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Noble–Metal Substitution in Hemoproteins: An Emerging Strategy for Abiological Catalysis

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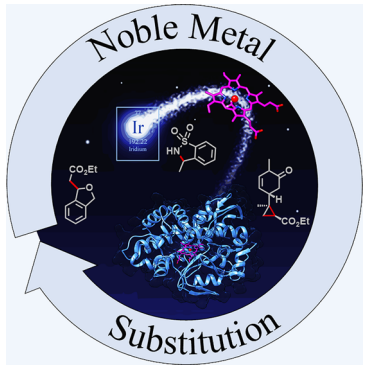
CONSPECTUS: Enzymes have evolved to catalyze a range of biochemical transformations with high efficiencies and unparalleled selectivities, including stereoselectivities, regioselectivities, chemoselectivities, and substrate selectivities, while typically operating under mild aqueous conditions. These properties have motivated extensive research to identify or create enzymes with reactivity that complements or even surpasses the reactivity of small-molecule catalysts for chemical reactions. One of the limitations preventing the wider use of enzymes in chemical synthesis, however, is the narrow range of bond constructions catalyzed by native enzymes. One strategy to overcome this limitation is to create artificial metalloenzymes (ArMs) that combine the molecular recognition of nature with the reactivity discovered by chemists.



This Account describes a new approach for generating ArMs by the formal replacement of the natural iron found in the porphyrin IX (PIX) of hemoproteins with noble metals. Analytical techniques coupled with studies of chemical reactivity have demonstrated that expression of apomyoglobins and apocytochrome P450s (for which "apo-" denotes the cofactor-free protein) followed by reconstitution with metal–PIX cofactors in vitro creates proteins with little perturbation of the native structure, suggesting that the cofactors likely reside within the native active site. By means of this metal substitution strategy, a large number of ArMs have been constructed that contain varying metalloporphyrins and mutations of the protein. The studies discussed in this Account encompass the use of ArMs containing noble metals to catalyze a range of abiological transformations with high chemoselectivity, enantioselectivity, diastereoselectivity, and regioselectivity. These transformations include intramolecular and intermolecular insertion of carbenes into C–H, N–H, and S–H bonds, cyclopropanation of vinylarenes and of internal and nonconjugated alkenes, and intramolecular insertions of nitrenes into C–H bonds. The rates of intramolecular insertions into C–H bonds catalyzed by thermophilic P450 enzymes reconstituted with an Ir(Me)–PIX cofactor are now comparable to the rates of reactions catalyzed by native enzymes and, to date, 1000 times greater than those of any previously reported ArM. This reactivity also encompasses the selective intermolecular insertion of the carbene from ethyl diazoacetate into C–H bonds over dimerization of the carbene to form alkenes, a class of carbene insertion or selectivity not reported to occur with small-molecule catalysts. These combined results highlight the potential of well-designed ArMs to catalyze abiological transformations that have been challenging to achieve with any type of catalyst. The metal substitution strategy described herein should complement the reactivity of native enzymes and expand the scope of enzyme-catalyzed reactions.

1. INTRODUCTION

The site-selective and stereoselective synthesis of organic molecules catalyzed by transition–metal complexes has been a major focus of many research groups, including ours.[1] However, one difficulty faced when developing catalysts based transition–metal complexes is to control the position and orientation of the substrate and to evolve and modify these catalysts in an iterative fashion to control reactivity and selectivity.

In parallel, our research group and others have investigated the potential of artificial metalloenzymes (ArMs) to be used for the synthesis of organic molecules, in many cases for the synthesis of enantioenriched products.[2,3] One difference between small-molecule catalysts and enzymes is the innate specificity of the enzymatic system for a native substrate and the ability of the catalyst to be readily evolved to react with similarly high selectivity for non-native substrates.[2,4,5] This ability to evolve enzymes for non-native functions was the subject of the 2018 Nobel Prize.

To illustrate this point, we highlight the oxidation of organic molecules catalyzed by P450 enzymes. Through the course of natural evolution, cytochrome P450s have evolved to oxidize a wide array of molecules. Some P450 enzymes catalyze

hydroxylation of a narrow scope of substrates with high regioselectivity and stereoselectivity; other P450s catalyze reactions of a broad scope of substrates with low selectivity as part of biological detoxification processes.[6,7] Through laboratory evolution, the site selectivity and enantioselectivity of these enzymes can be altered and controlled.[8−10] Moreover, the activities of certain P450 enzymes toward reactions other than hydroxylation can be tuned by directed evolution.[4]

A portion of our research group has been interested in combining the broad range of reactions catalyzed by transition metal complexes with the high selectivity and potential for evolution of a protein scaffold. If these systems could be combined in a productive fashion, then enzymes could catalyze the formation of C−N, C−C, and C−X (X = halogen) bonds between diverse functional groups. For example, if P450 enzymes could catalyze the formation of C−C bonds from C−H bonds, rather than being restricted to their natural formation of C−O bonds from C−H bonds, then these systems could be used for many synthetic applications.

Several research groups have been interested in creating ArMs that catalyze abiological reactions.[2] Ward first devoted a concerted effort to this goal by applying biotin−streptavidin complexation to create catalysts for a wide range of reactions catalyzed by transition−metal complexes tethered to biotin.[11] This versatile supramolecular anchoring has been used by Ward and co-workers to create abiological metalloenzymes that catalyze allylic alkylation, ring-closing metathesis, hydrogenation, and annulation.[12,13] Many others who have contributed to this virtual special issue have used alternative hosts, unnatural amino acids, or bioconjugation schemes to affix organometallic sites to a protein.[14−16]

We sought a different approach that would create a small but consequential perturbation of the composition of native cofactors. Our strategy was to change only the metal within the porphyrin IX (PIX) cofactor to minimally perturb the interaction and structure of the protein−cofactor complex and therefore leave a nearly native binding site for organic molecules within the protein. We substituted the biological metal in the PIX cofactor with metals more commonly employed in classical organometallic catalytic processes.

Prior to our initial reports, a few groups followed a related strategy for modifying metalloenzymes in this fashion. For example, Wantanabe and Lu independently modified the composition of myoglobin by installing porphyrin-like cofactors containing manganese in the active site.[17−19] Although they were catalytically active toward sulfoxidation of thioanisole, these ArMs did not react with high enantioselectivities or with activities that surpassed those of the natural iron-containing analogues.[18]

We proposed that the substitution of noble metals for iron could increase the activity of the resulting ArMs toward abiological reactions. If this were true, we would take advantage of the wide range of hemoproteins, including P450 enzymes, as hosts for unnatural cofactors containing Ru, Rh, Pd, Ag, and Ir.[20] Indeed, the reactivity of these noble−metal ArMs was distinct from that of the native enzymes.[21−23] Increased reaction rates and selectivities were achieved by directed evolution.

2. INTRAMOLECULAR AND INTERMOLECULAR INSERTION OF CARBENES INTO C−H BONDS CATALYZED BY ARMS

2.1. Metal Substitution in Myoglobin for Intramolecular Carbene Insertion into C−H Bonds

Our goals relied on a facile approach to generate a library of ArMs comprising porphyrin complexes of noble metals and a series of heme protein hosts. It is not possible to simply exchange the iron within the heme ligated to a protein. Thus, a ground-up synthetic strategy was needed to formally replace iron with other metals. Procedures have been published for the extraction of heme from stable proteins such as myoglobin, but these procedures can be laborious and are not applicable to less stable heme proteins.[24] Therefore, this method was unsuitable for high-throughput experimentation with noble−metal analogues of most heme proteins. Instead, we generated the apoprotein and added the noble metal−porphyrin complexes to them. To do so, we expressed apomyoglobin in media lacking the δ-aminolevulinic acid required for porphyrin synthesis and containing low concentrations of iron to minimize the formation of the Fe−PIX hemoprotein.[25] By this protocol, we reliably expressed and isolated a wide range of mutants of the apo form of myoglobin. To these apomyoglobins we added a series of metal−porphyrin cofactors containing Co, Cu, Mn, Rh, Ir, Ru, and Ag in vitro. In select cases these cofactors contained organic coligands bound to the noble metals (Figure 1a).[20]

Other methods can be used to generate these artificial metalloenzymes without purification of the apoprotein for more rapid analysis of catalytic activity. For example, recent work from the Brustad laboratory demonstrated that evolution of a P450 active site in which amino acids sterically inhibit binding of the

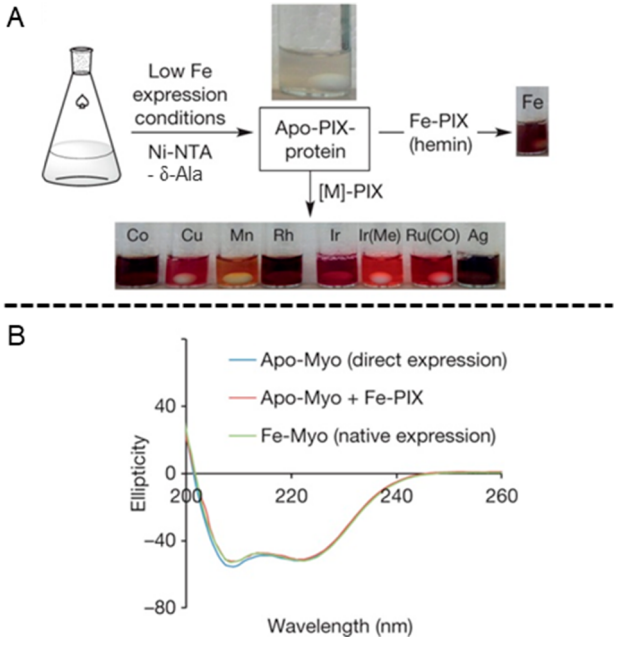


Figure 1. (A) Expression of apomyoglobin in minimal medium and reconstitution with transition metal−PIX cofactors. (B) CD spectra of the directly expressed apomyoglobin, natively expressed myoglobin, and directly expressed apomyoglobin reconstituted with heme, highlighting that the structure of Fe−PIX myoglobin is conserved regardless of preparation. Adapted with permission from ref 20. Copyright 2016 Nature Publishing Group.

native heme cofactor leads to higher-yielding in vivo expression of P450 proteins containing noble metal cofactors possessing a sterically smaller deuteroporphyrin IX ligand.[26] Prior to our work, heme proteins containing abiological metals for imaging applications were generated by approaches involving heme transporters.[26−29] As noted at the end of this Account, we simply expressed the apo form of the protein and added the artificial cofactors to the resulting cell lysates to generate the ArMs as part of directed-evolution experiments.

Studies were conducted to assess the structure of the holoprotein generated from the apoprotein and metal−porphyrin complexes. We found that the circular dichroism (CD) spectrum of the native heme-containing myoglobin was identical to those of the apo form of myoglobin and the reconstituted heme-containing myoglobin that was prepared by isolating the apomyoglobin and inserting the Fe−PIX cofactor (Figure 1b). Moreover, mass spectrometry showed that the artificially assembled hemoprotein contained a 1:1 stoichiometry of protein to cofactor. Finally, in a particularly revealing experiment, the enantioselectivities obtained from the cyclopropanations of styrene catalyzed by a mutant of myoglobin expressed in a medium that naturally incorporates heme was the same as that from the reaction catalyzed by the same mutant expressed in apo form and reconstituted in vitro with the Fe−PIX cofactor. This result suggests that the structure of the final catalyst is independent of how it is prepared.

Having developed a method to generate myoglobins containing porphyrins bound to a series of metals, we assessed the reactivities of these potential ArMs toward C−H activation. A series of myoglobins containing mutations at position H93, the amino acid residue that ligates to iron at the axial position, were generated and investigated as catalysts for the insertion of a carbene unit into the C−H bond of diazoacetate **1** to form chiral dihydrobenzofuran **2** (Figure 2). The reaction of a carbene

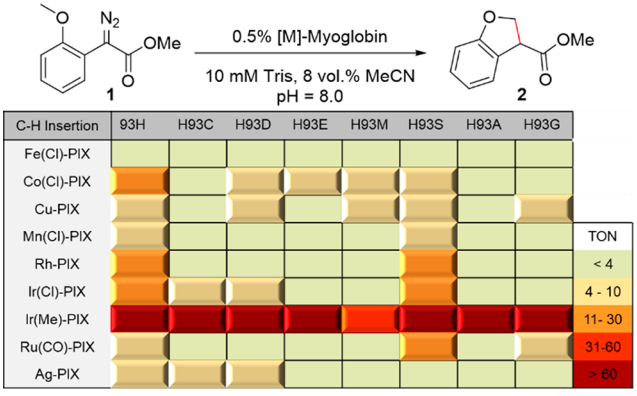


Figure 2. Intramolecular insertion of a carbene into a C−H bond to form a C−C bond (**1** → **2**) catalyzed by several metal−PIX reconstituted myoglobins containing the single mutation of the axial residue.

precursor such as a diazo ester with an enzyme to insert a carbene into a C−H bond is an abiological process and had not yet been achieved with an ArM or repurposed hemoprotein.[30] One can see from Figure 2 that the protein−cofactor assembly containing iridium in the active site and an axially bound methyl group attached to the iridium (Ir(Me)) was most active for this insertion of a carbene into the methoxy C−H bond.

Having observed activity for this C−H bond functionalization catalyzed by an ArM, we sought to determine whether directed evolution could improve the activity and selectivity of the initially generated constructs. The product obtained from the first experiments with the wild-type myoglobin and Ir(Me) was nearly racemic, indicating that the reaction did not occur within the active site of the protein or that the active site of the protein was not well-tuned to place the reacting methyl group of the methoxy substituent on one face of the metal−carbene unit over the other. To increase the reactivity and stereoselectivity, we mutated residues H63 and H93 above and below the porphyrin to hydrophobic amino acids of varying size (Figure 3A). We then modified residues F43 and V68 to hydrophobic and alcohol-containing residues. Finally, with the aid of an existing crystal structure of the wild-type myoglobin, we selected amino acid residues more distal to the binding site for modification. After four rounds of evolution, we increased the ee from 0% to over 80% for formation of one of the two enantiomers and identified a separate mutant that formed the cyclized product with 50% ee favoring the opposite enantiomer (Figure 3B).

This library of mutant ArMs catalyzed the insertion of a carbene unit into the C−H bonds of related substrates containing a series of groups on the ester (**2-Et**), at varying positions of the aromatic ring (**4**), and into a methylene position α to oxygen (**6**) (Figure 3C). In all cases, a mutant that selectively formed each enantiomer of each product was identified. These reactions could be achieved with turnover numbers (TONs) exceeding 7000. In addition to catalyzing insertions into C−H bonds, these Ir(Me)−myoglobins catalyzed the cyclopropanation of both internal and terminal vinylarenes and unconjugated aliphatic α-olefins with good diastereoselectivity and at least measurable enantioselectivity without additional rounds of evolution.

Although these ArMs based on myoglobin catalyzed carbene insertions and cyclopropanations that were unprecedented for an enzyme, they did have significant limitations. They did not react with a substrate containing a benzyl group on oxygen, even though the C−H bond would be weaker than that in the methoxy group. Moreover, they did not react with substrates lacking the oxygen atom of the methoxy group, which makes the methyl C−H bond more electron-rich and weaker than that at the terminus of an alkyl group. They did react with substantial enantioselectivity, but the enantioselectivity was not high enough for practical applications. Finally, although the reactions occurred with TONs of more than 7000 on a 50 μmol scale, reactions on a larger 120 μmol scale occurred with TONs closer to 200. These limitations of the catalytic activity of the ArMs based on the myoglobin scaffold might be expected because myoglobin is not an enzyme. The natural biological function of myoglobin is to bind oxygen, not organic substrates. Therefore, we envisioned that by following our original experimental design starting with a P450 enzyme, one of nature's natural heme-containing catalysts, we would create ArMs that could catalyze a wider range of reactions with higher activity and selectivity than those catalyzed by ArMs from myoglobin.

2.2. Metal Substitution in P450s for Carbene Insertions into C−H Bonds

Among the many P450s for which crystallographic data are available, the P450 called CYP119, from *Sulfolobus solfataricus*, was the protein from which we constructed mutant iridium-containing ArMs because this P450 originates from a thermophilic organism. It seemed plausible that this greater thermal stability would allow reactions to occur at elevated

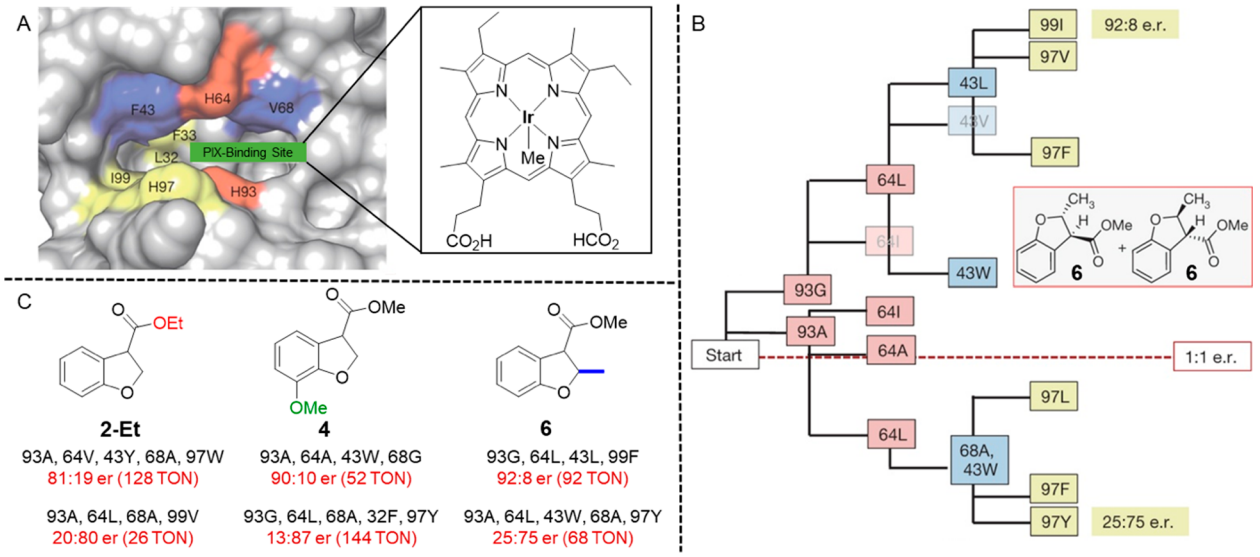


Figure 3. (A) Inner-sphere (red), middle-sphere (blue), and outer-sphere (yellow) residues in the active site. (The image of active site and its surroundings was produced in Chimera from PDB entry 1MBN.[30]) (B) Evolutionary tree exemplified by showing the enantioselectivies achieved for the formation of **6**. Mutants positioned above the dotted line formed predominantly the opposite enantiomer of those shown below the dotted line. (C) Additional substrates from the reaction catalyzed by Ir(Me)−myoglobins that form either enantiomer. Adapted with permission from ref 20. Copyright 2016 Nature Publishing Group.

temperature and that mutants of this enzyme would be more stable than those of other P450s.[31,32]

We constructed a mutant library of CYP119 proteins by following an approach similar to that used to generate the library of myoglobins. We modified the amino acids proximal and increasingly more distal to the metal active site,[21] making a conservative change of hydrophobic amino acids for other hydrophobic amino acids of various sizes. Studies of the reactivities and selectivities of these artificial P450 enzymes showed that the resulting enzymes reacted with higher enantioselectivities in almost all cases and with enantiomeric ratios (er's) as high as 97:3 in several cases (Figure 4). In addition, a P450 mutant was identified that accommodated the larger size of benzyl ether **7**. Moreover, the resulting enzymes reacted with the unactivated primary C−H bond in the ethyl group of **8**, a substrate lacking the oxygen α to the C−H bond at which insertion occurred in other substrates.

In addition to improving the intramolecular insertion reactions, mutants of CYP119 containing the Ir(Me)−PIX complex catalyzed, for the first time for an enzyme, the *intermolecular* insertion of a carbene into a C−H bond. The intermolecular insertion of a carbene into substrate **10** with ethyl diazoacetate (EDA) occurred in over 60% yield with high selectivity for insertion over dimerization of the carbene. This selectivity contrasts with that of the reaction with small-molecule, transition−metal complexes;[33] the reaction catalyzed by Ir(Me)-CYP119 occurred with ca. 100:1 selectivity for the C−H insertion over formal dimerization.

In addition to reacting with broader scope and higher enantioselectivities than ArMs based on myoglobin, the resulting enzyme reacted with higher TONs and was more suitable for reactions on a larger scale (Figure 5). For example, the reaction on a gram scale with less than 0.02 mol % catalyst formed the insertion product in 55% yield with 92% ee and a TON of 3235. On a smaller scale the enantioselectivity was equally high, but the yield was higher (76%) and the TON exceeded 30 000.

Kinetic studies of the reaction converting **1** to **2** began to reveal the value of directed evolution, the relationship between the reactivity of an ArM based on a P450 scaffold (CYP119) and that of an ArM based on a myoglobin scaffold, and the relationship between the reactivity of the protein−cofactor assembly and that of the free Ir(Me)−PIX complex. As shown in Figure 6, the reaction catalyzed by the free Ir(Me)−PIX complex was significantly faster than the reaction catalyzed by the Ir(Me)−myoglobin assembly. Thus, the selectivity of the myoglobin-based system rests on the stability of the protein−cofactor complex. Because the free Ir(Me)−PIX generates racemic product faster than the bound Ir(Me)−PIX forms enantioenriched product, the generation of free Ir(Me)−PIX reduces the enantioselectivity. In contrast, the reactivity of the Ir(Me)−CYP119 assembly was much higher than that of Ir(Me)−myoglobin and higher than that of the free Ir(Me)−PIX complex at substrate concentrations lower than those that saturate the protein. As shown in Figure 7, the catalytic efficiency of the Ir(Me)−CYP119-Max (C317G, T213G, L69V, V254L) system is about 2000 times higher than that of the analogous enzyme derived from myoglobin. Moreover, the binding of diazo ester **1** to the P450 system is stronger than it is to the myoglobin system. This difference in binding was expected on the basis of the natural functions and substrates of the two hemoproteins. Less anticipated are the striking differences between the k_{cat} values of the Ir(Me)−myoglobin system and the Ir(Me)−CYP119 systems and the large differences in the k_{cat} values of different mutants even though the cofactor and its methyl ligand are the same in each protein. Figure 7 shows a plot that places the kinetic parameters for this reaction in the context of those of native enzymes involved in the formation of biosynthetic intermediates and secondary metabolites.[34] These data show that the K_M and k_{cat} values of the Ir(Me)−CYP-Max enzyme are similar to the median K_M and k_{cat} values of native enzymes for their natural substrates.[35]

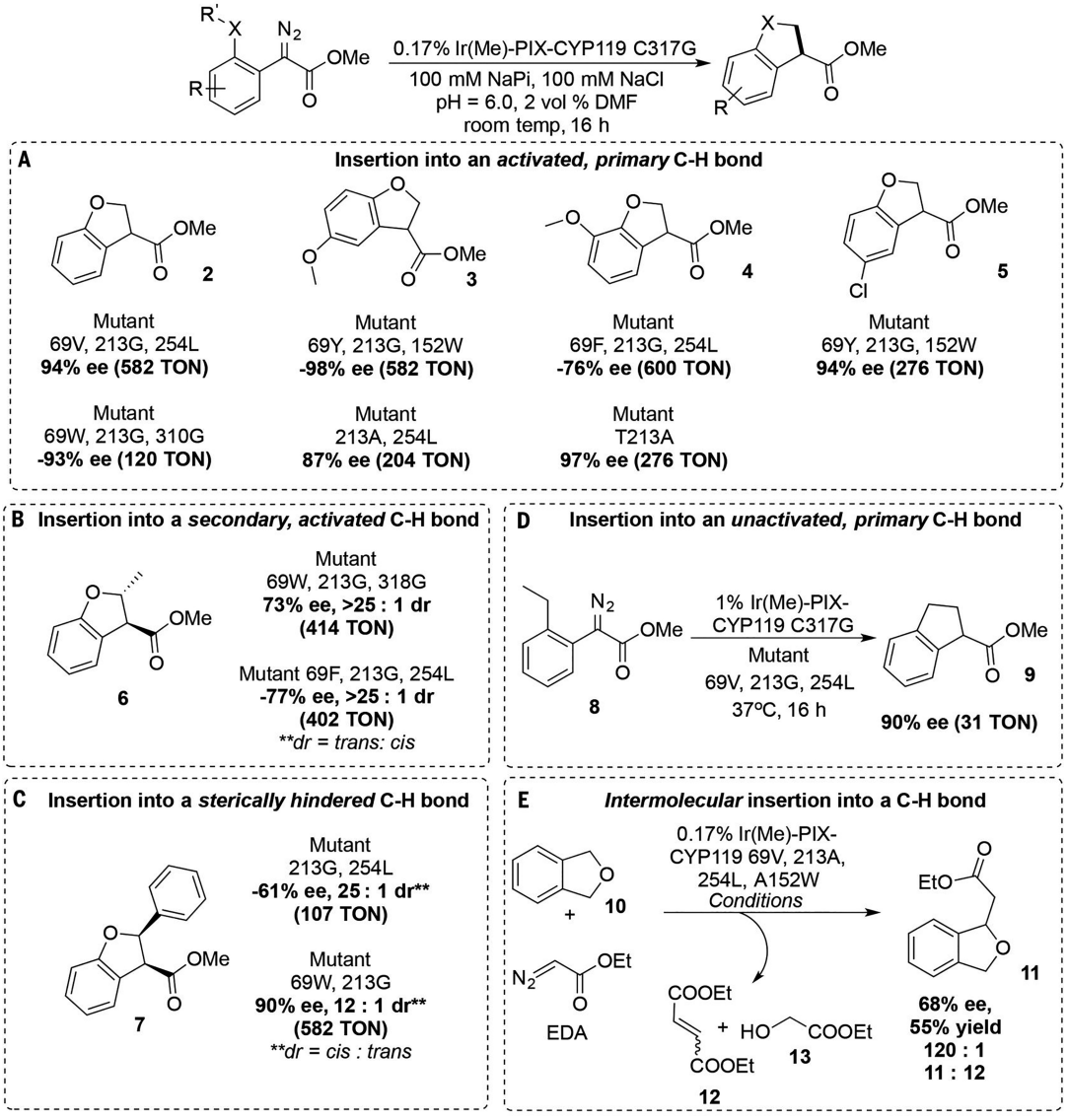
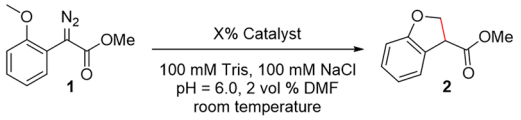


Figure 4. Variants of Ir(Me)−CYP119 catalyze enantioselective intramolecular and intermolecular C−H carbene insertion reactions (A to E). Adapted with permission from ref 21. Copyright 2016 American Association for the Advancement of Science.



mmol/ mg substrate	% Catalyst	TON	Yield	ee (%)
0.0025 mmol, 0.5 mg	0.17%	330	56% Yield (GC)	94%
200 mmol, 10 mg	0.00125	35,129	44% Yield (GC)	91%
0.2 mmol, 40 mg	0.25%	1060	48% Yield (Isolated)	92%
1.0 mmol, 206mg	0.017%	3529	60% Yield (Isolated)	93%
5.0 mmol, 1 gram	0.017%	3235	55% Yield (Isolated)	93%

Figure 5. Intramolecular insertion of the carbene from diazo ester **1** into the methyl C−H bond catalyzed by an Ir(Me)−CYP119 containing the mutations C317G, T213G, L69V, and V254L under synthetically relevant reaction conditions.

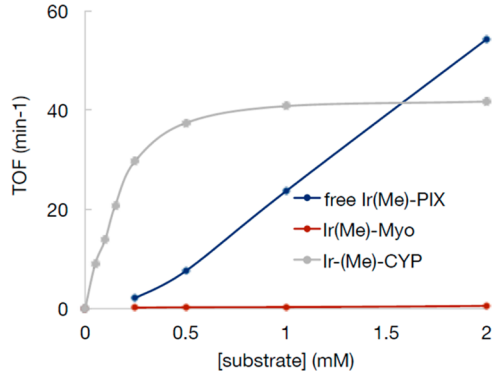


Figure 6. Rates for the intramolecular insertion of a carbene into a C−H bond to convert diazo ester **1** to dihydrobenzofuran **2** catalyzed by the free Ir(Me)−PIX cofactor (blue), Ir(Me)−myoglobin (red), and Ir(Me)−CYP-Max (gray). The reaction conditions are those in Figure 4 with catalyst loadings of 0.025%.

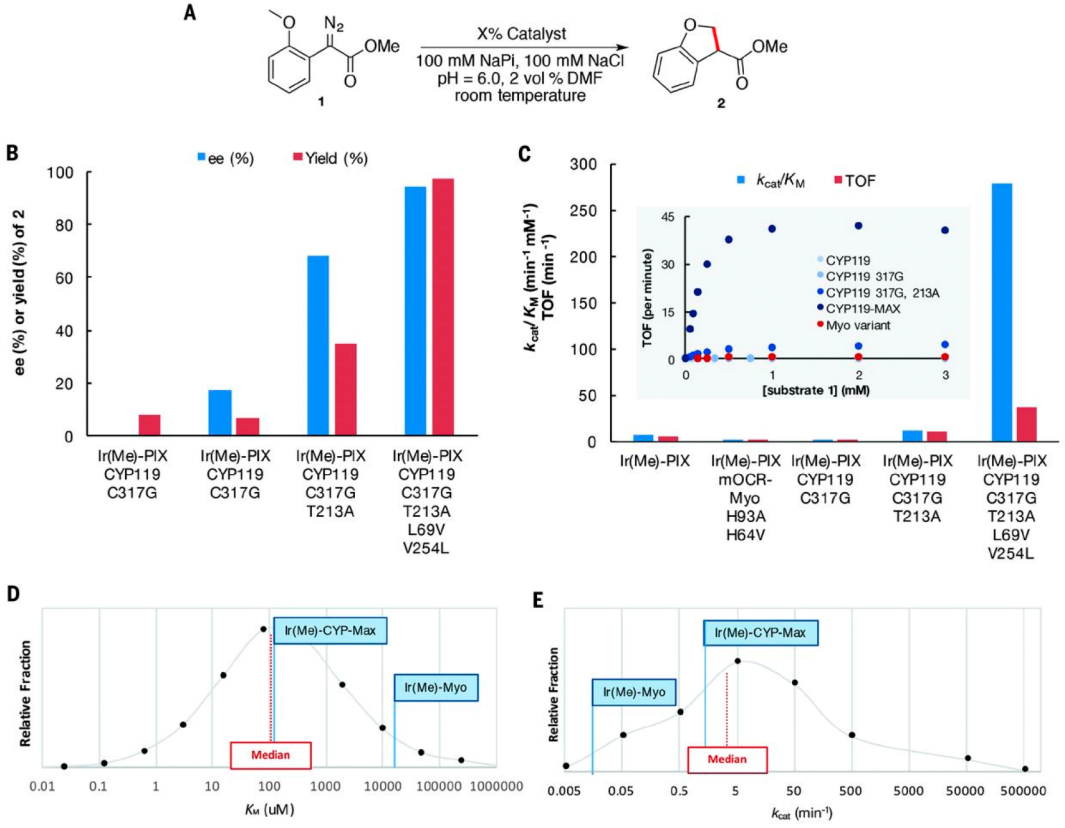


Figure 7. (A) Model reaction converting **1** to **2**. (B) Enantioselectivities and yields for the formation of **2** catalyzed by evolved variants of CYP119 (0.17% catalyst loading, 10 mM substrate). (C) Kinetic parameters for the formation of **2** by variants of CYP119 (0.1 mol % catalyst loading, 5 mM substrate). For free Ir(Me)−PIX, k_1 (the first-order kinetic constant) is listed instead of k_{cat}/K_M. The inset shows the dependence of the turnover frequency (TOF) on $[\mathbf{1}]_0$ for reactions conducted with 0.005 mM catalyst. (D, E) Comparison of K_M and k_{cat} values for CYP119-Max with those of natural enzymes involved in the metabolism of intermediate and secondary metabolites; for comparison, the kinetic parameters of an Ir(Me)−PIX myoglobin catalyzing the same transformation are shown. Adapted with permission from ref 21. Copyright 2016 American Association for the Advancement of Science.

2.3. Insertion of Carbenes into N−H and S−H Bonds with Noble−Metal ArMs

Following a different approach to the generation of apomyoglobin, Lehnert and co-workers reported the formal substitution of Ru(II) for Fe in sperm whale myoglobin.[36] These reconstituted Ru−PIX myoglobin systems catalyzed the insertion of carbenes into the N−H bonds of aniline and aniline derivatives. Specifically, these Ru-ArMs catalyzed the reaction of EDA with aniline with TONs greater than 500. Furthermore, the reaction of EDA with aniline derivatives containing both electron-withdrawing and electron-donating groups, such as 4-trifluoromethylaniline (TON = 451), 4-nitroaniline (TON = 29), 4-methoxyaniline (TON = 57), and 4-methylaniline (TON = 358), were catalyzed by the single Ru−PIX myoglobin mutant H64A. These Ru-ArMs were shown to catalyze the insertions of the carbene from EDA into N−H bonds over additions to the C=C bond of 4-vinylaniline with perfect chemoselectivity.

Contemporaneous with the work of Lehnert, Fasan and co-workers reported the application of myoglobins containing noble−metal cofactors for the insertion of carbenes into N−H bonds and S−H bonds.[29] Using a double mutant of sperm whale myoglobin (H64V and V68A), they generated ArMs housing porphyrins bound to Ru(CO), Rh, and Ir(Me) units. Using these ArMs, they reported the insertion of the carbene from EDA into the N−H bond of aniline with moderate TONs (as high as 168 in the case of the Ir(Me)-ArM). In the same paper, Fasan and co-workers also reported the reaction of thiophenol with EDA catalyzed by mutants of myoglobin. The iron hemoproteins catalyzed this carbene insertion into S−H bonds with TONs of 985. Myoglobin mutants reconstituted with metalloporphyrins containing Ru(CO) (TON = 795), Rh (TON = 95), and Ir(Me) (TON > 1000) also catalyzed this insertion reaction. It is clear from this work that the substitution of a noble metal for iron does not always generate a more active catalyst and is not required for all organic transformations. Also included in Fasan's report are the cyclopropanation of styrene and the intermolecular insertion of a carbene into a C−H bond catalyzed by an Ir(Me)−myoglobin construct.

3. CYCLOPROPANATION OF BOTH TERMINAL AND INTERNAL OLEFINS CATALYZED BY NOBLE−METAL ARMS

In addition to catalyzing the insertion of carbenes into C−H bonds, our group's Ir(Me)−CYP119 mutants catalyzed the cyclopropanation of alkenes, including internal, unconjugated alkenes. Reactions of alkenes that occur in low yield with low TONs when catalyzed by iron-based enzymes occur in high yield with high TONs when catalyzed by the Ir(Me)-ArM systems. For example, Ir(Me)-ArMs catalyze the cyclopropanation of both internal and terminal vinylarenes, unactivated α-olefins, and internal, unconjugated olefins, and they do so with good diastereoselectivity and enantioselectivity.[22]

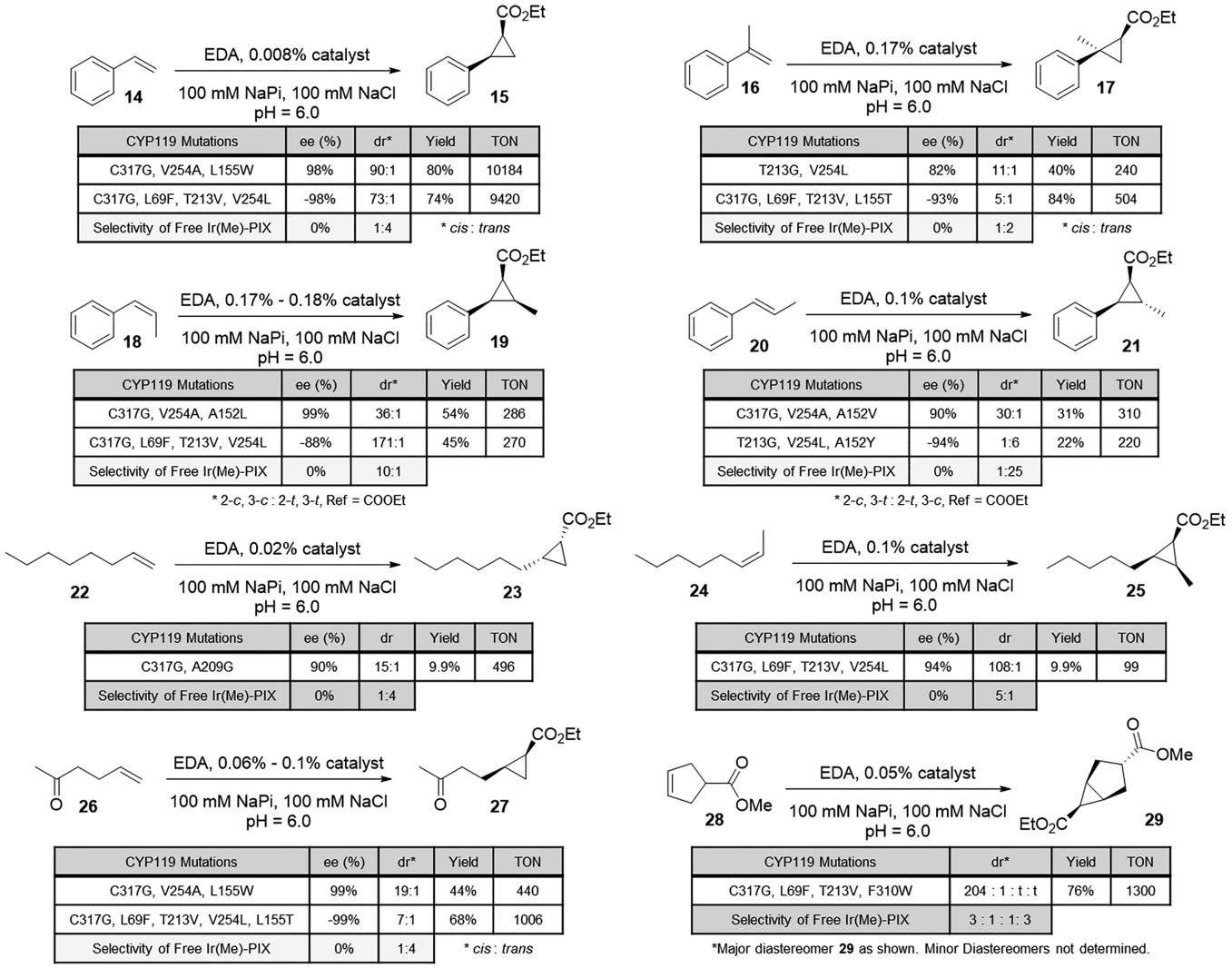


Figure 8. Addition of EDA to terminal and internal olefins catalyzed by variants of Ir(Me)−PIX−CYP119 and Ir(Me)−PIX.

Our studies to investigate the activity of the Ir(Me)-containing ArMs as catalysts for cyclopropanation were initiated with the library of mutants that we studied for carbene insertion into C−H bonds (see section 2.2). An assessment of the reactivity of these ArMs showed that the products were formed with varying diastereoselectivity and enantioselectivity. Just one additional round of directed evolution produced enzymes that catalyzed the cyclopropanation of styrene with EDA to give either enantiomer of **15** with nearly perfect enantioselectivity, high cis:trans diastereoselectivity, high yields, and TONs in the range of 10 000 (Figure 8). The cis:trans selectivity observed for the cyclopropanation of styrene catalyzed by the Ir(Me)−P450 assembly contrasts with the selectivity of the same reaction catalyzed by the free Ir(Me)−PIX cofactor. The reaction catalyzed by the Ir(Me)−P450 assembly forms the cis isomer as the major product, but the free cofactor forms the trans isomer as the major product. The cyclopropanations of the 1,1-disubstituted vinylarene (α-methylstyrene) with EDA catalyzed by these Ir(Me)-ArMs also occurred to form either enantiomer of **17** as the major enantiomer and the cis product as the major diastereomer. The TONs for reaction of α-methylstyrene were 240 to 500.

Reactions of internal alkenes also occurred with high enantioselectivities and diastereoselectivities. The reactions of the two isomers of β-methylstyrene gave distinct isomeric products. *cis*-β-Methylstyrene reacted to give either enantiomer of cis diastereomer **19** with TONs between 250 and 300. This diastereomer is the same as that formed by the free Ir(Me)−PIX cofactor. *trans*-β-Methylstyrene also reacted to give product **21** with high enantioselectivity and diastereoselectivity. In this case, the diastereoselectivity depended on the mutant, in one case favoring the isomer with the aryl substituent cis and the methyl substituent trans to the ester group with 30:1 selectivity and in another case favoring the isomer with the aryl substituent trans and the methyl group cis to the ester group with 6:1 selectivity. The free Ir(Me)−PIX complex favored the formation of the latter diastereomer, indicating that the active site can reverse the stereoselectivity of this process. Additional rounds of directed evolution would likely increase the stereoselectivities and TONs of these reactions.

These ArMs also catalyzed the first enzyme-catalyzed cyclopropanations of an unconjugated terminal alkene (**23**) and an unconjugated internal alkene (**25**). Although cyclopropanations of two nitrile-substituted internal vinylarenes catalyzed by a mutant of native myoglobin have been claimed recently, the TONs were based on conversion (2−5%), and the yields were not determined.[37] The reaction of allylacetone catalyzed by two different mutants of Ir(Me)−CYP119 gave

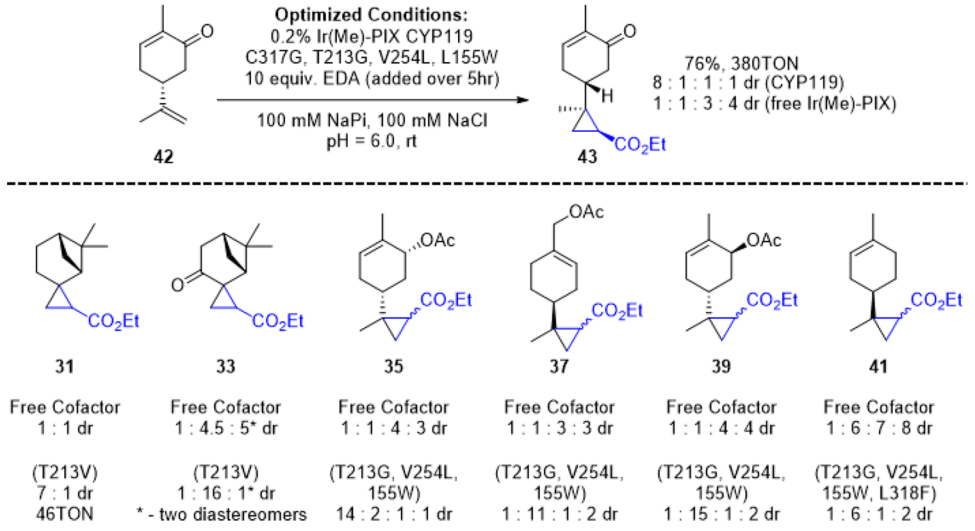


Figure 9. Cyclopropanation reactions of natural terpenes and their derivatives. For cases in which the TONs for the remaining terpenes are not given, the TONs were ≤20.

either enantiomer of the cyclopropane product with nearly perfect enantioselectivity of the cis diastereomer (**27**). The TONs ranged from 440 for one enantiomer to over 1000 for the other. The reactions of unconjugated internal alkenes are a particular challenge because of the greater HOMO−LUMO gap and unfavorable steric properties for approach to the catalyst. Nevertheless, a mutant of the Ir(Me)-ArM catalyzed the cyclopropanation of cyclic unconjugated internal alkenes. As a representative example, methyl cyclopentene-4-carboxylate reacted to give one of four stereoisomers of cyclopropane **29** with over 200:1 selectivity and greater than 1000 turnovers.

We envisioned in the long term that these kinds of enzymes can be used as parts of biosynthetic pathways. Thus, we investigated whether the kinds of alkenes that are the products of biosynthesis, such as unsaturated terpenes, would be amenable to cyclopropanation. Indeed, *β*-pinene (**30**), limonene (**40**), and carvone (**42**) all underwent cyclopropanation at the unconjugated disubstituted alkene (Figure 9). Studies to increase the TONs for the cyclopropanation of terpenes catalyzed by our Ir(Me)−CYP119 constructs are ongoing.

Although much of the work reported here focuses on stereoselectivity, we are equally interested in control of the regioselectivity and site selectivity. An initial experiment to assess whether such selectivities could be achieved involved a competition between 1-octene (**22**) and *cis*-2-octene (**24**) (Figure 10). The free Ir(Me)−PIX cofactor strongly favors cyclopropanation of the terminal alkene to give a 9:1 ratio of the cyclopropane derived from 1-octene over that derived from *cis*-2-octene. However, a mutant of CYP119 was identified that forms the cyclopropane derived from *cis*-2-octene in preference to that derived from 1-octene. This result indicates that ArMs can be evolved for a wide range of selectivities, even in the absence of functional groups that could interact with the amino acid side chains in the substrate binding site.

4. INTRAMOLECULAR INSERTION OF NITRENES INTO C−H BONDS

The Ir(Me)-ArMs also catalyze enantioselective aminations of C−H bonds. Directed evolution of hemoproteins has created iron-containing enzymes that catalyze C−H amination with good TONs.[4,38] However, these iron-containing enzymes

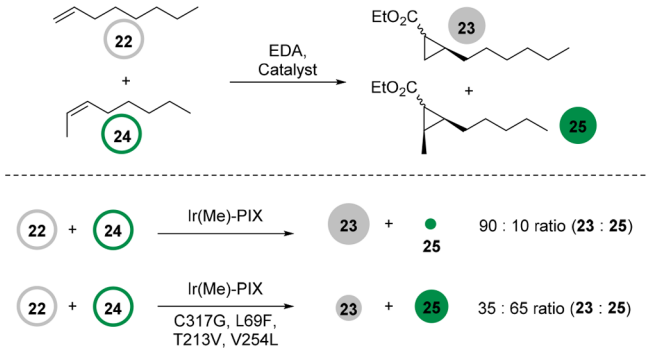


Figure 10. Substrate-selective cyclopropanation catalyzed by a variant of CYP119 in comparison with the same reaction catalyzed by the free cofactor. The amounts of **23** and **25** are the sums of all stereoisomers of the product. The reaction conditions and stereoselectivities are reported in ref 22

required significant bioengineering (more than nine mutations) to reach the desired reactivity, in part because of the low chemoselectivity of the heme unit for the insertion of a nitrene into a C−H bond over the reduction of the sulfonyl azide to the sulfonamide. In comparison, amination catalyzed by Ir(Me)-ArMs occurred with similar TONs but required fewer mutations to the P450 scaffold to achieve nitrene insertion over azide reduction because of the inherent selectivity of the Ir(Me) cofactor for nitrene insertion over azide reduction.[23]

Figure 11 shows the products from nitrene insertions into secondary and tertiary benzylic and non-benzylic C−H bonds catalyzed by Ir(Me)-ArMs and the selectivities of these reactions for insertion of the nitrene unit into a C−H bond versus reduction of the azide. These data show that several azides reacted without formation of the reduced sulfonamide by-product and formed the product from intramolecular nitrene insertion in yields of up to 98%. The products shown in Figure 11a from the intramolecular insertion of the nitrene unit into C−H bonds were formed with TONs exceeding 100 with up to 95:5 er. A survey of nitrene insertions catalyzed by a series of metalloporphyrins is shown in Figure 11b. This graph shows the high inherent selectivity of the Ir(Me)−PIX cofactor for the formation of the product from nitrene insertion into a C−H

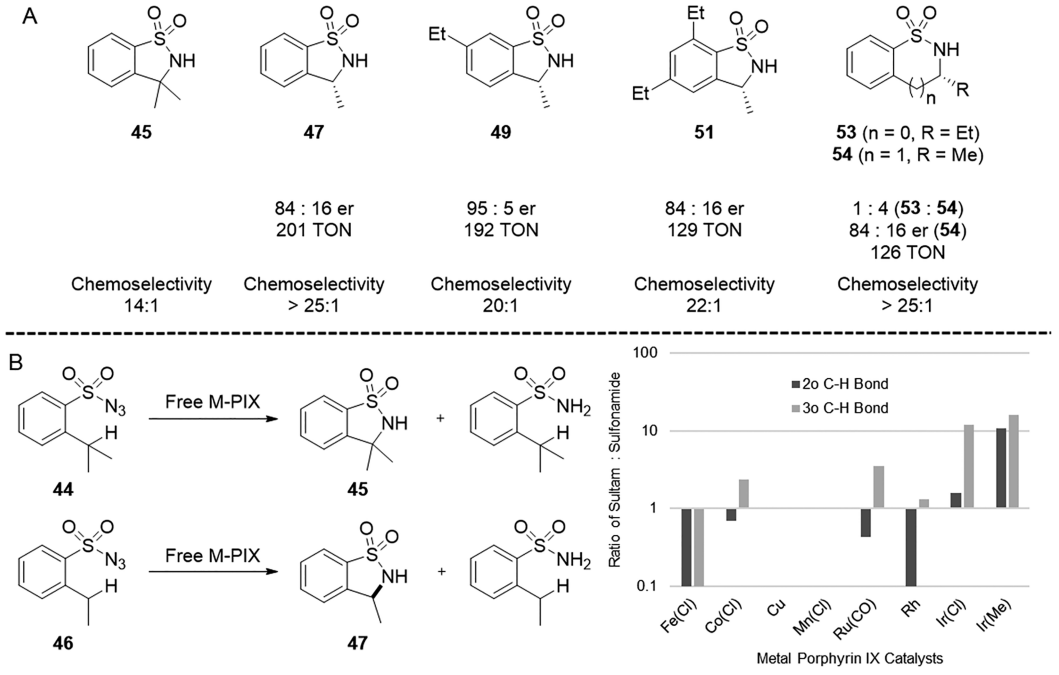


Figure 11. (A) Outcomes of C−H insertion reactions forming sultams **45**, **47**, **49**, **51**, **53**, and **54**. Chemoselectivity refers to the molar ratio of sultam to sulfonamide products. Yield and TON refer to the formation of the sultam. (B) Outcomes of C−H insertion reactions catalyzed by each metal−PIX complex. Bars reflect the molar ratios of the two products formed (sultam and sulfonamide), and a comparison of the outcomes for insertions into tertiary (**45**, light gray bars) and secondary (**47**, dark gray bars) C−H bonds is shown. The reaction conditions are reported in ref 23.

bond over that from reduction of the azide. Further studies of nitrene insertion are ongoing in our laboratory.

5. OUTLOOK AND CONCLUSIONS

The studies described in this Account point toward many future directions that would exploit the high chemoselectivity, regioselectivity, and stereoselectivity of ArMs for the synthesis of organic molecules. Much work must be done to show that ArMs can be used on large scales and to devise ways in which ArMs can be assembled in whole cells and used as both purified proteins and in whole-cell systems. However, the wide range of P450s with binding sites predisposed for specific large or small molecules and the known P450s that bind indiscriminately to organic molecules can be used as hosts following the strategies outlined in this Account. Moreover, one can envision that these ArMs could be used to intersect intermediates along biosynthetic pathways to catalyze unnatural transformations prior to the full processing of the intermediates to form the final biosynthetic product. Finally, cofactors that are distinct from porphyrin complexes can be envisioned that would create enzymes that catalyze processes with a scope beyond the group transfer reactions catalyzed by metalloporphyrin systems. These directions are part of the future research in our laboratory.

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Notes

The authors declare no competing financial interest.

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