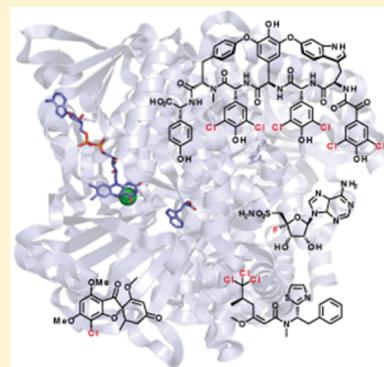


## Development of Halogenase Enzymes for Use in Synthesis

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**ABSTRACT:** Nature has evolved halogenase enzymes to regioselectively halogenate a diverse range of biosynthetic precursors, with the halogens introduced often having a profound effect on the biological activity of the resulting natural products. Synthetic endeavors to create non-natural bioactive small molecules for pharmaceutical and agrochemical applications have also arrived at a similar conclusion: halogens can dramatically improve the properties of organic molecules for selective modulation of biological targets *in vivo*. Consequently, a high proportion of pharmaceuticals and agrochemicals on the market today possess halogens. Halogenated organic compounds are also common intermediates in synthesis and are particularly valuable in metal-catalyzed cross-coupling reactions. Despite the potential utility of organohalogens, traditional nonenzymatic halogenation chemistry utilizes deleterious reagents and often lacks regiocontrol. Reliable, facile, and cleaner methods for the regioselective halogenation of organic compounds are therefore essential in the development of economical and environmentally friendly industrial processes. A potential avenue toward such methods is the use of halogenase enzymes, responsible for the biosynthesis of halogenated natural products, as biocatalysts. This Review will discuss advances in developing halogenases for biocatalysis, potential untapped sources of such biocatalysts and how further optimization of these enzymes is required to achieve the goal of industrial scale biohalogenation.



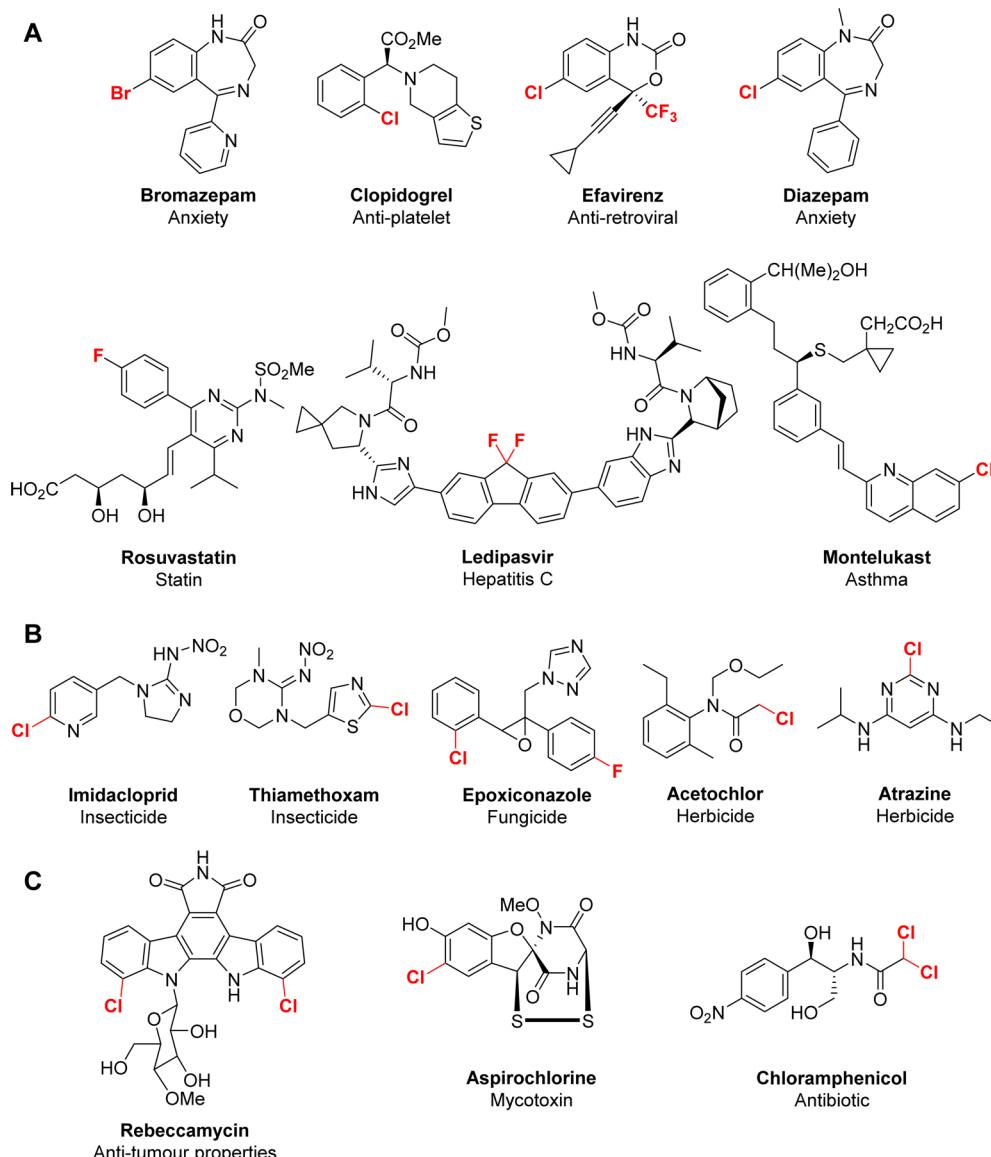
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**Figure 1.** (A) Examples of top-selling halogen-containing pharmaceuticals. (B) Halogenated agrochemicals. (C) Halogenated natural products with known potent bioactivity.

## 1. INTRODUCTION

Organohalogen moieties are present in many pharmaceutical and agrochemical products, as well as other valuable materials, and are also widely used in all sectors of the chemical industry as synthetic intermediates. Transition metal-catalyzed cross-coupling reactions have become indispensable tools for the synthesis of complex molecules because of the multitude of C–C, C–F, C–N, and other C–heteroatom couplings that are possible.<sup>1–9</sup> Many of these reactions utilize organohalogens because of the ability to metalate C–X bonds, and consequently halogenated compounds are now ubiquitous intermediates in organic synthesis.<sup>3,6</sup> Additionally, the introduction of a halogen atom can have a profound effect on the bioactivity and physicochemical properties of small molecules. This effect has been exploited in medicinal chemistry, with a large proportion of all drugs in clinical trials or on the market containing halogen atoms (Figure 1A).<sup>10–14</sup> In the case of the antibiotic vancomycin, for example, it has been demonstrated that the halogen substituents are important

for antimicrobial activity, with dechlorovancomycin derivatives exhibiting significantly reduced binding affinity for the peptidoglycan biological target.<sup>15</sup> The privileged effect of the halogen upon biological activity has also transcended to the design of agrochemicals, where many of the bestselling herbicides, pesticides, and insecticides are halogen-containing (Figure 1B).<sup>16,17</sup> Organohalogen compounds have also been found to have desirable properties in polymers and therefore are receiving increased attention for the next generations of materials.<sup>18–20</sup>

The impact of halogens upon bioactivity and bioavailability was thought to be due solely to modulation of lipophilicity and nonspecific hydrophobic interactions with protein targets. More recently, however, it has been shown that carbon–halogen bonds can form directional intermolecular interactions with proteins, called halogen bonds.<sup>10,11,21–25</sup> These come about because of the electron-deficient “sigma-hole” of the halogen in a C–X bond, which allows interaction of halogens with the lone pairs of heteroatoms like N, O and S in protein targets in a manner analogous to hydrogen bonding.<sup>23–28</sup> As such, the

incorporation of halogen atoms during medicinal chemistry efforts is a well-established practice as it can allow the introduction of additional ligand–target interactions without the need to significantly alter other interactions with the target. The C–F bond in particular is of long-standing importance in the development of pharmaceuticals; indeed fluorine is the most prevalent halogen found in drugs, and a number of the best-selling small molecule pharmaceutical compounds contain fluorine.<sup>12–14</sup> This effect is due in part to fluorine's similar size to hydrogen but significantly increased electronegativity. This combination facilitates the replacement of metabolically labile C–H bonds with C–F, without significantly disturbing interactions with the biological target, in addition to the formation of strong halogen bonds and significant changes in lipophilicity.

As with many synthetic endeavors, the development and application of organohalogen compounds has to a large extent been inspired by nature. A plethora of halogenated natural products have been isolated from a diverse range of microorganisms, many of which possess potent antimicrobial and antitumor activities, among others (Figure 1C).<sup>29–31</sup> In marine organisms, organobromine compounds prevail, while chlorinated compounds are found mostly from terrestrial sources and naturally occurring organoiodine is relatively rare. Elucidation of the biosynthetic pathways responsible for the production of these compounds has revealed a number of enzymes capable of halogenating both aliphatic and aromatic carbons on either protein-tethered or free-standing substrates.<sup>32–34</sup> These enzymes have received much interest since their first discoveries, and now a number of fundamentally different classes of halogenases are known, categorized on the basis of the mechanism by which they generate and utilize activated halide.

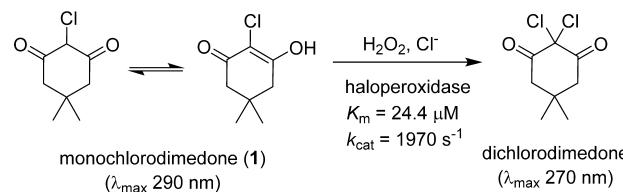
Given the critical importance of organohalogens, methods for the facile and selective installation of halogen substituents are necessary. Many of the traditional methods, especially for aromatic halogenation, require electrophilic halogen sources that are typically toxic and harmful to the environment.<sup>35</sup> The selectivity of these methods is usually poor, affording mixtures of halogenated regioisomers, limiting material efficiency and requiring careful disposal of persistent environmental pollutants.<sup>36</sup> More recent synthetic methods address some of these issues,<sup>37</sup> but often confer selectivity through subtle differences in the acidity of certain C–H bonds or require directing groups to control the position of functionalization, which can limit the substrate scope and number of regiochemistries accessible.<sup>38–42</sup> The use of halogenase enzymes from secondary metabolism for the installation of halogens into both natural and synthetic scaffolds is therefore an attractive prospect that has potential to develop greener and more selective processes for the production of important halogenated compounds. The use of enzymes in aqueous reaction media at ambient temperatures, in addition to reducing the waste generated from production of unwanted regioisomers, could have a profound effect on all sectors of the chemical industry. This Review will focus on the work, reported to date, that aims to develop halogenase enzymes toward reliable biocatalysts for industrial biotransformations, in addition to work on closely related monooxygenase enzymes that may aid their development.

## 2. HALOPEROXIDASES

Haloperoxidases were among the first halogenase enzymes to be discovered and characterized *in vitro*. Enzymes of this class

generate hypohalous acid as the halogenating agent from hydrogen peroxide and halide ions. Chlorination, bromination, and iodination can be observed with this type of enzyme; however, fluorination has yet to be detected, most likely due to the high redox potential and tight aqueous solvation of fluoride. These enzymes are classified by the most electronegative halide they can activate. For example, a chloroperoxidase can activate chloride, bromide, and iodide, whereas a bromoperoxidase can activate bromide and iodide but not chloride, although this does not always correlate with the rate of halogenation.<sup>43</sup> Haloperoxidases can be further classified on the basis of the cofactor that they utilize, and this will be discussed in the sections that follow.

Generally, haloperoxidase activity can be screened for using substrates whose spectral properties change upon halogenation. Monochlorodimedone (**1**) has been used in numerous assays to assess both bromination and chlorination activity following the change in UV absorbance maxima from 290 to 270 nm (Figure 2).<sup>43–46</sup> This assay does have its drawbacks with both false



**Figure 2.** Monochlorodimedone-based assay for detecting haloperoxidase activity. The kinetic parameters displayed are for the chlorination of **1** by CPO from *Caldariomyces fumago*.<sup>55</sup>

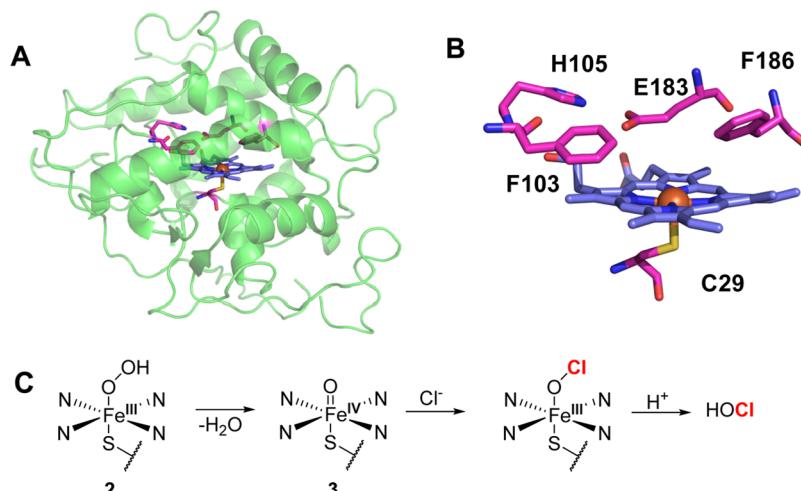
negatives and false positives reported for specific vanadium-dependent chloroperoxidases.<sup>47–49</sup> Other assays utilize phenol red and form bromophenol blue<sup>50–53</sup> or monitor the formation of triiodide.<sup>43,54</sup>

### 2.1. Heme-Iron-Dependent Haloperoxidases

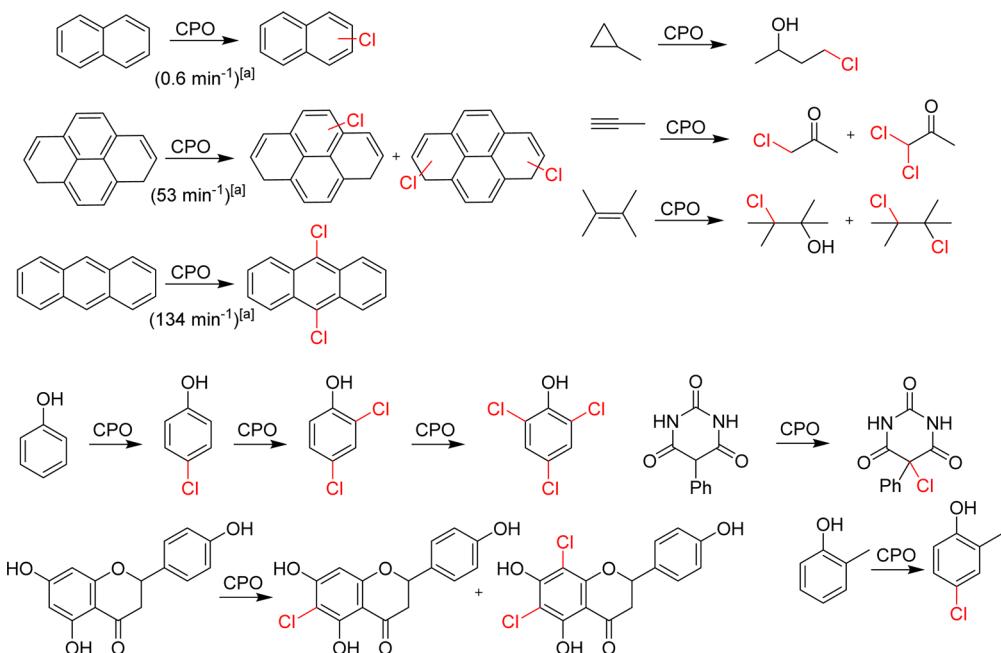
The first halogenating enzyme to be identified was the chloroperoxidase from the fungus *Caldariomyces fumago* (*Leptoxiphium fumago*), which was discovered during the investigation of caldariomycin biosynthesis.<sup>46,56,57</sup> The *C. fumago* chloroperoxidase dihalogenates 1,3-cyclopentanedione leading to caldariomycin; however, it is also capable of halogenating other electron-rich substrates.<sup>58</sup>

Crystallography revealed the *C. fumago* chloroperoxidase to be a 42 kDa glycoprotein with a heme cofactor and unusual tertiary structure dominated by 8 helices (Figure 3A).<sup>59–62</sup> The enzyme shares features of both cytochrome P450s and peroxidases.<sup>59</sup> The proximal heme ligand is cysteine (Cys-29), as in cytochrome P450s, but the distal side of the heme is polar like peroxidases (Figure 3B). A further difference between the traditional peroxidases and chloroperoxidase can be noted in the general acid–base catalytic group, which is a glutamate in chloroperoxidase, rather than a histidine, which is found in the peroxidases. Glutamate is thought to participate in peroxide O–O bond cleavage. When this glutamate residue is mutated to histidine, chlorination activity is severely reduced.<sup>63</sup>

The catalytic cycle of chloroperoxidase is initiated through the binding of peroxide to the axial position of the Fe<sup>III</sup> complex (**2**), followed by the heterolytic O–O bond cleavage leading to the formation of the Fe<sup>IV</sup>–oxo species (**3**), which is known as compound I. The halide ion is then oxidized by compound I, generating hypohalous acid and regenerating the



**Figure 3.** (A) Overall structure of chloroperoxidase from *C. fumago* (PDB 1CPO). (B) Active site of CPO from *C. fumago* (PDB 1CPO). Blue sticks represent heme, and orange spheres represent Fe. (C) Proposed catalytic cycle of hypochlorous acid generation by Fe<sup>III</sup>-heme halogenases.



**Figure 4.** Fe-heme halogenase-catalyzed biotransformations. [a] Specific activity of substrate halogenation by CPO.<sup>66</sup>

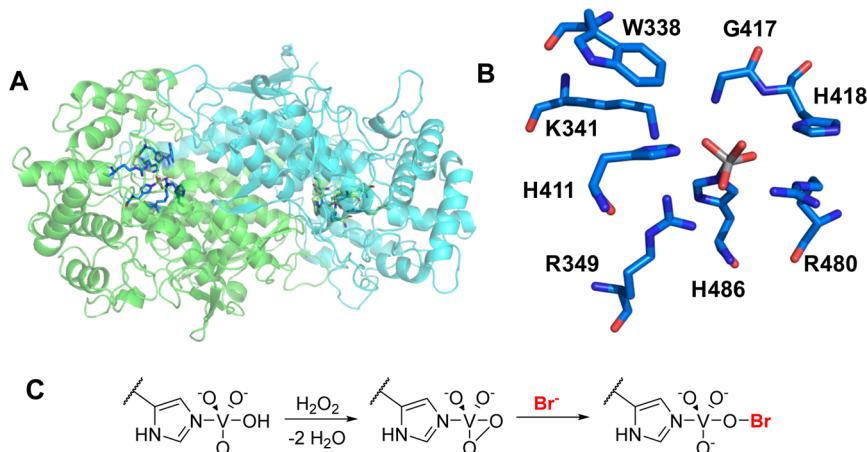
Fe<sup>III</sup> resting state (Figure 3C).<sup>60,64,65</sup> There has been considerable debate about how hypohalous acid then reacts with substrates; however, due to the lack of selectivity conferred by the enzyme, it is likely that hypohalous acid is freely diffusible and can therefore react with many substrates that are susceptible to electrophilic attack (Figure 4).<sup>66–72</sup>

## 2.2. Vanadium-Dependent Haloperoxidases

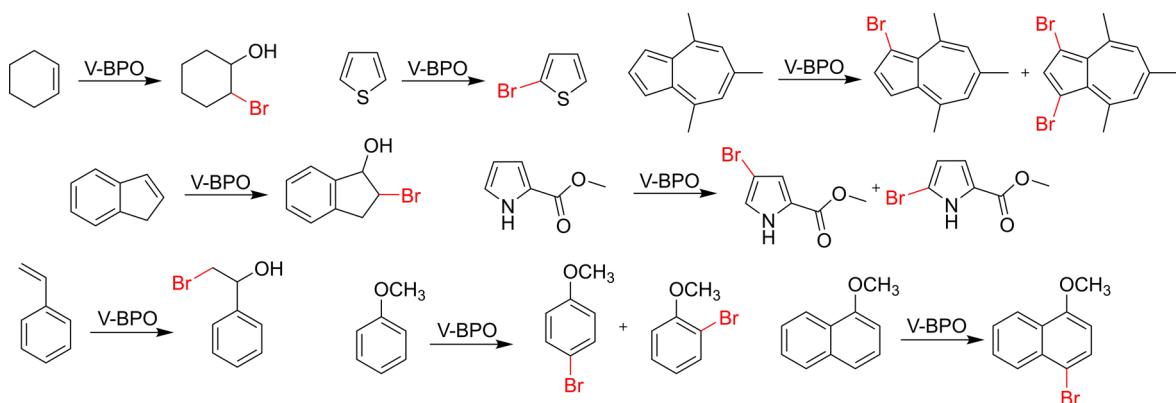
A number of vanadium-dependent haloperoxidases are also known, the first of which to be identified was from the brown algae *Ascophyllum nodosum*.<sup>73,74</sup> Vanadium-dependent haloperoxidases are mainly found in marine organisms with bromoperoxidases predominating.<sup>45,75–79</sup> Vanadium-dependent haloperoxidases have been found in algae, fungi, lichens, and more recently bacteria.<sup>48,73,80–83</sup> These enzymes can be recombinantly expressed in *E. coli* and yeast, and the bromoperoxidase from *A. nodosum* is commercially available.<sup>80,77,84–86</sup> Vanadium-dependent haloperoxidases have

been subject to mutagenesis to further improve their thermal stability as well as solvent and pH tolerance.<sup>50,87,88</sup>

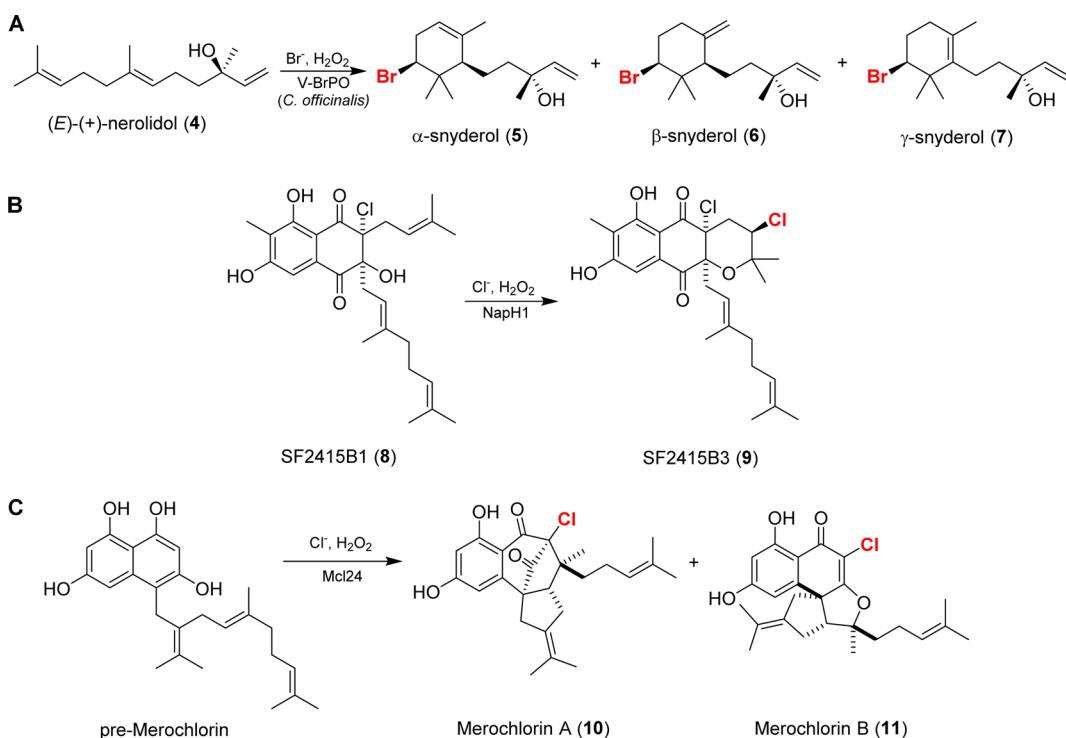
Structurally, the vanadium-dependent haloperoxidases are similar to the acid phosphatases; therefore, it is not surprising that these enzymes can bind phosphate in place of vanadate and exhibit phosphatase activity.<sup>89</sup> Phosphate buffers can be therefore useful for crystallization of the enzymes, but should be avoided in assays as phosphate can act as a competitive inhibitor.<sup>90,91</sup> The vanadate ion is bound at the bottom of a wide funnel, which is between 15 and 20 Å in length at the core of two four helix bundles. A conserved histidine bonds to the vanadium, while vanadate oxygens are coordinated to basic residues (one lysine and two arginines) (Figure 5B).<sup>92,93</sup> While the active site is highly conserved among the vanadium-dependent haloperoxidases, their quaternary state is highly variable. The vanadium-dependent chloroperoxidase from *Curvularia inaequalis* is monomeric, whereas the vanadium-dependent bromoperoxidases from *A. nodosum* and *Corallina*



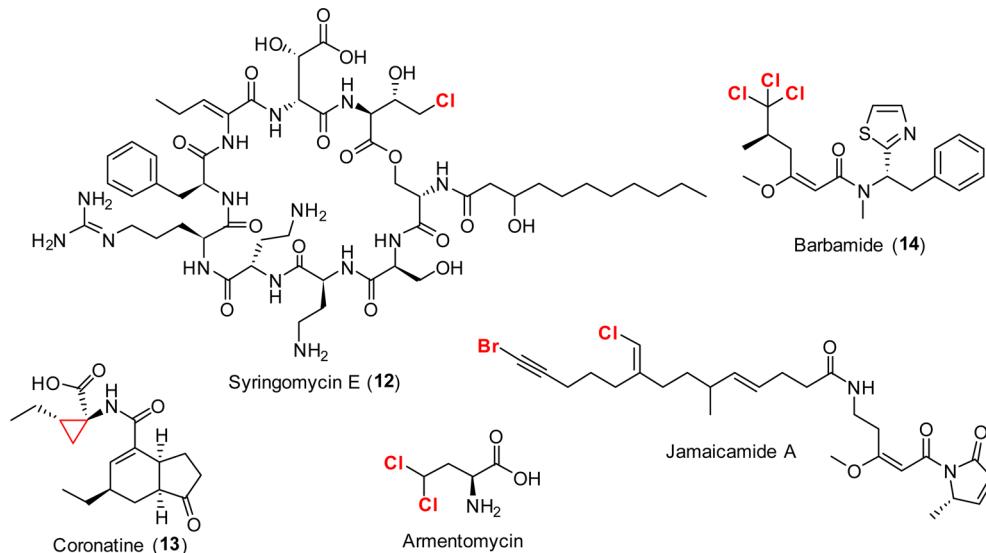
**Figure 5.** (A) Overall structure of C-BPO from *A. nodosum* (PDB 1QI9). (B) Active site of C-BPO from *A. nodosum* (PDB 1QI9). (C) Proposed catalytic cycle of hypobromous acid generation by vanadium-dependent haloperoxidases.



**Figure 6.** Typical substrates from vanadium-dependent haloperoxidases.



**Figure 7.** Examples of specific halogenations by vanadium-dependent haloperoxidases using (A) V-BrPO from *C. officinalis*, (B) NapH1, and (C) McI24.



**Figure 8.** Halogenated natural products produced using nonheme Fe(II)- $\alpha$ -KG-dependent halogenases. The coronatine cyclopropyl moiety is formed after  $\gamma$ -halogenation of L-allo-isoleucine followed by intramolecular ring closure.

*pilulifera* are a homodimer and a homododecamer, respectively.<sup>43,93–95</sup>

Unlike the heme-dependent haloperoxidases, the oxidation state of the catalytic vanadium center is maintained throughout the catalytic cycle, offering the advantage that these enzymes do not suffer from oxidative inactivation. Initially, hydrogen peroxide binds to the distal position of the vanadate complex, leading to a loss of water and the formation of peroxy-vanadate intermediate. Halide ion is then oxidized, leading to the formation of hypohalous acid (Figure 5C). Examples in the literature suggest that, like the heme-dependent haloperoxidases, the vanadium-dependent haloperoxidases can release free hypohalous acid, allowing halogenation of a variety of substrates (Figure 6),<sup>76,96–98</sup> although some examples of substrate specificity have been observed.<sup>92,99,100</sup>

The vanadium-dependent bromoperoxidase from *Corallina officinalis* was found to be selective and utilized to asymmetrically brominate and cyclize the sesquiterpene (*E*)-(+)–nerolidol (4) to the marine natural products  $\alpha$ -,  $\beta$ -, and  $\gamma$ -snyderol (5–7, Figure 7A). Nonenzymatic bromination of (*E*)-(+)–nerolidol (4) produced a mixture of diastereomers, whereas the bromoperoxidase only formed a single diastereomer. This study demonstrated the stereo- and regioselectivity of these enzymes for the first time, thereby suggesting that the substrate binds to the active site in a specific manner (Figure 7).<sup>76</sup>

More recently, the first example of a bacterial vanadium-dependent chloroperoxidase was reported from *Streptomyces* sp. CNQ-525. Three genes were identified, *napH1*, *H3*, and *H4*, with homology to other known vanadium-dependent haloperoxidases. It was hypothesized that at least one of these genes encoded for an enzyme that was involved in the halogenation and cyclization of SF2415B1 (8) to SF2415B3 (9) in a manner similar to that of (*E*)-(+)–nerolidol (4). All three enzymes were heterologously expressed in *E. coli*; however, NapH4 proved to be insoluble. NapH3 was found to be unable to catalyze the formation of SF2415B3 (9); however, NapH1 demonstrated the ability to catalyze the halocyclization in the presence of hydrogen peroxide and chloride at pH 6 (Figure 7B). Interestingly, NapH1 was not able to chlorinate non-native substrates, (±)-nerolidol or lapachol; however, nonspecific

activity was exhibited with (±)-nerolidol and lapachol in the presence of bromide ions.<sup>48</sup> Another bacterial vanadium-dependent haloperoxidase (Mcl24) has been identified from the marine bacterium *Streptomyces* CNH-189. Mcl24 is a vanadium-dependent chloroperoxidase involved in the late stages of merochlorin (10 and 11) biosynthesis catalyzing a site-selective napthol chlorination, followed by a sequence of oxidative dearomatization and terpene cyclization reactions (Figure 7C). Like NapH1, Mcl24 is specific for chloride; however, in the presence of bromide, monochlorodimedone was also brominated, suggesting that halogenation by freely diffusing hypohalous acid may also occur if substrate is not bound to the active site.<sup>49,83,101</sup>

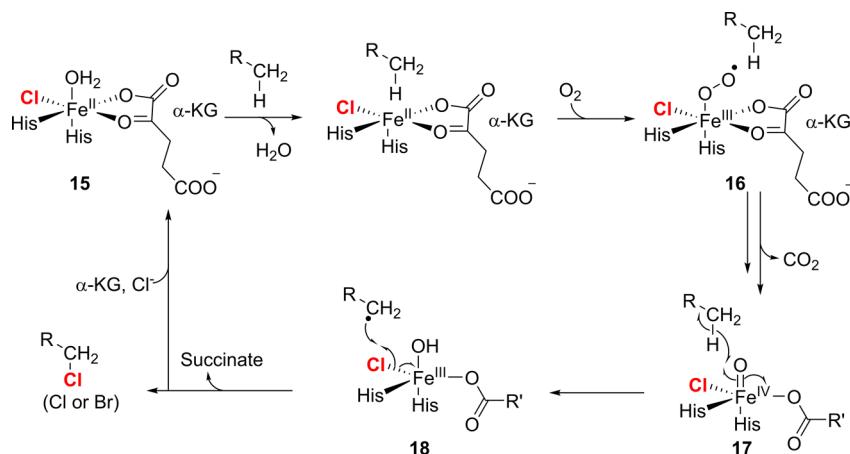
While haloperoxidases can halogenate a wide range of substrates under mild conditions, as shown in Figures 4 and 6, they generally either lack specificity or, as in the case of the bacterial vanadium-dependent haloperoxidases, are highly specific and therefore are limited in biocatalytic application.

### 3. $\alpha$ -KETOGLUTARATE-DEPENDENT HALOGENASES

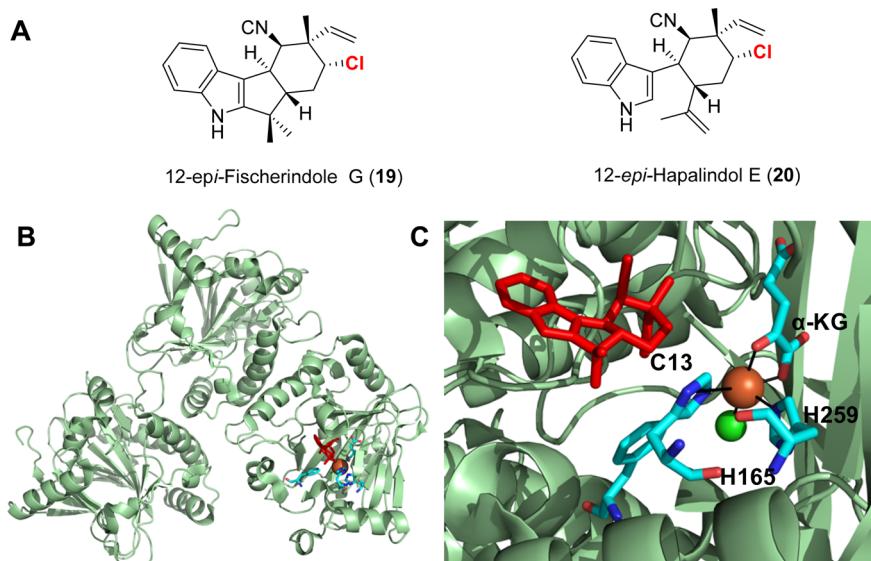
#### 3.1. Discovery of $\alpha$ -Ketoglutarate-Dependent Halogenases

Nonheme Fe(II)- $\alpha$ -ketoglutarate-dependent ( $\alpha$ -KG) halogenases are involved in the halogenation of unactivated carbon centers on aliphatic moieties such as terminal methyl groups and predominantly utilize substrates tethered to acyl or peptidyl carrier proteins (PCP). The enzyme class was first reported in 2005, with the first example forming part of the biosynthetic assembly line required for production of the nonribosomal peptide Syringomycin E (12) in *Pseudomonas syringae* (Figure 8). The carrier protein tethered L-threonine (L-Thr-S-SyrB1) from Syringomycin biosynthesis was demonstrated to act as a substrate for the SyrB2-mediated chlorination.<sup>102</sup>

The selectivity of SyrB1 for L-Thr was confirmed by kinetic comparison with other amino acids showing a 60-fold preference for threonine over serine (L-Thr,  $K_m = 3.1 \pm 0.2$  mM,  $k_{cat} = 29.1 \pm 0.9$  min<sup>-1</sup>; L-Ser,  $K_m = 7.7 \pm 0.5$  mM,  $k_{cat} = 1.23 \pm 0.05$  min<sup>-1</sup>). Isolation of the final product from SyrB2-mediated chlorinations confirmed the product to be 4-Cl-L-threonine. SyrB2 was found to not accept free L-threonine at all, meaning that substrate must be tethered to the phosphopante-



**Figure 9.** Proposed mechanism for Fe(II)/ $\alpha$ -KG-dependent halogenases.



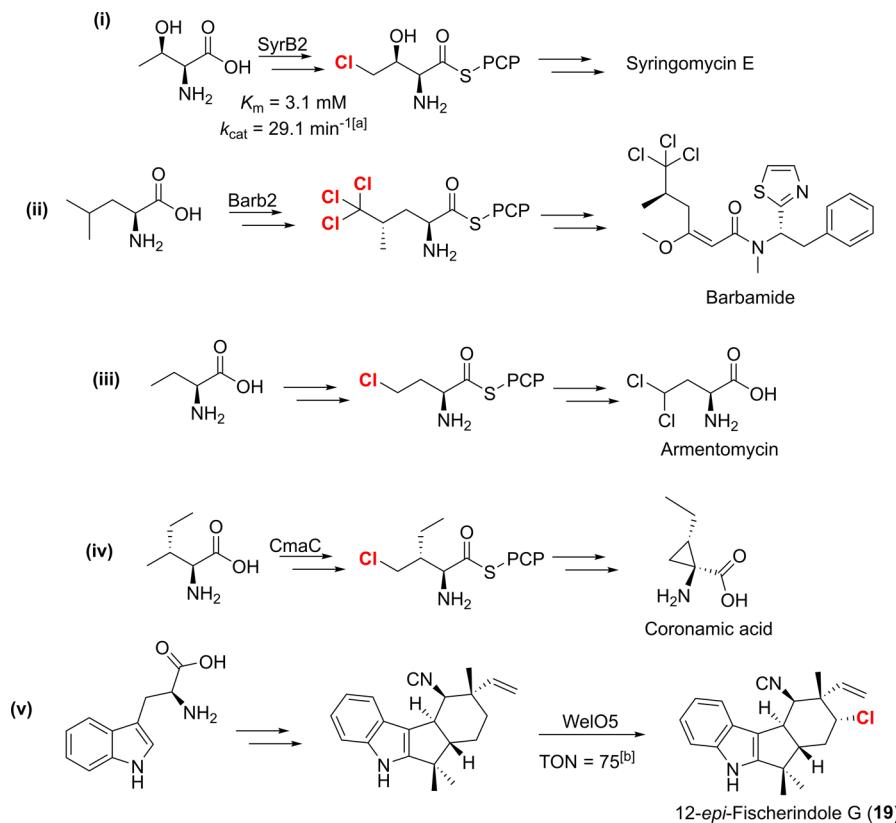
**Figure 10.** (A) Selectively chlorinated products from WelO5-catalyzed halogenation. (B) Crystal structure of WelO5 showing overall structure (PDB 5IQV). (C) Active site coordination of WelO5 (PDB 5IQV). Fe and Cl are represented by orange and green spheres, respectively.

theine arm of the halo-SyrB1 thiolation domain for chlorination to occur. Tethering of the substrate to SyrB1 was confirmed by the detection of [ $^{36}\text{Cl}$ ]-L-Thr-S-SyrB1 using the radioactive  $^{36}\text{Cl}$  and gel dosimetry. In vitro studies revealed a requirement for Fe(II),  $\text{O}_2$ , and  $\text{Cl}^-$  or halogenase activity. In addition, deactivation of halogenase activity was rapid such that no more than seven turnovers could be detected.<sup>102</sup>

On the basis of the observed cofactor and cosubstrate requirement, and by analogy to the  $\alpha$ -KG hydroxylases, a mechanism for the  $\alpha$ -KG halogenases was proposed involving radical abstraction of the methyl hydrogen by a high-valent Fe<sup>IV</sup>-oxido species. Recombination of this radical with Fe-coordinated  $\text{Cl}^-$  was then thought to afford 4-chloro-L-threonine. At a similar time, investigation of the biosynthetic pathway leading to coronatine (13) in *P. syringae* identified CmaB and CmaC to be involved in halogenation of the methyl group of L-allo-isoleucine, which undergoes subsequent cyclization to generate the cyclopropyl ring of the side chain amino acid (corronamic acid).<sup>103</sup> The trichloromethyl group of barbamide (14) derivatives from the marine cyanobacterium *Lyngbya majuscule* was also found to be derived from the pro-R methyl group of leucine.<sup>104,105</sup> Subsequent in vitro studies have

confirmed that two  $\alpha$ -KG-dependent halogenases (BarB1 and BarB2) are required to trichlorinate a carrier protein tethered L-Leu precursor in barbamide (14) biosynthesis (Figure 8).<sup>106</sup>

Crystallography of SyrB2 subsequently shed further light on the mechanistic aspects of the Fe(II)/ $\alpha$ -KG-dependent halogenases.<sup>107</sup> Analogous to the hydroxylation mechanism of TauD, a working mechanism was proposed for these terminal halogenations. SyrB2 was the first structurally characterized mononuclear iron protein which did not display the characteristic 2-His, 1-carboxylate iron coordination. Instead, two histidine ligands are present (His116 and His235), and the iron coordinates to a chloride ion rather than carboxylate. It was also observed that water and  $\alpha$ -KG coordinate to iron in the resting state (15) (Figure 9). Upon L-Thr-S-SyrB1 binding, water is displaced by dioxygen (16), and decarboxylation of  $\alpha$ -KG would then lead to a high valent Fe<sup>IV</sup>-oxido intermediate (17). This highly reactive species is then able to radically abstract a hydrogen atom from substrate (18), which can then recombine with a chloride ligand producing chlorinated L-Thr-S-SyrB1 and regenerated Fe(II). Although there is clearly the possibility of hydroxylation during the course of the reaction, chlorination seems to be greatly favored. One possible



**Figure 11.** Involvement of Fe(II)/ $\alpha$ -KG-dependent halogenases in the biosynthesis of halogenated natural products. [a] Refers to the loading of L-Thr onto SyrB1 as those for SyrB2 could not be obtained due to enzyme deactivation. TON for chlorination of L-Thr-S-PCP by SyrB2 is <7.<sup>102</sup> [b] Refers to the chlorination of free-standing substrate by WelO5.<sup>108</sup> PCP = peptidyl carrier protein. TON = turnover number.

explanation is the relative positioning of the chloride ligand toward the substrate, exclusively affording halogenation.

Recently, the Fe(II)/ $\alpha$ -KG-dependent halogenase WelO5 was discovered from the welwitindoline biosynthetic pathway in *Hapalosiphon welwitschii*.<sup>108–110</sup> It was shown that this enzyme can regioselectively monochlorinate an aliphatic carbons to afford 12-*epi*-Fischerindole G (19) and 12-*epi*-Hapalindol E (20, Figure 10A).<sup>111</sup> WelO5 is particularly notable as the first example of an Fe(II)/ $\alpha$ -KG-dependent halogenase that functions independently of a carrier protein. It is also the first example of a halogenase installing a halogen atom stereoselectively on a nonactivated position, C13 of 12-*epi*-fischerindole G, to create a new stereogenic center. When the WelO5 sequence was compared to other members of the  $\alpha$ -KG-dependent oxygenase superfamily, the D/E residue in the characteristic HX(D/E)XnH motif for iron binding was found to be absent and replaced by the glycine 166 residue. The two histidine residues H164 and H259 were found crucial for iron coordination. Site-directed mutagenesis of H259 to phenylalanine completely abolished activity toward the 12-*epi*-fischerindole U. The recent publication of the WelO5 crystal structure revealed the close proximity of the C13 halogenation site to the putative *cis*-halo-oxo-Fe<sup>IV</sup> complex, which confirms its preferred activity toward halogenation (Figure 10C).<sup>111</sup> Unlike the standard  $\alpha$ -KG hydroxylases, WelO5 contains a glycine (Gly166) at the sequence position of an aspartate or glutamate ligand. Accordingly, it was shown that the G166D variant exclusively gives C13 hydroxylation, as predicted. It was also demonstrated that the second-sphere mutant S189A produced a mixture of hydroxylated and halogenated product,

showing how outer sphere hydrogen bonding can influence chemoselectivity.<sup>111</sup>

### 3.2. Application of Fe(II)/ $\alpha$ -KG-Dependent Halogenases as Biocatalysts

The proteinaceous nature of substrates for the majority of Fe(II)/ $\alpha$ -KG halogenases forms a barrier for their application as biocatalysts. These enzymes also have to be purified under inert conditions, and their turnover numbers are generally low. In fact, kinetic parameters are difficult to determine due to auto-oxidative inactivation of the biocatalyst and limited substrate availability (maximum of 75 turnovers), which restricts their application in industrial biocatalysis. Moreover, the substrate scope identified to date seems to be largely limited to natural amino acids (Figure 11). However, the recent discovery of WelO5 allowing halogenations without the requirement of a carrier protein opens new opportunities for the application of those enzymes in synthesis. Moreover, given that mutagenesis studies have shown that the Fe(II)/ $\alpha$ -KG halogenases can be switched to hydroxylases,<sup>107,111,112</sup> it seems plausible that halogenases can be engineered from Fe(II)/ $\alpha$ -KG-dependent hydroxylases. Indeed, a recent report has demonstrated that it is possible to engineer a new halogenase enzyme from an Fe(II)/ $\alpha$ -KG-dependent hydroxylase using structure guided mutagenesis.<sup>113</sup> This approach<sup>113</sup> is attractive given that there are more known Fe(II)/ $\alpha$ -KG-dependent hydroxylases than halogenases, and the range of substrates that are processed by the hydroxylases is greater and more structurally diverse. Moreover, unlike halogenases, which, with the exception of WelO5, halogenate carrier protein-tethered substrates, the majority of hydroxylases utilize simple

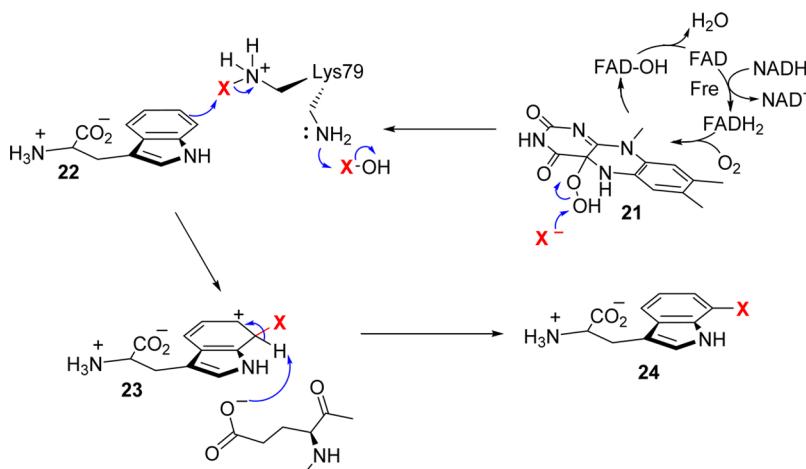


Figure 12. Proposed mechanism of flavin-dependent tryptophan halogenase-catalyzed halogenation.

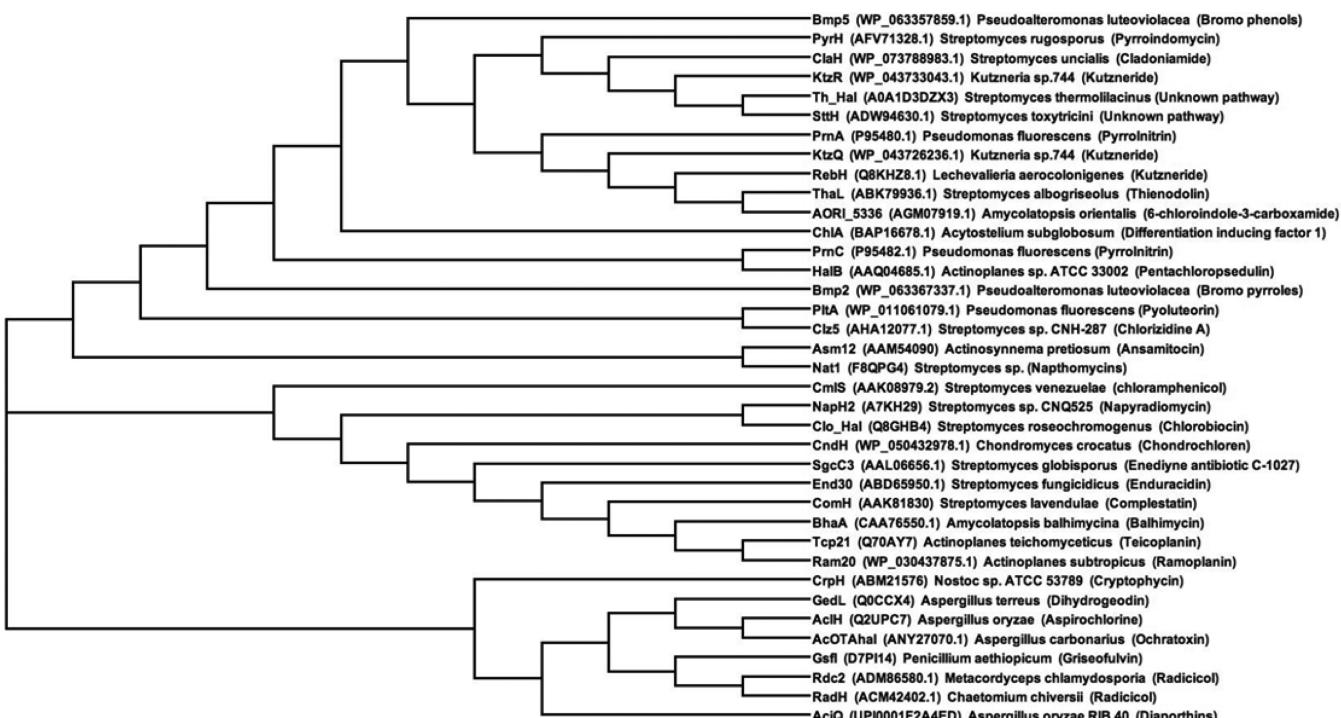


Figure 13. Phylogenetic tree of the Fl-Hals discussed herein. Generated using the neighbor-joining tree method without distance corrections via Dendroscope 3.

nontethered substrates. Despite their low catalytic activity, the fact that Fe(II)/ $\alpha$ -KG enzymes halogenate, or hydroxylate, unactivated aliphatic substrates with regio- and stereoselectivity is attractive for further investigation given that there are few viable synthetic alternatives.

#### 4. FLAVIN-DEPENDENT HALOGENASES

##### 4.1. Flavin-Dependent Halogenases in Nature

Flavin-dependent halogenase (Fl-Hal) enzymes belong to the superfamily of flavin-dependent monooxygenases.<sup>114,115</sup> A key feature of this class of enzymes is their activation of molecular oxygen using reduced flavin ( $\text{FADH}_2$ ) to generate C4a-hydroperoxy flavin (21),<sup>116–118</sup> which allows diverse reactions such as hydroxylation, epoxidation, Baeyer–Villiger oxidation, and heteroatom oxidations.<sup>119–121</sup> The flavin-dependent halogenases are typically classified as two-component mono-

oxygenases, meaning that they utilize freely diffusing reduced flavin cofactors produced by an additional flavin-reductase enzyme.<sup>115</sup> In addition to the flavin reductase, several Fl-Hals from NRPS and PKS pathways also require enzymes for substrate activation or tethering.<sup>32</sup> In the last two decades, a number of flavin-dependent halogenases from both bacterial and fungal biosynthetic pathways have been identified.<sup>114,122–126</sup>

The flavin-dependent halogenases were originally thought to operate via a mechanism analogous to those of other flavin-dependent monooxygenases through direct reaction of substrate with C4a-hydroperoxy-flavin (FAD-O<sub>OH</sub>, 21), followed by subsequent reaction with halide anion.<sup>123,127–129</sup> However, crystallography of the tryptophan (22) 7-halogenase PrnA revealed that the substrate and flavin binding sites were spatially distinct, separated by a 10 Å tunnel, and therefore

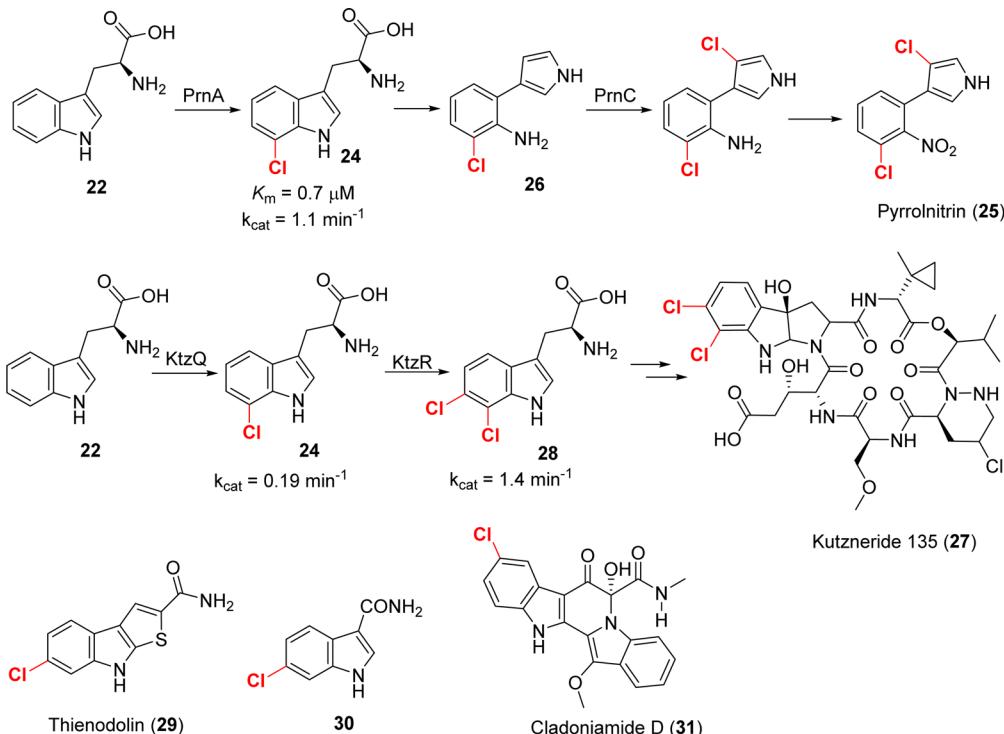


Figure 14. Halogenated natural products biosynthesized using flavin-dependent tryptophan halogenases.<sup>141,155</sup>

direct interaction of the substrate with the cofactor is not possible.<sup>130,131</sup> Mechanistic studies subsequently demonstrated that FAD-OOH (21) was generated prior to halogenation, even in the absence of substrate, suggesting that nucleophilic attack of chloride on 21 may result in hypochlorous acid generation in the flavin-binding domain.<sup>132</sup> Further work demonstrated the formation of a long-lived enzyme-chloride adduct, which, after identifying the vital importance of an active site lysine residue, is believed to be a covalent chloramine adduct or hydrogen-bonded lysine-hypochlorous acid species.<sup>133</sup> This electrophilic chlorine species is believed to be ultimately responsible for aromatic substitution of the substrate to generate the Wheland intermediate (23), which is then deprotonated by a conserved glutamate residue to afford chlorinated product (24, Figure 12).<sup>114,131,133–135</sup> Positioning of this active site lysine relative to substrate is therefore believed to control which position of the substrate is halogenated.<sup>130</sup> Direct monooxygenase-type activity is thought to be prevented in the tryptophan Fl-Hals by a conserved structural motif (WxWxIP), which blocks direct contact between FAD-OOH (21) and substrate.<sup>130,136</sup>

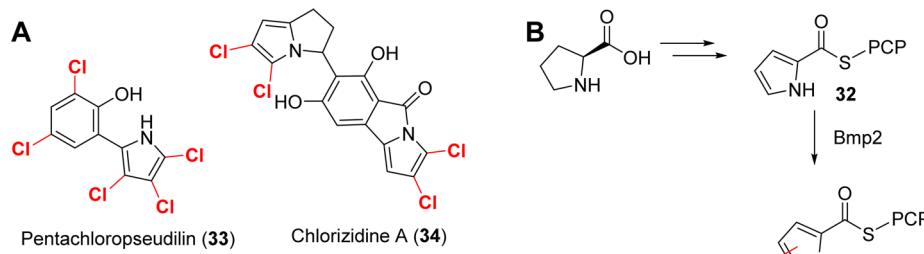
This highly conserved motif, along with the nucleoside-binding motif GxGxxG responsible for binding FADH<sub>2</sub>, are therefore considered to be the signature motifs for identifying putative Fl-Hal genes. These signature motifs have been used to identify flavin-dependent halogenases from diverse biosynthetic pathways that are responsible for the halogenation of pyroles, phenols, and other aromatic natural product precursors (Figure 13). Similar to other flavin-dependent monooxygenases, Fl-Hals of different substrate scope are thought to conserve the core flavin-binding domain with most structural changes coming about from the recruitment of different substrate binding domains.<sup>115</sup> Fl-Hals can therefore be further subdivided on the basis of their natural substrate.

#### 4.1.1. Flavin-Dependent Tryptophan Halogenases.

The flavin-dependent tryptophan halogenases are the most

extensively studied and characterized Fl-Hals.<sup>32–34</sup> The first tryptophan Fl-Hal to be identified was PrnA, which is required for the biosynthesis of pyrrolnitrin (25), a broad spectrum antifungal compound produced by *Pseudomonas fluorescens*.<sup>127,137,138</sup> In the pyrrolnitrin pathway, PrnA chlorinates the 7-position of tryptophan, while a second Fl-Hal, PrnC, chlorinates the pyrrolic intermediate 26 (Figure 14). Another tryptophan 7-halogenase RebH, with 55% sequence identity to PrnA, was subsequently identified from the rebeccamycin biosynthetic pathway in *Lechevalieria aerocolonigenes*.<sup>123,124,139</sup> X-ray crystal structures of these two Fl-Hals were subsequently determined which, along with further biochemical studies, provided the basis of our understanding of the mechanism and reactivity of Fl-Hals.<sup>114,123,127,132,134,135</sup>

Studies into the biosynthesis of the dichlorinated non-ribosomal peptide kutzneride (27), an antifungal from actinobacteria *Kutzneria* sp. 744, revealed that two tryptophan Fl-Hals (KtzQ and KtzR) and a cryptic Fe(II)/α-KG-dependent halogenase (KtzD) were required for kutzneride biosynthesis.<sup>140–142</sup> KtzQ and KtzR work sequentially, with KtzQ catalyzing the formation of 7-chlorotryptophan prior to halogenation at the 6-position by KtzR to produce 6,7-dichlorotryptophan (28, Figure 14).<sup>141</sup> In vitro studies showed that KtzR exhibits a 120-fold substrate preference for 7-chlorotryptophan (24) over nonchlorinated tryptophan.<sup>141</sup> A number of independent tryptophan 6-halogenases have also been identified recently.<sup>143–147</sup> For example, ThaL from the indole alkaloid thienodolin (29) pathway in *Streptomyces albovirens* is responsible for the 6-chlorination of tryptophan prior to thiophene ring formation.<sup>144,146,148,149</sup> SttH from *Streptomyces toxytricini* and Th\_Hal from the thermophile *Streptomyces violaceusniger* SPC6 have also been shown to function as tryptophan 6-halogenases.<sup>143,145</sup> Although both have been characterized in vitro and their structures determined,<sup>143,145,150</sup> the biosynthetic pathways in which they



**Figure 15.** Flavin-dependent pyrrole halogenases in natural product biosynthesis. (A) Natural products containing chlorinated pyrrole moieties. (B) Halogenation of pyrrole-S-PCP by Bmp2.

function are yet to be explored. Recently, a tryptophan 6-halogenase (AORI\_5336) was identified in a hybrid NRPS/PKS hybrid gene cluster of *Amycolatopsis orientalis*, which appears to be involved in the biosynthesis of the plant growth-regulating compound 30.<sup>147</sup>

The only fully characterized tryptophan 5-halogenase is PyrH from the pyrroindomycin producing strain *Streptomyces rugosporus*.<sup>130,151</sup> Although a tryptophan 5-halogenase is known to be involved in the biosynthesis of the bis-indole alkaloid cladoniamide (31) in *Streptomyces uncialis*, details of in vitro activity are still lacking, although in vivo studies suggest it may be of low activity compared to other tryptophan halogenases.<sup>152,153</sup> Additionally, a recent report suggests that a tryptophan 5-halogenase may be responsible for halogenation of a tryptophan moiety in a peptide chain, rather than freely diffusing tryptophan, in the biosynthesis of the lantibiotic NAI-107.<sup>154</sup> Structural and phylogenetic analyses (Figure 13) provide details of the similarities between halogenases of different regioselectivity. Many of the structural differences are present in a single loop region, with the tryptophan 7-halogenase PrnA containing a larger loop region (L443-W455) as compared to the 5- and 6-halogenases, suggesting that the 5-, 6-, and 7-tryptophan halogenases may have evolved from the same ancestral enzyme via loop insertions and deletions to give rise to different orientations of substrate in the active site.<sup>130,150</sup>

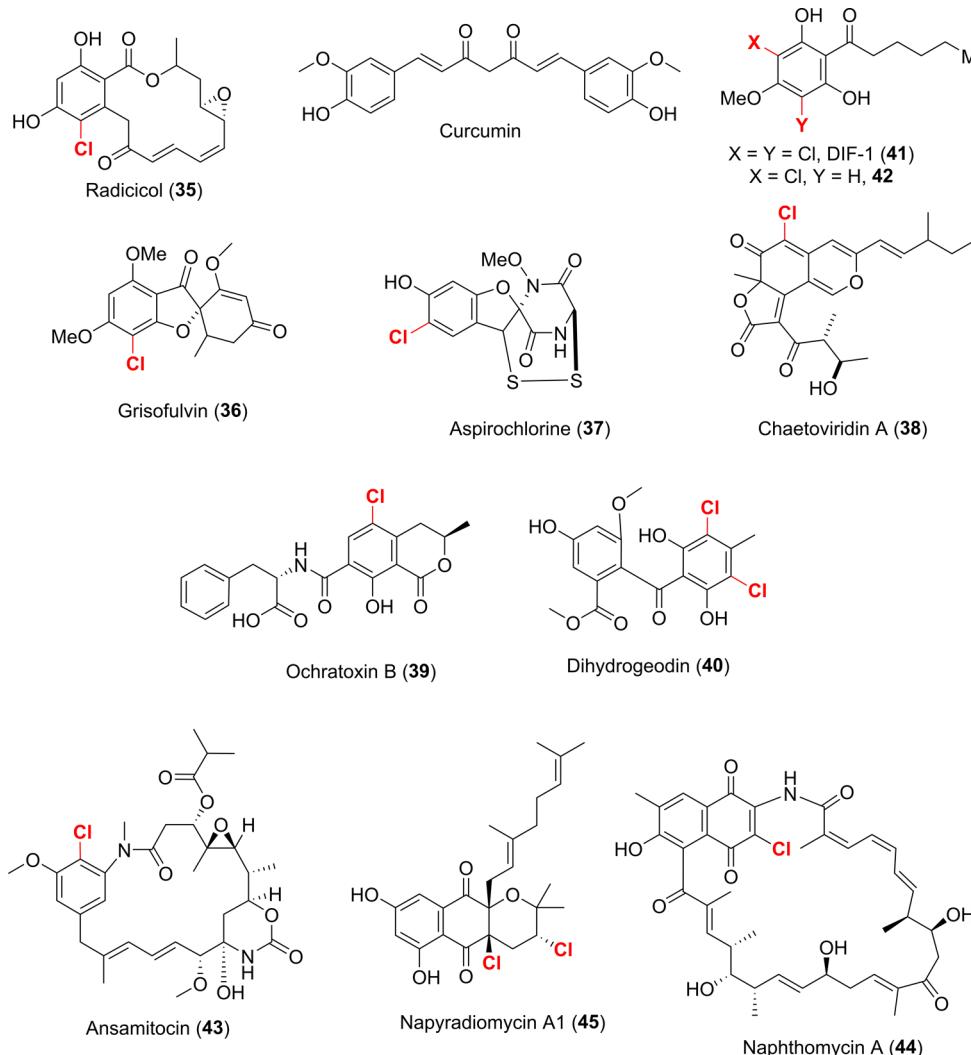
**4.1.2. Flavin-Dependent Pyrrole Halogenases.** In addition to the pyrrolnitrilin halogenase, PrnC, which halogenates a free-standing pyrrolic substrate, there are a number of Fl-Hals that process pyrrol-2-carboxy thioester substrates (32) tethered to peptidyl carrier proteins in NRPS or hybrid NRPS-PKS assembly lines (Figure 15).<sup>122,156</sup> This includes PltA from the pyoluteorin biosynthetic gene cluster.<sup>122,137,157</sup> The crystal structure of PltA revealed a unique helical region at the C-terminus, which blocks the substrate binding cleft. Binding of PltL-tethered substrate induces a conformation change, however, which allows access to the substrate-binding cleft.<sup>122</sup> Interestingly, PltA does not contain a residue analogous to the catalytic glutamate found with the tryptophan halogenases, suggesting a water molecule, or an as yet unidentified active site residue, might function to deprotonate the Wheland intermediate. Both PrnC and PltA appear to have relaxed regioselectivity as compared to the tryptophan halogenases, and mixtures of mono- and dichlorinated products are observed more frequently in vitro.<sup>122,158</sup> HalB is another pyrrole halogenase with high sequence similarity to PrnC and PltA, involved in the biosynthesis of pentachloropseuduolin (33) from *Actinoplanes* sp. ATCC3302.<sup>159,160</sup> ClzS from *Streptomyces* sp. CNQ-418 is also similar to PltA and has been identified as the pyrrole Fl-Hal in the biosynthesis of chlorizidine A (34, Figure 15A).<sup>161–163</sup>

The peptidyl carrier protein (PCP) tethered substrates of ClzS, PltA, and HalB are all derived from oxidation of proline-S-PCP intermediates in NRPS or NRPS-PKS assembly lines.<sup>162,163</sup>

Genome mining of the marine organisms responsible for the biosynthesis of brominated marine natural products has led to the identification of an interesting pyrrole Fl-Hal, Bmp2, from *Pseudoalteromonas luteoviolacea*.<sup>156,164,165</sup> Similar to the gene cluster containing PltA, Bmp2 is associated with a thioesterase (TE, Bmp1) and proline adenyltransferase (Bmp4).<sup>156,164</sup> Reconstitution of these enzymes in vitro showed Bmp2 to brominate pyrrole-2-carboxy-S-Bmp1 (32) to mono-, di-, and tribromo products (Figure 15B).<sup>164</sup> Unlike the other Fl-Hals studied to date, Bmp2 appeared capable of iodination of 32 in addition to bromination, but not chlorination.<sup>164</sup> Investigation of the structural features which allow Bmp2 to iodinate may be of interest in the engineering of other Fl-Hals, as the tryptophan Fl-Hals have been demonstrated to be inhibited by the presence of I<sup>-</sup>.<sup>114,166</sup> Biocatalytic iodination is attractive for synthetic applications, as aryl iodides are typically more reactive, and thus more readily derivatized, than the corresponding aryl chlorides or bromides. Comparison of Bmp2 to its closest analogue Myp16 and point mutations of Bmp2 to mimic this chlorinase did not afford a Bmp2 mutant capable of chlorination, suggesting that other, more subtle, features may be responsible for the observed halide preference.<sup>156</sup>

**4.1.3. Flavin-Dependent Phenolic Halogenases.** Three classes of phenolic flavin-dependent halogenase enzymes have been identified to date: those that act on free-standing substrates (class A); those that require substrates tethered to a carrier protein (class B); and halogenases that follow a decarboxylative halogenation mechanism (class C).

**4.1.3.1. Predicted Class A Phenolic Halogenases.** The fungal halogenase Rdc2 from *Pochonia chlamydosporia* and the similar enzyme RadH from *Chaetomium chiversi* are both involved in the biosynthesis of the resorcyclic acid lactone (RAL) radicicol (35) and act on free-standing phenolic substrates.<sup>125,167–170</sup> Radicicol is a potent inhibitor of heat shock protein 90 (Hsp90), a medically important target due to its involvement in many cancer-causing pathways, and also exhibits antifungal activity.<sup>170</sup> Rdc2 was heterologously expressed in *E. coli* and demonstrated to be capable of halogenating the macrocyclic natural products zearaleone, dihydrosorcyllide, curvularin, and curcumin in vitro.<sup>169–172</sup> Both chlorination and bromination reactions were found to form dihalogenated compounds after prolonged periods when multiple OH groups were present on the aromatic ring.<sup>169–172</sup> Rdc2 and RadH are both post-PKS tailoring enzymes, which is a plausible explanation for why these enzymes act on free-standing substrates. A number of other class A phenolic Fl-Hals



**Figure 16.** Natural products biosynthesized using phenolic flavin-dependent halogenases.

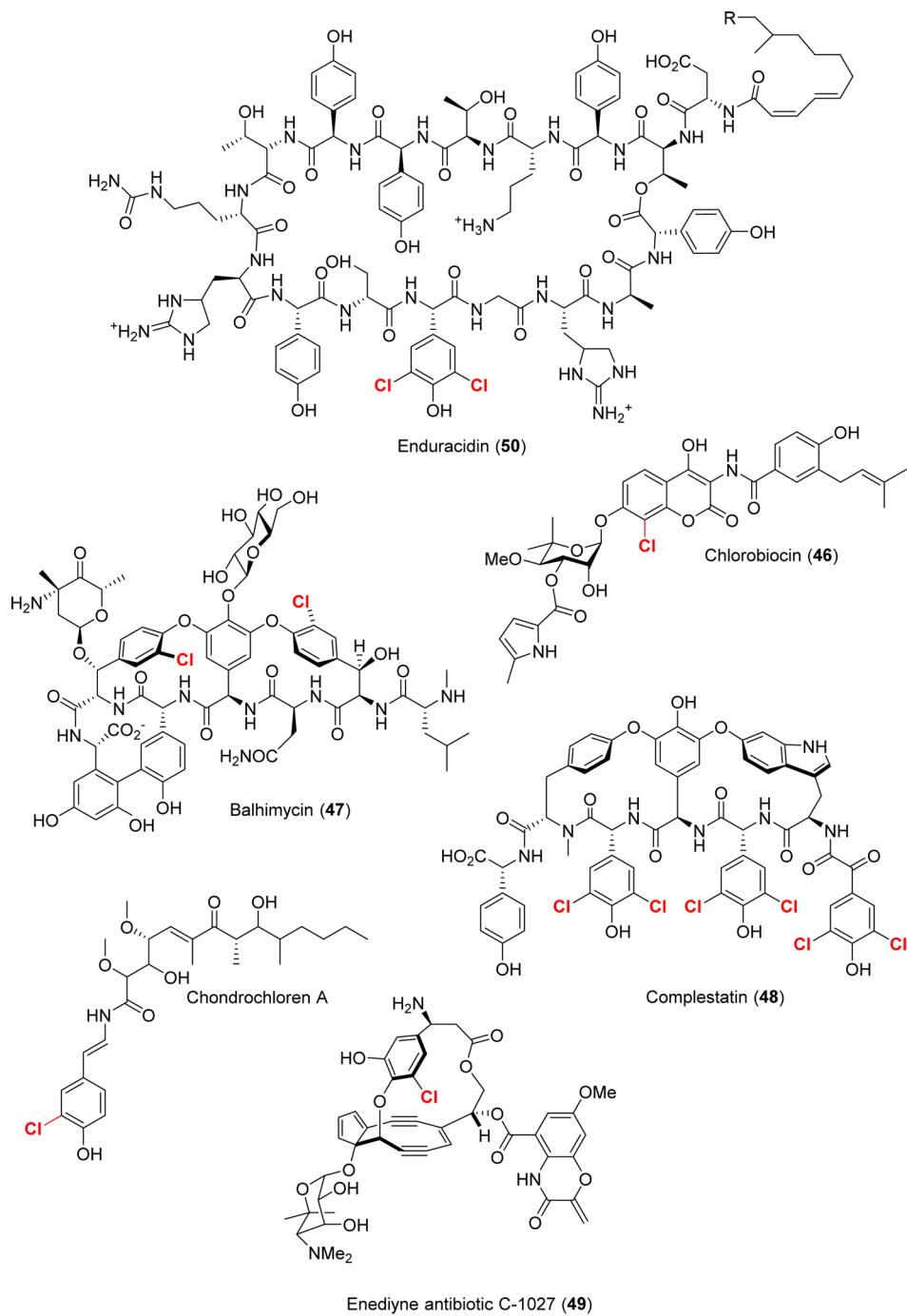
have been identified that are involved in the biosynthesis of a range of fungal natural products including griseofulvin (36, GsfI),<sup>173–175</sup> aspirochlorine (37, AclH),<sup>176–178</sup> chaetoviridin (38, CazI),<sup>179–181</sup> ochratoxin (39, AcOTAHal),<sup>126,182–184</sup> and dihydrogeodin (40, GedL) (Figure 16).<sup>185,186</sup> Like Rdc2 and RadH, these related fungal enzymes (GsfI, AclH, CazI, and GedL) halogenate *ortho* to a phenolic hydroxyl group of late-stage biosynthetic intermediates, except for the ochratoxin (39) halogenase (AcOTAHal) which installs a chloro substituent *para* to the hydroxyl.

Although not directly phylogenetically linked to Rdc2 or RadH, the differentiation-inducing factor 1 (DIF-1, 41) halogenase (ChlA) from *Dictyostelium discoideum* is a class A flavin-dependent phenolic halogenase.<sup>187–190</sup> ChlA was over-expressed and purified in *E. coli* and demonstrated in vitro to produce both mono- and dihalogenated DIF-1 (41 and 42) in the presence of an external flavin reductase.<sup>187</sup> The observation that many of the phenolic halogenases appear to require substrates with a hydroxyl group *ortho* to the halogenation site (*vide infra*) suggests that electrophilic aromatic substitution may be mediated through deprotonation of the *ortho*-hydroxyl.

Another halogenase, predicted to process a free-standing phenolic substrate, is Asm12 from ansamitocin (43) biosynthesis.<sup>191–193</sup> Asm12 shares 73% sequence identity with Nat1, a

halogenase involved in the biosynthesis of naphthomycins (44, NATs) by *Streptomyces* sp. CS.<sup>194</sup> Naphthomycins are 29-membered naphthalenic ansamacrolactam antibiotics with similarities to the ansamitocin type scaffold. Inactivation of the *nat1* gene in the naphthomycin biosynthetic gene cluster abolished production of the chlorinated naphthomycin A, which was restored on complementation with Asm12, demonstrating the functional similarity between the two enzymes.<sup>194</sup> Finally, NapH2 is the halogenase from the napyradiomycin (45) biosynthetic cluster (nap) in *Streptomyces* sp. CNQ-525.<sup>195</sup> In vivo studies suggest that NapH2 may halogenate the C2 position of a napthoquinone precursor, facilitating subsequent prenylation during napyradiomycin biosynthesis.<sup>195,196</sup>

**4.1.3.2. Predicted Class B Phenolic Halogenases.** A number of putative phenolic halogenases such as Clo-Hal (involved in the biosynthesis of chlorobiocin (46) in *Streptomyces roseochromogenes*),<sup>197,198</sup> BhaA (from the balhimycin (47) biosynthetic pathway in *Amycolatopsis mediterranei*),<sup>199–201</sup> and ComH (responsible for chlorination en route to complestatin (46) in *Streptomyces lavendulae*)<sup>202–204</sup> have been identified (Figure 17). While further in vitro studies are required to elucidate the true substrates of these enzymes, it is likely that Clo-Hal, BhaA, and ComH are all class B, carrier protein-dependent, phenolic halogenases. In chlorobiocin (59) biosyn-



**Figure 17.** Natural products biosynthesized using phenolic flavin-dependent halogenases.

thesis, it is suggested that Clo-Hal halogenates a PCP tethered tyrosine or  $\beta$ -hydroxy tyrosine ( $\beta$ -HT) intermediate prior to formation of the aminocoumarin ring system.<sup>197</sup> The location of the BhaA encoding gene in the balhimycin gene cluster alongside NRPS encoding genes, and in vitro experiments also suggest that BhaA halogenates a PCP tethered tyrosine or  $\beta$ -HT intermediate.<sup>199–205</sup> The halogenase (Tcp21) in the teicoplanin gene cluster of *Actinoplanes teichomyceticus* is similarly predicted to process a Tyr- or  $\beta$ -HT-S-PCP substrate.<sup>205–207</sup>

Crystallography of the related chondrochloren halogenase CndH revealed that the substrate for CndH may also be a tyrosine-related intermediate that is most likely PCP

tethered.<sup>208</sup> CndH has a large nonpolar surface patch that could accommodate the putative carrier protein, and it was also proposed that the catalytic base required for halogenation (Glu in tryptophan halogenases) might be supplied by C-terminal domain of the carrier protein.<sup>208</sup>

Other class B phenolic halogenases that require a carrier protein include SgcC3 involved in the biosynthesis of C-1027 (49), a chromoprotein antitumor antibiotic isolated from *Streptomyces globisporus*.<sup>209</sup> In vitro activity of SgcC3, overexpressed in *E. coli*, was observed only in the presence of SgcC2 peptidyl carrier protein-tethered substrates. SgcC3 was found to utilize both (S)- and (R)- $\beta$ -tyrosyl-S-SgcC2, but not 3-hydroxy- $\beta$ -tyrosyl-S-SgcC2.<sup>209–211</sup>

The halogenases Ram20 and End30 have also been described and are suggested to halogenate hydroxyphenylglycine (Hpg) residues during the biosynthesis of related lipopeptide antibiotics ramoplanin and enuracidin (**50**).<sup>212–215</sup> Despite exhibiting high sequence similarity, End30 is responsible for dichlorination of Hpg13 in enduracidin, while Ram20 monohalogenates the Hpg17 residue of ramoplanin. Interestingly, deletion of *ram20* from the ramoplanin biosynthetic gene cluster and complementation with *end30* resulted in mono-halogenation of Hpg17, while the same complementation experiment in a strain lacking the ramoplanin mannosyltransferase resulted in halogenation of Hpg13.<sup>212</sup> This suggests that halogenation occurs after peptide assembly and mannosylation of Hpg11, with the bulky mannosyl groups blocking halogenation at the more proximal Hpg13 residue. Further *in vitro* experiments are required to fully evaluate the regioselectivities of End30 and Ram20.

**4.1.3.3. Class C Phenolic Halogenases.** A decarboxylating phenol brominase enzyme, Bmp5, was discovered from *Pseudoalteromonas luteoviolacea* which was the first example of a class C phenolic Fl-Hal.<sup>156,164,165</sup> This enzyme lacks sequence similarity with other canonical flavin-dependent halogenases and has sequence homology to known single-component flavin-dependent monooxygenases.<sup>156,164,165</sup> Bmp5 does not require an external flavin reductase enzyme for *in vitro* activity, and activity was abolished in the absence of either bromide or NADPH, indicating Bmp5 alone is capable of flavin reduction and bromination. Reaction of Bmp5 with 4-hydroxybenzoic acid (**51**) in the presence of bromide, NADPH, and FAD led to the formation of 3-bromo-4-hydroxybenzoic acid **52**. A second bromination is then proceeded by decarboxylation to afford dibromo phenol **53** (*Figure 18A*). Bmp5 showed no

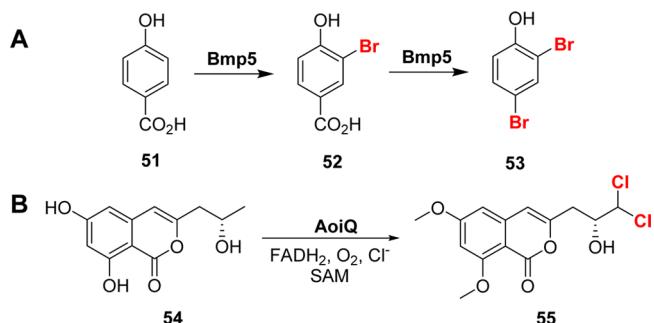
halogenase CmlS from *Streptomyces venezuelae*, for example, has been solved and displays a number of structural features not found in other Fl-Hals. A unique dynamic C-terminal domain creates a T-shaped tunnel leading to the active site. Most surprisingly, FAD appears to be covalently bound to the halogenase by the 8 $\alpha$  carbon, not found in other Fl-Hals.<sup>136</sup> A number of nonpolar surface patches suggest an activator or substrate-tethering protein is also involved, as with CndH.<sup>208</sup> ClmK, the proposed activator protein, has sequence homology to acyl-CoA synthetases leading to the postulation that ClmS may halogenate the CoA-thioester or free acyl group directly. The active site residue E44 is thought to catalyze HOCl generation, while Y350 is believed to stabilize the enolate intermediate, which can then act as a nucleophile to generate chlorinated product.<sup>136</sup>

A bifunctional methyl-transferase halogenase has recently been identified from the diapothins genome cluster of *Aspergillus oryzae* RIB40 (AoiQ) and found to be responsible for the geminal dichlorination of a methyl group in diaporthin (**54**) both *in vitro* and *in vivo* affording **55** (*Figure 18B*).<sup>216,217</sup> The position of halogenation suggests a mechanism different from both canonical Fl-Hals and CmlS as generation of an enolate from a secondary alcohol would likely require additional oxidoreductases.<sup>217</sup> Genomic analysis of AoiQ revealed that homologous enzymes are encoded in various other fungal biosynthetic gene clusters.<sup>217</sup> Identification of these aliphatic Fl-Hals, in addition to further *in vitro* structural and functional characterization, may provide exciting insight into their mechanism, as well as provide the potential for regio- and stereoselective aliphatic halogenation reactions for biocatalytic applications.

#### 4.2. Flavin-Dependent Halogenases as Biocatalysts

**4.2.1. Substrate Scope of Fl-Hals.** There has been significant interest in using flavin-dependent halogenases as biocatalysts because of their potential to regioselectively halogenate aromatic substrates under benign conditions. Thus far, most of this work has been focused on the tryptophan halogenases and has revealed a number of enzymes capable of halogenating tryptophan derivatives,<sup>218</sup> non-natural indolic substrates,<sup>150,155,219–221</sup> in addition to benzamides and benzoic acids<sup>150,155</sup> as well as naphthols and naphthyl amines (*Figure 19*).<sup>220,221</sup> In a number of cases, halogenation occurs with good regioselectivity. The tryptophan-7-halogenase RebH has been demonstrated to solely halogenate the 7-position of a number of non-natural indoles (**56–58**) and *ortho*- to the –NH<sub>2</sub> and –OH groups in **59** and **60**, respectively.<sup>220,221</sup> Interestingly, PrnA, another tryptophan-7-halogenase, was found to halogenate some of these indolic substrates at the more electronically favored C2 position.<sup>219</sup>

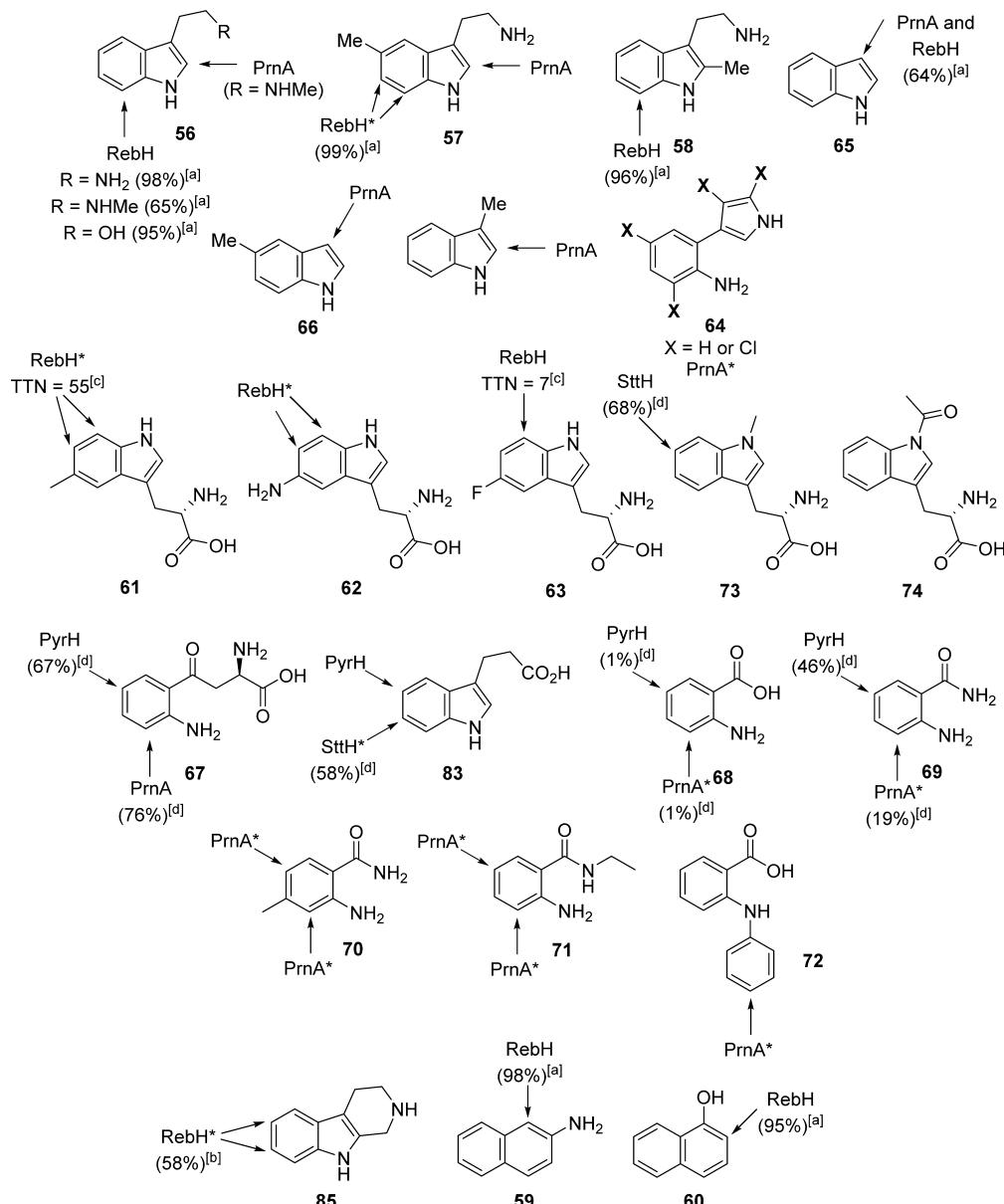
Introduction of electron-donating substituents onto the indole ring, which activate the substrate toward electrophilic aromatic substitution, led to the formation of dichlorinated products with RebH.<sup>220,221</sup> The formation of dichlorinated products and regioisomers has also been observed from the halogenation of tryptophan derivatives bearing electron-donating substituents with RebH (**61–63**), and in the halogenation of 3-(2'-aminophenyl)pyrrole (**64**) with PrnA,<sup>218,219</sup> demonstrating that substrate electronics influence regioselectivity when substrate positioning relative to the active site lysine is perturbed or flexible. Indeed, both RebH and PrnA have been shown to halogenate the most nucleophilic C3 position of indole (**65** and **66**) when this position is not



**Figure 18.** (A) Decarboxylative bromination catalyzed by Bmp5. (B) Halogenation and methylation activity of the proposed Fl-Hal-methyl transferase fusion protein AoiQ.

chlorination activity, while it forms iodophenols, indicating a highly evolved bromide or iodide binding site in the protein which is not present in other canonical flavin-dependent halogenases.<sup>164</sup> The full mechanistic and structural details of Bmp5 are still lacking, and the proposed decarboxylative flavin-dependent halogenase mechanism has no precedent. Whether the two steps are consecutive or require involvement of any specific catalytic residues is unclear; however, identification of Bmp5 homologues from different genomic clusters may shed light on those questions.<sup>164</sup>

**4.1.4. Aliphatic Flavin-Dependent Halogenases.** A small number of flavin-dependent halogenases have recently been identified, which are capable of halogenating aliphatic C–H bonds. The crystal structure of the chloramphenicol



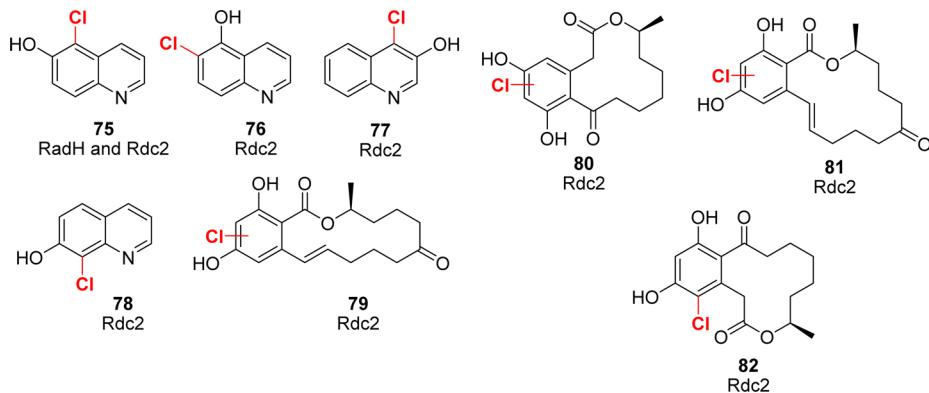
**Figure 19.** Known substrate scope and regioselectivity of the flavin-dependent tryptophan halogenases. \*Indicates examples where halogenation at multiple positions occurs. Conversions are shown in brackets. [a] Measured using 5 mol % of RebH.<sup>220</sup> [b] Measured using 10 mol % of RebH.<sup>220</sup> [c] TTN = total turnover number determined using 0.4 mol % RebH.<sup>218</sup> [d] Conversions are reported relative to the conversion of tryptophan by the relevant enzyme under the same conditions.<sup>155</sup>

functionalized.<sup>219–221</sup> Moreover, with the highly activated substrate **64**, seven halogenated products were formed (including regiosomers and dichlorinated products) due to the electron-rich nature of both the pyrrole and the aniline moieties.<sup>219</sup> Most of the tryptophan halogenases have also been shown to chlorinate both L- and D-tryptophan, with preference for the natural L-enantiomer.<sup>219–221</sup>

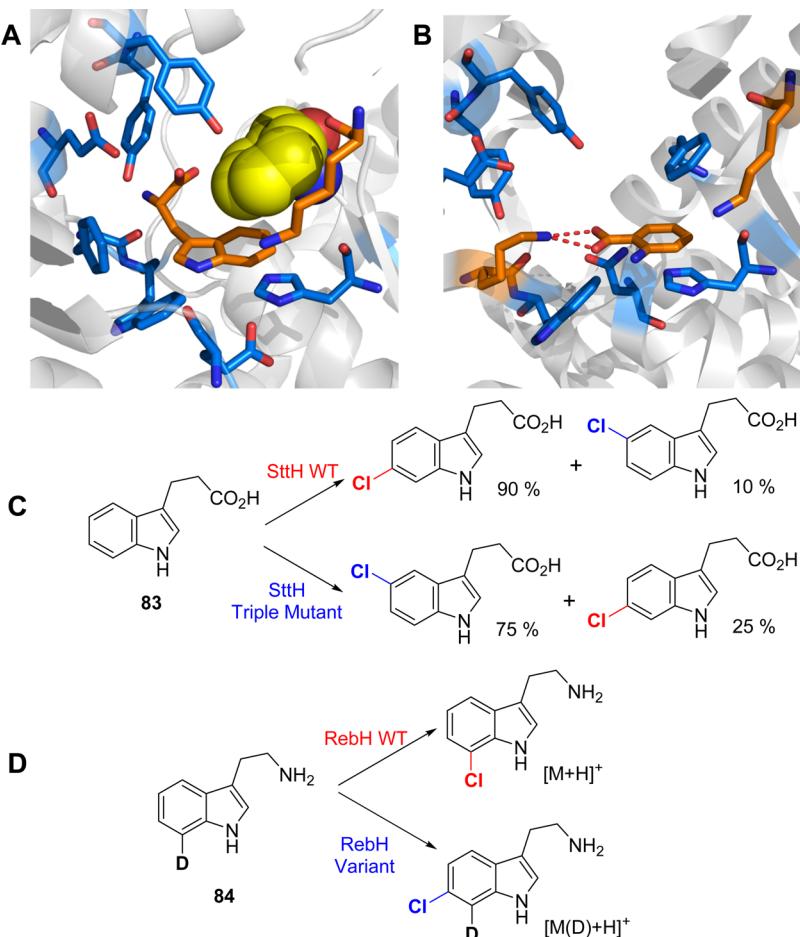
In an attempt to broaden substrate scope of the enzymatic halogenation, the tryptophan-5- and tryptophan-6-halogenases PyrH and SttH have also been studied. With a number of 2-amino benzamides and benzoic acids (**67–72**), PyrH and SttH were found to solely chlorinate *para* to the  $-\text{NH}_2$ , while PrnA afforded mixtures of both *ortho*- and *para*-chlorinated products in most cases.<sup>150,155</sup> The formation of regiosomers with the smaller substrates **68–72** is thought to be due to their flexibility upon binding within the active site and hence undefined

positioning relative to the catalytic lysine. Notably kynurenine (**67**), a larger substrate that can potentially form more hydrogen-bonding contacts with the active site, could be halogenated in a regio-divergent manner at the 3- or 5-position by PrnA or PyrH and SttH, respectively,<sup>150,155</sup> demonstrating the potential for these biocatalysts to offer a halogenation with catalyst-controlled regioselectivity. Additionally, SttH halogenates the 6-position of N-methylated tryptophan (**73**) with good efficiency,<sup>150</sup> while PrnA and RebH do not accept this or other N-functionalized tryptophans (**73** and **74**).<sup>219–221</sup>

Halogenases of different natural substrate scope have been explored for the halogenation of alternative aromatic scaffolds. Notably, Rdc2, a phenolic flavin-dependent halogenase, was shown to halogenate the hydroxyisoquinolines **75–78** in addition to a number of other macrocyclic lactones (**79–82**, Figure 20).<sup>125,167,168,172</sup> In most cases, these highly activated



**Figure 20.** Substrate scope and regioselectivity of the flavin-dependent phenolic halogenases.



**Figure 21.** (A) Active site of PrnA with tryptophan bound, with F103 highlighted as yellow spheres showing how this residue prevents approach of catalytic lysine at position 5- (PDB 2AQJ). (B) Active site of PrnA E450 K mutant with anthranilic acid placed showing how the lysine mutation could increase binding affinity of anthranilic acid through hydrogen bonding (PDB 4Z43). (C) Regiodivergent halogenation of indole-3-propionic acid by SttH and a triple mutant based upon structural differences between SttH and PyrH. (D) MALDI-ToF HTPS of halogenase variant regioselectivity using deuterated substrates.

phenolic compounds are halogenated regioselectively *ortho*- to the hydroxy functionality, suggesting that hydrogen bonding or deprotonation of this hydroxyl may be important in the control of regioselectivity and activity of Rdc2 and related phenolic Fl-Hals.

Each of the flavin-dependent halogenases mentioned above catalyzes the halogenation of an aromatic C–H. Interestingly, the bifunctional halogenase-methyl transferase fusion protein from *Aspergillus oryzae* RIB40 is believed to be responsible for

the dichlorination of **54** (Figure 18B).<sup>217</sup> While significant work is likely to be required to understand the mechanism of this aliphatic chlorination and develop this enzyme as a biocatalyst, there is an exciting prospect of regioselective and stereoselective chlorination of alkyl C–H's using this or related enzymes.

**4.2.2. Engineering Fl-Hals To Alter Substrate Scope and Regioselectivity.** To deliver biocatalysts with improved or altered regioselectivity, a number of attempts have been

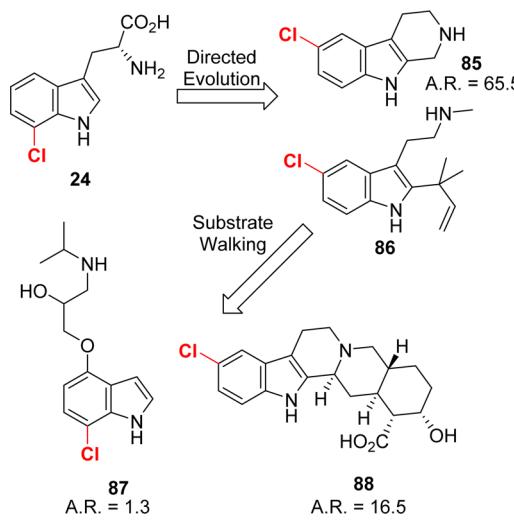
made using rational mutagenesis to change the regioselectivity of the flavin-dependent tryptophan halogenases.<sup>150,155,222</sup> The first example using PrnA found that mutation of the bulky active site residue F103, which would usually shield the C5 position of tryptophan from approach by active site lysine, to alanine led to the formation of 7- and 5-brominated tryptophan in a 2:1 ratio, while the wild-type afforded only 7-brominated product (Figure 21A).<sup>222</sup> Although a modest shift in regioselectivity, this work set the precedent that site-directed mutagenesis could be used to alter the regioselectivity of the tryptophan halogenases. Subsequent work used active site mutations to stabilize one postulated orientation of anthranilic acid (**68**) in the active site of PrnA and found a single mutant capable of increasing activity toward this substrate evidenced as a change in binding affinity by an almost 10-fold reduction in  $K_m$  as compared to wild-type (from 3161 to 384  $\mu\text{M}$ ).<sup>155</sup> The same work also identified a double mutant capable of shifting regioselectivity from predominantly 3- to 5-chlorination (Figure 21B), with further improved kinetic parameters ( $K_m = 205 \mu\text{M}$ ,  $k_{\text{cat}} = 1.82 \text{ min}^{-1}$ ) as compared to wild-type ( $k_{\text{cat}} = 0.51 \text{ min}^{-1}$ ) and the single mutant ( $k_{\text{cat}} = 0.93 \text{ min}^{-1}$ ).<sup>155</sup> A similar approach with SttH found a triple mutant capable of shifting the regioselectivity of halogenation from the 6- to the 5-position of 3-indolepropionate (**83**). Mutations were rationalized on the basis of comparison of the crystal structure of SttH with PyrH, which revealed key differences in a region close to the active site of both.<sup>150</sup> This example is the first to demonstrate that regioselectivity can be switched without altering the catalytic efficiency of the enzyme.

In addition to the rational and structure-guided approaches, random mutagenesis and directed evolution have been used to alter regioselectivity.<sup>223</sup> In this work, substrates with a single deuterium in place of an aromatic C–H were used in a MALDI-ToF-based screen to detect mutants that halogenated at positions other than the deuterated one (Figure 21C). Starting from a RebH mutant previously found to be more thermostable<sup>224</sup> and using a total of six rounds of screening, two RebH mutants were found, which halogenated the 6- and 5-positions of an indolic substrate (**84**) with good selectivity and reasonable conversion.<sup>223</sup> This method does not require structural information about the enzyme and can be used to modulate regioselectivity in a semi high-throughput manner, in contrast to most high-throughput screening (HTPS) methods, which focus on improving stability or activity of an enzyme.

Random mutagenesis has also been used in a “substrate walking” approach to allow the late-stage halogenation of a number of large bioactive substrates such as **85–88** (Figure 22).<sup>225</sup> Over a total of four generations of error-prone PCR, RebH was mutated to a quintuple mutant capable of halogenating the large C4-functionalized substrate **87** with complete regioselectivity. Together, these methods demonstrate the potential to develop a suite of biocatalysts for regiodivergent halogenation, either from a single parent enzyme or using halogenases of different natural regioselectivity.

#### 4.2.3. Engineering Fl-Hals To Improve Activity and Stability.

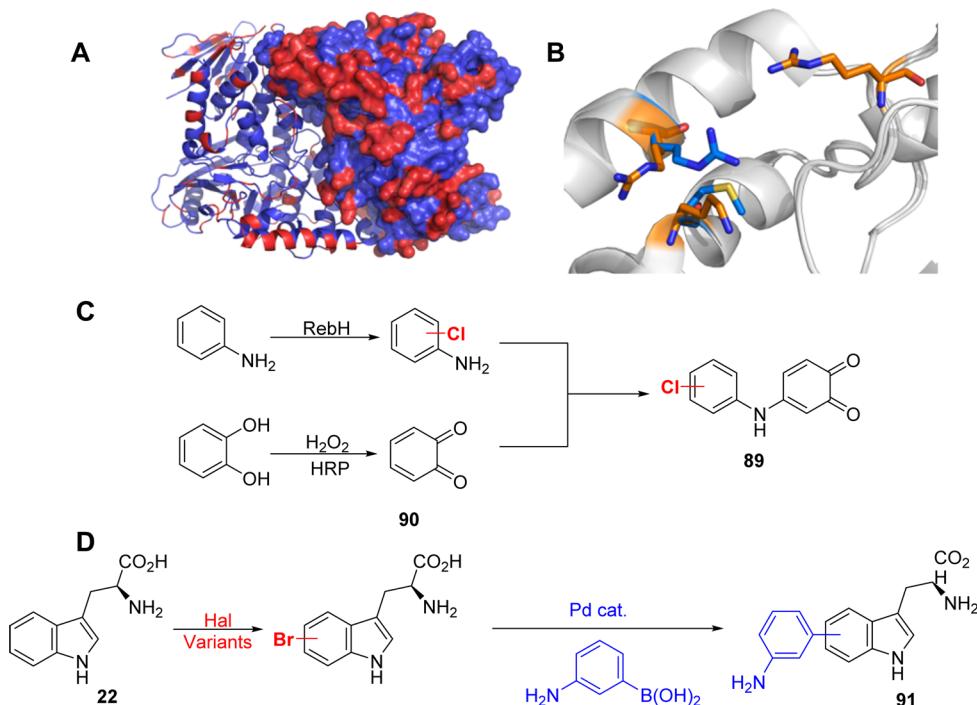
While the flavin-dependent halogenases are promising biocatalysts for regioselective and regiodivergent halogenation of aromatic compounds, their applications are still limited to the analytical and semipreparative scale because of low  $k_{\text{cat}}$  values, poor stability, and susceptibility to substrate–product inhibition.<sup>32–34,218,224,225</sup> This is likely to be an artifact of these enzymes evolving as part of the biosynthetic pathways to nonessential secondary metabolites. Halogenases are not



**Figure 22.** Products from the halogenation of large bioactive natural products by RebH variants generated through directed evolution and substrate walking. A.R. relates to the activity ratio of each substrate with the best variant RebH as compared to wild-type.<sup>225</sup> Substrate **86** showed negligible activity with wild-type, and therefore A.R. cannot be determined.

essential for the survival or growth of the native host, and hence there has been little evolutionary pressure for highly active halogenase enzymes.<sup>226</sup> Although attempts have been made to improve biocatalytic halogenations using Fl-Hals through reaction optimization, design-of-experiment approaches were required due to the large number of variables in such a system, and only modest improvements were obtained.<sup>227</sup> Significant work has therefore been focused on the engineering of Fl-Hals with improved biocatalytic properties.

One strategy to find more stable enzymes, which are therefore usually more amenable biocatalysts, is to look for analogous enzymes found in thermophilic organisms. Thermostable enzyme variants have been demonstrated to confer many advantages, including prolonged lifetime and increased tolerance to organic solvents and proteolysis.<sup>228–231</sup> This approach has been employed with the flavin-dependent halogenases. A thermophilic tryptophan 6-halogenase (Th\_Hal) was identified from *Streptomyces violaceusniger* SPC6, a thermophilic and halotolerant bacterium,<sup>232,233</sup> with a melting temperature ( $T_m$ ) almost 10 °C higher than that of the mesophilic tryptophan 6-halogenase SttH and a higher  $k_{\text{cat}}$  than a number of other tryptophan halogenases.<sup>145</sup> This thermophilic halogenase (Th\_Hal) was partnered with a flavin-reductase from a thermophilic *Bacillus* strain,<sup>234</sup> allowing biocatalytic halogenation reactions to occur at 45 °C in vitro with a number of non-natural substrates. The regioselectivity of Th\_Hal was the same as with SttH.<sup>150</sup> A crystal structure of Th\_Hal found that 40% of the total differences with the nearest homologue SttH were polar residues on the surface of Th\_Hal (Figure 23A), suggesting that increasing surface charge is likely to be largely responsible for the higher stability of Th\_Hal.<sup>145</sup> In the case of the naturally evolved thermophilic Fl-Hal, this is likely to be because it is easier to modify the surface of a protein rather than the inner catalytic core whilst retaining activity.<sup>235–237</sup> Increasing surface charge is thought to confer stability by deterring protein aggregation and increasing hydrogen bonding to water.<sup>235,237,238</sup>



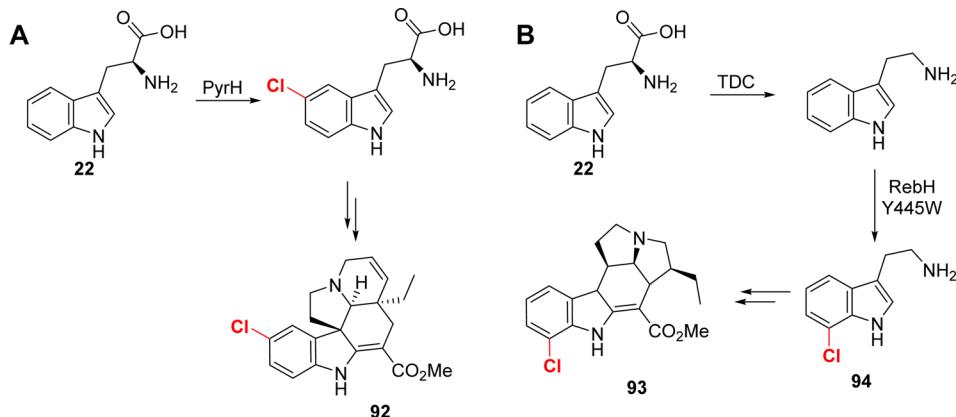
**Figure 23.** (A) Crystal structure of SttH with differences in amino acid sequence from *T*\_Hal highlighted in red (PDB 5HY5). (B) Crystal structures of wild-type (orange, PDB 2OA1) and 3-SLR (blue, PDB 4LU6) variant RebH overlaid to show some of the amino acid substitutions thought to contribute to increased stability. (C) UV-visible based screen for halogenase activity using either conjugation of aryl halide with *ortho*-quinones. (D) High-throughput halogenase activity screen through combination of enzymatic halogenation with palladium-catalyzed Suzuki chemistry to generate fluorescent adducts.

Other methods have involved using error-prone PCR (ePCR) and directed evolution to mutate a mesophilic enzyme toward increased stability using temperature as the selection pressure.<sup>224</sup> After three rounds of ePCR, two variants with seven and eight mutations possessing  $T_{opt}$  and  $T_m$  higher than the wild-type starting point were found, both of which proved to be stable over longer reaction times than wild-type, but with decreased turnover. Crystallography of one of these variants (PDB 4LU6) revealed a number of structural features, which may have contributed to increased enzyme stability.<sup>224</sup> Notably, the mutation of a surface glutamine to arginine resulting in increased surface charge, similar to the thermophilic *Th*\_Hal.<sup>145,238</sup> In other portions of the RebH variant, charge density was reduced to reduce repulsion with nearby residues (Figure 23B), and an N-terminal serine was mutated to proline and is believed to increase protein rigidity at this terminus and hence improve stability.

To achieve full synthetic utility, it is likely that the flavin-independent halogenases will need significantly more engineering than the reported low-throughput chromatographic methods of screening can offer in a reasonable time frame with economical resource consumption.<sup>224,225</sup> For this purpose, a number of high-throughput screens for halogenase activity have been developed (Figure 23C and D).<sup>239–241</sup> One such example relies upon the formation of aryl chloride-*ortho*-quinone adducts (89) with distinct UV/visible absorbance properties.<sup>241</sup> The generation of such adducts requires HRP (horseradish peroxidase) in addition to the halogenase and cofactor recycling enzymes to generate the *ortho*-quinone 90 from catechol. Michael addition of the aryl chloride and spontaneous reoxidation then generates the probe of interest (89, Figure 23C). Although this method does allow reliable monitoring of halogenase reactions using UV-vis in 96-well plates rather than chromatographic

methods, the need to quench reactions and use additional enzymes and substrates may limit its applicability. In a similar vein, a recent report of a fluorescence-based screen involves the combination of the enzymatic halogenation with palladium-catalyzed cross-coupling chemistry to create an adduct (91) with fluorescence sufficiently distinct from the cofactors and proteins required for biocatalytic halogenation, thereby allowing detection in a high-throughput manner using a fluorescence plate-reader (Figure 23D).<sup>240</sup> After applying this method to screen variants from error-prone PCR, a double mutant of Thal was found with a  $T_m$  10 °C higher than that of wild-type. The structural differences thought to be responsible for stabilization of the engineered and naturally occurring halogenase enzymes, as well as the methods used to discover them, could be used as the basis of engineering halogenase variants with further improved activity. The combination of these screening methods with strategies for targeting the generation of variant libraries to specific sites, as have been demonstrated to afford a minor improvement in RebH catalytic parameters by targeting mutations to the flavin-binding pocket,<sup>242</sup> could be used to focus such engineering efforts.

The stability and scalability of a number of Fl-Hals have been improved by their immobilization into heterogeneous cross-linked enzyme aggregates (CLEAs) of a Fl-Hal, flavin reductase, and alcohol dehydrogenase, for concurrent cofactor regeneration,<sup>150,243,244</sup> allowing enzymatic halogenation on a gram scale.<sup>244</sup> CLEAs are well-known to improve the efficiency of biocatalysts by improving catalyst lifetime, reducing the effects of substrate/product inhibition, and allowing more efficient biocatalyst removal and recycling.<sup>245–247</sup> In the case of the Fl-Hals, this cross-linking may help to stabilize interdomain interactions, or effectively “protect” some of the biocatalyst from high substrate concentration by shielding it within the



**Figure 24.** Integration of flavin-dependent halogenases into various biosynthetic pathways to yield chlorinated natural products. (A) Use of PyrH to afford 5-chlorinated indolocarbazoles. (B) Use of an engineered RebH variant to selectively catalyze 7-chlorination of tryptamine. TDC = tryptophan decarboxylase.

heterogeneous catalyst.<sup>244,248</sup> The preparation of such biocatalysts from crude cell lysates, rather than needing to purify enzymes,<sup>244</sup> is also advantageous.

**4.2.4. Integration of Fl-Hals into Non-Native Biosynthetic Pathways.** There are a number of examples where the Fl-Hals have been incorporated into natural product biosynthetic pathways, to increase the diversity of the products obtained and therefore attenuate bioactivity of the resulting compounds (Figure 24).<sup>33,249–254</sup> Generation of natural product analogues via this method is significantly more efficient, and therefore economical and ecologically friendly, than via laborious total synthesis.<sup>255</sup> A number of these reported examples rely upon the chlorination of tryptophan prior to incorporation into a biosynthetic pathway, thereby resulting in a natural product with a chlorinated tryptophan moiety.<sup>249,251,252</sup> The seminal work in this area involved the combinatorial reconstitution of genes from the biosynthesis of rebeccamycin and staurosporine (natural product antitumor agents) with genes encoding for tryptophan halogenases of different regioselectivity to afford different regioisomers of chlorinated indolocarbazole “non-natural” products (92, Figure 24A).<sup>252</sup> Subsequent work involved the introduction of a tryptophan-7-halogenase directly into a pacidamycin-producing *Streptomyces* strain, previously found to incorporate 7-halo tryptophans through substrate-directed biosynthesis,<sup>256</sup> to afford chlorinated pacidamycin.<sup>251</sup> Halogenase genes have also been incorporated into plant secondary metabolism to produce chlorinated monoterpene indole alkaloids (93) in *Catharanthus roseus* root cultures.<sup>249</sup> In this case, it was found that the tryptophan decarboxylase enzyme, in plants, was inefficient at turning over 7-chlorotryptophan to give the required 7-chlorotryptamine (94). Therefore, a RebH mutant capable of selectively catalyzing the chlorination of tryptamine was introduced to overcome this metabolic bottleneck and reduce accumulation of 7-chlorotryptophan (24), which has adverse effects on plant health (Figure 24B).<sup>250</sup> Other groups have since expressed the tryptophan halogenases SttH and RebH in the chloroplasts of tobacco plants, allowing the production of 6- and 7-halogenated tryptophans and, when partnered with a tryptophan decarboxylase, chlorinated tryptamines (94).<sup>257</sup>

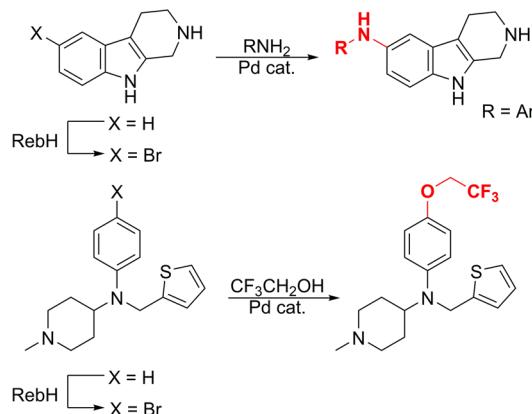
The ability of Rdc2 to catalyze the late-stage chlorination of a number of macrolactones, as well as smaller phenolic compounds, suggests it would be an ideal candidate for

introduction into non-indolic secondary metabolite biosynthetic pathways.<sup>125,167,168</sup> Rdc2, for example, has been incorporated into a reconstituted resveratrol biosynthetic pathway in *E. coli* to afford production of the 2-chlorinated resveratrol derivative, which has been shown to have increased antimicrobial and antioxidant activity as compared to the nonhalogenated parent compound.<sup>258,259</sup> CazI was recently demonstrated to be a flavin-dependent halogenase responsible for chlorination during the biosynthesis of chaetoviridin,<sup>179</sup> suggesting potential for the generation of halogenated polyketide non-natural products.

**4.2.5. Integration of Fl-Hal with Transition-Metal Catalysis.** The introduction of halogen atoms into complex natural products also provides a convenient moiety for further functionalization by transition-metal-catalyzed cross-coupling chemistry.<sup>260</sup> The number of C–C, C–N, C–F, and C–O bond-forming reactions possible using this chemistry has made it an indispensable tool in the synthesis of complex molecules of relevance in many sectors of the chemical industry.<sup>3</sup> As the carbon–halide bond is a key substrate for these transformations, it seems logical that the combination of the regioselective enzymatic methods for installation of halides onto aromatic substrates with this powerful cross-coupling chemistry may provide methodologies for the regioselective formation of C–C, C–N, C–F, and C–O bonds from unactivated C–H bonds. Current methods for such transformations, termed C–H activation, rely upon substrate control whereby either the difference in acidity of C–H bonds or intramolecular coordination is used to control the position of functionalization.<sup>42</sup> As such, this often means that certain positions of key moieties are inaccessible. Use of a halogenase, whereby the position of functionalization is controlled by coordinating effects between the substrate and a well-defined three-dimensional active site, however, represents an example of catalyst control as using different biocatalysts can functionalize different positions.

The sequential application of biosynthetic halogenation followed by a subsequent transition-metal-catalyzed cross-coupling was demonstrated on crude extracts of chloropacidamycin from *S. coeruleorubidus* expressing the tryptophan 7-halogenase PrnA to generate a number of 7-aryl pacidamycin derivatives through Suzuki–Miyaura chemistry.<sup>251</sup> A similar rationale, whereby halo-aryl containing crude extracts from halogenase biotransformations are used for Pd-catalyzed cross-

coupling chemistry, has been extended to allow the regioselective formation of C–C, C–N, and C–O bonds on a number of bioactive molecules (Figure 25).<sup>261</sup>



**Figure 25.** Integration of flavin-dependent halogenases with palladium-catalyzed cross-coupling chemistries to allow regioselective C–C and C–O cross-coupling reactions.

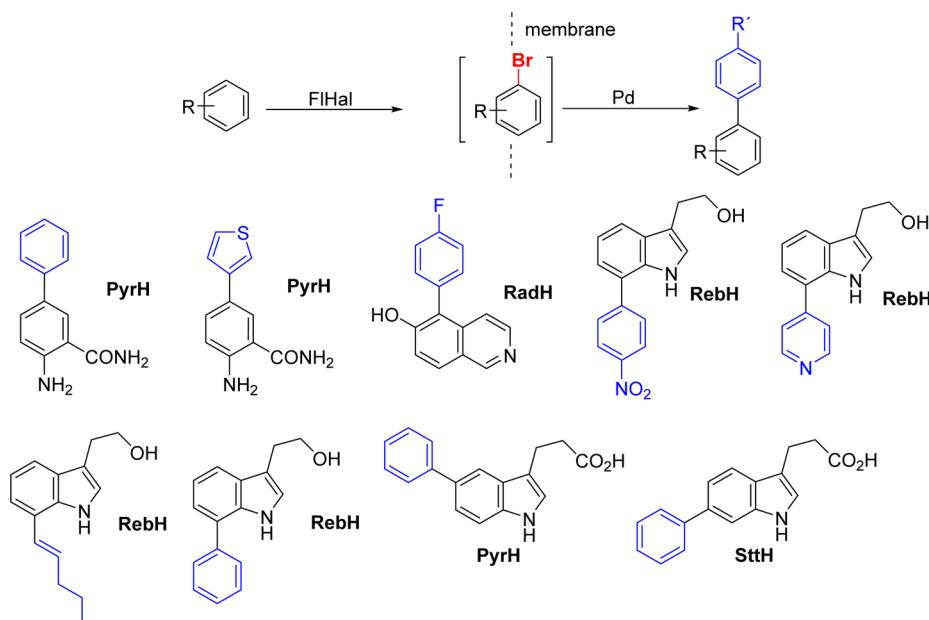
Although these two examples demonstrate the possibility of regioselective cross-coupling reactions using halogenases to generate an aryl halide precursor, the requirement for a two-step process including isolating the intermediate aryl halide is not ideal. The combination of multiple reactions into single-pot transformations represents a potential step-change in the efficiency of chemical synthesis by reducing solvent consumption and waste, eliminating the need for auxiliary chemicals and increasing space-time yield.<sup>262,263</sup> The combination of biocatalytic and chemocatalytic reactions into single pot aqueous transformations is often not straightforward, however, and requires the compartmentalization or removal of at least one of the components due to mutual deactivation of the bio- and chemocatalysts.<sup>264–266</sup> This has been realized to some extent by using the heterogeneous CLEAs of the flavin-

dependent halogenases (Figure 26).<sup>243,267</sup> As the heterogeneous biocatalyst is easily removed from the reaction, compartmentalization of biocatalysts and chemocatalysts can be achieved by filtration.<sup>243,267</sup> Methods where such intermediary processing is not required have also been reported.<sup>243</sup> Here, compartmentalization is achieved by using poly dimethylsiloxane (PDMS) membranes, which, due to their hydrophobic nature, allow only the nonpolar arylhalide to diffuse freely between compartments, while the charged chemocatalysts and biocatalysts are contained separately.<sup>264,268,269</sup> Such one-pot transformations have been applied to the arylation, heteroarylation, and alkenylation of isoquinolines and benzamides, in addition to the 5-, 6-, and 7-positions of indoles and tryptophan derivatives (Figure 26).<sup>243,267</sup> Notably, the direct functionalization of the 7- and 6-positions of indoles by nonenzymatic means requires the introduction and removal of directing groups.<sup>270,271</sup> The methods of compartmentalization used here set the stage for the integration of other transition metal-catalyzed processes and therefore additional regioselective transformations.

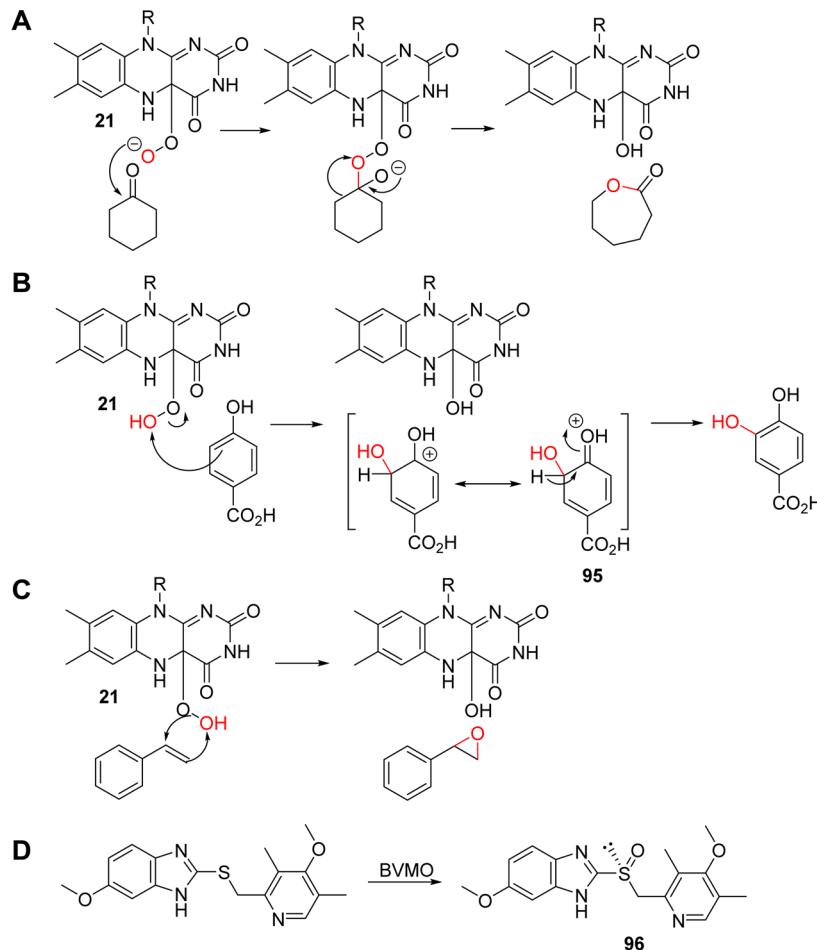
#### 4.3. Flavin-Dependent Monooxygenases

**4.3.1. Natural Occurrence and Mechanism of Flavin-Dependent Monooxygenases.** The flavin-dependent halogenases belong to the class of flavin-dependent monooxygenases, which operate via the activation of molecular oxygen using flavin cofactors. A number of other types of enzyme in this class have also been subject to significant interest as biocatalysts because of their ability to functionalize inert C–H bonds.<sup>115,272–275</sup> A number of recent reviews have extensively discussed these enzymes in terms of both structural biology and their applications as biocatalysts.<sup>115,116,275–277</sup> This Review will discuss how the methods employed to allow the application of some of these enzymes as industrial biocatalysts might be applied to improve the viability of using flavin-dependent halogenases in the same way.

The flavin-dependent hydroxylases and epoxidases in addition to the Bayer–Villager monooxygenases (BVMO)



**Figure 26.** Integration of flavin-dependent halogenases with palladium-catalyzed cross-coupling chemistries to allow regioselective and regiodivergent arylation, vinylation, and heteroarylation in one-pot reactions using membrane compartmentalization.



**Figure 27.** Reactivity of hydroperoxy-flavin (21) in (A) Bayer–Villager monooxygenases, (B) flavin-dependent hydroxylases, and (C) styrene monooxygenases. (D) Use of a Bayer–Villager monooxygenase for asymmetric sulfoxidation in the synthesis of esomeprazole (96).

operate via an analogous mechanism to Fl-Hals, except hydroperoxy-flavin (21) is believed to directly react with substrate to yield oxygenated product.<sup>118,120,278</sup> All of these enzymes therefore share the conserved flavin-binding motif GxGxxG. With the flavin-dependent hydroxylases and BVMOs, reduced flavin is found in the form of an FMN prosthetic group, which is reduced by a second domain using NADH, while the epoxidases utilize freely diffusing FADH<sub>2</sub> generated by a separate flavin-reductase in the same way as the Fl-Hals. Because of the mechanistic relationship between the Fl-Hals and the hydroxylases, epoxidases and BVMOs, useful insight may be gained as to how to apply the Fl-Hals as industrial biocatalysts by studying others in the class.

The flavin-dependent hydroxylases typically hydroxylate the 2-position of phenols,<sup>272,279</sup> most likely due to increased stabilization of intermediate 95 and positioning relative to hydroperoxy-flavin (21). These enzymes are responsible for the catabolism of xenobiotics as well as the biosynthesis of fatty acids and sterols.<sup>274,280–282</sup> Kynurenine-3-monooxygenase, on the other hand, hydroxylates *ortho*- to a NH<sub>2</sub> group, due to stabilization of the analogous ketamine intermediate.<sup>283</sup> This particular hydroxylase has attracted much academic attention due to its involvement in tryptophan catabolism and neurodegenerative disease.<sup>283–285</sup> Styrene monooxygenase is similarly involved in the microbial catabolism of styrene, catalyzing epoxidation of the vinyl group prior to ultimate incorporation to the TCA cycle,<sup>286</sup> while BVMOs are responsible for the

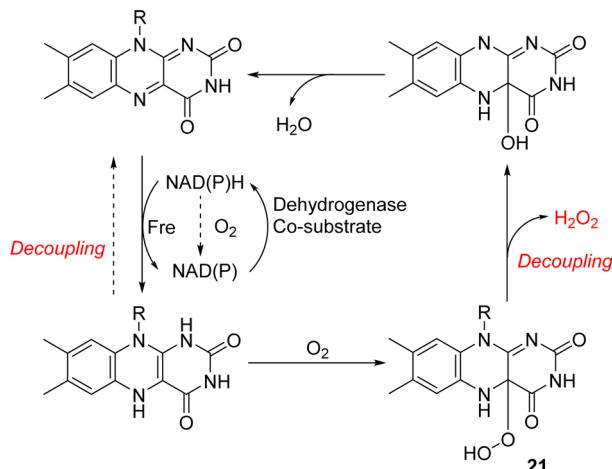
oxygenation of carbonyl-containing xenobiotics en route to innocuous products in addition to oxidation at heteroatoms such as S, N, and B.<sup>287,288</sup>

The different mechanisms of flavin-dependent monooxygenases serve to illustrate the versatility of the flavin cofactors (Figure 27). Notably, in BVMOs, the distal oxygen of hydroperoxy-flavin is deprotonated and functions as a nucleophile, in contrast to halogenases, hydroxylases, and the styrene epoxidase, where the distal oxygen is protonated and functions as an electrophile.

**4.3.2. Flavin-Dependent Monooxygenases as Biocatalysts.** There are a number of factors that limit the prospect of applying the flavin-dependent monooxygenases, including Fl-Hals, as industrial biocatalysts, including the requirement for stoichiometric reductants, substrate or product inhibition, the requirement for careful control of oxygenation, and poor enzyme stability.

Reduced flavin cofactors, required by all enzymes of this class, are unstable with respect to oxygen. Stoichiometric reductants must therefore be provided in the form of nicotinamide cofactors, which are more aerobically stable. With the hydroxylases and BVMOs, reduction of the flavin prosthetic group is carried out by a reductase domain of the monooxygenase.<sup>116,289,290</sup> With the epoxidases and Fl-Hals, freely diffusing reduced flavin is typically produced by a separate flavin-reductase enzyme before utilization by the monooxygenase,<sup>116</sup> with the exception of a recently discovered

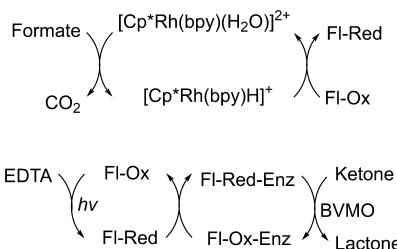
epoxidase-reductase fusion protein.<sup>291,292</sup> In the case of monooxygenases utilizing freely diffusing reduced flavin cofactors, it is possible for reduced flavin to react directly with oxygen in solution, resulting in hydroperoxy-flavin being generated outside of the active site.<sup>293</sup> Without stabilization through hydrogen bonding to an active site, this can break down to produce H<sub>2</sub>O<sub>2</sub> in solution (Figure 28).<sup>293,294</sup> In



**Figure 28.** Cycle of peroxy-flavin (**21**) production showing potential uncoupling processes.

addition to “uncoupling” the monooxygenase reaction and wasting the reductant cosubstrate, accumulation of such reactive species can impair the stability of the biocatalysts and therefore reduce their productivity.<sup>275</sup> Similar uncoupling reactions can also occur within the active site, including in those of enzymes that utilize flavin prosthetic groups, by inefficient transfer of oxygen from hydroperoxy-flavin to substrate and therefore degradation to H<sub>2</sub>O<sub>2</sub> and oxidized flavin. High concentrations of product are thought to promote uncoupling in this manner, by effectively blocking substrate from binding and preventing productive oxygen transfer.<sup>279,295</sup> Such uncoupling means that the reducing cosubstrate (NAD(P)H) must either be supplied in excess, which is undesirable due to cost, or regenerated in situ from inexpensive cosubstrates. Although oxygenation can promote uncoupling and impair productivity, it cannot simply be excluded from reaction media due to the requirement to generate hydroperoxy-flavin for productive cycles; consequently, careful control of oxygenation is often required.<sup>296,297</sup>

The use of additional enzymes *in vitro* to reduce nicotinamide cofactors using inexpensive cosubstrates such as glucose or isopropanol is widespread and has been applied to the Fl-Hals, in addition to a number of other monooxygenases, to allow the use of stoichiometric nicotinamide and flavin cofactors.<sup>155,220,298</sup> Methods for the direct regeneration of FAD, without using nicotinamide cofactors or additional enzymes, have also been reported and applied with the flavin-dependent monooxygenases (Figure 29).<sup>128,299–303</sup> These methods offer simplified electron-transfer cascades and therefore are envisioned to be a more robust alternative to enzymatic methods. A number of these examples utilize the organometallic complex [Cp\*Rh(bpy)(H<sub>2</sub>O)]<sup>2+</sup> and formate to generate the reduced complex [Cp\*Rh(bpy)H]<sup>+</sup>, which can then directly transfer hydride to oxidized FAD.<sup>128,302</sup> The use of transition metal complexes may not be ideal, however, because of their potential

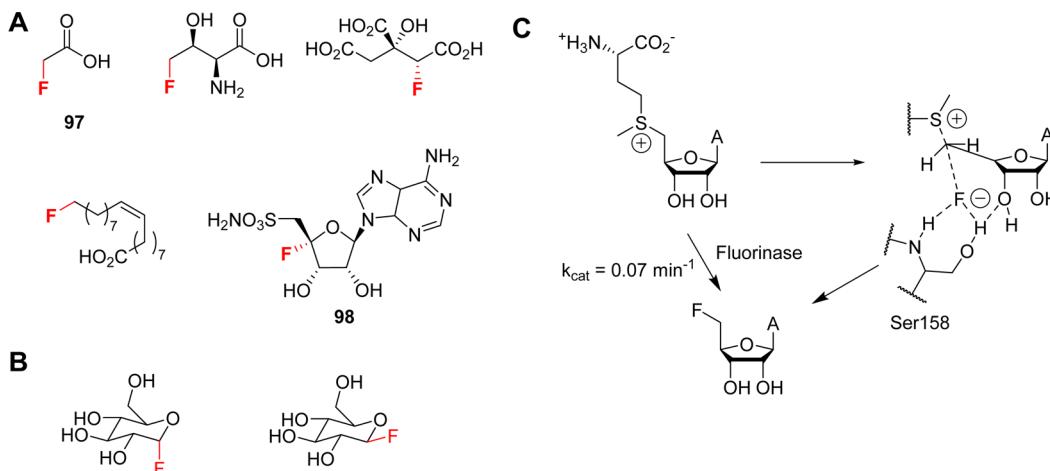


**Figure 29.** Nonenzymatic flavin recycling methods.

deactivation of the monooxygenase and expense. Methods of light-driven flavin reduction have therefore also been investigated, utilizing either water or EDTA as the sacrificial electron donor.<sup>300,304</sup> In one case, this method has been applied to the BVMO-catalyzed oxidation of cyclic ketones by using additional FAD in solution, to facilitate electron transport from EDTA to enzyme-bound FAD.<sup>300</sup> Further simplification has been achieved using electrochemical methods to directly reduce FAD for biocatalytic vinyl aromatic epoxidations without the need for nicotinamide cofactors.<sup>301</sup> Direct regeneration of FADH<sub>2</sub> using nicotinamide mimics has also been reported, which allows efficient biocatalytic oxidations using styrene monooxygenase without the flavin-reductase domain or nicotinamide cofactors.<sup>303</sup>

The inherent metabolism of a microbial host can also be used to regenerate the required cofactors, in addition to supplying enzymes such as superoxide dismutase or catalase, which can degrade reactive oxygen species.<sup>305</sup> Such an approach has the advantage of not requiring additional enzymes or cosubstrates, as well as ease-of-preparation of the biocatalyst when compared to pure protein. This was achieved to good effect with the flavin-dependent hydroxylase HBP1.<sup>306,307</sup> In these examples, a number of substituted phenols were successfully converted to catechols in gram amounts by using growing *E. coli* expressing the hydroxylase of interest.<sup>306,307</sup> The use of growing microbial host not only means that cofactors can be recycled by the inherent primary metabolism of the organism, but also that the biocatalyst is constantly being synthesized, which is useful in the case of particularly unstable enzymes.<sup>305–307</sup> The substrate phenols and subsequent catechols were found to be toxic to *E. coli* in fairly low concentrations, however, and therefore batch-feeding of phenol at or below the rate of bio-oxidation was used to limit accumulation of substrate. In combination with *in situ* product removal (ISPR) to adsorb catechol product onto a solid resin, the accumulation of substrate and product was limited to subtoxic levels, allowing constant bio-oxidation using the same whole cells.<sup>306,307</sup> The combination of controlling both substrate and product concentration is often termed substrate-feeding product-removal (SFPR). A similar rationale has been applied to the BVMOs, with a number of examples allowing between gram and kilogram production of enantiopure lactones from ketones.<sup>308–314</sup> In these examples, substrate and product are allowed to adsorb and desorb freely from a resin, therefore limiting their concentration in the reaction broth. Additionally, specially designed reactors are used to control the extent of oxygenation and therefore limit the effects of uncoupling, while allowing sufficient oxygenation for bio-oxidation.<sup>308,314</sup>

Biphasic reaction media can also allow the concentration of substrates and products to be controlled and therefore limit toxicity or inhibition by high concentrations of either. A water-immiscible organic cosolvent is used to create a substrate and



**Figure 30.** (A) Fluorinated natural products identified to date. (B) Fluorinated glycosides produced by mutants of glycosyl transferase enzymes. (C) Proposed mechanism of  $F^-$  binding to fluorinase and arrangement for  $S_N2$  displacement. Kinetic parameters shown refer to the fluorination of SAM to FDA by the fluorinase from *Streptomyces cattelya*.<sup>349</sup>

product reservoir, away from the biocatalyst. This has been successfully achieved with both the styrene monooxygenases and the BVMOs,<sup>315,316</sup> in one case allowing production of up to 388 g of styrene oxide.<sup>317</sup>

In addition to the methods of engineering monooxygenase-mediated biotransformations to overcome substrate/product inhibition, toxicity, oxygenation, and uncoupling discussed above, significant work has focused on engineering at the protein level and is responsible for some of the highly productive biocatalytic methods known. For example, the BMVO used by Codexis for sulfoxidation in the production of esomeprazole (96, Figure 27D) is a cyclohexanone monooxygenase (CHMO) variant containing 41 mutations.<sup>318</sup> Modulating activity of the BVMO in terms of substrate scope or enantioselectivity has been achieved using both rational (structure-guided) mutagenesis and directed evolution approaches. A widely used approach for this class is the “Complete Active Site Saturation Test” (CASTing) whereby amino acid positions close to the binding pocket are selected for saturated mutagenesis, and has allowed the evolution of BVMO variants with modified substrate scope and improved enantioselectivity using homology models as a guide for targeting saturated mutagnesis.<sup>319–323</sup> To facilitate directed evolution by allowing rapid screening of larger variant libraries, a number of high-throughput screens for BVMO and hydroxylase activity have also been developed. The majority of the most recent methods require further reaction of the monooxygenase product, either enzymatically or nonenzymatically, to either induce a pH change<sup>324</sup> or afford a compound with distinct UV-vis characteristics, which can then be easily detected.<sup>295,325–331</sup> A number of structure-guided approaches have also been used to modulate regioselectivity and substrate scope,<sup>332,333</sup> or improve oxidative and thermostability.<sup>324</sup>

## 5. FLUORINASES

### 5.1. Discovery of Fluorinases in Nature

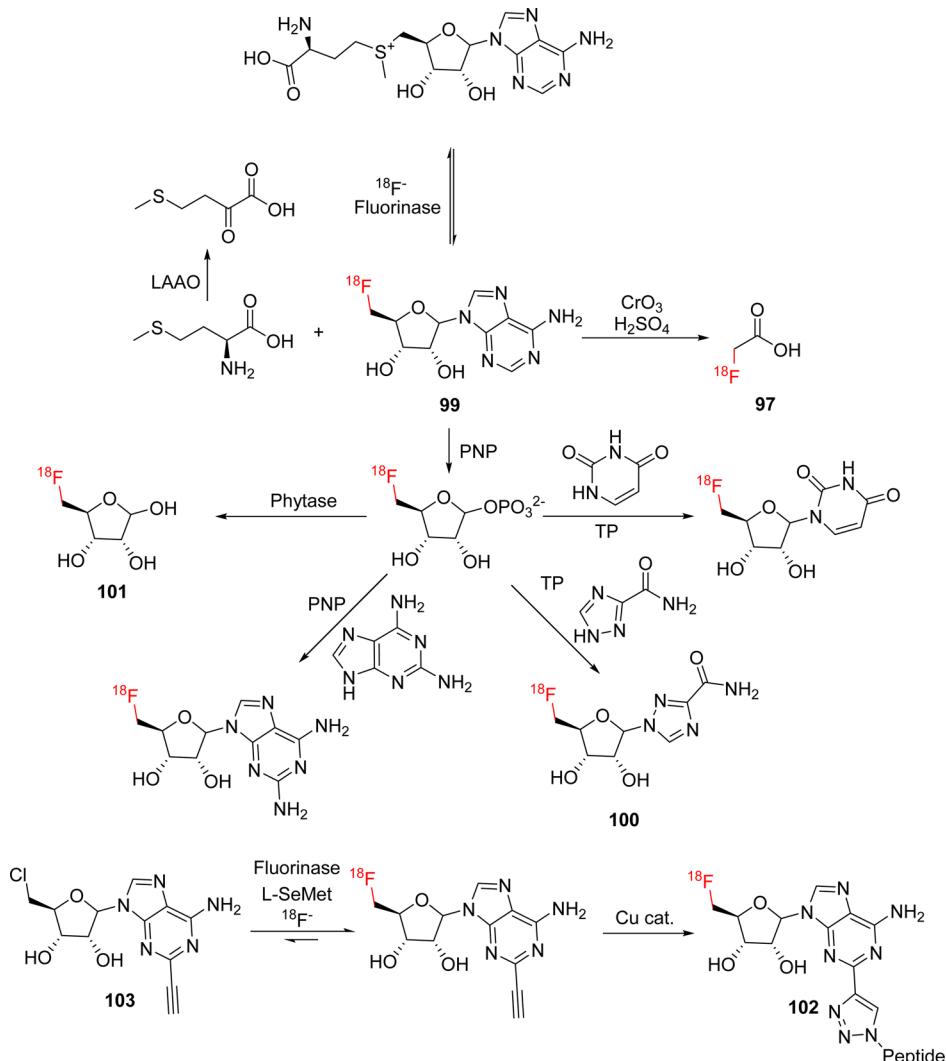
The enzymes responsible for biosynthesis of fluorinated natural products have remained relatively elusive, due in part to their rarity as compared to naturally occurring organochlorine and organobromine compounds.<sup>335–338</sup> Indeed, only five fluorinated natural products have been definitively identified to date (Figure 30A), with fluoroacetate (97) being the most

ubiquitous.<sup>339</sup> A recent review has thoroughly discussed the enzymes involved in the biosynthesis of these compounds;<sup>336</sup> however, they will be discussed herein briefly to complete the survey of biocatalytic halogenations, which might be applied to industrial or medical processes.

Enzymatic fluorination activity was first observed with mutants of the glycosyl transferase enzymes  $\beta$ -glucosidase and  $\beta$ -mannosidase from *Agrobacterium* sp. and *Cellulosmonas fimi*, respectively.<sup>340–342</sup> It was found that when catalytically essential nucleophilic residues were mutated, glycosyl transferase activity was abolished. Upon addition of high concentrations of fluoride, however, such activity was restored. NMR subsequently showed the production of fluorinated glycosides (Figure 30B) as intermediates, which would allow attack by nucleophilic moieties of incoming sugar residues due to the polarized nature of the C–F bond.<sup>340–342</sup>

Nucleocidin (98), an antibiotic produced by *Streptomyces calvus*, was one of the first fluorinated metabolites to be identified, although elucidation of its biosynthetic pathway was hampered by poor fluorometabolite production under laboratory fermentation conditions.<sup>343</sup> *Streptomyces cattelya*, which secretes fluoroacetate and 4-fluorothreonine,<sup>344</sup> however proved to be sufficiently practicable to allow identification of the enzyme responsible for fluorination.<sup>345</sup> This particular fluorinase has been shown to catalyze fluorination of S-adenosyl methionine (SAM), to generate 5'-deoxy-5'-fluoroadenosine (FDA) in an  $S_N2$ -type reaction (Figure 30C), and has informed much of the current mechanistic and functional understanding of these fluorinases.<sup>335,346–351</sup> The mechanism of enzymatic fluorination is particularly interesting because the high electronegativity of fluoride means that oxidation to an electrophilic species, as is commonplace with the other classes of halogenase (vide infra), would not be possible.<sup>336</sup> Moreover, tight solvation of  $F^-$  in water decreases its nucleophilicity and creates a great energetic penalty for nucleophilic substitution with an aqueous fluoride source.<sup>336,352</sup>

Crystallography of the *S. cattelya* fluorinase revealed that fluoride ion is bound in the active site through electrostatic interactions, positioned in a  $S_N2$ -like trajectory to the ribose 5'-C,<sup>348</sup> consistent with the observation that introduction of fluoride occurs with inversion of stereochemistry using isotopically labeled substrates.<sup>346,347</sup> The energetic penalty for



**Figure 31.** Preparation of <sup>18</sup>F labeled nucleotide analogues, fluoroacetate, and peptides using fluorinases in biobio and biochemo cascade reactions. LAAO = L-amino acid oxidase. PNP = purine nucleotide phosphorylase. PyNP = pyrimidine nucleotide phosphorylase.

desolvation of fluoride is thought to be compensated for by the retention of four hydrogen-bonded waters in the active site, in addition to the increased binding affinity of SAM.<sup>336,348–350,352</sup>

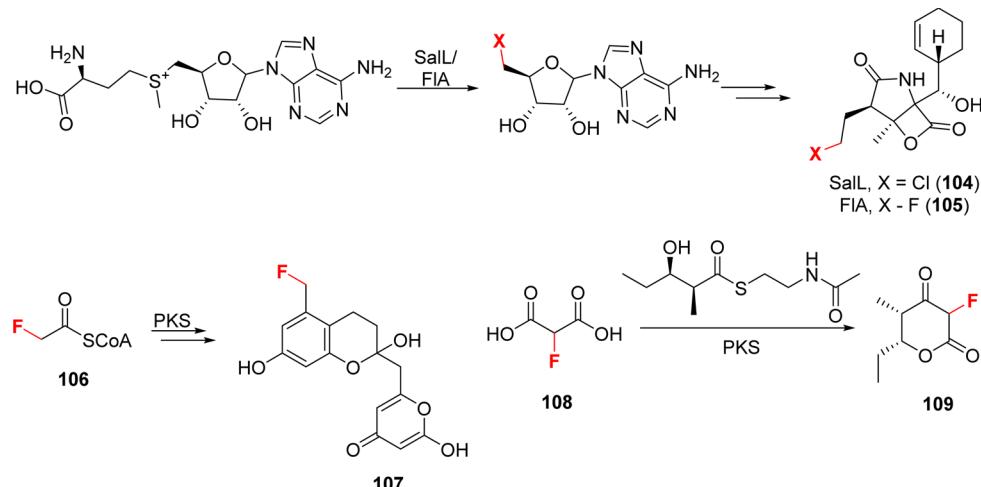
Since the seminal work on this fluorinase, a number of other enzymes have been identified with very high homology to the fluorinase from *S. cattelya*.<sup>353–355</sup> *Streptomyces* sp. MA37 was found to produce fluoroacetate and 4-fluorothreonine in culture, and subsequently a putative fluorinase gene with 87% sequence identity to the fluorinase from *S. cattelya* was identified.<sup>353,354</sup> Expression of this enzyme in *E. coli* confirmed fluorinase activity in vitro. Potential fluorinases have also been identified in *Nocardia brasiliensis* and *Actinoplanes* sp. N902-109, and similarly demonstrated to have fluorinase activity in vitro.<sup>353,354</sup> The marine organism *S. xinghaiensis* has been found to produce fluoroacetate, although in vitro activity of the fluorinase identified in this organism is yet to be demonstrated.<sup>355</sup>

A chlorinase, related mechanistically to the fluorinases, has also been identified in *Salinispora tropica*, which is responsible for chlorination during the biosynthesis of salinosporamide A (Figure 32).<sup>356–358</sup> Interestingly, this chlorinase cannot utilize fluoride as nucleophile in place of chloride, while the fluorinase from *S. cattelya* is capable of utilizing both halides.<sup>359</sup> This

selectivity is thought to be due to differences in active site organization around halide binding between the fluorinase and chlorinase.<sup>360,361</sup> Similarly, a putative fluorinase from *Pyrococcus horikoshii* was found to utilize hydroxide (from water) as nucleophile rather than either chloride or fluoride in vitro, affording adenosine and L-methionine.<sup>362,363</sup> Again, crystallography revealed significant differences in the way in which the nucleophile is bound to the active site.<sup>363</sup>

## 5.2. Application of Fluorinases as Biocatalysts

**5.2.1. <sup>18</sup>F Labeling.** The radioisotope <sup>18</sup>F is commonly used in positron-emission tomography (PET), a key medical imaging technique.<sup>336,344–366</sup> The <sup>18</sup>F required to produce the compounds used in this process is obtained from bombardment of H<sub>2</sub><sup>18</sup>O, which affords aqueous <sup>18</sup>F.<sup>336</sup> The potential to use fluorinases to prepare <sup>18</sup>F labeled compounds, which can use aqueous fluoride directly without the need for drying or coordination to specialist ligands to allow reactivity in organic solvents, could therefore yield expeditious routes to these compounds in higher radiochemical yield (RCY).<sup>336</sup> A great deal of the work on applying fluorinases for biocatalysis has therefore focused on this area (Figure 31).



**Figure 32.** Preparation of fluorinated natural product analogues through either substrate-directed biosynthesis of fluorinated building blocks or heterologous expression of fluorinases.

Early work found that using  $^{18}\text{F}^-$  equivalents could afford  $^{18}\text{F}$ -FDA (**99**) using L-AAO (L-amino acid oxidase) to oxidize L-methionine and therefore suppress the reverse reaction.<sup>367–369</sup> Various other radionucleotides have been generated using the above fluorinase-mediated  $^{18}\text{F}$  introduction, followed by “base-swapping” with PNP (purine nucleotide phosphorylase) or PyNP (pyrimidine nucleotide phosphorylase) enzymes to allow introduction or other purine or pyrimidine bases.<sup>369,370</sup> Interestingly, this method has also been applied to the one-pot synthesis of a fluorinated analogue of the antiviral agent ribavirin **100** from SAM.<sup>369</sup> A polymer-supported fluorinase has also been used for these radiofluorinations which, by allowing more facile removal of biocatalyst, may allow more efficient syntheses of these compounds with higher RCY.<sup>371,372</sup> The  $^{18}\text{F}$ -FDA nucleoside produced using fluorinase can also act as the substrate for a Kuhn–Roth oxidation, allowing the chemoenzymatic synthesis of  $^{18}\text{F}$  fluoroacetate (**97**), a common PET-imaging agent in neurology and oncology.<sup>365,366,373</sup>

Combination of the above cascade with a phytase enzyme allows the nucleobase portion of  $^{18}\text{F}$ -FDA to be replaced with a hydroxyl group, affording 5'- $^{18}\text{F}$  ribose (**101**).<sup>370,374</sup> **101** is of particular interest because of the potential to exploit this primed substrate in bioconjugation due to its propensity for ring-opening.<sup>375,376</sup> Bioconjugation has also been achieved by using nucleotide mimics containing chemical handles for conjugation onto a peptide target.<sup>377,378</sup> It was noted by crystallography that H2 of the adenine ring of SAM was positioned toward the surface of the enzyme, suggesting that analogues with substitution here may be accepted by the fluorinase.<sup>378</sup> Introduction of an acetylene group allows facile attachment of a tagged peptide to 5'-chloro adenosine using an azide–alkyne cycloaddition “click” reaction. Because of the orientation of this substitution, the reverse chlorinase reaction of fluorinase could then be used to afford fluorination of the adenosine portion, affording  $^{18}\text{F}$ -labeled peptide **102**, a good PET imaging agent in rats.<sup>378</sup> A simplified protocol, starting from “untagged” nucleoside **103**, was subsequently reported, which allowed the preparation of the same agent in 90% RCY.<sup>377</sup>

**5.2.2. Production of Fluorinated Natural Products.** Natural products are a common source of medicinal compounds. Given that fluorination can often have a profound effect upon the bioactivity of a compound,<sup>13,14</sup> methods for the

facile preparation of fluorinated natural product analogues through fermentation are therefore desirable.<sup>336</sup> In an attempt to achieve this goal, fluorinase enzymes have been incorporated into non-natural biosynthetic pathways (Figure 32).

A seminal example employing this rationale involved replacing the gene encoding salL with the fluorinase encoding gene flA in the salinosporamide (**104**) producer *Salinospira tropica*. The resulting mutant produced fluorosalinosporamide (**105**) directly by fermentation.<sup>379,380</sup> Fluorinated polyketide natural product analogues have also been obtained from substrate-directed biosynthesis. Through incubation of the reconstituted minimum components of the artinochodin PKS machinery with fluoroacetyl-CoA (**106**), a fluorinated octaketide could be produced, which is then subsequently cyclized to **107**.<sup>381</sup> R<sub>2</sub>CHF groups have also been introduced into polyketide natural products by feeding of fluoro-malonate.<sup>382</sup> It was demonstrated that a malonyl-CoA synthetase could also accept fluoromalonate **109** which, when combined with part of the assembly module of the erythromycin PKS, allowed generation of the fluorinated polyketide analogue **110**.<sup>382</sup> The ability to produce fluorinated substrates for PKS machinery, from fluoroacetate or similar starting materials generated by integration of fluorinases *in vivo*,<sup>383</sup> has the potential to allow the facile synthesis of fluorinated natural product analogues through fermentation.

## 6. FUTURE PERSPECTIVES

The enzymes discussed herein show great versatility for regio- and stereocontrolled halogenations of unactivated C–H bonds. Such transformations, affording highly bioactive compounds and ubiquitous synthetic intermediates, could have a great impact on the efficiency of synthesis in all sectors of the chemical industry. Given the importance of selectivity in these transformations, those classes of halogenases that utilize freely diffusing hypohalous acid intermediates (*vide infra*) are unlikely to afford transformations of great value, with the exception of the limited number of examples that appear to show some selectivity.<sup>76</sup> The  $\alpha$ -KG and flavin-dependent halogenases, which show exquisite control of their active halogenation species and hence afford regio- and stereoselective transformations, are the most promising. Together with fluorinases, the only class capable of introducing fluorine to organic

compounds, these classes of halogenase will likely attract the most attention for industrial biocatalytic applications.

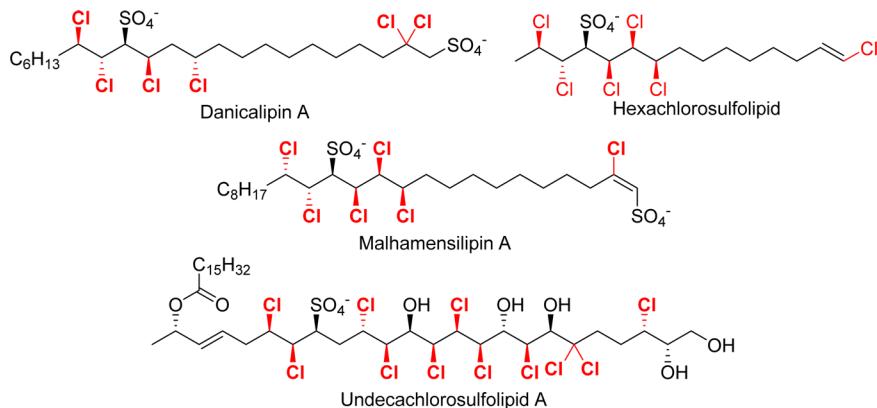
Despite this potential, however, there is still some way to go before these enzymes can be reliably used on an industrial scale. The fluorinases have been applied on a laboratory scale for the <sup>18</sup>F-labeling of small molecules and for the production of fluorinated natural product analogues,<sup>367–369,373,374,377–380</sup> although much research is still focused on the discovery of additional fluorinases, and little attempt has been made to determine the scalability of these transformations. Immobilization of the fluorinases, which would allow more facile removal of biocatalyst and therefore potentially higher RCY, in addition to higher purity products for medical applications and more productive enzyme preparations, is a promising direction.<sup>371</sup> Unlike the other classes of halogenase discussed herein, this class ultimately requires a methionine leaving group to be installed, which fluoride can then displace. Although this can be transiently installed by using the reverse chlorinase reaction of the fluorinases,<sup>377</sup> the need for prior functionalization could limit the efficiency of synthesis using these enzymes. The unique ability of this class to produce organofluorine compounds, unlike the other classes which require oxidation of halide to an activated species prior to chlorination, means that the transformations involving the class will still be of great value.

The Fe(II)/ $\alpha$ -KG-dependent halogenases are capable of halogenating aliphatic C–H bonds without the need for a leaving group. This class was initially believed to be of little value for biocatalysis due their selectivity for carrier-protein tethered amino acid substrates, although the recent discovery of a  $\alpha$ -Fe(II)/ $\alpha$ -KG-dependent halogenase capable of catalyzing the regio- and stereoselective halogenation of freely diffusing substrates offers fresh promise for this class of halogenase.<sup>108–111</sup> The substrate scope of this class is yet to be explored extensively, as is their amenability to engineering for modulation of substrate scope and selectivity, but the limited number of examples thus far is promising and is likely to broaden should further enzymes of this class be discovered. The demonstration that an Fe(II)/ $\alpha$ -KG dependent hydroxylase can be engineered to provide a halogenase biocatalyst is also promising because this could allow the engineering of other  $\alpha$ -KG/Fe(II)-dependent hydroxylases, which accept a broad range of freely diffusing substrates, into additional biocatalysts for the halogenation of aliphatic C–H bonds.<sup>111,113</sup>

The flavin-dependent halogenases are the most promising biocatalysts for the halogenation of aromatic C–H bonds and as such have received much attention. The tryptophan halogenases are the most extensively studied class to date, with a number of enzymes known which can halogenate complementary positions of a range of substrates.<sup>145,150,155,218,220,221,225</sup> Given the number of phenolic flavin-dependent halogenases demonstrated to be involved in the biosynthesis of natural products, a great deal of which can accept freely diffusing substrates (*vide infra*), the application of these enzymes will likely significantly broaden the substrate scope of these biotransformations. The Fl-Hals are still of limited scalability, however, due to low productivity and stability. The efforts described above on engineering other flavin-dependent monooxygenases demonstrate the strides that can be made in process development, and in using rational protein engineering or directed evolution, toward developing flavin-dependent hydroxylases, epoxidases, and BVMOs for practical biocatalysis and illustrates the need to apply such

rationale to the improvement of the less well-developed flavin-dependent halogenases. As the monooxygenase screening methods reported so far are dependent upon detection of a specific reaction product or sequence of reactions occurring, they are not appropriate for application to the flavin-dependent halogenases, although methods that could be applied to their directed evolution are slowly emerging and have been demonstrated feasible.<sup>223,240,241</sup> In combination with what is known about the modification of their substrate scope, regioselectivity, and stability through mutagenesis,<sup>150,155,222–225</sup> in addition to the discovery of naturally occurring thermostable Fl-Hals,<sup>145</sup> a number of reliable starting points for further engineering efforts are known. Other monooxygenases may also provide useful information about loci for targeting further engineering. Enzymes with lower rates of flavin decoupling, for example, could be used to focus engineering of Fl-Hals with improved stabilization of hydroperoxy-flavin, thereby reducing uncoupling and enzyme deactivation and potentially improving reaction efficiency and biocatalyst stability.<sup>275</sup> Mutation of such cofactor binding sites has been demonstrated to have a positive effect upon catalytic parameters with Fl-Hals and other enzymes.<sup>242,384</sup> Knowledge of the mechanisms of oxygen activation by the monooxygenases could also be used to promote formation and stabilization of this key intermediate.<sup>385</sup> It is possible however that a lack of productivity with the Fl-Hals could be due largely to the fact that the rate-determining step (electrophilic aromatic substitution) is very energetically demanding and therefore engineering could be focused upon stabilization of the Wheland intermediate or increasing electrophilicity of the halogenating species. The outcomes from the rational methods to improve the oxidative and thermostability of BVMO could also be useful to Fl-Hal engineering. As both classes suffer from deactivation from ROS, the mutation of active site and surface sulfur-containing residues to less oxidatively labile residues, found to improve the oxidative stability of BVMOs,<sup>334</sup> could develop halogenase biocatalysts more appropriate for industrial applications.

The methods used to improve monooxygenase practicability and productivity at the reaction and process engineering level may be more directly applicable to the Fl-Hals in the short term. For example, there has been little published work on the effect of oxygenation upon Fl-Hal-mediated biotransformations beyond mechanistic studies,<sup>128,132</sup> and therefore optimization in this regard may be a valuable avenue to pursue in their future development. It appears that the use of additional enzymes to destroy ROS is not commonplace in protocols for using Fl-Hals,<sup>227</sup> and therefore it may be useful to determine the extent of uncoupling in these reactions as well as its impact on enzyme productivity. Fairly straightforward and inexpensive methods of SFPR such as biphasic reactions and adsorbent resins do not seem to have been explored with the Fl-Hals either, and this may also provide some improvement of their scalability. Immobilization of the Fl-Hals does seem to have had an impact on their practicability,<sup>150,244,267</sup> and further work on immobilization methods may be promising, especially because heterogeneous materials may allow a flow-style biocatalytic halogenation, and hence SFPR. Such a system could allow the concentration of substrate, product, and ROS to be limited while allowing significant material to be halogenated with the same biocatalyst. Recovery of the biocatalyst in this manner may also allow more efficient halogenase-transition metal cascades. Additionally, work on adapting the Fl-Hals to accept



**Figure 33.** Chlorosulfolipid natural products identified to date identified from algae and toxic mussels.

larger, bioactive, substrates<sup>223,225</sup> may mean that ultimately they find application as a means of late-stage C–H activation.

Although this Review has focused largely upon halogenase enzymes and classes that are well understood and therefore closer to application in synthesis, halogenases that could be of significant utility may yet be discovered because there are halogenated natural products known with biosynthetic pathways that are yet to be fully elucidated. Study of the halogenation reactions involved in the biosynthesis of these compounds may lead to the discovery of synthetically relevant halogenation biocatalysts. Chlorosulfolipids, for example, were first isolated from algae in the 1970s,<sup>386–388</sup> but to date the enzymes responsible for installation of the halogen atoms have not been identified.<sup>389</sup> The halogenases from chlorosulfolipid biosynthesis may be of particular interest as biocatalysts because natural products from this class contain numerous chlorine atoms with defined stereochemistry,<sup>390–393</sup> and the halogenases involved appear to be capable of utilizing chloride and bromide interchangeably.<sup>394</sup> With these natural products thought to be derived from fatty acids,<sup>395,396</sup> the enzymes involved are likely capable of the stereo- and regioselective halogenation of aliphatic CH bonds, similar to the Fe(II)/ $\alpha$ -KG halogenases. Additionally, the range of halogenation patterns observed in this class of natural products (Figure 33) suggests exquisite control by a set of halogenase enzymes. Identification of the enzymes involved may therefore reveal biocatalysts of great synthetic utility. Study of these halogenases has likely been hampered by the difficulties associated with mining marine genomes, although the recent identification of a Fl-Hal from the metagenome of a marine sponge adds further promise to the successful identification and characterization of enzymes from such environments.<sup>397</sup>

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Eileen Brandenburger studied Chemistry at the Justus-Liebig-University in Germany where she obtained her Master's degree in 2013. In November 2013, she started her Ph.D. in Chemical Biology at the University of Manchester within the Manchester Institute of Biotechnology under the supervision of Prof. Jason Micklefield. Her research is focused on the development of novel halogenase enzymes for application in synthetic pathways.

Sarah Shepherd graduated from the University of Warwick in 2010 with a M.Chem. in Chemistry with Medicinal Chemistry. She then joined the Micklefield lab and received her Ph.D. in Chemical Biology in 2014. Her research interests include biocatalysis and biosynthesis.

Binuraj Menon has two Master's degrees in Pharmaceutical Chemistry (2003) and Biocatalysis (2006). He obtained his Ph.D. in 2009 on Molecular Enzymology with Prof. Nigel S. Scrutton at the University of Manchester. Before joining the Micklefield group in 2013, he was a postdoctoral researcher in structural biology with Dr. David Leys at the same university. Binuraj's research interests are in exploring novel proteins, and their structural, functional, and biophysical characterization for direct application in the pharmaceutical industry.

Jason Micklefield graduated from the University of Cambridge in 1993 with a Ph.D. in Chemistry, with Prof. Sir Alan R. Battersby FRS. He then moved to the University of Washington as a NATO postdoctoral fellow working with Prof. Heinz G. Floss. In 1995 he became a lecturer in organic chemistry at the University of London, Birkbeck College, before moving to the University of Manchester in 1998 where he is Professor of Chemical Biology within the School of Chemistry and the Manchester Institute of Biotechnology. His research interests include biosynthesis, biocatalysis, and riboswitches.

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