficient variants of bacterial Class I P450s.

Grogan and co-workers have also developed a ligation-independent cloning vector to generate a library of RhFRed-fused P450s and demonstrated the utility of this platform in the discovery of new hydroxylation enzymes.³⁰

PtmO5. Platensimycin is a diterpenoid antibiotic that acts by inhibiting the prokaryotic fatty acid biosynthesis.³¹ Studies by the Shen laboratory have elucidated many of the steps in platensimycin biosynthesis, including the terpenoid origin of the scaffold and some of the oxidative tailoring steps that modify the diterpenoid core.^{2,32} Following *ent*-kauranol formation by the terpene cyclase PtmT3, the P450 PtmO5 hydroxylates the carbocyclic core at C11,³³ which also results in the formation of the signature caged ether motif of platensimycin. Based on this insight, the Shen and the Renata laboratories sought to examine the biocatalytic application of PtmO5 in the chemoenzymatic synthesis of complex *ent*-kauranes (Scheme 4).³⁴ PtmO5 is

Scheme 4. Screening of Several PtmO5 Chimeras for the C11 Hydroxylation of *ent*-Kauronic Acid and the Synthetic Application of PtmO5-RhFRed in the Chemoenzymatic Synthesis of Fischericin B

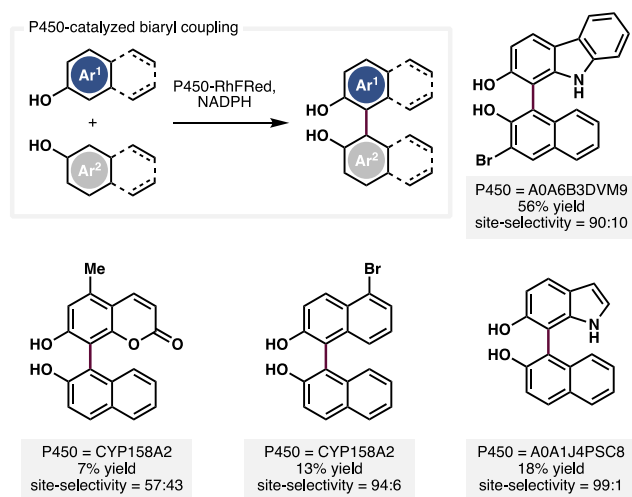


annotated as a Class I P450, and the biochemical characterization of this enzyme was initially performed with the CamAB reductase system. However, this approach yielded low conversions in large-scale reactions, which led the authors to investigate several fusion constructs. Three fusion chimeras were tested, namely PtmO5-BM3Red (through C-terminal fusion with P450BM3 reductase), PtmO5-RhFRed (through C-

terminal fusion with P450RhF reductase), and CamAB-PtmO5 (through *N*-terminal fusion with CamAB). PtmO5-RhFRed was found to provide the optimal conversion in the hydroxylation of steviol and *ent*-kaurenoic acid. Following further reaction optimization, an 88% isolated yield could be obtained in the hydroxylation of *ent*-kaurenoic acid on preparative scale. The synthetic utility of this biotransformation was demonstrated in the chemoenzymatic synthesis of fischericin B. Furthermore, PtmO5-RhFRed could also hydroxylate isosteviol at C12, which facilitated access to complex *ent*-atisane and *ent*-trachylobane diterpenoids through a series of skeletal rearrangements.

CYP158A2. The ubiquity of biaryl bonds in both natural³⁵ and man-made small molecules prompted the Narayan laboratory to investigate the biocatalytic utility of several P450s for oxidative biaryl coupling. Using yeast culture, the fungal P450 KtnC was shown to be capable of catalyzing not only the homodimerization but also the heterocoupling of a variety of phenolic substrates.³⁶ To complement this activity, several bacterial P450s were also tested. A sequence similarity network analysis³⁷ was performed to identify 23 P450s for initial screening, consisting of 20 uncharacterized and 3 characterized enzymes. These P450s were then linked at their C-termini to RhFRed to construct a small P450-RhFRed library. Among these enzymes, CYP158A2, previously implicated in the biosynthesis of biflavinol and triflavinol,³⁸ was able to produce several binaphthol heterodimers, and two previously uncharacterized P450s, A0A1J4PSC8 and A0A6B3DVM9, could catalyze the heterocoupling of 2-naphthol derivatives with indole- and carbazole-containing partners (Scheme 5).

Scheme 5. Oxidative Biaryl Couplings Catalyzed by P450 Chimeras from RhFRed Fusion



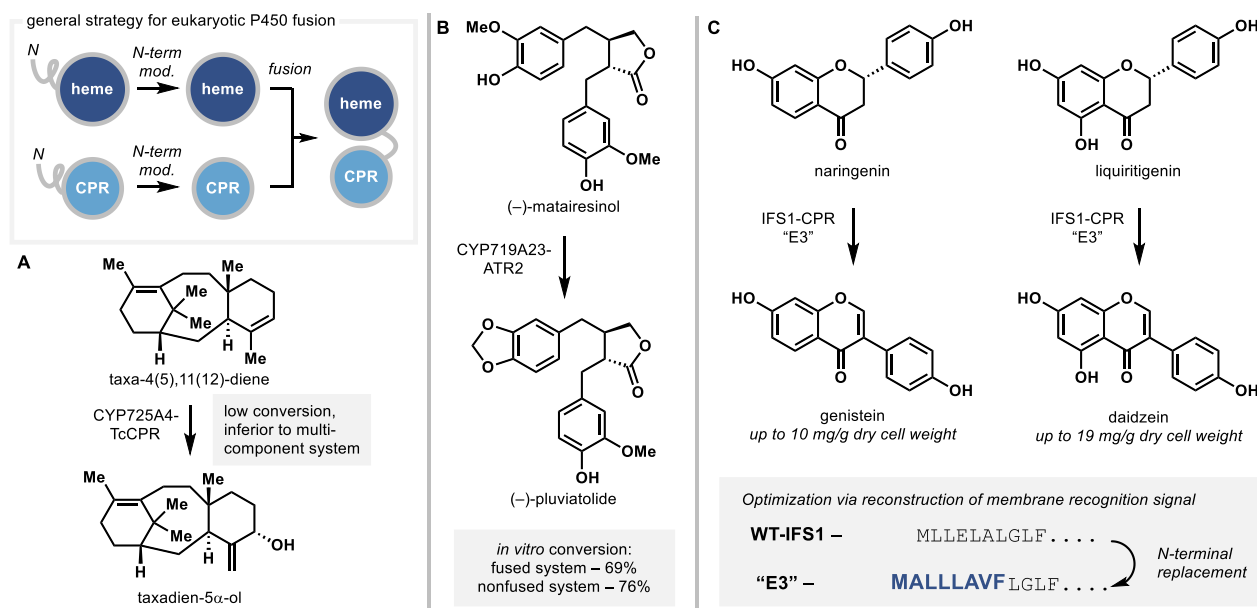
Artificial Chimeras of Eukaryotic P450s. The fusion protein approach to generate self-sufficient P450s is not limited to just bacterial P450s, and several eukaryotic P450s have been successfully rendered self-sufficient through a similar strategy. The membrane-bound nature³⁹ of eukaryotic P450s and their redox partners presents a significant challenge for their biochemical studies. Several studies have shown that excision or modification of the *N*-terminal transmembrane domain of some of these P450s can lead to soluble and functional proteins upon heterologous expression in prokaryotic hosts.⁴⁰ Furthermore, Gilardi and co-workers were able to combine this *N*-terminal modification with C-terminal fusion with P450BM3

reductase to generate several self-sufficient P450s that could be solubly expressed in *E. coli*.⁴¹ Such fusion strategy for eukaryotic P450s is not limited to the use of P450BM3 reductase and has been explored to varying degrees of success for biotechnological applications. For example, during the optimization of CYP725A4 activity for the production of oxygenated taxanes,⁴² De Mey, Ajikumar, and co-workers found that while the fusion of CYP725A4 with its cognate reductase partner TcCPR can provide low level production of oxygenated taxanes, the activity is in general inferior to the multicomponent counterpart, especially when the reductase partner is present in excess (Scheme 6A). Conversely, Urlacher and co-workers⁴³ found that *N*-terminally modified variants of the plant P450 CYP719A23 and the P450 reductase from *A. thaliana* ATR2 could be artificially fused to generate a chimera that provides comparable conversion to the multicomponent system in the *in vitro* conversion of matairesinol to pluviatolide (69% vs 76%), a key biosynthetic intermediate to the microtubule destabilizer podophyllotoxin (Scheme 6B). However, due to the poor heterologous expression of the chimera in *E. coli*, coexpression of CYP719A23 and ATR2 without physically linking them was found to be superior for *in vivo* production. A similar fusion approach was previously investigated by Leonard and Koffas for the biological production of isoflavones.⁴⁴ In this case, the P450 IFS1 was first linked to an *N*-terminally truncated variant of the *Catharanthus* P450 reductase. Next, several *N*-terminal modifications of the IFS1 domain were investigated, and the optimal construct “E3” was obtained by replacing the first 6 *N*-terminal residues of IFS1 with the first 8 *N*-terminal residues of the bovine 17 α -hydroxylase (Scheme 6C). Under optimal conditions, up to 10 mg/g dry cell weight of genistein and 19 mg/g dry cell weight of daidzein could be obtained.

DISCUSSION AND CONCLUSION

Despite the many successes outlined above, current fusion strategies still suffer from several limitations, especially with respect to the catalytic activity of the resulting enzymes. For example, though the self-sufficient PikC-RhFRed described previously was found to display an approximately 4-fold improvement in catalytic efficiency relative to the multicomponent PikC/Fdr/Fdx mixture, the rate of camphor hydroxylation by P450cam-RhFRed is significantly lower than the optimally reconstituted P450cam/Pdr/Pdx system. The Sherman laboratory recently reported a chemoenzymatic platform for the generation of cryptophycin analogs as potent antiproliferative agents.⁴⁵ The cryptophycins are high-affinity microtubule binding agents that have previously been investigated for the treatment of ovarian cancer and nonsmall-cell lung cancer in clinical trials.⁴⁶ Sherman’s approach leveraged two key enzymes from cryptophycin biosynthesis, namely CrpTE, the thioesterase that catalyzes the macrocycle formation, and CrpE, the P450 that catalyzes a late-stage epoxidation on the styrenyl olefin. The promiscuity of CrpTE was demonstrated through the formation of more than ten unnatural cryptophycin analogs, including those bearing heterocyclic rings. *In vitro* conversion of these analogs to the epoxide derivatives was performed with both CrpE-RhFRed chimera and a multicomponent system consisting of standalone CrpE and spinach Fdr/Fdx reductase. While the latter was able to provide low to moderate conversions to the desired products, no conversion could be detected with the former. Based on some of these case studies, one can surmise that artificial fusion could dramatically reduce coupling efficiency in the reaction or

Scheme 6. A. Investigation of CYP725A4-TcCPR Fusion for the Production of Taxadien-5 α -ol, B. *in Vitro* Conversion of (–)-Matairesinol to (–)-Pluviatolide with CYP719A23-ATR2 Fusion and Its Comparison with the Nonfused Counterpart, and C. Production of Genistein and Daidzein with IFS1-CPR Chimera Construct “E3”^a



^aGenerated by fusion of IFS1 with *Catharanthus* CPR, followed by N-terminal engineering.

introduce deleterious domain–domain interactions. Similar issues have been encountered in the reactions of natural P450s with non-native substrates and could be remedied through a domain-based engineering strategy⁴⁷ featuring separate evolution of the heme and reductase domains, followed by recombination. A collaborative work between the Saab-Rincon and the Fasan laboratories⁴⁸ also showed that a consensus-guided mutagenesis of P450BM3 featuring comparison of reductase domain residues among 16 of its homologues can lead to improved thermal stability and increased catalytic activity at elevated temperatures. Thus, it is conceivable that a similar evolution-based strategy can also be applied in the future to further improve the coupling efficiency and catalytic activity of self-sufficient P450 chimeras.

The two primary considerations for fusion generation are the order of arrangement of the different domains and the identity of the linker region. Many studies in the fundamentals of protein design⁴⁹ have pointed to the impact of the linker region on the fusion protein microenvironment, typically by using fluorescent proteins as model systems. However, contemporary linker design for self-sufficient P450s, as well as other artificially fused enzyme systems,⁵⁰ is largely reliant on the use of simple tethers (either flexible linkers with glycine-rich sequence or rigid linkers with proline- or alanine-rich sequence) of varying lengths or the direct adaptation of linker sequences found in naturally self-sufficient P450s. More recently, Minter and co-workers⁵¹ have demonstrated the viability of incorporating Rosetta-based modeling to aid with the linker placement and identity for the fusion of an alcohol and aldehyde dehydrogenase. Beyond artificial fusion, noncovalent interactions can also be exploited to generate self-assembling multidomain P450 constructs. In 2016, Urlacher and co-workers reported⁵² that the heme and the reductase domains of P450BM3 could be fused separately to the hydrophobin HFBI, a self-assembling protein from *Trichoderma reesei*, and the resulting proteins could undergo self-assembly to generate a multimeric system that is catalytically more active

than the nonfused system. Beyond HFBI self-assembly, there have also been a number of recent studies on the design of heteromeric assemblies with short protein sequences and the discovery of docking motifs from polyketide synthases (PKS) that facilitate domain assemblies. For example, Woolfson and co-workers reported⁵³ the *de novo* design of short peptide sequences (21–28 residues each) that can undergo heterodimeric coiled-coil assemblies with binding affinities in the micromolar to subnanomolar range. Conversely, Ellington, Keatinge-Clay, and co-workers⁵⁴ were able to mine a number of docking domain pairs that comprise ca. 25 residues from the N- and C-termini of modular PKS domains. They further showed that these domains can be used to connect non-PKS proteins through four-helix bundle formation with micromolar affinities. Each of these emerging technologies could potentially be adapted in the design of next-generation linkers for self-sufficient P450 chimeras.

To date, the artificial fusion approaches have also largely relied on reductases from P450s that have been well-studied in the past. However, recent explosion of genomic information has enabled the identification of a number of naturally self-sufficient P450s. In 2017, Tavanti et al. reported the discovery of thermostable homologues of P450RhF that also contain natural fusion with their reductase partners.^{14c} Though there have been no reports on the use of these reductases to create self-sufficient P450s, they share only 53–68% sequence identity to RhFRed and may display complementary protein–protein interactions to RhFRed. Similar to Grogan’s work, one could envision the development of a ligation-independent cloning vector to generate a library of reductases that are fused to a specific P450 of interest to rapidly screen for an optimal construct. The full-length crystal structure of one of these thermostable P450s, CYP116B46 (also known as P450TT), was recently solved,⁵⁵ which might prove useful for the generation of structural models to more rationally design artificial fusion constructs.

To summarize, the P450s hold enormous potential for a wide array of biotechnological and biocatalytic applications. However, their multidomain nature presents significant challenges for large-scale applications beyond simple biochemical characterization. The discovery of natural P450s that are catalytically self-sufficient has served as an inspiration for the engineering of artificial systems with the same properties by fusing multi-component P450s with noncognate reductase partners. Though a number of successful case studies have been recently reported, further advances are still needed to fully realize the potential of this engineering approach, especially to address the attenuated catalytic activity and coupling efficiency of the artificial systems. In addition to summarizing recent reports on the creation of self-sufficient P450 chimeras, we have also outlined several developments in the protein engineering field that can potentially be adapted to further optimize the current strategies. We anticipate that the incorporation of these emerging technologies will help maximize the biocatalytic and synthetic utility of the P450s.

AUTHOR INFORMATION

Corresponding Author

Hans Renata — Department of Chemistry, BioScience Research Collaborative, Rice University, Houston, Texas 77005, United States; orcid.org/0000-0003-2468-2328; Email: hr28@rice.edu

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acs.biochem.2c00336>

Notes

The author declares no competing financial interest.

ACKNOWLEDGMENTS

Our work in this area is supported, in part, by the National Institutes of Health (grant R35 GM128895) and the National Science Foundation CAREER award (grant #1945468).

ABBREVIATIONS

CPR, cytochrome P450 reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PCR, polymerase chain reaction; PDR, phthalate dioxygenase reductase.

REFERENCES

- (1) Poulos, T. L.; Johnson, E. F. Structures of Cytochrome P450 Enzymes. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Springer: Switzerland, 2015; pp 3–32, DOI: [10.1007/978-3-319-12108-6_1](https://doi.org/10.1007/978-3-319-12108-6_1).
- (2) Klingenberg, M. Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* **1958**, *75*, 376–386.
- (3) (a) Guengerich, F. P. Human Cytochrome P450 Enzymes. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Springer: Switzerland, 2015; pp 523–786, DOI: [10.1007/978-3-319-12108-6_9](https://doi.org/10.1007/978-3-319-12108-6_9). (b) Auchus, R. J.; Miller, W. L. P450 Enzymes in Steroid Processing. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Springer: Switzerland, 2015; pp 851–881, DOI: [10.1007/978-3-319-12108-6_12](https://doi.org/10.1007/978-3-319-12108-6_12).
- (4) (a) Podust, L. M.; Sherman, D. H. Diversity of P450 enzymes in the biosynthesis of natural products. *Nat. Prod. Rep.* **2012**, *29*, 1251–1266. (b) Rudolf, J. D.; Chang, C.-Y.; Ma, M.; Shen, B. Cytochromes P450 for natural product biosynthesis in *Streptomyces*: sequence, structure, and function. *Nat. Prod. Rep.* **2017**, *34*, 1141–1172. (c) Zhang, X.; Guo, J.; Cheng, F.; Li, S. Cytochrome P450 enzymes

in fungal natural product biosynthesis. *Nat. Prod. Rep.* **2021**, *38*, 1072–1099.

- (5) (a) Fasan, R. Tuning P450 enzymes as oxidation catalysts. *ACS Catal.* **2012**, *2*, 647–666. (b) Urlacher, V. B.; Girhard, M. Cytochrome P450 monooxygenases in biotechnology and synthetic biology. *Trends Biotechnol.* **2019**, *37*, 882–897. (c) Girvan, H. M.; Munro, A. W. Applications of microbial cytochrome P450 enzymes in biotechnology and synthetic biology. *Curr. Opin. Chem. Biol.* **2016**, *31*, 136–145. (d) Jung, S. T.; Lauchli, R.; Arnold, F. H. Cytochrome P450: taming a wild type enzyme. *Curr. Opin. Biotechnol.* **2011**, *22*, 809–817.
- (6) Munro, A. W.; Girvan, H. M.; Mason, A. E.; Dunford, A. J.; McLean, K. J. What makes a P450 tick? *Trends Biochem. Sci.* **2013**, *38*, 140–150.
- (7) (a) Narhi, L. O.; Fulco, A. J. Characterization of a catalytically self-sufficient 119,000-Dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **1986**, *261*, 7160–7169. (b) Wen, L.-P.; Fulco, A. J. Cloning of the gene encoding a catalytically self-sufficient cytochrome P-450 fatty acid monooxygenase induced by barbiturates in *Bacillus megaterium* and its functional expression and regulation in heterologous (*Escherichia coli*) and homologous (*Bacillus megaterium*) hosts. *J. Biol. Chem.* **1987**, *262*, 6676–6682. (c) Munro, A. W.; Leys, D. G.; McLean, K. J.; Marshall, K. R.; Ost, T. W. B.; Daff, S.; Miles, C. S.; Chapman, S. K.; Lysek, D. A.; Moser, C. C.; Page, C. C.; Dutton, P. L. P450 BM3: the very model of a modern flavocytochrome. *Trends Biochem. Sci.* **2002**, *27*, 250–257.
- (8) (a) Roberts, G. A.; Grogan, G.; Greter, A.; Flitsch, S. L.; Turner, N. J. Identification of a new class of cytochrome P450 from a *Rhodococcus* sp. *J. Bacteriol.* **2002**, *184*, 3898–3908. (b) Hunter, D. J. B.; Roberts, G. A.; Ost, T. W. B.; White, J. H.; Müller, S.; Turner, N. J.; Flitsch, S. L.; Chapman, S. K. Analysis of the domain properties of the novel cytochrome P450 RhF. *FEBS Lett.* **2005**, *579*, 2215–2220. (c) Roberts, G. A.; Celik, A.; Hunter, D. J. B.; Ost, T. W. B.; White, J. H.; Chapman, S. K.; Turner, N. J.; Flitsch, S. L. A self-sufficient cytochrome P450 with a primary structural organization that includes a flavin domain and a [2Fe-2S] redox center. *J. Biol. Chem.* **2003**, *278*, 48914–48920.
- (9) Munro, A. W.; Girvan, H. M.; McLean, K. J. Cytochrome P450–redox partner fusion enzymes. *Biochim. Biophys. Acta* **2007**, *1770*, 345–359.
- (10) Chen, C.-C.; Min, J.; Zhang, L.; Yang, Y.; Yu, X.; Guo, R.-T. Advanced understanding of the electron transfer pathway of cytochrome P450s. *ChemBioChem.* **2021**, *22*, 1317–1328.
- (11) Li, S.; Du, L.; Bernhardt, R. Redox partners: function modulators of bacterial P450 enzymes. *Trends Microbiol.* **2020**, *28*, 445–454.
- (12) Finnigan, J. D.; Young, C.; Cook, D. J.; Charnock, S. J.; Black, G. W. Cytochromes P450 (P450s): A review of the class system with a focus on prokaryotic P450s. *Adv. Protein Chem. Struct. Biol.* **2020**, *122*, 289–320.
- (13) Correddu, D.; Di Nardo, G.; Gilardi, G. Self-sufficient Class VII cytochromes P450: from full-length structure to synthetic biology applications. *Trends Biotechnol.* **2021**, *39*, 1184–1207.
- (14) (a) De Mot, R.; Parret, A. H. A novel class of self-sufficient cytochrome P450 monooxygenases in prokaryotes. *Trends Microbiol.* **2002**, *10*, 502–508. (b) Liu, L.; Schmid, R. D.; Urlacher, V. B. Cloning, expression, and characterization of a self-sufficient cytochrome P450 monooxygenase from *Rhodococcus ruber* DSM 44319. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 876–882. (c) Tavanti, M.; Porter, J. L.; Sabatini, S.; Turner, N. J.; Flitsch, S. L. Panel of new thermostable CYP116B self-sufficient cytochrome P450 monooxygenases that catalyze C–H activation with a diverse substrate scope. *ChemCatChem.* **2018**, *10*, 1042–1051. (d) Yin, Y. C.; Yu, H. L.; Luan, Z. J.; Li, R. J.; Ouyang, P. F.; Liu, J.; Xu, J. H. Unusually broad substrate profile of self-sufficient cytochrome P450 monooxygenase CYP116B4 from *Labrenzia aggregata*. *ChemBioChem.* **2014**, *15*, 2443–2449.
- (15) (a) Meinhold, P.; Peters, M. W.; Hartwick, A.; Hernandez, A. R.; Arnold, F. H. Engineering cytochrome P450 BM3 for terminal alkane hydroxylation. *Adv. Synth. Catal.* **2006**, *348*, 763–772. (b) Peters, M. W.; Meinhold, P.; Glieder, A.; Arnold, F. H. Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3. *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450. (c) Glieder, A.; Farinas, E. T.;

Arnold, F. H. Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase. *Nat. Biotechnol.* **2002**, *20*, 1135–1139.

(d) Farinas, E. T.; Schwaneberg, U.; Glieder, A.; Arnold, F. H. Directed evolution of a cytochrome P450 monooxygenase for alkane oxidation. *Adv. Synth. Catal.* **2001**, *343*, 601–606. (e) Fasan, R.; Meharena, Y. T.; Snow, C. D.; Poulos, T. L.; Arnold, F. H. Evolutionary history of a specialized P450 propane monooxygenase. *J. Mol. Biol.* **2008**, *383*, 1069–1080.

(16) (a) Whitehouse, C. J. C.; Bell, S. G.; Wong, L.-L. P450BM3 (CYP102A1): connecting the dots. *Chem. Soc. Rev.* **2012**, *41*, 1218–1260. (b) Alwaseem, H.; Giovani, S.; Crotti, M.; Welle, K.; Jordan, G. T.; Ghaemmaghami, S.; Fasan, R. Comprehensive structure-activity profiling of micheliolide and its targeted proteome in leukemia cells via probe-guided late-stage C–H functionalization. *ACS Cent. Sci.* **2021**, *7*, 841–857. (c) Alwaseem, H.; Frisch, B. J.; Fasan, R. Anticancer activity profiling of parthenolide analogs generated via P450-mediated chemoenzymatic synthesis. *Bioorg. Med. Chem.* **2018**, *26*, 1365–1373. (d) Tyagi, V.; Alwaseem, H.; O'Dwyer, K. M.; Ponder, J.; Li, Q. Y.; Jordan, C. T.; Fasan, R. Chemoenzymatic synthesis and antileukemic activity of novel C9- and C14-functionalized parthenolide analogs. *Bioorg. Med. Chem.* **2016**, *24*, 3876–3886. (e) Kolev, N. J.; Dwyer, K. M.; Jordan, C. T.; Fasan, R. Discovery of potent parthenolide-based antileukemic agents enabled by late-stage P450-mediated C–H functionalization. *ACS Chem. Biol.* **2014**, *9*, 164–173. (f) Zhang, K.; Shafer, B. M.; Demars, M. D.; II; Stern, H. A.; Fasan, R. Controlled oxidation of remote sp^3 C–H bonds in artemisinin via P450 catalysts with fine-tuned regio- and stereoselectivity. *J. Am. Chem. Soc.* **2012**, *134*, 18695–18704. (g) Zhang, K.; El Damaty, S.; Fasan, R. P450 fingerprinting method for rapid discovery of terpene hydroxylating P450 catalysts with diversified regioselectivity. *J. Am. Chem. Soc.* **2011**, *133*, 3242–3245.

(17) (a) Loskot, S. A.; Romney, D. K.; Arnold, F. H.; Stoltz, B. M. Enantioselective total synthesis of nigelladine A via late-stage C–H oxidation enabled by an engineered P450 enzyme. *J. Am. Chem. Soc.* **2017**, *139*, 10196–10199. (b) Li, J.; Li, F.; King-Smith, E.; Renata, H. Merging chemoenzymatic and radical-based retrosynthetic logic for rapid and modular synthesis of oxidized meroterpenoids. *Nat. Chem.* **2020**, *12*, 173–179. (c) Li, F.; Renata, H. A chiral-pool-based strategy to access trans-syn-fused drimane meroterpenoids: chemoenzymatic total syntheses of polysyn, *N*-acetyl-polyveoline and the chrodrimanins. *J. Am. Chem. Soc.* **2021**, *143*, 18280–18286. (d) Li, F.; Deng, H.; Renata, H. Remote B-ring oxidation of sclareol with an engineered P450 facilitates divergent access to complex terpenoids. *J. Am. Chem. Soc.* **2022**, *144*, 7616–7621.

(18) (a) Daiber, A.; Shoun, H.; Ulrich, V. Nitric oxide reductase (P450nor) from *Fusarium oxysporum*. *J. Inorg. Biochem.* **2005**, *99*, 185–193. (b) Takaya, N.; Suzuki, S.; Kuwazaki, S.; Shoun, H.; Maruo, F.; Yamaguchi, M.; Takeo, K. Cytochrome P450nor, a novel class of mitochondrial cytochrome P450 involved in nitrate respiration in the fungus *Fusarium oxysporum*. *Arch. Biochem. Biophys.* **1999**, *372*, 340–346.

(19) (a) Hughes, R. K.; De Domenico, S.; Santino, A. Plant cytochrome CYP74 family: biochemical features, endocellular localisation, activation mechanism in plant defence and improvements for industrial applications. *ChemBioChem.* **2009**, *10*, 1122–1133. (b) Brash, A. R. Mechanistic aspects of CYP74 allene oxide synthases and related cytochrome P450 enzymes. *Phytochemistry* **2009**, *70*, 1522–1531.

(20) (a) Rude, M. A.; Baron, T. S.; Brubaker, S.; Alibhai, M.; Del Cardayre, S. B.; Schirmer, A. Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid decarboxylase from *Jeotgalicoccus* species. *Appl. Environ. Microbiol.* **2011**, *77*, 1718–1727. (b) Liu, Y.; Wang, C.; Yan, J.; Zhang, W.; Guan, W.; Lu, X.; Li, S. Hydrogen peroxide-independent production of α -alkenes by OleT_{JE} P450 fatty acid decarboxylase. *Biotechnol. Biofuels* **2014**, *7*, 28. (c) Dennig, A.; Kuhn, M.; Tassoti, S.; Thiessenhusen, A.; Gilch, S.; Büttler, T.; Haas, T.; Hall, M.; Faber, K. Oxidative decarboxylation of short-chain fatty acids to 1-alkenes. *Angew. Chem., Int. Ed.* **2015**, *54*, 8819–8822. (d) Wang, J. B.; Lonsdale, R.; Reetz, M. T. Exploring substrate scope and stereoselectivity of P450

peroxygenase OleT_{JE} in olefin-forming oxidative decarboxylation. *Chem. Commun.* **2016**, *52*, 8131–8133.

(21) Lu, C.; Shen, F.; Wang, S.; Wang, Y.; Liu, J.; Bai, W.-J.; Wang, X. An engineered self-sufficient biocatalyst enables scalable production of linear α -olefins from carboxylic acids. *ACS Catal.* **2018**, *8*, 5794–5798.

(22) Dus, K.; Katagiri, M.; Yu, C.-A.; Erbes, D. L.; Gunsalus, I. C. Chemical characterization of cytochrome P-450cam. *Biochem. Biophys. Res. Commun.* **1970**, *40*, 1423–1430.

(23) (a) Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem.* **1985**, *260*, 16122–16130. (b) Poulos, T. L.; Finzel, B. C.; Howard, A. J. High-resolution crystal structure of cytochrome P450cam. *J. Mol. Biol.* **1987**, *195*, 687–700.

(24) Sibbesen, O.; De Voss, J. J.; Ortiz de Montellano, P. R. Putidaredoxin reductase-putidaredoxin-cytochrome P450cam triple fusion protein. *J. Biol. Chem.* **1996**, *271*, 22462–22469.

(25) Hirakawa, H.; Kamiya, N.; Tanaka, T.; Nagamune, T. Intramolecular electron transfer in a cytochrome P450cam system with a site-specific branched structure. *Protein Eng. Des. Sel.* **2007**, *20*, 453–459.

(26) Johnson, E. O.; Wong, L.-L. Partial fusion of a cytochrome P450 system by carboxy-terminal attachment of putidaredoxin reductase to P450cam (CYP101A1). *Catal. Sci. Technol.* **2016**, *6*, 7549–7560.

(27) (a) Kubota, M.; Nodate, M.; Yasumoto-Hirose, M.; Uchiyama, T.; Kagami, O.; Shizuri, Y.; Misawa, N. Isolation and functional analysis of cytochrome P450 CYP153A genes from various environments. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 2421–2430. (b) Nodate, M.; Kubota, M.; Misawa, N. Functional expression system for cytochrome P450 genes using the reductase domain of self-sufficient P450RhF from *Rhodococcus* sp. NCIMB 9784. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 455–462.

(28) Li, S.; Podust, L. M.; Sherman, D. H. Engineering and analysis of a self-sufficient biosynthetic cytochrome P450 PikC fused to the RhFRED reductase domain. *J. Am. Chem. Soc.* **2007**, *129*, 12940–12941.

(29) Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-W.; Sherman, D. H. Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the pikC-encoded cytochrome P450 in *Streptomyces venezuelae*. *Chem. Biol.* **1998**, *5*, 661–667.

(30) Sabbadin, F.; Hyde, R.; Robin, A.; Hilgarth, E.-M.; Delenne, M.; Flitsch, S.; Turner, N. J.; Grogan, G.; Bruce, N. C. LICRED: A versatile drop-in vector for rapid generation of redox-self-sufficient cytochrome P450s. *ChemBioChem.* **2010**, *11*, 987–994.

(31) Rudolf, J. D.; Dong, L.-B.; Shen, B. Platensimycin and platencin: Inspirations for chemistry, biology, enzymology, and medicine. *Biochem. Pharmacol.* **2017**, *133*, 139–151.

(32) (a) Rudolf, J. D.; Dong, L.-B.; Manoogian, K.; Shen, B. Biosynthetic origin of the ether ring in platensimycin. *J. Am. Chem. Soc.* **2016**, *138*, 16711–16721. (b) Smanski, M. J.; Yu, Z.; Casper, J.; Lin, S.; Peterson, R. M.; Chen, Y.; Wendt-Pienkowski, E.; Rajski, S. R.; Shen, B. Dedicated ent-kaurane and ent-atiserene synthases for platensimycin and platencin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 13498–13503. (c) Dong, L.-B.; Zhang, X.; Rudolf, J. D.; Deng, M.-R.; Kalkreuter, E.; Cepeda, A. J.; Renata, H.; Shen, B. Cryptic and stereospecific hydroxylation, oxidation, and reduction in platensimycin and platencin biosynthesis. *J. Am. Chem. Soc.* **2019**, *141*, 4043–4050.

(33) Rudolf, J. D.; Dong, L.-B.; Zhang, X.; Renata, H.; Shen, B. Cytochrome P450-catalyzed hydroxylation initiating ether formation in platensimycin biosynthesis. *J. Am. Chem. Soc.* **2018**, *140*, 12349–12353.

(34) Zhang, X.; King-Smith, E.; Dong, L.-B.; Yang, L.-Y.; Rudolf, J. D.; Shen, B.; Renata, H. Divergent synthesis of complex diterpenes through a hybrid oxidative approach. *Science* **2020**, *369*, 799–806.

(35) Aldemir, H.; Richarz, R.; Gulder, T. A. M. The biocatalytic repertoire of natural biaryl formation. *Angew. Chem., Int. Ed.* **2014**, *53*, 8286–8293.

(36) Zetzsche, L. E.; Yazarians, J. A.; Chakrabarty, S.; Hinze, M. E.; Murray, L. A. M.; Lukowski, A. L.; Joyce, L. A.; Narayan, A. R. H. Biocatalytic oxidative cross-coupling reactions for biaryl bond formation. *Nature* **2022**, *603*, 79–85.

(37) Zallot, R.; Oberg, N.; Gerlt, J. A. The EFI web resource for genomic enzymology tools: Leveraging protein, genome, and metagenome databases to discover novel enzymes and metabolic pathways. *Biochemistry* **2019**, *58*, 4169–4182.

(38) Zhao, B.; Guengerich, F. P.; Bellamine, A.; Lamb, D. C.; Izumikawa, M.; Lei, L.; Podust, L. M.; Sundaramoorthy, M.; Kalaitzis, J. A.; Reddy, L. M.; Kelly, S. L.; Moore, B. S.; Stec, D.; Voehler, M.; Falck, J. R.; Shimada, T.; Waterman, M. R. Binding of two flavin substrate molecules, oxidative coupling, and crystal structure of *Streptomyces coelicolor* A3(2) cytochrome P450 158A2. *J. Biol. Chem.* **2005**, *280*, 11599–11607.

(39) (a) Hausjell, J.; Halbwirth, H.; Spadiut, O. Recombinant production of eukaryotic cytochrome P450s in microbial cell factories. *Biosci. Rep.* **2018**, *38*, BSR20171290. (b) Durairaj, P.; Li, S. Functional expression and regulation of eukaryotic cytochrome P450 enzymes in surrogate microbial cell factories. *Eng. Microbiol.* **2022**, *2*, 100011.

(40) (a) Gillam, E. M.; Guo, Z.; Guengerich, F. P. Expression of modified human cytochrome P450 2E1 in *Escherichia coli*, purification, and spectral and catalytic properties. *Arch. Biochem. Biophys.* **1994**, *312*, 59–66. (b) Barnes, H. J.; Arlotto, M. P.; Waterman, M. R. Expression and enzymatic activity of recombinant cytochrome P450 17 α -hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5597–5601.

(41) (a) Fairhead, M.; Giannini, S.; Gillam, E. M. J.; Gilardi, G. Functional characterisation of an engineered multidomain human P450 2E1 by molecular Lego. *J. Biol. Inorg. Chem.* **2005**, *10*, 842–853. (b) Dodhia, V. R.; Fantuzzi, A.; Gilardi, G. Engineering human cytochrome P450 enzymes into catalytically self-sufficient chimeras using molecular Lego. *J. Biol. Inorg. Chem.* **2006**, *11*, 903–916.

(42) Biggs, B. W.; Lim, C. G.; Sagliani, K.; Shankar, S.; Stephanopoulos, G.; De Mey, M.; Ajikumar, P. K. Overcoming heterologous protein interdependency to optimize P450-mediated Taxol precursor synthesis in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113*, 3209–3214.

(43) Decembrino, D.; Ricklefs, E.; Wohlgemuth, S.; Girhard, M.; Schullehner, K.; Jach, G.; Urlacher, V. Assembly of plant enzymes in *E. coli* for the production of the valuable (–)-podophyllotoxin precursor (–)-pluviatolide. *ACS Synth. Biol.* **2020**, *9*, 3091–3103.

(44) Leonard, E.; Koffas, M. A. G. Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by *Escherichia coli*. *Appl. Environ. Microbiol.* **2007**, *73*, 7246–7251.

(45) Schmidt, J. J.; Khatri, Y.; Brody, S. L.; Zhu, C.; Pietraszkiewicz, H.; Valeriote, F. A.; Sherman, D. H. A versatile chemoenzymatic synthesis for the discovery of potent cryptophycin analogs. *ACS Chem. Biol.* **2020**, *15*, 524–532.

(46) (a) Smith, C. D.; Zhang, X. Q.; Mooberry, S. L.; Patterson, G. M. L.; Moore, R. E. Cryptophycin – a new antimicrotubule agent active against drug-resistant cells. *Cancer Res.* **1994**, *54*, 3779–3784. (b) D'Agostino, G.; del Campo, J.; Mellado, B.; Izquierdo, M. A.; Minarik, T.; Cirri, L.; Marini, L.; Perez-Garcia, J. L.; Scambia, G. A multicenter phase II study of the cryptophycin analog LY355703 in patients with platinum-resistant ovarian cancer. *Int. J. Gynecol. Cancer* **2006**, *16*, 71–76.

(47) Fasan, R.; Chen, M. M.; Crook, N. C.; Arnold, F. H. Engineered alkane-hydroxylating cytochrome P450BM3 exhibiting native-like catalytic properties. *Angew. Chem., Int. Ed.* **2007**, *46*, 8414–8418.

(48) Saab-Rincon, G.; Alwaseem, H.; Guzman-Luna, B.; Olvera, L.; Fasan, R. Stabilization of the reductase domain in the catalytically self-sufficient cytochrome P450BM3 via consensus-guided mutagenesis. *ChemBioChem.* **2018**, *19*, 622–632.

(49) Chen, X.; Zaro, J. L.; Shen, W.-C. Fusion protein linkers: Property, design and functionality. *Adv. Drug Delivery Rev.* **2013**, *65*, 1357–1369.

(50) Aalbers, F. S.; Fraaije, M. W. Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes. *ChemBioChem.* **2019**, *20*, 20–28.

(51) Kummer, M. J.; Lee, Y. S.; Yuan, M.; Alkotaini, B.; Zhao, J.; Blumenthal, E.; Minter, S. D. Substrate channeling by a rationally

designed fusion protein in a biocatalytic cascade. *JACS Au* **2021**, *1*, 1187–1197.

(52) Schulz, S.; Schumacher, D.; Raszkowski, D.; Girhard, M.; Urlacher, V. B. Fusion to hydrophobin HFBI improves the catalytic performance of a cytochrome P450 system. *Front. Bioeng. Biotechnol.* **2016**, *4*, 57.

(53) Thomas, F.; Boyle, A. L.; Burton, A. J.; Woolfson, D. N. A set of de novo designed parallel heterodimeric coiled coils with quantified dissociation constants in the micromolar to sub-nanomolar regime. *J. Am. Chem. Soc.* **2013**, *135*, 5161–5166.

(54) Meinke, J. L.; Simon, A. J.; Wagner, D. T.; Morrow, B. R.; You, S.; Ellington, A. D.; Keatinge-Clay, A. T. Employing 25-residue docking motifs from modular polyketide synthases as orthogonal protein connectors. *ACS Synth. Biol.* **2019**, *8*, 2017–2024.

(55) Zhang, L.; Xie, Z.; Liu, Z.; Zhou, S.; Ma, L.; Liu, W.; Huang, J.-W.; Ko, T.-P.; Li, X.; Hu, Y.; Min, J.; Yu, X.; Guo, R.-T.; Chen, C.-C. Structural insight into the electron transfer pathway of a self-sufficient P450 monooxygenase. *Nature Commun.* **2020**, *11*, 2676.

Recommended by ACS

Rationally Controlling Selective Steroid Hydroxylation via Scaffold Sampling of a P450 Family

Xiaodong Zhang, Aitao Li, *et al.*

JANUARY 06, 2023

ACS CATALYSIS

READ 

Cooperative Substrate Binding Controls Catalysis in Bacterial Cytochrome P450terp (CYP108A1)

Jessica A. Gable, Alec H. Follmer, *et al.*

FEBRUARY 13, 2023

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

READ 

Development of a P450 Fusion Enzyme for Biaryl Coupling in Yeast

Lara E. Zetzsche, Alison R. H. Narayan, *et al.*

OCTOBER 31, 2022

ACS CHEMICAL BIOLOGY

READ 

Non-active Site Residue in Loop L4 Alters Substrate Capture and Product Release in d-Arginine Dehydrogenase

Daniel Ouedraogo, Giovanni Gadda, *et al.*

FEBRUARY 16, 2023

BIOCHEMISTRY

READ 

Get More Suggestions >