Non-Native Site-Selective Enzyme Catalysis

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Abstract

The ability to a site-selectively modify equivalent functional groups in a molecule has the potential to streamline syntheses and increase product yields by lowering step counts. Enzymes catalyze site-selective transformations throughout primary and secondary metabolism, but leveraging this capability for non-native substrates and reactions requires a detailed understanding of the potential and limitations of enzyme catalysis and how these bounds can be extended by protein engineering. In this review, we discuss representative examples of site-selective enzyme catalysis involving functional group manipulation and C-H bond functionalization. We include illustrative examples of native catalysis, but our focus is on cases involving non-native substrates and reactions often using engineered enzymes. We then discuss the use of these enzymes for chemoenzymatic transformations and target-oriented synthesis and conclude with a survey of tools and techniques that could expand the scope of non-native site-selective enzyme catalysis.

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Keywords

Site-selective catalysis, biocatalysis, chemoenzymatic, C-H functionalization

1. Introduction

Controlling the selectivity of a chemical reaction is as central to synthetic organic chemistry as bond formation itself. From a practical perspective, forming a new bond is only useful if it provides a desired product with the correct connectivity between atoms (i.e. constitutional selectivity) and with the correct arrangement of those atoms in space (i.e. stereoselectivity).[1] From a fundamental perspective, navigating the energy surfaces that govern selectivity constitutes a powerful test of our understanding of reactivity.[2] This challenge can be simplified by exploiting the relative reactivity, or chemoselectivity,[3] of reagents and catalysts toward different functional groups.[4] If reactivity toward multiple functional groups is observed, protecting groups can be used to eliminate the chemoselectivity challenge, albeit at the expense of added operations and wasted material. Indeed, much of organic methodology involves generating a reactive intermediate and either blocking problematic functional groups or designing a synthesis so that they are not present to ensure that the intermediate reacts primarily with a desired functional group. This substrate-controlled approach reduces the problem of controlling constitutional selectivity, if it remains a factor, to controlling regioselectivity, that is, the "preferential making or breaking of bonds in one of two possible orientations"[5] at that desired functional group (Scheme 1A).[1]

But what happens when the chemoselectivity simplification fails? As molecular complexity increases, the reactivities of different functional groups may be attenuated or exaggerated due to their molecular context such that they overlap. What if *chemoselectivity* is not relevant because one is dealing with identical functional groups? Carbon-hydrogen bonds can present a particularly

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challenging case in this regard since they are ubiquitous in organic molecules. The latter question poses the challenge of site selectivity, a sub-classification of constitutional selectivity that "differentiates unrelated functional groups in a molecule which can undergo the same type of reaction" (Scheme 1A).[1, 4] While molecular context, including proximity to directing groups, may allow for differentiation of identical functional groups,[6] this form of substrate control is limiting (what if you need to functionalize a less reactive or non-directed site?).
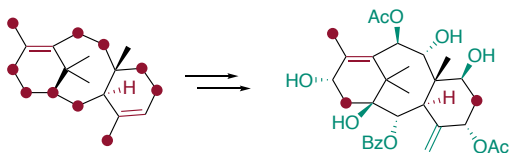
A *Regioselectivity: reaction at a functional group in different orientations*
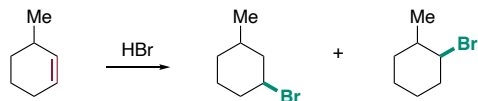
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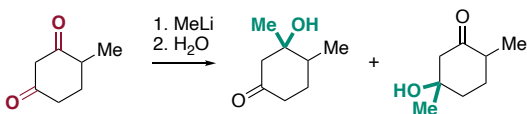
B *Site- and enantioselective enzymatic C-H functionalization*
(and site-selective O-H functionalization) in paclitaxel biosynthesis

Scheme 1. A) Definitions of regioselectivity and site selectivity with representative examples of each (adapted from reference 1).[1] B) Examples of site-selective reactions in the biosynthesis of paclitaxel.[7] Throughout this review, crimson is used to highlight functional groups (A/B) or sites (B, filled circles) undergoing site-selective reactions, and mint is used to highlight the functionalized sites.

Site-selective catalysis emerged in nature long ago when enzymes evolved to achieve this capability presumably due to its benefit to organismal fitness. Site-selective oxidation, reduction, and phosphorylation are common throughout primary metabolism, and examples of site-selective hydroxylation in secondary metabolism are among the most-cited examples of the potential synthetic utility of site-selective catalysis. The extensive hydroxylation and acylation/benzoylation of taxadiene *en route* to paclitaxel, for example, involves discrimination of multiple secondary, tertiary, and allylic C-H bonds, olefins, and secondary alcohols, functionality that comprises nearly the entire molecule (Scheme 1B).[7] Many of these enzymes catalyze the formation of reactive intermediates, but rather than requiring blocking/directing groups or circuitous synthetic routes, they use catalyst control to override inherent substrate reactivity. Critical to this capability is the extensive, redundant, and dynamic molecular recognition exerted by enzyme active sites. Extensive because enzymes use many different intermolecular forces to bind substrates, redundant because often one or more of these can be disrupted without completely ablating catalysis, and dynamic because enzymes can change conformations to suit different states along catalytic cycles. Chemists have long sought to mimic these properties.[8] Early efforts used cyclodextrins that formed a hydrophobic pocket for substrate binding and that provided scaffold on which catalysts could be appended to enable site-selective functionalization of the bound substrate.[9, 10] A variety of substrate binding and functionalization strategies have since been explored within this bifunctional catalyst framework.[11]

It is interesting that these "bio-inspired" synthetic catalysts find themselves at a stage of development that wild type enzymes *appeared* to occupy in what has been termed the "first wave" of biocatalysis.[12] That is, the former are and the latter were known to catalyze site-selective functionalization of a relatively narrow range of substrates; a notable similarity given that substrate scope is often an advantage of synthetic catalysts over enzymes. We say that first wave enzymes *appeared* to be limited in scope, however, because they have proven remarkably adept at site-selective catalysis when researchers decided to *look* for that reactivity outside the confines of the native substrate and reaction scope.[13, 14] Moreover, directed evolution has enabled rapid tuning of enzymes to enable site-selective reactions of diverse substrates.[15, 16] Will it be possible to design or otherwise engineer synthetic site-selective catalysts with molecular recognition abilities as versatile and adaptable as enzymes? How complex must such systems be to achieve these emergent properties? There is no doubt that learning the answers to these questions will be endlessly fascinating. Moreover, it is likely that both classes of catalysts, hybrids thereof,[17] and others that have not yet been developed will be required to achieve the ultimate goal of site-selective catalysis: *selective* reaction at *any* site on *any* substrate.

With the goal of helping to frame the pursuit of this goal from the perspective of enzyme catalysis, we begin this review by discussing enzymes that control the site selectivity of functional group manipulation and C-H functionalization reactions. We then cover applications of these enzymes in the context of chemoenzymatic methods and target-oriented synthesis, and we conclude with a brief overview of site-selective modification of macromolecules. While we occasionally cover early studies involving native substrates, our focus is on non-native substrates, non-native reactions, and artificial enzymes that, collectively, illustrate the potential to use enzymes for site-selective catalysis in situations far beyond what they evolved for in nature. Because the definition of site selectivity provided above does not account for the fact that a given functional group can behave differently depending on its molecular context, we draw particular attention to cases involving groups in similar contexts or in which expected reactivity patterns are overridden. Also, while site selectivity is fundamentally different than stereoselectivity, we highlight systems that are site- and stereoselective since both are required to achieve the above-stated goal. We note that our focus differs from late-stage functionalization, which has been reviewed elsewhere,[18] in that it often involves relatively simple substrates and excludes cases in which chemoselectivity alone is the key challenge. The basics of the techniques that were used to identify or engineer enzymes discussed in this review (e.g. directed evolution, genome mining, etc.) are also not extensively discussed since there are many reviews in this regard, but we do discuss key advances that are needed in these areas to advance the field of non-native site-selective enzyme catalysis.

2. Functional Group Manipulation

As noted above, functional groups lie at the heart of synthetic methodology, and while chemoselectivity can often be used to ensure reaction of one functional group over others, this is not always possible. As functional group reactivity and molecular context become more similar, addressing this challenge becomes harder so that one must use either protecting groups or an alternate synthetic strategy to avoid the chemoselectivity issue entirely. Enzymes capable of site-selectively manipulating similar or identical function groups using catalyst control provide an alternate strategy that enables otherwise impossible synthetic routes.

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2.1. Carboxylic Acid Derivative Hydrolysis and Formation

2.1.1. Carboxylic Ester Hydrolysis

Hydrolysis of carboxylic acid derivatives, including esters, amides, and nitriles, enables a range of subsequent reactions to elaborate compounds. Classical methods to hydrolyze these functional groups use strong acids or bases, but these reagents often lead to side reactions, making site-selective hydrolysis difficult. More recent methods correct this problem to varying extents,[19-21] but the need for reactions that operate under mild conditions, including ambient temperature and neutral pH, have led to broad use of esterases and lipases as catalysts for carboxylic acid derivative hydrolysis. Indeed, these were among the first enzymes commonly used for organic synthesis.[22] Particularly important in this regard are serine hydrolases, which do not require a cofactor and instead rely largely on a Ser–Asp/Glu–His catalytic triad and an H-bonding by backbone amide N-H bonds in a so-called "oxyanion hole" to catalyze hydrolysis (Scheme 2).[23] These enzymes often tolerate high concentrations of organic solvents,[24] which allows for high substrate loadings in an aqueous solution. Extensive development of serine hydrolases has established that they can exhibit broad substrate scope while maintaining high site and stereoselectivity.
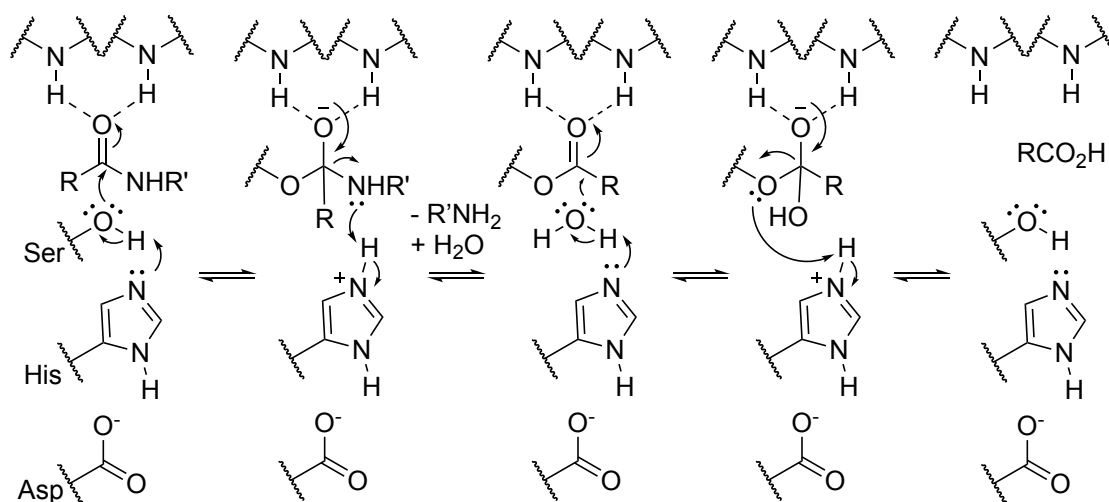
Scheme 2. Simplified mechanism of serine hydrolases showing the roles of the so-called "catalytic triad" and "oxyanion hole" residues on catalysis.[23]

The serine hydrolase *C. antarctica* lipase B (CALB) has been used in both fine and commodity chemical synthesis,[25] and several examples of site-selective CALB-catalysis have been reported. For example, hydrolysis of the terminal ester in **1** without hydrolysis of the more reactive phenolic ester linkage is challenging under conditions typically used for ester hydrolysis. Site-selective hydrolysis of the former was possible using Novozym 435, which is a resin-supported version of CALB,[26] in 90/10 v/v *t*-BuOH/H$_2$O at 20 °C to give pro-drug **2** in 84% yield (Scheme 3A).[27] Novozym 435 has also been used for site-selective hydrolysis of triethylcitrate (**3**) to give **4** in 45% yield (Scheme 3B),[28, 29] and this compound can be converted to value-added products like rhizoferrin.[29]
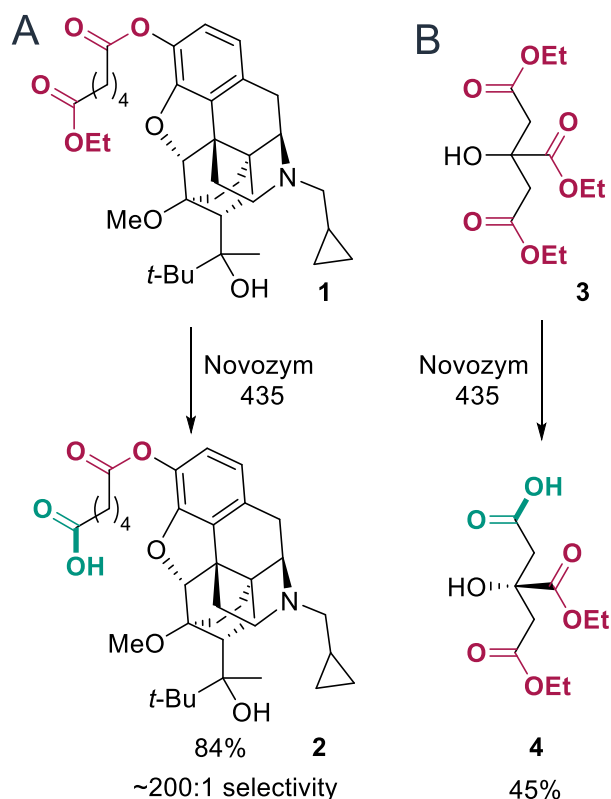
Scheme 3. A) Hydrolysis of **1** in 90/10 v/v *t*-BuOH/H$_2$O using Novozym 435 to give **2**.[27] B) Mono hydrolysis of triethylcitrate using Novozym 435 to give **4**.[29]

Pig liver esterase (PLE) has also been used for site-selective hydrolysis. For example, hydrolysis of methyl-(Z)-2-methyl-butenedioate **5** was achieved with high selectivity for the ester distal to the 2-methyl group (Scheme 4A).[30] Castanospermine, an indolizidine alkaloid isolated from the seeds of *Castanospermum austral* and a potent inhibitor of dengue virus infection, bears four similarly reactive hydroxyl groups. To differentiate these groups, the natural product was selectively di-butyrylated to give **8**.[31] PLE preferentially hydrolyzes the butyryl group from the C-7 position of **8** to give **9**, whereas a different serine hydrolase, subtilisin, hydrolyzes the ester bond at the C-1 position to give **10** (Scheme 4B).[32] Finally, while poor site selectivity was observed for hydrolysis of (R)-aspartate dimethyl ester (**11**) using acid or base catalysis, PLE afforded **12a** with a selectivity of 98:2 (Scheme 4C).[33, 34] This selectivity was maintained even for a gram scale reaction, and both (R)- and (S)-dimethyl aspartate were viable substrates.
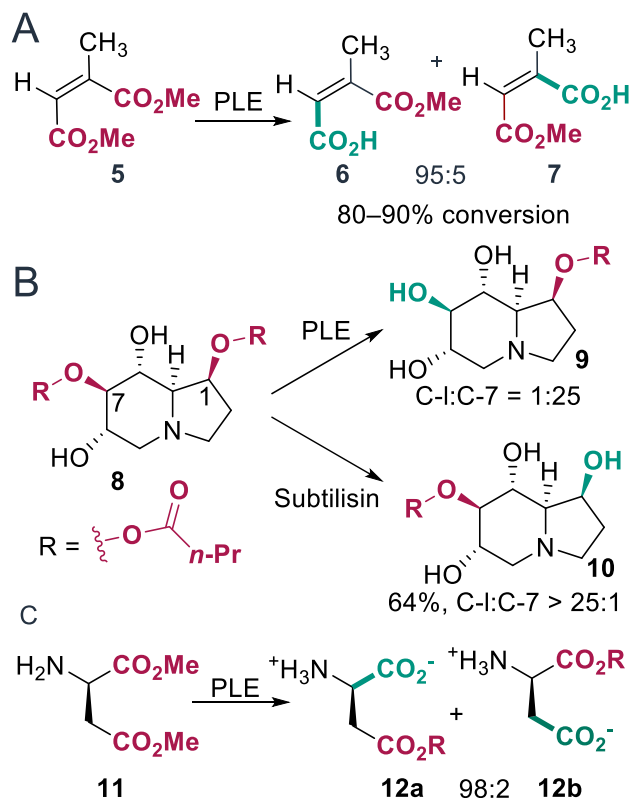
Scheme 4. A) PLE-catalyzed hydrolysis of of (Z)-2-methyl-butenedioic acid esters.[30] B) PLE-catalyzed the hydrolysis of the butyryl group at C-7 of **8** (C-l:C-7 = 1:25). Subtilisin-catalyzed hydrolysis of the butyryl group at C-1 of **8** (C-l:C-7 > 25:1).[32] C) Hydrolysis of (R)-aspartate dimethyl ester **11** using PLE to obtain monoester **12a**.[34]

Several other hydrolases have also been used for site-selective catalysis involving polyacylated sugars. For example, Wong used a lipase from *Candida cylindracea* to deacylate compound **13** to give the 6-OH derivative **14** in high yield (80-90%, Scheme 4A).[35] This same group also examined lipase-catalyzed deacylation of other monosaccharides, including furanoses, pyranoses, and anomers of compounds in both classes.[36] Lipases from *Rhizopus japonics* and *Mucor sp.*, crude porcine pancreatic lipase, *Aspergillus niger* lipase (ANL)*,* and *Candida cylindracea* lipase (CCL) were used to site-selectively hydrolyze acylated furanoses. ANL and CCL provided the best results as shown in Scheme 5B, and hydrolysis of the pyranose series also proceeded via preferential hydrolysis at the primary positions.[36] A separate study by the Crout group reported similar site-selective C-6 deacetylation of peracetylated α-D-hexopyranoses **17** (54-88% yield) and tetra-O-acetyl-α-D- hexopyranoses **19** (70-85% yield) using an esterase from *Rhodosporidium toruloides* (Scheme 5C).[37] A more recent effort showed that C-1 of per-acylated glucose can be selectively hydrolized in preference to C-6 using porcine pancreatic lipase.[38]
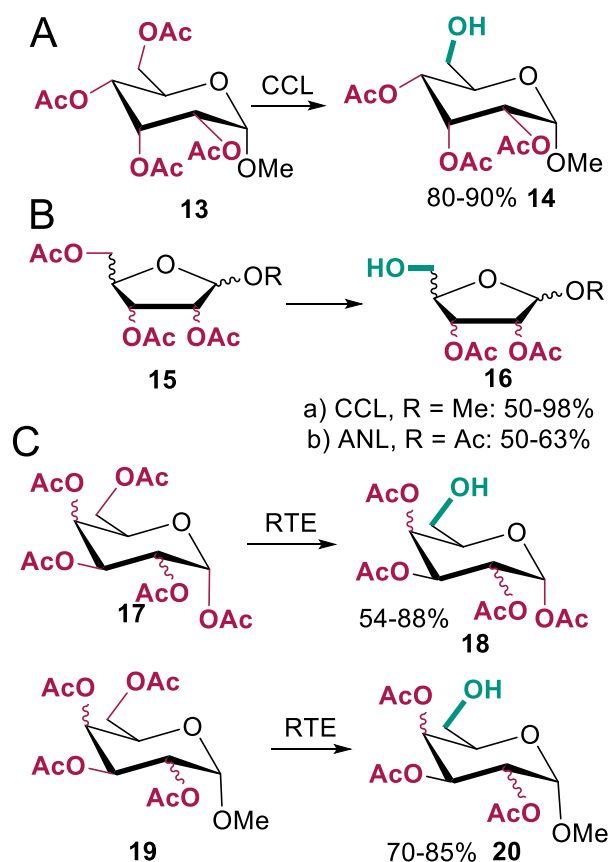
Scheme 5. A/B) Selective hydrolysis of peracylated sugars from the pyranose and furanose series by *Aspergillus niger* lipase (ANL) and *Candida cylindracea* lipase (CCL). C) *Rhodosporidium toruloides* esterase-catalyzed hydrolysis of peracetylated compound **17** and **19**.

2.1.2. Carboxylic Amide Formation

Amide bond formation is among the most frequently performed organic transformations, and under typical chemical conditions, it requires the orchestration of different activating reagents and protecting group chemistries.[39] Enzymatic amide bond formation is catalyzed by a variety of enzyme classes that activate carboxylic acid substrates under mild reaction conditions and without protecting groups. Many different classes of enzymes utilize ATP as a cofactor for activating carboxylic acids for amide bond formation(Scheme 6A),[40] so application of these enzymes were initially limited by the requirement for super-stoichiometric ATP, but efficient ATP recycling systems have alleviated this issue.[41] ATP-dependent amide bond forming enzymes use different mechanisms to activate carboxylic acid substrates toward attack by amines. For example, acyl/aryl CoA synthetases form an acyl-AMP phosphoester that is attacked by acyl-CoA to form the corresponding thioester. This acyl-CoA thioester is subsequently coupled to an amine via an *N*-acyl/aryltransferases (NATs). Historically, the expense of the CoA cofactor and the relatively limited substrate scope of NATs limited applications of these enzymes for non-native amide bond formation. As with ATP recycling, CoA recycling allows for use of CoA and truncated derivatives in sub-stoichiometric quantities for acylation of lysine residues in a histone-derived peptide with non-native amine donors.[42]

Some amide bond forming enzymes catalyze the intermolecular reaction between two unprotected amino acids.[43] The lack of homo-dimerization in these reactions highlights the inherent selectivity of these enzymes. ATP grasp enzymes are a class of amino acid ligases that release ADP to form an acyl-phosphate intermediate, which is attacked by an amine nucleophile to produce the amide. While ATP grasp enzymes have been engineered using site-directed mutagenesis,[44] can have broad substrate scope,[43] and have been used for the synthesis of macrocyclic peptides[45] and natural product synthesis,[46] no site-selective examples have been reported.
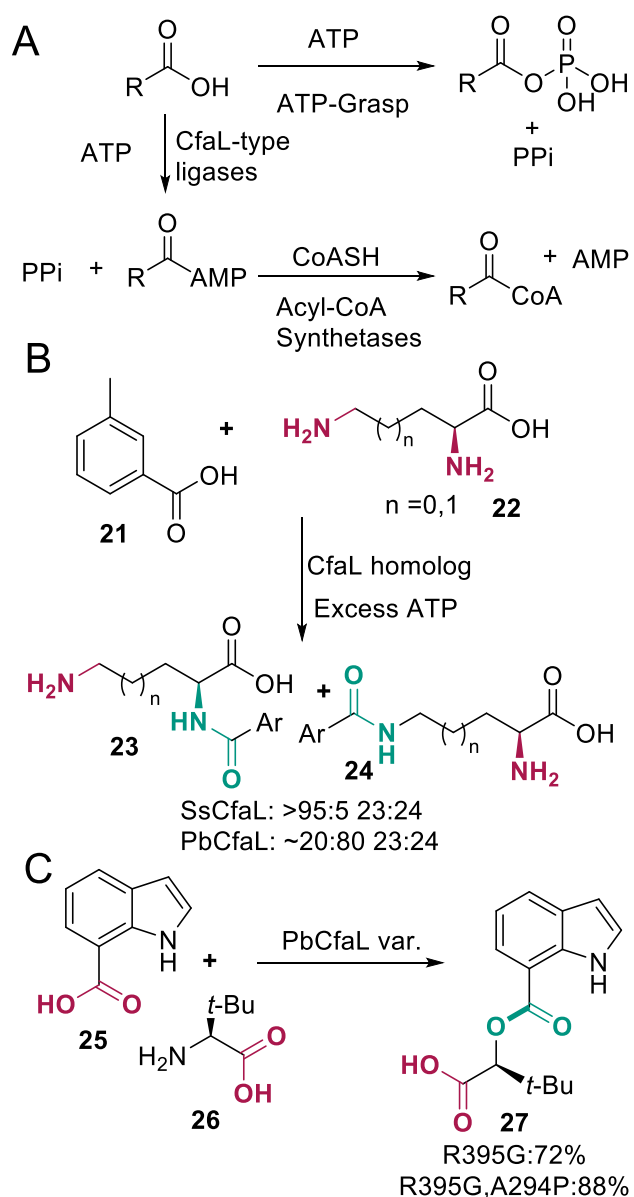
Scheme 6. A) Activated carboxylic acid intermediates. Enzymes listed below the arrow utilize that activated species for subsequent amidation. **B)** CfaL and related enzymes catalyze site-selective amidation of a diamine substrate. **C)** PbCfaL can be engineered for improved yields in the coupling of acids **25** and **26** with no observed homocoupling.

The enzyme CfaL belongs to a subset of amide bond synthetases that do not require an additional *N*-acyltransferase to affect amide bond formation. This enzyme and its homologs coupled a range of aliphatic and aromatic carboxylic acids to isoleucine, and various amines to 3-methyl benzoic acid (Scheme 6A).[47] These ligases also tolerate different amino acid coupling partners, including 2,4-diaminobutyrate (DAB) and L-ornithine, which underwent selective amide bond formation with *m*-methylbenzoic acid at the α-amino group using SsCfaL (Scheme 6B), obviating the need for sidechain protecting groups. Notably, while PbCfaL gave low yield and selectivity, it preferentially acylated the sidechain amino group in DAB and L-ornithine, highlighting the catalyst-controlled site selectivity of this enzyme class. Improved activity of CfaL ligase variants generated via site-directed mutagenesis (i.e. Scheme 6B) also suggests that further protein engineering could be used to expand the scope and selectivity of these enzymes.

Increasing recognition of the importance of macrocyclic peptides[48] has driven the use of thioesterases (TEs) to enable protecting group free macrocylization via amide bond formation.[49] The application of this enzyme class *in vitro* was historically limited by the requirement for a C-terminal *N*-acetylcysteamine (SNAC) peptide thioester to enable TE biocatalysis following solid phase peptide synthesis.[50] This penultimate coupling step introduces an additional expense by requiring coupling with *N*-acetylcysteamine, leads to epimerization of the α-position, and complicates HPLC purification. Recently, it was found that the TE SurE tolerates peptide substrates with a simple C-terminal diol in place of the SNAC group required by most TE cyclases. This modification simplified macrocycle synthesis and purification, and substrate profiling showed SurE accepted a variety of peptides with an L-amino acid at the *N*-terminus and D-amino acid at the C-terminus (Scheme 7). SurE G235L was found to fully cyclized a peptide substrate with a C-terminal glycine residue, eliminating the native requirement for the less common C-terminal D-amino acid. Other TE enzymes maintained high activity toward their native substrates upon incorporation of a C-terminal ethylene glycol group, showing the generality of this approach.
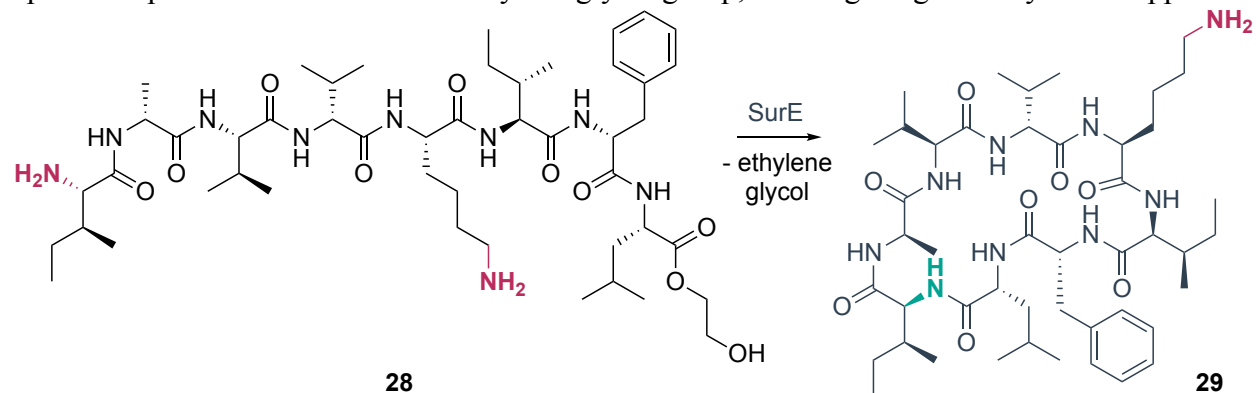
Scheme 7. Site-selective macrocyclization of peptidic substrate **28** to Surugamide **29** using a peptide substrate with a C-terminal ethylene glycol group.[50]

2.2. Alcohol and Amine Functionalization

2.2.1. Alcohol Acylation

Many serine hydrolases also catalyze the acylation of alcohols with carboxylic acid derivatives to form esters. As in the hydrolysis reactions noted above, enzymes can provide these compounds with high selectivity and specificity relative to chemical methods.[51, 52] For example,

polyhydroxylated steroids exhibit a wide range of biological activities, and while selective modification of the hydroxyl groups in these compounds is often desirable, their similar reactivity complicates this goal. *Candida rugosa* lipase was able to overcome this challenge to enable site-selective acylation of substrates **30** and **32** (Scheme 8A).[53] Likewise, antisense oligonucleotides are used to selectively inhibit translation of disease-associated genes, but manipulating the hydroxyl groups in these compounds requires several protection/deprotection steps and with low yield.[54] CALB-catalyzed transesterification, on the other hand, provides 5'-O-benzoyl-2'-deoxynucleosides in high yields (Scheme 8B).[55] CALB has also been used for site-selective esterification in addition to trans-esterification. Naringin is a natural product with several similarly reactive alcohols on the sugar moiety and two phenol hydroxyl groups, but immobilized CALB allowed selective esterification of the primary hydroxyl group of the sugar (Scheme 8C).[56]
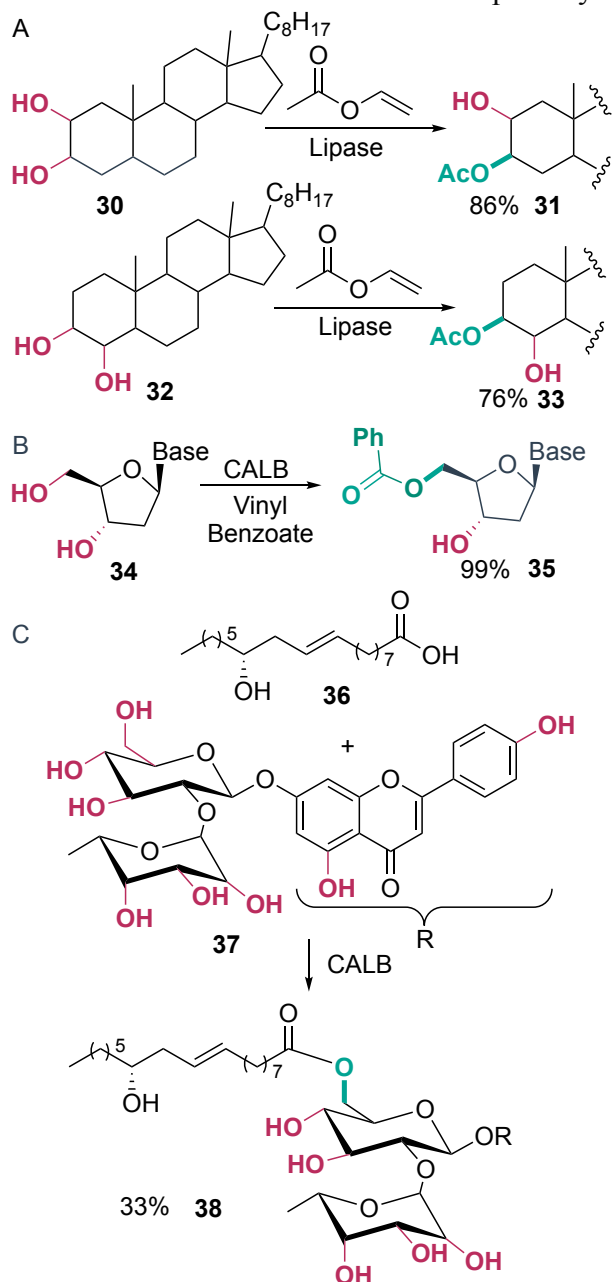
Scheme 8. A) Lipase-catalyzed site-selective acylation of vicinal diols of steroid molecules.[53] B) site-selective acylation of deoxynucleosides by CALB at an elevated temperature of 60°C.[55] C) Site-selective esterification of the sugar moiety of naringin using immobilized lipase from *C. antarctica*.[56]

Acyltransferases also catalyze site-selective acylation. For example, ArmB from *Armillaria mellea* is responsible for site-selective acylation of a single secondary hydroxyl group in the biosynthesis of melleolide (Scheme 9A).[57] ArmB also catalyzes transesterification of orsellinic acid (OA) esters with various other alcohols (Scheme 9B). LovD is an acyltransferase found in *Aspergillus terreus* that converts the inactive monacolin J acid into the lovastatin by the selective acylation of the hydroxy group.[58] Wild type LovD is inefficient and poor stability, but directed evolution was used to engineer a LovD variant with activity 11-fold higher than the wild type enzyme (Scheme 9C).[58]
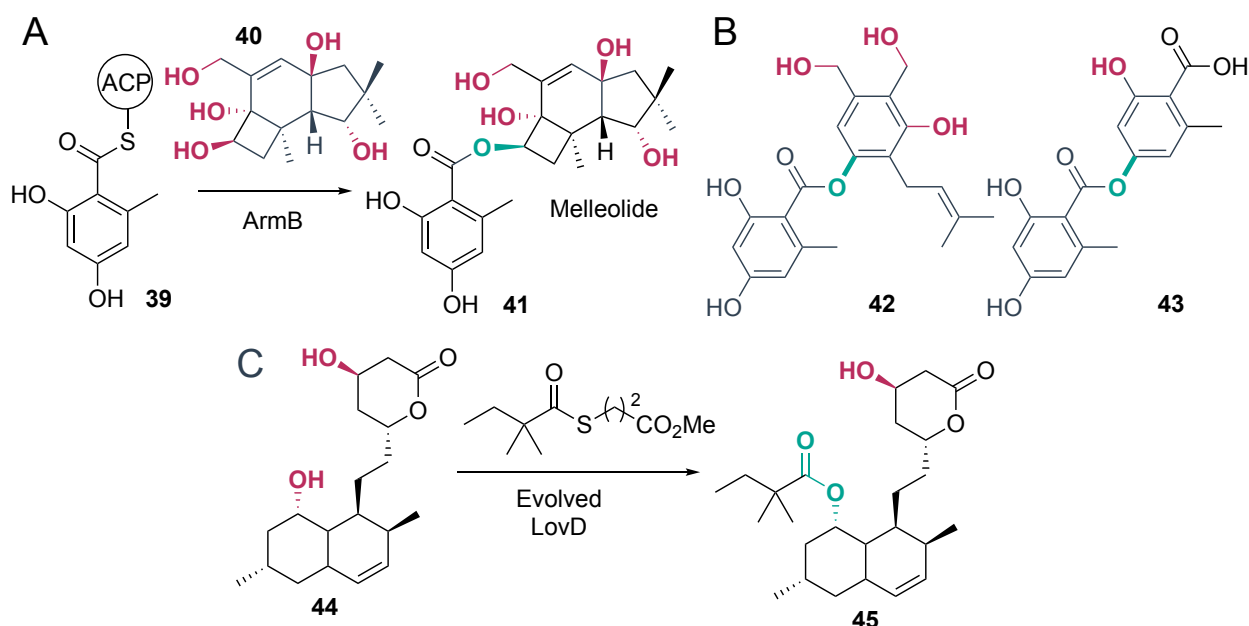
Scheme 9. A) ArmB-catalyzed site-selective acylation to generate melleolide.[57] B) Representative orsellinic acid esters found in fungi.[57] C) Evolved acyltransferase LovD catalyze the acylation of monacolin J acid (MJA) to generate lovastatin.[59]

2.2.2. Alcohol Alkylation

Selective alkylation of alcohols is critical to the function of natural products[60] and pharmaceuticals.[61] While a recent report describes a site-selective PLP dependent *O*-alkylase capable of site-selective alkylation of a catechol moiety,[62] this important transformation is typically catalyzed by *S*-adenosylmethionine (SAM)-dependent methyltransferases (MTases), which act by transferring a methyl group from the SAM cofactor to oxygen, carbon, sulfur, or nitrogen groups (Scheme 10A).[63, 64] *O*-methyl transferases (OMTs) are particularly prevalent in natural product biosynthesis, as exemplified by the methylation of naringenin to the 7-*O*-methylated natural product sakuranetin **47** or of **48** to give reticuline (Scheme 10B/C).[65] The widespread presence of OMTs in biosynthetic pathways has facilitated the development of these enzymes for site-selective alkylation of hydroxyl groups on a variety of non-native substrates.
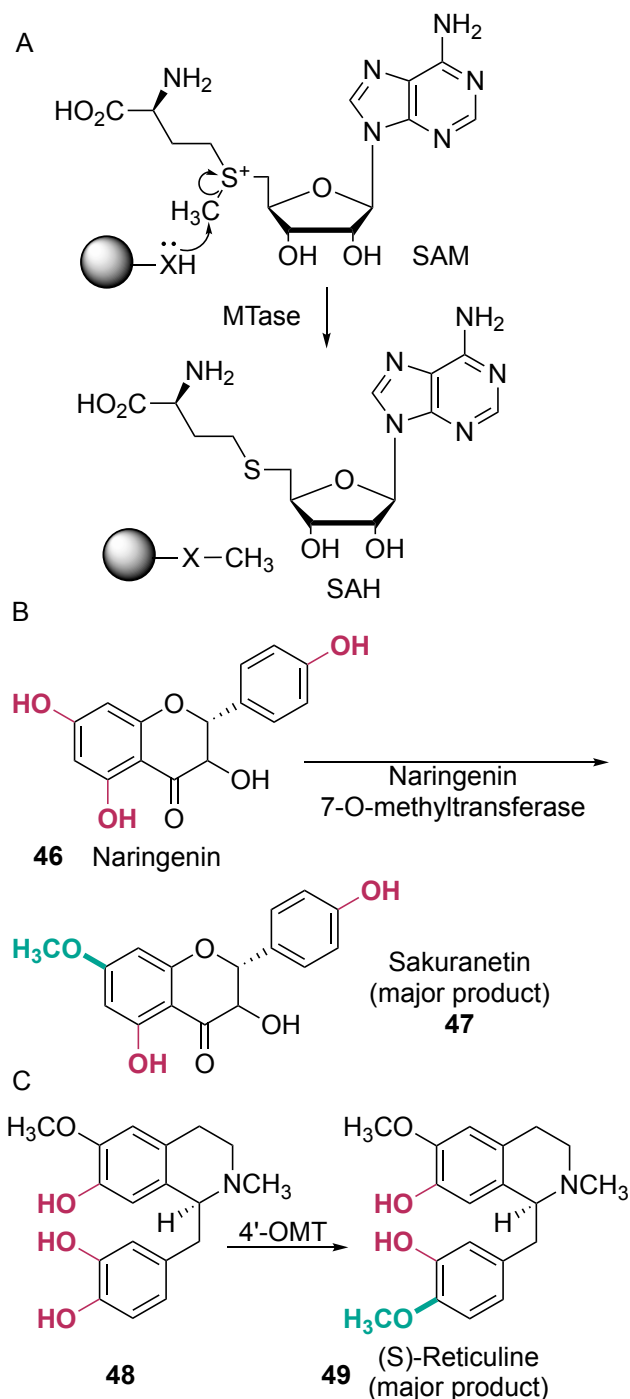
Scheme 10. A) Mechanism for methylation by the SAM cofactor. B) For some methyl transferases, alkyl transfer follows an S_N2 type mechanism and can transfer the alkyl group to O-, N-, C-, S-, Se- and As-centred nucleophiles.[66] C) Representative OMTs involved in sakuranetin and reticuline[67] biosynthesis.

In one representative example of this capability, a catechol *O*-methyl transferase (COMT) and variants of an isoeugenol *O*-methyltransferase (IOMT) from *C. breweri* were used to selectively methylate either the *para*- or *meta*-hydroxyl group of a series of substituted catechols (Scheme 11

A/B).[68] Although these enzymes share ~83% amino acid sequence identity, a triple mutant of IOMT (T133M/A134N/T135Q) obtained through site-directed mutagenesis could alter the site selectivity to provide *para* methylation of caffeic acid. A different COMT from *C. roseus* (CaRo_MT) containing the same three mutations at the homologues residues provided the *meta* products (Scheme 11).[68]
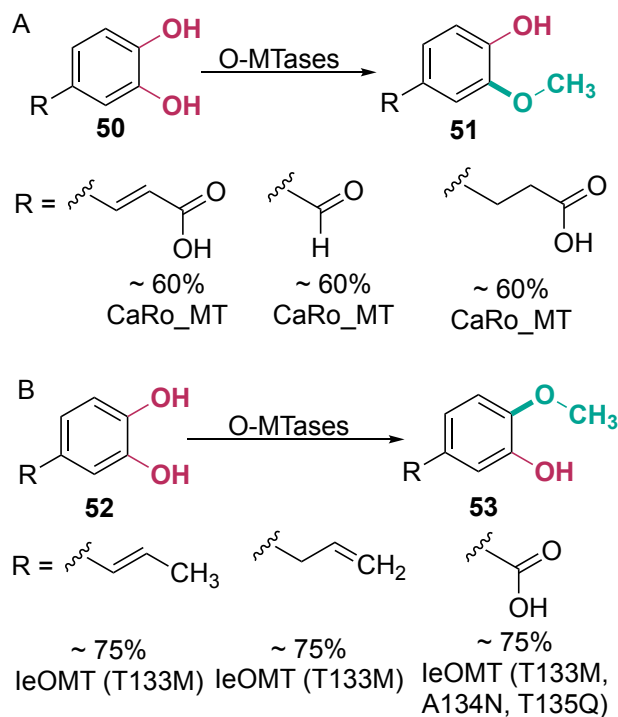
Scheme 11. A) Methylation of phenols catalyzed by OMTs to give *meta* (**51**) and *para* (**53**) products.[68]

OMTs can also catalyze selective methylation of the 2,4-dihydroxybenzophenone core in polyketide derived natural products.[69] [70] A panel of these substrates was screened against wild-type and evolved variants of methyl transferases LtOMT and HsOMT, which were obtained from *Lasiodiplodia theobromae* and *Hypomyces subiculosus*. These enzymes methylated either the *ortho*- or *para*-hydroxyl group (relative to the ketone moiety), respectively, with high site selectivity. These site preferences were maintained despite different substitution patterns on the benzophenone core. These same OMTs have also been used as models to understand and modify the site selectivity of the alkyl transfer reaction through protein engineering.[71] Docking desmethyl lasiodiplodin into the active site of LtOMT and HsOMT suggested four residues that might affect substrate positioning relative to the SAM cofactor. Residues in LtOMT were mutated to the corresponding residues in HsOMT (Q384K, G386R, W387H, and Q388H, numbering taken from HsOMT), and the resulting OMT (M1) was capable of producing the *para* product as a minor byproduct in the reaction Scheme 12A. A variant of M1 (HM1) constructed by installing segment 330−366 of HsOMT into M1 afforded exclusively the *para*-methylated product, indicating that this short region is critical for the site selectivity of the enzymes.[71]
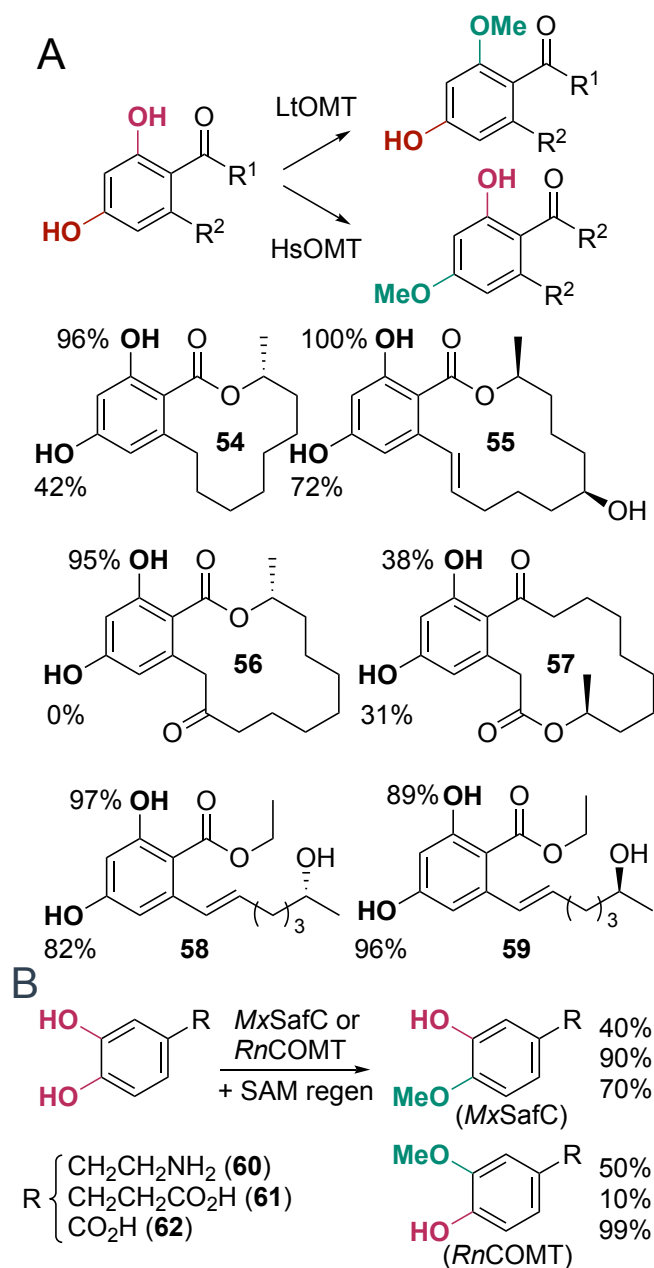
Scheme 12. A) Methylation of novel benzenediol lactones catalyzed by HsOMT and LtOMT variants.[71] B) Selective methylation of **60-62** by *Rn*COMT and *Mx*SafC using a SAM regeneration system.[72]

As noted above, the high cost of SAM necessitates regeneration of this cofactor for efficient biocatalysis in alkyl transfer reactions.[73-75] In one approach to this goal, an archaeal methionine adenosyltransferase (*Tk*MAT) was used to catalyze the synthesis of SAM from L-methionine and ATP.[76] This system was evaluated using OMTs with complementary site selectivity. Reaction of 3,4-dihydroxyphenethylamine (**60**), dihydrocaffeic acid (**61**), or 3,4-dihydroxybenzoic acid (**62**), catalyzed by *Rn*COMT from *R. norvegicus*[77] or *Mx*SafC from *M. xanthus*[78] afforded complementary methylated products using substoichiometric SAM (Scheme 12B).[72]

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Recent studies also show that MTases can accept synthetic SAM analogues, such as *S*-alkyl, -allyl, or -propargyl substituents, facilitating alkylation reactions beyond methylation.[72, 79-81] For example, *Rn*COMT accepts carboxy-*S*-adenosyl-l-methionine (cxSAM) to enable carboxymethylation of 3,4-dihydroxybenzaldehyde with roughly 4:3 *m-/p*-selectivity.[82, 83] The Y200L variant of this enzyme displayed improved *m-/p*-selectivity (**64a'** 64% and **65a'** 3%), and similar selectivity trends were observed for 4-nitrocatechol. In a more recent study, an engineered halide methyltransferase (HMT) was evolved to accept ethyliodide as the alkyl donor to enable ethylation reactions.[74] A variant with improved kinetics for ethylation of *S*-adenosyl-L-homocysteine (SAH) was coupled with *O*-methyltransferases (IeOMT and COMT) to achieve site-selective ethylation of luteolin and allylation of 3,4-dihydroxybenzaldehyde (Scheme 13B).[74] The ability of SAM cofactor regeneration systems to turn over SAH over 1000 times, and the more recent advent of thiomethyltransferases for catalytic SAM production using sulfonate donors bode well for the advances in biocatalytic alkylations.[84]
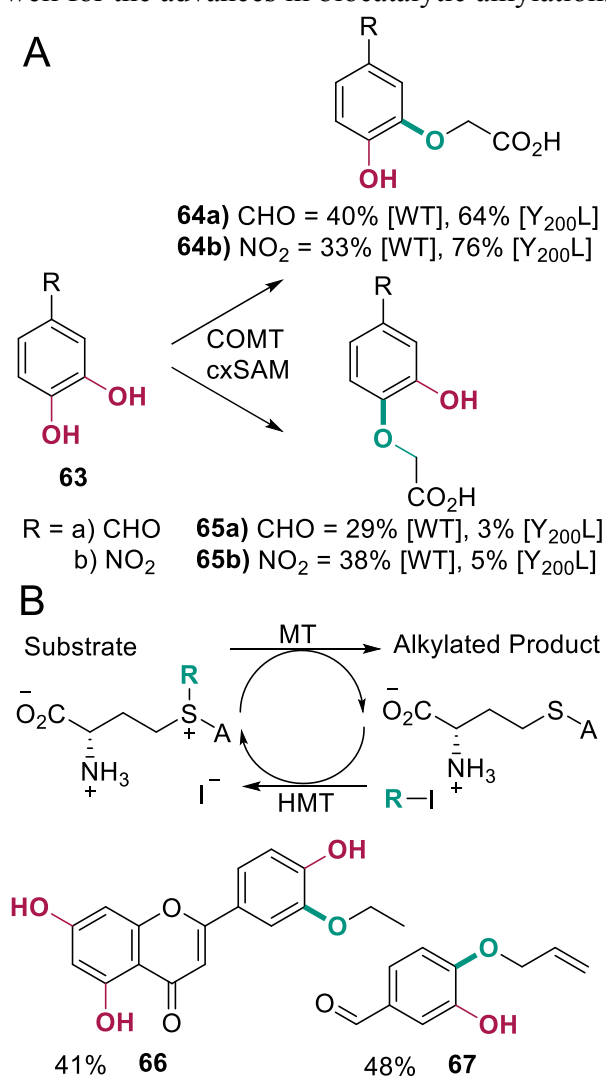
Scheme 13. A) Site-selective carboxymethylation of **63** using carboxy-*S*-adenosyl-L-methionine (cxSAM) catalyzed by COMT. B) Regeneration of alkyl-*S*-adenoxyl-L-methionines using HMT and representative ethyl and allyl products generated by IOMT and COMT variants, respectively.

2.2.2. Amine Alkylation

Selective alkylation of amines is a highly desirable yet challenging reaction.[85] SAM-dependent enzymes also catalyze amine alkylation, but most reported substrates possess only a single amine substituent, so site selectivity is not an issue.[86] Pyrazoles can be alkylated at either N-1 or N-2 as a result of tautomerization between these sites, so selective alkylation of these compounds is particularly challenging.[87] Screening several promiscuous *N*-methyl transferases (NMTs) revealed that human nicotinamide NMT catalyzed methylation of 3-methylpyrazole in 14% yield and roughly 2:1 N-1/N-2 site selectivity (Scheme 14A).[86]. Computational library design was used to generate a diverse panel of NMT active site variants, and several of these variants were found to possess improved activity and complementary site selectivity toward several substituted pyrazoles (Scheme 14B). The engineered enzymes from this study were subsequently found to catalyze selective alkylation of a variety of other *N*-heterocycles (Scheme 14C).[88]
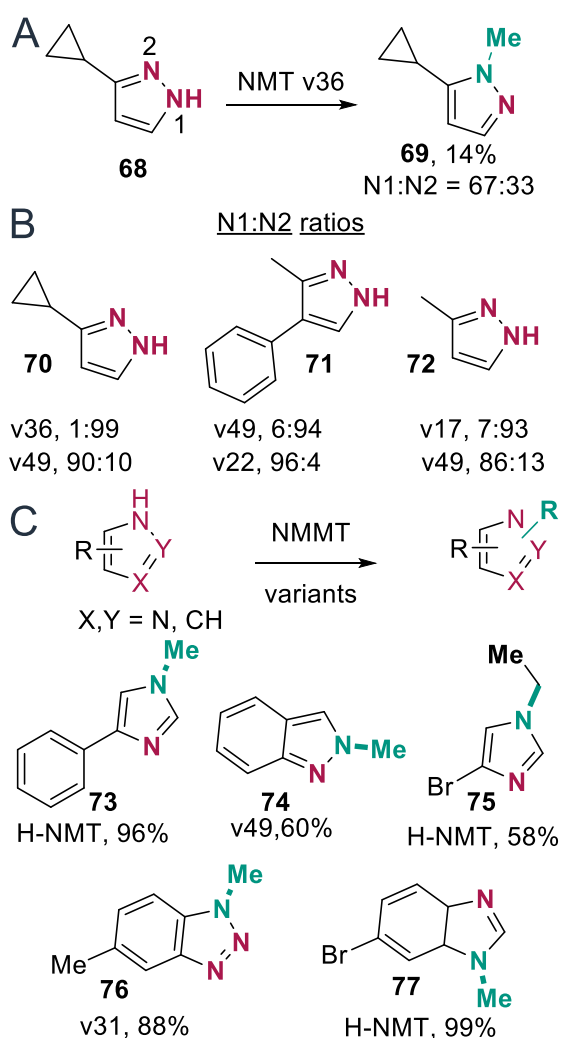
Scheme 14. A) Site-selective alkylation catalyzed by NMT variant v36 and haloalkanes. B) Site-selective methylation by variants obtained through *in silico* mutational studies.[86] C) Representative examples of site-selective alkylation catalyzed by variants of the NMTs.[88]

2.2.3. Hydroxyl Group Phosphorylation

Phosphorylated molecules play critical roles in metabolism, signaling, and a wide array of other biological processes.[89-91] Classes of phosphorylating enzymes include phosphotransferases, phosphorylases, phosphohydrolases, and phosphomutases.[92] Purine nucleoside phosphorylases (PNPs) catalyze the reaction between a purine nucleoside and phosphate to generate the free nucleobase and ribose-1-phosphate.[93] Recently, a panel of PNPs was examined to identify an enzyme capable of converting **78** to **79** via transfer of a phosphate group from a primary hydroxyl to a more sterically encumbered secondary hydroxyl.[94] A PNP from *E. coli* provided the highest activity on **78**, and four rounds of directed evolution were used to improve the activity of this enzyme.[95] This study also described an enzyme that catalyzes selective phosphorylation of the primary alcohol over the tertiary alcohol in substrate **80**. Screening a range of kinases that naturally act on sugars and primary alcohols revealed that pantothenate kinase (PanK) from *E. coli* could accomplish this task, albeit with low activity. Directed evolution was used to improve this activity 100-fold to enable the desired transformation on the (*R*)-enantiomer of aldehyde **81**. Directed evolution was also used to engineer a variant of 5-S-methylthioribose kinase from *Klebsiella sp.* that could catalyze selective phosphorylation of the C-1 hydroxyl on **82** over the two other secondary alcohols on this substrate to give **83**.[94, 96]
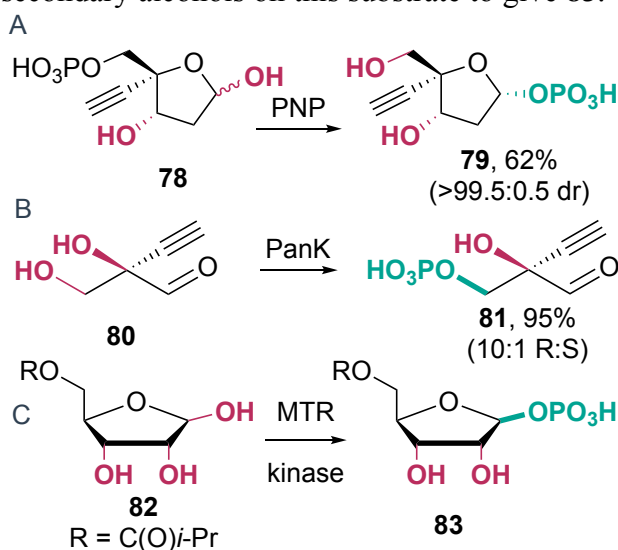
Scheme 15. Evolved variants of A) *E. coli* purine nucleoside phosphorylase (PNP)[94] B) *E. coli* pantothenate kinase (PanK),[94] and C) *Klebsiella sp.* 5-S-methylthioribose kinase catalyze site-selective phosphorylation of different non-native substrates.[96]

2.2.4. Hydroxyl Group Glycosylation

Glycosylation of small molecules can also dramatically alter their biological function.[97] Many natural glycosyl acceptors, including polyphenols, catechols, and sugars, often bear multiple similarly reactive hydroxyl groups, so site-selective glycosylation is required to ensure that only the biologically relevant isomer is formed.[98, 99] Glycosyl transferases (GTs) must also control the stereoselectivity at the anomeric carbon of the glycosyl donor. While this selectivity is typically accompanied by relatively high substrate specificity, researchers have found that some GTs and engineered variants can accept a range of glycosyl donors and acceptors to enable site-selective,

non-native glycosylation.[100-102] For example, while UGT71A15 was found to catalyze non-selective glycosylation of some polyphenolic acceptors, it catalyzes site-selective addition of glucose to **84** (Scheme 16A).[103] In terms of donor variation, the antibiotic-modifying GT GtfE was shown to install a variety of monosaccharides onto vancomycin aglycone.[104] The GTs OleD, OleI, and MGT accepted a variety of substrates from a panel of 64 acceptors and 18 sugar donors. Selective glycosylation of flavanols, coumarins, and 3,4-dichloroaniline was possible using these enzymes (Scheme 16B/C).[105]
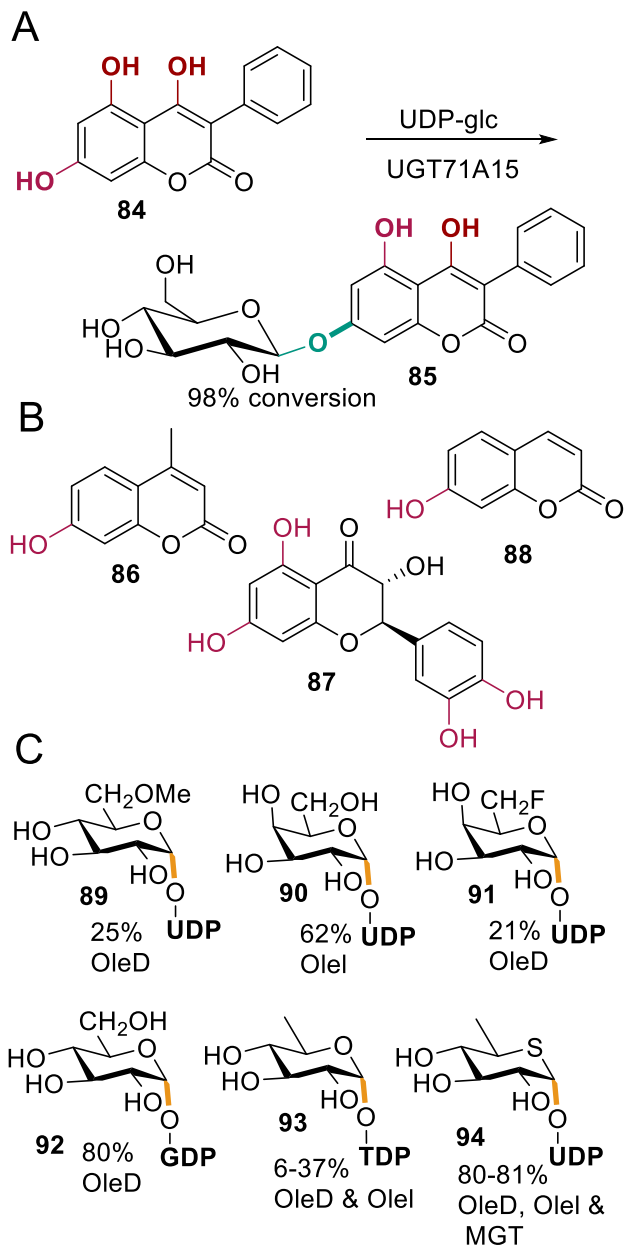
Scheme 16. A) Glycosylation of **84** by glycosyl transferase UGT71A15.[103] Representative scope of B) glycosyl acceptors and C) glycosyl donors for glycosylation of oleandomycin with OleD, OleI and MGT. UDP = uridine diphosphate, GDP = guanosine diphosphate, and TDP = thymidine diphosphate glucose.[105]

Different protein engineering strategies have been used to expand the substrate scope and utility of GTs for site-selective biocatalysis. For example, directed evolution was used to expand the acceptor scope of CgtB, a β-1,3-galactosyltransferase.[106] Fluorescence-activated cell sorting was used to screen large libraries of CgtB variants (from ~10^7 colonies) generated via error-prone PCR. This assay involved the use of fluorescently labeled glycosyl acceptors that are trapped within cells following GT-catalyzed reaction with a suitable donor. Improved sequences were subjected to DNA shuffling, and ~10^6 colonies were screened. This process provided variant S42, which contained five amino acid mutations, provided significant improvements in activity toward different glycosyl acceptors and even UDP-Glu as a glycosyl donor, and maintained high site selectivity in all cases reported.

Substrate engineering has also been used to expand the scope of site-selective glycosylation using GTs.[107] For example, LgtC from *Neisseria meningitidis* is an α-1,4-galactosyltransferase that catalyzes the transfer of galactose from UDP Gal to lactose-containing acceptors.[108] Different anomeric protecting groups were shown to enable glycosyl transfer involving several monosaccharides in place of the native disaccharide (lactose) substrate.[107] Varying the protecting group could also be used to improve yields on a given monosaccharide while still maintaining high site selectivity.

A

95 + 96

↓ CgtB and variants

97

B

98

↓ CgtB-S42
UDP-galactopyranose

99
Specific activity 300-fold
higher than parent

C

100
101
102
103

1) Lgtc
UDP Gal
───────→
2) Ac₂O, Py

αAc₄Gal
104

αAc₄Gal
105

αAc₄Gal
106

αAc₄Gal
107
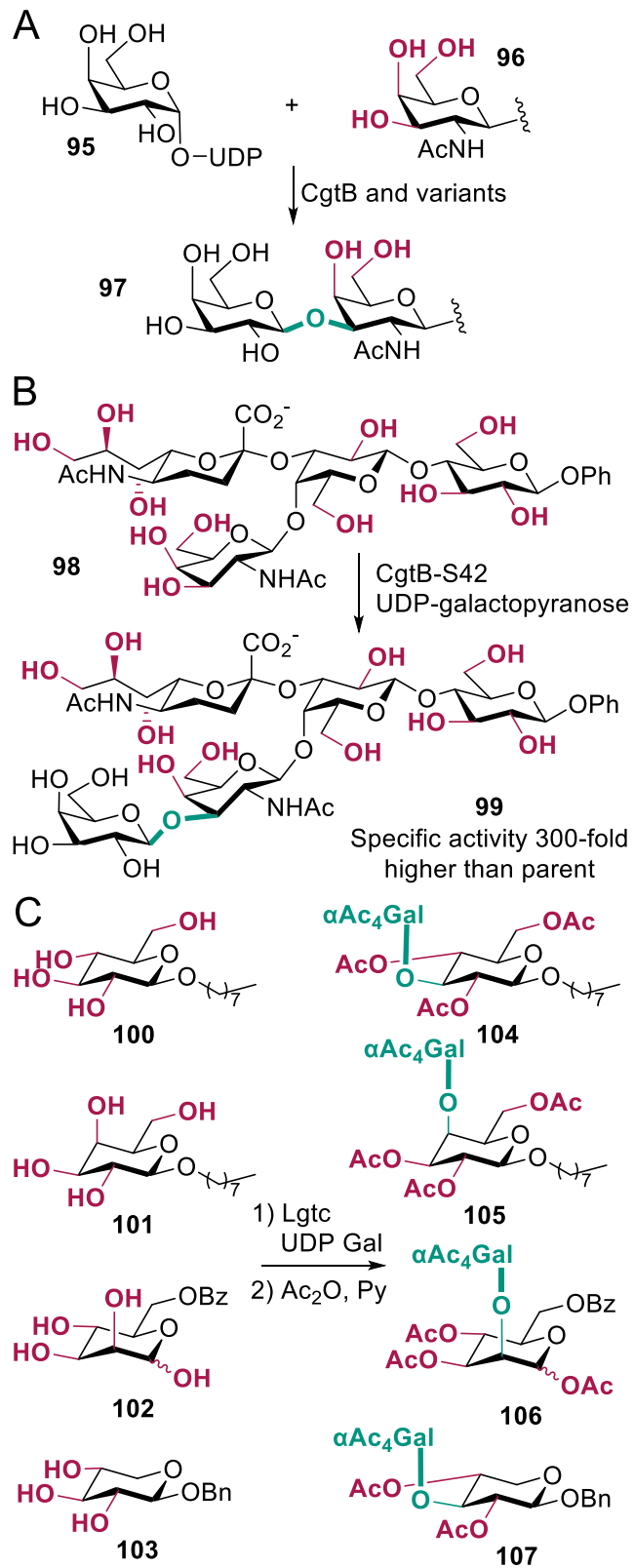
Scheme 17. Site-selective glycosylation by A) β-1,3-galactosyltransferase CgtB and B) CgtB variant S42. C) Substrate scope for glycosylation of non-natural sugar acceptors and uridine 5'-diphosphogalactose (UDP Gal) donors catalyzed by LgtC.

Finally, site-selective *C*-glycosylation has been achieved using GT catalysis. For example, the CGT responsible for the biosynthesis of flavone-*C*-glycosides found in rice and wheat was shown to accept a range of non-natural substrates to generate the corresponding *C*-glycosylated products (Scheme 18A).[109] Likewise, TcCGT1 catalyzes selective 8-*C*-glycosylation of several flavones and flavonoids.[110] This enzyme could also be engineered through site directed mutagenesis (I94E and G284K) to switch the chemoselectivity from *C*- to *O*-glycosylation, while maintaining high site selectivity for the latter reaction (Scheme 18B).
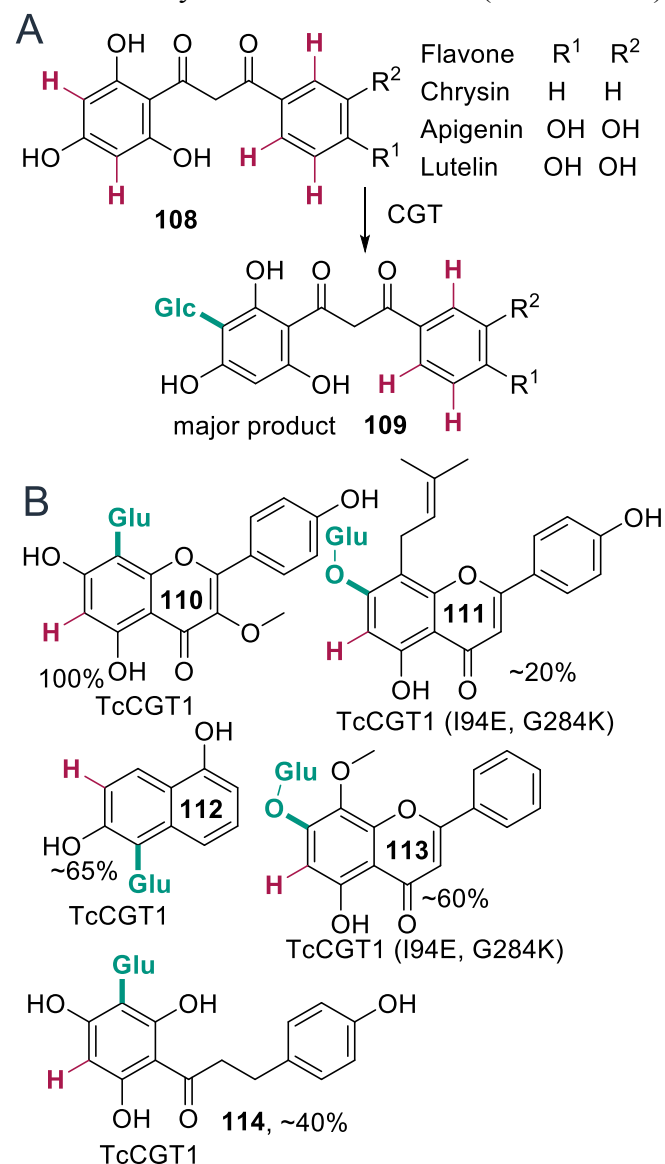
Scheme 18. A) 2-hydroxyflavanones exists in equilibrium with its open chain form compound (108). These open chain intermediates get glycosylated by the C-glucosyltransferase. B) Site-selective catalytic promiscuity of TcCGT1. Point mutation I94E and G284K switched C- to O-glycosylation.[110]

Glycosidases, which natively catalyze the cleavage of glycosylated substrates,[102] have also been engineered to catalyze site-selective transglycosylation, but these reactions are thermodynamically disfavored, and the reaction products are susceptible to the native reverse reaction.[111] Withers found that mutating the active site Glu/Asp of several different glycosidases to alanine ablated the undesired hydrolysis reaction to enable site-selective glycosylation of substrates using glycosyl fluoride acceptors.[111, 112] Representative glycosidases on which this approach has proven successful are *T. thermophilus* β-glycosidase,[113] *Thermotoga maritima* β-glucuronidase,[114] and β-endoglycosidase from *Rhodococcus* sp.[115] One example of site-selective catalysis by such a system involved the E197S mutant of *Humicola insolens* Cel7B.[116] This enzyme was found to catalyze site-selective glycosylation of different flavonoids using the glycosyl acceptor LacF (Scheme 19B).
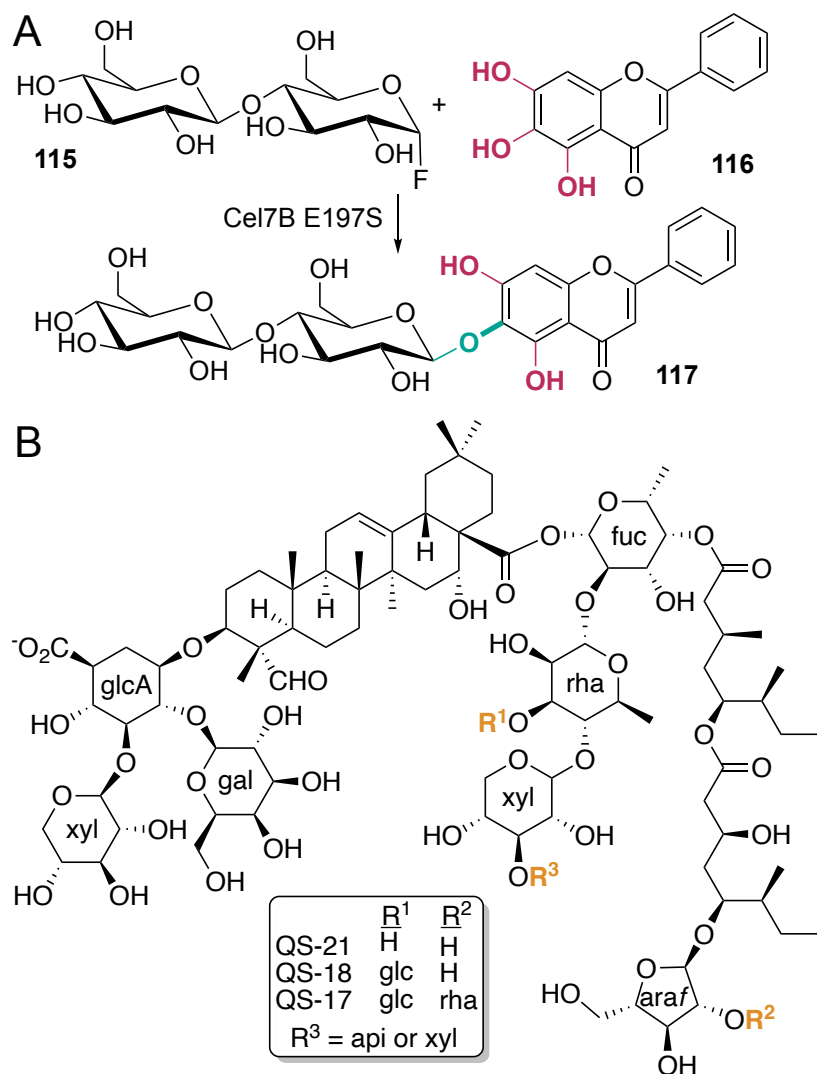
Scheme 19. Site-selective glycosidase catalysis. A) Synthesis of glycoflavonoids catalyzed by Cel7B–E197S with disaccharide donor lactosyl fluoride (LacF).[116] B) Saponin QS-21 can be enriched from a mixture of QS-17, -18, and -21 via site-selective removal of glc and rha residues.

Very recently, researchers at GSK used a combination of genome mining and directed evolution to engineer glycosidases for site-selective cleavage of sugars from a complex mixture of saponins obtained from bark extracts of the evergreen tree *Quillaja Saponaria* Molina (Scheme 19B).[117] Saponins QS-17, QS-18, and QS-21 from *Quillaja* extracts are used as adjuvants, but they must be purified by tedious reversed phase HPLC; removal of Q-18 is particularly important since it possesses high toxicity in mice. QS-21 can be obtained from QS-18 via loss of a specific β-D-glucose, and QS-18 can be obtained from QS-17 via loss of a specific rhamnose. Researchers envisioned that they could improve the yield of QS-21, eliminate toxic QS-18, and simplify the saponin mixture to facilitate isolation of QS-21 by identifying enzymes that could achieve these transformations. An extensive genome mining effort led to the identification of a glucosidase and a rhamnosidase with activity on Q-18 and Q-17, respectively. Concurrent directed evolution of these enzymes led to site-selective variants with improved activity that achieved the desired enrichment of QS-21 from *Quillaja* extracts when the engineered enzymes were used together.

2.2.5. Hydroxyl Group Epimerization

In addition to enabling site-selective functionalization of hydroxyl groups via phosphorylation and glycosylation, enzymes can also epimerize hydroxyl groups. This is an especially desirable application in carbohydrate chemistry in that it enables converting readily available sugars to rare ones.[118, 119] Rare sugars can serve as low calorie sweeteners to replace more common sugars, influence cell wall biosynthesis,[120] and attenuate or increase immune response.[121] While chemical methods for site-selective epimerization have been reported,[122, 123] enzymes from several different classes of epimerases often offer superior selectivity.[124] These enzymes possess different site-selectivity, but only those that have been applied to non-natural catalysis are highlighted below.

Uracil diphosphate (UDP) hexose-4-epimerases are NAD dependent enzymes that epimerize specific hydroxyl groups on substrates, as dictated by the orientation of the substrate relative to the oxidized cofactor.[125, 126] Hydride transfer from the carbohydrate results in ketone formation, and rotation of the sugar within the active site of the epimerase repositions the ketone such that hydride transfer from the reduced cofactor produces the C4 epimer of the original compound. A few examples of evolving UDP hexose epimerases with improved activity or expanded scope have been reported.[118, 119] The requirement for a UDP group on the substrates of these enzymes limits their synthetic utility, but GalE was found to catalyze site-selective 4-epimerization of free sugars.[127] Site-saturation mutagenesis of residues predicted to interact with nucleoside portion of UDP-galactose in this enzyme showed that GalE N179S displayed improved activity for epimerization of the 4-position of free fructose and tagatose (i.e. lacking a UDP group). Further improvement of this activity could significantly improve the synthetic utility of these enzymes.

D-tagatose epimerases have been extensively explored for non-native catalysis.[128-131] These enzymes enable C-3 epimerization of D/L-tagatose, -fructose, -psicose, and –sorbose. Both C-3 and C-4 epimerization involves both Lewis acid catalysis by an M^{2+} ion and general acid/base catalysis involving a glutamic or aspartic acid residue. In the case of C-3 epimerization,[132] these species catalyze formation of a *cis*-enediolate, and subsequent protonation from the opposite face of the enolate by a second aspartic acid residue leads to epimizeration. C-4 epimerization[133] proceeds via a retro-aldol reaction of the keto-sugar substrate involving a M^{2+} ion and a glutamic acid residue in the epimerase active site. Rotation of the resulting C-4 aldehyde presents the

opposite face of the carbonyl to the intermediate M^{2+}-bound enolate so that the aldol reaction gives the opposite stereoisomer at C-4.

By exploiting a thermostabilized variant of the D-tagatose 3-epimerase from *P. cichorii,*[134] divergent evolutions were carried out for epimerization of L-sorbose and D-fructose to L-tagatose and D-psicose respectively.[128] By coupling production of the product sugar to a NADH dependent dehydrogenase, a high-throughput assay for detection of cofactor oxidation was developed that ultimately allowed for efficient production L-tagatose in 6 rounds of evolution and D-psicose in 8.[134] (Scheme 20A). Crystal structure analysis of the two final variants showed minor impacts on the overall structure and indicated that the mutations seemed to influence charge at the surface of the protein where the substrate entered the active site.

While use of D-tagatose-3-epimerases for production of non-native sugars is common, methods to produce D-tagatose from commodity sugars are underdeveloped. D-tagatose is a low-calorie alternative to more abundant natural sugars but is rarely present in nature, making scalable isolation a challenge, and natural enzymes capable of epimerizing D-fructose to D-tagatose have proven elusive.[133] An enzyme that catalyzes C-3 epimerization of a related substrate,[135] was engineered to achieve this goal using several rounds of structure-buided site-directed mutagenesis (Scheme 20B). The final variant 5V was capable of capable of producing D-tagatose at 200 g/L titers and had reduced activity on the native D-tagaturonate substrate compared to the wild-type variant, indicating a significant modification of substrate scope.
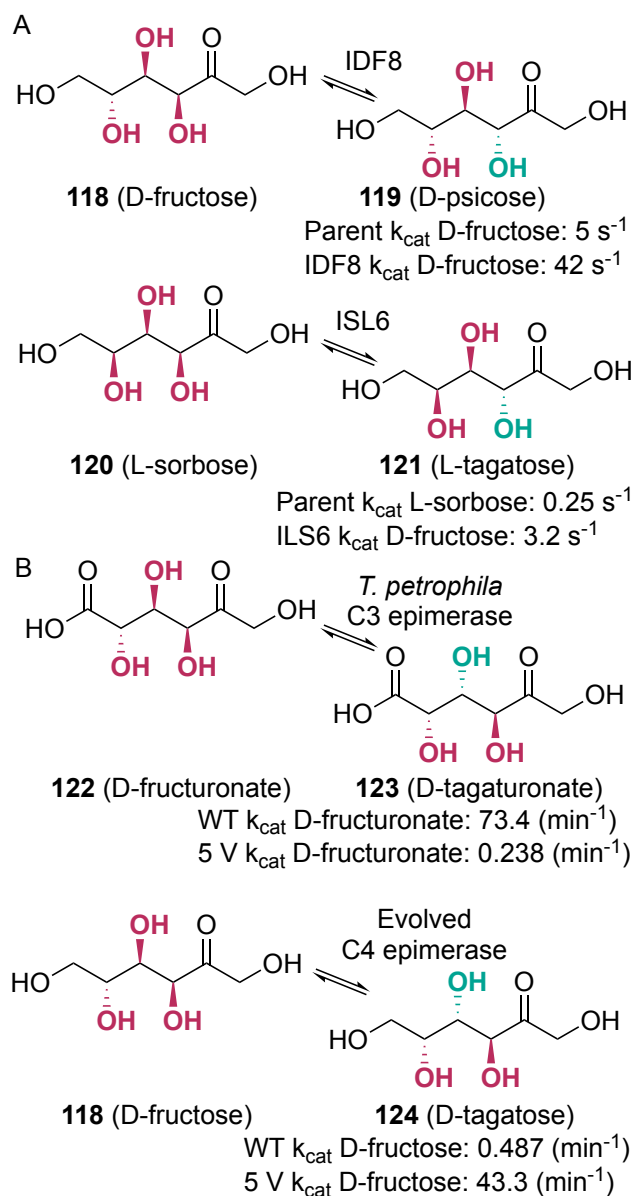
Scheme 20. Epimerization of abundant carbohydrates to furnish rare sugars. A.) Divergent evolution of a thermostable D-fructose epimerase results in efficient variants for D-psicose and L-tagatose production. B.) A natural C3 epimerase for D-frucuronate is converted into a C4 D-fructose epimerase capable of 200 g/L production of D-tagatose.

2.2.6. Alcohol Oxidation

Alcohol oxidation is common in biosynthetic pathways, and several classes of enzymes, including alcohol oxidases have been used to catalyze oxidation of primary alcohols over secondary alcohols.[136] Other enzymes, including NAD(P)(H)-dependent hydroxysteroid dehydrogenases (HSDHs), are capable of discriminating between secondary alcohols in steroids and bile acids.[137, 138] HSDHs from *Bacteroides fragilis* and *Clostridium* absonum DSM 599, for example, were used to develop a chemoenzymatic method for converting cholic acid to ursodeoxycholic acid, which

is used to treat gallstones.[139] A one-pot strategy for this transformation used 7α- and 12α-HSDHs to oxidize the hydroxyl groups at positions C-7 and C-12 of cholic acid; a 7β-HSDH was then used to reduce the ketone at position C-7 to a β-hydroxyl group (Scheme 21). A more recent study focused on the epimerization of chenodeoxycholic acid to produce ursodeoxycholic acid in another one-pot experiment where a NAD(H)-dependent 7α-HSDH and a NADP(H)-dependent 7β-HSDH were employed.[140] In this case, the oxidative step was coupled to a flavin reductase to oxidize NADH to NAD⁺, and the reaction was driven towards the production of ursodeoxycholic acid thanks to the auto-oxidation of FMN under aerobic conditions.
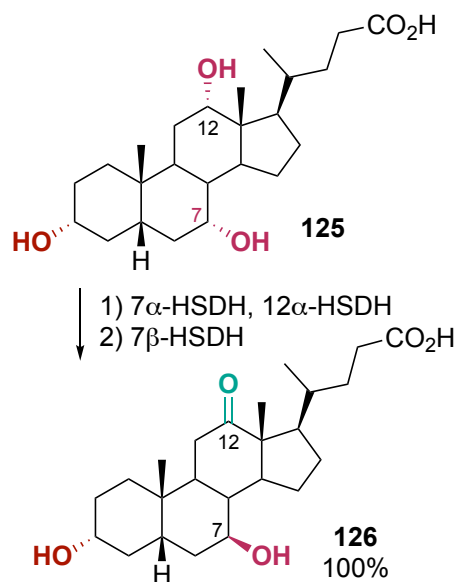
Scheme 21. One-pot synthesis of 12-ketoursodeoxycholic acid from cholic acid using HSDHs.[139]

2.3. Carbonyl Transformations

2.3.1. Carbonyl Reduction

In nature, enzymes from the aldo-keto reductase (AKR) superfamily catalyze the interconversion of wide range of carbonyl compounds and the corresponding alcohols.[141] Representative AKRs include aldose reductases, aldehyde reductases, hydroxysteroid dehydrogenases, ketoreductases (KREDs), and alcohol dehydrogenases (ADHs).[142] KREDs and ADHs have been widely used and engineered to synthesize chiral alcohols.[143] One example of site-selective KRED biocatalysis involves reduction of tri-ketone **127** to the corresponding dihydro iso-alpha acid **128** (Scheme 22A). Directed evolution was used to engineer a site-selective KRED for this transformation.[144] Compound **129** presents an interesting challenge for site-selective ketone reduction since reduction of the trifluoromethyl ketone would be expected based on electronic considerations (Scheme 22B). Indeed, chemoselective reduction of **129** to give racemic **131** has been reported.[145] Several commercially available KREDs (KRED-112, KRED-129, KRED-131) were found to catalyze this same reaction with high enantioselectivity (>99% ee). Notably, an alcohol dehydrogenase from *Candida parapsilosis* (ADH-CP) and KRED-A1p demonstrated enantioselectivity (98%) and chemoselectivity (100% and 87%, respectively) toward the reduction of the methyl carbonyl producing the methyl hydroxyketone **130**.[146]
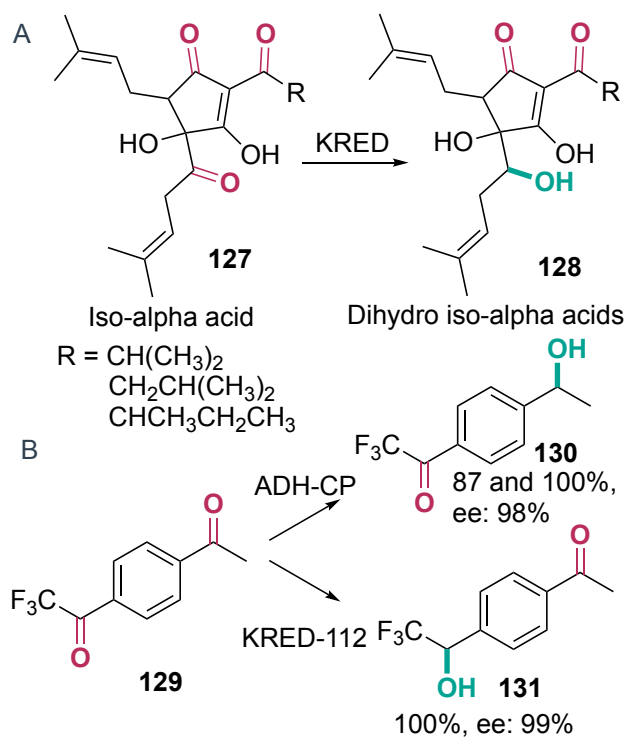
Scheme 22. Site-selective reduction of A) sterically and electronically similar ketones in **127** and B) either ketone in **129**, overcoming electronic bias, using engineered KREDs.

The commercial availability of KREDs and ADHs has facilitated several additional examples of site-selective ketone reduction. Screening 20 commercially available KREDs for the reduction of α-alkyl-1,3-diketones, for example, led to the site-selective reduction 3-methyl-2,4 hexadione with 100% yield after 24 hours and >99% diastereomeric ratio (Scheme 23A).[147] Similarly, more than 400 KREDs, both proprietary and commercial, were screened for the reduction of 5-androstene-3,17-dione (5-AD) to dehydroepiandrosterone (DHEA).[148] This study identified a KRED from *Sphingomonas wittichii* capable of fully converting 5-AD to DHEA with excellent site- and diastereoselectivity (>99% d.e., Scheme 23B). Additionally, a more recent study evaluated more than 500 commercial KREDs for the reduction of diketone **136** as part of the development of a chemoenzymatic strategy for the synthesis of navoximod.[149] KRED ADH 430 selectively reduced the cyclohexanone over the acyclic ketone in this substrate with a 95% yield and >99% selectivity for the *trans* isomer **137** Scheme 23C). Finally, ADHs from different origins showed high site selectivity towards the reduction of *tert*-butyl 4-methyl-3,5-dioxohexanoate to *tert*-butyl 5-hydroxy-4-methyl-3-oxohexanoates.[150, 151] ADHs from *Lactobacillus brevis* (LBADH) and *Saccharomyces cerevisiae* (YGL157w) both showed activity on the (*S*)-enantiomer with complementary diastereoselectivities, while ADH from *Rhodococcus* sp. (RS 1-ADH) was able to reduce the (*R*)-enantiomer (Scheme 23D), providing a platform to access most stereoisomers of this synthetically attractive building block via enzymatic dynamic kinetic resolution.
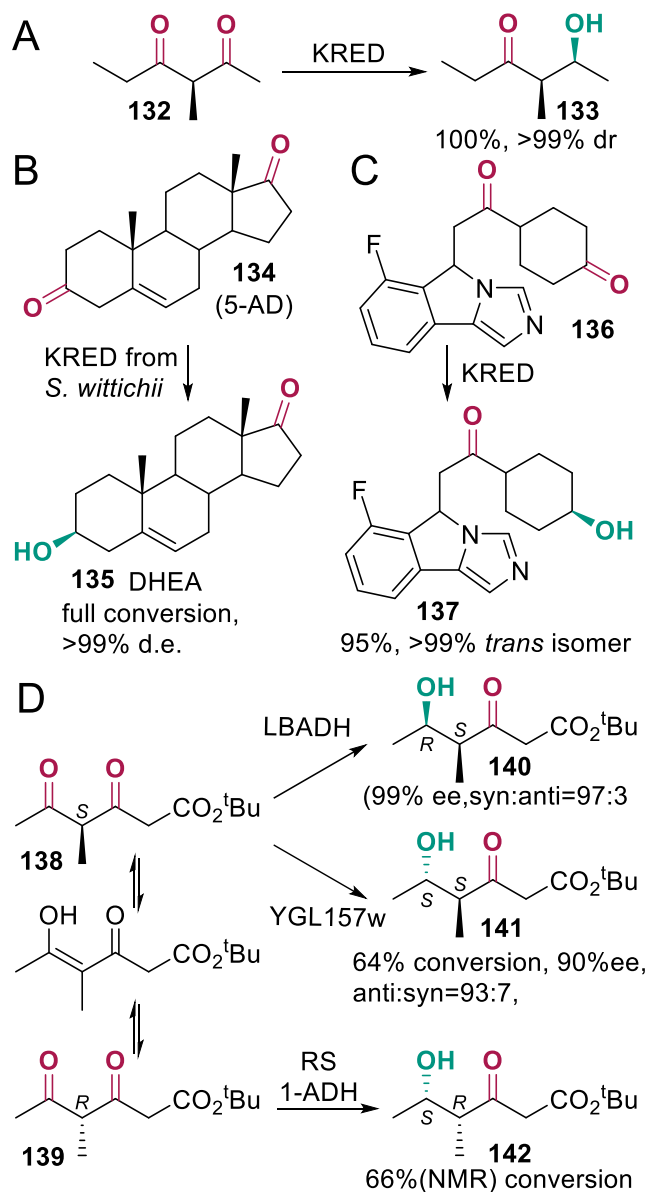
Scheme 23. Site-selective carbonyl reductions. (A) Reduction of 3-methyl-2,4 hexadione.[147] (B) Formation of dehydroepiandrosterone (DHEA) by a KRED from *Sphingomonas wittichii*.[148] (C) Site-selective reduction of a diketone as part of the chemoenzymatic synthesis of navoximod.[149] (D) Kinetic resolution of *tert*-butyl 4-methyl-3,5-dioxohexanoate by using substrate-specific alcohol dehydrogenases.[150, 151]

2.3.2. Carbonyl Reductive Amination

Biocatalytic methods for amine formation have been widely adopted in both industry and academia. This capability was first facilitated by the development of transaminases (TAs), which catalyze the formation of primary amines from ketones, as exemplified at industrial scale by the biocatalytic synthesis of sitagliptin.[152] More recently, reductive aminases (RedAMs) and imine reductases (IREDS) have expanded this capability significantly to allow for the formation and/or

enantioselective reduction of secondary and tertiary amines.[153, 154] Both TAs and IREDs are now available as commercial enzyme panels to facilitate easy screening. TAs use an amine donor and the pyridoxal phosphate (PLP) cofactor while IREDs require a reducing equivalent provided by NAD(P)H to reduce an imine to the corresponding amine.[154] These enzymes typically have restricted active sites formed upon dimerization of the enzymes in solution. This active site structure has been exploited to enable site-selective catalysis in cases where two ketones are distinguished by their relative steric environments (Scheme 24A). Even for examples where sterically similar groups, like methyl and *n*-propyl, are used, a panel of S- and R- selective TA's was nearly entirely 99:1 selective for the formation of the less-hindered amine (Scheme 24B). [155]
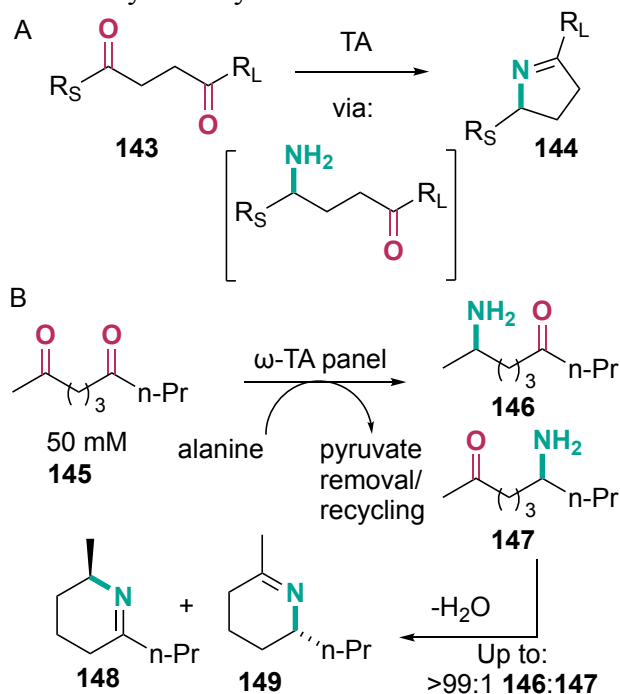
Scheme 24: ω-Transaminase catalyzed cyclization of diketones. A.) General scheme for cyclization with TAs. B.) Cyclization of nonane-2,6-dione with a panel of ω-TAs showed >90:10 selectivity for amination of the less-hindered ketone.

ω-TAs have also been used for the asymmetric synthesis of 2-methyl-5-phenylpyrrolidines via enzymatic and chemoenzymatic cascades. In the latter approach, a ω-TA was used to site-selectively aminate the 4-position of 1-phenylpentane-1,4-dione, and the resulting amine underwent spontaneous cyclization to form an imine (Scheme 25A).[156] This intermediate was non-specifically reduced to the racemic pyrrolidine by ammonia borane, and the *trans* pyrrolidine was selectively re-oxidized to the imine by a monoamine oxidase leading to enrichment of the *cis* isomer. By replacing the ammonia borane and MAO-N with an imine reductase (Scheme 25B), this cascade was rendered fully enzymatic with up to 68% yield and >98% d.r. and e.r.[157] This predictable preference for amination of less hindered ketones also extends beyond diketones, as shown in the synthesis of the bicyclic alkaloid xenovenine.[158] In this study, the first key asymmetric step was accomplished by screening a panel of wild-type (R) and (S)-selective transaminases. The best variants provided the desired products with perfect site-selectivity for the less hindered ketone and 99% enantioselectivity, enabling the most efficient synthesis of the xenovenine enatiomers at the time.
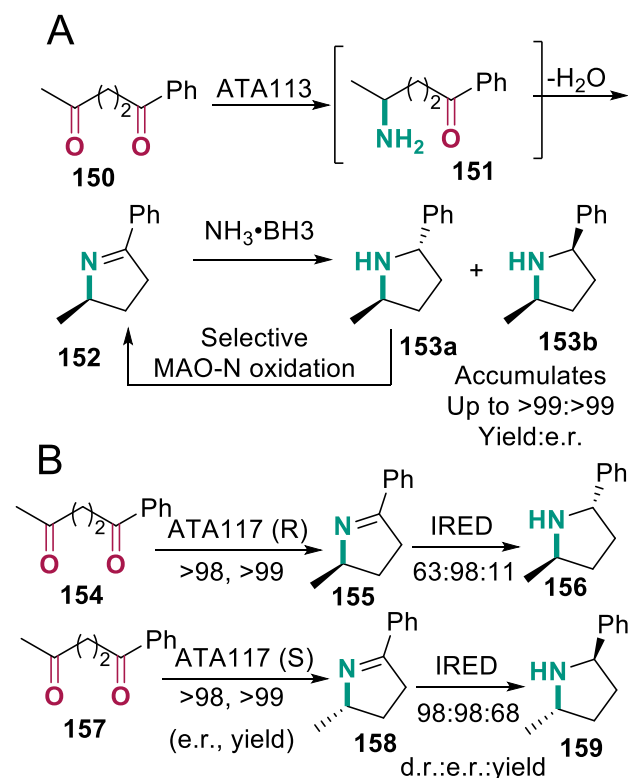
Scheme 25. Biocatalytic or chemoenzymatic cascade reactions to furnish pyrrolidines from diketones. A) By coupling ω-TA enzymes for amination of the less hindered ketone with non-selective ammonia borane reduction and trans-pyrrolidine selective MAO-N oxidation, the cis-pyrrolidine accumulates. B.) A similar reaction is accomplished using an IRED for the reduction, providing direct access three enantiomers starting from a simple diketone.

2.4. Alkene Transformations

2.4.1. Alkene Hydration

Alkenes are substrates for a wide range of catalytic asymmetric transformations like hydrogenation, hydroformylation, dihydroxylation, and epoxidation. While differentiating the prochiral faces of a given olefin is often possible in many of these reactions, site-selective reaction when multiple olefins are present remains challenging. This task is particularly difficult for olefin hydration to produce the corresponding alcohols since this reaction is traditionally carried out using catalysts like mineral acids, metal oxides, and zeolites under harsh conditions.[159] Several hydratases have been discovered that can activate unactivated alkenes to achieve stereoselective hydration.[159-161] While site-selective hydration of non-native olefin substrates remains rare, linalool dehydratase isomerase (LinD)[162] from β- protobacterium *Castellaniella defragrans* highlights the potential utility of this transformation in biocatalysis. The native substrate of this enzyme, β-myrcene, is a monoterpene that is produced by a variety of plants, and it is a key ingredient in the flavor and food industries. LinD catalyzes site-selective hydration of the 1,1-disubstituted olefin in this compound to generate (*S*)-linalool in 24% conversion after 24 h.[163] Preliminary analysis of LinD variants confirmed that importance of active site residues believed to play a role in water

31

activation, and altered product distributions in the reverse reaction of geraniol to give myrcene and linalool. These efforts suggest that LinD may be a viable platform for biocatalytic olefin hydration.

160   **161** **(S)-linalool** **24% conversion**   **162**
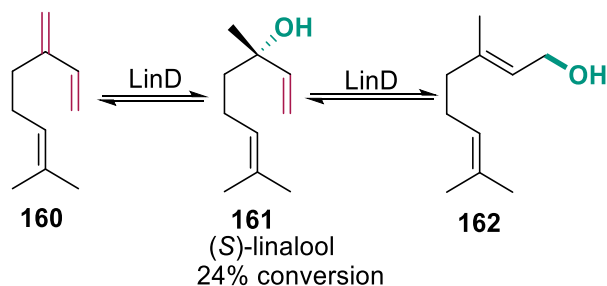
Scheme 26. Linalool dehydratase isomerase (LinD) enzyme catalyzes the reversible (de)hydration of the tertiary alcohol (S)-linalool to β-myrcene and its isomerization to the primary alcohol geraniol.[163]

2.4.2. Alkene Epoxidation

While site-selective olefin epoxidation can be achieving using directing groups or electronic bias,[164] methods to achieve this transformation on diverse substrates remain highly desirable due to its synthetic utility. Cytochromes P450 have found particular utility toward this end due to the reactivity of the intermediate compound I, a ferryl porphyrin cation radical species (*vide infra*), toward different types of olefins.[165] The high reactivity of olefins toward this species relative to C-H bonds enables chemoselective epoxidation of substrates that might otherwise undergo C-H hydroxylation by these enzymes. For example, the antitumor sesquiterpene lactone parthenolide, which contains two alkenes and multiple C-H bonds that could potentially be hydroxylated, gave a mixture of epoxidation and allylic hydroxylation using P450-BM3 variant FL#62.[166] Three active site mutations were found to provide an enzyme that is 90% selective for formation of epoxide product **164** (Scheme 27A) with only minor formation of the hydroxylated side reactions and nearly 5-fold improved TTN. A variant of BM3 also catalyzed site-selective epoxidation of the terpenoid β-cembrenediol, albeit as a side product in a reaction that produced many oxidized products.[167]

Several other P450s have also been used for site-selective epoxidation. For example, TamI oxidizes its native substrate **165** in a cascade of reactions to form the tirandamycins, including epoxide formation of a bicyclic core in the presence of several similarly reactive alkenes (Scheme 27B).[168] Active site mutations showed that the order and degree of TamI-catalyze oxidations could be modified, as exemplified by TamI L101A_L295I, which catalyzes the epoxidation as the first reaction before later showing improved hydroxylation of the adjacent C-H bond and decreased hydroxylation at other sites usually transformed during the native oxidative cascade. Theobromine is a substrate of the human P450 enzyme CYP 3A4,[169] which was selected as a catalyst to screen for epoxidation of a polyolefin appended to the N3 in the theobromine core (Scheme 27C). In the cases of substrate **168** and **172**, the epoxidation was perfectly selective for the terminal alkene, whereas for the 1,3-hex-diene modified theobromine **170** site selectivity for the epoxidation was measured at 53%, although the enantioselectivity for the major product was higher than for the corresponding 2,4-hex-diene **1**. This study not only shows an effective site and enantioselective

epoxidation reaction but also cleverly uses a known substrate for P450s to anchor the modified compounds in the active site, spurring the promiscuous activity.
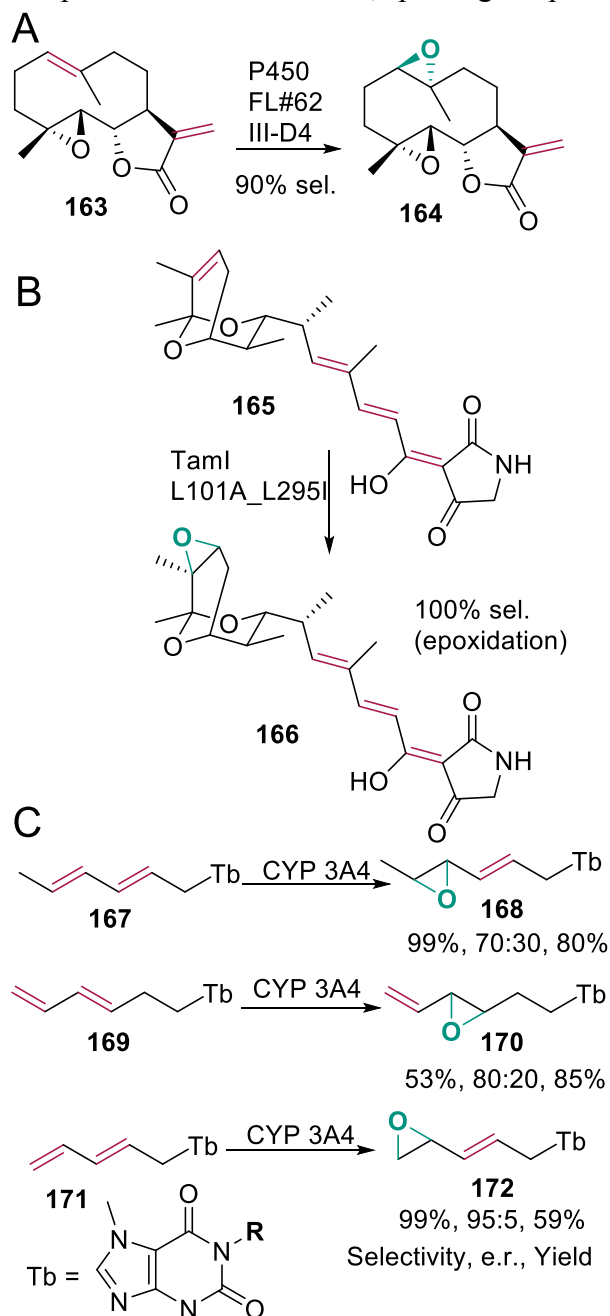
Scheme 27: Alkene epoxidation by P450 enzymes. A.) Epoxidation of parthenolide with evolved BM3 variant III-D4 leads to 90% selectivity for epoxidation of the internal alkene. B.) A TamI variant first epoxidizes the terminal alkene in the native substrate instead of hydroxylation of an adjacent C-H bond. C.) CYP 3A4 is capable of selective epoxidation of alkenes appended to theobromine.

2.5 Miscellaneous Functional Group Transformations

2.5.1. Nitrile Hydration

Hydration of nitriles to the corresponding amide bond is a valuable transformation, and nitrile hydratase (NHases) have been used to catalyze this transformation under mild conditions even for commodity chemicals like acrylamide.[170, 171] NHases are metalloenzymes that typically contain an active site Co or Fe ion, although an exact mechanism has not been fully described for this enzyme class.[172, 173] NHase reactions are also frequently performed using whole-cells to reduce the cost associated with biocatalyst preparation. The sequestration of the NHase enzyme within cells also allows for the incorporation of NHases into chemoenzymatic cascades where the free enzyme may be inhibited by other cofactors or cocatalysts in solution.[174] This was demonstrated in the synthesis of receptor agonist compound **174**,[175] which was performed in a two-step, one-pot reaction wherein nitrile bond hydration was catalyzed by the Co-dependent CGA009 NHase (Scheme 28). The enzyme was highly selective for the less hindered nitrile which allowed for the formation of the receptor agonist in 49% yield, providing a much shorter and higher yielding synthesis than the best previous reported effort. In addition to this site-selective example, the same work also describes several enantioselective kinetic resolutions, showing the wide utility of this highly active class of biocatalysts.
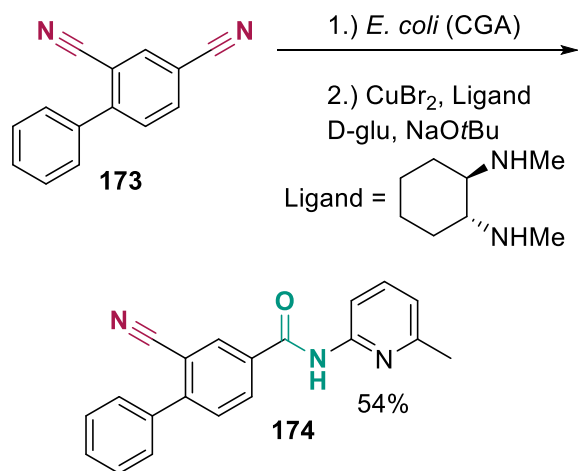
Scheme 28. Chemoenzymatic cascade to compound **174** enabled by site-selective hydration of polynitrile precursor.

2.5.2. Nucleophilic Demethylation

Demethylation of methyl phenyl ethers is an important transformation in synthetic organic chemistry,[176, 177] but traditional synthetic methods rely on harsh reagents or/and reaction conditions that limit site selectivity.[178, 179] Multi-component B12-dependent methyl transferases (MTs) catalyze this reaction with concomitant methylation of an acceptor substrate.[180] While the native methyl donors for these enzymes include substrates like CH_3-H_4folate, methanol, methylamine, and dimethylsulfide,[180-182] some can also accept various methyl phenyl ethers as substrates. Demethylation proceeds via attack of the methyl group by the nucleophilic cob(I)alamin form of a cobalamin-binding protein (e.g. vdmB) catalyzed by a suitable carrier protein (e.g. dhaf4611) to form the methyl cob(III)alamin form of the protein and the demethylated substrate. The reverse

reaction then occurs using an acceptor substrate to drive the reaction in the desired direction.[181] For example, site-selective monodemethylation of papaverine (**175**) to form **176** was achieved using a veratrol-*O*-demethylase from *A. dehalogenans* (vdmB) in combination with the cobalamin carrier protein dhaf4611 from *D. hafniense*.[183] This same system also enabled selective monodemethylation of **177** to give **178** (6% conversion), and several other aromatic substrates with methyl ether substituents provided preferences in the site selectivity of demethylation.
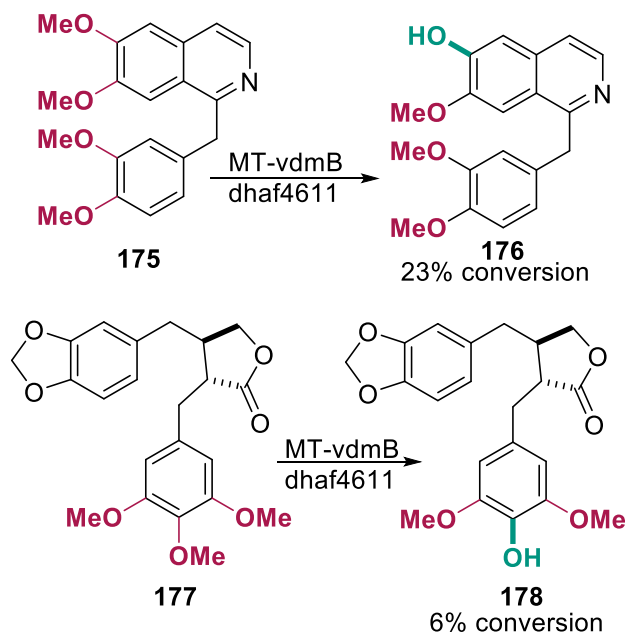
Scheme 29. Papaverine and *Rac*-yatein are both natural products and are of biological significance. Site-selective demethylation of these compounds were achieved by cobalamin-dependent methyltransferase MT-vdmB.

3. C-H Functionalization

Site-selective functionalization of C-H bonds constitutes a long-standing challenge in synthetic chemistry since such transformations could eliminate the need for pre-functionalized starting materials and enable new synthetic routes.[184, 185] The same difficulties associated with site-selective functional group manipulation apply to C-H bonds, but at least at first glance, the challenge appears greater since C-H bonds are ubiquitous in organic molecules. The molecular context of a given C-H bond, however, leads to differences in proximity to blocking or directing groups, acidity, bond-dissociation energy, and stereoelectronic properties that can be differentiated by reagents and catalysts.[186] Indeed, the reactivity of C-H bonds is so diverse and approaches to functionalize them so varied that Dyker once recounted in an early review of the field that "C-H functionalization begins just below one's own results".[187] Nonetheless, there are many cases where similar C-H bond reactivity leads to poor site selectivity using small molecule catalysts. Just as importantly, selective functionalization of a single C-H bond on a given substrate is just the start of the challenge since, ideally, one would be able to functionalize *any* C-H bond on that substrate. Only enzymes have been able to achieve this feat,[188, 189] enabling sequential functionalization of C-H bonds in natural product biosyntheses, for example,[190] that far surpass current synthetic methods. Leveraging this power for synthetic chemistry provides a means to complement our

ability to exploit differences in C-H bond reactivity with differences in substrate binding and molecular recognition to expand the scope of site-selective C-H functionalization.

3.1. C–H Hydroxylation

Several classes of enzymes catalyze C-H hydroxylation due to the broad importance of hydroxylation for the function and metabolism of natural products and xenobiotic compounds.[191] As noted above, alcohols are also important for the function of pharmaceuticals, natural products, and agrochemicals, and they serve as useful intermediates and building blocks. The diversity of enzymes that catalyze this transformation has led to several options for site-selective hydroxylation of different substrate classes, so these are grouped below based on enzyme class.

3.1.1 Cytochromes P450

Cytochrome P450 monooxygenases (P450s) catalyze a variety of oxidative reactions, including C–H hydroxylation.[192] These enzymes possess a heme cofactor with a cysteine thiolate acting as a ligand to the iron center on the proximal face, leaving the distal face free for O_2 activation. P450-catalyzed hydroxylation is initiated by substrate binding, which promotes electron transfer from a NAD(P)H cofactor through a P450 reductase or ferredoxin to the iron center of the heme cofactor (Scheme 30).[192, 193] Heme reduction is followed by O_2 binding to the distal face of heme, transfer of a second electron by a P450 reductase or ferredoxin to produce an Fe(III)-peroxo species, and protonolysis of this intermediate to give a highly reactive Fe(IV)-oxo complex known as "compound I". This intermediate can abstract a hydrogen atom from primary, secondary, or tertiary C–H bonds (or react with olefins as noted in section 2.4.2). C-H abstraction is followed by a radical rebound step between the substrate radical and the newly formed Fe(III)-hydroxo species, resulting in the hydroxylated product. Perhaps not surprisingly, given their ability to react with such a broad range of C-H bonds in diverse substrates, protein engineering has proven central to the utility of P450s for site-selective catalysis. Because of the extensive effort that this field has attracted, the examples below are meant to highlight early studies in the field and recent examples of the diversity of substrates that can now be site-selectively functionalized. Reviews on P450 biocatalysis should be consulted for a more exhaustive coverage.[194-196]
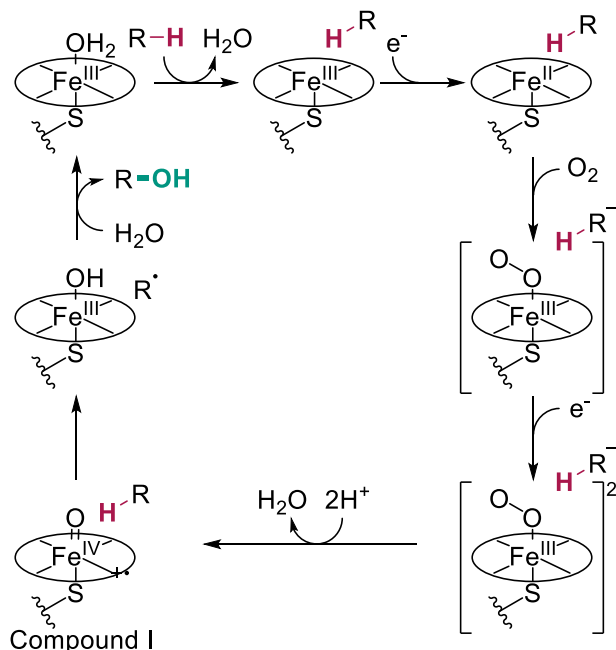
Scheme 30. Simplified scheme of the catalytic cycle for the hydroxylation of a substrate R–H by a cytochrome P450.

Many P450s have been used for site-selective hydroxylation,[197, 198] but cytochrome P450$_{BM3}$ (BM3), a fatty acid hydroxylase from *Bacillus megaterium*, has attracted particular attention because it has a fused reductase domain that leads to high reaction rates and obviates the need for a separate reductase.[199] This architecture also simplifies protein engineering, and several groups have developed BM3 variants that provide high yields and site selectivity on a range of different substrates. Early studies by Arnold used directed evolution to develop BM3 variants with activity on *n*-alkanes,[200, 201] and subsequent mutagenesis of these variants led to enzymes with high site selectivity on these substrates, including 86% selectivity for 2-nonanol with 1-12G and 67% selectivity for 4-nonanol with 9-10A A82L (Scheme 31A).[202] Further evolution of variant 139-3 led to the development of propane monooxygenase (PMO) P450$_{PMO}$R2, capable of carrying out the challenging hydroxylation of propane with native-like efficiency with a 9:1 2-propanol:1-propanol product ratio.[203]

A

P450$_{BM3}$
variant

H$_n$ ⟶ H$_{n-1}$ OH R

H ← 1-12G: 77%
C$_2$H$_5$
179 H ← 9-10A: 95%

H ← 1-12G: 82%
C$_4$H$_9$
180

1-12G: 86% → H H ← 9-10A-A82L: 67%
C$_5$H$_{11}$
181

B

P450$_{BM3}$
variant

(OMe)$_4$ OMe ⟶ OH O (OMe)$_3$ OMe

9-10A A82L →
9-10A →
OMe
MeO
MeO
MeO OMe
12-10C
182

9-10A F87I →
OMe
MeO O
MeO
MeO
OMe
183

MeO OMe
MeO
MeO OMe
5E5 →
184
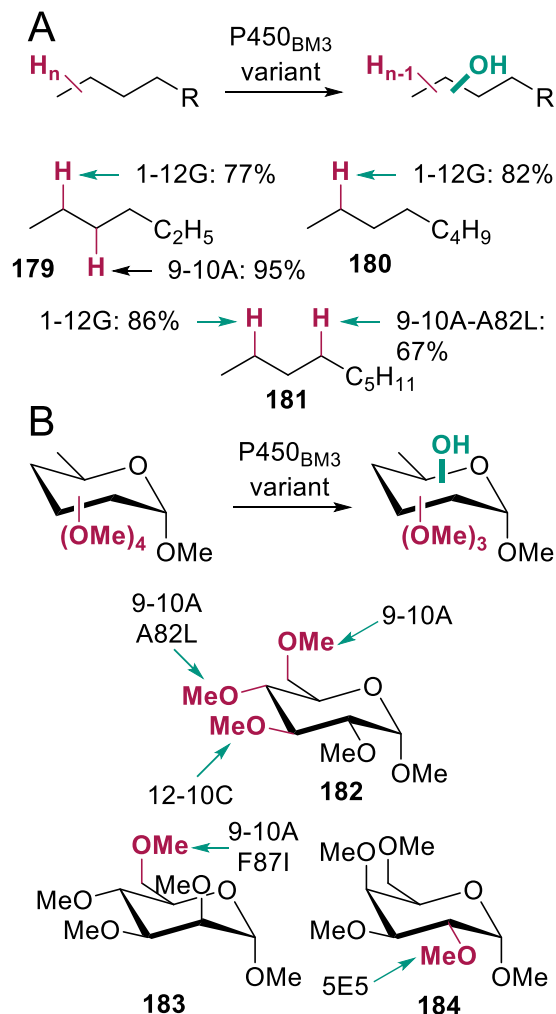
Scheme 31. A) Site-selective hydroxylation of *n*-alkanes by P450$_{BM3}$ variants.[202] B) Demethylation of protected monosaccharides by P450$_{BM3}$ variants shown as the major product for each reaction.[204]

The P450 variants obtained via these evolution efforts proved to be remarkably versatile for site-selective hydroxylation of different compounds, including drugs[205], natural products,[206] and other small molecules. In a particularly notable example that illustrated the ability to tune site selectivity to different C–H bonds using a combination of protein engineering and substrate engineering, P450$_{BM3}$ was used for site-selective demethylation of permethylated monosaccharides (Scheme 31B). This capability enabled chemoenzymatic functionalization of different sugars via subsequent manipulation of the single deprotected hydroxyl group.[204] Screening previously engineered P450$_{BM3}$ variants to identify hits for different sugars followed by further directed evolution yielded a panel of enzymes with high selectivity (~50-100% in terms of product distribution) for the demethylation of protected hexoses (**182-184**).

Hydroxylation of steroid substrates is of particular interest for pharmaceutical applications, but this goal requires distinguishing several non-activated C–H bonds. Additionally, while various eukaryotic P450s can perform these transformations,[207] these are often membrane proteins, which makes them unattractive as biocatalysts. The previously engineered P450$_{BM3}$ variant 9-10A F87V

TS was therefore further evolved using combinatorial alanine scanning and random mutagenesis to enable selective C-2 hydroxylation of 11α-hydroxyprogesterone (**185**) and demethylation of the alkaloids thebaine and dextromethorphan (Scheme 32A).[208] Site-selective hydroxylation of a range of steroids using different BM3 variants was subsequently reported by other groups.[209-212]
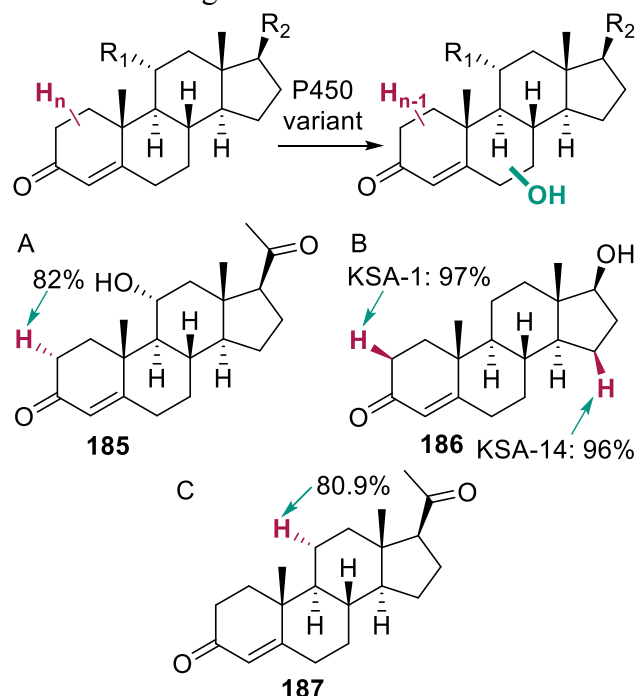
Scheme 32. Site selectivity expressed as percent in product distribution in the hydroxylation of steroids by engineered P450 enzymes. Remaining products consist of hydroxylation at different sites and other oxidation products. Reactions shown include A) 11α-hydroxyprogesterone (**185**) with P450$_{BM3}$ F1,[208] B) progesterone (**186**) with CYP106A2 T89N/A395I,[213] and C) testosterone (**187**) with P450$_{BM3}$ variants KSA-1, KSA-14, LG-23, WWV-M, and LIFI-WQM.[209, 210, 214]

Wong also conducted early studies focusing on the hydroxylation of compounds containing aromatic moieties, and these efforts led to the development of BM3 variant KT5.[215] While hydroxylation of propylbenzene (**188**) with wild-type P450$_{BM3}$ resulted in a product distribution of 99% 1-phenyl-1-propanol, variant KT5 yielded 78% 1-phenyl-2-propanol and 20% 1-phenyl-1-propanol (Scheme 33A). This effect was even more pronounced when using toluene as substrate, resulting in 95% benzyl alcohol and 5% *o*-cresol while the product distribution for BM3 consisted of 98% *o*-cresol. Further computational studies on KT5 linked the change in site selectivity in the hydroxylation of toluene to changes in the active site caused by mutations F87A/A330P, which allow for toluene to bind in previously inaccessible orientations.[216]

Reetz and coworkers used P450$_{BM3}$ to catalyze site-selective hydroxylation of cyclohexenes such as cyclohexene-1-carboxylic acid methyl ester (**189**).[217] Wild-type P450$_{BM3}$ provided a product distribution comprising 84% (*R*)-3-hydroxycyclohexene-1-carboxylic acid methyl ester (34% e.e.) and 16% isomeric alcohols and other oxidation products. Targeted mutagenesis of the active site resulted in variants with comparable conversion levels and improved selectivities, including one (F87V/A328N) that provided the desired (*R*)-3-hydroxycyclohexene product with 93% site selectivity and 96% e.e. A second variant (I263G/A328S) gave the corresponding *S*-enantiomer

with 97% site selectivity and 94% e.e. (Scheme 33B). The variants engineered for the reaction of the model cyclohexene **189** were successfully tested on other substrates, with cyclopentene-1-carboxylic acid methyl ester (**191**) showing significant improvements in site selectivity, from a 58% of side products with wild-type P450$_{BM3}$ to 13% and 17% with the *R*- and *S*-selective variants, respectively.
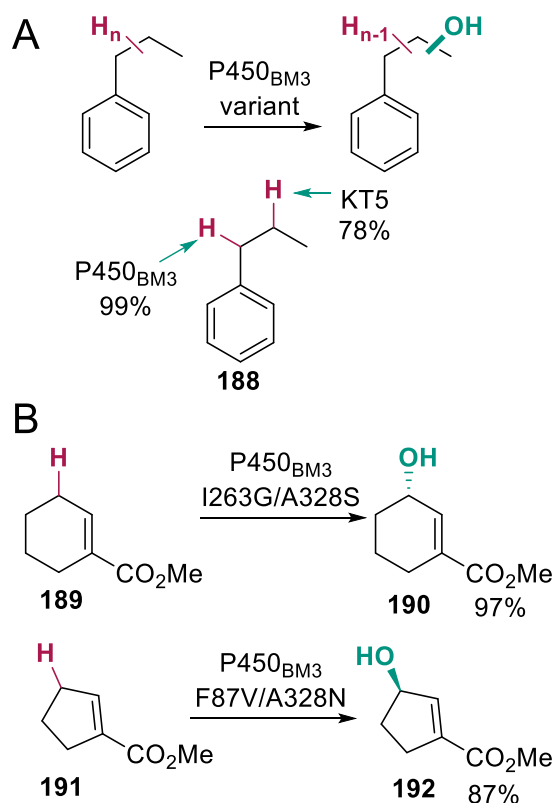
Scheme 33. Site-selective hydroxylation reactions carried out with engineered P450$_{BM3}$ enzymes and A) propylbenzene (**188**),[215] B) cyclohexene-1-carboxylic acid methyl ester (**189**), and cyclopentene-1-carboxylic acid methyl ester (**191**).[217] Product distribution is presented as the percentage of the major product in each reaction.

Several P450$_{BM3}$ biocatalysts have been developed for the site-selective hydroxylation of testosterone (**187**, Scheme 32C). BM3 variant F87A was used in two different studies to engineer variants KSA-1 with 97% selectivity towards 2β-hydroxylation, KSA-14 with 96% selectivity towards 15β-hydroxylation, LIFI-WQM with 98% selectivity towards 6α-hydroxylation, and WWV-M with 92% selectivity towards 16β-hydroxylation.[209, 214] Additionally, the triple mutant P450$_{BM3}$ F87G/A328G/A330W was evolved for hydroxylation at the C-7 position of **187**, with final variant LG-23 having an improved site selectivity of 90% 7β-hydroxytestosterone.[210]

Finally, Fasan has engineered BM3 variants that catalyze site- and enantioselective hydroxylation of artemisinin (**193**) using the promiscuous variant FL#62 as a starting point.[218, 219] While the parent enzyme showed a product distribution of 83% 7(*S*)-hydroxyartemisinin, 10% 7(*R*)-hydroxyartemisinin, and 7% 6α-hydroxyartemisinin, highly selective variants were obtained resulting in IV-H4 producing 100% 7(*S*)-hydroxyartemisinin, II-H10 producing 100 % 7(*R*)-

hydroxyartemisinin, and X-E12 producing 94% 6α-hydroxyartemisinin (Scheme 34A). FL#62 variants that exhibit site-selective hydroxylation of parthenolide analogs were also developed, showcasing the value of promiscuous variants that can be further engineered into selective biocatalysts.[166, 220, 221]

A variety of other prokaryotic P450s have also been the focus of extensive engineering efforts. For instance, CYP106A2 has been developed as a platform for selective steroid hydroxylation, with mutant T89N/A395I showcasing the potential of this enzyme by having its site selectivity switched to 80.9% 11α-hydroxyprogesterone from wild-type 27.7% (Scheme 32B).[213, 222] Additionally, the P450 PikC, from the pikromycin biosynthetic pathway,[223] has been engineered into PikC$_{D50N}$, a single mutant with increased activity on the native macrolide substrates YC-17 and narbomycin.[224, 225] This variant was also used to show that the native site selectivity between C-10 and C-12 hydroxylation could be modulated by modifying the desosamine sugar group, with a site selectivity C-10:C-12 of 1:1 and >20:1 for YC-17 and an analogue with a benzylic amine as anchoring group, respectively (Scheme 34B).[226]
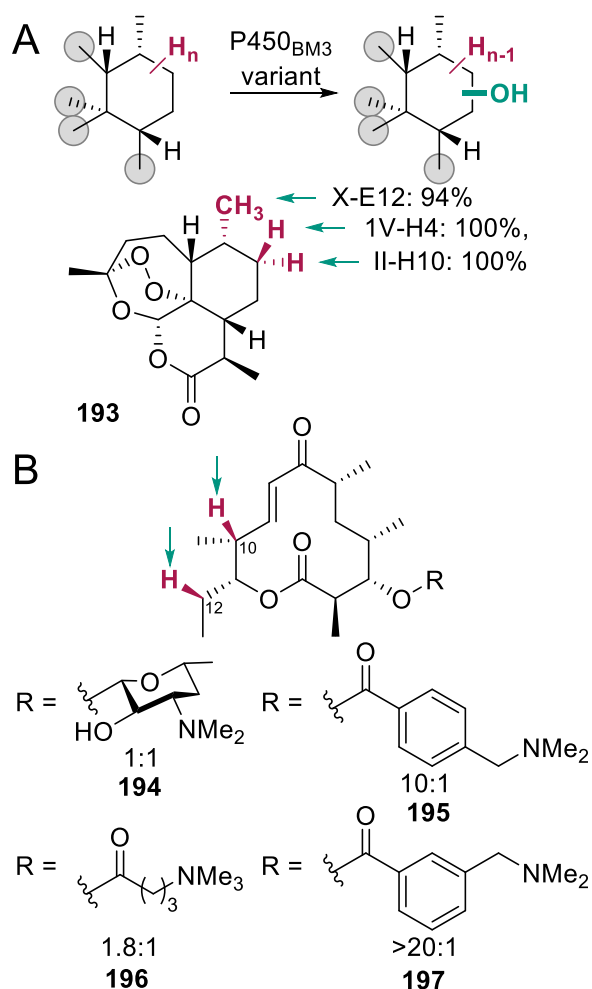
Scheme 34. A) Selectivity in product distribution for the hydroxylation of artemisinin (**193**) by engineered P450$_{BM3}$ variants.[219] B) Site selectivity of hydroxylation shown as the ratio of C-10:C-12 hydroxylated products for YC-17 analogues with varying anchoring group with PikC.[226]

While aliphatic hydroxylation occurs via abstraction of the C–H bond, aromatic hydroxylation by P450s is believed take place via the formation of an arene oxide or electrophilic attack of compound I on the aromatic substrate.[227-229] Site-selective aromatic hydroxylation by engineered P450$_{BM3}$ variant M2 (P450$_{BM3}$ R47S/Y51W/I401W)[230] was shown to have high selectivity for *o*-hydroxylation of monosubstituted benzenes, including substrates that were not accepted by the wild-type enzyme (Scheme 35A).[231] A more recent study showed that CYP199A4 from *Rhodopseudomonas palustris* could be engineered to hydroxylate 4-phenylbenzoic acid (**202**) to 4-(2'-hydroxyphenyl)benzoic acid (**203**) with a 83% site selectivity, a reaction that was not catalyzed by wild-type CYP199A4 (Scheme 35B).[232]
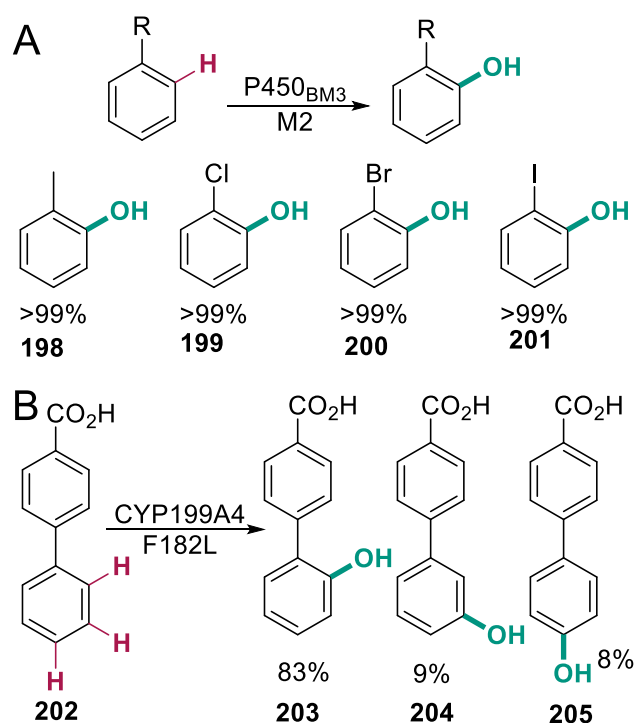
Scheme 35. Aromatic hydroxylation reactions carried out by P450s. A) Product distribution in the hydroxylation of monosubstituted benzenes by P450$_{BM3}$ M2.[231] B) Selective hydroxylation of 4-phenylbenzoic acid (**202**) by variant CYP199A4 F182L with percentages depicting product distribution.[232]

3.1.2. Fe(II)- and α-Ketoglutarate-Dependent Oxygenases

A second class of enzymes that hydroxylate C-H bonds site-selectively is the Fe(II)-/α-ketoglutarate-dependent oxygenases (FeDOs). The consensus mechanism of FeDO-catalyzed C-H hydroxylation is initiated via bidentate binding of α-ketoglutarate to a Fe(II) ion that is coordinated by a conserved His-Asp/Glu-His facial triad and three water ligands, resulting in the displacement of two water molecules (Scheme 36).[233] Substrate binding causes loss of the last water ligand and creates an open coordination site for O$_2$ to binding and activation to form a Fe(III)-superoxo intermediate. Oxidative decarboxylation of α-ketoglutarate by the superoxo

species leads to formation of a Fe(IV)-oxo intermediate, succinate, and CO_2. As in P450s, the Fe(IV)-oxo species is responsible for abstracting a hydrogen atom from the primary substrate to generate a Fe(III)-OH intermediate and a substrate radical, which react via radical rebound to form the hydroxylated product and regenerate the $Fe(II)(H_2O)_3$ center.
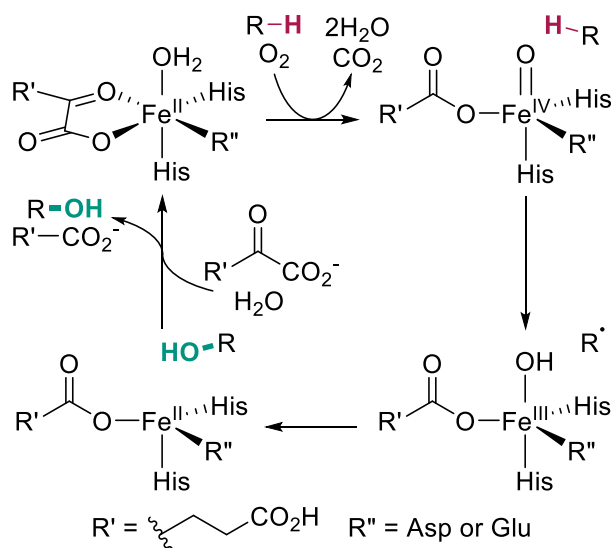
Scheme 36. Simplified scheme of the catalytic cycle for the hydroxylation of substrate R–H by a FeDO.

The use of FeDOs for site-selective hydroxylation has primarily focused on amino acid substrates. These enzymes exhibit relatively high substrate specificity compared to P450s, so they tend to be used on compounds similar to their native substrates. For example, bacterial and fungal L-proline hydroxylases can selectively produce *cis*-3-, *cis*-4-, *trans*-3-, and *trans*-4-hydroxy-L-proline (Scheme 37A).[234] These enzymes also accept other cyclic amino acids, including L-pipecolic acids. However, native pipecolic acid hydroxylases, such as GetF and PiFa, have been shown to have poor activity on L-proline, with efforts to increase substrate promiscuity via protein engineering being unsuccessful.[235, 236] A patent by Merck & Co. reports the evolution of a proline hydroxylase over twelve rounds of evolution for the production of (2*S*, 5*S*)-hydroxypipecolic acid at 180 g/L substrate loading.[237] The evolution approach included several rounds aimed at mitigating self-deactivation of the enzyme by pre-incubating it with reaction components before adding substrate into the bioconversion. Additionally, a FeDO from the same gene cluster as GetF, GetI, was characterized as a citrulline hydroxylase and engineered to selectively hydroxylate arginine to 4-hydroxyarginine, with an activity high enough to allow for incorporation in the synthesis of novel dipeptides.[238, 239]
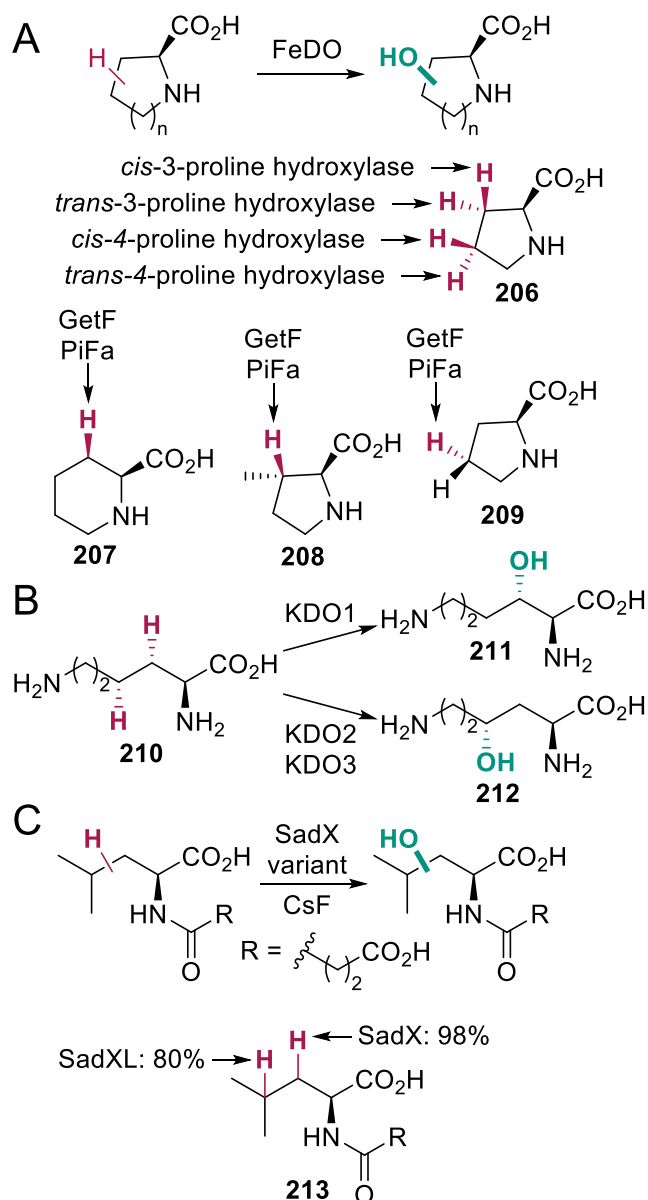
Scheme 37. Examples of site-selective hydroxylation of amino acids with FeDOs. A) Proline and pipecolic acid hydroxylases.[236] B) Lysine hydroxylases.[240] C) Engineered SadA variants. Percentages correspond to relative amounts in product distribution.

Genome mining has been used to discover new FeDOs with novel selectivities. A series of L-lysine (KDO1–3) and L-ornithine (ODO) hydroxylases obtained via such a strategy resulted in biocatalysts with the less common C-4 site selectivity (KDO2 and KDO3) as opposed to the more frequent C-3 hydroxylation of polar amino acids like lysine (**210**, Scheme 37B).[240] This capability allowed for the synthesis of dihidroxylated L-lysine via the sequential use of KDO1 and KDO2/3. These lysine hydroxylases have been used in combination with a lysine decarboxylase to form an enzymatic cascade for the synthesis of chiral amino alcohols from L-lysine.[241] Furthermore, the lysine hydroxylase GlbB from the glidobactin biosynthetic cluster in *Polyangium brachysporum* was shown to hydroxylate C-4 of both L-lysine and L-leucine.[242]

Finally, a recent study by our group showed that the site selectivity of the FeDO SadA can be altered.[243] Variants SadX (MBP-fused SadA D157G) and SadXL (SadX F152L) lack the native glutamic acid in their facial triad, allowing for exogenous anions to bind to the Fe(II) center and rescue native activity. The F152L mutation in SadXL produced a distinct change in the site selectivity of the hydroxylation of N-succinyl-L-leucine (**213**), from 98% β-hydroxylation with SadX to 80% γ-hydroxylation when CsF is added to a reaction. The identity of the anion also has a significant impact, with the product distribution of SadXL being 57% γ-hydroxylation in the presence of sodium formate (Scheme 37C).

3.1.3. Other Site-Selective Metalloenzyme Oxygenases and Dioxygenases

Heme peroxidases are widely distributed enzymes that carry out oxidative transformations by reducing peroxide (H_2O_2) through their heme prosthetic group.[244, 245] The promiscuous unspecific peroxidase (UPO) is an aromatic peroxygenase known to perform oxyfunctionalization of C–H bonds that has been extensively studied and engineered.[244] For example, a fungal UPO from *Agrocybe aegerita* (*Aae*UPO) was engineered to enable selective hydroxylation of naphthalene to give 1-naphthol.[246] This effort resulted in a two-fold increase in activity from the parent enzyme and a final ratio of 1-napthol:2-napthol of 97:3. *Aae*UPO also catalyzes site-selective hydroxylation short alkanes such as propane and *n*-butane to the corresponding 2-alcohols, and flavonoids like apigenin and luteolin to the corresponding 6-hydroxyflavonoids, showcasing the broad substrate scope of this enzyme (Scheme 38A).[247, 248] Finally, engineered *Aae*UPO variant *Aae*UPO Fett was recently reported to show improved selectivity for the functionalization of sub-terminal positions of fatty acid substrates.[249] The crystal structure of the Fett variant showed a narrower channel into the active site, restricting the access of bulkier substrates and resulting in a higher site selectivity towards the ω-1 position of lauric (**218**), myristic (**219**), palmitic (**220**), and stearic (**221**) acids. While the ω-1 selectivity of *Aae*UPO ranged from 20% to 56%, the Fett variant provided >92% selectivity in all cases (Scheme 38B).
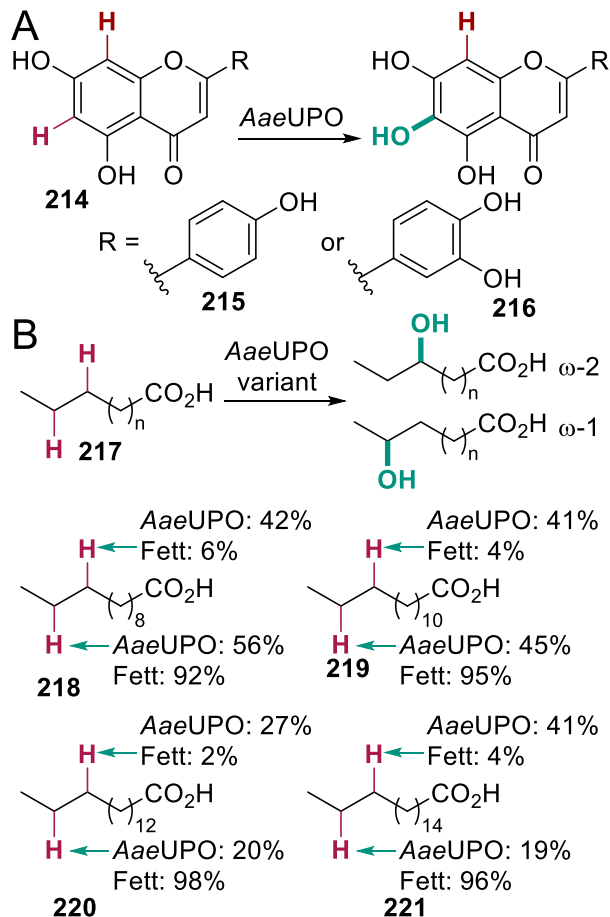
Scheme 38. A) Flavonoid site-selective aromatic hydroxylation by heme peroxidase *Aae*UPO.[248] B) Hydroxylation of unsaturated fatty acids by *Aae*UPO variants. Products are shown as percentages of total product pool, with remaining products being ω-hydroxylated fatty acids and overoxidation products.[249]

Rieske non-heme iron-dependent oxygenases are iron-sulfur cluster-containing enzymes involved in the degradation of aromatic compounds by bacteria via *cis*-dihydroxylation reactions.[250] The iron-sulfur cluster transfers electrons from a reductase to the catalytic non-heme iron center, allowing for O_2 reduction and the formation of a high-valent iron intermediate. This species is responsible for oxidizing the substrate via a series of radical intermediates. A set of Rieske dioxygenases from *Pseudomonas* strains were shown to oxidize alkenes and aromatic compounds with complementary selectivity; naphthalene dioxygenase (NDO) catalyzed dihydroxylation of the olefin in styrene exclusively, while cumene dioxygenase (CDO) provided >99% selectivity for the corresponding arene-1,2-dihydrodiol (Scheme 39A).[251] Additionally, a single point mutation was found to switch the selectivity of CDO from 0.3% to 92% for olefin dihydroxylation, highlighting the potential for these enzymes to be engineered for non-native selectivities. More recent studies focused on characterizing enzymes from the saxitoxin biosynthetic pathway showed that Rieske monooxygenases SxtT and GxtA give different hydroxylation patterns on a series of tricyclic natural products derived from saxitoxin (Scheme 39B).[252, 253]
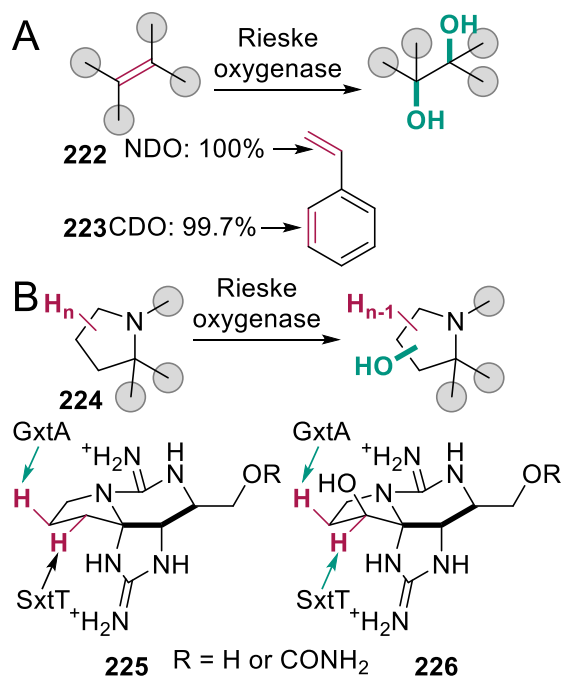
Scheme 39. A) Site selectivity in hydroxylation expressed as percentage of major product in product distribution for Rieske dioxygenases NDO and CDO.[251] B) Site-selective monooxygenation of saxitoxin-derived natural products by Rieske monooxygenases SxtT and GxtA.[252]

Bacterial multicomponent monooxygenases (BMMs) have also been used for site-selective hydroxylation. These enzymes comprise three or four components: a carboxylate-bridged diiron(III) center-containing hydroxylase enzyme, a NADH reductase, an effector protein required for the coupling of electron consumption and substrate oxidation, and, in some cases, a Rieske-type [2Fe-2S] ferredoxin. Variants of the BMM toluene 4-monooxygenase (T4MO), which natively hydroxylates toluene (**227**) to *p*-cresol (**228**), were engineered to provide different site selectivity toward aromatic substrates (Scheme 40A).[254, 255] For example, T4MO G103L catalyzes toluene hydroxylation to give *o*-cresol (**229**) as the major product. This superfamily of enzymes also includes methane monooxygenases (MMOs) from methanotrophic bacteria. Soluble MMO (sMMO) from *Methylococcus capsulatus*, for example, is capable of hydroxylating aliphatic C–H bonds selectively (Scheme 40B), and this enzyme catalyzes other oxidative reactions on alkanes, alkenes, and aromatic substrates.[256, 257] While sMMOs are produced only by some methanotrophs, nearly all methanotrophic bacteria produce particulate MMO (pMMO).[258] These membrane-bound, copper-dependent enzymes possess a narrower substrate scope than sMMO, hydroxylating mostly linear alkanes (Scheme 40B).[259] Whereas the mechanism of sMMOs has been studied extensively, with most of its intermediates being identified,[260] characterization of the catalytic cycle of pMMOs is actively under study.[261]
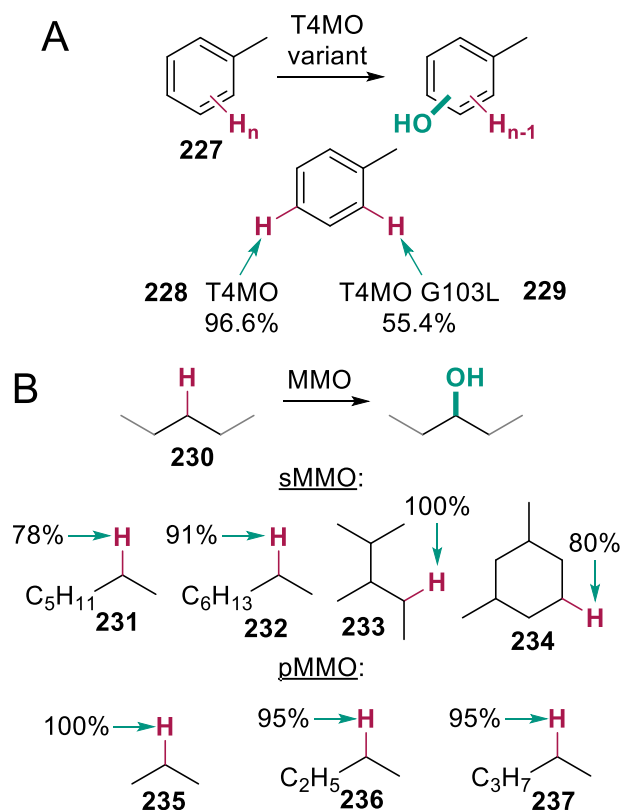
Scheme 40. Major products in hydroxylation as percentage in product pool for A) bacterial multicomponent monooxygenase T4MO,[255] and B) methane monooxygenases sMMO[256, 262] and pMMO[259] from *Methylococcus capsulatus* (Bath).

3.1.4. Flavin-dependent Monooxygenases

Flavin-dependent monooxygenases (FMOs) are part of the FAD-oxidoreductase family and catalyze, among other reactions hydroxylation of aromatic sp^2 C–H bonds.[263, 264] These enzymes can be classified as either single-component FMOs that tightly bind FAD as a prosthetic group, or two-component FMOs that require a second reductase enzyme to provide the reduced flavin as a co-substrate. The mechanism of single-component FMOs involves the reduction of FAD by NAD(P)H in the enzyme, followed by reaction with O_2 to generate the reactive hydroperoxyflavin intermediate. This species can react with electron rich aromatic substrates via electrophilic aromatic substitution to generate the hydroxylated arene and hydroxyflavin, which undergoes elimination to form oxidized FAD. Depending on the class of single-component FMO, substrate binding may be required for the reduction of FAD (Scheme 41), or it may happen after formation of the hydroperoxyflavin intermediate. In the case of two-component FMOs, the flavin is reduced in the reductase by NAD(P)H and transferred to the oxygenase, where reaction with O_2 occurs to form the hydroperoxyflavin intermediate. The mechanism then follows a similar path to the one described for single-component FMOs.
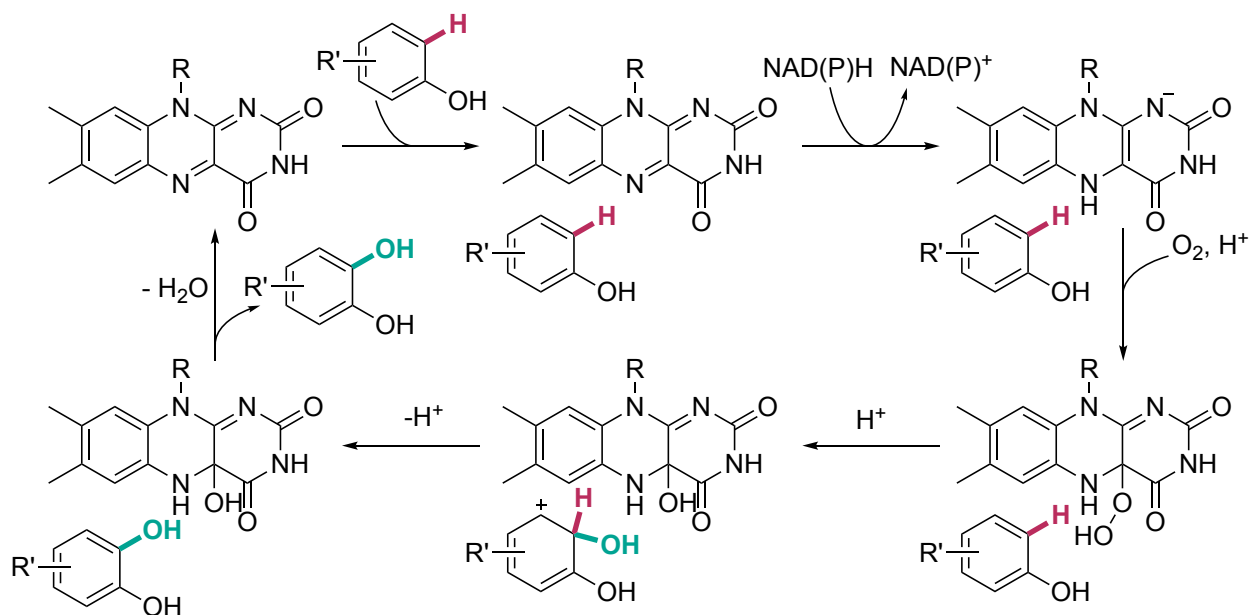
Scheme 41. Catalytic cycle for hydroxylation of a phenolic compound by a single-component FMO triggered by substrate binding.

The native activity and substrates of many FMOs have been thoroughly characterized,[263] and this has facilitated efforts to explore site-selective non-native FMO catalysis. For example, the single-component 4-hydroxybenzoate 3-hydroxylase (PHBH), which natively converts 4-hydroxybenzoate into 3,4-dihydroxybenzoate, can also hydroxylate a limited scope of phenolic acids. A study focused on PHBH enzymes from *Rhodococcus rhodnii* 135 and *Rhodococcus opacus* 557 showed that while both enzymes hydroxylate 2,4-dihydroxybenzoate at C-3, they hydroxylate 2-chloro-4-hydroxybenzoate with C-3:C-5 ratios of 40:60 and 77:23, respectively.[265] 2-Hydroxybiphenyl 3-monooxygenase (HbpA), which natively hydroxylates 2-hydroxybiphenyl (**238**) at C-3, was engineered to hydroxylate *tert*-butyl-2-hydroxybiphenyl.[266] A more recent study showed that HbpA M321A catalyzes selective C-4 hydroxylation of 3-hydroxybiphenyl (**240**), a substrate not accepted by wild-type HbpA (Scheme 42A).[267]

The 4-hydroxyphenylacetate 3-hydroxylases (HPAHs) are two component FMOs that natively catalyze hydroxylation of 4-hydroxyphenylacetate to 3,4-dihydroxyphenylacetate and accept a range of additional substrates.[268] The S146A variant of HPAH from *Acinetobacter baumannii* provides improved activity on 4-aminophenylacetic acid to give 3-hydroxy-4-aminophenylacetic acid when compared to wild-type.[269] HpaBC from *Pseudomonas aeruginosa* catalyzes site-selective hydroxylation of cinnamic acid derivatives such as ferulic acid, *p*-coumaric acid, and caffeic acid.[270] HpaBC was also used to synthesize the natural antioxidant piceatannol from resveratrol.[271] More recent studies on HpaB from *E. coli*,[272] showed that this enzyme could be engineered to site-selectively hydroxylate bulkier molecules like naringenin (**242**, Scheme 42B).[273]

Some FMOs, such as TropB, AzaH, and SorbC, natively catalyze the oxidative dearomatization of resorcinol derivatives to quinol products via site-selective hydroxylation.[274-276] These enzymes showed complementary substrate scope and orthogonal site selectivity toward a panel of resorcinol substrates with varying steric and electronic features. For example, TropB and AzaH prefer C-3 hydroxylation while SorbC prefers C-5 hydroxylation (Scheme 42C).[277] Further study of SorbC

showed that the substituent at the C-1 position of its native substrate, sorbicillin, plays an important role in placing the substrate in a productive pose. Replacing this substituent with a crotyl ester led to expanded substrate scope, and the crotyl group could be removed to yield the corresponding carboxylic acid.[278]
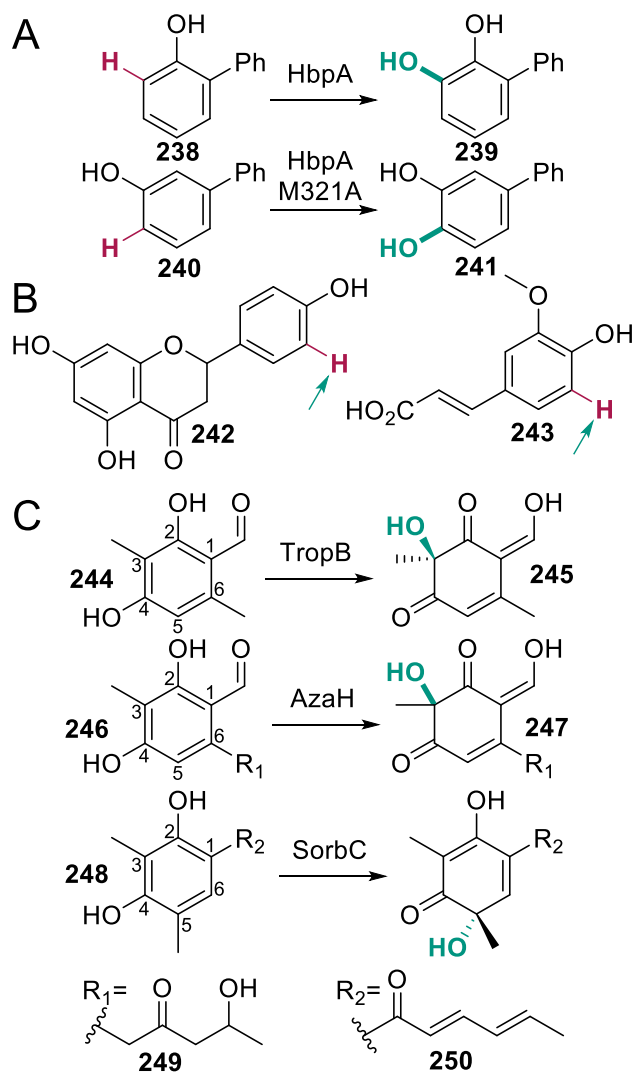
Scheme 42. Hydroxylase activity of native and engineered FMOs with strict site selectivity. A) Mutation M321A in HbpA increases substrate scope.[267] Reactions result in single products. B) Site-selective hydroxylation by engineered HpaB variants in non-native substrates.[273] Hydroxylation sites are denoted with arrows. C) Dearomatization reactions with distinct site-selectivities.[277]

3.2. C–H Halogenation

Just as different classes of hydroxylases evolved to act on different types of C-H bonds, so too did different classes of halogenases emerge in nature. Aromatic and aliphatic C-H halogenation

50

proceed via electrophilic aromatic substitution and radical rebound mechanisms, respectively, and unique mechanisms for generating the relevant halogenating species evolved to accommodate these pathways.

3.2.1. Aromatic C-H Halogenation

Early studies on chloroperoxidase (CPO) from *C. fumago* showed that this enzyme could promote aromatic C-H halogenation,[279] but the selectivity of CPO[280] and the later characterized vanadium-dependent haloperoxidases[281] is substrate-controlled. This reactivity was found to result from peroxidase-catalyzed oxidation of halide ions to generate free hypohalous acid (HOX, X = Cl, Br, I), which was responsible for the observed halogenation reactions. In the early 2000's, however, it was established that site-selective chlorination of the 7-position of tryptophan is catalyzed by PrnA (Scheme 44A),[282] the first characterized flavin-dependent halogenase (FDH). Following this discovery, FDHs capable of halogenating different sites of tryptophan[283, 284] and various indole-,[285, 286] pyrrole-,[287-289] and phenol-containing[290-295] natural products were characterized, showing that these enzymes are responsible for site-selective halogenation of aromatic C-H bonds on a variety of substrates in nature, and many subsequent examples further illustrate this point.[296-299] These enzymes bind reduced flavin,[300] which reacts with oxygen to form a hydroperoxyflavin intermediate analogous to that formed in FMOs (Scheme 41). Rather than reacting with an organic substrate, however, this species reacts with a halide ion (X = I, Br, or Cl) bound proximal to the hydroperoxyflavin to generate the corresponding hypohalous acid (Scheme 43B).[301] Unlike the case for peroxidases, this species then travels through a tunnel within the enzyme to access the active site.[302] Recent computational evidence supports the original suggestion[302, 303] that HOX is activated toward electrophilic attack via general acid catalysis by an active site lysine residue.[304, 305] Structural studies show that substrate positioning relative to this activated species then controls the site of halogenation (Scheme 43B).[306, 307]
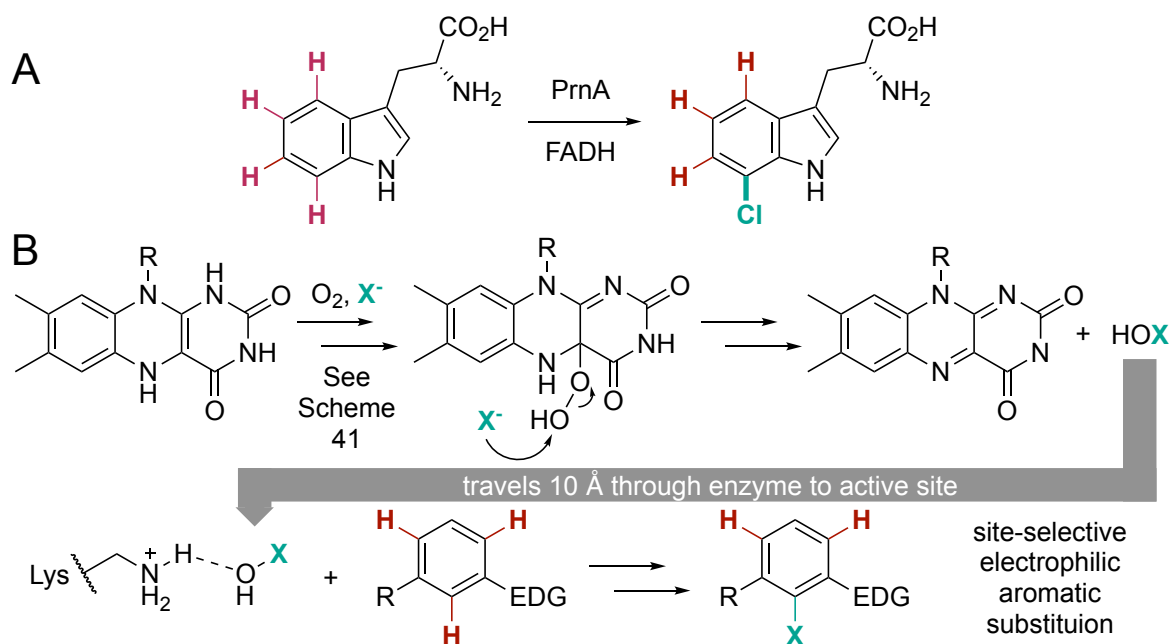
Scheme 43. A) Simplified FDH mechanism. B) PrnA and RebH natively chlorinate L-tryptophan site-selectively at C7 of the indole ring.

Early studies showed that the native site selectivity of tryptophan halogenases could be used to access various halogenated natural product derivatives by incorporating these enzymes into organisms expressing genes for different metabolic pathways.[308, 309] Halogenation of substrates other than tryptophan by PrnA was reported to proceed with substrate-controlled selectivity,[310] but RebH catalyzed 7-halogenation of tryptamine,[311] showing that at least certain FDHs could exert catalyst controlled selectivity toward non-native substrates. It was then established that RebH,[312] Rdc2,[313] and later several additional FDHs[314-317] exhibit catalyst-controlled selectivity toward a number of non-native substrates (Scheme 44A).

Early efforts to expand FDH reactivity beyond that offered by native enzymes showed that site directed mutagenesis of active site residues could be used to change RebH substrate preference to favor tryptamine over tryptophan.[311] Directed evolution was used to evolve a thermostable RebH variant,[318] and this enzyme was further evolved to expand substrate scope to encompass larger, biologically active compounds (Scheme 44B)[319]. This effort involved a substrate walking approach in which iterative rounds of mutagenesis and screening were conducted on progressively larger and more complex substrates. Variant 3-SS possessed 65-fold improved activity for the tetrahydrocarbazole derivatives tryptoline and eleagnine relative to RebH, and variant 4-V exhibited 40-fold improved chlorination for yohimbine. Variants 3-SS, 4-V, and related active site mutants were later shown to catalyze site- and enantioselective halogenation of methylene dianilines substrates.[320] Directed evolution also enabled site- and atroposelective halogenation of a 3-aryl-4(3*H*)-quinazolinones (Scheme 44C).[321] While most FDH variants examined in preliminary screening halogenated probe substrates at the less sterically hindered C4 site, one RebH variant 6-TLP gave the desired C6-brominated product with high enantioselectivity. Three rounds of evolution were required to evolve this enzyme for improved site selectivity and yield, and the final variant 3-T demonstrated excellent site- and atroposelectivity for a panel of related quinazolinones.

A

FDH
NaX, Cofactor regen

251 **53**
RebH

252 95%
RebH

253 Rdc2

254 Rdc2

255 Rdc2

99%
RebH **256**

B

substrate walking

1-PVM   2-T   3-SS   3S   → 4V

L-Trp   Tryptoline   d-dfBr

257

4V - 54%

258

3-SS, 79%

C

3-T

% yield, e.r.
site sel.

259, 92% (>99.5:0.5)
25:1

260, 78% (2.2:97.8)
25:1

261, 33% (97.5:2.5)
20:1

262, 15% (95:5)
3:1, *E* =22
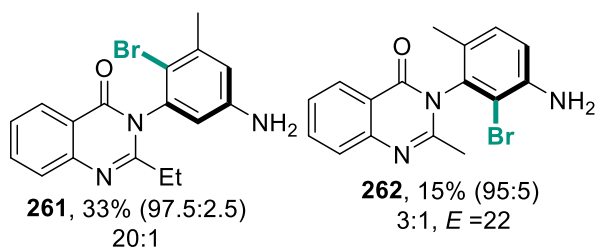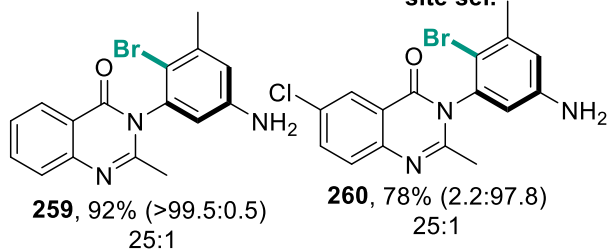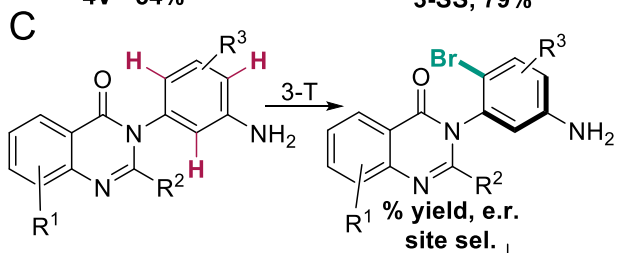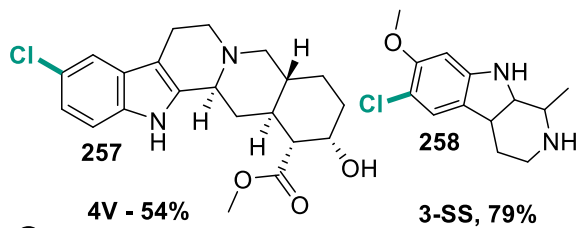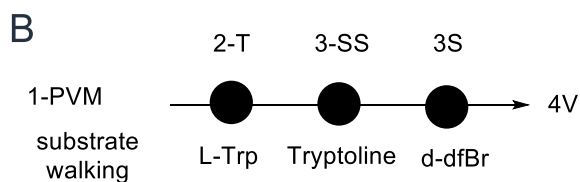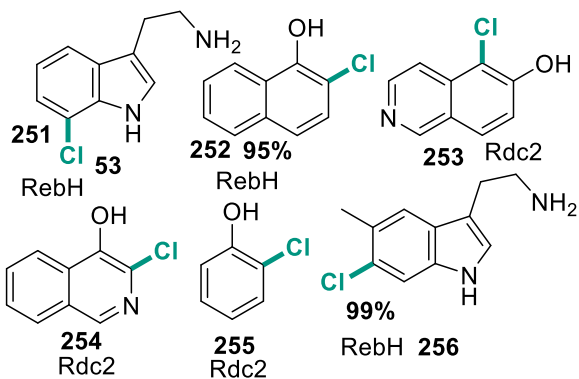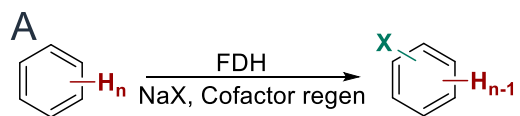
Scheme 44. A) Biocatalysis with wild-type RebH and Rdc2 for halogenation of non-native substrates. Number refers to HPLC yield when provided. B) Directed evolution of a thermostable RebH variant for halogenation of biologically active molecules. C) Site- and atroposelective catalysis with FDHs.

In addition to expanding FDH substrate scope, researchers have sought to alter the site selectivity of FDHs on both native and non-native substrates.[322] For example, incorporating arginine and lysine residues in the active site of PrnA was used to reshape the size and charge distribution of the active site to modify regioselectivity and improve activity for halogenation of anthranilic acid derivatives.[323] In a separate study, the similarity of the structures for the 5- and 6-tryptophan halogenases PyrH and SttH was exploited to engineer a 5-selective SttH variant by mutating three active site residues in SttH to the homologous residues in PyrH.[324] While wild type SttH halogenated tryptophan and 3-indolepropionic acid with 6-selectivities of >99% and 90%, respectively, the triple mutant gave the 5-chlorinated compounds in 32% and 75% selectivity, respectively. In a similar fashion, the crystal structures of tryptophan 6- and 7-halogenases Thal and RebH were used to identify key residues for positioning the substrate in the active site.[325] A total of 5 residues in the active site chosen based upon their interactions with substrate were switched to produce variant Thal-RebH-5, which generated the 7-chlorinated tryptophan product in 30% yield and with a 7:6 selectivity of 19:1, representing the most complete switch in regioselectivity for an FDH for a structure-guided approach (Scheme 45A). Structure based mutagenesis was also used to alter the site selectivity of MalA.[326]
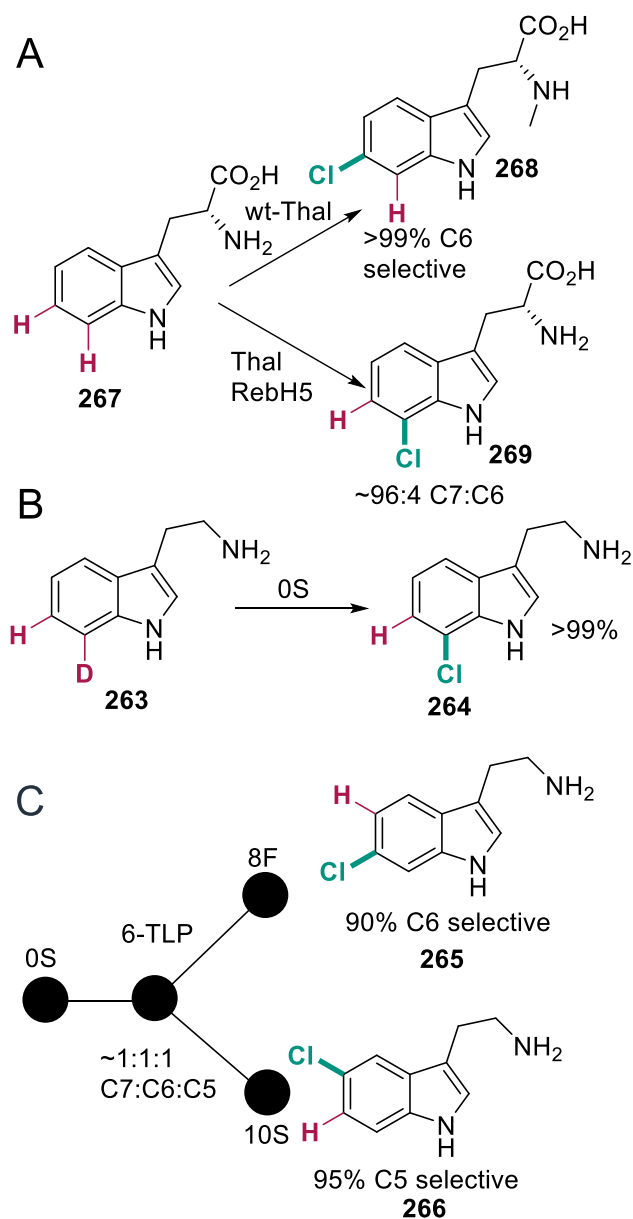
Scheme 45. Strategies for modifying the site selectivity of tryptophan halogenases. A) Use of a deuterated tryptamine probe substrate allows for MALDI screening based on m/z values B) Directed evolution of RebH into 5- and 6- selective chlorinases 10S and 8F. C) Using the crystal structures of Thal and RebH, substitution of key active site residues from RebH into Thal results in modified site selectivity.

These structure-guided approaches show that key active site residues identified in crystal structures of FDHs with different selectivity toward a co-crystallized substrate can be mutated to achieve the corresponding switch in site selectivity. To avoid the need for crystal structures and to provide a means to switch site selectivity to any desired site, a directed evolution approach to altering FDH site selectivity was developed.[327] A MALDI-based screen using deuterated tryptamine probe substrates enabled direct evaluation FDH variant site selectivity (Scheme 45B). Several rounds of

evolution led to the generation of 7-selective variant 0S, which possessed increased rate relative to WT and 5- and 6-selective halogenases 8F and 10S that provided their respective products (relative to all others) with 90:10 and 95:5 site selectivity (Scheme 45C). Variants from this lineage were later used in conjunction with the native 6-halogenase Thal and two fungal phenol halogenases to compare a panel of substrates for chemical and biocatalytic halogenation.[328] Characterization of products generated by biocatalytic halogenation using RebH variants 0S, 8F, and 10S showed that mutations designed to modify site selectivity for tryptamine carried over to other substrates as well. In many cases the site selectivity of the FDH-catalyzed halogenation differed from the chemical NCS reaction, showing the ability of the enzyme scaffold to override the inherent reactivity of the substrate. Crystal structures of these variants coupled with reversion mutations and MD simulations were used to rationalize the observed changes in site selectivity and to develop a computational model to predict FDH site selectivity.[305]
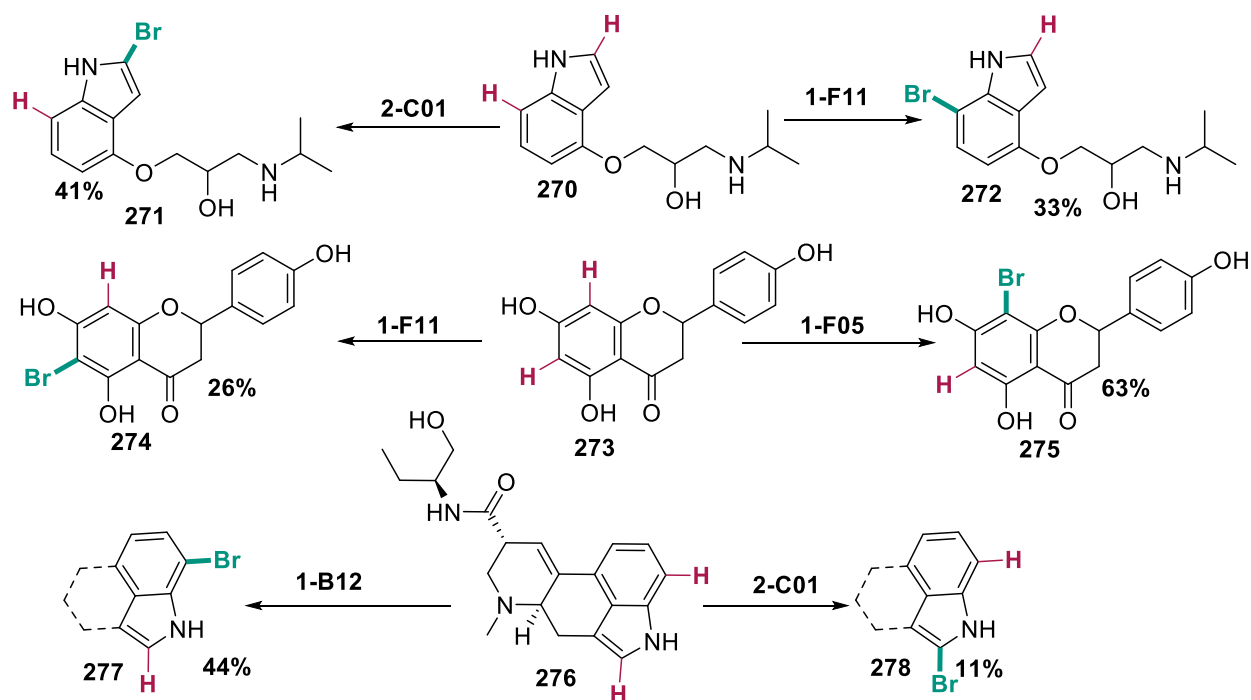
Scheme 46. Genome mining for FDHs enables site-selective catalysis on biologically active compounds. Numbers represent isolated yields.

Genome mining has often been used to identify individual FDHs with novel properties from diverse sources.[295, 315, 329] Our group pursued a family-wide genome mining approach to build a panel of FDHs with novel site selectivity toward diverse non-native substrates.[330] A sequence-similarity network (SSN) was constructed, and the substrate preferences for known FDHs was mapped onto the SSN. FDH sequences with like substrate preferences were found to cluster, providing a means to probe FDH sequence space for novel function. Ultimately, 80 new FDHs were obtained from this analysis, and the chlorinase and brominase activity of these enzymes on a panel of 12 probe substrates was examined using a mass spectrometry-based assay. Several of the

active enzymes identified from this analysis catalyze site-complementary bromination of diverse substrates (Scheme 46).

The FDHs discussed above require a separate flavin reductase (FRED) to supply the FADH$_2$ required for mechanism shown in Scheme 43. This requirement complicates FDH biocatalysis since these enzymes are not widely available, and any FDH evolution done to improve process compatibility is ultimately limited by the FRED unless it is also engineered. The FDH Bmp5 is notable in that it is a single-component FRED/FDH, but its reported activity is limited to substrate-controlled site selectivity on phenols and aryl ethers.[331] This contrasts with the recently described single-component FRED/FDH AetF,[332] which natively catalyzes site-selective 5-/7-dibromination of L-tryptophan.[333] This novel site selectivity led to a recent effort to evaluate the activity and selectivity of AetF toward a diverse range of substrates.[334] AetF accepted not only indoles and phenols analogous to those halogenated by many conventional FDHs, but also heterocycles and less electronically activated substrates that provide low or no yield using other FDHs (Scheme 47A). Remarkably, AetF also catalyzed site-selective iodination of the benzene ring of L-tryptophan and 6-fluorotryptamine with high yield. Previous reports of iodination reactions involving FDHs have typically involved substrate controlled site selectivity,[315, 331, 335] and further analysis of iodination activity has shown that FREDs can release hydrogen peroxide into solution that oxidizes I$^-$ to HOI, leading to substrate-controlled iodination selectivity.[336] The broad substrate and halide scope of AetF, coupled with its catalyst controlled site selectivity therefore makes this and related single component FRED/FDHs promising tools for biocatalytic halogenation.

Scheme 47. Substrate scope of AetF for site-selective A) bromination and B) iodination
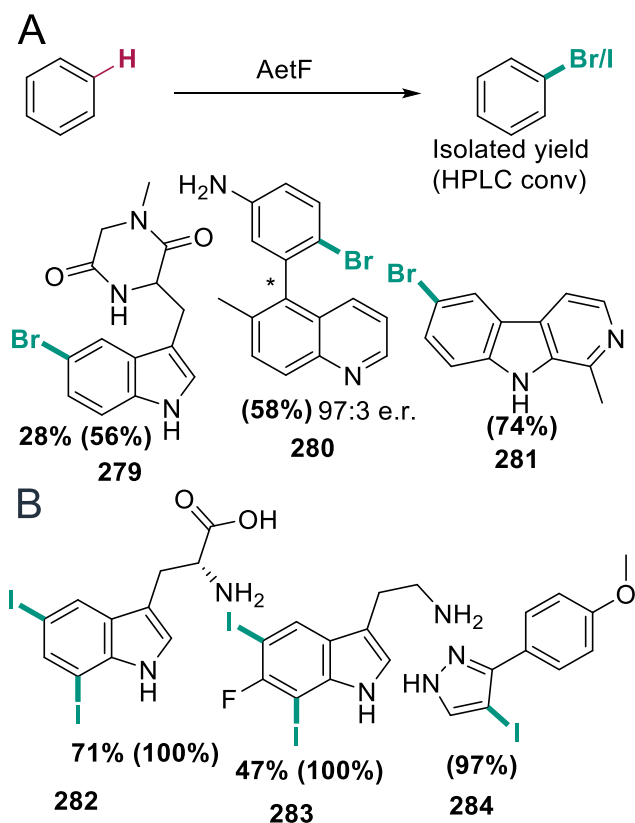
3.2.2. Aliphatic C-H Halogenation

Even in comparison to the relatively recent development of FDH biocatalysis, biocatalytic halogenation and of aliphatic C-H bonds is in its infancy. To date, only Fe(II)-/α-ketoglutarate dependent halogenases (FeDHs) can achieve this challenging reaction. These enzymes are similar to the analogous FeDOs discussed above, but their Fe(II) center lacks a conserved Asp/Glu residue found in FeDOs (Scheme 36). The resulting open coordination site allows FeDHs to bind halide ions (X = Cl, Br). FeDH proceeds in analogy to FeDO catalysis in that substrate binding leads to O_2 activation and formation of a reactive Fe(IV)(X)(oxo) intermediate capable of abstracting unactivated C-H bonds to generate a Fe(III)(X)(OH) intermediate and a carbon-centered radical. The presence of the X ligand leads to the possibility for X$^\bullet$ rebound to generate the halogenated product, which proceeds with high specificity in FeDHs.
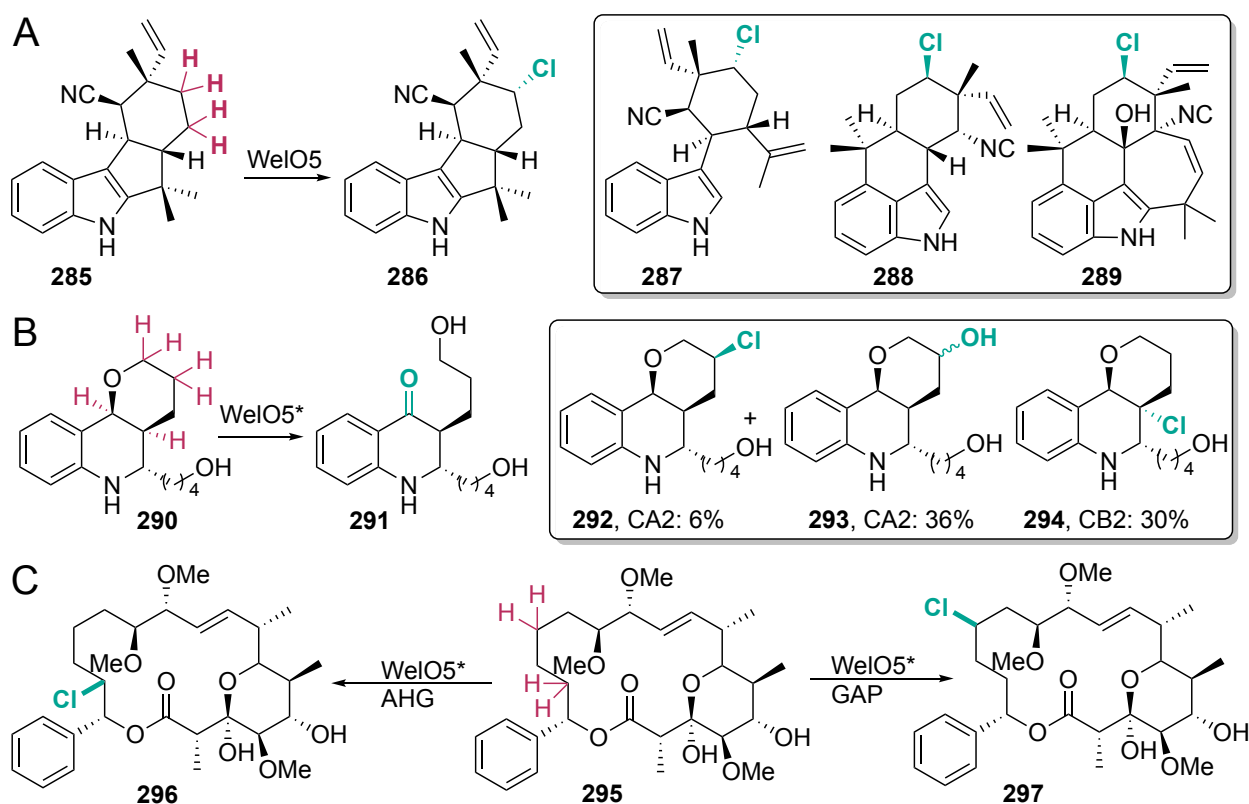
Scheme 48. Site-selective halogenation of fisherindole (**285**), hapalindole (**287** and **288**), and ambiguine (**289**) substrates by WelO5 and AmbO5. B) WelO5* and evolved variants catalyze halogenation of a martinelline-derived substrate to give products **291**, a mixture of **292** and **293**, and **294**.[337] C) Site-selective halogenation of soraphen A by evolved WelO5* variants.

The first FeDHs characterized acted on substrates linked to a carrier protein,[338-340] so it was unclear whether this class of enzymes would be useful for biocatalysis involving small molecule substrates. Eventually, however, it was established that two FeDHs, WelO5 and AmBO5, were responsible for site-selective installation of the chlorine substituents in the natural products welwitindolinone[341] and ambiguine[342], respectively. These enzymes could accept fisherindole,

hapalindole, and ambiguine substrates related to their native substrates (Scheme 48A), and WelO5 catalyzed selective bromination of its native substrate in the presence of excess bromide.[343]

WelO5 was the subject of the first efforts to engineer FeDH variants with substrate scope beyond close analogues of the natural fischerindole substrates. Buller used site-saturation mutagenesis of residues suspected to be involved in substrate binding to generate variants CA2 and CB2, which provided different chlorinated isomers of martinelline-derived substrate **290** (Scheme 48B).[337] On the other hand, CA2 provided only 6% yield of the chlorinated product, and the reaction was dominated by hydroxylation (36% yield of two diastereomers). CB2 gave 30% of a chlorinated product, but the site selectivity had changed to an adjacent tertiary position. This same group later screened libraries of WelO5* variants for activity on the macrolide soraphen A to identify the initial hit WelO5*-GAP.[344] Subsequent mutagenesis of active site residues established that a triple mutant dubbed SLP (WelO5*-GAP V81S, A88L, I161P) possessed 13-fold activity for formation of a single product isomer relative to the parent enzyme (Scheme 48C). Using the data from this initial library, machine learning was applied to predict functional sequences, which were then characterized experimentally. It was found that all seven ML-derived sequences were active with four outperforming the previous best SLP enzyme, and one ML-derived sequence also showed altered site selectivity, with enzyme variant WelO5*-AHG being highly selective for chlorination of C16 in soraphen A whereas the parent enzyme GAP was largely a C14 selective halogenase.
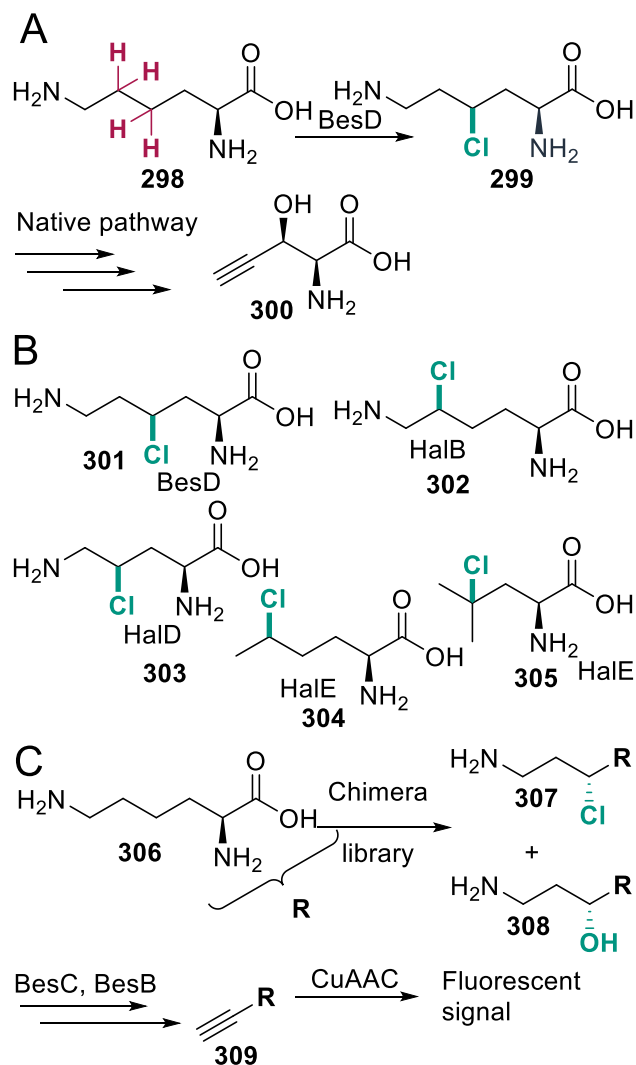
Scheme 49. BesD site-selective chlorination of amino acids and conversion of terminal chloride to alkyne. A) Natural product pathway of BesD results in alkyne formation.[345] B) Homologs of BesD site-selectively halogenate amino acid substrates. C) A fluorogenic click assay developed for high-throughput screening of chimera Hydox/Hal libraries.[346]

In an effort to expand the substrate scope of WelO5, a directed evolution campaign was conducted via screening for halogenation on compounds that contained a ketone rather than the typical isonitrile group present in the natural products accepted by these enzymes.[347] Although the wild-type enzyme had no activity for the substrate with the ketone replacing the isonitrile, a library constructed by an active-site scan of 7 residues deemed likely to influence substrate binding resulted in a variant capable of 30% halogenation. The activity was further improved by three additional rounds of evolution incorporating mutations found to be beneficial as single mutants, ultimately providing a panel of variants capable of preparative halogenation for a panel of related ketone containing haplaindole compounds with high site, chemo, and enantioselectivity.

The lysine halogenase BesD (Scheme 49A) has been successfully used as a template for the study and identification of FeDHs.[345] While the substrate scope of BesD proved limited in a full screen of L-amino acids, this enzyme was used as a template gene sequence in a BLAST search to identify active halogenases with activity on small aliphatic amino acids (Scheme 49B). Further exploration of the gene clusters identified in this study led to the identification of two closely related enzymes dubbed Hal (WP_122981682) and Hydrox (WP_107105619) which had over 70% sequence identity and both functionalized lysine with the same site selectivity favoring either hydroxylation or halogenation (Scheme 49C).[346] The native alkyne-forming pathway of BesABCD was used to enable a high-throughput screen for lysine chlorination, which led to the identification of residues crucial for favoring halogenation over hydroxylation. By comparing the location of the conserved residues in halogenase variants, two adjacent beta loops were identified as being crucial for maintaining both high chemoselectivity and activity. Such structural insights into this enzyme class may inform future engineering efforts.

3.3. C–H Amination, Azidation, Amidation

Remarkably, several anionic species can bind to the Fe(II) center in FeDHs and FeDOs facial triad mutants, and the resulting X-type ligand can undergo rebound with the substrate radical.[348] Transformations such as nitration, azidation, and cyanation have been demonstrated, although thus far only azidation has been successfully demonstrated for functionalization of small-molecules.[243] The ability FeDHs to accept peusdo-halides varies among the enzymes studied to date. Most FeDHs which are natively known for chlorination accept non-native anions but do so only in low yield or with poor chemoselectivity in the presence of chloride in the media.[349]

The FeDH SyrB2 was the first enzyme of this family to successfully carry out C-H functionalization with nitrogenous groups, catalyzing both nitration and azidation reactions.[348] The engineered FeDO SadX appears to be relatively broadly accepting of anions, demonstrating site- and chemoselective rebound for chloride, bromide, azide, and isocyanate.[243, 350] SadX was also used as the starting point in a directed evolution effort to evolve site- and chemo-selective azidation biocatalysts.[351] Over four rounds of evolution, the chemoselectivity increased 7.5-fold over the parent enzyme and total yield of the azidated product increased 6.7-fold. In keeping with the need for precise substrate positioning relative to the reactive iron center, the substrate scope for individual mutants from this lineage was narrow. Instead, a panel of enzymes from the lineage was used to site-selectively azidate a variety of *N*-succinylated amino acids (Scheme 50)**,** which were then readily functionalized to the reduced amine and the desuccinyalated products to produce modified amino acids. Azidation chemistry in FeDHs has also been accessed through an anaerobic abiological radical-relay reaction as well. While no site-selective examples were presented, a key development from this work was the synthesis and implementation of a pro-fluorogenic alkyne probe which enabled rapid, high-throughput screening of azidase libraries through the copper-catalyzed azide-alkyne cycloaddition.[352] Given the substrate-agnostic nature of the alkyne probe, this high-throughput screening platform should lay a foundation for future azidase evolution.
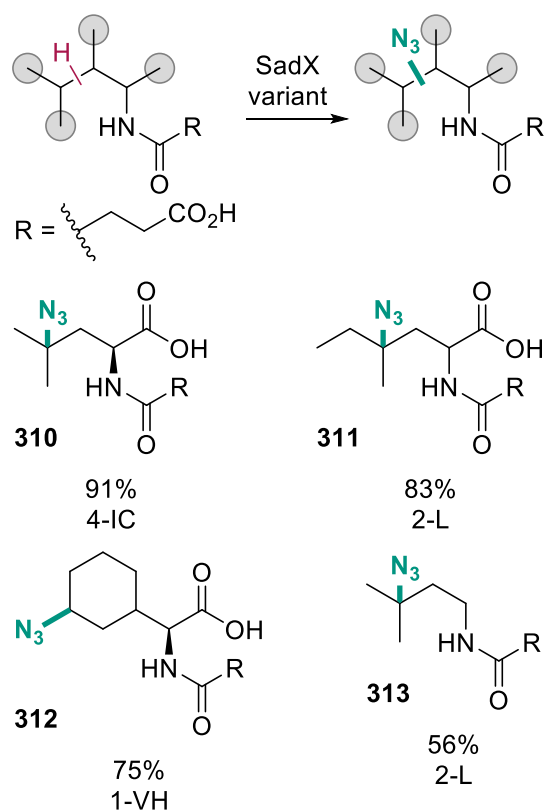
Scheme 50. Site-selective azidation by engineered SadX variants.[351]

Heme-containing proteins have also been used for site-selective C-H functionalization via C-N bond formation. For example, a BM3 variant (P411$_{BM3}$-CIS-T438S) containing key mutations that reduce monooxygenation activity (T268A) and alter the primary coordination sphere of the heme cofactor (C400S) displayed robust nitrene transfer activity.[353] Site-saturation mutagenesis of active-site residues in P411$_{BM3}$-CIS-T438S was then used to engineer variants with orthogonal site selectivity toward functionalization of benzylic and homobenzylic bonds, both with high enantioselectivity (Scheme 51A).[354] The variants developed in this study maintained the desired site selectivity for a small panel of related compounds. A similar reaction was catalyzed by an artificial metalloenzyme generated from the CYP119 enzyme from the thermophilic *S. solfataricus*.[355] In the absence of the protein scaffold, the free iridium cofactor generated the 5- (**317**) and 6-membered (**318**) rings in a 60:40 ratio, presumably dominated by the lower BDE of the benzylic C-H bond. When the Ir(Me)-PIX cofactor was encapsulated within the CYP119 variant this selectivity switched to 20:80 towards the less favored 6-membered ring **318** with a 84:16 e.r. (Scheme 51B).
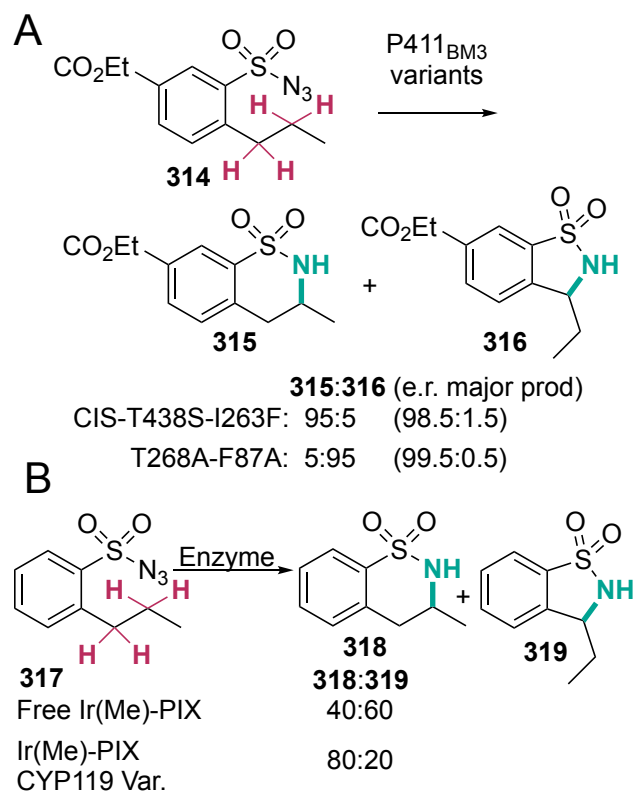
Scheme 51. Heme protein catalyzed intramolecular sulfamidation reactions. A) P411 enzymes evolved for regio-divergent intramolecular sulfamidation. B) A non-natural iridium heme cofactor exhibits modified site selectivity when incorporated into a CYP119 protein scaffold compared to free cofactor.

Intermolecular C-H amination reactions have also been developed. For example, site saturation mutagenesis of a P411$_{BM3}$ variant led to variant P411$_{BPA}$, which catalyzes site-selective amination of both *m*- and *p*-ethylmethylbenzene (among many other substrates) using hydroxylamine ester as a nitrene source (Scheme 52A).[356] A second evolution effort was used to engineer an allylic C-H aminase. The resulting P411$_{APA}$ enzyme demonstrated remarkable site selectivity for allylic C-H bonds, with up to 20:1 site selectivity observed for substrate bearing benzylic C-H bonds. Similarly high selectivity was obtained for α-terpinene, although usually P411$_{APA}$ had somewhat limited selectivity in the presence of multiple primary alkene C-H bonds. Intermolecular amidation of benzylic C-H bonds using P411 enzymes was also demonstrated,[43] and constitutional isomers were not detected in examples bearing both secondary and primary C-H bonds (Scheme 52B).
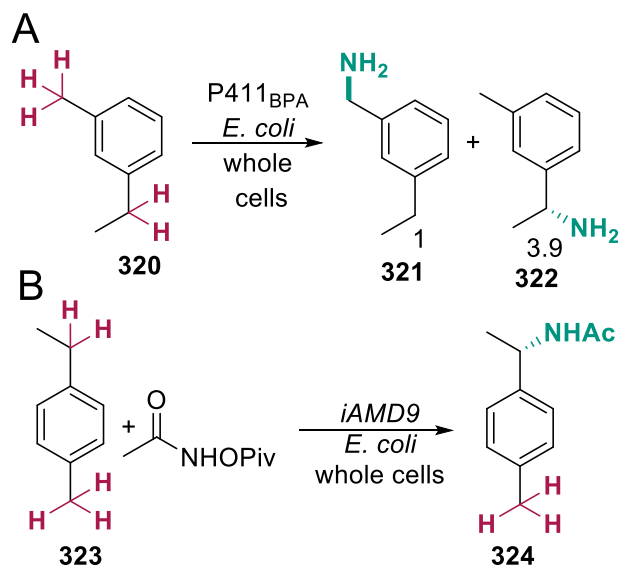
Scheme 52. Intermolecular amination reactions with heme proteins. A.) P411$_{BPA}$ and B) uAMD9 expressed in whole-cells are capable of site-selective functionalization amination and amidation of benzyl C-H bonds.

Perhaps most impressively, BM3 variants capable of functionalizing unactivated C-H bonds via amidation or amination was recently reported.[357] The latter reaction constitutes the nitrogen analogue of the native P450 reaction. By screening variants previously developed for nitrene insertion chemistry, researchers from the Arnold lab identified two variants with low activity for 3-amidation and 2-amination of methylcyclohexanone. Over 9 rounds of evolution, the activity and selectivity of these enzymes were improved to ultimately provide two variants uPA9 and uAMD9, which were 86% and 91% site selective for the 3- and 2- position respectively (Scheme 53). These enzymes showed high site selectivity toward several cyclic and acylic compounds, and promising activity (though low selectivity) was observed on many additional substrates. Through the kinetic isotope effect and computational efforts, site selectivity was found to be largely determined by the steric environment of the protein active site, whereas the stereoselectivity was determined by both an irreversible HAT and the subsequent radical rebound.
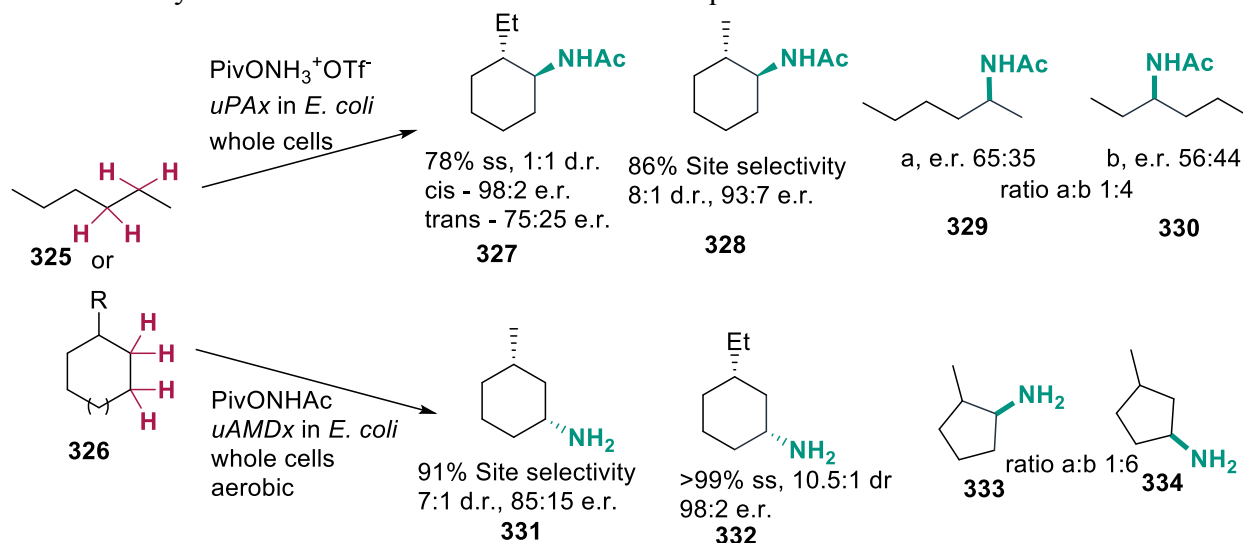
Scheme 53. Evolution of P450 variants for site-selective amidation and amination of unactivated C-H bonds.

3.4. C–H Deuteration

Deuteration is a nearly traceless way to modify a compound that has both biochemical and therapeutic applications. Recently, the isoleucine epimerization enzyme DsaD and the partner protein DsaE were used to enable selective αβ, α, and β-deuteration of amino acids via selective epimerization.[358] Reaction of the amino acid with DsaD in D_2O resulted in selective α-deuteration, whereas by reactions with DsaD and excess DsaE led to deuteration of both the α- and β- positions (Scheme 54A). Incubating the α- and β-trideuterated compound with DsaD in protic water led to selective α-protonation, resulting in the selectively β-deuterated product (Scheme 54B). The DsaD/DsaE system showed efficient α-deuteration for non-polar amino acids, although the trends for DsaE assisted β-deuteration were less clear. Derivatization of the resulting deuterated amino acids with Marfey's reagent revealed that in most cases, perfect stereoretention was observed regardless of whether the α-, β-, or α,β-deuterated product was obtained. Given the increasing importance of peptide-based drugs, the ability to site-selectively deuterate these building blocks could prove useful for future drug development.
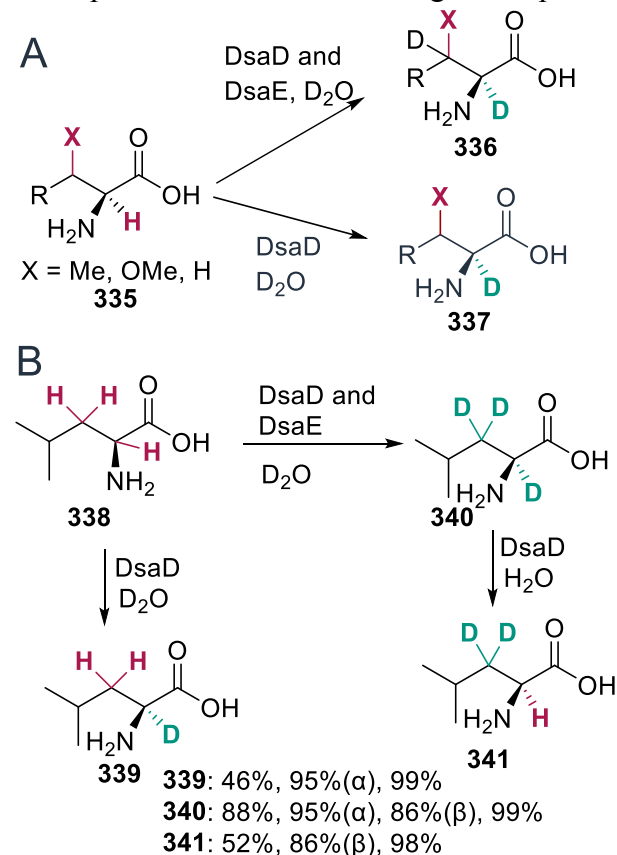
Scheme 54. Biocatalytic deuteration of amino acids. A) & B) By using DsaD alone or in combination with DsaE, selective deuteration of either α, β, or both can be accomplished by incubation in D_2O or H_2O; reaction outcomes are formatted as % recovery, % deuteration (site), and $ ee.[358]

3.5. C–C Bond Forming Reactions

3.5.1 Oxidative Coupling

Cross-coupling reactions are widely used to form biaryl compounds from pre-functionalized coupling partners like aryl halides and aryl boronic acids.[359] A wide range of C-H functionalization methods have been developed to form biaryl compounds while avoiding the need for functionalization of one or both coupling partners.[360, 361] One approach to accomplish the latter involves oxidative coupling, in which the biaryl bond is forged with formal loss of H_2 from the reacting aromatic coupling partners. Chemical methods for oxidative coupling are often limited by poor site selectivity.[362, 363] Biaryl bond formation in nature often proceeds via oxidative coupling, but enzymes enforce remarkable site-, chemo- and enantioselectivities on these reactions. Extending the scope of these reactions to enable site-selective non-native oxidative coupling is therefore highly desirable.

One of the most well-studied examples of intramolecular biaryl bond formation via oxidative coupling in natural product biosynthesis is the OxyC-catalyzed coupling of the A and B rings in vancomycin.[364] OxyC and other P450s[365] involved in the formation of vancomycin have been studied for biocatalytic synthesis of related compounds, but the need for a PCP-tethered substrate limits the practical application of these enzymes. It was recently discovered that the enzyme AryC accomplishes a similar intramolecular C–C coupling reaction using only a lipophilic chain instead of a PCP tether to anchor the substrate within the enzyme active site.[366] Recently, a P450 from *Streptomyces* sp. MG-AR, originally identified for its ability to hydroxylate testosterone, was engineered to enable gram-scale oxidative coupling of the arylomycin core, demonstrating scalable production of this medicinally relevant motif (Scheme 55A).[367] This study constitutes the first example of a native hydroxylase being engineered for oxidative C–C bond formation, suggesting that other P450s originally studied for oxygenation could be used for site-selective oxidative coupling.
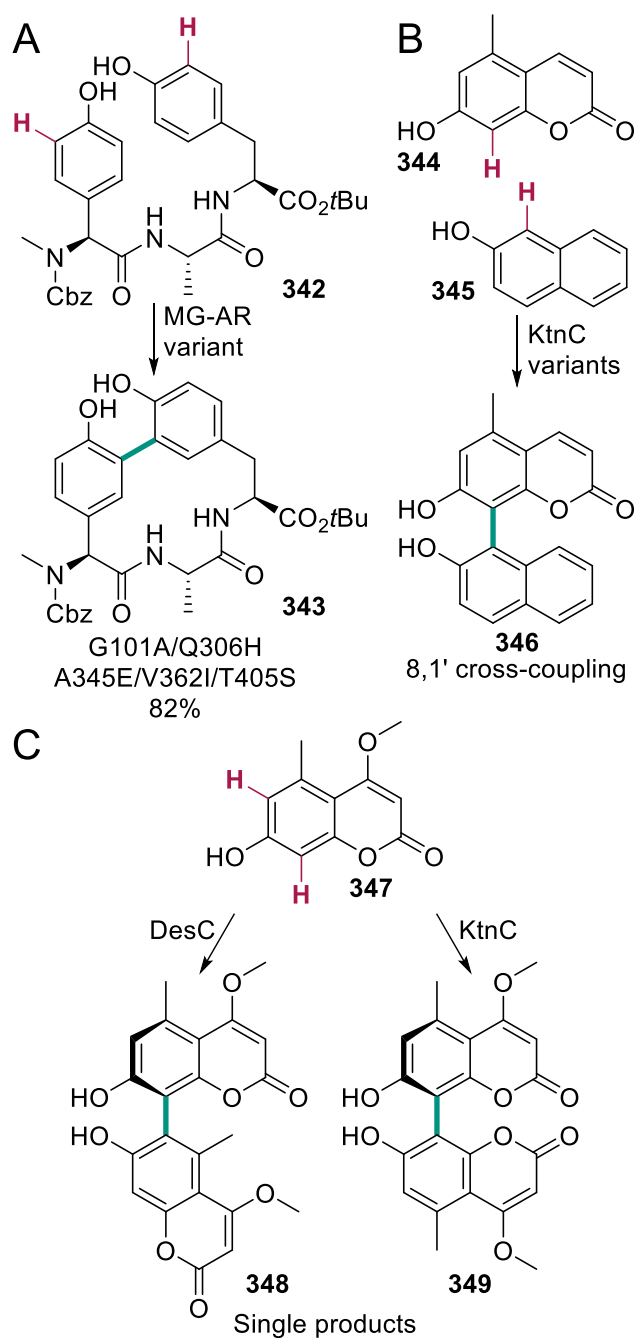
Scheme 55. Intra- and intermolecular site-selective cross-coupling reactions. A) Percent of product pool in the synthesis of the arylomycin core by P450 MG-AR variant.[367] B) Intermolecular cross-coupling of non-identical monomers by KtnC.[368] C) Reactions of 7-demethylsiderin (**347**) with enzymes KtnC and DesC.[369]

Most efforts aimed at understanding P450-catalyzed oxidative couplings focus on identifying enzymes that natively catalyze these reactions. For example, the enzyme DesC from *E. desertorum* was found to catalyze site- and atroposelective oxidative homo-coupling of 7-demethylsiderin (**347**) to produce the unsymmetrical 6,8'-dimer **348**, showing strong catalyst control of selectivity.[369] Similar catalyst-controlled site selectivity was observed for the enzymes AunB and

BfoB, which produce 10,7'- and 10,10'-coupled products of fonsecin B and rubrofusarin B.[370] A related P450, KtnC was shown to catalyze site- and atroposelective intermolecular coupling of **347** to produce the natural product P-orlandin via 8-8' coupling (**349**, Scheme 55C), but related substrates were not successfully coupled to the corresponding dimers.[369] More recently, directed evolution targeting residues in the active site of KtnC was used to improve the activity and site selectivity for the 8,1'-cross coupled product **346**, albeit at the expense of enantioselectivity (Scheme 55B).[368] Further evolution led to variant LxC7 with improved site selectivity and enantioselectivity comparable to the parent enzyme (80:20 e.r. wild-type KtnC, 77:23 e.r. LxC7), but this variant had lower total activity than previous enzymes in the lineage for the substrate used in the evolution. Genome mining of the sequence space around KtnC identified several additional P450s capable of intermolecular coupling, including several examples where KtnC had no activity.

3.5.2. Prenylation

Prenyl-, gerenyl-, and farnesyltransferases catalyze nucleophilic substitution of the respective organic phosphates by electronically activated aromatic compounds or heteroatom substituents. The former reactivity is typified by the site-selective functionalization of tryptophan (**350**) by a class of prenyltransferases known as dimethylallyltryptophan synthases (DMATSs, Scheme 56A). The enzymes DmaW[371] and FgaPT2[372] have also been used to produce 4-prenyl-L-tryptophan, and shortly after its discovery, the substrate scope of FgaPT2 was elucidated by modifying both the alkyl and aryl substituents.[373] While these substrates were typically prenylated in low yield, this enzyme was later found to be competent for the site-selective prenylation of cyclo-L-Trp-L-Tyr and other diketopiperazines, albeit at higher enzyme loading.[374] A related 4-DMATS enzyme from the organism *A. japonicus* was recently found to accept a variety of tryptophan derivatives with exceptional site selectivity in high yields even at relatively high substrate loadings.[375]

The 4-DMATS enzymes were the first prenyltransferases to be characterized, and shortly thereafter the 7-DMATS analogue from *A. fumigatus* was discovered.[376] This enzyme has a broader substrate scope than FgaPT2.[377] The first 6-DMATS enzyme characterized was IptA, which selectively prenylates the C-6 position of L-tryptophan and various indole compounds.[378] These C-6 prenyltransferases were present in many organisms, and two homologs of these enzymes from *S. ambofaciens* (6-DMATS$_{Sa}$) and *S. violaceusniger* (6-DMATS$_{Sv}$) were used as catalysts for prenylation of a variety of hydroxynapthalenes and tryptophan derivatives.[379] Unlike previously described DMATS enzymes, 6-DMATS$_{Sa}$ was also capable of using GPP as an alkyl donor, though it did not accept FPP. A unique 6-DMATS designated PriB was found to accept a variety of prenyl acceptors, including phenazine-2,3-diamine, 1,4-diaminoanthraquinone, and pindolol, and even different alkyl donors, including geranyl diphosphate, farnesyl diphosphate, and a variety of farnesyl diphosphate analogs with phenoxy-, anilino-, phenylsulfanyl-, and benzyl-substitutions.[380]

Scheme 56. A) General scheme of the native prenyltransferase reaction by dimethylallyltryptophan synthases (DMATSs) and representative non-native products for DMATSs with different site selectivity.[373, 381] B) Geranylation reactions on genistein (**359**) by AtaPT single mutants.[382]
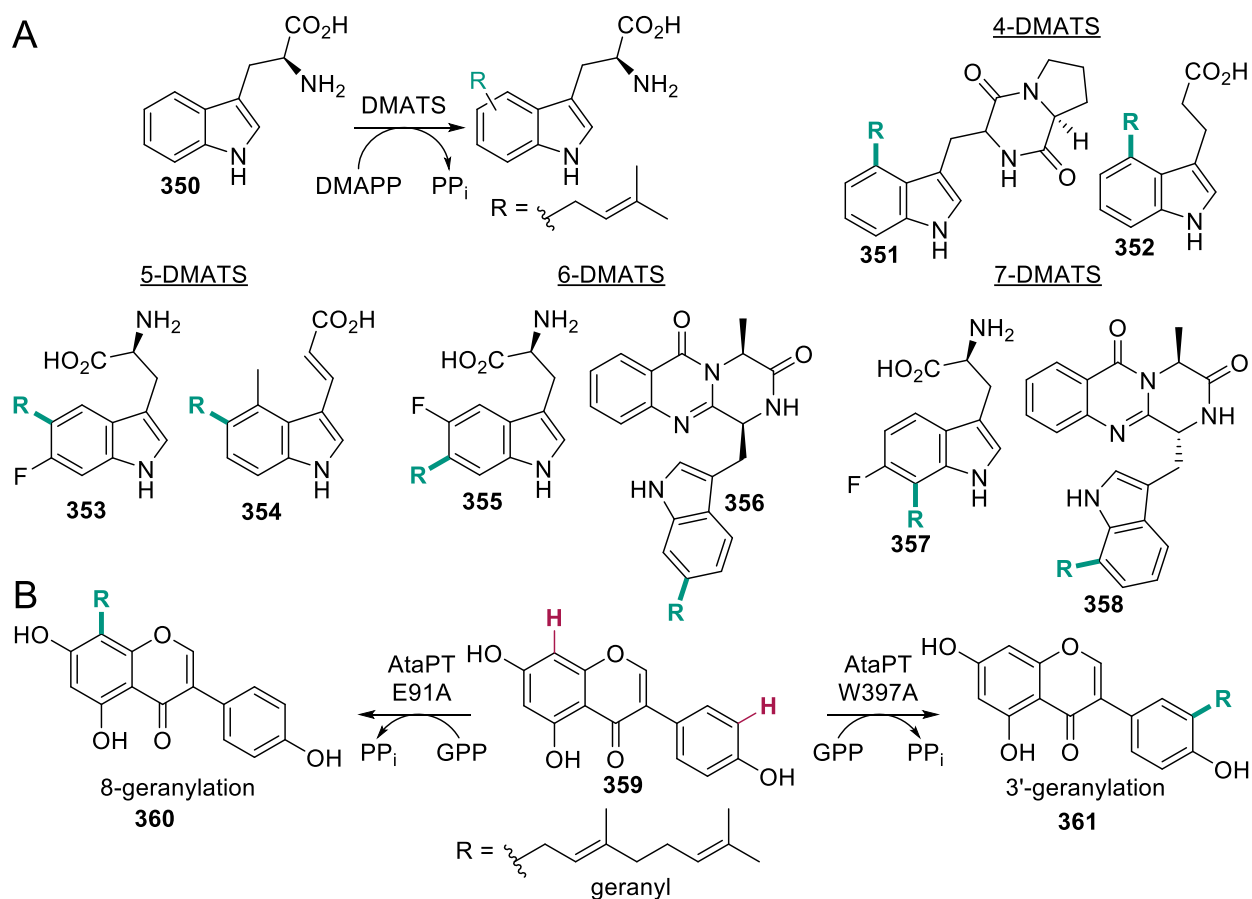
DMATS-catalyzed functionalization of the remaining position on the benzene ring of indoles was enabled by the discovery of the C-5 prenyltransferase from *A. clavatus*.[383] This enzyme and the later-discovered C-5 prenyltransferase 5-DMATS$_{Sc}$ can also transfer non-native alkyl chains, including benzyl and 2-pentenyl groups.[384] Yields for these reactions were typically 5-15% those involving the native DMAPP donor, but they provide viable starting points for further engineering. While 5-DMATS remain relatively rare, the 4-DMATS FgaPT2 catalyzes C-5 alkylation when non-native alkyl donors like 2-pentenyl, 1-methyl, and benzyl phosphate are used.[385] The ability to control site selectivity was also shown in an effort to functionalize different sites on the four diastereomers of a fumiquinazoline substrate with a DMATS panel.[381] This effort also highlighted the stereoselectivity of DMATS catalysis. For example, perfect site selectivity was observed for C-7 prenylation of the (11S,14S) diastereomer using a 7-DMATS, but a 2.5:1 C-6:C-7 ratio was observed for (11R,14R) diastereomer.

DMATS-catalyzed alkylation of substrates other than those containing indole cores has also been demonstrated. For example, the activity of a prenyl transferase from *A. terreus* (AtaPT) was examined on over 100 substrates, including lignanoids, quinoline alkaloids, xanthones, coumarins, and flavonoid glycosides. Of these substrates, 46 were prenylated in over 5% conversion, and 26

additional substrates provided measurable levels of product.[382] While AtaPT generally prenylated alcohols, activated aromatic sites on several flavonoid compounds and umbelliferone were also prenylated. This enzyme also showed both evolvability and a broader than usual acceptance for prenyl donors, as the single mutants W397A and E91A were capable of selectively geranylating genistein (**359**) whereas the wild-type enzyme digeranylated the compound (Scheme 56B).

3.5.3. Radical Coupling Reactions

Radical SAM enzymes catalyze a wide range of reactions, including site-selective sp^3 C–C bond formation,[386] as exemplified by the sequential methylation of carbapenam scaffold **362** by Tokk to forge an isopropyl group (Scheme 57A).[387] While some radical SAM enzymes, including TsrM, which was actually found to employ a polar mechanism for methylation,[388] have been explored as biocatalysts, such applications remain rare. To facilitate radical SAM biocatalysis by eliminating the need for stoichiometric SAM in these reactions, a SAM regeneration system described above (Scheme 12B)[72] was further developed and used for selective alkylation of a glutamine residue in a 24-mer polypeptide (**366** Scheme 57B).[389] This one-pot system involves ribophosphorylation of adenine and two subsequent phosphorylations to produce ATP, which is used by a methionine adenosyltransferase to produce SAM. This regeneration system was used with cobalamin-dependent glutamine C-methyltransferase (QCMT) to alkylate **366**, showing the ability of this approach to generate SAM analogues by replacing the addition of methionine by ethionine (Scheme 57B).
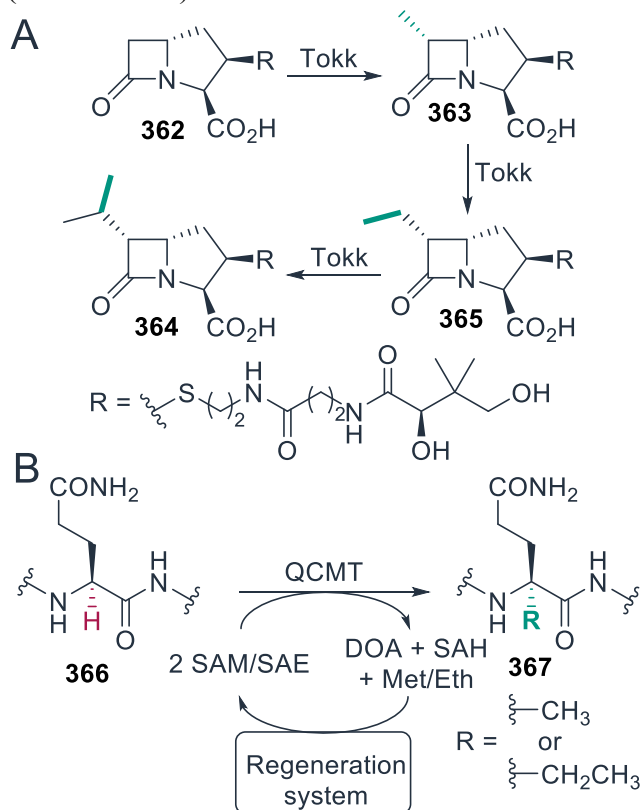
Scheme 57. A) Sequential methylation of carbapenem substrate **362** by SAM-dependent methylase Tokk.[387] B) Alkylation of the glutamine residue in a 24-mer polypeptide substrate by

QCMT employing a one-pot SAM regeneration system.[389] SAE: S-adenosylethionine, DOA: 5'-deoxyadenosine, SAH: S-adenosylhomocysteine, Met: L-methionine, Eth: L-ethionine.

Although they lack catalytic activity themselves, dirigent proteins (DIRs) also mediate site- and enantioselectivity of radical C–C coupling reactions.[390] DIRs are involved in lignan biosynthesis by binding and controlling the reactivity of free radical lignol intermediates that would otherwise result in a mixture of products.[391] The DIR from *Podophyllum hexandrum* (*Ph*DIR) was used in a study that focused on the heterocoupling of coniferyl alcohol analogues when used with the fungal laccase *Tv*Lac.[392] The addition of *Ph*DIR resulted in an increase of the 8-8' isomer both for the homo- and heterodimer products when compared to the 8-5' isomer (Scheme 58A). This study not only showcased the control that DIRs can exert on radical coupling reactions, but also the synthetic relevance of *Ph*DIR due to its substrate flexibility.
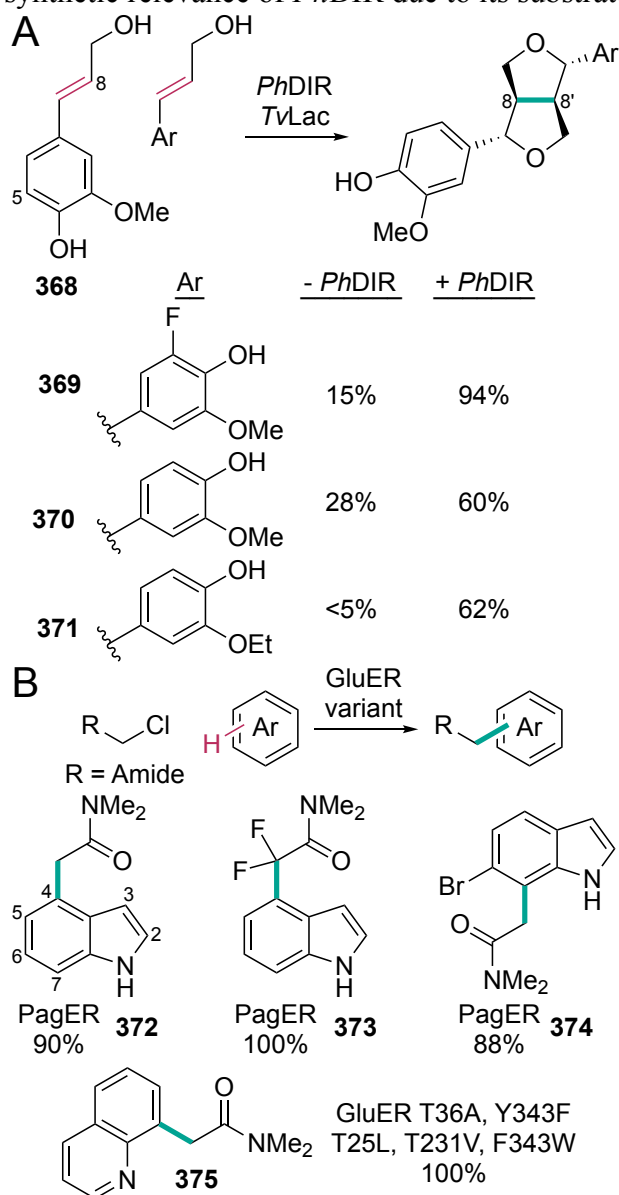
Scheme 58. A) Radical heterocoupling of coniferyl alcohol analogues by laccase *Tv*Lac and the effect of dirigent protein *Ph*DIR in the 8-8' to 5-8' products ratio.[392] Percentages represent the

amount of 8-8' heterodimer product compared to the 5-8' heterodimer product. B) Non-native heteroarene alkylation by ene-reductases.[393] Site selectivity expressed as the percentage of the shown compound in the total product pool.

In recent years, flavin-dependent ene-reductases (EREDs) have been engineered to carry out non-native light-promoted C–C bond forming reactions.[394-396] A recent study used a previously optimized variant of the ERED from *Gluconobacter oxydans*, GluER T36A/Y343F, as the parent enzyme for a directed evolution campaign aimed at developing a biocatalyst for the selective alkylation of heteroarene substrates.[393] The final enzyme, PagER, accumulated five additional mutations and was able to override the initial preference for alkylation of indole at the C-2 and C-3 positions in favor of C-4, with a ratio of 9:1 for C-4:(C-2 + C-3) (Scheme 58B). PagER and other GluER variants derived from its lineage were shown to have high site selectivity on a panel of both electron rich and electron poor arenes.

3.5.4. Carbene and Nitrene Insertion into C-H Bonds

The utility of cytochromes P450 for site-selective C-H hydroxylation and olefin epoxidation were outlined above, but these enzymes have more recently be explored as catalysts for non-native carbene and nitrene insertion reactions.[397] Early studies toward this end established that cytochrome P450$_{BM3}$ variant P411, whose native cysteine axial ligand was substituted for serine as noted above, provide selective olefin cyclopropanation over the native hydroxylation chemistry.[398] Several rounds of directed evolution and removal of the P411 FAD domain led to variant P411-CHF, which catalyzes carbene insertion into sp^3 C-H bonds at benzylic, allylic, propargylic, and α-amino sites.[399]

Most importantly for this discussion, P411-CHF catalyzes site-selective alkylation of *N*-methyltetrahydroquinoline with a selectivity of 9:1 for alkylation of the 2-position of the tetrahydroquinoline moiety over alkylation of the methyl group (Scheme 59A). A variant from the same lineage, P411-gen5, showed superior selectivity (>50:1) and formed the opposite enantiomer, demonstrating the utility in developing a panel of enzymes for a given transformation. A similar lineage of P411 enzymes was later developed and found to catalyze alkylation of a simple *N*-methylpyrrole with orthogonal site selectivity depending on the variant used.[400] Four rounds of evolution of P411-C10 generated variant L9, which catalyzes site-selective functionalization of *N*-ethyl-*N*-methylaniline (**379**) with a preference of up to 99:1 for the methyl carbon over the ethyl carbon (Scheme 59B).[401] A second variant developed from this lineage, P411-L10, had a significantly lower selectivity of 71:29 for the methyl carbon but showed high diastereoselectivity and enantioselectivity for the minor product, suggesting this enzyme could be further evolved for this minor product if desired.
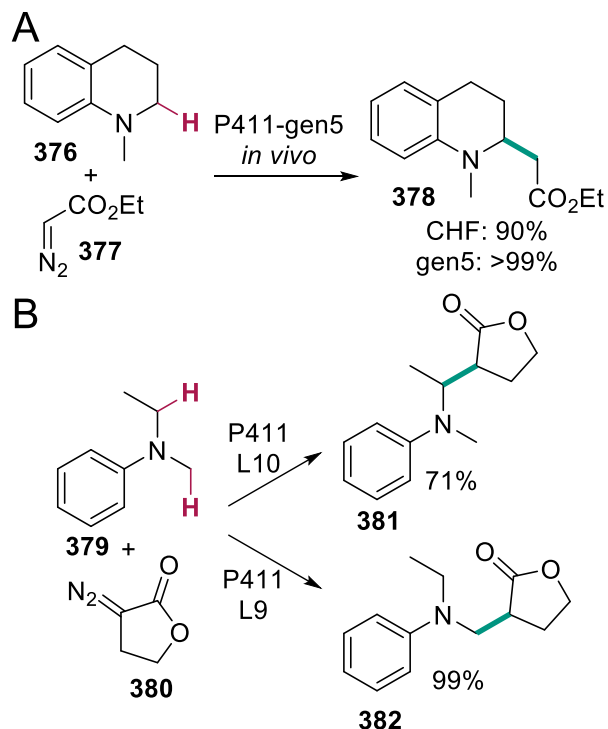
Scheme 59. Site-selective carbene transfer reactions catalyzed by P411 variants to functionalize sp³ C–H bonds. Major products for each reaction are presented with the corresponding percent of product pool. A) Alkylation of the 2-position of the tetrahydroquinoline moiety of *N*-methyltetrahydroquinoline (**376**), with remaining product pool being the alkylation of the methyl group.[399] B) P411 variants selective for methyl and ethyl alkylation of *N*-ethyl-*N*-methylaniline (**379**).[401]

Another approach to site-selective enzymatic carbene insertion involves replacing the native heme cofactor with metal-substituted protoporphyrin IX derivatives.[402, 403] A recent report showed that directed evolution of an iridum-containing P450 artificial metalloenzyme enabled site-selective alkylation of substituted benzofurans and demonstrated alkylation with divergent selectivities for *meta* and *para* positions to various substituents, with an intermediate variant from the lineage showing high enantioselectivities in a subset of select reactions (up to 97:3 e.r., Scheme 60A).[404] Streptavidin-based artificial metalloenzymes have also been used for site-selective carbene insertion. Specifically, a streptavidin variant containing a biotinylated copper(I) heteroscorpionate complex catalyzed cyclization of aryl amides resulting in the formation of β- and γ-lactams. Site-saturation mutagenesis of hotspots within the streptavidin scaffold resulted in two variants for selective formation of the 4- and 5- membered lactam (Scheme 60B).[405]
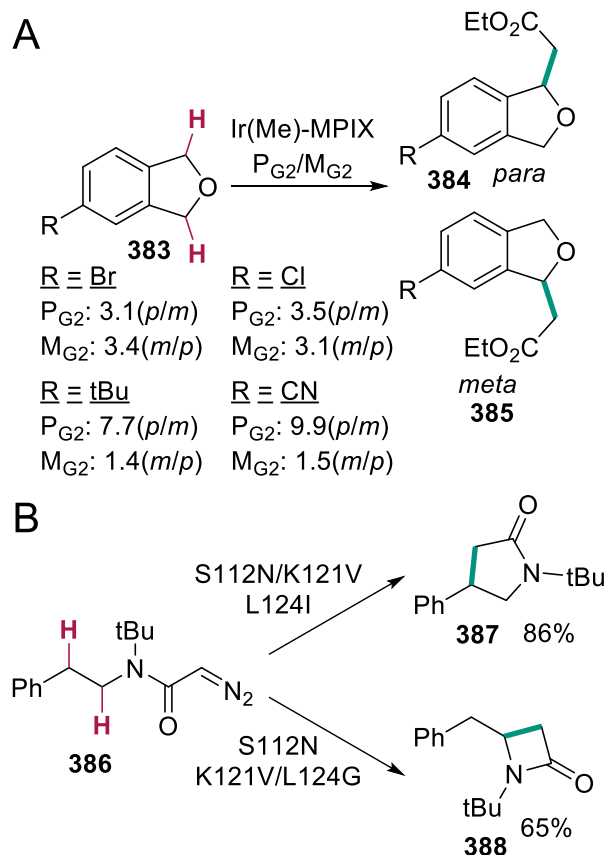
Scheme 60. Site-selective alkylation via carbene transfer by artificial metalloenzymes. A) Distribution of *para* and *meta* products in the alkylation of several benzofurans by artificial iridium-containing P450s.[404] B) Lactam formation via intramolecular cyclization by engineered artificial copper-streptavidin metalloenzymes with major products displayed with the corresponding percent of product pool.[405]

4. Applications of Site-Selective Enzyme Catalysis

The ability to insert functional groups into molecules at will opens up a variety of subsequent bond forming capabilities. When paired with the vast arsenal of chemoselective methods available to synthetic chemists, site-selective catalysis enables a range of chemoenzymatic methods and novel synthetic disconnections for target-oriented synthesis.

4.1. Chemoenzymatic Methods

Nearly all transformations discussed above result in functionality that can be used in subsequent transformations. Some, including hydroxylation, halogenation, and azidation, are particularly notable in that they have been paired with chemical transformations to enable bond disconnections that are not known for any enzymes. For example, P450-catalyzed C-H hydroxylation has been coupled with deoxyfluorination to enable chemoenzymatic fluorination of unactivated C-H bonds (Scheme 61A).[204, 206, 219] While fluorinases are known, these enzymes act on the adenoside fragment of their SAM cofactor via an S_N^2 reaction that defines their site selectivity, so the chemoenzymatic approach provides far greater generality.[406] P450-catalyzed demethylation has

also been paired with either Barton-McCombie deoxygenation or glycosylation to enable site-selective sugar modification.[204] Site-selective azidation catalyzed by the engineered Fe(II)/KG dependent oxygenase SadX (Scheme 50) has also been combined with both reduction and click chemistry to enable access to amines and triazoles.[351]

Given the importance of halogenation in a wide range of transformations, particularly transition metal catalyzed cross-coupling, it is perhaps not surprising that enzymatic halogenation has been paired with a variety of reactions to enable site-selective formation of carbon-carbon and carbon heteroatom bonds. Early efforts towads this end focused on using wild type FDHs to access natural product derivatives with halide substitution that enabled subsequent Suzuki-Miura coupling to access biaryl derivatives (Scheme 61B).[407, 408] The use of engineered FDHs allowed for extension of this approach to diverse substrates and different cross-coupling reactions,[409] and the use of membrane-divided reactors allowed for the halogenation and cross-coupling steps to be conducted in a two-phase "one-pot" fashion.[410] Concurrent halogenation/cross-coupling was achieving using a whole-cell biocatalyst and a Pd catalyst in the culture media.[411] This transformation was also used to enable a high throughput screen for FDH activity,[412] and cross-linked enzyme aggregates comprised of different FDHs, a FRED, and an alcohol dehydrogenase were shown to be effective catalysts.[411] Extending this approach even further, enzymatic halogenation and Pd-catalyzed cyanation has been followed by either nitrile hydratase or nitrilase catalysis to enable site-selective installation of nitrile, amide, or carboxylic acid groups.[174]
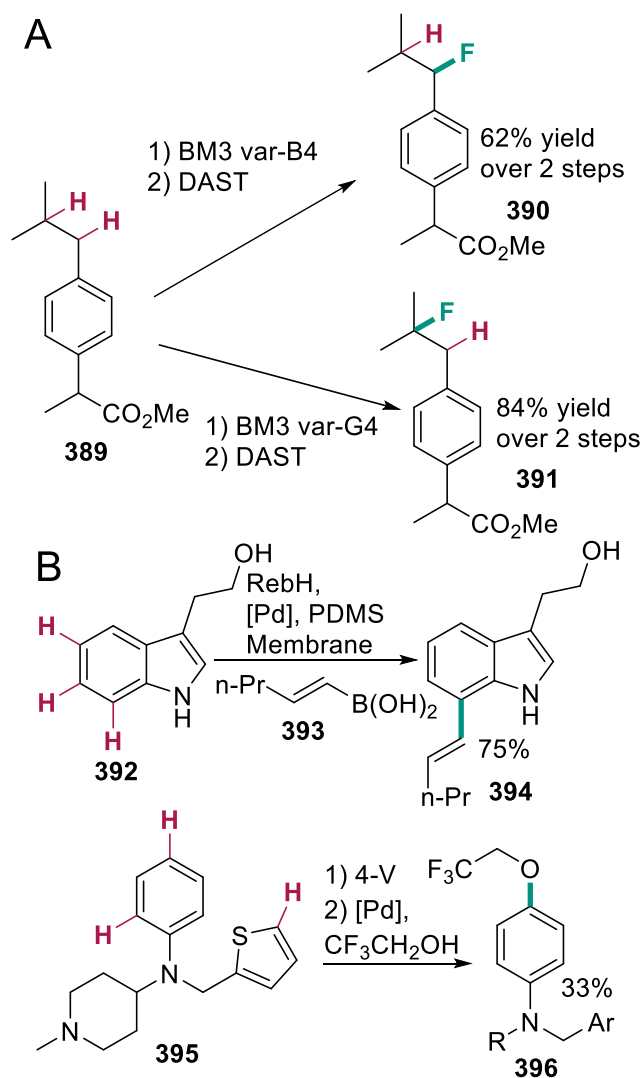
Scheme 61. A) Representative examples of site-selective chemoenzymatic hydroxylation/deoxy fluorination and B) halogenation/cross-coupling.

4.2. Target-Oriented Synthesis

Site-selective enzyme catalysis has also been used to increase the efficiency of synthetic routes to natural products and other compounds. Importantly, however, these efforts have been limited relative to chemoselective enzyme catalysis due to the fundamental challenge of identifying enzymes with the *correct* site selectivity and substrate scope, since the native activity of many enzymes often provides little information regarding their non-native catalytic potential and scope. This is particularly challenging for enzymes like cytochromes P450 and flavin dependent halogenases, both of which have been shown to functionalize a broad range of substrates at different sites.[15, 328, 330, 413] In methodology efforts, this capability is a highlight since it shows the versatility of these enzymes, but target oriented synthesis demands *specific* site selectivity that might prove elusive using known enzymes. Despite these difficulties, several promising examples

of target-oriented synthesis enabled by enzymatic site-selective functionalization show the potential of this approach.
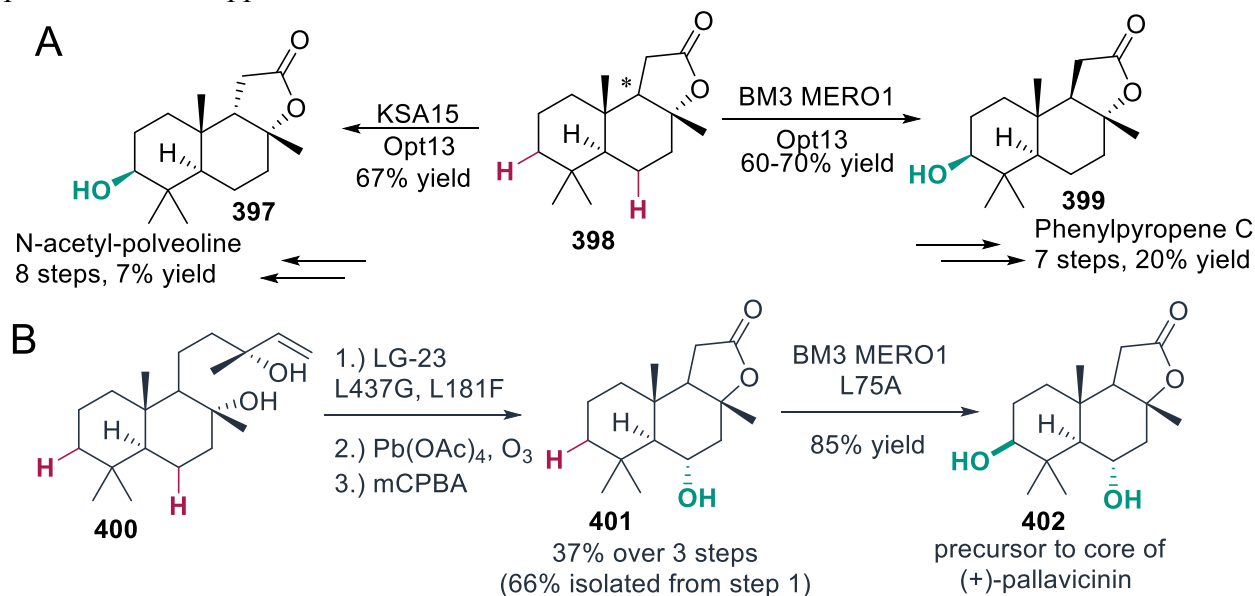
Scheme 62. P450 BM3 variants utilized for site-selective C-H oxyfunctionalization in the total synthesis of terpenoid products. A) BM3 MERO1 and KSA 15 are both highly selective for the C3 hydroxylation of sclareolide and episclareolide respectively. These selectively functionalized compounds were then used for the concise construction of natural products. B) Sequential gram-scale oxidation of sclareol with BM3 variants LG-23 and MERO1 provide a precursor for the core of (+)-pallavincin.

To date, target-oriented site-selective enzyme catalysis has largely focused on C-H oxyfunctionalization reactions catalyzed by enzymes such as P450s and Fe(II)-dependent hydroxylases. For P450s, these efforts are facilitated by decades of research that has revealed key residues responsible for selectivity and engineered variants that provide excellent starting points for site-selective catalysis even on relatively complex scaffolds. For example, based on previous data regarding the substrate scope of BM3 variant IL-H8,[218] which bears 15 mutations from wild-type BM3, a panel of BM3 variants with mutations similar to IL-H8 were screened to identify variants with activity on sclareolide.[414] Alanine scanning of the top hit from this panel provided BM3 MERO1, which hydroxylated C-3 of sclareolide in 60-70% isolated yield on gram scale, enabling the total synthesis of phenylpropene C. A related BM3 variant dubbed KSA15 exhibited the same site selectivity for episclareolide, providing a precursor for the synthesis of *N*-acetyl-polveoline (Scheme 62A).

BM3 variant LG-23 was similarly found to enable hydroxylation of C-6 of sclareol.[210, 214] This enzyme was further improved through two rounds of directed evolution.[415] The final variant contained two additional mutations and allowed for the gram-scale production of the 6-hydroxylated compound **401** coupled to oxidative chemistry. Using the complementary hydroxylase BM3 MERO then afforded product **402** (Scheme 62 B). These sequential oxidation reactions allowed for the efficient conversion of this compound into product **402**, a precursor to the core moiety found in (+)-pallavincin. Demonstrating incorporation into longer synthetic

sequences, MERO1 L437A was used to produce 3-hydroxylated sclareolide in the first total synthesis of gedunin, accomplished in 13 steps in 5.5% yield.[416]
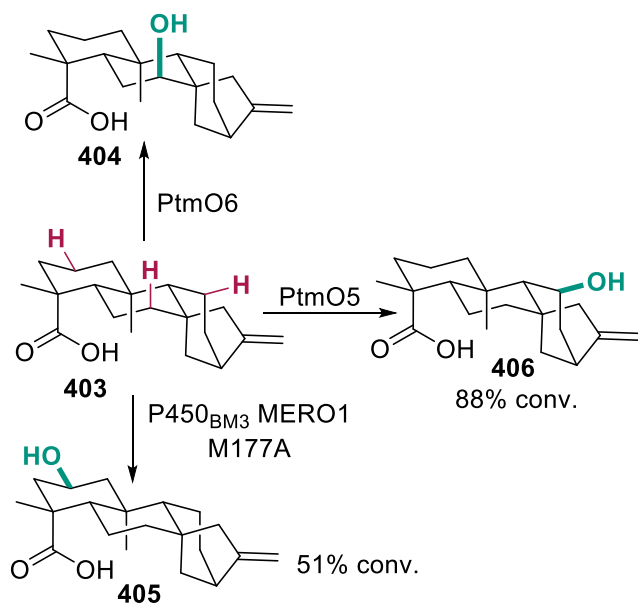
Scheme 63. Site-selective oxidation of a complex diterpenoid using P450 and FeDO enzymes. Integrating these enzymes into synthetic logic for this substrate class enabled the construction of 9 natural products in 10 or fewer steps.

Complementing utility of engineered P450 BM3 variants, the diversity of enzymes that catalyze C-H hydroxylation provides a means to rapidly identify enzymes that might be better suited to different targets. For example, BM3 MERO1, a fusion of the P450 PtmO5 with the reductase RHFRed, and the FeDO PtmO6, were used to enable site-selective oxidation of stevioside aglycone **403** at three different sites (Scheme 63).[417] These selectively oxidized compounds provided functional handles for further diversification through traditional organic chemistry and ultimately provided access to nine natural products. The reported syntheses often included sequential biocatalytic oxidations using evolved enzymes and shortened reported syntheses for these compounds significantly. While bacterial P450s such as PtmO5 and BM3 are often easier to handle than enzymes from eukaryotic sources, enzymes sourced from higher organisms can yield catalysts bearing new function. This was demonstrated in the synthesis of several cardiotonic steroids using a fungal P450 in *C. lunata*,[418] capable of producing more than 5 g of 14α-hydroxy-androst-4-ene-3,17-dione. This hydroxylated intermediate was used to produce bufogenin B and digitoxigenin in 11 and 12 steps respectively.
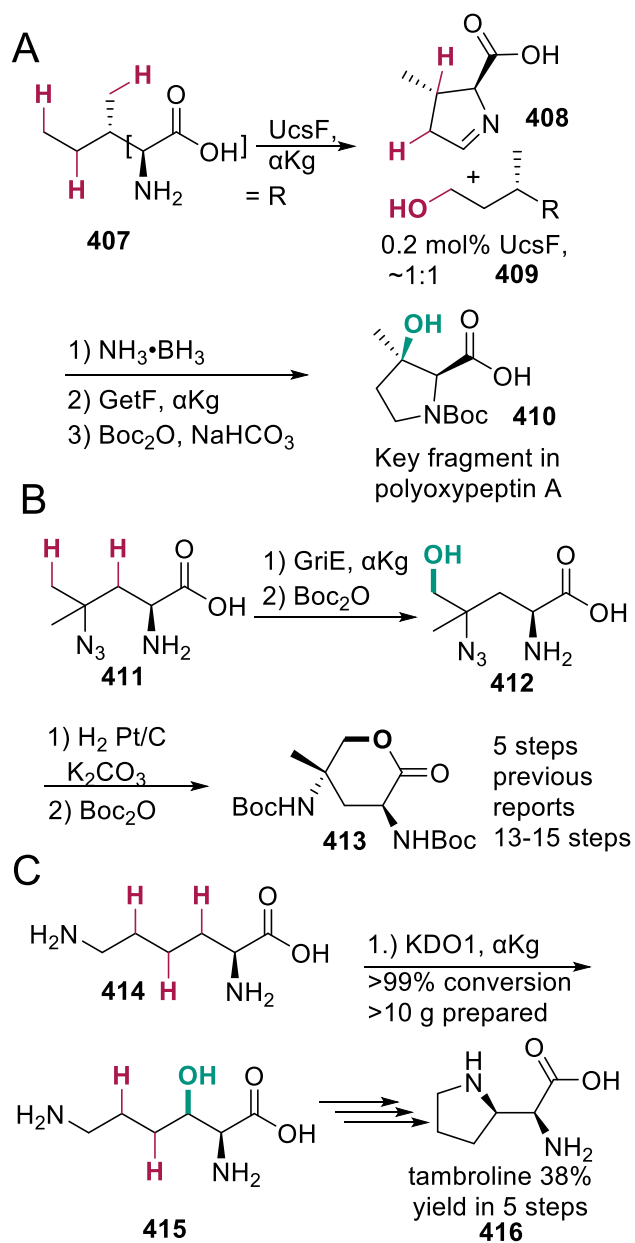
Scheme 64. Site-selective hydroxylation of amino acids with FeDOs. A) Hydroxylation with UscF and GetF is used to construct a core unnatural amino acid fragment in polyoxpeptin A. B) GriE hydroxylation of an azidated substrate establishes the chirality of lactam X. C) In the total synthesis of tambromycin, the first step in construction of the tambroline core is the KDO1 catalyzed hydroxylation of lysine.

Unnatural amino acids are often used as building blocks for synthesis of both natural products and pharmaceuticals, so methods to access these compounds from proteinogenic amino acids and related compounds are highly sought after. FeDOs and FeDHs have proven particularly useful in this regard since their native activity often involves site-selective amino acid functionalization. For example, GetF was used in the synthesis of (2S, 3R)-3-hydroxy-3-methylproline, a key monomer in several peptide derived natural products.[419] By coupling GetF in a cascade with FeDO UcsF,

sequential site-selective C-H hydroxylations enabled the isolation of the boc-protected amino acid **410** in 28% over 3 steps providing 300 mg of the product (Scheme 64 A). Similarly, the FeDO GriE was found to accept additional bulk at the gamma position of the native substrate L-leucine. Consequently, hydroxylation of an azidated L-leucine derivative provided a quick way to access the lactone core of manzacidin C in significantly fewer steps than some previous reports (Scheme 64 B).

While some applications of FeDOs in target-oriented synthesis utilize non-native substrates, most take advantage of native enzyme-substrate pairs. For example, the aforementioned hydroxylation of leucine by GriE was also used to produce (2S, 4R)-4-methylproline *en route* to the total synthesis of cavinafungin B.[420] The enzymatically produced compound was used as a monomer in solid-phase peptide synthesis as the penultimate coupling partner before hydrolysis of the product from the resin, providing the target compound entirely from building blocks derived from proteinogenic amino-acids. A similar approach was used to access the pyrrolidine-containing amino acid tambroline in tambromycin.[421] The native reactivity of a 3-selective lysine hydroxylase KDO1 was used to provide 3-hydroxylysine on gram scale with perfect site- and stereoselectivity (Scheme 64 C).[240] This enabled gram-scale production of *N*-boc protected tambroline (**416**) and ultimately the efficient synthesis of tambromycin in 11 total steps demonstrating an efficient use of biocatalytic retrosynthesis.
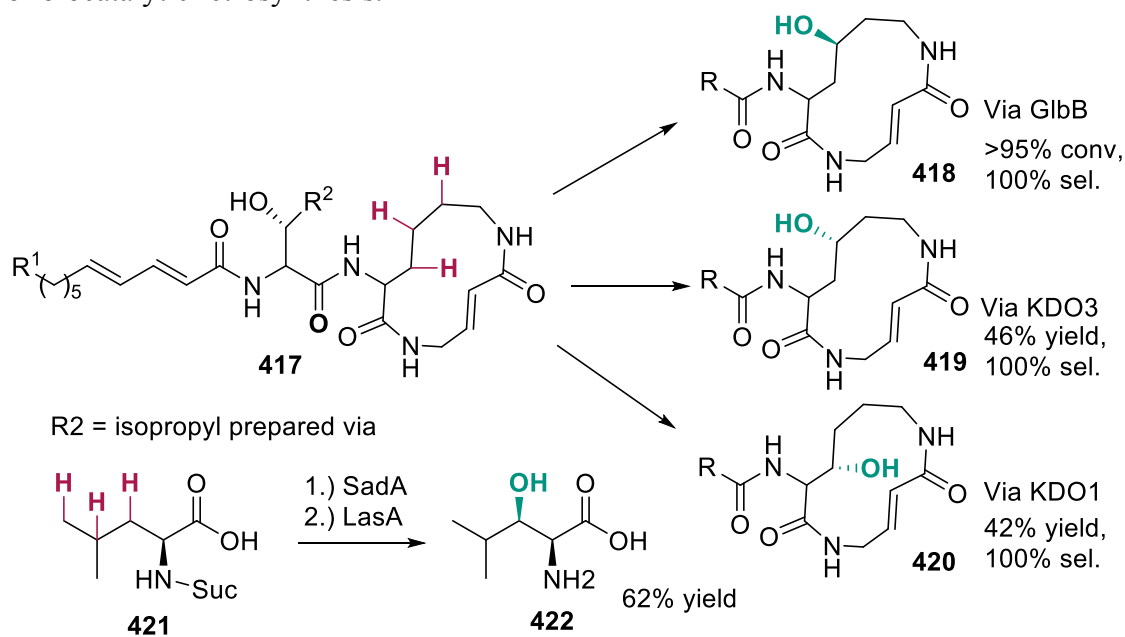
Scheme 65. Chemoenzymatic synthesis of cepafungin I. A lysine-derived fragment can be modified with various lysine hydroxylases to study the impact of modification. For KDO1 and KDO3 derived peptides, yield refers to the Boc-protected monomer. The monomer for the middle portion of the fragment bearing an isopropyl group was prepared using the hydroxylase SadA in combination with the desuccinylase LasA, yield for **422** refers to the Fmoc protected monomer.

The native substrate scope of new FeDOs can be leveraged to diversify natural products in programmable ways. For example, the biosynthetic gene cluster of the terpenoid natural product GE81112 contains two FeDOs, GetI and GetF, that respectively catalyze site- and enantioselective

hydroxylation of L-citrulline and L-pipecolic acid.[422] These enzymatically produced unnatural amino acids could then be used as monomers in the chemical synthesis of the final GE81112 product. To expand this approach beyond total synthesis of GE81112, the native reactivity of other FeDOs was used to produce diverse unnatural lysine monomers. These were incorporated into the GE81112 backbone to produce novel analogues, identifying modifications that could be tolerated without ablating antimicrobial activity. A similar approach was recently used for profiling the antimicrobial activity of cepafungin I, wherein retrosynthetic analysis identified 4-hydroxylysine as a potential synthon for macrolactam **417** (Scheme 65).[423] A panel of lysine hydroxylases with complementary selectivities could be used to provide diversified northern fragments of the macrocyclic core. Furthermore, SadA in combination with the desuccinylase LasA were used to provide a modified center amino acid fragment further proving the utility of biocatalysts for product diversification.
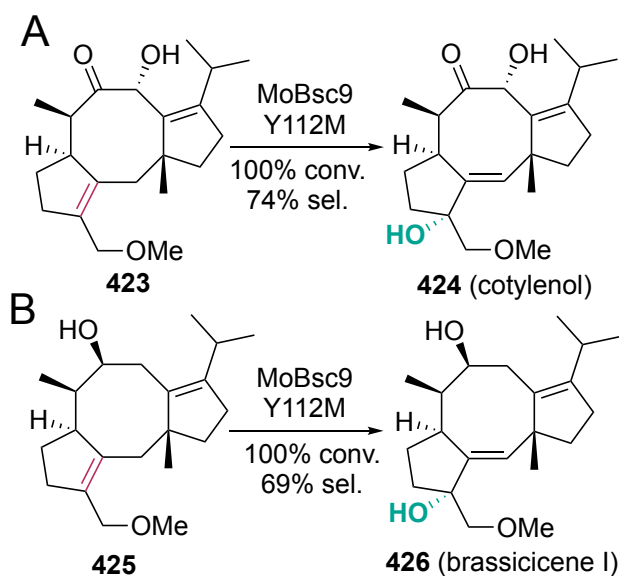
Scheme 66. Hydroxylation of cotylenol and brassicicene I precursors with evolved variants of MoBsc9, a homolog of the native hydroxylase.

In a similar way to how GetF and GetI were identified from analyzing biosynthetic gene clusters, BscD and Bsc9 were found to be involved in the formation of a tertiary alcohol late in cotylenol biosynthesis.[424] These enzymes were initially investigated for *in vitro* late-stage hydroxylation of compound **423** but could not be solubly expressed in *E. coli*. Screening of related Bsc9 homologs led to the identification of MoBsc9, which was found to catalyze the desired transformation with roughly 1:1 selectivity for the desired alcohol **424** and a ketone byproduct. Site-saturation mutagenesis of active-site residues was used to generate MoBsc9 L110A, Y112R, which provided the desired tertiary alcohol and allowed for synthesis of over 100 mg brassicicene I **426** in 10 total steps (Scheme 66 A). A second variant with the mutation Y112M maintained high activity and selectivity for hydroxylation of an analog of this compound bearing different oxygenation patterns on the cyclooctene fragment (Scheme 66 B). This example shows how bioinformatic approaches coupled with protein engineering in a total synthesis context can enable powerful transformations.
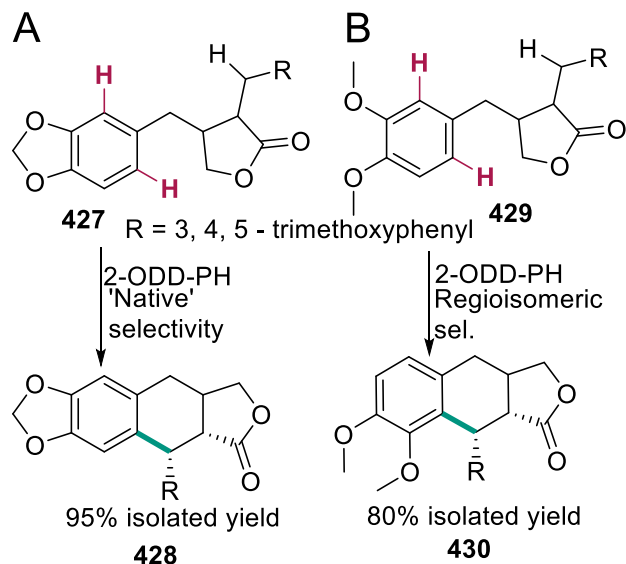
Scheme 67. Oxidative coupling of etoposide precursors with FeDOs. A) The native reaction. B) A closely related non-native substrate shows altered selectivity for the ring closure.

The FeDO 2-ODD-PH natively catalyzes the C-C bond-forming cyclization of the C-ring in (-)-podophyllotoxin, which serves as a precursor for the chemotherapeutic compound etoposide.[425] Substrate profiling of 2-ODD-PH showed that this enzyme accepted modifications of the native substrate, especially in the "A" ring of the compound, although in the absence of electron donating groups the enzyme affects a C-H hydroxylation reaction at the benzylic position. When an electron donating moiety was present enantioselective C-C bond formation was accomplished instead, providing homologs of podophyllotoxin. Later exploration of 2-ODD-PH reactivity showed that product outcomes with 2-ODD-PH were highly susceptible to minor modifications of the substrate as shown by the difference between the selectivity observed for the methylenedioxy substrate **427** and corresponding dimethoxy compound **429** (Scheme 67 A&B).[426] Ultimately by utilizing 2-ODD-PH for the key cyclization step in a chemoenzymatic synthesis, (-)-podophyllotoxin was produced in 28% yield over 5 steps.

A
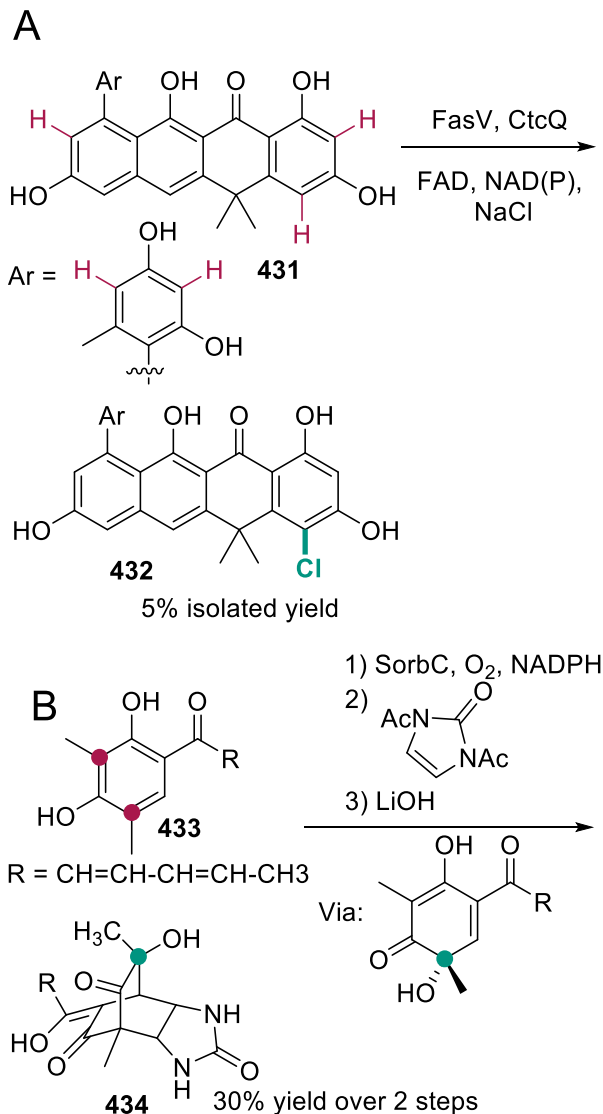
Scheme 68. Flavin dependent enzymes as tools in total synthesis. A) FasV was used as the final step in the total synthesis of fasamycin A to affect site-selective halogenation of napthacemycin B1. B) SorbC was used in the first total synthesis of sorbicillactone A.

Flavin-dependent enzymes have also been used for target-oriented synthesis. For example, the site-selective chlorination of napthacemycin B1 by flavin-dependent halogenase FasV provided fasamycin A **432** in 5% isolated yield as the final step in a chemoenzymatic approach starting from prenol and a simple phenol.[427] Confirming the activity of FasV for chlorination of napthacemycin B1 both supports this enzyme as being responsible for late-stage functionalization in its native context and enables a competitive route to this product step-wise compared to the previous synthesis. Other than this example of target-oriented synthesis with halogenases, most flavin-dependent enzymes used for synthetic purposes catalyze oxidative chemistry. As noted above, the flavin-dependent oxidase SorbC catalyzes a site- and enantioselective hydroxylation reaction to produce sorbicillinol in biosynthesis of sorbicillactone A (Scheme 68 B).[277] This sorbicillinol intermediate has been used to synthesize several natural products,[428, 429] for example by cyclization

with a bisacyl urea to produce natural product sorbacatechol in 30% yield over 2 steps, the first total synthesis of this natural product (Scheme 68 B).[428]

5. Site-Selective Functionalization of Macromolecules

The use of protein-based pharmaceuticals has increased dramatically in recent years, leading to a commensurate increase in the need for generalizable methods for site-selective modification of these macromolecules.[430] Site-selective modification of proteins is particularly challenging given that they are of course comprised of only 20 amino acid building blocks. Most enzymes that catalyze site-selective protein functionalization, including glycosyltransferases, lipoic acid ligases, sortases, and transpeptidases, therefore rely on the presence of specific amino acid sequence motifs that are recognized by the biocatalyst, a form of substrate control. While the respective motifs for these enzymes can often be recognized in different proteins, this requires modification of the original protein, which might not be desirable, and the context of that motif can have a significant impact on its modification efficiency (just as with directing groups in small molecule substrates). Overcoming this limitation with sequence-independent, site-selective protein modification is therefore a major challenge to this area. Here, we also note that DNA base-editing technologies such as the Crispr-Cas9 system have shown a remarkable ability to effect site-selective transformations both *in vitro* and *in vivo,* but since the site selectivity of these methods is dictated by a guide-RNA, it is not relevant to other biopolymers, and we refer readers to other excellent reviews of this area.[431, 432]

5.1. Hydrolases for peptide modification

Insulin is a peptidic natural product with inter- and intramolecular bonds between the peptide fragments. The enormous clinical relevance of this protein makes it a prime target for the development of site-selective methods for biomolecule modification. In addition to the two free amines in the *N*-terminal glycine and phenylalanine residues (labeled A1 and B1 respectively, Scheme 69) a reactive lysine residue must also be protected before chemical modification, presenting a challenge for selective modification. Early studies showed that the penicillin G acylase from *E. coli* (*Ec*PGA) could be used to deprotect the B29 lysine residue with a phenylacetyl protecting group on an insulin derived peptide without disrupting the peptide structure.[433] This finding was exploited by researchers at Merck to develop a comprehensive chemoenzymatic strategy for site-selective protection and deprotection of the insulin peptide. A penicillin G acylase enzyme from *K. cryocrescens* (*Kc*PGA) was found to exhibit differential rates of hydrolysis for a triacylated insulin peptide and could be modified to express well in *E. coli*. After divergent directed evolution campaigns resulted in selective B29 and A1 deacylating enzymes, the reverse acylating reaction starting from unprotected insulin was developed to provide a route to A1-PAc in high selectivity. Ultimately, five distinct variants of this enzyme were obtained which in conjunction with the wild-type PGA from *Achromobacter* sp. allowed for the site-selective modification of both N-terminal residues and the internal lysine sidechain. For global protection and deprotection, further evolution of the starting enzyme produced a variant capable of efficient non-selective modification of each exposed amine group, finishing a fully biocatalytic approach to mono, di, and tri protected insulin (Scheme 69).[434]
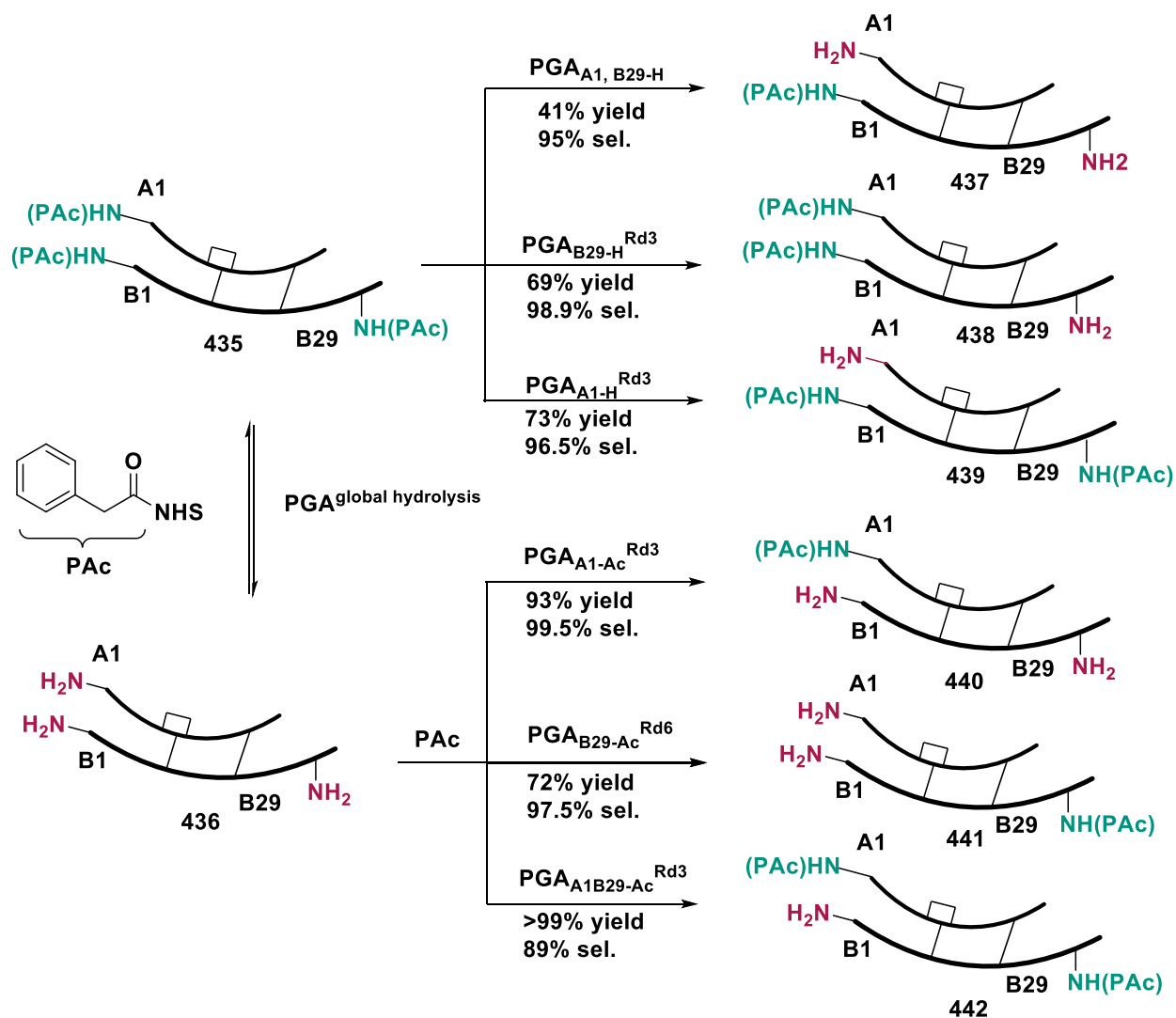
Scheme 69. A toolbox for insulin modification from penicillin acylases. Starting from a panel of *Ec*PGA enzymes, a complete biocatalytic, regiocomplementary strategy for insulin protection and deprotection was developed.

This so-called insulin toolbox of PGA enzymes greatly streamlined the existing syntheses for several clinical candidates and provided the starting point for evolving an enzyme capable of modifying the insulin dimer MK-5160 in up to twice the yield of the previous chemical synthesis with high purity.[435] The PGA enzymes evolved for acylation of insulin were investigated for their promiscuous activity on other peptides and in several cases were found to be highly selective for modification of N-terminal residues. In the case of the small peptide somatostatin which contains several lysine residues in addition to the N-terminal alanine, evolved acylase enzymes were capable of differential acylation reminiscent of the differential hydrolysis that inspired selection of *Kc*PGA as the template. Taken together this study is a significant step forward in the development of site-selective peptide modifications and demonstrates a marked improvement over existing pKa based strategies exploited by chemical synthesis.[436]

5.2. Microbial transglutaminases

Microbial transglutatminases (mTGs) catalyze the intermolecular formation of an amide bond between the γ-amine in glutamine and the primary amine from lysine or a similar amine donor. A cysteine residue in the active site of the mTG forms an acyl enzyme-substrate intermediate, which is then attacked by the primary amine of the amine donor resulting in the release of ammonia and the formation of a cross-linking bond. Unlike transpeptidases, mTGs can recognize glutamine residues in unique primary and secondary structures without a consistent motif. Furthermore, studies focusing on incorporation of an acyl donor containing a fluorescent probe revealed that mTG was competent for forming amide bonds on a variety of secondary structures including α-helices, β-sheets and unstructured portions when glutamine residues were genetically incorporated into Protein G.[437] This combined site-selective ligation without absolute motif or structural requirements underpins the potential of transglutaminases, but while this can provide a benefit by obviating the need to genetically incorporate a specific motif into the substrate, it also results in site selectivity that must often be determined experimentally and is largely not programmable. This difficulty in predicting and screening for site selectivity in biomolecule modification means that even in instances where directed evolution of mTGs is attempted, the targets are largely activity or thermoselectivity.[438, 439]

Many non-naturally occurring amines including aromatic and aliphatic compounds can act as acyl acceptors in mTG catalyzed reactions,[440] although mTGs exhibit a distinct preference for less sterically hindered primary amines. Early studies with mTGs showed that even for proteins containing multiple surface-exposed glutamine residues, addition of Boc-PEG-NH_2 or PEG-NH_2 resulted in only mono- or di-PEGylation of the target protein sequences.[441, 442] Analysis of the glutamine residues pegylated by mTG showed only glutamine residues located on flexible portions of the target protein surface were functionalized with high selectivity compared to glutamine residues on more rigid portions of the surface. A critical study found that for humanized, aglycosylated antibodies an mTG enzyme was highly site-selective for Q295 in the heavy chain portion of the protein. This high selectivity has been used to produce antibody-drug conjugates using primary amines connected to a payload via a defined linker which usually contains a site designed to be readily hydrolyzed when uptaken into the target cells.[443] Incorporation of a linker connected to both azide and tetrazine moieties enabled the production of bifunctionally labeled ADCs which proved advantageous for the reduction of hetereogeneous tumors, demonstrating benefits over the mono-labeled antibody treatment.[444]

6. Outlook

The examples outlined above highlight the current state of the art in our ability to use and engineer enzymes for non-native site-selective enzyme catalysis. With this information in mind, we can consider what types of advances might have the potential to expand the substrate and reaction scope of these enzymes and thus applications for which they could be used. As for biocatalysis and protein engineering more broadly,[445] tight integration of tools and techniques from disparate research areas will be required, but the added challenge of site selectivity will provide an interesting context for their development.

Identifying new enzymes is central to biocatalysis. This task has historically been accomplished by characterizing enzymes involved in natural product biosynthesis.[12] Looking at the diversity of natural product structures, we can infer that enzymes with remarkable biosynthetic capabilities exist in nature.[446] The unique reactivity of enzymes involved in anaerobic secondary metabolism are notable in this regard, particularly in light of the dearth of studies examining their utility for biocatalysis.[447] Even relatively common enzymatic activities like sp^3 C-H hydroxylation and aromatic halogenation can belie the fact that they may involve novel enzymes with untapped potential, like recently reported Mn/Fe-dependent hydroxylases[448] and single-component flavin reductase/halogenases.[332, 333] For these enzymes to be broadly useful, however, it is equally important to establish whether these capabilities extend to non-native substrates and reactions. Metagenomic and family-wide genome mining have also been used to identify new enzymes,[449] and these efforts allow for direct analysis of non-native activity on probe substrates[330, 450] to identify enzymes with broad substrate scope. Advances in *de novo* enzyme design continue to emerge, but these remain limited to relatively simple chemical processes.[451] Efforts to enable the design of enzymes that catalyze complex multi-step reactions involving cofactors, including the metal cofactors responsible for much of the reactivity outlined above, are needed. For all of these systems, chemical and mechanistic intuition is critical to judge whether a given enzyme might reasonably catalyze a desired transformation and how probe substrates might be designed to identify novel activity and site selectivity.

Artificial metalloenzymes (ArMs)[17] represent a particularly interesting source of enzymes for site-selective catalysis since they could be used to impart site selectivity to synthetic catalysts, thus avoiding the blocking and protecting group strategies noted in the introduction. To date, there have been relatively few examples of this capability,[404, 405] and these involved the generation of carbene intermediates. The ArM scaffold is primarily involved in binding and orienting substrates relative to these intermediates to impart selectivity, which mimics the mechanisms of P450s, FeDOs, FDHs, and other enzymes that generate intermediates capable of reacting with a range of functional groups and C-H bonds in organic substrates (e.g. Schemes 30 and 36). This approach simplifies ArM (and natural enzyme) evolution since one need not worry about mutations disrupting complex networks of amino acids involved in substrate activation, and any compound reactive toward the intermediate could potential serve as a substrate. On the other hand, it requires robust methods to orient the catalyst within the scaffold and to avoid diffusion of reactive intermediates since both could lead to non-selective reactions. Native substrate activation modes in ArM scaffolds mitigate these issues and can also be used to enable non-native catalysis,[452, 453] but this approach is inherently limited to substrates that contain suitable functionality for activation, such as a carbonyl compound in the case of serine hydrolases (Scheme 2). Beyond the significant technical challenges to ArM engineering,[454-456] perhaps the biggest limitation to broadening their utility is the fact that the vast majority of transition metal catalysis is incompatible with water. The benefits to developing transition metal-catalyzed reactions that tolerate water are manifold, and the potential to incorporate water-tolerant catalysts into ArMs and to interface them with biocatalysts more broadly should certainly be counted among these.[457]

Identifying an enzyme with novel activity and/or selectivity is a necessary but not sufficient requirement for biocatalysis since extensive directed evolution is often needed for synthetic utility and essentially always required for commercial applications.[458, 459] The successful examples of directed evolution outlined above should not distract from the fact that enzymes are not equally

evolvable [460] and that technical hurdles can derail evolution efforts. Moreover, the field has now moved on from evolving individual enzymes to evolving entire synthetic pathways,[445] so the scope and speed of directed evolution must be increased. Tools like laboratory automation, *in vitro* transcription/translation,[461] and *in vivo* continuous evolution can increase library size and ease of generation. Importantly, however, improved screening throughput is required to realize the potential of these tools, and screening for site selectivity is often challenging since isomeric products are formed. These products are difficult to resolve using chromatographic methods, and while microfluidic ESI-MS[462, 463] and DESI-MS[464] can increase screening throughput, particularly using pooled reactions,[330] site selectivity can only be distinguished if the products have different ionization patterns. Selectively deuterated probe substrates can be used to directly screen for site selectivity using mass spectrometry, but synthesis of such probes can be difficult, and the selectivity analysis must account for possible deuterium kinetic isotope effects.[327] Biosensors, including ligand-dependent transcription factors, can provide a direct readout of formation of a specific product isomers, but these must be engineered to achieve the desired substrate and site specificity.[465] New, rapid methods for assaying the site selectivity of chemical reactions are therefore needed.

Computational tools also have the potential to accelerate both directed evolution and genome mining, and these areas have been extensively reviewed.[466] In the context of site-selective catalysis, methods to rapidly model[467] enzyme structures and analyze differential substrate binding in enzyme active sites can be used to guide enzyme selection or library design. Molecular dynamics simulations have been used to rationalize size selectivity in cases where docking simulations fail to provide useful insights,[305] so decreasing the computational cost of these simulations or finding ways to use machine learning to avoid them entirely would be beneficial. Machine learning directed evolution[468] could also prove particularly useful for site-selective catalysis since deep learning methods are adept at handling complex data,[469] such as the selectivity data associated with substrates that can undergo reaction at multiple sites with variable stereoselectivity. This approach also requires efficient methods to pair sequence information for entire enzyme libraries,[469] not just improved variants, with enzyme activities from high throughput assays such as those noted above.

Given the scope and limitation of these tools and the diversity of molecules to which they could be applied, it is difficult at this time to envision a "general" approach to generating enzymes for biocatalysis and non-native site-selective enzyme catalysis in particular. Careful consideration parent enzymes and protein scaffolds; target reactivity, selectivity, and process compatibility; and methods for library generation and analysis will likely be required for some time. Site-selective catalysis is therefore not just a challenging goal for organic synthesis, it is a driving force for fundamental developments in protein engineering.

Biographies

Jared C. Lewis was born and raised in Effingham, IL. He obtained his B.S. in chemistry from the University of Illinois (2002, Prof. Eric Oldfield), earned his Ph.D. in chemistry from the University of California, Berkeley (2007, Profs. Jonathan Ellman and Robert Bergman), and conducted postdoctoral studies at Caltech (2010, Prof. Frances Arnold). He started his independent career at the University of Chicago in 2011 and moved to Indiana University as an Associate Professor in 2018. His group engineers enzymes and develops new protein engineering tools to enable selective

chemical transformations.

Harrison M. Snodgrass is a native of Audubon, PA. He obtained his B.S. in chemistry from West Chester University of Pennsylvania where he worked with Professors Felix Goodson and Kurt Kolasinski. After starting graduate school in 2016, he earned his Ph.D. from the University of Chicago (2022, Prof. Jared Lewis) studying biocatalysis with a focus on flavin-dependent halogenases. He is currently a protein engineer at Merck & Co working at Merck Research Laboratories.

Christian A. Gomez was born and raised in Buenos Aires, Argentina. He obtained his B.S. in chemistry from the University of Buenos Aires, where he worked with Dr. Marta B. Mazzetti. In 2016 he began his graduate studies at the University of Chicago with Prof. Jared C. Lewis and moved to Indiana University in 2018 to continue his work on developing Fe(II)- and α-ketoglutarate-dependent enzymes as selective biocatalysts for C–H functionalization reactions.

Dibyendu Mondal was born in West Bengal, India. He received his M.Sc. in organic chemistry from the University of Pune. Following graduation, he spent few years in the drug discovery division of Takeda and Daiichi Sankyo Pharmaceuticals. In 2012, he began his graduate studies at the University of Iowa (Prof Amnon Koehn) where his research has focused on understanding the mechanism of Flavin Dependent Thymidylate Synthase. In 2018, he took up a postdoctoral fellowship at the Indiana University (Prof. Jared C. Lewis) and spent three years engineering flavin dependent halogenase for selective catalysis. In 2021, he joined the discovery group of Kalsec Inc. as a Lead Scientist.

Acknowledgement

References

(1) Helmchen, G. *Stereoselective Synthesis*; Helmchen, G. S. S.; Helmchen, G., Hoffmann, R. W., Mulzer, J., Schaumann, E., Eds.; Methods of Organic Chemistry (Houben-Weyl); Georg Thieme Verlag: New York, 1996; Vol. Workbench Edition E21 Volume 1, p45-63.

(2) Mahatthananchai, J.; Dumas, A. M.; Bode, J. W. Catalytic Selective Synthesis. *Angewandte Chemie International Edition* **2012**, *51* (44), 10954-10990. DOI: https://doi.org/10.1002/anie.201201787.

(3) Trost, B. M.; Salzmann, T. N.; Hiroi, K. New synthetic reactions. Sulfenylations and dehydrosulfenylations of esters and ketones. *Journal of the American Chemical Society* **1976**, *98* (16), 4887-4902. DOI: 10.1021/ja00432a034.

(4) Afagh, N. A.; Yudin, A. K. Chemoselectivity and the Curious Reactivity Preferences of Functional Groups. *Angewandte Chemie International Edition* **2010**, *49* (2), 262-310. DOI: https://doi.org/10.1002/anie.200901317.

(5) Hassner, A. Correction. Regiospecificity. A Useful Terminology in Addition and Elimination Reactions. *The Journal of Organic Chemistry* **1970**, *35* (11), 4004-4004. DOI: 10.1021/jo00836a604.

(6) Brown, C. J.; Toste, F. D.; Bergman, R. G.; Raymond, K. N. Supramolecular Catalysis in Metal–Ligand Cluster Hosts. *Chemical Reviews* **2015**, *115* (9), 3012-3035. DOI: 10.1021/cr4001226.

(7) Ishihara, Y.; Baran, P. S. Two-Phase Terpene Total Synthesis: Historical Perspective and Application to the Taxol® Problem. *Synlett* **2010**, *2010* (12), 1733-1745. DOI: 10.1055/s-0030-1258123.

(8) Breslow, R. Biomimetic Chemistry and Artificial Enzymes: Catalysis by Design. *Accounts of Chemical Research* **1995**, *28* (3), 146-153. DOI: 10.1021/ar00051a008.

(9) Breslow, R.; Yang, J.; Yan, J. Biomimetic hydroxylation of saturated carbons with artificial cytochrome P-450 enzymes—liberating chemistry from the tyranny of functional groups. *Tetrahedron* **2002**, *58* (4), 653-659. DOI: https://doi.org/10.1016/S0040-4020(01)01098-5.

(10) Yang, J.; Gabriele, B.; Belvedere, S.; Huang, Y.; Breslow, R. Catalytic Oxidations of Steroid Substrates by Artificial Cytochrome P-450 Enzymes. *The Journal of Organic Chemistry* **2002**, *67* (15), 5057-5067. DOI: 10.1021/jo020174u.

(11) Toste, F. D.; Sigman, M. S.; Miller, S. J. Pursuit of Noncovalent Interactions for Strategic Site-Selective Catalysis. *Accounts of Chemical Research* **2017**, *50* (3), 609-615. DOI: 10.1021/acs.accounts.6b00613.

(12) Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K. Engineering the third wave of biocatalysis. *Nature* **2012**, *485* (7397), 185-194. DOI: 10.1038/nature11117.

(13) Khersonsky, O.; Tawfik, D. S. Enzyme Promiscuity: A Mechanistic and Evolutionary Perspective. *Annual Review of Biochemistry* **2010**, *79* (1), 471-505. DOI: 10.1146/annurev-biochem-030409-143718.

(14) Prier, C. K.; Arnold, F. H. Chemomimetic Biocatalysis: Exploiting the Synthetic Potential of Cofactor-Dependent Enzymes To Create New Catalysts. *Journal of the American Chemical Society* **2015**, *137* (44), 13992-14006. DOI: 10.1021/jacs.5b09348.

(15) Yang, Y.; Arnold, F. H. Navigating the Unnatural Reaction Space: Directed Evolution of Heme Proteins for Selective Carbene and Nitrene Transfer. *Accounts of Chemical Research* **2021**, *54* (5), 1209-1225. DOI: 10.1021/acs.accounts.0c00591.

(16) Andorfer, M. C.; Lewis, J. C. Understanding and Improving the Activity of Flavin-Dependent Halogenases via Random and Targeted Mutagenesis. *Annual Review of Biochemistry* **2018**, *87* (1), 159-185. DOI: 10.1146/annurev-biochem-062917-012042.

(17) Schwizer, F.; Okamoto, Y.; Heinisch, T.; Gu, Y.; Pellizzoni, M. M.; Lebrun, V.; Reuter, R.; Köhler, V.; Lewis, J. C.; Ward, T. R. Artificial Metalloenzymes: Reaction Scope and Optimization Strategies. *Chemical Reviews* **2018**, *118* (1), 142-231. DOI: 10.1021/acs.chemrev.7b00014.

(18) Romero, E.; Jones, B. S.; Hogg, B. N.; Rué Casamajo, A.; Hayes, M. A.; Flitsch, S. L.; Turner, N. J.; Schnepel, C. Enzymatic Late-Stage Modifications: Better Late Than Never. *Angewandte Chemie International Edition* **2021**, *60* (31), 16824-16855. DOI: https://doi.org/10.1002/anie.202014931.

(19) Niwayama, S. Highly Efficient Selective Monohydrolysis of Symmetric Diesters. *The Journal of Organic Chemistry* **2000**, *65* (18), 5834-5836. DOI: 10.1021/jo0001986.

(20) Niwayama, S.; Cho, H.; Lin, C. Highly efficient selective monohydrolysis of dialkyl malonates and their derivatives. *Tetrahedron Letters* **2008**, *49* (28), 4434-4436. DOI: https://doi.org/10.1016/j.tetlet.2008.05.007.

(21) Nicolaou, K. C.; Estrada, A. A.; Zak, M.; Lee, S. H.; Safina, B. S. A Mild and Selective Method for the Hydrolysis of Esters with Trimethyltin Hydroxide. *Angewandte Chemie International Edition* **2005**, *44* (9), 1378-1382. DOI: https://doi.org/10.1002/anie.200462207.

(22) Domínguez de María, P.; García-Burgos, C. A.; Bargeman, G.; van Gemert, R. W. Pig Liver Esterase (PLE) as Biocatalyst in Organic Synthesis: From Nature to Cloning and to Practical Applications. *Synthesis* **2007**, *2007* (10), 1439-1452. DOI: 10.1055/s-2007-966024.

(23) Hedstrom, L. Serine Protease Mechanism and Specificity. *Chemical Reviews* **2002**, *102* (12), 4501-4524. DOI: 10.1021/cr000033x.

(24) Bornscheuer, U. T. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiology Reviews* **2002**, *26* (1), 73-81. DOI: 10.1111/j.1574-6976.2002.tb00599.x (acccessed 1/30/2023).

(25) Chandra, P.; Enespa; Singh, R.; Arora, P. K. Microbial lipases and their industrial applications: a comprehensive review. *Microbial Cell Factories* **2020**, *19* (1), 169. DOI: 10.1186/s12934-020-01428-8.

(26) Ortiz, C.; Ferreira, M. L.; Barbosa, O.; dos Santos, J. C. S.; Rodrigues, R. C.; Berenguer-Murcia, Á.; Briand, L. E.; Fernandez-Lafuente, R. Novozym 435: the "perfect" lipase immobilized biocatalyst? *Catalysis Science & Technology* **2019**, *9* (10), 2380-2420, 10.1039/C9CY00415G. DOI: 10.1039/C9CY00415G.

(27) Carey, J. S.; McCann, E. Lipase-Catalyzed Regioselective Ester Hydrolysis as a Key Step in an Alternative Synthesis of a Buprenorphine Pro-Drug. *Organic Process Research & Development* **2019**, *23* (5), 771-774. DOI: 10.1021/acs.oprd.9b00026.

(28) Chênevert, R.; Ngatcha, B. T.; Rose, Y. S.; Goupil, D. Regio- and enantioselectivity of the enzyme-catalyzed hydrolysis of citric acid derivatives. *Tetrahedron: Asymmetry* **1998**, *9* (24), 4325-4329. DOI: https://doi.org/10.1016/S0957-4166(98)00448-0.

(29) Serbian, I.; Wiese, J.; Raschke, A.; Deising, H. B.; Csuk, R. Streamlined synthesis of (R, R)-rhizoferrin, (S, S)-rhizoferrin and (R, S, R)-staphyloferrin A. *Phytochemistry Letters* **2019**, *33*, 64-69. DOI: https://doi.org/10.1016/j.phytol.2019.07.012.

(30) Schmid, R.; Partali, V.; Anthonsen, T.; Anthonsen, H. W.; Kvittingen, L. Regioselective hydrolysis of diesters of (Z)- and (E)-2-methyl-butenedioic acids by PLE. *Tetrahedron Letters* **2001**, *42* (48), 8543-8545. DOI: https://doi.org/10.1016/S0040-4039(01)01834-2.

(31) Zaks, A.; Dodds, D. R. Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals. *Drug Discovery Today* **1997**, *2* (12), 513-531. DOI: https://doi.org/10.1016/S1359-6446(97)01078-7.

(32) Margolin, A. L.; Delinck, D. L. Enzymic synthesis of biologically active compounds: synthesis of castanospermine derivatives. *Biotechnology Progress* **1990**, *6* (3), 203-204. DOI: 10.1021/bp00003a007.

(33) Gmeiner, P.; Feldman, P. L.; Chu-Moyer, M. Y.; Rapoport, H. An efficient and practical total synthesis of (+)-vincamine from L-aspartic acid. *The Journal of Organic Chemistry* **1990**, *55* (10), 3068-3074. DOI: 10.1021/jo00297a023.

(34) Stein, K. A.; Toogood, P. L. Enzyme-catalyzed regioselective hydrolysis of aspartate diesters. *The Journal of Organic Chemistry* **1995**, *60* (24), 8110-8112.

(35) Sweers, H. M.; Wong, C. H. Enzyme-catalyzed regioselective deacylation of protected sugars in carbohydrate synthesis. *Journal of the American Chemical Society* **1986**, *108* (20), 6421-6422. DOI: 10.1021/ja00280a068.

(36) Hennen, W. J.; Sweers, H. M.; Wang, Y. F.; Wong, C. H. Enzymes in carbohydrate synthesis. Lipase-catalyzed selective acylation and deacylation of furanose and pyranose derivatives. *The Journal of Organic Chemistry* **1988**, *53* (21), 4939-4945. DOI: 10.1021/jo00256a008.

(37) Horrobin, T.; Hao Tran, C.; Crout, D. Esterase-catalysed regioselective 6-deacylation of hexopyranose per-acetates, acid-catalysed rearrangement to the 4-deprotected products and conversions of these into hexose 4- and 6-sulfates. *Journal of the Chemical Society, Perkin Transactions 1* **1998**, (6), 1069-1080, 10.1039/A708596F. DOI: 10.1039/A708596F.

(38) Wang, J.-b.; Li, G.; Reetz, M. T. Enzymatic site-selectivity enabled by structure-guided directed evolution. *Chemical Communications* **2017**, *53* (28), 3916-3928, 10.1039/C7CC00368D. DOI: 10.1039/C7CC00368D.

(39) Massolo, E.; Pirola, M.; Benaglia, M. Amide Bond Formation Strategies: Latest Advances on a Dateless Transformation. *European Journal of Organic Chemistry* **2020**, *2020* (30), 4641-4651. DOI: https://doi.org/10.1002/ejoc.202000080.

(40) Winn, M.; Richardson, S. M.; Campopiano, D. J.; Micklefield, J. Harnessing and engineering amide bond forming ligases for the synthesis of amides. *Current Opinion in Chemical Biology* **2020**, *55*, 77-85. DOI: https://doi.org/10.1016/j.cbpa.2019.12.004.

(41) Andexer, J. N.; Richter, M. Emerging Enzymes for ATP Regeneration in Biocatalytic Processes. *ChemBioChem* **2015**, *16* (3), 380-386. DOI: https://doi.org/10.1002/cbic.201402550.

(42) Schnepel, C.; Pérez, L. R.; Yu, Y.; Angelastro, A.; Heath, R. S.; Lubberink, M.; Falcioni, F.; Mulholland, K.; Hayes, M. A.; Turner, N. J.; et al. Thioester-mediated biocatalytic amide bond synthesis with in situ thiol recycling. *Nature Catalysis* **2023**, *6* (1), 89-99. DOI: 10.1038/s41929-022-00889-x.

(43) Arai, T.; Arimura, Y.; Ishikura, S.; Kino, K. l-Amino Acid Ligase from Pseudomonas syringae Producing Tabtoxin Can Be Used for Enzymatic Synthesis of Various Functional Peptides. *Applied and Environmental Microbiology* **2013**, *79* (16), 5023-5029. DOI: doi:10.1128/AEM.01003-13.

(44) Tsuda, T.; Asami, M.; Koguchi, Y.; Kojima, S. Single Mutation Alters the Substrate Specificity of l-Amino Acid Ligase. *Biochemistry* **2014**, *53* (16), 2650-2660. DOI: 10.1021/bi500292b.

(45) Lee, H.; Choi, M.; Park, J.-U.; Roh, H.; Kim, S. Genome Mining Reveals High Topological Diversity of ω-Ester-Containing Peptides and Divergent Evolution of ATP-Grasp Macrocyclases. *Journal of the American Chemical Society* **2020**, *142* (6), 3013-3023. DOI: 10.1021/jacs.9b12076.

(46) Petchey, M. R.; Rowlinson, B.; Lloyd, R. C.; Fairlamb, I. J. S.; Grogan, G. Biocatalytic Synthesis of Moclobemide Using the Amide Bond Synthetase McbA Coupled with an ATP Recycling System. *ACS Catalysis* **2020**, *10* (8), 4659-4663. DOI: 10.1021/acscatal.0c00929.

(47) Winn, M.; Rowlinson, M.; Wang, F.; Bering, L.; Francis, D.; Levy, C.; Micklefield, J. Discovery, characterization and engineering of ligases for amide synthesis. *Nature* **2021**, *593* (7859), 391-398. DOI: 10.1038/s41586-021-03447-w.

(48) Vinogradov, A. A.; Yin, Y.; Suga, H. Macrocyclic Peptides as Drug Candidates: Recent Progress and Remaining Challenges. *Journal of the American Chemical Society* **2019**, *141* (10), 4167-4181. DOI: 10.1021/jacs.8b13178.

(49) Kobayashi, M.; Fujita, K.; Matsuda, K.; Wakimoto, T. Streamlined Chemoenzymatic Synthesis of Cyclic Peptides by Non-ribosomal Peptide Cyclases. *Journal of the American Chemical Society* **2023**, *145* (6), 3270-3275. DOI: 10.1021/jacs.2c11082.

(50) Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A.; Walsh, C. T. Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase. *Nature* **2000**, *407* (6801), 215-218. DOI: 10.1038/35025116.

(51) Stergiou, P.-Y.; Foukis, A.; Filippou, M.; Koukouritaki, M.; Parapouli, M.; Theodorou, L. G.; Hatziloukas, E.; Afendra, A.; Pandey, A.; Papamichael, E. M. Advances in lipase-catalyzed esterification reactions. *Biotechnology Advances* **2013**, *31* (8), 1846-1859. DOI: https://doi.org/10.1016/j.biotechadv.2013.08.006.

(52) Vilas Bôas, R. N.; de Castro, H. F. A review of synthesis of esters with aromatic, emulsifying, and lubricant properties by biotransformation using lipases. *Biotechnology and Bioengineering* **2022**, *119* (3), 725-742. DOI: https://doi.org/10.1002/bit.28024.

(53) Cruz Silva, M. M.; Riva, S.; Sá e Melo, M. L. Regioselective enzymatic acylation of vicinal diols of steroids. *Tetrahedron* **2005**, *61* (12), 3065-3073. DOI: https://doi.org/10.1016/j.tet.2005.01.104.

(54) Oyo, M.; Junji, K.; Yoshiyuki, F. Studies on Nucleosides and Nucleotides. II. Selective Acylation of 5'-Hydroxyl Group of Thymidine. *Bulletin of the Chemical Society of Japan* **1972**, *45* (1), 245-247. DOI: 10.1246/bcsj.45.245.

(55) García, J.; Fernández, S.; Ferrero, M.; Sanghvi, Y. S.; Gotor, V. A mild, efficient and regioselective enzymatic procedure for 5′-O-benzoylation of 2′-deoxynucleosides. *Tetrahedron Letters* **2004**, *45* (8), 1709-1712. DOI: https://doi.org/10.1016/j.tetlet.2003.12.098.

(56) Almeida, V. M.; Branco, C. R. C.; Assis, S. A.; Vieira, I. J. C.; Braz-Filho, R.; Branco, A. Synthesis of naringin 6"-ricinoleate using immobilized lipase. *Chemistry Central Journal* **2012**, *6* (1), 41. DOI: 10.1186/1752-153X-6-41.

(57) Lackner, G.; Bohnert, M.; Wick, J.; Hoffmeister, D. Assembly of Melleolide Antibiotics Involves a Polyketide Synthase with Cross-Coupling Activity. *Chemistry & Biology* **2013**, *20* (9), 1101-1106. DOI: https://doi.org/10.1016/j.chembiol.2013.07.009.

(58) Gao, X.; Xie, X.; Pashkov, I.; Sawaya, M. R.; Laidman, J.; Zhang, W.; Cacho, R.; Yeates, T. O.; Tang, Y. Directed Evolution and Structural Characterization of a Simvastatin Synthase. *Chemistry & Biology* **2009**, *16* (10), 1064-1074. DOI: https://doi.org/10.1016/j.chembiol.2009.09.017.

(59) Antonopoulou, I.; Varriale, S.; Topakas, E.; Rova, U.; Christakopoulos, P.; Faraco, V. Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application.

Applied Microbiology and Biotechnology **2016**, *100* (15), 6519-6543. DOI: 10.1007/s00253-016-7647-9.

(60) Zhang, Q.; van der Donk, W. A. Catalytic promiscuity of a bacterial α-N-methyltransferase. *FEBS Letters* **2012**, *586* (19), 3391-3397. DOI: https://doi.org/10.1016/j.febslet.2012.07.050.

(61) Cha, B.; Jho, E.-H. Protein arginine methyltransferases (PRMTs) as therapeutic targets. *Expert Opinion on Therapeutic Targets* **2012**, *16* (7), 651-664. DOI: 10.1517/14728222.2012.688030.

(62) Yee, D. A.; Niwa, K.; Perlatti, B.; Chen, M.; Li, Y.; Tang, Y. Genome mining for unknown–unknown natural products. *Nature Chemical Biology* **2023**. DOI: 10.1038/s41589-022-01246-6.

(63) Liao, C.; Seebeck, F. P. S-adenosylhomocysteine as a methyl transfer catalyst in biocatalytic methylation reactions. *Nature Catalysis* **2019**, *2* (8), 696-701. DOI: 10.1038/s41929-019-0300-0.

(64) Abdelraheem, E.; Thair, B.; Varela, R. F.; Jockmann, E.; Popadić, D.; Hailes, H. C.; Ward, J. M.; Iribarren, A. M.; Lewkowicz, E. S.; Andexer, J. N.; et al. Methyltransferases: Functions and Applications. *ChemBioChem* **2022**, *23* (18), e202200212. DOI: https://doi.org/10.1002/cbic.202200212.

(65) Shimizu, T.; Lin, F.; Hasegawa, M.; Okada, K.; Nojiri, H.; Yamane, H. Purification and Identification of Naringenin 7-O-Methyltransferase, a Key Enzyme in Biosynthesis of Flavonoid Phytoalexin Sakuranetin in Rice *. *Journal of Biological Chemistry* **2012**, *287* (23), 19315-19325. DOI: 10.1074/jbc.M112.351270 (acccessed 2023/01/30).

(66) Schmidberger, J. W.; James, A. B.; Edwards, R.; Naismith, J. H.; O'Hagan, D. Halomethane Biosynthesis: Structure of a SAM-Dependent Halide Methyltransferase from Arabidopsis thaliana. *Angewandte Chemie International Edition* **2010**, *49* (21), 3646-3648. DOI: https://doi.org/10.1002/anie.201000119.

(67) Minami, H.; Kim, J.-S.; Ikezawa, N.; Takemura, T.; Katayama, T.; Kumagai, H.; Sato, F. Microbial production of plant benzylisoquinoline alkaloids. *Proceedings of the National Academy of Sciences* **2008**, *105* (21), 7393-7398. DOI: doi:10.1073/pnas.0802981105.

(68) Tang, Q.; Bornscheuer, U. T.; Pavlidis, I. V. Specific Residues Expand the Substrate Scope and Enhance the Regioselectivity of a Plant O-Methyltransferase. *ChemCatChem* **2019**, *11* (14), 3227-3233. DOI: https://doi.org/10.1002/cctc.201900606.

(69) Reeves, C. D.; Hu, Z.; Reid, R.; Kealey, J. T. Genes for the Biosynthesis of the Fungal Polyketides Hypothemycin from <i>Hypomyces subiculosus</i> and Radicicol from <i>Pochonia chlamydosporia</i>. *Applied and Environmental Microbiology* **2008**, *74* (16), 5121-5129. DOI: doi:10.1128/AEM.00478-08.

(70) Xu, Y.; Zhou, T.; Espinosa-Artiles, P.; Tang, Y.; Zhan, J.; Molnár, I. Insights into the Biosynthesis of 12-Membered Resorcylic Acid Lactones from Heterologous Production in Saccharomyces cerevisiae. *ACS Chemical Biology* **2014**, *9* (5), 1119-1127. DOI: 10.1021/cb500043g.

(71) Wang, X.; Wang, C.; Duan, L.; Zhang, L.; Liu, H.; Xu, Y.-m.; Liu, Q.; Mao, T.; Zhang, W.; Chen, M.; et al. Rational Reprogramming of O-Methylation Regioselectivity for Combinatorial Biosynthetic Tailoring of Benzenediol Lactone Scaffolds. *Journal of the American Chemical Society* **2019**, *141* (10), 4355-4364. DOI: 10.1021/jacs.8b12967.

(72) Mordhorst, S.; Siegrist, J.; Müller, M.; Richter, M.; Andexer, J. N. Catalytic Alkylation Using a Cyclic S-Adenosylmethionine Regeneration System. *Angewandte Chemie International Edition* **2017**, *56* (14), 4037-4041. DOI: https://doi.org/10.1002/anie.201611038.

(73) Struck, A.-W.; Thompson, M. L.; Wong, L. S.; Micklefield, J. S-Adenosyl-Methionine-Dependent Methyltransferases: Highly Versatile Enzymes in Biocatalysis, Biosynthesis and Other

Biotechnological Applications. *ChemBioChem* **2012**, *13* (18), 2642-2655. DOI: https://doi.org/10.1002/cbic.201200556.

(74) Tang, Q.; Grathwol, C. W.; Aslan-Üzel, A. S.; Wu, S.; Link, A.; Pavlidis, I. V.; Badenhorst, C. P. S.; Bornscheuer, U. T. Directed Evolution of a Halide Methyltransferase Enables Biocatalytic Synthesis of Diverse SAM Analogs. *Angewandte Chemie International Edition* **2021**, *60* (3), 1524-1527. DOI: https://doi.org/10.1002/anie.202013871.

(75) Popadić, D.; Mhaindarkar, D.; Dang Thai, M. H. N.; Hailes, H. C.; Mordhorst, S.; Andexer, J. N. A bicyclic S-adenosylmethionine regeneration system applicable with different nucleosides or nucleotides as cofactor building blocks. *RSC Chemical Biology* **2021**, *2* (3), 883-891, 10.1039/D1CB00033K. DOI: 10.1039/D1CB00033K.

(76) Siegrist, J.; Aschwanden, S.; Mordhorst, S.; Thöny-Meyer, L.; Richter, M.; Andexer, J. N. Regiocomplementary O-Methylation of Catechols by Using Three-Enzyme Cascades. *ChemBioChem* **2015**, *16* (18), 2576-2579. DOI: https://doi.org/10.1002/cbic.201500410.

(77) Vidgren, J.; Svensson, L. A.; Liljas, A. Crystal structure of catechol O-methyltransferase. *Nature* **1994**, *368* (6469), 354-358. DOI: 10.1038/368354a0.

(78) Nelson, J. T.; Lee, J.; Sims, J. W.; Schmidt, E. W. Characterization of SafC, a Catechol 4-*O*-Methyltransferase Involved in Saframycin Biosynthesis. *Applied and Environmental Microbiology* **2007**, *73* (11), 3575-3580. DOI: doi:10.1128/AEM.00011-07.

(79) Dalhoff, C.; Lukinavičius, G.; Klimašauskas, S.; Weinhold, E. Direct transfer of extended groups from synthetic cofactors by DNA methyltransferases. *Nature Chemical Biology* **2006**, *2* (1), 31-32. DOI: 10.1038/nchembio754.

(80) Wang, R.; Zheng, W.; Yu, H.; Deng, H.; Luo, M. Labeling Substrates of Protein Arginine Methyltransferase with Engineered Enzymes and Matched S-Adenosyl-l-methionine Analogues. *Journal of the American Chemical Society* **2011**, *133* (20), 7648-7651. DOI: 10.1021/ja2006719.

(81) Singh, S.; Zhang, J.; Huber, T. D.; Sunkara, M.; Hurley, K.; Goff, R. D.; Wang, G.; Zhang, W.; Liu, C.; Rohr, J.; et al. Facile Chemoenzymatic Strategies for the Synthesis and Utilization of S-Adenosyl-L-Methionine Analogues. *Angewandte Chemie International Edition* **2014**, *53* (15), 3965-3969. DOI: https://doi.org/10.1002/anie.201308272.

(82) Herbert, A. J.; Shepherd, S. A.; Cronin, V. A.; Bennett, M. R.; Sung, R.; Micklefield, J. Engineering Orthogonal Methyltransferases to Create Alternative Bioalkylation Pathways. *Angewandte Chemie International Edition* **2020**, *59* (35), 14950-14956. DOI: https://doi.org/10.1002/anie.202004963.

(83) Law, B. J. C.; Bennett, M. R.; Thompson, M. L.; Levy, C.; Shepherd, S. A.; Leys, D.; Micklefield, J. Effects of Active-Site Modification and Quaternary Structure on the Regioselectivity of Catechol-O-Methyltransferase. *Angewandte Chemie International Edition* **2016**, *55* (8), 2683-2687. DOI: https://doi.org/10.1002/anie.201508287.

(84) Wen, X.; Leisinger, F.; Leopold, V.; Seebeck, F. P. Synthetic Reagents for Enzyme-Catalyzed Methylation. *Angewandte Chemie International Edition* **2022**, *61* (41), e202208746. DOI: https://doi.org/10.1002/anie.202208746.

(85) Blakemore, D. C.; Castro, L.; Churcher, I.; Rees, D. C.; Thomas, A. W.; Wilson, D. M.; Wood, A. Organic synthesis provides opportunities to transform drug discovery. *Nature Chemistry* **2018**, *10* (4), 383-394. DOI: 10.1038/s41557-018-0021-z.

(86) Bengel, L. L.; Aberle, B.; Egler-Kemmerer, A.-N.; Kienzle, S.; Hauer, B.; Hammer, S. C. Engineered Enzymes Enable Selective N-Alkylation of Pyrazoles With Simple Haloalkanes. *Angewandte Chemie International Edition* **2021**, *60* (10), 5554-5560. DOI: https://doi.org/10.1002/anie.202014239.

(87) Faheem, M.; Rathaur, A.; Pandey, A.; Kumar Singh, V.; Tiwari, A. K. A Review on the Modern Synthetic Approach of Benzimidazole Candidate. *ChemistrySelect* **2020**, *5* (13), 3981-3994. DOI: https://doi.org/10.1002/slct.201904832.

(88) Ospina, F.; Schülke, K. H.; Soler, J.; Klein, A.; Prosenc, B.; Garcia-Borràs, M.; Hammer, S. C. Selective Biocatalytic N-Methylation of Unsaturated Heterocycles. *Angewandte Chemie International Edition* **2022**, *61* (48), e202213056. DOI: https://doi.org/10.1002/anie.202213056.

(89) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The Protein Kinase Complement of the Human Genome. *Science* **2002**, *298* (5600), 1912-1934. DOI: doi:10.1126/science.1075762.

(90) Saier, M. H. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. *Microbiological Reviews* **1989**, *53* (1), 109-120. DOI: doi:10.1128/mr.53.1.109-120.1989.

(91) Westheimer, F. H. Why Nature Chose Phosphates. *Science* **1987**, *235* (4793), 1173-1178. DOI: doi:10.1126/science.2434996.

(92) Wohlgemuth, R. The Power of Biocatalysts for Highly Selective and Efficient Phosphorylation Reactions. *Catalysts* **2022**, *12* (11), 1436.

(93) Tozzi, M. G.; Camici, M.; Mascia, L.; Sgarrella, F.; Ipata, P. L. Pentose phosphates in nucleoside interconversion and catabolism. *The FEBS Journal* **2006**, *273* (6), 1089-1101. DOI: https://doi.org/10.1111/j.1742-4658.2006.05155.x.

(94) Huffman, M. A.; Fryszkowska, A.; Alvizo, O.; Borra-Garske, M.; Campos, K. R.; Canada, K. A.; Devine, P. N.; Duan, D.; Forstater, J. H.; Grosser, S. T.; et al. Design of an in vitro biocatalytic cascade for the manufacture of islatravir. *Science* **2019**, *366* (6470), 1255-1259. DOI: doi:10.1126/science.aay8484.

(95) Kilstrup, M.; Hammer, K.; Ruhdal Jensen, P.; Martinussen, J. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiology Reviews* **2005**, *29* (3), 555-590. DOI: 10.1016/j.fmrre.2005.04.006 (acccessed 2/19/2023).

(96) McIntosh, J. A.; Benkovics, T.; Silverman, S. M.; Huffman, M. A.; Kong, J.; Maligres, P. E.; Itoh, T.; Yang, H.; Verma, D.; Pan, W.; et al. Engineered Ribosyl-1-Kinase Enables Concise Synthesis of Molnupiravir, an Antiviral for COVID-19. *ACS Central Science* **2021**, *7* (12), 1980-1985. DOI: 10.1021/acscentsci.1c00608.

(97) Reily, C.; Stewart, T. J.; Renfrow, M. B.; Novak, J. Glycosylation in health and disease. *Nature Reviews Nephrology* **2019**, *15* (6), 346-366. DOI: 10.1038/s41581-019-0129-4.

(98) Ouadhi, S.; López, D. M. V.; Mohideen, F. I.; Kwan, D. H. Engineering the enzyme toolbox to tailor glycosylation in small molecule natural products and protein biologics. *Protein Engineering, Design and Selection* **2022**, *36*. DOI: 10.1093/protein/gzac010 (acccessed 3/7/2023).

(99) Danieli, B.; Falcone, L.; Monti, D.; Riva, S.; Gebhardt, S.; Schubert-Zsilavecz, M. Regioselective Enzymatic Glycosylation of Natural Polyhydroxylated Compounds: Galactosylation and Glucosylation of Protopanaxatriol Ginsenosides1. *The Journal of Organic Chemistry* **2001**, *66* (1), 262-269. DOI: 10.1021/jo001424e.

(100) He, B.; Bai, X.; Tan, Y.; Xie, W.; Feng, Y.; Yang, G.-Y. Glycosyltransferases: Mining, engineering and applications in biosynthesis of glycosylated plant natural products. *Synthetic and Systems Biotechnology* **2022**, *7* (1), 602-620. DOI: https://doi.org/10.1016/j.synbio.2022.01.001.

(101) McArthur, John B.; Chen, X. Glycosyltransferase engineering for carbohydrate synthesis. *Biochemical Society Transactions* **2016**, *44* (1), 129-142. DOI: 10.1042/bst20150200 (acccessed 2/19/2023).

(102) Shaikh, F. A.; Withers, S. G. Teaching old enzymes new tricks: engineering and evolution of glycosidases and glycosyl transferases for improved glycoside synthesisThis paper is one of a selection of papers published in this Special Issue, entitled CSBMCB — Systems and Chemical Biology, and has undergone the Journal's usual peer review process. *Biochemistry and Cell Biology* **2008**, *86* (2), 169-177. DOI: 10.1139/o07-149 %m 18443630.

(103) Gutmann, A.; Schiller, M.; Gruber-Khadjawi, M.; Nidetzky, B. An ortho C-methylation/O-glycosylation motif on a hydroxy-coumarin scaffold, selectively installed by biocatalysis. *Organic & Biomolecular Chemistry* **2017**, *15* (37), 7917-7924, 10.1039/C7OB01513E. DOI: 10.1039/C7OB01513E.

(104) Losey, H. C.; Jiang, J.; Biggins, J. B.; Oberthür, M.; Ye, X.-Y.; Dong, S. D.; Kahne, D.; Thorson, J. S.; Walsh, C. T. Incorporation of Glucose Analogs by GtfE and GtfD from the Vancomycin Biosynthetic Pathway to Generate Variant Glycopeptides. *Chemistry & Biology* **2002**, *9* (12), 1305-1314. DOI: https://doi.org/10.1016/S1074-5521(02)00270-3.

(105) Yang, M.; Proctor, M. R.; Bolam, D. N.; Errey, J. C.; Field, R. A.; Gilbert, H. J.; Davis, B. G. Probing the Breadth of Macrolide Glycosyltransferases: In Vitro Remodeling of a Polyketide Antibiotic Creates Active Bacterial Uptake and Enhances Potency. *Journal of the American Chemical Society* **2005**, *127* (26), 9336-9337. DOI: 10.1021/ja051482n.

(106) Yang, G.; Rich, J. R.; Gilbert, M.; Wakarchuk, W. W.; Feng, Y.; Withers, S. G. Fluorescence Activated Cell Sorting as a General Ultra-High-Throughput Screening Method for Directed Evolution of Glycosyltransferases. *Journal of the American Chemical Society* **2010**, *132* (30), 10570-10577. DOI: 10.1021/ja104167y.

(107) Lairson, L. L.; Watts, A. G.; Wakarchuk, W. W.; Withers, S. G. Using substrate engineering to harness enzymatic promiscuity and expand biological catalysis. *Nature Chemical Biology* **2006**, *2* (12), 724-728. DOI: 10.1038/nchembio828.

(108) Ly, H. D.; Lougheed, B.; Wakarchuk, W. W.; Withers, S. G. Mechanistic Studies of a Retaining α-Galactosyltransferase from Neisseria meningitidis. *Biochemistry* **2002**, *41* (16), 5075-5085. DOI: 10.1021/bi012031s.

(109) Brazier-Hicks, M.; Evans, K. M.; Gershater, M. C.; Puschmann, H.; Steel, P. G.; Edwards, R. The C-Glycosylation of Flavonoids in Cereals *^{♦}. *Journal of Biological Chemistry* **2009**, *284* (27), 17926-17934. DOI: 10.1074/jbc.M109.009258 (acccessed 2023/01/30).

(110) He, J.-B.; Zhao, P.; Hu, Z.-M.; Liu, S.; Kuang, Y.; Zhang, M.; Li, B.; Yun, C.-H.; Qiao, X.; Ye, M. Molecular and Structural Characterization of a Promiscuous C-Glycosyltransferase from Trollius chinensis. *Angewandte Chemie International Edition* **2019**, *58* (33), 11513-11520. DOI: https://doi.org/10.1002/anie.201905505.

(111) Hancock, S. M.; Vaughan, M. D.; Withers, S. G. Engineering of glycosidases and glycosyltransferases. *Current Opinion in Chemical Biology* **2006**, *10* (5), 509-519. DOI: https://doi.org/10.1016/j.cbpa.2006.07.015.

(112) Mackenzie, L. F.; Wang, Q.; Warren, R. A. J.; Withers, S. G. Glycosynthases: Mutant Glycosidases for Oligosaccharide Synthesis. *Journal of the American Chemical Society* **1998**, *120* (22), 5583-5584. DOI: 10.1021/ja980833d.

(113) Drone, J.; Feng, H.-y.; Tellier, C.; Hoffmann, L.; Tran, V.; Rabiller, C.; Dion, M. Thermus thermophilus Glycosynthases for the Efficient Synthesis of Galactosyl and Glucosyl β-(1→3)-Glycosides. *European Journal of Organic Chemistry* **2005**, *2005* (10), 1977-1983. DOI: https://doi.org/10.1002/ejoc.200500014.

(114) Müllegger, J.; Chen, H.-M.; Chan, W. Y.; Reid, S. P.; Jahn, M.; Warren, R. A. J.; Salleh, H. M.; Withers, S. G. Thermostable Glycosynthases and Thioglycoligases Derived from Thermotoga maritima β-Glucuronidase. *ChemBioChem* **2006**, *7* (7), 1028-1030. DOI: https://doi.org/10.1002/cbic.200600028.

(115) Vaughan, M. D.; Johnson, K.; DeFrees, S.; Tang, X.; Warren, R. A. J.; Withers, S. G. Glycosynthase-Mediated Synthesis of Glycosphingolipids. *Journal of the American Chemical Society* **2006**, *128* (19), 6300-6301. DOI: 10.1021/ja058469n.

(116) Yang, M.; Davies, G. J.; Davis, B. G. A Glycosynthase Catalyst for the Synthesis of Flavonoid Glycosides. *Angewandte Chemie International Edition* **2007**, *46* (21), 3885-3888. DOI: https://doi.org/10.1002/anie.200604177.

(117) Brown, M.; Chapman, E.; Collis, A.; Fuerst, D.; Hosford, J.; Macdermaid, C.; Morrison, J.; SAPONINS; WO2022122830A2. December 8, 2021.

(118) Beerens, K.; Desmet, T.; Soetaert, W. Enzymes for the biocatalytic production of rare sugars. *Journal of Industrial Microbiology and Biotechnology* **2012**, *39* (6), 823-834. DOI: 10.1007/s10295-012-1089-x (acccessed 3/10/2023).

(119) Izumori, K. Izumoring: A strategy for bioproduction of all hexoses. *Journal of Biotechnology* **2006**, *124* (4), 717-722. DOI: https://doi.org/10.1016/j.jbiotec.2006.04.016.

(120) Lafite, P.; Daniellou, R. Rare and unusual glycosylation of peptides and proteins. *Natural Product Reports* **2012**, *29* (7), 729-738, 10.1039/C2NP20030A. DOI: 10.1039/C2NP20030A.

(121) Nishimura, S.-I.; Hato, M.; Hyugaji, S.; Feng, F.; Amano, M. Glycomics for Drug Discovery: Metabolic Perturbation in Androgen-Independent Prostate Cancer Cells Induced by Unnatural Hexosamine Mimics. *Angewandte Chemie International Edition* **2012**, *51* (14), 3386-3390. DOI: https://doi.org/10.1002/anie.201108742.

(122) Wang, Y.; Carder, H. M.; Wendlandt, A. E. Synthesis of rare sugar isomers through site-selective epimerization. *Nature* **2020**, *578* (7795), 403-408. DOI: 10.1038/s41586-020-1937-1.

(123) Angyal, S. J. The Lobry de Bruyn-Alberda van Ekenstein Transformation and Related Reactions. In *Glycoscience: Epimerisation, Isomerisation and Rearrangement Reactions of Carbohydrates*, Stütz, A. E. Ed.; Springer Berlin Heidelberg, 2001; pp 1-14.

(124) Allard, S. T. M.; Giraud, M. F.; Naismith, J. H. Epimerases: structure, function and mechanism. *Cellular and Molecular Life Sciences CMLS* **2001**, *58* (11), 1650-1665. DOI: 10.1007/PL00000803.

(125) Fushinobu, S. Molecular evolution and functional divergence of UDP-hexose 4-epimerases. *Current Opinion in Chemical Biology* **2021**, *61*, 53-62. DOI: https://doi.org/10.1016/j.cbpa.2020.09.007.

(126) Beerens, K.; Soetaert, W.; Desmet, T. UDP-hexose 4-epimerases: a view on structure, mechanism and substrate specificity. *Carbohydrate Research* **2015**, *414*, 8-14. DOI: https://doi.org/10.1016/j.carres.2015.06.006.

(127) Kim, H.-J.; Kang, S. Y.; Park, J. J.; Kim, P. Novel Activity of UDP-Galactose-4-Epimerase for Free Monosaccharide and Activity Improvement by Active Site-Saturation Mutagenesis. *Applied Biochemistry and Biotechnology* **2011**, *163* (3), 444-451. DOI: 10.1007/s12010-010-9052-7.

(128) Bosshart, A.; Hee, C. S.; Bechtold, M.; Schirmer, T.; Panke, S. Directed Divergent Evolution of a Thermostable D-Tagatose Epimerase towards Improved Activity for Two Hexose Substrates. *ChemBioChem* **2015**, *16* (4), 592-601. DOI: https://doi.org/10.1002/cbic.201402620.

(129) Bosshart, A.; Wagner, N.; Lei, L.; Panke, S.; Bechtold, M. Highly efficient production of rare sugars D-psicose and L-tagatose by two engineered D-tagatose epimerases. *Biotechnology and Bioengineering* **2016**, *113* (2), 349-358. DOI: https://doi.org/10.1002/bit.25547.

(130) Zhu, Z.; Gao, D.; Li, C.; Chen, Y.; Zhu, M.; Liu, X.; Tanokura, M.; Qin, H.-M.; Lu, F. Redesign of a novel d-allulose 3-epimerase from Staphylococcus aureus for thermostability and efficient biocatalytic production of d-allulose. *Microbial Cell Factories* **2019**, *18* (1), 59. DOI: 10.1186/s12934-019-1107-z.

(131) Shompoosang, S.; Yoshihara, A.; Uechi, K.; Asada, Y.; Morimoto, K. Enzymatic production of three 6-deoxy-aldohexoses from l-rhamnose. *Bioscience, Biotechnology, and Biochemistry* **2014**, *78* (2), 317-325. DOI: 10.1080/09168451.2014.878217.

(132) Yoshida, H.; Yamada, M.; Nishitani, T.; Takada, G.; Izumori, K.; Kamitori, S. Crystal Structures of d-Tagatose 3-Epimerase from Pseudomonas cichorii and Its Complexes with d-Tagatose and d-Fructose. *Journal of Molecular Biology* **2007**, *374* (2), 443-453. DOI: https://doi.org/10.1016/j.jmb.2007.09.033.

(133) Shin, K.-C.; Lee, T.-E.; Seo, M.-J.; Kim, D. W.; Kang, L.-W.; Oh, D.-K. Development of Tagaturonate 3-Epimerase into Tagatose 4-Epimerase with a Biocatalytic Route from Fructose to Tagatose. *ACS Catalysis* **2020**, *10* (20), 12212-12222. DOI: 10.1021/acscatal.0c02922.

(134) Bosshart, A.; Panke, S.; Bechtold, M. Systematic Optimization of Interface Interactions Increases the Thermostability of a Multimeric Enzyme. *Angewandte Chemie International Edition* **2013**, *52* (37), 9673-9676. DOI: https://doi.org/10.1002/anie.201304141.

(135) Rodionova, I. A.; Scott, D. A.; Grishin, N. V.; Osterman, A. L.; Rodionov, D. A. Tagaturonate–fructuronate epimerase UxaE, a novel enzyme in the hexuronate catabolic network in Thermotoga maritima. *Environmental Microbiology* **2012**, *14* (11), 2920-2934. DOI: https://doi.org/10.1111/j.1462-2920.2012.02856.x.

(136) Heath, R. S.; Turner, N. J. Recent advances in oxidase biocatalysts: Enzyme discovery, cascade reactions and scale up. *Current Opinion in Green and Sustainable Chemistry* **2022**, *38*, 100693. DOI: https://doi.org/10.1016/j.cogsc.2022.100693.

(137) Bianchini, E.; Chinaglia, N.; Dean, M.; Giovannini, P. P.; Medici, A.; Pedrini, P.; Poli, S. Regiospecific oxidoreductions catalyzed by a new Pseudomonas paucimobilis hydroxysteroid dehydrogenase. *Tetrahedron* **1999**, *55* (5), 1391-1398. DOI: https://doi.org/10.1016/S0040-4020(98)01111-9.

(138) Secundo, F.; Carrea, G.; De Amici, M.; Joppolo di Ventimiglia, S.; Dordick, J. S. A combinatorial biocatalysis approach to an array of cholic acid derivatives. *Biotechnology and Bioengineering* **2003**, *81* (4), 391-396. DOI: https://doi.org/10.1002/bit.10486.

(139) Monti, D.; Ferrandi, E. E.; Zanellato, I.; Hua, L.; Polentini, F.; Carrea, G.; Riva, S. One-Pot Multienzymatic Synthesis of 12-Ketoursodeoxycholic Acid: Subtle Cofactor Specificities Rule the Reaction Equilibria of Five Biocatalysts Working in a Row. *Advanced Synthesis & Catalysis* **2009**, *351* (9), 1303-1311. DOI: https://doi.org/10.1002/adsc.200800727.

(140) Chen, X.; Cui, Y.; Feng, J.; Wang, Y.; Liu, X.; Wu, Q.; Zhu, D.; Ma, Y. Flavin Oxidoreductase-Mediated Regeneration of Nicotinamide Adenine Dinucleotide with Dioxygen and Catalytic Amount of Flavin Mononucleotide for One-Pot Multi-Enzymatic Preparation of Ursodeoxycholic Acid. *Advanced Synthesis & Catalysis* **2019**, *361* (11), 2497-2504. DOI: https://doi.org/10.1002/adsc.201900111.

(141) Barski, O. A.; Tipparaju, S. M.; Bhatnagar, A. The Aldo-Keto Reductase Superfamily and its Role in Drug Metabolism and Detoxification. *Drug Metabolism Reviews* **2008**, *40* (4), 553-624. DOI: 10.1080/03602530802431439.

(142) Hyndman, D.; Bauman, D. R.; Heredia, V. V.; Penning, T. M. The aldo-keto reductase superfamily homepage. *Chemico-Biological Interactions* **2003**, *143-144*, 621-631. DOI: https://doi.org/10.1016/S0009-2797(02)00193-X.

(143) France, S. P.; Lewis, R. D.; Martinez, C. A. The Evolving Nature of Biocatalysis in Pharmaceutical Research and Development. *JACS Au* **2023**. DOI: 10.1021/jacsau.2c00712.

(144) Whalen, K.; Berdahl, D. R.; Patrick, B.; Jones, M. B.; Williams, K.; WO2020069139A2. Enzymatic process for production of modified hop products. USA April 2, 2020.

(145) Sasaki, S.; Yamauchi, T.; Kubo, H.; Kanai, M.; Ishii, A.; Higashiyama, K. Chemoselective reduction of ketones: trifluoromethylketones versus methylketones. *Tetrahedron Letters* **2005**, *46* (9), 1497-1500. DOI: https://doi.org/10.1016/j.tetlet.2005.01.013.

(146) Grau, B. T.; Devine, P. N.; DiMichele, L. N.; Kosjek, B. Chemo- and Enantioselective Routes to Chiral Fluorinated Hydroxyketones Using Ketoreductases. *Organic Letters* **2007**, *9* (24), 4951-4954. DOI: 10.1021/ol701810v.

(147) Kalaitzakis, D.; Rozzell, J. D.; Kambourakis, S.; Smonou, I. Highly Stereoselective Reductions of α-Alkyl-1,3-diketones and α-Alkyl-β-keto Esters Catalyzed by Isolated NADPH-Dependent Ketoreductases. *Organic Letters* **2005**, *7* (22), 4799-4801. DOI: 10.1021/ol051166d.

(148) Fryszkowska, A.; Peterson, J.; Davies, N. L.; Dewar, C.; Evans, G.; Bycroft, M.; Triggs, N.; Fleming, T.; Gorantla, S. S. C.; Hoge, G.; et al. Development of a Chemoenzymatic Process for Dehydroepiandrosterone Acetate Synthesis. *Organic Process Research & Development* **2016**, *20* (8), 1520-1528. DOI: 10.1021/acs.oprd.6b00215.

(149) St-Jean, F.; Angelaud, R.; Bachmann, S.; Carrera, D. E.; Remarchuk, T.; Piechowicz, K. A.; Niedermann, K.; Iding, H.; Meier, R.; Hou, H.; et al. Stereoselective Synthesis of the IDO Inhibitor Navoximod. *The Journal of Organic Chemistry* **2022**, *87* (7), 4955-4960. DOI: 10.1021/acs.joc.1c02994.

(150) Ji, A.; Wolberg, M.; Hummel, W.; Wandrey, C.; Müller, M. Dynamic kinetic resolution of -butyl 4-methyl-3,5-dioxohexanoate through enzymatic reduction. *Chemical Communications* **2001**, (1), 57-58, 10.1039/B007448I. DOI: 10.1039/B007448I.

(151) Lüdeke, S.; Richter, M.; Müller, M. Stereoselective Synthesis of Three Isomers of tert-Butyl 5-Hydroxy-4-methyl-3-oxohexanoate through Alcohol Dehydrogenase-Catalyzed Dynamic Kinetic Resolution. *Advanced Synthesis & Catalysis* **2009**, *351* (1-2), 253-259. DOI: https://doi.org/10.1002/adsc.200800619.

(152) Savile, C. K.; Janey, J. M.; Mundorff, E. C.; Moore, J. C.; Tam, S.; Jarvis, W. R.; Colbeck, J. C.; Krebber, A.; Fleitz, F. J.; Brands, J.; et al. Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture. *Science* **2010**, *329* (5989), 305-309. DOI: doi:10.1126/science.1188934.

(153) Aleku, G. A.; France, S. P.; Man, H.; Mangas-Sanchez, J.; Montgomery, S. L.; Sharma, M.; Leipold, F.; Hussain, S.; Grogan, G.; Turner, N. J. A reductive aminase from Aspergillus oryzae. *Nature Chemistry* **2017**, *9* (10), 961-969. DOI: 10.1038/nchem.2782.

(154) Gilio, A. K.; Thorpe, T. W.; Turner, N.; Grogan, G. Reductive aminations by imine reductases: from milligrams to tons. *Chemical Science* **2022**, *13* (17), 4697-4713, 10.1039/D2SC00124A. DOI: 10.1039/D2SC00124A.

(155) Simon, R. C.; Zepeck, F.; Kroutil, W. Chemoenzymatic Synthesis of All Four Diastereomers of 2,6-Disubstituted Piperidines through Stereoselective Monoamination of 1,5-Diketones. *Chemistry – A European Journal* **2013**, *19* (8), 2859-2865. DOI: https://doi.org/10.1002/chem.201202793.

(156) O'Reilly, E.; Iglesias, C.; Ghislieri, D.; Hopwood, J.; Galman, J. L.; Lloyd, R. C.; Turner, N. J. A Regio- and Stereoselective ω-Transaminase/Monoamine Oxidase Cascade for the Synthesis of Chiral 2,5-Disubstituted Pyrrolidines. *Angewandte Chemie International Edition* **2014**, *53* (9), 2447-2450. DOI: https://doi.org/10.1002/anie.201309208.

(157) France, S. P.; Hussain, S.; Hill, A. M.; Hepworth, L. J.; Howard, R. M.; Mulholland, K. R.; Flitsch, S. L.; Turner, N. J. One-Pot Cascade Synthesis of Mono- and Disubstituted Piperidines and Pyrrolidines using Carboxylic Acid Reductase (CAR), ω-Transaminase (ω-TA), and Imine Reductase (IRED) Biocatalysts. *ACS Catalysis* **2016**, *6* (6), 3753-3759. DOI: 10.1021/acscatal.6b00855.

(158) Payer, S. E.; Schrittwieser, J. H.; Grischek, B.; Simon, R. C.; Kroutil, W. Regio- and Stereoselective Biocatalytic Monoamination of a Triketone Enables Asymmetric Synthesis of Both Enantiomers of the Pyrrolizidine Alkaloid Xenovenine Employing Transaminases. *Advanced Synthesis & Catalysis* **2016**, *358* (3), 444-451. DOI: https://doi.org/10.1002/adsc.201500781.

(159) Resch, V.; Hanefeld, U. The selective addition of water. *Catalysis Science & Technology* **2015**, *5* (3), 1385-1399, 10.1039/C4CY00692E. DOI: 10.1039/C4CY00692E.

(160) Hiseni, A.; Arends, I. W. C. E.; Otten, L. G. New Cofactor-Independent Hydration Biocatalysts: Structural, Biochemical, and Biocatalytic Characteristics of Carotenoid and Oleate Hydratases. *ChemCatChem* **2015**, *7* (1), 29-37. DOI: https://doi.org/10.1002/cctc.201402511.

(161) Demming, R. M.; Fischer, M.-P.; Schmid, J.; Hauer, B. (De)hydratases—recent developments and future perspectives. *Current Opinion in Chemical Biology* **2018**, *43*, 43-50. DOI: https://doi.org/10.1016/j.cbpa.2017.10.030.

(162) Brodkorb, D.; Gottschall, M.; Marmulla, R.; Lüddeke, F.; Harder, J. Linalool Dehydratase-Isomerase, a Bifunctional Enzyme in the Anaerobic Degradation of Monoterpenes *. *Journal of Biological Chemistry* **2010**, *285* (40), 30436-30442. DOI: 10.1074/jbc.M109.084244 (acccessed 2023/01/30).

(163) Cuetos, A.; Iglesias-Fernández, J.; Danesh-Azari, H.-R.; Zukic, E.; Dowle, A.; Osuna, S.; Grogan, G. Mutational Analysis of Linalool Dehydratase Isomerase Suggests That Alcohol and Alkene Transformations Are Catalyzed Using Noncovalent Mechanisms. *ACS Catalysis* **2020**, *10* (19), 11136-11146. DOI: 10.1021/acscatal.0c02958.

(164) Katsuki, T.; Martin, V. Asymmetric Epoxidation of Allylic Alcohols: the Katsuki–Sharpless Epoxidation Reaction. In *Organic Reactions*, pp 1-299.

(165) Coleman, T.; Kirk, A. M.; Chao, R. R.; Podgorski, M. N.; Harbort, J. S.; Churchman, L. R.; Bruning, J. B.; Bernhardt, P. V.; Harmer, J. R.; Krenske, E. H.; et al. Understanding the Mechanistic Requirements for Efficient and Stereoselective Alkene Epoxidation by a Cytochrome P450 Enzyme. *ACS Catalysis* **2021**, *11* (4), 1995-2010. DOI: 10.1021/acscatal.0c04872.

(166) Kolev, J. N.; O'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 Chemical Biology* **2014**, *9* (1), 164-173. DOI: 10.1021/cb400626w.

(167) Le-Huu, P.; Heidt, T.; Claasen, B.; Laschat, S.; Urlacher, V. B. Chemo-, Regio-, and Stereoselective Oxidation of the Monocyclic Diterpenoid β-Cembrenediol by P450 BM3. *ACS Catalysis* **2015**, *5* (3), 1772-1780. DOI: 10.1021/cs5020404.

(168) Espinoza, R. V.; Maskeri, M. A.; Turlik, A.; Nangia, A.; Khatri, Y.; Montgomery, J.; Houk, K. N.; Sherman, D. H. Epoxidation and Late-Stage C–H Functionalization by P450 TamI Are Mediated by Variant Heme-Iron Oxidizing Species. *ACS Catalysis* **2022**, *12* (6), 3731-3742. DOI: 10.1021/acscatal.2c00364.

(169) Polic, V.; Cheong, K. J.; Hammerer, F.; Auclair, K. Regioselective Epoxidations by Cytochrome P450 3A4 Using a Theobromine Chemical Auxiliary to Predictably Produce N-Protected β- or γ-Amino Epoxides. *Advanced Synthesis & Catalysis* **2017**, *359* (22), 3983-3989. DOI: https://doi.org/10.1002/adsc.201700637.

(170) Kobayashi, M.; Nagasawa, T.; Yamada, H. Enzymatic synthesis of acrylamide: a success story not yet over. *Trends in Biotechnology* **1992**, *10*, 402-408. DOI: https://doi.org/10.1016/0167-7799(92)90283-2.

(171) Yamada, H.; Kobayashi, M. Nitrile Hydratase and Its Application to Industrial Production of Acrylamide. *Bioscience, Biotechnology, and Biochemistry* **1996**, *60* (9), 1391-1400. DOI: 10.1271/bbb.60.1391 (acccessed 2/21/2023).

(172) Kobayashi, M.; Shimizu, S. Metalloenzyme nitrile hydratase: Structure, regulation, and application to biotechnology. *Nature Biotechnology* **1998**, *16* (8), 733-736. DOI: 10.1038/nbt0898-733.

(173) Cheng, Z.; Xia, Y.; Zhou, Z. Recent Advances and Promises in Nitrile Hydratase: From Mechanism to Industrial Applications. *Frontiers in Bioengineering and Biotechnology* **2020**, *8*, Review. DOI: 10.3389/fbioe.2020.00352.

(174) Craven, E. J.; Latham, J.; Shepherd, S. A.; Khan, I.; Diaz-Rodriguez, A.; Greaney, M. F.; Micklefield, J. Programmable late-stage C−H bond functionalization enabled by integration of enzymes with chemocatalysis. *Nature Catalysis* **2021**, *4* (5), 385-394. DOI: 10.1038/s41929-021-00603-3.

(175) Bering, L.; Craven, E. J.; Sowerby Thomas, S. A.; Shepherd, S. A.; Micklefield, J. Merging enzymes with chemocatalysis for amide bond synthesis. *Nature Communications* **2022**, *13* (1), 380. DOI: 10.1038/s41467-022-28005-4.

(176) Farnberger, J. E.; Richter, N.; Hiebler, K.; Bierbaumer, S.; Pickl, M.; Skibar, W.; Zepeck, F.; Kroutil, W. Biocatalytic methylation and demethylation via a shuttle catalysis concept involving corrinoid proteins. *Communications Chemistry* **2018**, *1* (1), 82. DOI: 10.1038/s42004-018-0083-2.

(177) Wuts, P. G. M. G., T. W. *Greene's Protective Groups in OrganicSynthesis*; John Wiley & Sons, Inc., , 2006.

(178) Selva, M.; Perosa, A. Green chemistry metrics: a comparative evaluation of dimethyl carbonate, methyl iodide, dimethyl sulfate and methanol as methylating agents. *Green Chemistry* **2008**, *10* (4), 457-464, 10.1039/B713985C. DOI: 10.1039/B713985C.

(179) Zhang, M.-X.; Hu, X.-H.; Xu, Y.-H.; Loh, T.-P. Selective Dealkylation of Alkyl Aryl Ethers. *Asian Journal of Organic Chemistry* **2015**, *4* (10), 1047-1049. DOI: https://doi.org/10.1002/ajoc.201500196.

(180) and, R. B.; Ragsdale, S. W. The Many Faces of Vitamin B12: Catalysis by Cobalamin-Dependent Enzymes. *Annual Review of Biochemistry* **2003**, *72* (1), 209-247. DOI: 10.1146/annurev.biochem.72.121801.161828.

(181) Richter, N.; Zepeck, F.; Kroutil, W. Cobalamin-dependent enzymatic O-, N-, and S-demethylation. *Trends in Biotechnology* **2015**, *33* (7), 371-373. DOI: https://doi.org/10.1016/j.tibtech.2015.03.011.

(182) Pompei, S.; Grimm, C.; Schiller, C.; Schober, L.; Kroutil, W. Thiols Act as Methyl Traps in the Biocatalytic Demethylation of Guaiacol Derivatives. *Angewandte Chemie International Edition* **2021**, *60* (31), 16906-16910. DOI: https://doi.org/10.1002/anie.202104278.

(183) Grimm, C.; Lazzarotto, M.; Pompei, S.; Schichler, J.; Richter, N.; Farnberger, J. E.; Fuchs, M.; Kroutil, W. Oxygen-Free Regioselective Biocatalytic Demethylation of Methyl-phenyl Ethers

via Methyltransfer Employing Veratrol-O-demethylase. *ACS Catalysis* **2020**, *10* (18), 10375-10380. DOI: 10.1021/acscatal.0c02790.

(184) Davies, H. M. L.; Morton, D. Guiding principles for site selective and stereoselective intermolecular C–H functionalization by donor/acceptor rhodium carbenes. *Chemical Society Reviews* **2011**, *40* (4), 1857-1869, 10.1039/C0CS00217H. DOI: 10.1039/C0CS00217H.

(185) White, M. C. Adding Aliphatic C–H Bond Oxidations to Synthesis. *Science* **2012**, *335* (6070), 807-809. DOI: doi:10.1126/science.1207661.

(186) Newhouse, T.; Baran, P. S. If C H Bonds Could Talk: Selective C H Bond Oxidation. *Angewandte Chemie International Edition* **2011**, *50* (15), 3362-3374. DOI: https://doi.org/10.1002/anie.201006368.

(187) Dyker, G. Transition Metal Catalyzed Coupling Reactions under C−H Activation. *Angewandte Chemie International Edition* **1999**, *38* (12), 1698-1712. DOI: https://doi.org/10.1002/(SICI)1521-3773(19990614)38:12<1698::AID-ANIE1698>3.0.CO;2-6.

(188) Lewis, J. C.; Coelho, P. S.; Arnold, F. H. Enzymatic functionalization of carbon–hydrogen bonds. *Chemical Society Reviews* **2011**, *40* (4), 2003-2021, 10.1039/C0CS00067A. DOI: 10.1039/C0CS00067A.

(189) Cochrane, R. V. K.; Vederas, J. C. Highly Selective but Multifunctional Oxygenases in Secondary Metabolism. *Accounts of Chemical Research* **2014**, *47* (10), 3148-3161. DOI: 10.1021/ar500242c.

(190) Li, F.; Zhang, X.; Renata, H. Enzymatic CH functionalizations for natural product synthesis. *Current Opinion in Chemical Biology* **2019**, *49*, 25-32. DOI: https://doi.org/10.1016/j.cbpa.2018.09.004.

(191) Nozaki, M. Oxygenases and dioxygenases. *Top Curr Chem* **1979**, *78*, 145-186. DOI: 10.1007/BFb0048193 From NLM.

(192) Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. Structure and Chemistry of Cytochrome P450. *Chemical Reviews* **2005**, *105* (6), 2253-2278. DOI: 10.1021/cr0307143.

(193) Groves, J. T. Models and Mechanisms of Cytochrome P450 Action. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Ortiz de Montellano, P. R. Ed.; Springer US, 2005; pp 1-43.

(194) McIntosh, J. A.; Farwell, C. C.; Arnold, F. H. Expanding P450 catalytic reaction space through evolution and engineering. *Current Opinion in Chemical Biology* **2014**, *19*, 126-134. DOI: https://doi.org/10.1016/j.cbpa.2014.02.001.

(195) Wei, Y.; Ang, E. L.; Zhao, H. Recent developments in the application of P450 based biocatalysts. *Current Opinion in Chemical Biology* **2018**, *43*, 1-7. DOI: https://doi.org/10.1016/j.cbpa.2017.08.006.

(196) Urlacher, V. B.; Girhard, M. Cytochrome P450 Monooxygenases in Biotechnology and Synthetic Biology. *Trends in Biotechnology* **2019**, *37* (8), 882-897. DOI: 10.1016/j.tibtech.2019.01.001 (acccessed 2023/02/18).

(197) Bernhardt, R. Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology* **2006**, *124* (1), 128-145. DOI: https://doi.org/10.1016/j.jbiotec.2006.01.026.

(198) Ortiz de Montellano, P. R. Hydrocarbon Hydroxylation by Cytochrome P450 Enzymes. *Chemical Reviews* **2010**, *110* (2), 932-948. DOI: 10.1021/cr9002193.

(199) Warman, A. J.; Roitel, O.; Neeli, R.; Girvan, H. M.; Seward, H. E.; Murray, S. A.; McLean, K. J.; Joyce, M. G.; Toogood, H.; Holt, R. A.; et al. Flavocytochrome P450 BM3: an update on structure and mechanism of a biotechnologically important enzyme. *Biochem Soc Trans* **2005**, *33* (Pt 4), 747-753. DOI: 10.1042/bst0330747 From NLM.

(200) Farinas, Edgardo T.; Schwaneberg, U.; Glieder, A.; Arnold, Frances H. Directed Evolution of a Cytochrome P450 Monooxygenase for Alkane Oxidation. *Advanced Synthesis & Catalysis* **2001**, *343* (6-7), 601-606. DOI: https://doi.org/10.1002/1615-4169(200108)343:6/7<601::AID-ADSC601>3.0.CO;2-9.

(201) Glieder, A.; Farinas, E. T.; Arnold, F. H. Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase. *Nature Biotechnology* **2002**, *20* (11), 1135-1139. DOI: 10.1038/nbt744.

(202) Peters, M. W.; Meinhold, P.; Glieder, A.; Arnold, F. H. Regio- and Enantioselective Alkane Hydroxylation with Engineered Cytochromes P450 BM-3. *Journal of the American Chemical Society* **2003**, *125* (44), 13442-13450. DOI: 10.1021/ja0303790.

(203) Fasan, R.; Chen, M. M.; Crook, N. C.; Arnold, F. H. Engineered Alkane-Hydroxylating Cytochrome P450BM3 Exhibiting Nativelike Catalytic Properties. *Angewandte Chemie International Edition* **2007**, *46* (44), 8414-8418. DOI: https://doi.org/10.1002/anie.200702616.

(204) Lewis, J. C.; Bastian, S.; Bennett, C. S.; Fu, Y.; Mitsuda, Y.; Chen, M. M.; Greenberg, W. A.; Wong, C.-H.; Arnold, F. H. Chemoenzymatic elaboration of monosaccharides using engineered cytochrome P450BM3 demethylases. *Proceedings of the National Academy of Sciences* **2009**, *106* (39), 16550-16555. DOI: doi:10.1073/pnas.0908954106.

(205) Sawayama, A. M.; Chen, M. M. Y.; Kulanthaivel, P.; Kuo, M.-S.; Hemmerle, H.; Arnold, F. H. A Panel of Cytochrome P450 BM3 Variants to Produce Drug Metabolites and Diversify Lead Compounds. *Chemistry – A European Journal* **2009**, *15* (43), 11723-11729. DOI: https://doi.org/10.1002/chem.200900643.

(206) Rentmeister, A.; Arnold, F. H.; Fasan, R. Chemo-enzymatic fluorination of unactivated organic compounds. *Nature Chemical Biology* **2009**, *5* (1), 26-28. DOI: 10.1038/nchembio.128.

(207) Wrighton, S. A.; Stevens, J. C. The Human Hepatic Cytochromes P450 Involved in Drug Metabolism. *Critical Reviews in Toxicology* **1992**, *22* (1), 1-21. DOI: 10.3109/10408449209145319.

(208) Lewis, J. C.; Mantovani, S. M.; Fu, Y.; Snow, C. D.; Komor, R. S.; Wong, C.-H.; Arnold, F. H. Combinatorial Alanine Substitution Enables Rapid Optimization of Cytochrome P450BM3 for Selective Hydroxylation of Large Substrates. *ChemBioChem* **2010**, *11* (18), 2502-2505. DOI: https://doi.org/10.1002/cbic.201000565.

(209) Acevedo-Rocha, C. G.; Gamble, C. G.; Lonsdale, R.; Li, A.; Nett, N.; Hoebenreich, S.; Lingnau, J. B.; Wirtz, C.; Fares, C.; Hinrichs, H.; et al. P450-Catalyzed Regio- and Diastereoselective Steroid Hydroxylation: Efficient Directed Evolution Enabled by Mutability Landscaping. *ACS Catalysis* **2018**, *8* (4), 3395-3410. DOI: 10.1021/acscatal.8b00389.

(210) Li, A.; Acevedo-Rocha, C. G.; D'Amore, L.; Chen, J.; Peng, Y.; Garcia-Borràs, M.; Gao, C.; Zhu, J.; Rickerby, H.; Osuna, S.; et al. Regio- and Stereoselective Steroid Hydroxylation at C7 by Cytochrome P450 Monooxygenase Mutants. *Angewandte Chemie International Edition* **2020**, *59* (30), 12499-12505. DOI: https://doi.org/10.1002/anie.202003139.

(211) Peng, Y.; Gao, C.; Zhang, Z.; Wu, S.; Zhao, J.; Li, A. A Chemoenzymatic Strategy for the Synthesis of Steroid Drugs Enabled by P450 Monooxygenase-Mediated Steroidal Core Modification. *ACS Catalysis* **2022**, *12* (5), 2907-2914. DOI: 10.1021/acscatal.1c05776.

(212) Zhang, X.; Shen, P.; Zhao, J.; Chen, Y.; Li, X.; Huang, J.-W.; Zhang, L.; Li, Q.; Gao, C.; Xing, Q.; et al. Rationally Controlling Selective Steroid Hydroxylation via Scaffold Sampling of a P450 Family. *ACS Catalysis* **2023**, *13* (2), 1280-1289. DOI: 10.1021/acscatal.2c04906.

(213) Nguyen, K. T.; Virus, C.; Günnewich, N.; Hannemann, F.; Bernhardt, R. Changing the Regioselectivity of a P450 from C15 to C11 Hydroxylation of Progesterone. *ChemBioChem* **2012**, *13* (8), 1161-1166. DOI: https://doi.org/10.1002/cbic.201100811.

(214) Kille, S.; Zilly, F. E.; Acevedo, J. P.; Reetz, M. T. Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. *Nature Chemistry* **2011**, *3* (9), 738-743. DOI: 10.1038/nchem.1113.

(215) Whitehouse, C. J. C.; Bell, S. G.; Tufton, H. G.; Kenny, R. J. P.; Ogilvie, L. C. I.; Wong, L.-L. Evolved CYP102A1 (P450BM3) variants oxidise a range of non-natural substrates and offer new selectivity options. *Chemical Communications* **2008**, (8), 966-968, 10.1039/B718124H. DOI: 10.1039/B718124H.

(216) Wu, X.; Chen, Y.; Wang, X.; Wei, W.; Liang, Y. Origin of Site Selectivity in Toluene Hydroxylation by Cytochrome P450 Enzymes. *The Journal of Organic Chemistry* **2021**, *86* (19), 13768-13773. DOI: 10.1021/acs.joc.1c01295.

(217) Agudo, R.; Roiban, G.-D.; Reetz, M. T. Achieving Regio- and Enantioselectivity of P450-Catalyzed Oxidative CH Activation of Small Functionalized Molecules by Structure-Guided Directed Evolution. *ChemBioChem* **2012**, *13* (10), 1465-1473. DOI: https://doi.org/10.1002/cbic.201200244.

(218) Zhang, K.; El Damaty, S.; Fasan, R. P450 Fingerprinting Method for Rapid Discovery of Terpene Hydroxylating P450 Catalysts with Diversified Regioselectivity. *Journal of the American Chemical Society* **2011**, *133* (10), 3242-3245. DOI: 10.1021/ja109590h.

(219) Zhang, K.; Shafer, B. M.; Demars, M. D., II; Stern, H. A.; Fasan, R. Controlled Oxidation of Remote sp3 C–H Bonds in Artemisinin via P450 Catalysts with Fine-Tuned Regio- and Stereoselectivity. *Journal of the American Chemical Society* **2012**, *134* (45), 18695-18704. DOI: 10.1021/ja3073462.

(220) 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. *Bioorganic & Medicinal Chemistry* **2016**, *24* (17), 3876-3886. DOI: https://doi.org/10.1016/j.bmc.2016.06.028.

(221) Alwaseem, H.; Frisch, B. J.; Fasan, R. Anticancer activity profiling of parthenolide analogs generated via P450-mediated chemoenzymatic synthesis. *Bioorganic & Medicinal Chemistry* **2018**, *26* (7), 1365-1373. DOI: https://doi.org/10.1016/j.bmc.2017.08.009.

(222) Schmitz, D.; Zapp, J.; Bernhardt, R. Steroid conversion with CYP106A2 – production of pharmaceutically interesting DHEA metabolites. *Microbial Cell Factories* **2014**, *13* (1), 81. DOI: 10.1186/1475-2859-13-81.

(223) Xue, Y.; Zhao, L.; Liu, H.-w.; Sherman, D. H. A gene cluster for macrolide antibiotic biosynthesis in <i>Streptomyces venezuelae</i>: Architecture of metabolic diversity. *Proceedings of the National Academy of Sciences* **1998**, *95* (21), 12111-12116. DOI: doi:10.1073/pnas.95.21.12111.

(224) Sherman, D. H.; Li, S.; Yermalitskaya, L. V.; Kim, Y.; Smith, J. A.; Waterman, M. R.; Podust, L. M. The Structural Basis for Substrate Anchoring, Active Site Selectivity, and Product Formation by P450 PikC from Streptomyces venezuelae*. *Journal of Biological Chemistry* **2006**, *281* (36), 26289-26297. DOI: 10.1074/jbc.M605478200 (acccessed 2023/01/30).

(225) Li, S.; Ouellet, H.; Sherman, D. H.; Podust, L. M. Analysis of Transient and Catalytic Desosamine-binding Pockets in Cytochrome P-450 PikC from Streptomyces venezuelae *. *Journal of Biological Chemistry* **2009**, *284*, 5723 - 5730.

(226) Negretti, S.; Narayan, A. R. H.; Chiou, K. C.; Kells, P. M.; Stachowski, J. L.; Hansen, D. A.; Podust, L. M.; Montgomery, J.; Sherman, D. H. Directing Group-Controlled Regioselectivity in an Enzymatic C–H Bond Oxygenation. *Journal of the American Chemical Society* **2014**, *136* (13), 4901-4904. DOI: 10.1021/ja5016052.

(227) de Visser, S. P.; Shaik, S. A Proton-Shuttle Mechanism Mediated by the Porphyrin in Benzene Hydroxylation by Cytochrome P450 Enzymes. *Journal of the American Chemical Society* **2003**, *125* (24), 7413-7424. DOI: 10.1021/ja034142f.

(228) Asaka, M.; Fujii, H. Participation of Electron Transfer Process in Rate-Limiting Step of Aromatic Hydroxylation Reactions by Compound I Models of Heme Enzymes. *Journal of the American Chemical Society* **2016**, *138* (26), 8048-8051. DOI: 10.1021/jacs.6b03223.

(229) Stok, J. E.; Chow, S.; Krenske, E. H.; Farfan Soto, C.; Matyas, C.; Poirier, R. A.; Williams, C. M.; De Voss, J. J. Direct Observation of an Oxepin from a Bacterial Cytochrome P450-Catalyzed Oxidation. *Chemistry – A European Journal* **2016**, *22* (13), 4408-4412. DOI: https://doi.org/10.1002/chem.201600246.

(230) Dennig, A.; Marienhagen, J.; Ruff, A. J.; Guddat, L.; Schwaneberg, U. Directed Evolution of P450 BM3 into a p-Xylene Hydroxylase. *ChemCatChem* **2012**, *4* (6), 771-773. DOI: https://doi.org/10.1002/cctc.201200092.

(231) Dennig, A.; Lülsdorf, N.; Liu, H.; Schwaneberg, U. Regioselective o-Hydroxylation of Monosubstituted Benzenes by P450 BM3. *Angewandte Chemie International Edition* **2013**, *52* (32), 8459-8462. DOI: https://doi.org/10.1002/anie.201303986.

(232) Coleman, T.; Lee, J. Z. H.; Kirk, A. M.; Doherty, D. Z.; Podgorski, M. N.; Pinidiya, D. K.; Bruning, J. B.; De Voss, J. J.; Krenske, E. H.; Bell, S. G. Enabling Aromatic Hydroxylation in a Cytochrome P450 Monooxygenase Enzyme through Protein Engineering. *Chemistry – A European Journal* **2022**, *28* (67), e202201895. DOI: https://doi.org/10.1002/chem.202201895.

(233) Martinez, S.; Hausinger, R. P. Catalytic Mechanisms of Fe(II)- and 2-Oxoglutarate-dependent Oxygenases*. *Journal of Biological Chemistry* **2015**, *290* (34), 20702-20711. DOI: https://doi.org/10.1074/jbc.R115.648691.

(234) Klein, C.; Hüttel, W. A Simple Procedure for Selective Hydroxylation of L-Proline and L-Pipecolic Acid with Recombinantly Expressed Proline Hydroxylases. *Advanced Synthesis & Catalysis* **2011**, *353* (8), 1375-1383. DOI: https://doi.org/10.1002/adsc.201000863.

(235) Hibi, M.; Mori, R.; Miyake, R.; Kawabata, H.; Kozono, S.; Takahashi, S.; Ogawa, J. Novel Enzyme Family Found in Filamentous Fungi Catalyzing <i>trans</i>-4-Hydroxylation of l-Pipecolic Acid. *Applied and Environmental Microbiology* **2016**, *82* (7), 2070-2077. DOI: doi:10.1128/AEM.03764-15.

(236) Mattay, J.; Hüttel, W. Pipecolic Acid Hydroxylases: A Monophyletic Clade among cis-Selective Bacterial Proline Hydroxylases that Discriminates l-Proline. *ChemBioChem* **2017**, *18* (15), 1523-1528. DOI: https://doi.org/10.1002/cbic.201700187.

(237) Haibin Chen, Y. K. B., Fabien L. Cabirol, Anupam Gohel Prafulchandra, Tao Li, Jeffrey C. Moore, Martina Quintanar-Audelo,Yang Hong, Steven J. Collier, Derek Smith. biocatalysts and methods for hydroxylation of chemical compounds.

(238) Binz, T. M.; Maffioli, S. I.; Sosio, M.; Donadio, S.; Müller, R. Insights into an Unusual Nonribosomal Peptide Synthetase Biosynthesis: IDENTIFICATION AND CHARACTERIZATION OF THE GE81112 BIOSYNTHETIC GENE CLUSTER *. *Journal of Biological Chemistry* **2010**, *285* (43), 32710-32719. DOI: 10.1074/jbc.M110.146803 (acccessed 2023/03/10).

(239) Zwick III, C. R.; Sosa, M. B.; Renata, H. Characterization of a Citrulline 4-Hydroxylase from Nonribosomal Peptide GE81112 Biosynthesis and Engineering of Its Substrate Specificity for the Chemoenzymatic Synthesis of Enduracididine. *Angewandte Chemie International Edition* **2019**, *58* (52), 18854-18858. DOI: https://doi.org/10.1002/anie.201910659.

(240) Baud, D.; Saaidi, P.-L.; Monfleur, A.; Harari, M.; Cuccaro, J.; Fossey, A.; Besnard, M.; Debard, A.; Mariage, A.; Pellouin, V.; et al. Synthesis of Mono- and Dihydroxylated Amino Acids with New α-Ketoglutarate-Dependent Dioxygenases: Biocatalytic Oxidation of C H Bonds. *ChemCatChem* **2014**, *6* (10), 3012-3017. DOI: https://doi.org/10.1002/cctc.201402498.

(241) Baud, D.; Peruch, O.; Saaidi, P.-L.; Fossey, A.; Mariage, A.; Petit, J.-L.; Salanoubat, M.; Vergne-Vaxelaire, C.; de Berardinis, V.; Zaparucha, A. Biocatalytic Approaches towards the Synthesis of Chiral Amino Alcohols from Lysine: Cascade Reactions Combining alpha-Keto Acid Oxygenase Hydroxylation with Pyridoxal Phosphate- Dependent Decarboxylation. *Advanced Synthesis & Catalysis* **2017**, *359* (9), 1563-1569. DOI: https://doi.org/10.1002/adsc.201600934.

(242) Amatuni, A.; Renata, H. Identification of a lysine 4-hydroxylase from the glidobactin biosynthesis and evaluation of its biocatalytic potential. *Organic & Biomolecular Chemistry* **2019**, *17* (7), 1736-1739, 10.1039/C8OB02054J. DOI: 10.1039/C8OB02054J.

(243) Chan, N. H.; Gomez, C. A.; Vennelakanti, V.; Du, Q.; Kulik, H. J.; Lewis, J. C. Non-Native Anionic Ligand Binding and Reactivity in Engineered Variants of the Fe(II)- and α-Ketoglutarate-Dependent Oxygenase, SadA. *Inorganic Chemistry* **2022**, *61* (36), 14477-14485. DOI: 10.1021/acs.inorgchem.2c02872.

(244) Beltrán-Nogal, A.; Sánchez-Moreno, I.; Méndez-Sánchez, D.; Gómez de Santos, P.; Hollmann, F.; Alcalde, M. Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases. *Current Opinion in Structural Biology* **2022**, *73*, 102342. DOI: https://doi.org/10.1016/j.sbi.2022.102342.

(245) Hobisch, M.; Holtmann, D.; Gomez de Santos, P.; Alcalde, M.; Hollmann, F.; Kara, S. Recent developments in the use of peroxygenases – Exploring their high potential in selective oxyfunctionalisations. *Biotechnology Advances* **2021**, *51*, 107615. DOI: https://doi.org/10.1016/j.biotechadv.2020.107615.

(246) Molina-Espeja, P.; Cañellas, M.; Plou, F. J.; Hofrichter, M.; Lucas, F.; Guallar, V.; Alcalde, M. Synthesis of 1-Naphthol by a Natural Peroxygenase Engineered by Directed Evolution. *ChemBioChem* **2016**, *17* (4), 341-349. DOI: https://doi.org/10.1002/cbic.201500493.

(247) Peter, S.; Kinne, M.; Wang, X.; Ullrich, R.; Kayser, G.; Groves, J. T.; Hofrichter, M. Selective hydroxylation of alkanes by an extracellular fungal peroxygenase. *The FEBS Journal* **2011**, *278* (19), 3667-3675. DOI: https://doi.org/10.1111/j.1742-4658.2011.08285.x.

(248) Barková, K.; Kinne, M.; Ullrich, R.; Hennig, L.; Fuchs, A.; Hofrichter, M. Regioselective hydroxylation of diverse flavonoids by an aromatic peroxygenase. *Tetrahedron* **2011**, *67* (26), 4874-4878. DOI: https://doi.org/10.1016/j.tet.2011.05.008.

(249) Gomez de Santos, P.; González-Benjumea, A.; Fernandez-Garcia, A.; Aranda, C.; Wu, Y.; But, A.; Molina-Espeja, P.; Maté, D. M.; Gonzalez-Perez, D.; Zhang, W.; et al. Engineering a Highly Regioselective Fungal Peroxygenase for the Synthesis of Hydroxy Fatty Acids. *Angewandte Chemie International Edition* **2023**, *62* (9), e202217372. DOI: https://doi.org/10.1002/anie.202217372.

(250) Barry, S. M.; Challis, G. L. Mechanism and Catalytic Diversity of Rieske Non-Heme Iron-Dependent Oxygenases. *ACS Catalysis* **2013**, *3* (10), 2362-2370. DOI: 10.1021/cs400087p.

(251) Gally, C.; Nestl, B. M.; Hauer, B. Engineering Rieske Non-Heme Iron Oxygenases for the Asymmetric Dihydroxylation of Alkenes. *Angewandte Chemie International Edition* **2015**, *54* (44), 12952-12956. DOI: https://doi.org/10.1002/anie.201506527.

(252) Lukowski, A. L.; Ellinwood, D. C.; Hinze, M. E.; DeLuca, R. J.; Du Bois, J.; Hall, S.; Narayan, A. R. H. C–H Hydroxylation in Paralytic Shellfish Toxin Biosynthesis. *Journal of the American Chemical Society* **2018**, *140* (37), 11863-11869. DOI: 10.1021/jacs.8b08901.

(253) Lukowski, A. L.; Liu, J.; Bridwell-Rabb, J.; Narayan, A. R. H. Structural basis for divergent C–H hydroxylation selectivity in two Rieske oxygenases. *Nature Communications* **2020**, *11* (1), 2991. DOI: 10.1038/s41467-020-16729-0.

(254) Lippard, S. J. Hydroxylation of C–H bonds at carboxylate-bridged diiron centres. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* **2005**, *363* (1829), 861-877. DOI: doi:10.1098/rsta.2004.1532.

(255) Mitchell, K. H.; Studts, J. M.; Fox, B. G. Combined Participation of Hydroxylase Active Site Residues and Effector Protein Binding in a Para to Ortho Modulation of Toluene 4-Monooxygenase Regiospecificity. *Biochemistry* **2002**, *41* (9), 3176-3188. DOI: 10.1021/bi012036p.

(256) Colby, J.; Stirling, D. I.; Dalton, H. The soluble methane mono-oxygenase of Methylococcus capsulatus (Bath). Its ability to oxygenate n-alkanes, n-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. *Biochemical Journal* **1977**, *165* (2), 395-402. DOI: 10.1042/bj1650395 (acccessed 2/21/2023).

(257) Green, J.; Dalton, H. Substrate specificity of soluble methane monooxygenase. Mechanistic implications. *J Biol Chem* **1989**, *264* (30), 17698-17703. From NLM.

(258) Ross, M. O.; Rosenzweig, A. C. A tale of two methane monooxygenases. *JBIC Journal of Biological Inorganic Chemistry* **2017**, *22* (2), 307-319. DOI: 10.1007/s00775-016-1419-y.

(259) Elliott, S. J.; Zhu, M.; Tso, L.; Nguyen, H. H. T.; Yip, J. H. K.; Chan, S. I. Regio- and Stereoselectivity of Particulate Methane Monooxygenase from Methylococcus capsulatus (Bath). *Journal of the American Chemical Society* **1997**, *119* (42), 9949-9955. DOI: 10.1021/ja971049g.

(260) Banerjee, R.; Jones, J. C.; Lipscomb, J. D. Soluble Methane Monooxygenase. *Annual Review of Biochemistry* **2019**, *88* (1), 409-431. DOI: 10.1146/annurev-biochem-013118-111529.

(261) Peng, W.; Qu, X.; Shaik, S.; Wang, B. Deciphering the oxygen activation mechanism at the CuC site of particulate methane monooxygenase. *Nature Catalysis* **2021**, *4* (4), 266-273. DOI: 10.1038/s41929-021-00591-4.

(262) Green, J.; Dalton, H. Substrate specificity of soluble methane monooxygenase: Mechanistic implications*. *Journal of Biological Chemistry* **1989**, *264* (30), 17698-17703. DOI: https://doi.org/10.1016/S0021-9258(19)84627-6.

(263) Chenprakhon, P.; Wongnate, T.; Chaiyen, P. Monooxygenation of aromatic compounds by flavin-dependent monooxygenases. *Protein Science* **2019**, *28* (1), 8-29. DOI: https://doi.org/10.1002/pro.3525.

(264) Reis, R. A. G.; Li, H.; Johnson, M.; Sobrado, P. New frontiers in flavin-dependent monooxygenases. *Archives of Biochemistry and Biophysics* **2021**, *699*, 108765. DOI: https://doi.org/10.1016/j.abb.2021.108765.

(265) Jadan, A. P.; Moonen, M. J. H.; Boeren, S.; Golovleva, L. A.; Rietjens, I. M. C. M.; van Berkel, W. J. H. Biocatalytic Potential of p-Hydroxybenzoate Hydroxylase from Rhodococcus rhodnii 135 and Rhodococcus opacus 557. *Advanced Synthesis & Catalysis* **2004**, *346* (2-3), 367-375. DOI: https://doi.org/10.1002/adsc.200303146.

(266) Meyer, A.; Schmid, A.; Held, M.; Westphal, A. H.; Röthlisberger, M.; Kohler, H.-P. E.; van Berkel, W. J. H.; Witholt, B. Changing the Substrate Reactivity of 2-Hydroxybiphenyl 3-Monooxygenase from Pseudomonas azelaica HBP1 by Directed Evolution*. *Journal of Biological Chemistry* **2002**, *277* (7), 5575-5582. DOI: https://doi.org/10.1074/jbc.M110018200.

(267) Bregman-Cohen, A.; Deri, B.; Maimon, S.; Pazy, Y.; Fishman, A. Altering 2-Hydroxybiphenyl 3-Monooxygenase Regioselectivity by Protein Engineering for the Production of a New Antioxidant. *ChemBioChem* **2018**, *19* (6), 583-590. DOI: https://doi.org/10.1002/cbic.201700648.

(268) Chaiyen, P.; Suadee, C.; Wilairat, P. A novel two-protein component flavoprotein hydroxylase. *European Journal of Biochemistry* **2001**, *268* (21), 5550-5561. DOI: https://doi.org/10.1046/j.1432-1033.2001.02490.x.

(269) Dhammaraj, T.; Pinthong, C.; Visitsatthawong, S.; Tongsook, C.; Surawatanawong, P.; Chaiyen, P. A Single-Site Mutation at Ser146 Expands the Reactivity of the Oxygenase Component of p-Hydroxyphenylacetate 3-Hydroxylase. *ACS Chemical Biology* **2016**, *11* (10), 2889-2896. DOI: 10.1021/acschembio.6b00402.

(270) Furuya, T.; Kino, K. Catalytic activity of the two-component flavin-dependent monooxygenase from Pseudomonas aeruginosa toward cinnamic acid derivatives. *Applied Microbiology and Biotechnology* **2014**, *98* (3), 1145-1154. DOI: 10.1007/s00253-013-4958-y.

(271) Furuya, T.; Kino, K. Regioselective synthesis of piceatannol from resveratrol: catalysis by two-component flavin-dependent monooxygenase HpaBC in whole cells. *Tetrahedron Letters* **2014**, *55* (17), 2853-2855. DOI: https://doi.org/10.1016/j.tetlet.2014.03.076.

(272) Deng, Y.; Faivre, B.; Back, O.; Lombard, M.; Pecqueur, L.; Fontecave, M. Structural and Functional Characterization of 4-Hydroxyphenylacetate 3-Hydroxylase from Escherichia coli. *ChemBioChem* **2020**, *21* (1-2), 163-170. DOI: https://doi.org/10.1002/cbic.201900277.

(273) Herrmann, S.; Dippe, M.; Pecher, P.; Funke, E.; Pietzsch, M.; Wessjohann, L. A. Engineered Bacterial Flavin-Dependent Monooxygenases for the Regiospecific Hydroxylation of Polycyclic Phenols. *ChemBioChem* **2022**, *23* (6), e202100480. DOI: https://doi.org/10.1002/cbic.202100480.

(274) Abood, A.; Al-Fahad, A.; Scott, A.; Hosny, A. E.-D. M. S.; Hashem, A. M.; Fattah, A. M. A.; Race, P. R.; Simpson, T. J.; Cox, R. J. Kinetic characterisation of the FAD dependent monooxygenase TropB and investigation of its biotransformation potential. *RSC Advances* **2015**, *5* (62), 49987-49995, 10.1039/C5RA06693J. DOI: 10.1039/C5RA06693J.

(275) Zabala, Angelica O.; Xu, W.; Chooi, Y.-H.; Tang, Y. Characterization of a Silent Azaphilone Gene Cluster from Aspergillus niger ATCC 1015 Reveals a Hydroxylation-Mediated Pyran-Ring Formation. *Chemistry & Biology* **2012**, *19* (8), 1049-1059. DOI: https://doi.org/10.1016/j.chembiol.2012.07.004.

(276) Fahad, A. a.; Abood, A.; Fisch, K. M.; Osipow, A.; Davison, J.; Avramović, M.; Butts, C. P.; Piel, J.; Simpson, T. J.; Cox, R. J. Oxidative dearomatisation: the key step of sorbicillinoid biosynthesis. *Chemical Science* **2014**, *5* (2), 523-527, 10.1039/C3SC52911H. DOI: 10.1039/C3SC52911H.

(277) Baker Dockrey, S. A.; Lukowski, A. L.; Becker, M. R.; Narayan, A. R. H. Biocatalytic site- and enantioselective oxidative dearomatization of phenols. *Nature Chemistry* **2018**, *10* (2), 119-125. DOI: 10.1038/nchem.2879.

(278) Dockrey, S. A. B.; Suh, C. E.; Benítez, A. R.; Wymore, T.; Brooks, C. L., III; Narayan, A. R. H. Positioning-Group-Enabled Biocatalytic Oxidative Dearomatization. *ACS Central Science* **2019**, *5* (6), 1010-1016. DOI: 10.1021/acscentsci.9b00163.

(279) Hager, L. P.; Morris, D. R.; Brown, F. S.; Eberwein, H. Chloroperoxidase. II. Utilization of halogen anions. *J Biol Chem* **1966**, *241* (8), 1769-1777. From NLM.

(280) Getrey, L.; Krieg, T.; Hollmann, F.; Schrader, J.; Holtmann, D. Enzymatic halogenation of the phenolic monoterpenes thymol and carvacrol with chloroperoxidase. *Green Chemistry* **2014**, *16* (3), 1104-1108, 10.1039/C3GC42269K. DOI: 10.1039/C3GC42269K.

(281) Fernández-Fueyo, E.; van Wingerden, M.; Renirie, R.; Wever, R.; Ni, Y.; Holtmann, D.; Hollmann, F. Chemoenzymatic Halogenation of Phenols by using the Haloperoxidase from Curvularia inaequalis. *ChemCatChem* **2015**, *7* (24), 4035-4038. DOI: https://doi.org/10.1002/cctc.201500862.

(282) Keller, S.; Wage, T.; Hohaus, K.; Hölzer, M.; Eichhorn, E.; van Pée, K.-H. Purification and Partial Characterization of Tryptophan 7-Halogenase (PrnA) from Pseudomonas fluorescens. *Angewandte Chemie International Edition* **2000**, *39* (13), 2300-2302. DOI: https://doi.org/10.1002/1521-3773(20000703)39:13<2300::AID-ANIE2300>3.0.CO;2-I.

(283) Seibold, C.; Schnerr, H.; Rumpf, J.; Kunzendorf, A.; Hatscher, C.; Wage, T.; Ernyei, A. J.; Dong, C.; Naismith, J. H.; Van Pée, K.-H. A flavin-dependent tryptophan 6-halogenase and its use in modification of pyrrolnitrin biosynthesis. *Biocatalysis and Biotransformation* **2006**, *24* (6), 401-408. DOI: 10.1080/10242420601033738.

(284) Heemstra, J. R., Jr.; Walsh, C. T. Tandem Action of the O2- and FADH2-Dependent Halogenases KtzQ and KtzR Produce 6,7-Dichlorotryptophan for Kutzneride Assembly. *Journal of the American Chemical Society* **2008**, *130* (43), 14024-14025. DOI: 10.1021/ja806467a.

(285) Zehner, S.; Kotzsch, A.; Bister, B.; Süssmuth, R. D.; Méndez, C.; Salas, J. A.; van Pée, K.-H. A Regioselective Tryptophan 5-Halogenase Is Involved in Pyrroindomycin Biosynthesis in Streptomyces rugosporus LL-42D005. *Chemistry & Biology* **2005**, *12* (4), 445-452. DOI: 10.1016/j.chembiol.2005.02.005 (acccessed 2023/01/30).

(286) Foulston, L. C.; Bibb, M. J.; Demain, A. L. Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes. *Proceedings of the National Academy of Sciences of the United States of America* **2010**, *107* (30), 13461-13466. (acccessed 2023/03/04/).JSTOR.

(287) Wynands, I.; van Pée, K.-H. A novel halogenase gene from the pentachloropseudilin producer Actinoplanes sp. ATCC 33002 and detection of in vitro halogenase activity. *FEMS Microbiology Letters* **2004**, *237* (2), 363-367. DOI: 10.1111/j.1574-6968.2004.tb09718.x (acccessed 3/5/2023).

(288) Dorrestein, P. C.; Yeh, E.; Garneau-Tsodikova, S.; Kelleher, N. L.; Walsh, C. T. Dichlorination of a pyrrolyl-S-carrier protein by FADH₂-dependent halogenase PltA during pyoluteorin biosynthesis. *Proceedings of the National Academy of Sciences* **2005**, *102* (39), 13843-13848. DOI: doi:10.1073/pnas.0506964102.

(289) Heide, L.; Westrich, L.; Anderle, C.; Gust, B.; Kammerer, B.; Piel, J. Use of a Halogenase of Hormaomycin Biosynthesis for Formation of New Clorobiocin Analogues with 5-Chloropyrrole Moieties. *ChemBioChem* **2008**, *9* (12), 1992-1999. DOI: https://doi.org/10.1002/cbic.200800186.

(290) Yu, T.-W.; Bai, L.; Clade, D.; Hoffmann, D.; Toelzer, S.; Trinh, K. Q.; Xu, J.; Moss, S. J.; Leistner, E.; Floss, H. G. The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from <i>Actinosynnema</i> <i>pretiosum</i>. *Proceedings of the National Academy of Sciences* **2002**, *99* (12), 7968-7973. DOI: doi:10.1073/pnas.092697199.

(291) Lin, S.; Van Lanen, S. G.; Shen, B. Regiospecific Chlorination of (S)-β-Tyrosyl-S-Carrier Protein Catalyzed by SgcC3 in the Biosynthesis of the Enediyne Antitumor Antibiotic C-1027.

Journal of the American Chemical Society **2007**, *129* (41), 12432-12438. DOI: 10.1021/ja072311g.

(292) Wang, S.; Xu, Y.; Maine, E. A.; Wijeratne, E. M. K.; Espinosa-Artiles, P.; Gunatilaka, A. A. L.; Molnár, I. Functional Characterization of the Biosynthesis of Radicicol, an Hsp90 Inhibitor Resorcylic Acid Lactone from Chaetomium chiversii. *Chemistry & Biology* **2008**, *15* (12), 1328-1338. DOI: https://doi.org/10.1016/j.chembiol.2008.10.006.

(293) Buedenbender, S.; Rachid, S.; Müller, R.; Schulz, G. E. Structure and Action of the Myxobacterial Chondrochloren Halogenase CndH: A New Variant of FAD-dependent Halogenases. *Journal of Molecular Biology* **2009**, *385* (2), 520-530. DOI: https://doi.org/10.1016/j.jmb.2008.10.057.

(294) Podzelinska, K.; Latimer, R.; Bhattacharya, A.; Vining, L. C.; Zechel, D. L.; Jia, Z. Chloramphenicol Biosynthesis: The Structure of CmlS, a Flavin-Dependent Halogenase Showing a Covalent Flavin–Aspartate Bond. *Journal of Molecular Biology* **2010**, *397* (1), 316-331. DOI: https://doi.org/10.1016/j.jmb.2010.01.020.

(295) Agarwal, V.; Blanton, J. M.; Podell, S.; Taton, A.; Schorn, M. A.; Busch, J.; Lin, Z.; Schmidt, E. W.; Jensen, P. R.; Paul, V. J.; et al. Metagenomic discovery of polybrominated diphenyl ether biosynthesis by marine sponges. *Nature Chemical Biology* **2017**, *13* (5), 537-543. DOI: 10.1038/nchembio.2330.

(296) Agarwal, V.; Miles, Z. D.; Winter, J. M.; Eustáquio, A. S.; El Gamal, A. A.; Moore, B. S. Enzymatic Halogenation and Dehalogenation Reactions: Pervasive and Mechanistically Diverse. *Chemical Reviews* **2017**, *117* (8), 5619-5674. DOI: 10.1021/acs.chemrev.6b00571.

(297) Latham, J.; Brandenburger, E.; Shepherd, S. A.; Menon, B. R. K.; Micklefield, J. Development of Halogenase Enzymes for Use in Synthesis. *Chemical Reviews* **2018**, *118* (1), 232-269. DOI: 10.1021/acs.chemrev.7b00032.

(298) Gkotsi, D. S.; Dhaliwal, J.; McLachlan, M. M. W.; Mulholand, K. R.; Goss, R. J. M. Halogenases: powerful tools for biocatalysis (mechanisms applications and scope). *Current Opinion in Chemical Biology* **2018**, *43*, 119-126. DOI: https://doi.org/10.1016/j.cbpa.2018.01.002.

(299) Weichold, V.; Milbredt, D.; van Pée, K.-H. Specific Enzymatic Halogenation—From the Discovery of Halogenated Enzymes to Their Applications In Vitro and In Vivo. *Angewandte Chemie International Edition* **2016**, *55* (22), 6374-6389. DOI: https://doi.org/10.1002/anie.201509573.

(300) Yeh, E.; Cole, L. J.; Barr, E. W.; Bollinger, J. M.; Ballou, D. P.; Walsh, C. T. Flavin Redox Chemistry Precedes Substrate Chlorination during the Reaction of the Flavin-Dependent Halogenase RebH. *Biochemistry* **2006**, *45* (25), 7904-7912. DOI: 10.1021/bi060607d.

(301) Yeh, E.; Garneau, S.; Walsh, C. T. Robust <i>in vitro</i> activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis. *Proceedings of the National Academy of Sciences* **2005**, *102* (11), 3960-3965. DOI: doi:10.1073/pnas.0500755102.

(302) Dong, C.; Flecks, S.; Unversucht, S.; Haupt, C.; van Pée, K.-H.; Naismith, J. H. Tryptophan 7-Halogenase (PrnA) Structure Suggests a Mechanism for Regioselective Chlorination. *Science* **2005**, *309* (5744), 2216-2219. DOI: doi:10.1126/science.1116510.

(303) Flecks, S.; Patallo, E. P.; Zhu, X.; Ernyei, A. J.; Seifert, G.; Schneider, A.; Dong, C.; Naismith, J. H.; van Pée, K.-H. New Insights into the Mechanism of Enzymatic Chlorination of Tryptophan. *Angewandte Chemie International Edition* **2008**, *47* (49), 9533-9536. DOI: https://doi.org/10.1002/anie.200802466.

(304) Karabencheva-Christova, T. G.; Torras, J.; Mulholland, A. J.; Lodola, A.; Christov, C. Z. Mechanistic Insights into the Reaction of Chlorination of Tryptophan Catalyzed by Tryptophan 7-Halogenase. *Scientific Reports* **2017**, *7* (1), 17395. DOI: 10.1038/s41598-017-17789-x.

(305) Andorfer, M. C.; Evans, D.; Yang, S.; He, C. Q.; Girlich, A. M.; Vergara-Coll, J.; Sukumar, N.; Houk, K. N.; Lewis, J. C. Analysis of laboratory-evolved flavin-dependent halogenases affords a computational model for predicting halogenase site selectivity. *Chem Catalysis* **2022**, *2* (10), 2658-2674. DOI: https://doi.org/10.1016/j.checat.2022.07.003.

(306) Zhu, X.; De Laurentis, W.; Leang, K.; Herrmann, J.; Ihlefeld, K.; van Pée, K.-H.; Naismith, J. H. Structural Insights into Regioselectivity in the Enzymatic Chlorination of Tryptophan. *Journal of Molecular Biology* **2009**, *391* (1), 74-85. DOI: https://doi.org/10.1016/j.jmb.2009.06.008.

(307) Phintha, A.; Prakinee, K.; Chaiyen, P. Chapter Eleven - Structures, mechanisms and applications of flavin-dependent halogenases. In *The Enzymes*, Chaiyen, P., Tamanoi, F. Eds.; Vol. 47; Academic Press, 2020; pp 327-364.

(308) Sánchez, C.; Zhu, L.; Braña, A. F.; Salas, A. P.; Rohr, J.; Méndez, C.; Salas, J. A. Combinatorial biosynthesis of antitumor indolocarbazole compounds. *Proceedings of the National Academy of Sciences* **2005**, *102* (2), 461-466. DOI: doi:10.1073/pnas.0407809102.

(309) Runguphan, W.; Qu, X.; O'Connor, S. E. Integrating carbon–halogen bond formation into medicinal plant metabolism. *Nature* **2010**, *468* (7322), 461-464. DOI: 10.1038/nature09524.

(310) Gray, Kevin A.; Richardson, Toby H.; Kretz, K.; Short, Jay M.; Bartnek, F.; Knowles, R.; Kan, L.; Swanson, Paul E.; Robertson, Dan E. Rapid Evolution of Reversible Denaturation and Elevated Melting Temperature in a Microbial Haloalkane Dehalogenase. *Advanced Synthesis & Catalysis* **2001**, *343* (6-7), 607-617. DOI: https://doi.org/10.1002/1615-4169(200108)343:6/7<607::AID-ADSC607>3.0.CO;2-M.

(311) Glenn, W. S.; Nims, E.; O'Connor, S. E. Reengineering a Tryptophan Halogenase To Preferentially Chlorinate a Direct Alkaloid Precursor. *Journal of the American Chemical Society* **2011**, *133* (48), 19346-19349. DOI: 10.1021/ja2089348.

(312) Payne, J. T.; Andorfer, M. C.; Lewis, J. C. Regioselective Arene Halogenation using the FAD-Dependent Halogenase RebH. *Angewandte Chemie International Edition* **2013**, *52* (20), 5271-5274. DOI: https://doi.org/10.1002/anie.201300762.

(313) Zeng, J.; Lytle, A. K.; Gage, D.; Johnson, S. J.; Zhan, J. Specific chlorination of isoquinolines by a fungal flavin-dependent halogenase. *Bioorganic & Medicinal Chemistry Letters* **2013**, *23* (4), 1001-1003. DOI: https://doi.org/10.1016/j.bmcl.2012.12.038.

(314) Frese, M.; Guzowska, P. H.; Voß, H.; Sewald, N. Regioselective Enzymatic Halogenation of Substituted Tryptophan Derivatives using the FAD-Dependent Halogenase RebH. *ChemCatChem* **2014**, *6* (5), 1270-1276. DOI: https://doi.org/10.1002/cctc.201301090.

(315) Gkotsi, D. S.; Ludewig, H.; Sharma, S. V.; Connolly, J. A.; Dhaliwal, J.; Wang, Y.; Unsworth, W. P.; Taylor, R. J. K.; McLachlan, M. M. W.; Shanahan, S.; et al. A marine viral halogenase that iodinates diverse substrates. *Nature Chemistry* **2019**, *11* (12), 1091-1097. DOI: 10.1038/s41557-019-0349-z.

(316) Smith, D. R. M.; Uria, A. R.; Helfrich, E. J. N.; Milbredt, D.; van Pée, K.-H.; Piel, J.; Goss, R. J. M. An Unusual Flavin-Dependent Halogenase from the Metagenome of the Marine Sponge Theonella swinhoei WA. *ACS Chemical Biology* **2017**, *12* (5), 1281-1287. DOI: 10.1021/acschembio.6b01115.

(317) Patallo, E. P.; Walter, A.; Milbredt, D.; Thomas, M.; Neumann, M.; Caputi, L.; O'Connor, S.; Ludwig-Müller, J.; van Pée, K.-H. Strategies to Produce Chlorinated Indole-3-Acetic Acid and

Indole-3-Acetic Acid Intermediates. *ChemistrySelect* **2017**, *2* (34), 11148-11153. DOI: https://doi.org/10.1002/slct.201701933.

(318) Poor, C. B.; Andorfer, M. C.; Lewis, J. C. Improving the Stability and Catalyst Lifetime of the Halogenase RebH By Directed Evolution. *ChemBioChem* **2014**, *15* (9), 1286-1289. DOI: https://doi.org/10.1002/cbic.201300780.

(319) Payne, J. T.; Poor, C. B.; Lewis, J. C. Directed Evolution of RebH for Site-Selective Halogenation of Large Biologically Active Molecules. *Angewandte Chemie International Edition* **2015**, *54* (14), 4226-4230. DOI: https://doi.org/10.1002/anie.201411901.

(320) Payne, J. T.; Butkovich, P. H.; Gu, Y.; Kunze, K. N.; Park, H. J.; Wang, D.-S.; Lewis, J. C. Enantioselective Desymmetrization of Methylenedianilines via Enzyme-Catalyzed Remote Halogenation. *Journal of the American Chemical Society* **2018**, *140* (2), 546-549. DOI: 10.1021/jacs.7b09573.

(321) Snodgrass, H. M.; Mondal, D.; Lewis, J. C. Directed Evolution of Flavin-Dependent Halogenases for Site- and Atroposelective Halogenation of 3-Aryl-4(3H)-Quinazolinones via Kinetic or Dynamic Kinetic Resolution. *Journal of the American Chemical Society* **2022**, *144* (36), 16676-16682. DOI: 10.1021/jacs.2c07422.

(322) Lang, A.; Polnick, S.; Nicke, T.; William, P.; Patallo, E. P.; Naismith, J. H.; van Pée, K.-H. Changing the Regioselectivity of the Tryptophan 7-Halogenase PrnA by Site-Directed Mutagenesis. *Angewandte Chemie International Edition* **2011**, *50* (13), 2951-2953. DOI: https://doi.org/10.1002/anie.201007896.

(323) Shepherd, S. A.; Karthikeyan, C.; Latham, J.; Struck, A.-W.; Thompson, M. L.; Menon, B. R. K.; Styles, M. Q.; Levy, C.; Leys, D.; Micklefield, J. Extending the biocatalytic scope of regiocomplementary flavin-dependent halogenase enzymes. *Chemical Science* **2015**, *6* (6), 3454-3460, 10.1039/C5SC00913H. DOI: 10.1039/C5SC00913H.

(324) Shepherd, S. A.; Menon, B. R. K.; Fisk, H.; Struck, A.-W.; Levy, C.; Leys, D.; Micklefield, J. A Structure-Guided Switch in the Regioselectivity of a Tryptophan Halogenase. *ChemBioChem* **2016**, *17* (9), 821-824. DOI: https://doi.org/10.1002/cbic.201600051.

(325) Moritzer, A.-C.; Minges, H.; Prior, T.; Frese, M.; Sewald, N.; Niemann, H. H. Structure-based switch of regioselectivity in the flavin-dependent tryptophan 6-halogenase Thal. *Journal of Biological Chemistry* **2019**, *294* (7), 2529-2542. DOI: 10.1074/jbc.RA118.005393 (acccessed 2023/01/30).

(326) Fraley, A. E.; Garcia-Borràs, M.; Tripathi, A.; Khare, D.; Mercado-Marin, E. V.; Tran, H.; Dan, Q.; Webb, G. P.; Watts, K. R.; Crews, P.; et al. Function and Structure of MalA/MalA′, Iterative Halogenases for Late-Stage C–H Functionalization of Indole Alkaloids. *Journal of the American Chemical Society* **2017**, *139* (34), 12060-12068. DOI: 10.1021/jacs.7b06773.

(327) Andorfer, M. C.; Park, H. J.; Vergara-Coll, J.; Lewis, J. C. Directed evolution of RebH for catalyst-controlled halogenation of indole C–H bonds. *Chemical Science* **2016**, *7* (6), 3720-3729, 10.1039/C5SC04680G. DOI: 10.1039/C5SC04680G.

(328) Andorfer, M. C.; Grob, J. E.; Hajdin, C. E.; Chael, J. R.; Siuti, P.; Lilly, J.; Tan, K. L.; Lewis, J. C. Understanding Flavin-Dependent Halogenase Reactivity via Substrate Activity Profiling. *ACS Catalysis* **2017**, *7* (3), 1897-1904. DOI: 10.1021/acscatal.6b02707.

(329) Neubauer, P. R.; Widmann, C.; Wibberg, D.; Schröder, L.; Frese, M.; Kottke, T.; Kalinowski, J.; Niemann, H. H.; Sewald, N. A flavin-dependent halogenase from metagenomic analysis prefers bromination over chlorination. *PLOS ONE* **2018**, *13* (5), e0196797. DOI: 10.1371/journal.pone.0196797.

(330) Fisher, B. F.; Snodgrass, H. M.; Jones, K. A.; Andorfer, M. C.; Lewis, J. C. Site-Selective C–H Halogenation Using Flavin-Dependent Halogenases Identified via Family-Wide Activity Profiling. *ACS Central Science* **2019**, *5* (11), 1844-1856. DOI: 10.1021/acscentsci.9b00835.

(331) Agarwal, V.; El Gamal, A. A.; Yamanaka, K.; Poth, D.; Kersten, R. D.; Schorn, M.; Allen, E. E.; Moore, B. S. Biosynthesis of polybrominated aromatic organic compounds by marine bacteria. *Nature Chemical Biology* **2014**, *10* (8), 640-647. DOI: 10.1038/nchembio.1564.

(332) Breinlinger, S.; Phillips, T. J.; Haram, B. N.; Mareš, J.; Martínez Yerena, J. A.; Hrouzek, P.; Sobotka, R.; Henderson, W. M.; Schmieder, P.; Williams, S. M.; et al. Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy. *Science* **2021**, *371* (6536), eaax9050. DOI: doi:10.1126/science.aax9050.

(333) Adak, S.; Lukowski, A. L.; Schäfer, R. J. B.; Moore, B. S. From Tryptophan to Toxin: Nature's Convergent Biosynthetic Strategy to Aetokthonotoxin. *Journal of the American Chemical Society* **2022**, *144* (7), 2861-2866. DOI: 10.1021/jacs.1c12778.

(334) Jiang, Y.; Snodgrass, H. M.; Zubi, Y. S.; Roof, C. V.; Guan, Y.; Mondal, D.; Honeycutt, N. H.; Lee, J. W.; Lewis, R. D.; Martinez, C. A.; et al. The Single-Component Flavin Reductase/Flavin-Dependent Halogenase AetF is a Versatile Catalyst for Selective Bromination and Iodination of Arenes and Olefins**. *Angewandte Chemie International Edition* **2022**, *61* (51), e202214610. DOI: https://doi.org/10.1002/anie.202214610.

(335) Mori, S.; Pang, A. H.; Thamban Chandrika, N.; Garneau-Tsodikova, S.; Tsodikov, O. V. Unusual substrate and halide versatility of phenolic halogenase PltM. *Nature Communications* **2019**, *10* (1), 1255. DOI: 10.1038/s41467-019-09215-9.

(336) Zhang, Y.; Chen, L.; Chen, H.; Huang, T.; Shi, Q.; Wang, X.; Wang, Y.; Tang, M.-C.; Zhou, N.-Y.; Lin, S. Aryl C-H iodination: are there actual flavin-dependent iodinases in nature? *Science China Chemistry* **2021**, *64* (10), 1730-1735. DOI: 10.1007/s11426-021-1018-0.

(337) Hayashi, T.; Ligibel, M.; Sager, E.; Voss, M.; Hunziker, J.; Schroer, K.; Snajdrova, R.; Buller, R. Evolved Aliphatic Halogenases Enable Regiocomplementary C−H Functionalization of a Pharmaceutically Relevant Compound. *Angewandte Chemie International Edition* **2019**, *58* (51), 18535-18539. DOI: https://doi.org/10.1002/anie.201907245.

(338) Ueki, M.; Galonić, D. P.; Vaillancourt, F. H.; Garneau-Tsodikova, S.; Yeh, E.; Vosburg, D. A.; Schroeder, F. C.; Osada, H.; Walsh, C. T. Enzymatic Generation of the Antimetabolite γ,γ-Dichloroaminobutyrate by NRPS and Mononuclear Iron Halogenase Action in a Streptomycete. *Chemistry & Biology* **2006**, *13* (11), 1183-1191. DOI: https://doi.org/10.1016/j.chembiol.2006.09.012.

(339) Vaillancourt, F. H.; Yin, J.; Walsh, C. T. SyrB2 in syringomycin E biosynthesis is a nonheme Fe^{II} α-ketoglutarate- and O$_2$-dependent halogenase. *Proceedings of the National Academy of Sciences* **2005**, *102* (29), 10111-10116. DOI: doi:10.1073/pnas.0504412102.

(340) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T. Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis. *Nature* **2005**, *436* (7054), 1191-1194. DOI: 10.1038/nature03797.

(341) Hillwig, M. L.; Liu, X. A new family of iron-dependent halogenases acts on freestanding substrates. *Nature Chemical Biology* **2014**, *10* (11), 921-923. DOI: 10.1038/nchembio.1625.

(342) Hillwig, M. L.; Zhu, Q.; Ittiamornkul, K.; Liu, X. Discovery of a Promiscuous Non-Heme Iron Halogenase in Ambiguine Alkaloid Biogenesis: Implication for an Evolvable Enzyme Family for Late-Stage Halogenation of Aliphatic Carbons in Small Molecules. *Angewandte Chemie International Edition* **2016**, *55* (19), 5780-5784. DOI: https://doi.org/10.1002/anie.201601447.

(343) Zhu, Q.; Hillwig, M. L.; Doi, Y.; Liu, X. Aliphatic Halogenase Enables Late-Stage C−H Functionalization: Selective Synthesis of a Brominated Fischerindole Alkaloid with Enhanced Antibacterial Activity. *ChemBioChem* **2016**, *17* (6), 466-470. DOI: https://doi.org/10.1002/cbic.201500674.

(344) Büchler, J.; Malca, S. H.; Patsch, D.; Voss, M.; Turner, N. J.; Bornscheuer, U. T.; Allemann, O.; Le Chapelain, C.; Lumbroso, A.; Loiseleur, O.; et al. Algorithm-aided engineering of aliphatic halogenase WelO5* for the asymmetric late-stage functionalization of soraphens. *Nature Communications* **2022**, *13* (1), 371. DOI: 10.1038/s41467-022-27999-1.

(345) Neugebauer, M. E.; Sumida, K. H.; Pelton, J. G.; McMurry, J. L.; Marchand, J. A.; Chang, M. C. Y. A family of radical halogenases for the engineering of amino-acid-based products. *Nature Chemical Biology* **2019**, *15* (10), 1009-1016. DOI: 10.1038/s41589-019-0355-x.

(346) Neugebauer, M. E.; Kissman, E. N.; Marchand, J. A.; Pelton, J. G.; Sambold, N. A.; Millar, D. C.; Chang, M. C. Y. Reaction pathway engineering converts a radical hydroxylase into a halogenase. *Nature Chemical Biology* **2022**, *18* (2), 171-179. DOI: 10.1038/s41589-021-00944-x.

(347) Duewel, S.; Schmermund, L.; Faber, T.; Harms, K.; Srinivasan, V.; Meggers, E.; Hoebenreich, S. Directed Evolution of an FeII-Dependent Halogenase for Asymmetric C(sp3)–H Chlorination. *ACS Catalysis* **2020**, *10* (2), 1272-1277. DOI: 10.1021/acscatal.9b04691.

(348) Matthews, M. L.; Chang, W.-c.; Layne, A. P.; Miles, L. A.; Krebs, C.; Bollinger, J. M. Direct nitration and azidation of aliphatic carbons by an iron-dependent halogenase. *Nature Chemical Biology* **2014**, *10* (3), 209-215. DOI: 10.1038/nchembio.1438.

(349) Kim, C. Y.; Mitchell, A. J.; Glinkerman, C. M.; Li, F.-S.; Pluskal, T.; Weng, J.-K. The chloroalkaloid (−)-acutumine is biosynthesized via a Fe(II)- and 2-oxoglutarate-dependent halogenase in Menispermaceae plants. *Nature Communications* **2020**, *11* (1), 1867. DOI: 10.1038/s41467-020-15777-w.

(350) Mitchell, A. J.; Dunham, N. P.; Bergman, J. A.; Wang, B.; Zhu, Q.; Chang, W.-c.; Liu, X.; Boal, A. K. Structure-Guided Reprogramming of a Hydroxylase To Halogenate Its Small Molecule Substrate. *Biochemistry* **2017**, *56* (3), 441-444. DOI: 10.1021/acs.biochem.6b01173.

(351) Gomez, C. A.; Mondal, D.; Du, Q.; Chan, N.; Lewis, J. C. Directed Evolution of an Iron(II)- and α-Ketoglutarate-Dependent Dioxygenase for Site-Selective Azidation of Unactivated Aliphatic C-H Bonds. *Angewandte Chemie International Edition n/a* (n/a). DOI: https://doi.org/10.1002/anie.202301370.

(352) Rui, J.; Zhao, Q.; Huls, A. J.; Soler, J.; Paris, J. C.; Chen, Z.; Reshetnikov, V.; Yang, Y.; Guo, Y.; Garcia-Borràs, M.; et al. Directed evolution of nonheme iron enzymes to access abiological radical-relay C(sp^3)−H azidation. *Science* **2022**, *376* (6595), 869-874. DOI: doi:10.1126/science.abj2830.

(353) McIntosh, J. A.; Coelho, P. S.; Farwell, C. C.; Wang, Z. J.; Lewis, J. C.; Brown, T. R.; Arnold, F. H. Enantioselective Intramolecular C H Amination Catalyzed by Engineered Cytochrome P450 Enzymes In Vitro and In Vivo. *Angewandte Chemie International Edition* **2013**, *52* (35), 9309-9312. DOI: https://doi.org/10.1002/anie.201304401.

(354) Hyster, T. K.; Farwell, C. C.; Buller, A. R.; McIntosh, J. A.; Arnold, F. H. Enzyme-Controlled Nitrogen-Atom Transfer Enables Regiodivergent C–H Amination. *Journal of the American Chemical Society* **2014**, *136* (44), 15505-15508. DOI: 10.1021/ja509308v.

(355) Dydio, P.; Key, H. M.; Hayashi, H.; Clark, D. S.; Hartwig, J. F. Chemoselective, Enzymatic C–H Bond Amination Catalyzed by a Cytochrome P450 Containing an Ir(Me)-PIX Cofactor. *Journal of the American Chemical Society* **2017**, *139* (5), 1750-1753. DOI: 10.1021/jacs.6b11410.

(356) Jia, Z.-J.; Gao, S.; Arnold, F. H. Enzymatic Primary Amination of Benzylic and Allylic C(sp3)–H Bonds. *Journal of the American Chemical Society* **2020**, *142* (23), 10279-10283. DOI: 10.1021/jacs.0c03428.

(357) Athavale, S. V.; Gao, S.; Das, A.; Mallojjala, S. C.; Alfonzo, E.; Long, Y.; Hirschi, J. S.; Arnold, F. H. Enzymatic Nitrogen Insertion into Unactivated C–H Bonds. *Journal of the American Chemical Society* **2022**, *144* (41), 19097-19105. DOI: 10.1021/jacs.2c08285.

(358) Doyon, T. J.; Buller, A. R. Site-Selective Deuteration of Amino Acids through Dual-Protein Catalysis. *Journal of the American Chemical Society* **2022**, *144* (16), 7327-7336. DOI: 10.1021/jacs.2c00608.

(359) Kohei, T.; Miyaura, N. Introduction to Cross-Coupling Reactions. In *Cross-Coupling Reactions: A Practical Guide*, Miyaura, N. Ed.; Springer Berlin Heidelberg, 2002; pp 1-9.

(360) Kuhl, N.; Hopkinson, M. N.; Wencel-Delord, J.; Glorius, F. Beyond Directing Groups: Transition-Metal-Catalyzed C H Activation of Simple Arenes. *Angewandte Chemie International Edition* **2012**, *51* (41), 10236-10254. DOI: https://doi.org/10.1002/anie.201203269.

(361) Ackermann, L. Carboxylate-Assisted Transition-Metal-Catalyzed C−H Bond Functionalizations: Mechanism and Scope. *Chemical Reviews* **2011**, *111* (3), 1315-1345. DOI: 10.1021/cr100412j.

(362) Kozlowski, M. C. Oxidative Coupling in Complexity Building Transforms. *Accounts of Chemical Research* **2017**, *50* (3), 638-643. DOI: 10.1021/acs.accounts.6b00637.

(363) Shalit, H.; Dyadyuk, A.; Pappo, D. Selective Oxidative Phenol Coupling by Iron Catalysis. *The Journal of Organic Chemistry* **2019**, *84* (4), 1677-1686. DOI: 10.1021/acs.joc.8b03084.

(364) Forneris, C. C.; Seyedsayamdost, M. R. In Vitro Reconstitution of OxyC Activity Enables Total Chemoenzymatic Syntheses of Vancomycin Aglycone Variants. *Angewandte Chemie International Edition* **2018**, *57* (27), 8048-8052. DOI: https://doi.org/10.1002/anie.201802856.

(365) Pylypenko, O.; Vitali, F.; Zerbe, K.; Robinson, J. A.; Schlichting, I. Crystal Structure of OxyC, a Cytochrome P450 Implicated in an Oxidative C–C Coupling Reaction during Vancomycin Biosynthesis *. *Journal of Biological Chemistry* **2003**, *278* (47), 46727-46733. DOI: 10.1074/jbc.M306486200 (acccessed 2023/01/30).

(366) Aldemir, H.; Shu, S.; Schaefers, F.; Hong, H.; Richarz, R.; Harteis, S.; Einsiedler, M.; Milzarek, T. M.; Schneider, S.; Gulder, T. A. M. Carrier Protein-Free Enzymatic Biaryl Coupling in Arylomycin A2 Assembly and Structure of the Cytochrome P450 AryC**. *Chemistry – A European Journal* **2022**, *28* (2), e202103389. DOI: https://doi.org/10.1002/chem.202103389.

(367) Molinaro, C.; Kawasaki, Y.; Wanyoike, G.; Nishioka, T.; Yamamoto, T.; Snedecor, B.; Robinson, S. J.; Gosselin, F. Engineered Cytochrome P450-Catalyzed Oxidative Biaryl Coupling Reaction Provides a Scalable Entry into Arylomycin Antibiotics. *Journal of the American Chemical Society* **2022**, *144* (32), 14838-14845. DOI: 10.1021/jacs.2c06019.

(368) 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* (7899), 79-85. DOI: 10.1038/s41586-021-04365-7.

(369) Mazzaferro, L. S.; Hüttel, W.; Fries, A.; Müller, M. Cytochrome P450-Catalyzed Regio- and Stereoselective Phenol Coupling of Fungal Natural Products. *Journal of the American Chemical Society* **2015**, *137* (38), 12289-12295. DOI: 10.1021/jacs.5b06776.

(370) Obermaier, S.; Müller, M. Biaryl-Forming Enzymes from Aspergilli Exhibit Substrate-Dependent Stereoselectivity. *Biochemistry* **2019**, *58* (22), 2589-2593. DOI: 10.1021/acs.biochem.9b00291.

(371) Coyle, C. M.; Panaccione, D. G. An Ergot Alkaloid Biosynthesis Gene and Clustered Hypothetical Genes from <i>Aspergillus fumigatus</i>. *Applied and Environmental Microbiology* **2005**, *71* (6), 3112-3118. DOI: doi:10.1128/AEM.71.6.3112-3118.2005.

(372) Unsöld, I. A.; Li, S.-M. Overproduction, purification and characterization of FgaPT2, a dimethylallyltryptophan synthase from Aspergillus fumigatus. *Microbiology* **2005**, *151* (5), 1499-1505. DOI: https://doi.org/10.1099/mic.0.27759-0.

(373) Steffan, N.; Unsöld, I. A.; Li, S.-M. Chemoenzymatic Synthesis of Prenylated Indole Derivatives by Using a 4-Dimethylallyltryptophan Synthase from Aspergillus fumigatus. *ChemBioChem* **2007**, *8* (11), 1298-1307. DOI: https://doi.org/10.1002/cbic.200700107.

(374) Steffan, N.; Li, S.-M. Increasing structure diversity of prenylated diketopiperazine derivatives by using a 4-dimethylallyltryptophan synthase. *Archives of Microbiology* **2009**, *191* (5), 461-466. DOI: 10.1007/s00203-009-0467-x.

(375) Eggbauer, B.; Schrittwieser, J. H.; Kerschbaumer, B.; Macheroux, P.; Kroutil, W. Regioselective Biocatalytic C4-Prenylation of Unprotected Tryptophan Derivatives. *ChemBioChem* **2022**, *23* (17), e202200311. DOI: https://doi.org/10.1002/cbic.202200311.

(376) Kremer, A.; Westrich, L.; Li, S.-M. A 7-dimethylallyltryptophan synthase from Aspergillus fumigatus: overproduction, purification and biochemical characterization. *Microbiology* **2007**, *153* (10), 3409-3416. DOI: https://doi.org/10.1099/mic.0.2007/009019-0.

(377) Kremer, A.; Li, S.-M. Potential of a 7-dimethylallyltryptophan synthase as a tool for production of prenylated indole derivatives. *Applied Microbiology and Biotechnology* **2008**, *79* (6), 951-961. DOI: 10.1007/s00253-008-1505-3.

(378) Takahashi, S.; Takagi, H.; Toyoda, A.; Uramoto, M.; Nogawa, T.; Ueki, M.; Sakaki, Y.; Osada, H. Biochemical Characterization of a Novel Indole Prenyltransferase from <i>Streptomyces</i> sp. SN-593. *Journal of Bacteriology* **2010**, *192* (11), 2839-2851. DOI: doi:10.1128/JB.01557-09.

(379) Winkelblech, J.; Li, S.-M. Biochemical Investigations of Two 6-DMATS Enzymes from Streptomyces Reveal New Features of L-Tryptophan Prenyltransferases. *ChemBioChem* **2014**, *15* (7), 1030-1039. DOI: https://doi.org/10.1002/cbic.201400046.

(380) Elshahawi, S. I.; Cao, H.; Shaaban, K. A.; Ponomareva, L. V.; Subramanian, T.; Farman, M. L.; Spielmann, H. P.; Phillips, G. N.; Thorson, J. S.; Singh, S. Structure and specificity of a permissive bacterial C-prenyltransferase. *Nature Chemical Biology* **2017**, *13* (4), 366-368. DOI: 10.1038/nchembio.2285.

(381) Mai, P.; Zocher, G.; Ludwig, L.; Stehle, T.; Li, S.-M. Actions of Tryptophan Prenyltransferases Toward Fumiquinazolines and their Potential Application for the Generation of Prenylated Derivatives by Combining Chemical and Chemoenzymatic Syntheses. *Advanced Synthesis & Catalysis* **2016**, *358* (10), 1639-1653. DOI: https://doi.org/10.1002/adsc.201600064.

(382) Chen, R.; Gao, B.; Liu, X.; Ruan, F.; Zhang, Y.; Lou, J.; Feng, K.; Wunsch, C.; Li, S.-M.; Dai, J.; et al. Molecular insights into the enzyme promiscuity of an aromatic prenyltransferase. *Nature Chemical Biology* **2017**, *13* (2), 226-234. DOI: 10.1038/nchembio.2263.

(383) Yu, X.; Liu, Y.; Xie, X.; Zheng, X.-D.; Li, S.-M. Biochemical Characterization of Indole Prenyltransferases: FILLING THE LAST GAP OF PRENYLATION POSITIONS BY A 5-DIMETHYLALLYLTRYPTOPHAN SYNTHASE FROM ASPERGILLUS CLAVATUS *. *Journal of Biological Chemistry* **2012**, *287* (2), 1371-1380. DOI: 10.1074/jbc.M111.317982 (acccessed 2023/01/30).

(384) Winkelblech, J.; Liebhold, M.; Gunera, J.; Xie, X.; Kolb, P.; Li, S.-M. Tryptophan C5-, C6- and C7-Prenylating Enzymes Displaying a Preference for C-6 of the Indole Ring in the Presence

of Unnatural Dimethylallyl Diphosphate Analogues. *Advanced Synthesis & Catalysis* **2015**, *357* (5), 975-986. DOI: https://doi.org/10.1002/adsc.201400958.

(385) Liebhold, M.; Li, S.-M. Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase. *Organic Letters* **2013**, *15* (22), 5834-5837. DOI: 10.1021/ol4029012.

(386) Yokoyama, K.; Lilla, E. A. C–C bond forming radical SAM enzymes involved in the construction of carbon skeletons of cofactors and natural products. *Natural Product Reports* **2018**, *35* (7), 660-694, 10.1039/C8NP00006A. DOI: 10.1039/C8NP00006A.

(387) Knox, H. L.; Sinner, E. K.; Townsend, C. A.; Boal, A. K.; Booker, S. J. Structure of a B12-dependent radical SAM enzyme in carbapenem biosynthesis. *Nature* **2022**, *602* (7896), 343-348. DOI: 10.1038/s41586-021-04392-4.

(388) Soualmia, F.; Guillot, A.; Sabat, N.; Brewee, C.; Kubiak, X.; Haumann, M.; Guinchard, X.; Benjdia, A.; Berteau, O. Exploring the Biosynthetic Potential of TsrM, a B12-dependent Radical SAM Methyltransferase Catalyzing Non-radical Reactions. *Chemistry – A European Journal* **2022**, *28* (31), e202200627. DOI: https://doi.org/10.1002/chem.202200627.

(389) Gericke, L.; Mhaindarkar, D.; Karst, L. C.; Jahn, S.; Kuge, M.; Mohr, M. K. F.; Gagsteiger, J.; Cornelissen, N. V.; Wen, X.; Mordhorst, S.; et al. Biomimetic S-Adenosylmethionine Regeneration Starting from Multiple Byproducts Enables Biocatalytic Alkylation with Radical SAM Enzymes. *ChemBioChem* *n/a* (n/a), e202300133. DOI: https://doi.org/10.1002/cbic.202300133.

(390) Pickel, B.; Schaller, A. Dirigent proteins: molecular characteristics and potential biotechnological applications. *Applied Microbiology and Biotechnology* **2013**, *97* (19), 8427-8438. DOI: 10.1007/s00253-013-5167-4.

(391) Davin, L. B.; Lewis, N. G. Dirigent Proteins and Dirigent Sites Explain the Mystery of Specificity of Radical Precursor Coupling in Lignan and Lignin Biosynthesis1. *Plant Physiology* **2000**, *123* (2), 453-462. DOI: 10.1104/pp.123.2.453 (acccessed 2/22/2023).

(392) Kim, S. S.; Sattely, E. S. Dirigent Proteins Guide Asymmetric Heterocoupling for the Synthesis of Complex Natural Product Analogues. *Journal of the American Chemical Society* **2021**, *143* (13), 5011-5021. DOI: 10.1021/jacs.0c13164.

(393) Hyster, T.; Page, C.; Cao, J.; Oblinsky, D.; MacMillan, S.; Dahagam, S.; Lloyd, R.; Charnock, S.; Scholes, G. Regioselective Radical Alkylation of Arenes Using Evolved Photoenzymes. *Res Sq* **2023**. DOI: 10.21203/rs.3.rs-2602958/v1 From NLM.

(394) Biegasiewicz, K. F.; Cooper, S. J.; Gao, X.; Oblinsky, D. G.; Kim, J. H.; Garfinkle, S. E.; Joyce, L. A.; Sandoval, B. A.; Scholes, G. D.; Hyster, T. K. Photoexcitation of flavoenzymes enables a stereoselective radical cyclization. *Science* **2019**, *364* (6446), 1166-1169. DOI: doi:10.1126/science.aaw1143.

(395) Clayman, P. D.; Hyster, T. K. Photoenzymatic Generation of Unstabilized Alkyl Radicals: An Asymmetric Reductive Cyclization. *Journal of the American Chemical Society* **2020**, *142* (37), 15673-15677. DOI: 10.1021/jacs.0c07918.

(396) Nicholls, B. T.; Oblinsky, D. G.; Kurtoic, S. I.; Grosheva, D.; Ye, Y.; Scholes, G. D.; Hyster, T. K. Engineering a Non-Natural Photoenzyme for Improved Photon Efficiency**. *Angewandte Chemie International Edition* **2022**, *61* (2), e202113842. DOI: https://doi.org/10.1002/anie.202113842.

(397) Brandenberg, O. F.; Fasan, R.; Arnold, F. H. Exploiting and engineering hemoproteins for abiological carbene and nitrene transfer reactions. *Current Opinion in Biotechnology* **2017**, *47*, 102-111. DOI: https://doi.org/10.1016/j.copbio.2017.06.005.

(398) Coelho, P. S.; Wang, Z. J.; Ener, M. E.; Baril, S. A.; Kannan, A.; Arnold, F. H.; Brustad, E. M. A serine-substituted P450 catalyzes highly efficient carbene transfer to olefins in vivo. *Nature Chemical Biology* **2013**, *9* (8), 485-487. DOI: 10.1038/nchembio.1278.

(399) Zhang, R. K.; Chen, K.; Huang, X.; Wohlschlager, L.; Renata, H.; Arnold, F. H. Enzymatic assembly of carbon–carbon bonds via iron-catalysed sp3 C–H functionalization. *Nature* **2019**, *565* (7737), 67-72. DOI: 10.1038/s41586-018-0808-5.

(400) Brandenberg, O. F.; Chen, K.; Arnold, F. H. Directed Evolution of a Cytochrome P450 Carbene Transferase for Selective Functionalization of Cyclic Compounds. *Journal of the American Chemical Society* **2019**, *141* (22), 8989-8995. DOI: 10.1021/jacs.9b02931.

(401) Zhou, A. Z.; Chen, K.; Arnold, F. H. Enzymatic Lactone-Carbene C–H Insertion to Build Contiguous Chiral Centers. *ACS Catalysis* **2020**, *10* (10), 5393-5398. DOI: 10.1021/acscatal.0c01349.

(402) Dydio, P.; Key, H. M.; Nazarenko, A.; Rha, J. Y.-E.; Seyedkazemi, V.; Clark, D. S.; Hartwig, J. F. An artificial metalloenzyme with the kinetics of native enzymes. *Science* **2016**, *354* (6308), 102-106. DOI: doi:10.1126/science.aah4427.

(403) Natoli, S. N.; Hartwig, J. F. Noble−Metal Substitution in Hemoproteins: An Emerging Strategy for Abiological Catalysis. *Accounts of Chemical Research* **2019**, *52* (2), 326-335. DOI: 10.1021/acs.accounts.8b00586.

(404) Gu, Y.; Natoli, S. N.; Liu, Z.; Clark, D. S.; Hartwig, J. F. Site-Selective Functionalization of (sp3)C−H Bonds Catalyzed by Artificial Metalloenzymes Containing an Iridium-Porphyrin Cofactor. *Angewandte Chemie International Edition* **2019**, *58* (39), 13954-13960. DOI: https://doi.org/10.1002/anie.201907460.

(405) Rumo, C.; Stein, A.; Klehr, J.; Tachibana, R.; Prescimone, A.; Häussinger, D.; Ward, T. R. An Artificial Metalloenzyme Based on a Copper Heteroscorpionate Enables sp3 C–H Functionalization via Intramolecular Carbene Insertion. *Journal of the American Chemical Society* **2022**, *144* (26), 11676-11684. DOI: 10.1021/jacs.2c03311.

(406) O'Hagan, D.; Deng, H. Enzymatic Fluorination and Biotechnological Developments of the Fluorinase. *Chemical Reviews* **2015**, *115* (2), 634-649. DOI: 10.1021/cr500209t.

(407) Roy, A. D.; Grüschow, S.; Cairns, N.; Goss, R. J. M. Gene Expression Enabling Synthetic Diversification of Natural Products: Chemogenetic Generation of Pacidamycin Analogs. *Journal of the American Chemical Society* **2010**, *132* (35), 12243-12245. DOI: 10.1021/ja1060406.

(408) Runguphan, W.; O'Connor, S. E. Diversification of Monoterpene Indole Alkaloid Analogs through Cross-Coupling. *Organic Letters* **2013**, *15* (11), 2850-2853. DOI: 10.1021/ol401179k.

(409) Durak, L. J.; Payne, J. T.; Lewis, J. C. Late-Stage Diversification of Biologically Active Molecules via Chemoenzymatic C–H Functionalization. *ACS Catalysis* **2016**, *6* (3), 1451-1454. DOI: 10.1021/acscatal.5b02558.

(410) Latham, J.; Henry, J.-M.; Sharif, H. H.; Menon, B. R. K.; Shepherd, S. A.; Greaney, M. F.; Micklefield, J. Integrated catalysis opens new arylation pathways via regiodivergent enzymatic C–H activation. *Nature Communications* **2016**, *7* (1), 11873. DOI: 10.1038/ncomms11873.

(411) Frese, M.; Schnepel, C.; Minges, H.; Voß, H.; Feiner, R.; Sewald, N. Modular Combination of Enzymatic Halogenation of Tryptophan with Suzuki–Miyaura Cross-Coupling Reactions. *ChemCatChem* **2016**, *8* (10), 1799-1803. DOI: https://doi.org/10.1002/cctc.201600317.

(412) Schnepel, C.; Minges, H.; Frese, M.; Sewald, N. A High-Throughput Fluorescence Assay to Determine the Activity of Tryptophan Halogenases. *Angewandte Chemie International Edition* **2016**, *55* (45), 14159-14163. DOI: https://doi.org/10.1002/anie.201605635.

(413) Lewis, J. C.; Arnold, F. H. Catalysts on Demand: Selective Oxidations by Laboratory-Evolved Cytochrome P450 BM3. *CHIMIA* **2009**, *63* (6), 309. DOI: 10.2533/chimia.2009.309 (acccessed 2023/03/05).

(414) Li, J.; Li, F.; King-Smith, E.; Renata, H. Merging chemoenzymatic and radical-based retrosynthetic logic for rapid and modular synthesis of oxidized meroterpenoids. *Nature Chemistry* **2020**, *12* (2), 173-179. DOI: 10.1038/s41557-019-0407-6.

(415) Li, F.; Deng, H.; Renata, H. Remote B-Ring Oxidation of Sclareol with an Engineered P450 Facilitates Divergent Access to Complex Terpenoids. *Journal of the American Chemical Society* **2022**, *144* (17), 7616-7621. DOI: 10.1021/jacs.2c02958.

(416) Li, J.; Chen, F.; Renata, H. Concise Chemoenzymatic Synthesis of Gedunin. *Journal of the American Chemical Society* **2022**, *144* (42), 19238-19242. DOI: 10.1021/jacs.2c09048.

(417) Zhang, X.; King-Smith, E.; Dong, L.-B.; Yang, L.-C.; Rudolf, J. D.; Shen, B.; Renata, H. Divergent synthesis of complex diterpenes through a hybrid oxidative approach. *Science* **2020**, *369* (6505), 799-806. DOI: doi:10.1126/science.abb8271.

(418) Zhao, Y.; Zhang, B.; Sun, Z. Q.; Zhang, H.; Wang, W.; Wang, Z. R.; Guo, Z. K.; Yu, S.; Tan, R. X.; Ge, H. M. Biocatalytic C14-Hydroxylation on Androstenedione Enabled Modular Synthesis of Cardiotonic Steroids. *ACS Catalysis* **2022**, *12* (16), 9839-9845. DOI: 10.1021/acscatal.2c02185.

(419) Zhang, X.; Renata, H. Efficient chemoenzymatic synthesis of (2S,3R)-3-hydroxy-3-methylproline, a key fragment in polyoxypeptin A and FR225659. *Tetrahedron* **2019**, *75* (24), 3253-3257. DOI: https://doi.org/10.1016/j.tet.2019.04.009.

(420) Zwick, C. R.; Renata, H. A one-pot chemoenzymatic synthesis of (2S, 4R)-4-methylproline enables the first total synthesis of antiviral lipopeptide cavinafungin B. *Tetrahedron* **2018**, *74* (45), 6469-6473. DOI: https://doi.org/10.1016/j.tet.2018.09.046.

(421) Zhang, X.; King-Smith, E.; Renata, H. Total Synthesis of Tambromycin by Combining Chemocatalytic and Biocatalytic C−H Functionalization. *Angewandte Chemie International Edition* **2018**, *57* (18), 5037-5041. DOI: https://doi.org/10.1002/anie.201801165.

(422) Zwick, C. R., III; Sosa, M. B.; Renata, H. Modular Chemoenzymatic Synthesis of GE81112 B1 and Related Analogues Enables Elucidation of Its Key Pharmacophores. *Journal of the American Chemical Society* **2021**, *143* (3), 1673-1679. DOI: 10.1021/jacs.0c13424.

(423) Amatuni, A.; Shuster, A.; Abegg, D.; Adibekian, A.; Renata, H. Comprehensive Structure–Activity Relationship Studies of Cepafungin Enabled by Biocatalytic C–H Oxidations. *ACS Central Science* **2023**, *9* (2), 239-251. DOI: 10.1021/acscentsci.2c01219.

(424) Jiang, Y.; Renata, H. *Eleven-Step Chemoenzymatic Synthesis of Cotylenol*; 2022. DOI: 10.26434/chemrxiv-2022-7nvn3.

(425) Lazzarotto, M.; Hammerer, L.; Hetmann, M.; Borg, A.; Schmermund, L.; Steiner, L.; Hartmann, P.; Belaj, F.; Kroutil, W.; Gruber, K.; et al. Chemoenzymatic Total Synthesis of Deoxy-, epi-, and Podophyllotoxin and a Biocatalytic Kinetic Resolution of Dibenzylbutyrolactones. *Angewandte Chemie International Edition* **2019**, *58* (24), 8226-8230. DOI: https://doi.org/10.1002/anie.201900926.

(426) Li, J.; Zhang, X.; Renata, H. Asymmetric Chemoenzymatic Synthesis of (−)-Podophyllotoxin and Related Aryltetralin Lignans. *Angewandte Chemie International Edition* **2019**, *58* (34), 11657-11660. DOI: https://doi.org/10.1002/anie.201904102.

(427) Li, J.; Renata, H. Concise Chemoenzymatic Synthesis of Fasamycin A. *The Journal of Organic Chemistry* **2021**, *86* (16), 11206-11211. DOI: 10.1021/acs.joc.1c00526.

(428) Sib, A.; Gulder, T. A. M. Chemo-enzymatic Total Synthesis of Oxosorbicillinol, Sorrentanone, Rezishanones B and C, Sorbicatechol A, Bisvertinolone, and (+)-Epoxysorbicillinol. *Angewandte Chemie International Edition* **2018**, *57* (44), 14650-14653. DOI: https://doi.org/10.1002/anie.201802176.

(429) Sib, A.; Gulder, T. A. M. Stereoselective Total Synthesis of Bisorbicillinoid Natural Products by Enzymatic Oxidative Dearomatization/Dimerization. *Angewandte Chemie International Edition* **2017**, *56* (42), 12888-12891. DOI: https://doi.org/10.1002/anie.201705976.

(430) Hoyt, E. A.; Cal, P. M. S. D.; Oliveira, B. L.; Bernardes, G. J. L. Contemporary approaches to site-selective protein modification. *Nature Reviews Chemistry* **2019**, *3* (3), 147-171. DOI: 10.1038/s41570-019-0079-1.

(431) Rees, H. A.; Liu, D. R. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nature Reviews Genetics* **2018**, *19* (12), 770-788. DOI: 10.1038/s41576-018-0059-1.

(432) Porto, E. M.; Komor, A. C.; Slaymaker, I. M.; Yeo, G. W. Base editing: advances and therapeutic opportunities. *Nature Reviews Drug Discovery* **2020**, *19* (12), 839-859. DOI: 10.1038/s41573-020-0084-6.

(433) Žáková, L.; Zyka, D.; Ježek, J.; Hančlová, I.; Šanda, M.; Brzozowski, A. M.; Jiráček, J. The use of Fmoc-Lys(Pac)-OH and penicillin G acylase in the preparation of novel semisynthetic insulin analogs. *Journal of Peptide Science* **2007**, *13* (5), 334-341. DOI: https://doi.org/10.1002/psc.847.

(434) Fryszkowska, A.; An, C.; Alvizo, O.; Banerjee, G.; Canada, K. A.; Cao, Y.; DeMong, D.; Devine, P. N.; Duan, D.; Elgart, D. M.; et al. A chemoenzymatic strategy for site-selective functionalization of native peptides and proteins. *Science* **2022**, *376* (6599), 1321-1327. DOI: doi:10.1126/science.abn2009.

(435) Pissarnitski, D. A.; Kekec, A.; Yan, L.; Zhu, Y.; Feng, D. D.; Huo, P.; Madsen-Duggan, C.; Moyes, C. R.; Nargund, R. P.; Kelly, T.; et al. Discovery of Insulin Receptor Partial Agonists MK-5160 and MK-1092 as Novel Basal Insulins with Potential to Improve Therapeutic Index. *J Med Chem* **2022**, *65* (7), 5593-5605. DOI: 10.1021/acs.jmedchem.1c02073 From NLM.

(436) Wu, M.; Carballo-Jane, E.; Zhou, H.; Zafian, P.; Dai, G.; Liu, M.; Lao, J.; Kelly, T.; Shao, D.; Gorski, J.; et al. Functionally selective signaling and broad metabolic benefits by novel insulin receptor partial agonists. *Nature Communications* **2022**, *13* (1), 942. DOI: 10.1038/s41467-022-28561-9.

(437) Rachel, N. M.; Quaglia, D.; Lévesque, É.; Charette, A. B.; Pelletier, J. N. Engineered, highly reactive substrates of microbial transglutaminase enable protein labeling within various secondary structure elements. *Protein Science* **2017**, *26* (11), 2268-2279. DOI: https://doi.org/10.1002/pro.3286.

(438) Marx, C. K.; Hertel, T. C.; Pietzsch, M. Random mutagenesis of a recombinant microbial transglutaminase for the generation of thermostable and heat-sensitive variants. *Journal of Biotechnology* **2008**, *136* (3), 156-162. DOI: https://doi.org/10.1016/j.jbiotec.2008.06.005.

(439) Deweid, L.; Neureiter, L.; Englert, S.; Schneider, H.; Deweid, J.; Yanakieva, D.; Sturm, J.; Bitsch, S.; Christmann, A.; Avrutina, O.; et al. Directed Evolution of a Bond-Forming Enzyme: Ultrahigh-Throughput Screening of Microbial Transglutaminase Using Yeast Surface Display. *Chemistry – A European Journal* **2018**, *24* (57), 15195-15200. DOI: https://doi.org/10.1002/chem.201803485.

(440) Zhou, L.; Kooy-Winkelaar, Y. M. C.; Cordfunke, R. A.; Dragan, I.; Thompson, A.; Drijfhout, J. W.; van Veelen, P. A.; Chen, H.; Koning, F. Abrogation of Immunogenic Properties

of Gliadin Peptides through Transamidation by Microbial Transglutaminase Is Acyl-Acceptor Dependent. *Journal of Agricultural and Food Chemistry* **2017**, *65* (34), 7542-7552. DOI: 10.1021/acs.jafc.7b02557.

(441) Fontana, A.; Spolaore, B.; Mero, A.; Veronese, F. M. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. *Advanced Drug Delivery Reviews* **2008**, *60* (1), 13-28. DOI: https://doi.org/10.1016/j.addr.2007.06.015.

(442) Mero, A.; Spolaore, B.; Veronese, F. M.; Fontana, A. Transglutaminase-Mediated PEGylation of Proteins: Direct Identification of the Sites of Protein Modification by Mass Spectrometry using a Novel Monodisperse PEG. *Bioconjugate Chemistry* **2009**, *20* (2), 384-389. DOI: 10.1021/bc800427n.

(443) Anami, Y.; Yamazaki, C. M.; Xiong, W.; Gui, X.; Zhang, N.; An, Z.; Tsuchikama, K. Glutamic acid–valine–citrulline linkers ensure stability and efficacy of antibody–drug conjugates in mice. *Nature Communications* **2018**, *9* (1), 2512. DOI: 10.1038/s41467-018-04982-3.

(444) Yamazaki, C. M.; Yamaguchi, A.; Anami, Y.; Xiong, W.; Otani, Y.; Lee, J.; Ueno, N. T.; Zhang, N.; An, Z.; Tsuchikama, K. Antibody-drug conjugates with dual payloads for combating breast tumor heterogeneity and drug resistance. *Nature Communications* **2021**, *12* (1), 3528. DOI: 10.1038/s41467-021-23793-7.

(445) Bell, E. L.; Finnigan, W.; France, S. P.; Green, A. P.; Hayes, M. A.; Hepworth, L. J.; Lovelock, S. L.; Niikura, H.; Osuna, S.; Romero, E.; et al. Biocatalysis. *Nature Reviews Methods Primers* **2021**, *1* (1), 46. DOI: 10.1038/s43586-021-00044-z.

(446) van der Donk, W. A. Introduction: Unusual Enzymology in Natural Product Synthesis. *Chemical Reviews* **2017**, *117* (8), 5223-5225. DOI: 10.1021/acs.chemrev.7b00124.

(447) Booker, S. J. Anaerobic functionalization of unactivated C–H bonds. *Current Opinion in Chemical Biology* **2009**, *13* (1), 58-73. DOI: https://doi.org/10.1016/j.cbpa.2009.02.036.

(448) Powell, M. M.; Rao, G.; Britt, R. D.; Rittle, J. Enzymatic Hydroxylation of Aliphatic C-H Bonds by a Mn/Fe Cofactor. *bioRxiv* **2023**, 2023.2003.2010.532131. DOI: 10.1101/2023.03.10.532131.

(449) Ziemert, N.; Alanjary, M.; Weber, T. The evolution of genome mining in microbes – a review. *Natural Product Reports* **2016**, *33* (8), 988-1005, 10.1039/C6NP00025H. DOI: 10.1039/C6NP00025H.

(450) Marshall, J. R.; Yao, P.; Montgomery, S. L.; Finnigan, J. D.; Thorpe, T. W.; Palmer, R. B.; Mangas-Sanchez, J.; Duncan, R. A. M.; Heath, R. S.; Graham, K. M.; et al. Screening and characterization of a diverse panel of metagenomic imine reductases for biocatalytic reductive amination. *Nature Chemistry* **2021**, *13* (2), 140-148. DOI: 10.1038/s41557-020-00606-w.

(451) Vaissier Welborn, V.; Head-Gordon, T. Computational Design of Synthetic Enzymes. *Chemical Reviews* **2019**, *119* (11), 6613-6630. DOI: 10.1021/acs.chemrev.8b00399.

(452) Biegasiewicz, K. F.; Cooper, S. J.; Emmanuel, M. A.; Miller, D. C.; Hyster, T. K. Catalytic promiscuity enabled by photoredox catalysis in nicotinamide-dependent oxidoreductases. *Nature Chemistry* **2018**, *10* (7), 770-775. DOI: 10.1038/s41557-018-0059-y.

(453) Devamani, T.; Rauwerdink, A. M.; Lunzer, M.; Jones, B. J.; Mooney, J. L.; Tan, M. A. O.; Zhang, Z.-J.; Xu, J.-H.; Dean, A. M.; Kazlauskas, R. J. Catalytic Promiscuity of Ancestral Esterases and Hydroxynitrile Lyases. *Journal of the American Chemical Society* **2016**, *138* (3), 1046-1056. DOI: 10.1021/jacs.5b12209.

(454) Yang, H.; Swartz, A. M.; Park, H. J.; Srivastava, P.; Ellis-Guardiola, K.; Upp, D. M.; Lee, G.; Belsare, K.; Gu, Y.; Zhang, C.; et al. Evolving artificial metalloenzymes via random mutagenesis. *Nature Chemistry* **2018**, *10* (3), 318-324. DOI: 10.1038/nchem.2927.

(455) Jeschek, M.; Reuter, R.; Heinisch, T.; Trindler, C.; Klehr, J.; Panke, S.; Ward, T. R. Directed evolution of artificial metalloenzymes for in vivo metathesis. *Nature* **2016**, *537* (7622), 661-665. DOI: 10.1038/nature19114.

(456) Markel, U.; Sauer, D. F.; Schiffels, J.; Okuda, J.; Schwaneberg, U. Towards the Evolution of Artificial Metalloenzymes—A Protein Engineer's Perspective. *Angewandte Chemie International Edition* **2019**, *58* (14), 4454-4464. DOI: https://doi.org/10.1002/anie.201811042.

(457) Gröger, H.; Gallou, F.; Lipshutz, B. H. Where Chemocatalysis Meets Biocatalysis: In Water. *Chemical Reviews* **2022**. DOI: 10.1021/acs.chemrev.2c00416.

(458) Introduction: Biocatalysis in Industry. *Chemical Reviews* **2018**, *118* (1), 1-3. DOI: 10.1021/acs.chemrev.7b00741.

(459) Wu, S.; Snajdrova, R.; Moore, J. C.; Baldenius, K.; Bornscheuer, U. T. Biocatalysis: Enzymatic Synthesis for Industrial Applications. *Angewandte Chemie International Edition* **2021**, *60* (1), 88-119. DOI: https://doi.org/10.1002/anie.202006648.

(460) Romero, P. A.; Arnold, F. H. Exploring protein fitness landscapes by directed evolution. *Nature Reviews Molecular Cell Biology* **2009**, *10* (12), 866-876. DOI: 10.1038/nrm2805.

(461) Quertinmont, L. T.; Orru, R.; Lutz, S. RApid Parallel Protein EvaluatoR (RAPPER), from gene to enzyme function in one day. *Chemical Communications* **2015**, *51* (1), 122-124, 10.1039/C4CC08240K. DOI: 10.1039/C4CC08240K.

(462) Holland-Moritz, D. A.; Wismer, M. K.; Mann, B. F.; Farasat, I.; Devine, P.; Guetschow, E. D.; Mangion, I.; Welch, C. J.; Moore, J. C.; Sun, S.; et al. Mass Activated Droplet Sorting (MADS) Enables High-Throughput Screening of Enzymatic Reactions at Nanoliter Scale. *Angewandte Chemie International Edition* **2020**, *59* (11), 4470-4477. DOI: https://doi.org/10.1002/anie.201913203.

(463) Diefenbach, X. W.; Farasat, I.; Guetschow, E. D.; Welch, C. J.; Kennedy, R. T.; Sun, S.; Moore, J. C. Enabling Biocatalysis by High-Throughput Protein Engineering Using Droplet Microfluidics Coupled to Mass Spectrometry. *ACS Omega* **2018**, *3* (2), 1498-1508. DOI: 10.1021/acsomega.7b01973.

(464) Wleklinski, M.; Loren, B. P.; Ferreira, C. R.; Jaman, Z.; Avramova, L.; Sobreira, T. J. P.; Thompson, D. H.; Cooks, R. G. High throughput reaction screening using desorption electrospray ionization mass spectrometry. *Chemical Science* **2018**, *9* (6), 1647-1653, 10.1039/C7SC04606E. DOI: 10.1039/C7SC04606E.

(465) Mahr, R.; Frunzke, J. Transcription factor-based biosensors in biotechnology: current state and future prospects. *Applied Microbiology and Biotechnology* **2016**, *100* (1), 79-90. DOI: 10.1007/s00253-015-7090-3.

(466) Derat, E.; Kamerlin, S. C. L. Computational Advances in Protein Engineering and Enzyme Design. *The Journal of Physical Chemistry B* **2022**, *126* (13), 2449-2451. DOI: 10.1021/acs.jpcb.2c01198.

(467) Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596* (7873), 583-589. DOI: 10.1038/s41586-021-03819-2.

(468) Yang, K. K.; Wu, Z.; Arnold, F. H. Machine-learning-guided directed evolution for protein engineering. *Nature Methods* **2019**, *16* (8), 687-694. DOI: 10.1038/s41592-019-0496-6.

(469) Freschlin, C. R.; Fahlberg, S. A.; Romero, P. A. Machine learning to navigate fitness landscapes for protein engineering. *Current Opinion in Biotechnology* **2022**, *75*, 102713. DOI: https://doi.org/10.1016/j.copbio.2022.102713.