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Engineered and artificial metalloenzymes for selective C–H functionalization

Xinkun Ren and Rudi Fasan

The direct functionalization of C–H bonds constitutes a powerful strategy to construct and diversify organic molecules. However, controlling the chemo- and site-selectivity of this transformation, particularly in complex molecular settings, represents a significant challenge. Metalloenzymes are ideal platforms for achieving catalyst-controlled selective C–H bond functionalization as their reactivities can be tuned by protein engineering and/or redesign of their cofactor environment. In this review, we highlight recent progress in the development of engineered and artificial metalloenzymes for C–H functionalization, with a focus on biocatalytic strategies for selective C–H oxyfunctionalization and halogenation as well as C–H amination and C–H carbene insertion via abiological nitrene and carbene transfer chemistries. Engineered heme and nonheme iron dependent enzymes have emerged as promising scaffolds for executing these transformations with high chemo-, regio-, and stereocontrol as well as tunable selectivity. These emerging systems and methodologies have expanded the toolbox of sustainable strategies for organic synthesis and created new opportunities for the generation of chiral building blocks, the late-stage C–H functionalization of complex molecules, and the total synthesis of natural products.

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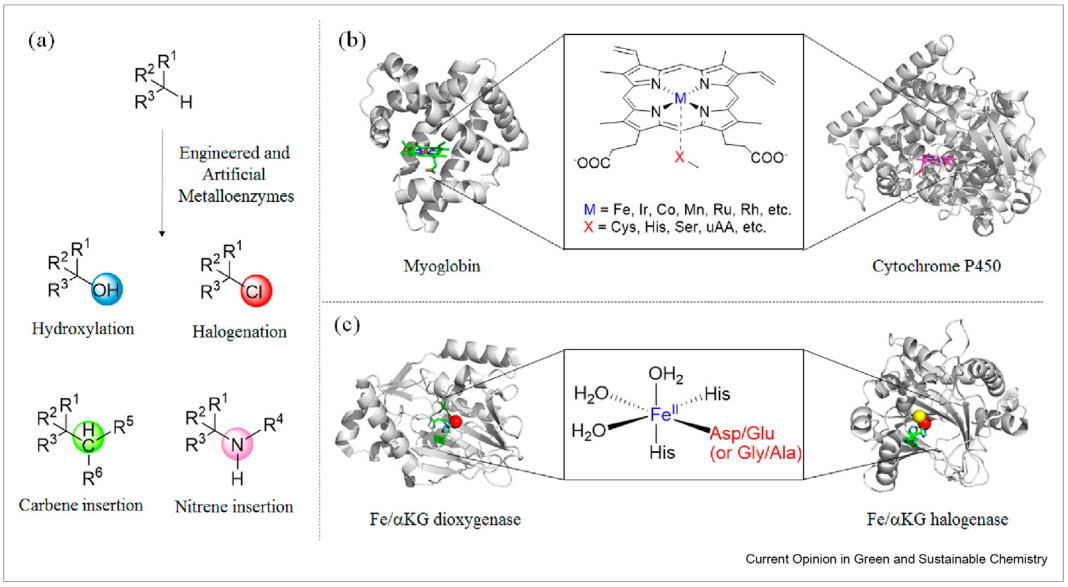
Introduction

The development of catalytic methods for selective C–H functionalization constitutes an area of intense research owing to the well-established potential of these transformations toward streamlining the synthesis and late-stage functionalization of complex molecules, including biologically active natural products and drugs [1–4]. Synthetic approaches to this important transformation often involve the use of high-energy reactants (e.g., peroxides, oxaziridines, iminoiodanes) in combination with transition metal catalysts to target (stereo) electronically activated C–H bonds in a substrate molecule [1–4]. Alternatively, 'directing' groups are exploited to functionalize C–H bonds proximal to a preexisting functional group (e.g., carboxylic group) [1–4]. Given the abundance of C–H bonds in complex molecules, however, controlling the chemoselectivity, regioselectivity, and stereoselectivity of these transformations, particularly toward 'isolated' and/or unactivated C–H bonds, remains an outstanding challenge. In this context, exploiting engineered enzymes has represented an attractive avenue for achieving tunable catalyst-controlled selectivity in C–H functionalization (Figure 1a) [5–9].

In nature, enzyme-mediated C–H oxyfunctionalization and halogenation reactions are implicated in the biosynthesis of a myriad of secondary metabolites and natural products [10]. Major enzyme classes involved in these transformations include members of the cytochromes P450 and nonheme iron (NHI)- and α-ketoglutarate (αKG)-dependent dioxygenases/halogenases superfamilies. These enzyme classes utilize a distinct catalytic machinery (Figure 1b), consisting of a heme cofactor and mononuclear NHI center, respectively, to produce high-valent iron-oxo intermediates capable of abstracting a H atom from a substrate, ultimately resulting in the selective oxyfunctionalization (or halogenation) of a specific C–H bond within the molecule [11–14]. Through natural evolution, these enzymes have specialized to recognize a broad range of structurally diverse substrates and to perform C–H oxyfunctionalization (or halogenation) reactions, under mild reactions, with an excellent degree of chemo-, regio-, and stereoselectivity.

Inspired by the remarkable functional versatility of these metalloenzymes in the context of biosynthetic pathways, the past decade has witnessed increasing efforts and progress toward adapting these enzymes, via protein engineering, to recognize non-native substrate and/or tuning their regioselectivity and stereoselectivity properties to generate biocatalysts for synthetic applications and sustainable chemistry [5–9]. In this review article, we highlight recent examples of selective C–H oxyfunctionalization and halogenation reactions achieved by means of engineered P450s and NHI-dependent enzymes (Figure 1), with an emphasis on their applications for the late-stage functionalization and synthesis of complex

Figure 1



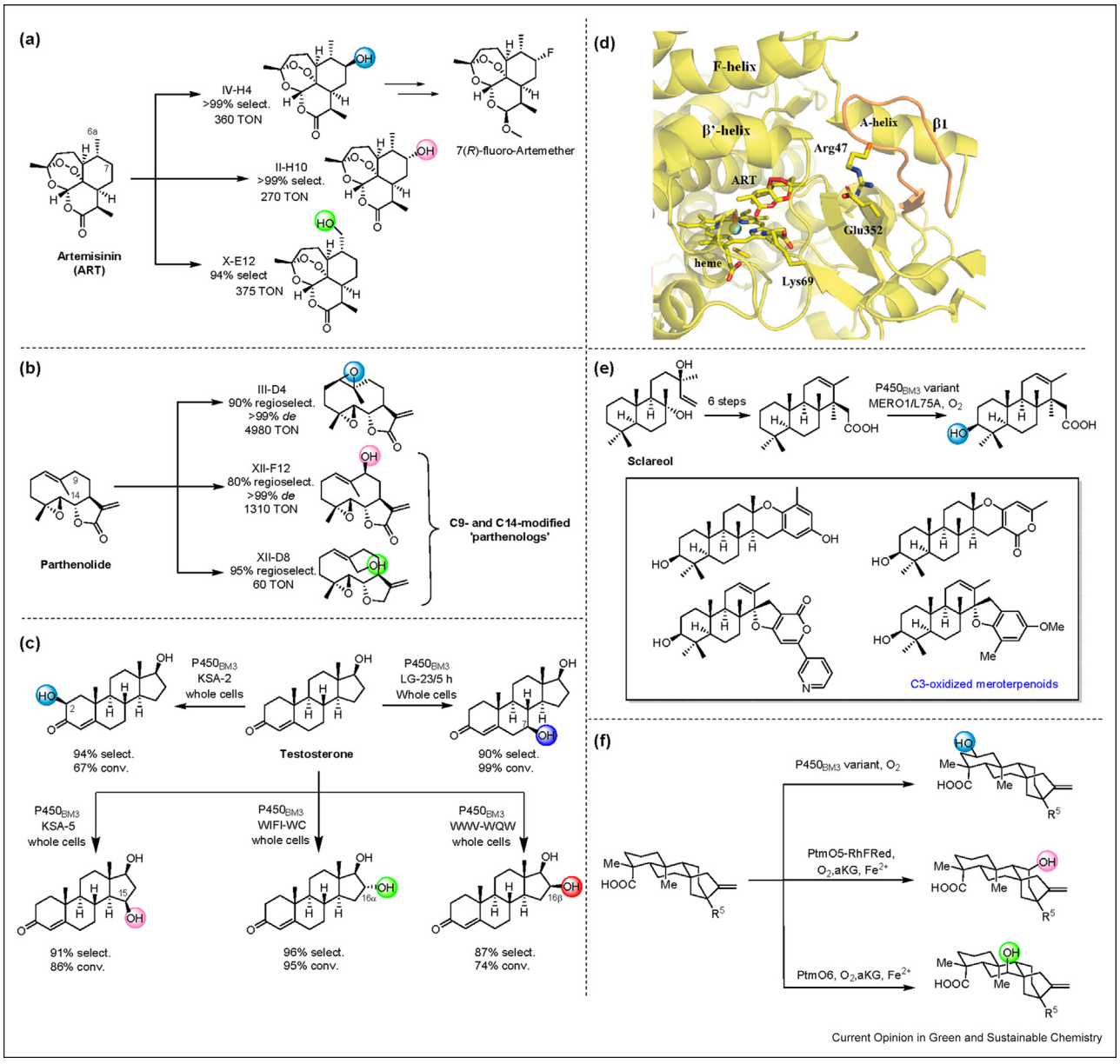
(a) C–H functionalization reactions catalyzed by engineered and artificial metalloenzymes. (b) Structure and catalytic center of representative hemoproteins (myoglobin and cytochrome P450) and NHI-/αKG-dependent enzymes.

molecules and natural products. In addition to these transformations, which rely on the inherent reactivity of the parent enzymes, significant progress has also been made in the development of engineered and artificial metalloenzymes for realizing new types of C–H functionalization transformations not found (or previously known) in the biological world. Seminal contributions from the Arnold group and our own laboratory have recently demonstrated that heme-dependent enzymes and proteins such as cytochrome P450s, myoglobin, and cytochrome *c*, can be adapted and exploited to catalyze non-native carbene and nitrene group transfer reactions involving a high-valent iron-carbenoid or iron-nitrenoid species [15]. Via protein engineering, efficient biocatalysts for a growing number of 'abiological' reactions including olefin cyclopropanations [16–20] and aziridination [21], carbene Y–H bond (Y = N, S, Si, B) insertions [22–26], sigmatropic rearrangements [27,28], and aldehyde olefinations [29] have been reported. Important ramifications of this research have recently enabled expansion of the reaction scope and toolbox of engineered and artificial nitrene and carbene transferases for the selective conversion of C–H bonds into new carbon–nitrogen and carbon–carbon bonds (Figure 1a) in both intramolecular and intermolecular settings, which will be surveyed here. Altogether, the classes of biocatalysts highlighted here are expanding the repertoire of sustainable, biocatalytic strategies for the catalyst-controlled selective functionalization of C–H bonds in organic molecules and complex natural products. While not covered here, readers are directed to excellent reviews on other enzymes classes (e.g., flavin-dependent enzymes) of synthetic relevance for selective C–H oxyfunctionalization and halogenation [13,30–32], while more comprehensive reviews on artificial metalloenzymes can be found elsewhere [33].

Selective C–H hydroxylation by engineered oxygenases

Hydroxylation is one of the most prevalent C–H functionalization reactions found in nature, being involved in biosynthesis of steroid hormones, antibiotics, and a variety of secondary metabolites in plants, fungi, and microbes as well as in the breakdown of xenobiotics and metabolisms of drugs in both humans and other organisms [10]. A central role in these oxyfunctionalization reactions is played by cytochromes P450 monooxygenases, which have been evolved to recognize and oxidize a diverse range of substrates across all kingdoms of life. Inspired by the functional versatility of cytochromes P450, increasing efforts have focused on engineering and tuning these biological catalysts for the late-stage C–H functionalization of complex scaffolds, including biologically active natural products. One of the earliest examples in this area entailed the development of engineered P450-based catalysts for the late-stage C–H hydroxylation of artemisinin (Figure 2a), a complex sesquiterpene lactone with antimalarial activity [34]. Zhang et al. initially identified a promiscuous variant of the bacterial fatty acid hydroxylase $P450_{BM3}$

Figure 2



Representative examples of selective C–H hydroxylations mediated by engineered P450s and Fe/αKG-dependent dioxygenases. (a–c) Regioselective and stereoselective oxyfunctionalization of artemisinin (a), parthenolide (b), and testosterone (c) with engineered $P450_{BM3}$ variants. (d) Modeled complex of 7(*S*)-selective $P450_{BM3}$ variant IV-H4 with artemisinin (ART). Key secondary structural elements in the P450 enzymes are labeled. Adapted from Ref. [38]. (e) Chemoenzymatic synthesis of meroterpenoids. (f) Site-selective oxyfunctionalization of diterpenes with natural Fe/αKG-dependent dioxygenases and an engineered $P450_{BM3}$ variant.

from *Bacillus megaterium* [35] that is capable of hydroxylating this natural product with poor selectivity at the aliphatic position C7 and C6a. Starting from this variant, a panel of highly regioselective and stereoselective biocatalysts for the synthesis of 7(*S*)-, 7(*R*), and 14-hydroxy-artemisinin at a preparative scale were obtained via re-engineering of the enzyme active site in combination with a 'P450 fingerprinting' strategy [36] for rapidly identifying P450 variants that feature diverse regioselectivity and stereoselectivity properties (Figure 2a). Via chemoenzymatic fluorination [37], the 7(S)-selective P450 variant could be applied to produce artemisinin-derived drugs (e.g., artemether) in which a metabolically labile C–H bond is protected via a H→F substitution (Figure 2a). Recently, a computational study by Huang and coworkers has provided insights

into the origin of the divergent regioselectivity and stereoselectivity in these artemisinin hydroxylating biocatalysts [38]. Using a combination of molecular dynamics simulations and QM/MM calculations, it was found that a conformational change of a β hairpin at the entrance of the substrate channel (β1 motif) and an α-helical region (β′-helix motif) next to the heme cofactor were critical for reshaping the binding pocket and repositioning of the substrate within the enzyme active site (Figure 2d), resulting in the divergent site-selectivity observed experimentally. In another study, Kolev et al. applied a similar strategy based on P450 fingerprint-based predictions and active site mutagenesis, for the development of three regioselective and stereoselective $P450_{BM3}$-based catalysts for hydroxylation of position C9 and C14 and epoxidation of the C1, C10 double bond in parthenolide, a plant-derived terpene with antileukemic activity (Figure 2b) [39]. While the parent enzyme strongly favored the epoxidation reaction (77% select.), the site-selectivity of the P450 could be efficiently steered to favor either C9 or C14 hydroxylation with over 80−90% regioselectivity and excellent stereoselectivity (C9: >99% *de*) by means of three to seven active site mutations. The enzymatically produced C9(*S*)- and C14-hydroxy-parthenolides served as key intermediates for further chemoenzymatic diversification via acylation, carbamoylation, alkylation, and O−H carbene insertion chemistries to yield a panel of novel 'parthenologs' [40,41]. By profiling their activity against multiple human cancer cell lines, parthenologs with significantly enhanced antileukemic and anticancer activity were identified, highlighting the value of P450-mediated chemoenzymatic C−H late-stage functionalization for tuning the pharmacological properties of a bioactive natural product and for drug discovery applications. More recently, the You group reported the engineering of two $P450_{BM3}$ variants for the site-selective hydroxylation of two aliphatic sites (C9: 90% select.; C7: 49% select.) in cyperenoic acid, a sesquiterpenoid with antiangiogenic activity [42].

The selective C−H oxyfunctionalization of steroid substrates has also attracted considerable attention, owing to the relevance of these compounds for hormone therapy and other pharmacological applications. Achieving selective hydroxylation in steroid molecules poses a significant challenge due to large number of unactivated and energetically similar $C(sp^3)$−H bonds in these molecules. Engineered variants of $P450_{BM3}$ and other P450s (e.g., CYP106) have provided a valuable source of biocatalysts for steroid hydroxylation [43−48]. Targeting testosterone, the Reetz group was able to optimize the modest regioselectivity (1:1 ratio) of an initial $P450_{BM3}$ variant ($P450_{BM3}$(F87A)) to achieve 2β- and 15β-hydroxylation with high selectivity (91−94%) on screening about 9000 active site enzyme variants by HPLC (Figure 2c) [45]. Using a similar approach but combined with mutability landscape analysis (= systematic analysis of all 19 amino acid substitutions at 20 active site positions), the same group more recently reported the development of $P450_{BM3}$ variants capable of catalyzing the highly regioselective and diastereoselective hydroxylation of testosterone at the C16 position with both α- and β-stereoselectivity [48]. Subsequent work from this group further enabled the directed evolution of a $P450_{BM3}$ variant with high 7β-selectivity (90%) for testosterone hydroxylation [49]. In this case, site-saturation mutagenesis of 15 active site positions followed by multisite mutagenesis using a binary (= 2 amino acid) code was effective toward refining the desired 7β-selectivity (3 → 90%). Importantly, the aforementioned P450-based catalysts were found to retain comparable regioselectivity and stereoselectivity for the hydroxylation of other related steroid molecules, including androstenedione, nandrolone, and boldenone [49]. Using a protein engineering strategy based on 'glycine scanning mutagenesis' guided by comparison of $P450_{BM3}$ active site with that of steroid C19-demethylase CYP19A1, Chen et al successfully engineered a small library of $P450_{BM3}$ variants (~30) that are capable of oxidizing androstenedione and dehydroepiandrostenedione at a wide range of aliphatic C−H sites (C2, C6, C7, C15, and C16) with good to excellent regioselectivity and stereoselectivity (48−97%) [50]. Notably, this approach also led to the identification of stereodivergent biocatalysts for dehydroepiandrosterone oxidation at C7 (7α:93% selectivity, 7β: 97% selectivity; up to 970 TON) and for dihydroxylation of these steroid substrates (e.g., C2/C16 and C7/C15) with good activity and selectivity.

As a complementary approach to protein engineering, substrate engineering has also been investigated for altering and tuning the selectivity of P450-catalyzed hydroxylations. The biosynthetic P450 enzyme PikC catalyzes the hydroxylation of macrolides YC-17 and narbomycin, which are recognized and bound by the enzyme through a key desosamine group [51]. By swapping this moiety with alternative amine-containing 'anchoring' groups, the Sherman group was able to obtain different regioselectivity patterns for the hydroxylation of YC-17 analogs in combination with a $PikC_{D50N}$ variant [52]. Expanding upon this concept and utilizing a panel of 13 triazole anchors, the same group later reported the late-stage hydroxylation of a 11-membered macrolactone at up to three different aliphatic sites and with 67−96% regioselectivity using a single engineered PikC variant [53]. A related strategy was investigated by Lange et al using a series of nitrophenylsulfonamide (nosyl)-based anchoring groups and $P450_{BM3}$ as the biocatalyst [54]. This approach was shown to enable the hydroxylation of a model substrate (vabicaserin) at multiple aromatic and aliphatic C−H sites with variable selectivity depending on the nature of the anchoring group. Overall, this substrate engineering approach provides an attractive complement to

protein engineering for tuning the selectivity of P450-catalyzed hydroxylations, although it requires the installation and removal of the anchoring group as additional steps toward the desired transformation.

In addition to late-stage C–H functionalization, selective P450 catalysts can offer new opportunities for the chemoenzymatic synthesis of complex molecules and natural products [5]. In a first example of this application, an engineered $P450_{BM3}$ variant (called 8C7) was applied to exert a regioselective allylic oxidation (~60% selectivity) useful for completing the total synthesis of nigelladine A [55]. The enzymatic hydroxylation step overcome limitations of chemical oxidation methods which showed poor selectivity and led to a mixture of products. Leveraging the propensity of $P450_{BM3}$ variants to favor the regioselective and stereoselective hydroxylation of sclareolide at C3 [36], Renata et al combined gram-scale P450-catalyzed C3 hydroxylation of sclareolide and sclareol to produce key intermediates for the concise total synthesis of eight oxidized meroterpenoid natural products (Figure 2e) [56•].

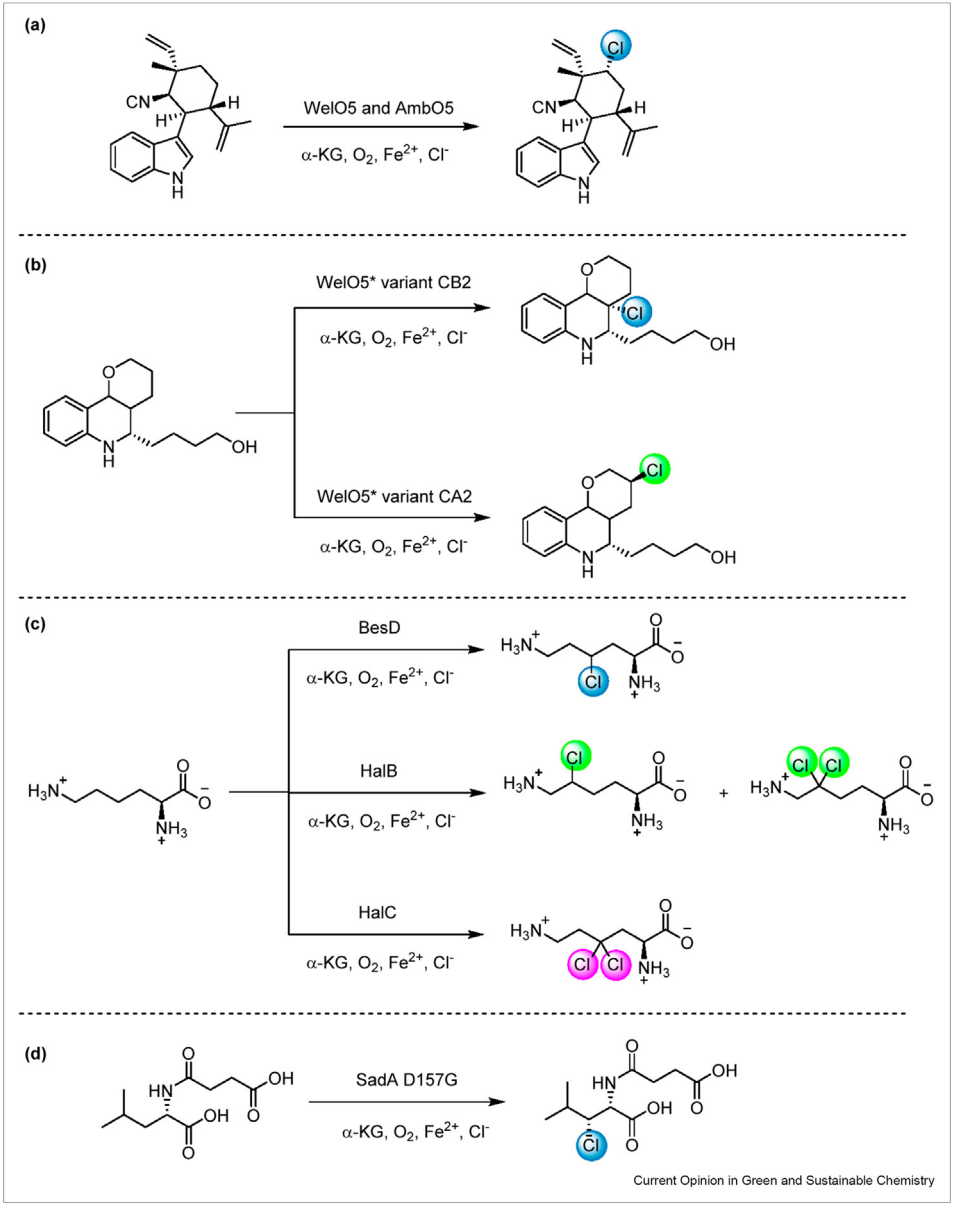
Along with cytochromes P450, mononuclear NHI-dependent oxidases such as αKG-dependent dioxygenases and Rieske dioxygenases participate in a broad range of oxidative processes implicated in the biosynthesis of natural products and metabolic degradation of xenobiotics [57,58]. While being well characterized from a structural and biochemical standpoint, the synthetic potential of NHI-/αKG-dependent dioxygenases has remained relatively underexplored. In early studies, Hüttel and coworkers demonstrated the value of members of this enzyme superfamily for the preparative scale of *cis*- and *trans*-3-hydroxy and 4-hydroxy-proline using proline hydroxylases from different microbial strains [59]. These enzymes along with related pipecolic acid hydroxylases (GetF, PiFa) could be used for the regioselective and stereoselective hydroxylation of L-pipecolic acid and 3- and 4-methyl-proline derivatives [60]. Previously, a L-isoleucine dioxygenase was utilized by the Shimizu group for the synthesis of 4(*S*)-hydroxyisoleucine from L-isoleucine [61]. More recently, the Renata group investigated the substrate scope of leucine 5-hydroxylase GriE, which is involved in the biosynthesis of griselimycin [62], and found that in addition to the native substrate (leucine), this enzyme is capable of catalyzing selective δ-hydroxylations in several aliphatic amino acids (11), supporting 150 to 10,000 turnovers and resulting in the isolation of desired oxidized products in 18–92% yields [63]. In addition, this biocatalytic reaction could be applied to generate a key intermediate for the chemoenzymatic total synthesis of manzacidin C at a subgram scale. Further exploiting the ability of wild-type GriE to perform a double C5 oxidation in leucine derivatives, various γ-substituted proline analogs could be prepared with high stereocontrol via a concise two-step chemoenzymatic route. The same enzyme was later exploited to implement an even shorter (5 vs. 9 steps) route for the total synthesis of manzacidin C [64]. In subsequent studies, stereoselective C–H hydroxylations catalyzed by a naturally occurring lysine 3-hydroxylase (KDO1) [65] and lysine 4-hydroxylase (GlbB) [66] were exploited for realizing concise routes for the total synthesis of tambromycin [67] and the proteasome inhibitor cepafungin I [68], respectively. The GlbB-catalyzed hydroxylation reaction was carried out at the multigram scale, demonstrating the scalability of the biocatalytic transformation [69]. In another recent study, Narayan et al used two Fe/αKG-dependent enzymes, CitB and ClaD, for the selective benzylic hydroxylation of a variety of *o*-cresol substrates [70]. The resulting products were found to undergo dehydration to generate reactive o-quinone methide derivatives, which could be further diversified chemically by means of a Michael addition or an inverse electron-demand Diels–Alder reaction. In addition, this biocatalytic protocol could be applied to enable the chemoenzymatic synthesis of the chroman natural product (−)-xyloketal D. In another important contribution, Zhang et al. reported a chemoenzymatic strategy to access a total of nine diterpene natural products belonging to the subfamilies of *ent*-kauranes, *ent*-atisanes, and enr-trachylobanes (Figure 2f) [71••]. In this case, two biosynthetic *ent*-kaurane hydroxylating enzymes, namely the Fe/αKG-dependent dioxygenase PtmO6 and class I P450 monooxygenase PtmO5, along with an engineered $P450_{BM3}$ variant (MERO1(M177A)), were applied to execute highly regioselective and stereoselective hydroxylations on a precursor terpene scaffold (*ent*-steviol) as key steps for affording the target diterpene natural products by chemoenzymatic means. While the aforementioned studies have relied on wild-type Fe/αKG-dependent dioxygenases, Zwick et al. recently demonstrated the feasibility of tailoring the selectivity of these enzymes by protein engineering to fit the desired synthetic needs [72]. After recognizing the ability of GetI to catalyze the selective γ-hydroxylation if citrulline, this enzyme was re-engineered into a functional arginine 4-hydroxylase via swapping four active site residues found in some homologous arginine C3/C4 hydroxylases. Albeit featuring modest catalytic activity (94 TTN), the engineered GetI variant maintained excellent stereoselectivity for the desired lysine γ-hydroxylation reaction and proved useful for the synthesis of a dipeptide fragment of the antibiotic enduracidin [72].

Metalloenzyme-catalyzed C(sp^3)–H halogenation

Enzymatic chlorination/bromination provides an attractive and environmentally friendly alternative to the installation of halogen groups in a target substrate [13,30,31]. In addition to modulating the physicochemical properties of a molecule, C–H halogenation

can provide useful synthetic handles for further elaboration and structural diversification (e.g., via metal-catalyzed cross coupling, nucleophilic substitution, etc.). In nature, two major classes of enzymes, i.e., flavin-dependent and NHI-dependent halogenases, mediate C−H halogenation reactions in a regioselective and stereoselective manner. While a detailed account of flavin-containing halogenases can be found elsewhere [13,30−32], here we will focus on recent progress in C(sp^3)−H halogenations by Fe/αKG-dependent halogenases. Similar to Fe/αKG-dependent dioxygenases, these enzymes rely on a high-valent Fe(IV)-oxo species to form a substrate carbon radical (C•) via H atom abstraction which undergoes halogenation via rebound to an iron-coordinated chloride/bromide ligand. A conserved glycine or alanine residue in place of aspartate or glutamate residue in the Fe/αKG dioxygenases provides an open coordination site for halide binding to the iron active center (Figure 1c).

Figure 3



Representative applications of C(sp^3)−H halogenation mediated by natural and engineered Fe/αKG-dependent halogenases. (a) Halogenation of 12-*epi*-hapalindole C catalyzed by WelO5 and AmbO5. (b) Regiodivergent chlorination of a martinelline-derived fragment mediated by engineered WelO5* variants. (c) Amino acid chlorination mediated by BesD and related halogenases. (d) SadA(D157G)-mediated halogenation of *N*-succinyl-L-leucine.

The synthetic application of NHI-/αKG-dependent halogenases has been limited by the fact that their substrates are typically tethered to an acyl or peptidyl carrier protein [73]. In a recent study, Liu et al discovered two biosynthetic αKG-dependent halogenases, namely WelO5 from *Hapalosiphon welwitschii* and AmbO5 from cyanobacterium *Fischerella ambigua*, that are capable of processing 12-*epi*-hapalindole C and analogs thereof as free-standing substrates [74,75] (Figure 3a). While these enzymes share an overall 79% sequence identity, AmbO5 was found to exhibit a broader substrate scope which included various hapalindole-type alkaloids [76]. More recently, the Buller group subjected a related halogenase, WelO5*, to a protein engineering campaign aimed at optimizing its activity and altering its regioselectivity for the chlorination of a core analog of martinelline, a potent bradykinin receptor agonist [77•]. After two round of active site engineering, a WelO5* variant (CB2) with enhanced catalytic activity (0.1 → 33 TON) for the regioselective chlorination of this substrate at the C9 position was obtained (Figure 3b). In addition, a regiodivergent variant (CA1) capable of chlorinating the same substrate at the C12 position with high stereoselectivity was isolated. Although a competing hydroxylation reaction was found to dominate in the latter case, this study provided an important proof-of-principle demonstration of the possibility of tuning the activity and regioselectivity of αKG-dependent halogenases via protein engineering.

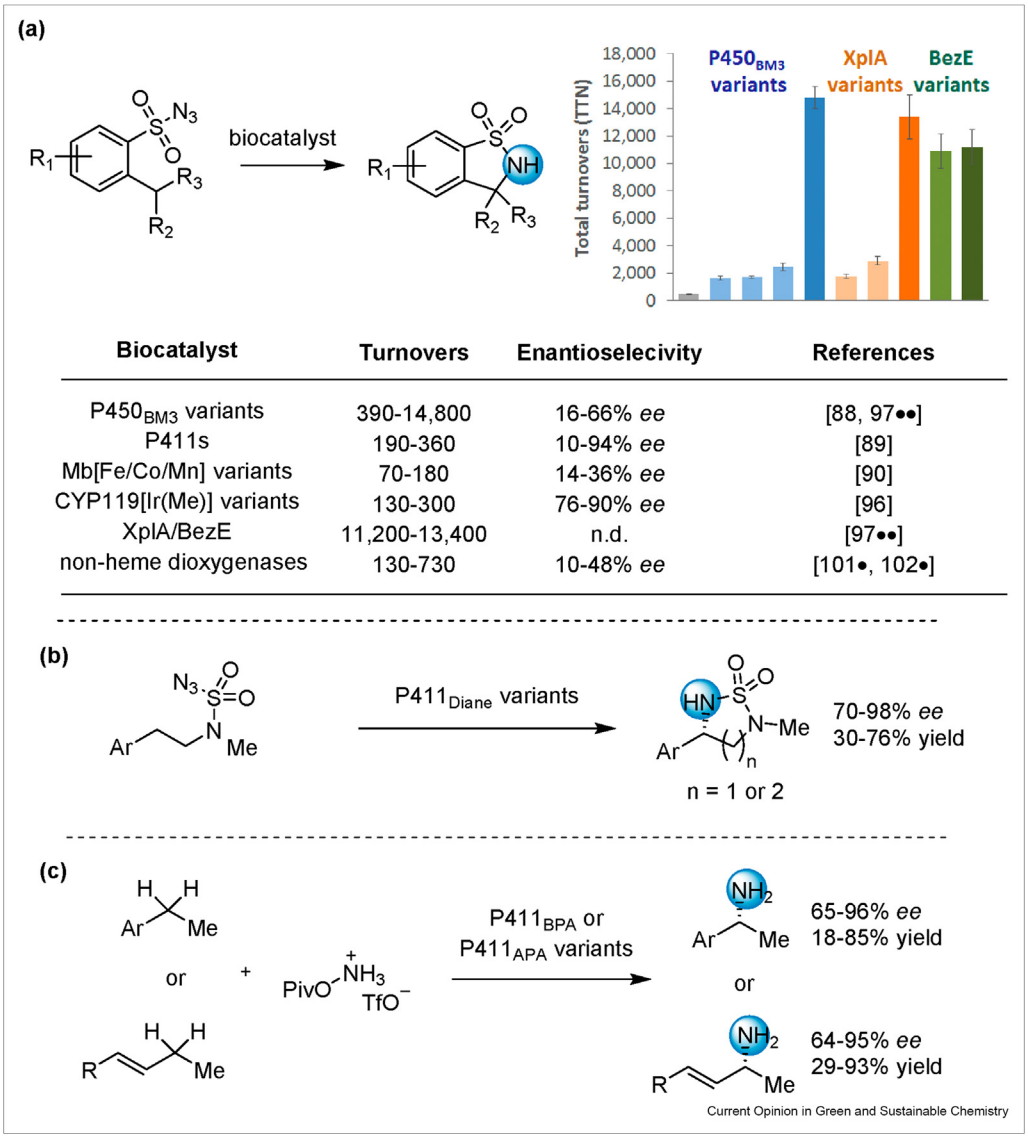
In another study, the Chang group discovered a radical halogenase, BesD, that catalyzes the regioselective γ-chlorination of lysine without the requirement of a carrier protein, showcasing the first example of the NHI-/αKG-dependent halogenase acting on a free-standing amino acid [78] (Figure 3c). Via bioinformatic analyses, the same authors later identified a BesD-related halogenases which are capable of chlorinating lysine and ornithine at the γ and δ position to produce both monochlorinated and dichlorinated products (e.g., HalB-D) [79] or capable of mediating subterminal chlorination of the aliphatic side chain in leucine and derivatives thereof (e.g., HalE). Although these reactions lacked stereoselectivity and their scalability was not investigated, these studies highlighted the potential of Fe/αKG-dependent halogenases for the C(sp^3)—H halogenation of amino acid substrates. Finally, Mitchell et al. reported the successful reprogramming of the αKG hydroxylase SadA into a halogenase [80]. After replacing a conserved iron-binding aspartate residue (Asp157) with a glycine residue typically found in αKG halogenases [81], the resulting SadA(D157G) variant was shown to be able to catalyze the γ chlorination (or bromination) of its native *N*-succinyl-L-leucine substrate with conserved regioselectivity and stereoselectivity (Figure 3d). This study suggests that a similar strategy could be applied to convert other αKG dioxygenases into selective halogenases.

Engineered and artificial metalloenzymes for C−H amination

The development of biocatalysts for selective C−H amination reactions is attractive to synthetic chemists due to the ubiquitous presence of nitrogen-based functionalities in pharmaceuticals and bioactive compounds. Biocatalytic systems for C−N bond formation include transaminases, ammonia lyases, and imine reductases which involve the transformation of oxidized or activated carbon centers [82−87]. A major breakthrough toward the direct conversion of C−H bonds into C−N bonds involved the discovery of the ability of heme-dependent enzymes and proteins to serve as biocatalysts for nitrene transfer reactions [21,88−92], a transformation previously limited to transition metal catalysts. In particular, engineered P450s as well as myoglobin and other heme-dependent enzymes were found to activate azide-containing substrates to catalyze intramolecular C−H amination reactions [88] as well as aziridination [21], sulfimidation [92] and other nitrene-mediated transformations [93]. These reactions were determined to be mediated by an electrophilic iron-nitrenoid intermediate, with P450-catalyzed C−H amination involving a stepwise H atom abstraction/radical rebound mechanism [91,94•]. Interestingly, the naturally occurring P450 BezE from *Streptomyces* sp. was later discovered to exploit a similar nitrene transfer mechanism as part of the biosynthesis of benzastatins [95].

Important contributions have recently enhanced the efficiency and expanded the scope of these biocatalytic C−H amination reactions. Both engineered P450s and myoglobin variants [88,90], along with metallo-substituted derivatives thereof [90,96], were found to be able to catalyze the intramolecular C−H amination of arylsulfonyl azides to produce sultam products (Figure 4a). The efficiency of these reactions is however significantly lower than that typically exhibited by P450 enzymes as monooxygenases. Based on mechanistic considerations, Steck et al. recently applied a rational design strategy to generate $P450_{BM3}$-based C−H aminases with dramatically enhanced C−H amination efficiency (460 → 14,800 TON) [97••]. Specifically, mutations directed at disrupting the native proton relay network and other conserved structural elements in the enzyme active site were shown to suppress an unproductive (reductive) pathway in the catalytic cycle, leading to a significant enhancement of the desired C−H amination reactivity. This approach also guided the discovery of two atypical P450s, XplA, and BezE, as efficient C−H aminases capable of supporting over 12,000 TON in this reaction (Figure 4a) [97••]. In another study, Moore et al. investigated the impact of substituting the conserved heme-coordinating histidine residue in myoglobin, with both proteinogenic (Cys, Ser, Tyr, Asp) and noncanonical amino acids (3-(3′-pyridyl)-

Figure 4



Biocatalyst	Turnovers	Enantioselecivity	References
$P450_{BM3}$ variants	390-14,800	16-66% *ee*	[88, 97••]
P411s	190-360	10-94% *ee*	[89]
Mb[Fe/Co/Mn] variants	70-180	14-36% *ee*	[90]
CYP119[Ir(Me)] variants	130-300	76-90% *ee*	[96]
XplA/BezE	11,200-13,400	n.d.	[97••]
non-heme dioxygenases	130-730	10-48% *ee*	[101•, 102•]

C(sp^3)–H amination reactions mediated by engineered and artificial metalloenzymes. (a) Intramolecular C–H amination of sulfonyl azides catalyzed by various engineered P450s, P411s, metallosubstituted myoglobins/P450s, and NHI-dependent enzymes with representative TON and enantioselectivity values (n.d. = not determined). The graph describes the activity of engineered $P450_{BM3}$, XplA and BezE variants for the C–H amination of triisopropylbenzenesulfonyl azide reported by Steck et al. [97••]. (b) Intramolecular C–H amination of azidosulfonylamines with engineered P411s. (c) P411-catalyzed intermolecular amination of benzylic and allylic C–H bonds.

alanine, *p*-aminophenylalanine, and β-(3-thienyl)-alanine). The resulting myoglobin variants were found to be able to catalyze the intramolecular C–H amination of triisopropylbenzenesulfonyl azide with up to 650 TON, showcasing the feasibility of exploiting pyridine-, thiophene-, and aniline-based noncanonical amino acids for metalloprotein engineering (Figure 4a) [98].

In terms of reaction scope, Yang et al. leveraged the intramolecular nitrene transfer reactivity of engineered serine-ligated P450 enzymes (also dubbed 'P411s') [21,89,92] to catalyze the intramolecular C–H amination of a broad range of sulfamoyl azides to produce protected diamine products (Figure 4b) [94•]. The best biocatalyst evolved for this reaction, called $P411_{Diane1}$, allowed for the efficient and enantioselective (70–99% *ee*) amination of benzylic and allylic C–H bonds to afford both 1,2- and 1,3-diamines, some of which were not accessible with small molecule catalysts [94•]. Furthermore, using directed evolution, the authors obtained two $P411_{Diane1}$-derived variants capable of catalyzing the stereodivergent amination of unactivated

secondary aliphatic C(sp^3)–H bonds. Mechanistic studies showed the involvement of a stepwise, radical-based nitrene C–H insertion mechanism similar to that previously observed for P450-catalyzed intramolecular C–H amination [91].

In another notable contribution, the Arnold group evolved a P411-based catalyst, called $P411_{CHA}$, for the intermolecular C–H amination of benzylic C–H bonds in the presence of tosyl azide [99]. This reaction could be applied for the aminofunctionalization of several ethylarene substrates with variable yield (16–85%) but with high enantioselectivity (92–99% *ee*). More recently, the same group further expanded the scope of this reaction to enable the amination of benzylic and allylic C–H bonds using a hydroxylamine ester as the nitrene precursor [100]. Starting from an initial P411 variant catalyzing this reaction with negligible activity (<1 TTN), two significantly improved biocatalysts were obtained through several rounds of directed evolution (up to 3930 TTN) [100]. The evolved enzymes, called $P411_{BPA}$ and $P411_{APA}$, displayed good to excellent levels of chemoselectivity, regioselectivity, and enantioselectivity (64–96% *ee*) toward the amination of benzylic and allylic C–H bonds (Figure 4c). Compared with the method involving tosyl azide as nitrene precursor [99], this strategy has the key advantage of providing direct access to primary amines as the products.

In addition to heme-dependent enzymes and proteins, recent studies showed that NHI-dependent enzymes are also able to catalyze non-native nitrene transfer reactions [101•,102•]. Goldberg et al. found that PsEFE, an ethylene-forming αKG-dependent iron dioxygenase, exhibit a basal, promiscuous activity toward the aziridination of styrene with tosyl azide. Via multiple rounds of directed evolution, an evolved PsEFE variant carrying five mutations was identified that shows improved activity and enantioselectivity for the styrene aziridination reaction (120 TON, 88% ee) as well as for the C–H amination of arylsulfonyl azides (Figure 4a) [101•]. Interestingly, the catalytic efficiency of the enzyme in both reactions could be significantly improved (130 → 730 TON) by replacing αKG with *N*-oxalylglycine as the iron coordinating ligand. In a separate study, Vila et al. reported that different types of mononuclear NHI enzymes, including Rieske dioxygenases (e.g., naphthalene dioxygenase) as well as αKG-dependent dioxygenases (TauD, Gab, AsqJ, H6H) and halogenases (WelO5) can catalyze the intramolecular C–H amination of sulfonyl azides (Figure 4a) [102•]. The naphthalene dioxygenase–catalyzed reaction could be carried out at a gram scale in a bioreactor, demonstrating the scalability of this transformation. Importantly, these studies demonstrated that different members of the NHI-dependent enzyme superfamily can exhibit non-native nitrene transferase activity, laying the basis for their further development and optimization in the context of other C–H aminations and nitrene-mediated transformations.

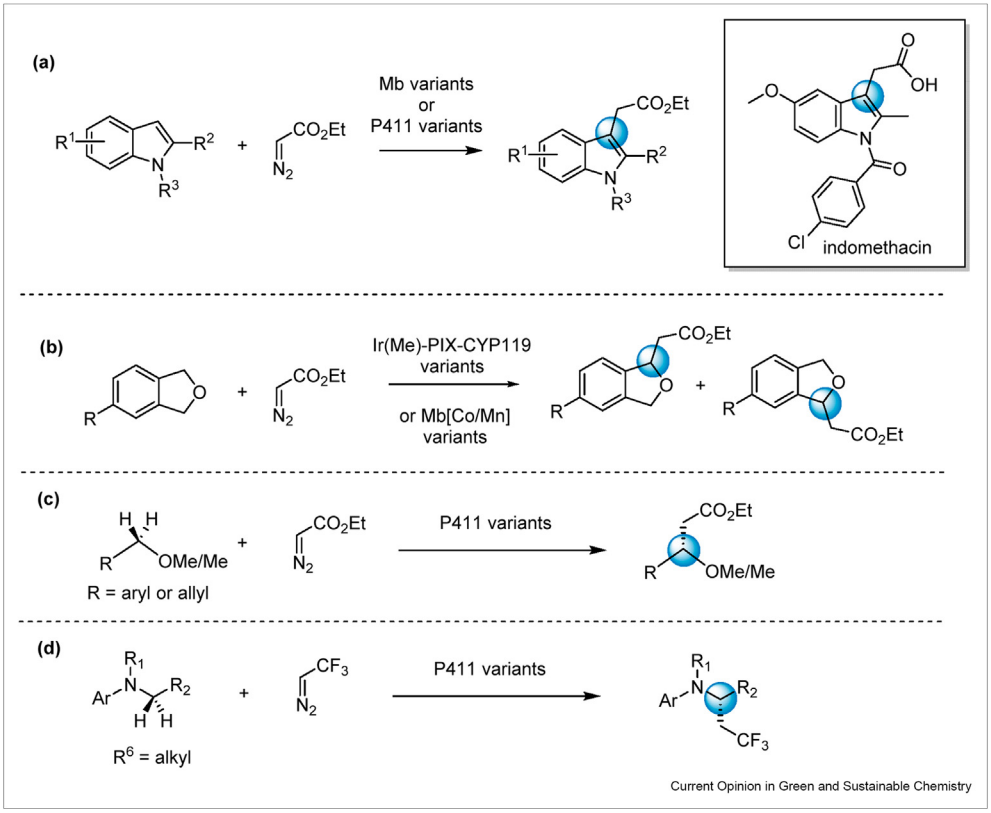
C–C bond formation via C–H functionalization

While various classes of enzymes are known to catalyze the formation of C–C bonds (e.g., aldolases, SAM-dependent methyl transferases, PLP-dependent enzymes) [103–106], these transformations encompass a narrow range of chemistries. Over the past few years, important progress has been made toward expanding the reaction scope of biocatalysts to enable new types of C–C bond forming transformations via direct C–H functionalization.

Engineered myoglobins have proven to be efficient and versatile biocatalysts for olefin cyclopropanations and other abiological carbene transfer reactions [15–17,24,107]. Recently, Vargas et al. demonstrated that engineered myoglobin-based carbene transferases are capable of functionalizing unprotected indoles in the presence of ethyl diazoacetate, furnishing a broad range of C3-functionalized indole derivatives in high yield (up to 99%) and with excellent chemoselectivity and regioselectivity [108•]. This biocatalytic method could be leveraged to implement a more streamlined (3 vs. 4–5 steps) chemoenzymatic route for the synthesis of the nonsteroidal anti-inflammatory drug indomethacin (Figure 5a) compared with previously available methods. Mechanistic studies established that this indole C–H functionalization reaction involves an electrophilic substitution reaction via a zwitterionic intermediate [109]. A similar reaction was later reported using engineered variants of YfeX, a hemoprotein found in *E. coli* [110], and P411 enzymes [111]. The latter biocatalysts could be applied for the regiodivergent functionalization of C2 and C3 position in N-methyl indole (98% and 91% regioselect., respectively).

The functionalization of C(sp^3)–H bonds via carbene C–H insertion are challenging transformations which have typically required the use of noble metals (e.g., Rh, Ir) as catalysts. By substituting the native heme cofactor with iridium-porphyrin in a thermophilic P450 enzyme (CYP119), Hartwig et al generated artificial metalloenzymes capable of promoting intermolecular carbene C–H insertion in a series of phthalan derivatives (Figure 5b) [112,113]. By protein engineering, these biocatalysts could be optimized to catalyze this reaction with up to 68% *ee* and 18:1 regioselectivity in the case of substituted phthalans. In another study, Sreenilayam et al. reported the intermolecular C–H functionalization of phthalan with EDA using Mn- and Co-containing myoglobin variants, thus demonstrating that metalloenzymes containing first-row transition metals are also

Figure 5



C–H functionalization reactions via metalloenzyme-catalyzed carbene transfer. (a) Indole functionalization by engineered myoglobin and P411 variants. (b) Intermolecular carbene insertion by iridium-based P450s. (c) P411-catalyzed carbene insertion into benzylic/allylic C(sp^3)–H bonds. (d) P411 catalyzed α-amino C–H carbene insertion with trifluorodiazoethane.

capable of mediating carbene C(sp^3)–H insertion chemistry [114]. More recently, another major breakthrough in this area was reported by the Arnold group through the development of engineered cytochrome P411 variants useful for the intermolecular alkylation of benzylic, allylic, and α-amino C(sp^3)–H bonds via carbene C–H insertion [115••]. Using ethyl diazoacetate as carbene donor, a P411-based biocatalyst optimized through multiple rounds of directed evolution (dubbed P411-CHF) enabled the functionalization a broad range of substrates with up to 3750 turnovers and up to 99% *ee* (Figure 5c) [115••]. In subsequent studies, the scope of these P411-based biocatalysts could be expanded to enable the functionalization of α-amino C(sp^3)–H bonds in the presence of 2-diazo-1,1,1-trifluoroethane or 3-diazodihydrofuran-2(3H)-one (Figure 5d) [116], two carbene donor reagents previously applied in the context of other hemoprotein-catalyzed carbene transfer reactions [117,118]. In both cases, the screening of a library of P411 active site variants against a panel of target substrates yielded substrate-matched biocatalysts with high enantioselectivity and, in some cases, stereocomplementary selectivity for the transformation of the substrate of interest. Overall, the studies highlighted above have begun to demonstrate the potential of engineered hemoproteins for C–H functionalization via carbene transfer chemistry. Despite this progress, the scope of these transformations is currently limited to electronically activated C(sp^3)–H bonds (i.e., benzylic/allylic C–H bonds or C–H bonds in alpha to heteroatoms) and simple substrates. An unmet challenge in this area thus includes gaining the ability to functionalize less activated C(sp^3)—H bonds and their application in more complex molecular settings.

Outlook

As outlined above, the toolbox of biocatalytic methods for the conversion of C–H bonds into new C–O, C–N, and C–C bonds have grown significantly over the past few years, creating new opportunities for the sustainable synthesis of chiral building blocks, drug molecules and complex molecules. The integration of metalloenzyme-catalyzed oxyfunctionalizations into chemoenzymatic scheme has provided a means to expedite the late-stage C–H functionalization and total synthesis of complex

molecules, such as bioactive natural products. Importantly, these approaches have enabled chemists to access new C–H sites and/or exploit disconnection strategies previously inaccessible with purely chemical methods, highlighting the value of these hybrid approaches. In the future, we expect these chemoenzymatic strategies will continue to expand to include new classes of enzymes as well as new-to-nature enzyme-catalyzed transformations such as biocatalytic C–H amination and C–H carbene insertion reactions, which are currently limited to small molecule substrates. Initial successes in the application of P450-catalyzed C–H aminations for the late-stage elaboration and synthesis of natural products offer an encouraging preview of these future opportunities [91,94•]. As noted previously, the scope of biocatalytic C(sp^3)–H carbene insertion reactions are currently limited to electronically activated C–H bonds and extending their scope for the functionalization to less activated C–H sites in both small and complex molecules remains an unmet challenge. Finally, the studies highlighted in this review illustrate the potential of various classes of metalloenzymes, namely heme and various types of NHI-dependent enzymes, to be repurposed for new-to-nature chemistry. This should continue to inspire the investigation of metalloenzymes and metalloproteins for novel chemical transformations and synthetic applications. Directed evolution and mechanism-guided rational design and cofactor re-engineering can offer powerful and complementary tools toward the development of new and more efficient biocatalysts for these applications. Ultimately, these systems will fulfil the demand for new, sustainable methods for organic synthesis as well as provide valuable new tools for applications in medicinal chemistry, drug discovery, and chemical biology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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