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Recent Advances in Synthetic Application and Engineering of Halogenases

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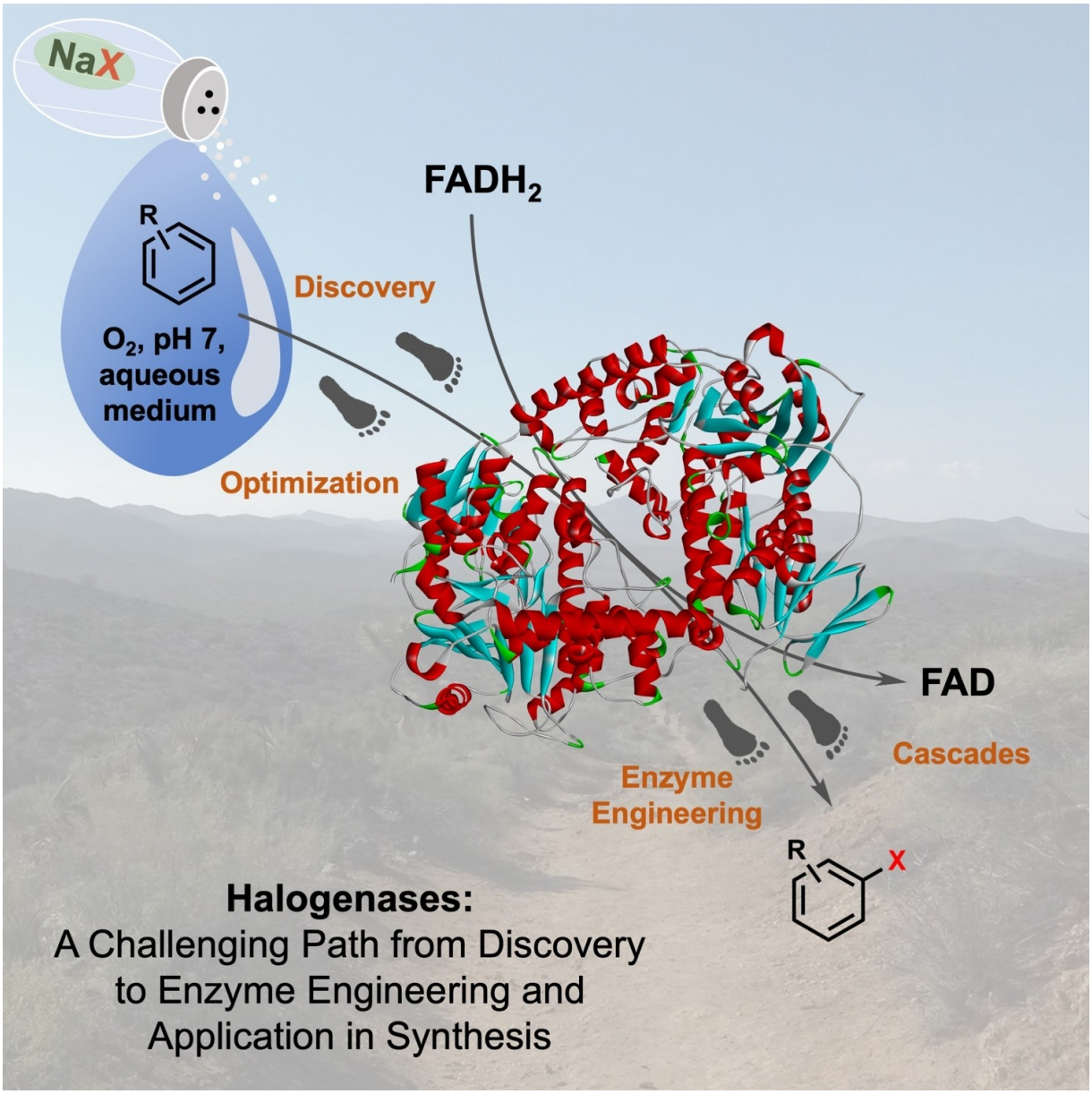

Halogenating enzymes are able to introduce halogen substituents under ambient conditions using non-hazardous reagents with intriguing selectivity, which is highly desired in green chemistry. Although C–H functionalization such as halogenation is a well-known transformation in synthetic chemistry, the selective incorporation of halogens using conventional chemical approaches often remains challenging. Therefore, enzyme-based strategies have been emerging as valuable alternatives in recent years. Inspired by manifold developments of enzymatic halogenation, this review focuses on advances of halogenating enzymes and their application with particular emphasis on FAD-dependent halogenases (FDHs). Catalytic strategies, application scope and engineering of FDHs are outlined pointing to the increasing utility of halogenases as promising biocatalysts. Current limitations as well as potential future developments of their synthetic utility are being discussed.

1. Introduction

1.1. Occurrence and Value of Halogenated Compounds

Halogenation attracts increasing interest from academia and industry as halogen substituents significantly impact the function of organic compounds and open up a vast repertoire of subsequent functionalization strategies. Up to now over 5000 natural products have been identified containing a halogen substituent that often is decisive for biological activity (Figure 1).[1]

For instance, the halogen motif represents a valuable strategy for improvement of bioactivity and bioavailability in pharmaceuticals. In view of their optimal efficacy organohalogens constitute over 34% of the registered drugs and continuously attract widespread attention for improving binding affinity or optimization of the ADME/T parameters (absorption, distribution, metabolism, excretion, and toxicity).[2] Today, around two thirds of all known agrochemicals contain halogen-substituted aryl and hetaryl moieties which underlines the widespread utility and increasing demand for these versatile compounds.[3,4]

Apart from their pharmacological value, the synthetic interest in halogenated compounds lies in their ability to serve as building blocks and starting material for a broad array of synthetic transformations. In particular, a large range of valuable products is made accessible from aryl halides by making use of metal-catalyzed cross-coupling reactions.[5]

Generally, C–H functionalization using halogenation is, therefore, an essential transformation to assemble complex organic scaffolds. However, activation of non-activated carbons often requires noble metal catalysts, toxic reagents and frequently proceeds under harsh conditions. Less activated positions are difficult to address while considerable amounts of by-products and waste being produced must not be neglected.[6] The desired organohalogens can be accessed via a wide range of strategies, including radical-based mechanisms, electrophilic addition or electrophilic aromatic substitution (S_EAr). S_EAr is a widely-applied reaction type for arene functionalization that is of major importance for the synthesis of bulk and fine chemicals, yet control of regioselectivity and introduction of the halogen atom at electronically and mechanistically disfavored positions remain challenging.[7–9] In recent years, identification, application and engineering of halogenases has attracted growing interest with an increasing significance for site-selective C–H functionalization.[10,11] The broad utility of halogenated compounds in synthesis has promoted extensive efforts in development and optimization of alternative routes towards halogenation providing a safe and selective approach for the synthesis of haloarenes under benign reaction

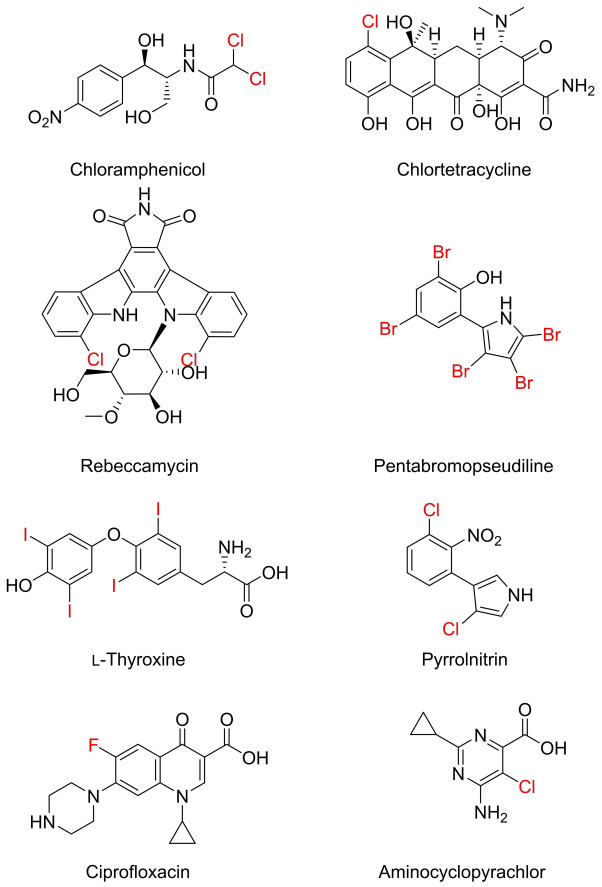

Figure 1. Selection of halogenated secondary metabolites as representatives for chlorinated, brominated, or iodinated natural products as well as the fluorinated synthetic antibiotic (ciprofloxacin) and herbicide (aminocyclopyrachlor).

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conditions. In living systems, a diverse array of halogenases catalyzes the incorporation of halogens. Biohalogenation occurs on a large variety of organic scaffolds, for instance aliphatic carbons, olefins as well as aromatic and heterocyclic rings.[9]

2. Halogenases: A Diverse Enzyme Class

2.1. Subclasses of Halogenating Enzymes

Halogenases form a heterogenous enzyme class and can be divided into O_2-dependent halogenases, haloperoxidases as well as fluorinases (Table 1).[16]

2.2. Fluorinating Enzymes

Most frequently, chlorine and bromine are found in natural products, whilst iodinated or fluorinated natural compounds are more rare.[19] Only a few fluorinated metabolites have been identified so far as chemical properties and natural abundance of fluorine restrict the biosynthesis and biochemical significance of organofluorine compounds. Even though fluoride is highly abundant in the earth's mantle and crust, its occurrence in the ocean is very low compared to chloride and bromide ($F^- =$ 1.3 ppm; $Cl^- = 18800$ ppm; $Br^- = 67$ ppm).[19–21] In contrast to the oxidative mechanism of the C–Cl, C–I and C–Br bond formation, enzymatic C–F bond formation is performed by nucleophilic substitution. Apart from fluorine's high electronegativity that prevents an oxidative mechanism, the difficulty of desolvating the fluoride ion in aqueous media, which is an essential feature to act as a naked nucleophile, as well as the toxicity of accumulating fluorinated metabolites in the producer organism are considered as reasons for the limited amount of known fluorinated metabolites.[9] Only a few native fluorinating enzymes have been characterized in detail so far, such as the fluorinase FIA from *Streptomyces cattleya*, catalyzing nucleophilic attack of fluoride to *S*-adenosyl-L-methionine (SAM), thus generating 5′-fluorodeoxyadenosine (5′-FDA) and L-methionine as a byproduct (Scheme 1a).[22,23] The biotechnological application of fluorinases is limited by the organism's sensitivity towards fluoride ions and thus hinders large-scale fermentation of fluorinated metabolites. Nevertheless, engineering approaches of FIA into a related host organism demonstrated the successful production of fluorinated pharmaceutically relevant molecules. A striking feature is that fluorinases are capable of selectively introducing the isotope ^{18}F into metabolites which serves as a probe for radiolabeling in positron emission tomography (PET). Simply by choosing ^{18}F as a fluoride source the desired isotope can be introduced into the target molecule albeit requiring high stoichiometric quantities of fluorinase compared to ^{18}F.[23]

2.3. Haloperoxidases: Heme-Dependent and Vanadium-Dependent Enzymes

Haloperoxidases are subdivided into heme-dependent and vanadium-dependent enzymes that follow an oxidative reaction pathway and rely on H_2O_2 as oxidizing agent.

The generated iron(IV)-oxo species (Scheme 1b) or vanadate intermediate (Scheme 1c) reacts with chloride, bromide or iodide forming diffusible hypohalous acid (HOX) that is released into the medium and serves as halogenating agent in the exterior.[9] As a consequence, HOCl reacts with electron-rich compounds in a purely chemical, nonspecific manner, thus addressing multiple halogenation sites and lacking selectivity.[13,24,25]

2.4. Mechanism and Engineering of Non-Heme-Iron Dependent Halogenases

O_2-dependent halogenases require oxygen as a reducible co-substrate and are subdivided into non-heme-iron-dependent halogenases and flavin-dependent halogenases.[9] Non-heme iron-dependent halogenases follow a radical-based mechanism coupled to the decarboxylation of α-ketoglutarate (α-KG). This induces formation of an Fe(IV)-oxo intermediate that acts as a hydrogen-abstracting species and enables the halogenation of non-activated, aliphatic C–H sites (Scheme 1d).[9,16,27–29]

These enzymes predominantly act on substrates tethered to acyl carrier proteins, such as SyrB2 from *Pseudomonas syringae pv. syringae*B301D that catalyzes the chlorination of threonine in syringomycin E biosynthesis.[30] Interestingly, characterization of WelO5 revealed that this non-heme-iron dependent halogenase also accepts free small-molecule substrates, as the latter catalyzes the chlorination of aliphatic carbons in fischerindoles.

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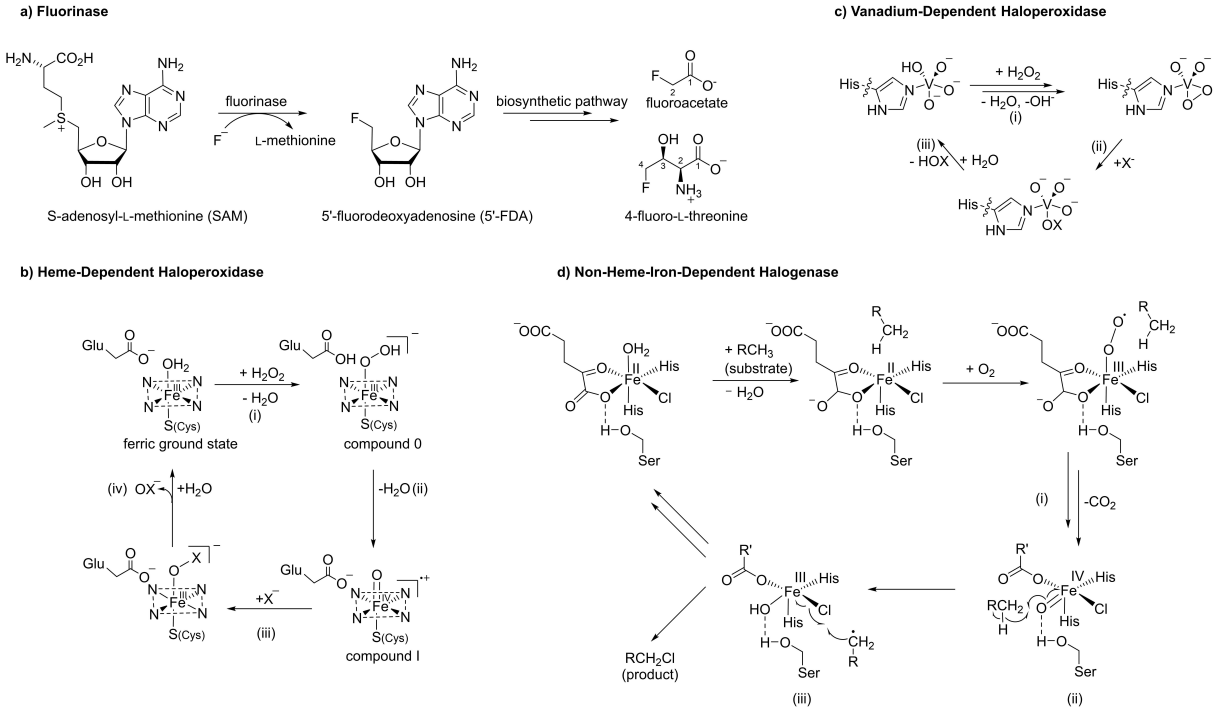
Norbert Sewald studied chemistry at the Technical University of Munich. Having obtained his PhD degree in organic chemistry, he worked in the group of Prof. J. E. Baldwin from 1991 to 1992 at the Dyson Perrins Laboratory, Oxford University. In 1998 he finished his habilitation at the University of Leipzig. He was appointed Professor of Organic and Bioorganic Chemistry at Bielefeld University in 1999.

Table 1. List of different halogenase subclasses and their associated characteristics.

Enzyme class	Subclass (reaction type)	Co-substrate	Halogenating species/specificity	Substrate requirements	Example
Haloperoxidase	Heme-dependent (oxidative)	H_2O_2/halide	Diffusible HOX; nonspecific halogenation	Aromatic and electron rich	Halogenase from *Caldariomyces fumago*[12]
	Vanadium-dependent (oxidative)	H_2O_2/halide	Diffusible HOX; nonspecific halogenation	Aromatic and electron rich	Fungal chloroperoxidase (V–CPO) from *Curvularia inaequalis*[13]; Vanadium haloperoxidase genes from *Streptomyces* sp. CNQ-525[14]
O_2-dependent halogenase	Non-heme, Iron-dependent (radical)	O_2 /halide/α-ketoglutarate	Halogen radical species; regio-and stereospecific halogenation on carrier-bound and free substrates	Non-activated carbon centres, aliphatic moieties	SyrB2 from *Pseudomonas syringae pv. syringae* B301D[15]; WelO5 from *H. welwitschii* UTEX B1830[16]
	Flavin-dependent (oxidative)	O_2/Cl^- or Br^-/$FADH_2$	HOCl trapped in active site; regiospecific halogenation on free and carrier bound substrates	Aromatic and electron rich	PrnA from *Pseudomonas fluorescens*[17]
Fluorinases	(nucleophilic)	SAM/F^-	Nucleophilic halide	Electrophilic, good leaving group	Fluorinase FlA from *Streptomyces cattleya*[18]



Scheme 1. a) Enzymatic fluorination from the biosynthetic pathway of fluoroacetate and 4-fluorothreonine in *Streptomyces cattleya*. The fluorinase catalyzes nucleophilic attack of F^- on SAM to produce 5′-FDA, thereby exploiting L-Met as an excellent leaving group for an S_N2-type reaction.[23] b) Catalytic mechanism of heme-dependent haloperoxidases: The ferric ground state is constituted of a water bound iron(III)-heme complex. (i) The substrate H_2O_2 enters the active site and displaces the distal water thereby forming the hydroperoxo intermediate Fe(III)–OOH (compound 0). (ii) The reactive iron(IV) oxo species (compound I) is generated by a heterolytic cleavage of the O–O bond initiated by preceding protonation of compound 0. (iii) Compound I oxidizes a halide anion (X^-) and releases hypohalite (OX^-). (iv) The ferric ground state is regenerated by addition of water.[26] c) Catalytic cycle of vanadium-dependent haloperoxidases: (i) The substrate H_2O_2 coordinates to the vanadium center leading to loss of H_2O and formation of a peroxo-vanadium intermediate. (ii,iii) The halide anion (X^-) is being oxidized, resulting in the formation of hypohalous acid (HOX). The oxidation state of vanadium is maintained throughout the catalytic cycle.[26] d) Catalytic cycle of non-heme-iron-dependent halogenases based on the crystal structure of WelO5: (i) O_2-dependent decarboxylation of α-KG results in formation of an iron(IV)oxo species that was presumed to relocate into the ligand plane. (ii) Radical abstraction of a hydrogen atom from the substrate. (iii) Recombination of the substrate radical with the halogen radical gives rise to the chlorinated product.[27]

Structural analysis of WelO5 provided further details on the reaction mechanism suggesting the migration of the Fe-oxo species upon decarboxylation of α-KG.[27] Even though WelO5 has a limited substrate scope, its close homologue AmbO5, to which it shares 79% sequence identity, was shown to chlorinate several structurally distinct ambiguine, fischerindole, and hapalindole alkaloids (Figure 2a–c). AmbO5's significantly broader substrate scope is related to its C-terminal region that contains

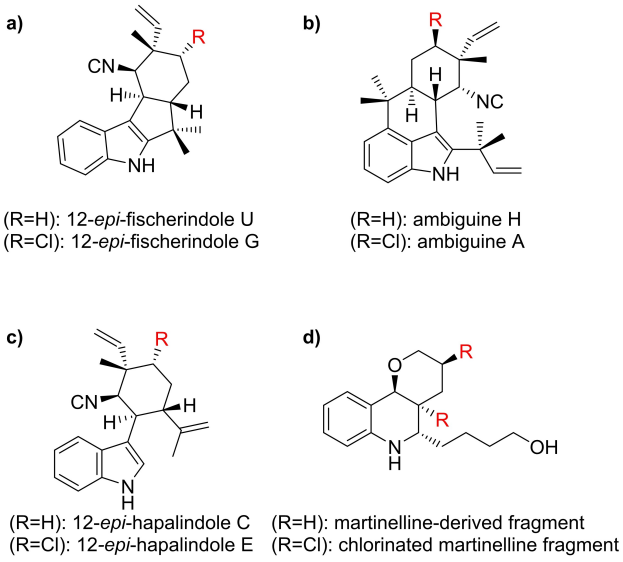


Figure 2. Selection of small-molecule substrates (a–c) of non-heme-iron dependent halogenases WelO5 and AmbO5. In addition, two engineered variants of WelO5* regioselectively chlorinate a martinelline-derived fragment (d) at distinct positions.

18 of the 32 differing amino acid residues compared to WelO5. Generation and characterization of WelO5/AmbO5 chimera further underlined the relevance of a signature C-terminal sequence motif that confers substrate promiscuity and specificity.[16,27] Recent identification of the third WelO5-type protein, WelO5*, sharing 95% sequence identity to WelO5, revealed an enhanced specificity for substrates that were merely poorly processed by WelO5. As previously reported for AmbO5, WelO5*'s C-terminal sequence motif harboring 11 varied amino acids is proposed to be the origin for the observed substrate specificity and enhanced activity for selected substrates compared to WelO5.[31] Hayashi *et al.* aimed to expand the substrate scope of WelO5* towards a pharmaceutically relevant martinelline-derived fragment that was neither accepted by WelO5 nor AmbO5 (Figure 2d). Based on low promiscuous activity of WelO5* a structure-guided evolution approach was pursued. After two rounds of mutagenesis two regio-complementary WelO5* single mutants were identified addressing different positions of the aliphatic cycle. The evolved variants were endowed with increased chlorination activity for the target substrate whereas the undesired hydroxylation activity observed for the WT enzyme was significantly reduced or even diminished. Thereby their effort provides the first example of enzyme engineering of a non-heme iron-dependent halogenase amenable for halogenation of non-activated carbons.[32] Very recently Duewel *et al.* made use of structure-guided evolution to evolve Wi-WelO15 where smart library design yielded an improved mutant capable of addressing non-activated C–H bonds for a panel of non-native hapalindoles. In light of these recent engineering efforts, it is apparent that Fe/α-KG dependent halogenases gain increasing importance due to their ability to address non-activated carbons. It will be of particular interest how the scope of these enzymes will be further broadened in future.[33]

3. Flavin-Dependent Halogenases

Among the halogenating enzymes, FAD-dependent halogenases (FDHs) currently provide suitable candidates for synthetic use. They are particularly suitable for halogenation of electron rich arenes owing to their impressive selectivity in conjunction with benign reaction conditions. These biocatalysts merely require halide salts, molecular oxygen and reduced flavin-adenine dinucleotide ($FADH_2$) to catalyze electrophilic aromatic substitution. Owing to their facile handling at room temperature, neutral pH and high functional group tolerance without requiring activating or protecting groups, enzymatic halogenation emerged as a versatile methodology for synthetic purposes.[34]

During the past decade extensive research was in particular carried out by the groups of van Pée and Walsh to gain deeper insight into FDHs. Despite recent efforts to improve their *in vitro* compatibility, FDHs still suffer from severe drawbacks such as low activity, insufficient stability and their restriction to relatively electron rich aromatic compounds. However, advances in the field of halogenase engineering and subsequent integration into chemical transformations corroborated their versatility as environmentally friendly biocatalysts.

3.1. Pioneering Identification and Characterization of Flavin-Dependent Tryptophan Halogenases (Trp-FDHs)

In 2000, van Pée and coworkers isolated and characterized PrnA, the first member of the tryptophan (Trp) halogenase family, from *Pseudomonas fluorescens*.[17] Subsequently, further Trp-FDHs, such as Trp 7-halogenase RebH from *Lechevalieria aerocolonigenes*,[35] the Trp 5-halogenase PyrH from *Streptomyces rugosporus*,[36] and the Trp 6-halogenase Thal from *Streptomyces albogriseolus*[37] were identified.[38,39] Moreover, SttH from *Streptomyces toxytricini NRRL* 15443,[40] thermophilic Th-Hal from *Streptomyces violaceusniger*,[41] thermophilic BorH from uncultured bacteria[42,43] as well as KtzR from *Kutzneria* sp. 744[44] also act as Trp 6-halogenases. Noteworthy, KtzR and KtzQ are acting in a tandem manner and catalyze the regiospecific dichlorination of L-Trp yielding L-6,7-dichloro-Trp. Both enzymes act sequentially on free L-Trp with the first chlorination introduced by KtzQ followed by KtzR that exhibits a 120-fold preference for L-7-Cl-Trp. In 2019, Domergue *et al.* reported the identification of *Xszen*FHal, a novel Trp-5 halogenase from *X. szentirmanii*.[45] For its identification a collection of putative enzymes homologous to halogenases was generated based on the protein sequence of 22 known FDHs and a reduced number of 148 enzymes tested against a panel of different aromatic substrates. Here, only *Xszen*FHal, sharing 60% identity with Trp 5-halogenase PyrH, confirmed halogenation activity on substituted Trp, indole and indole derivatives. Though halogenation exclusively occurred at C5, a clear enantioselectivity was noted

for L-Trp. In accordance to other Trp halogenases *Xszen*FHal prefers chlorination over bromination, whereas no incorporation of iodide was observed.

With the exceptions of Bmp2 and Bmp5[46] which only accept bromide but not chloride, most FDHs described so far are able to use both halides. Owing to increased reactivity in cross-coupling reactions, aryl bromides have a higher relevance for chemoenzymatic synthesis compared to their chlorinated analogues. However, chlorination prevails over bromination. Enzymatic bromination is possible for promiscuous enzymes if a large excess of bromide is supplied. However, even in such cases chlorination is commonly observed as a side reaction because contamination with small amounts of chloride is inevitable in enzyme preparation. For a long time it was assumed that neither fluoride nor iodide are competent for halogenation, though Bmp5 is reported to catalyze iodination of its substrates *in vivo*.[35,36,46] However, the group of Goss recently reported the identification of a iodinating viral FDH exhibiting a natural preference for iodination *in vitro*.[47]

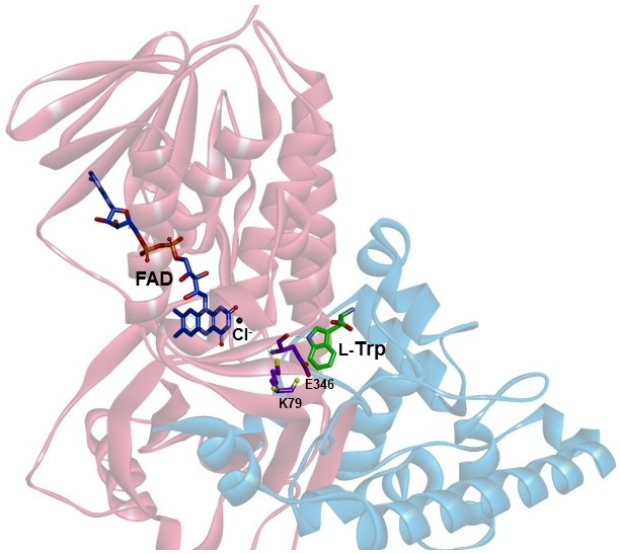


Figure 3. Crystal structure of Trp 7-halogenase PrnA monomer in complex with substrate FAD, L-Trp and chloride (PDB: 2AQJ). The conserved amino acid residues K79 and E346 are shown as purple stick models. The box-shaped flavin binding site is shown in red, the pyramidal subdomain is depicted as a blue ribbon model. The picture was generated and further modified using Pymol.

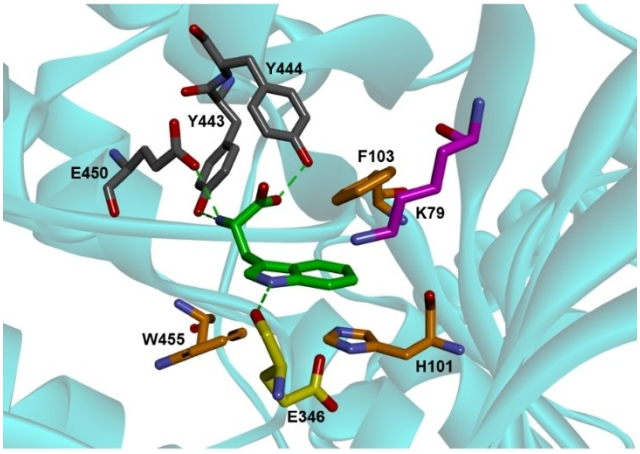


Figure 4. Active-site residues of PrnA (PDB: 2AQJ) containing bound L-Trp and highlighting crucial residues responsible for catalytic activity and forming interactions with the substrate. The picture was generated and further modified using Pymol.

3.2. Crystal Structure of FDHs

The X-ray single crystal structure of the Trp 7-halogenase PrnA involved in pyrrolnitrin biosynthesis was the first one to be published.[48] As has been observed also for other Trp halogenases, PrnA forms a dimer in the crystal consisting of two pyramid-shaped monomers (Figure 3; Figure 4).[41,49–53] Each monomer contains a variable pyramidal module and a conserved-box shaped subdomain harboring the FAD-binding site (GxGxxG motif, where "x" is any amino acid).[48,54] An additional conserved motif of FDHs (WxWxIP) is suggested to prevent the binding of organic substrates close to the isoalloxazine ring of $FADH_2$, suppressing potential monooxygenase activity.[51,55] Recently, the group of Goss identified another sequence motif characteristic for halogenases, Fx.Px.Sx.G, where each "." represents a varying number of amino acid residues between each conserved residue.[47,56] The overall FDH structure reveals that the cofactor is bound in a solvent-exposed groove whereas the substrate Trp is located in a distinct enzyme module at the interface of the FAD-binding site and the pyramidal subdomain.

X-ray single crystal structures of PrnA,[51] RebH,[49] PyrH[50] and ThaI[57] in presence of the substrate L-Trp are in accordance with a suggested mechanism for FDHs based on mechanistic[52,58] and theoretical evidence[59] (Figure 4).

4. Halogenation Mechanism

4.1. General Overview on Halogenation Mechanism

As proposed by Dong *et al.*, it is indisputable that $FADH_2$ reacts with molecular oxygen giving a peroxy-flavin intermediate as shown for flavin-dependent monooxygenases. In case of monooxygenases, this intermediate further reacts by nucleophilic attack of a nearby substrate resulting in oxygen transfer.[51,60] In case of FDHs there is no space for an organic molecule to bind adjacent to the isoalloxazine moiety. The crystal structure of PrnA revealed that the substrate Trp and cofactor FAD are located in different binding sites separated by a 10 Å tunnel.[51]

This strict separation is a conserved motif observed in all known Trp halogenases. Interestingly, the halide ion is positioned on the opposite face of FAD near to the tunnel entrance, allowing a nucleophilic attack on the flavin(C4a) hydroperoxide, leading to the formation of FAD-OH and hypochlorous acid (HOCl) (Figure 5). In contrast to haloperoxidases, diffusion of HOCl into the surrounding medium is prevented by the protein structure. Thus, it enters the tunnel

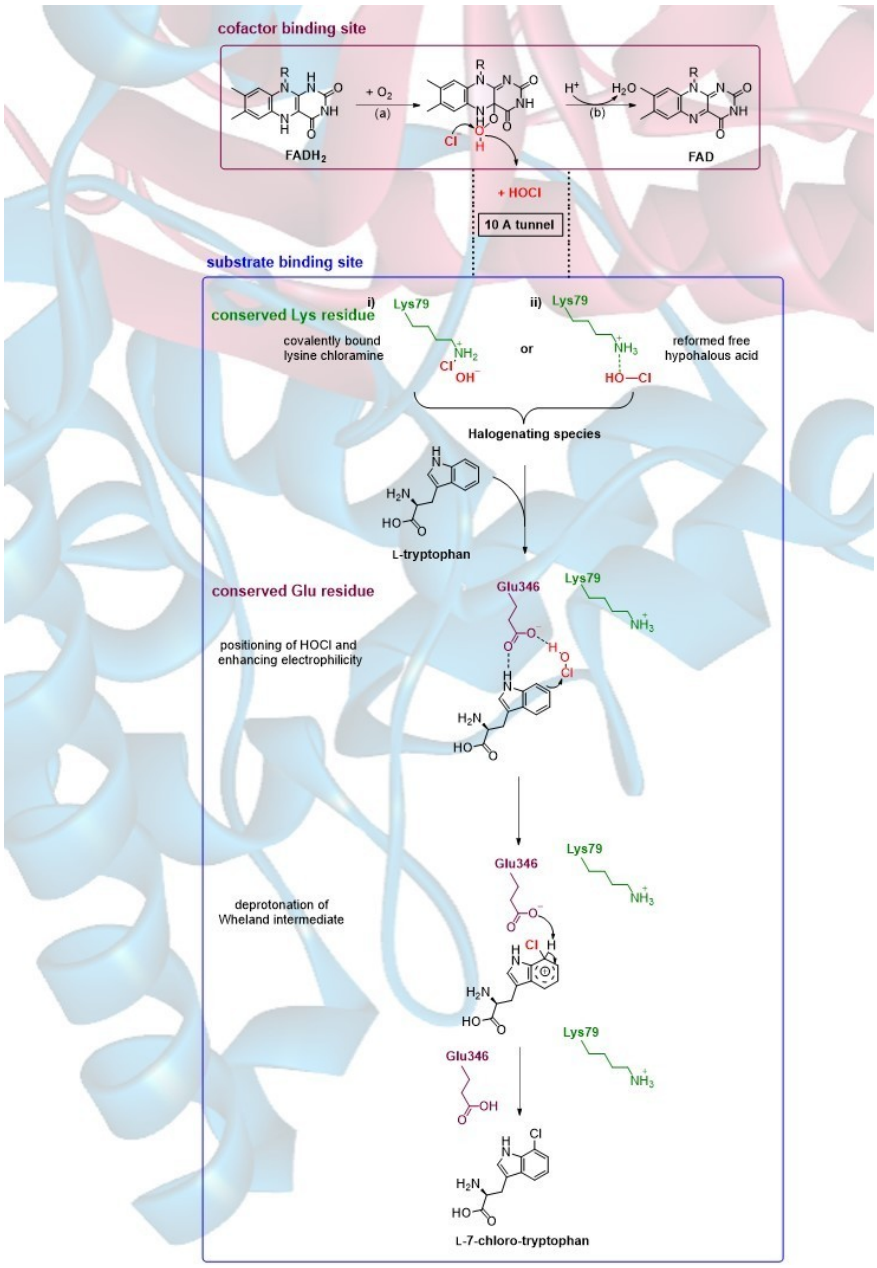


Figure 5. Proposed halogenation cycle of PrnA. (a) $FADH_2$ is oxidized in the cofactor binding site by O_2 to form the flavin hydroperoxide FAD(C4a)-OOH which reacts with a chloride ion giving HOCl (b). The hypochlorous acid is guided via a 10 Å tunnel to the substrate binding site. (i) Subsequently, HOCl interacts with the ε-amino group of K79 to form a chloroamine as proposed by Yeh *et al.*[52] This chloroamine is assumed to re-compose (ii), according to Flecks *et al.*,[58] to HOCl that performs the electrophilic aromatic substitution. After deprotonation of the Wheland intermediate L-7-Cl-Trp is released from the active site.

and is guided to the substrate binding site.[51] This tunnel connecting the surface exposed flavin binding site with the deeply buried substrate is lined by amino acids residues resistant to oxidation by HOCl. The halogenating agent passes the tunnel to the Trp-binding site. At the end of the tunnel, HOCl is supposed to interact with a lysine residue (PrnA: K79) highly conserved in all known FDHs. Mutation of this residue to alanine leads to a complete loss of activity as observed for RebH (K79),[52] PrnA (K79),[51] BrvH (K83),[61] and RadH (K74).[62] Its role will be discussed below. E346 is closely located to K79 in PrnA, and positioning of the substrate Trp is supported by a hydrogen bond between the NH group of the indole ring and the carbonyl group of peptide bond between E346 and S347. Furthermore, the Trp amino group forms a salt bridge with the carboxyl group of E450 and a hydrogen bond to the hydroxyl group of Y443. The carboxyl group of L-Trp is also stabilized by hydrogen bonds to the hydroxyl group of Y444. π-stacking interactions of the substrate indole moiety with H101, F103 and W455 add together to reach a rather rigid and fixed position of Trp decisive for high regioselectivity of the halogenation (Figure 4).

Recent approaches reported by Moritzer *et al.* to obtain crystal structures of Trp 6-halogenase ThaI either with bound cofactor FAD or with bound FAD and substrate L-Trp, indicated a decreased affinity of FAD upon substrate binding.[63] As previously observed for RebH, binding of L-Trp probably reduces the affinity of the oxidized cofactor that might facilitate its release and subsequent regeneration.[49,52] Although the X-ray singles crystal structure analyses of ThaI, RebH and PyrH hint towards negative coupling between FAD and substrate binding, PrnA provides no hints for negative coupling between binding of substrate and cofactor, which, therefore, cannot be assumed as general feature of FDHs yet.

4.2. Early Investigations on the Halogenating Species

The strict conservation of the lysine residue (PrnA: K79) in FDHs suggests that this residue adopts a crucial role in the catalytic cycle, but its particular function has been controversially discussed. Yeh *et al.* proposed that the rapid reaction of HOCl with the ε-amino group of K79 leads to the formation of a long-lived chloroamine as evidenced by radiolabeling using ^{36}Cl. This chloroamine could be only detected in absence of L-Trp, and attempts to characterize this intermediate by X-ray crystallography, NMR spectroscopy, and mass spectroscopy remained unsuccessful. Nevertheless, remarkable stability of the enzyme-bound chlorinating intermediate was detected in RebH, exhibiting $t_{1/2} = 2.3$ days. Therefore, it was concluded to be the actual halogenation species as it seems unlikely that HOCl would be sequestered within the active site for such a long time without diffusing away and participating on side reactions with other biomolecules.

It was emphasized that Lys79 is positioned between the binding pockets of FAD and Trp. In this arrangement HOCl reaches Lys79 before encountering the substrate. Consequently, reactive HOCl forms a covalently bound lysine chloroamine thereby storing its oxidative potential. Furthermore, Lys79 would be ideally oriented for delivering a chloronium ion equivalent for electrophilic aromatic substitution of the indole ring at C7. Yeh *et al.* point out that – albeit exhibiting reduced activity compared to HOCl – chloroamines are reported to be milder and more selective towards their target.[52,64] In contrast, Flecks *et al.* proposed that their electrophilicity is too weak to achieve electrophilic substitution of the indole moiety as quantum mechanical (QM) calculations of *N*-chloroamine revealed reduced charge on the Cl atom of HOCl compared to free HOCl. Consequently, chloroamines are weaker halogenating agents than HOCl and, therefore, not sufficient for chlorination in this context.[58,59] Instead of involving a covalently bound chloroamine reacting with the substrate, they proposed that free hypochlorous acid is being reformed upon substrate binding. Interactions of HOCl with proximal E346 are responsible for proper orientation of the halide species and might increase electrophilicity in order to allow for electrophilic aromatic substitution. Closer mechanistic studies revealed that one of the oxygens of the carboxyl group of E346 plays the role of proton acceptor, while the other oxygen is involved in a hydrogen bond interaction with the nearby NH group of Trp. Mutation of this conserved glutamate residue in PrnA (E346Q) causes significantly reduced activity with a decrease of k_{cat} by about two orders of magnitude.[51,59] As the presented assumptions rely on differing data no final conclusion concerning the actual halogenation species can be drawn.

5. Classification and Examples of Unusual FDHs

5.1. FDHs Acting on Free or Carrier Bound Substrates

In general, FDHs can be subdivided into two distinct subfamilies represented by PrnA and PrnC, respectively. The PrnA group, comprising the most prominent members such as Trp halogenases RebH,[65] PrnA,[66] and ThaI[37] catalyze the halogenation of Trp or indole derivatives, whereas the other group only accepts more reactive phenols and pyrrole derivatives (Table 2).[50,67] The majority of FDHs accepts non-carrier bound substrates and probably these enzymes are involved in the biosynthetic halogenation of secondary metabolites. In contrast, the FAD-dependent halogenase CndH from chondrochloren biosynthesis acts on substrates bound to a carrier protein.[68]

Table 2. Representative overview and classification of FDHs into tryptophan, pyrrole, or phenol halogenases. Classification is based on major features and an overlap to another class is not excluded.

Flavin-dependent Halogenases					
Flavin-dependent Trp Halogenases		Flavin-dependent Pyrrole Halogenases		Flavin-dependent Phenol Halogenases	
Trp 7-halogenase	PrnA,[17] RebH,[35] KtzQ[44]	untethered substrates	PrnC[66]	Class A: untethered substrates	Rdc2,[70] GedL,[72] RadH,[62] ChlA,[74] GsFl,[75] Asm12,[76] AclH,[77] Nat1,[78] CazI,[79] NapH2,[14] AcOTAhal[80]
Trp 6-halogenase	KtzR,[44] ThaI,[37] SttH,[40] ThHal,[41] BorH,[42,43] AORl_5336[82]	carrier-tethered substrates	PltA,[69] HalbB,[71] Clz5,[73] Bmp2[46]	Class B: carrier protein tethered substrates	CndH,[68] Clo-Hal,[83] End30,[84,85] BhaA,[86,87] Ram20,[88] ComH,[89] SgcC3,[90] Tcp12[91]
Trp 5-halogenase	PyrH,[36] XszenFHal[45]			Class C: decarboxylative halogenation	Bmp5[46]
Novel Halogenases from Genome Mining					
BrvH,[61] Iodinase VirX1,[47] Xcc_B100_4156, Xcc_B100_1333, Xcc_B100_4345[81]					

Structure-based sequence alignment of CndH with PrnA and RebH revealed a comparatively low level of conservation in the C-terminal region, allowing speculations for the altered substrate preference. As similarly observed for other Trp halogenases, the active site in PrnA is covered by a lid formed from the 120C-terminal residues that closes the substrate off.[68] Even though differences of this structural motif exist among Trp halogenases, a conformational change occurs upon Trp binding leading to the entire accommodation of bound Trp in the enzyme cavity. This contrasts the active site structure of CndH which lacks a structured C-terminal chain.[49,57] Here, the active site remains open and solvent exposed, presenting a large non-polar patch that probably acts as docking site of the putative substrate carrier.[68]

5.2. FDHs Exhibiting Altered Ion Selectivity and Substrate Scope

As disclosed by Neubauer *et al.*, the FAD-dependent halogenase BrvH, identified from a marine metagenome of *Brevundimonas* sp. BAL3, does not accept Trp as a substrate. Comparison of its crystal structure to other FDHs such as RebH revealed that the BrevH substrate binding site is notably more open and lacks side chains to position the amino and carboxylate groups of Trp. Although the native substrate could not yet be elucidated, halogenation of free indole in C3 position was proven. BrvH exhibits a remarkably high preference for bromination over chlorination, but though this unusual halide specificity could not yet be explained.[61]

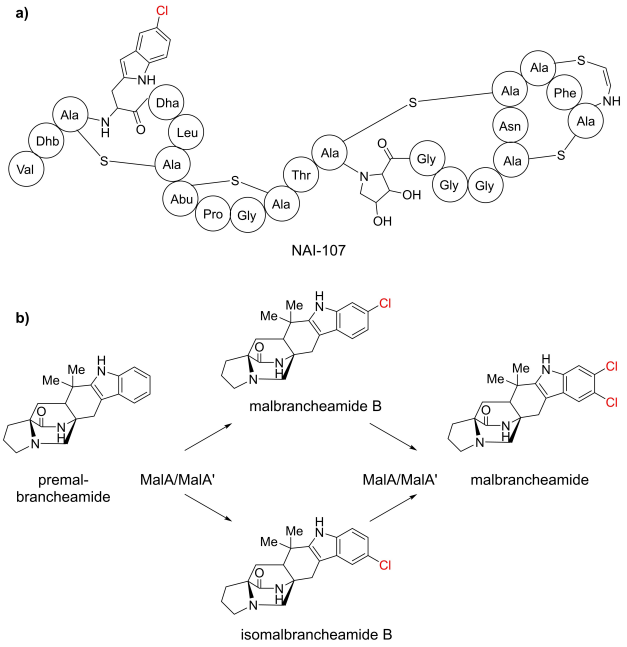
Likewise, an exclusive preference for bromination over chlorination has also been reported for three FDHs from *Xanthomonas campestris*. While exhibiting high similarity to Trp halogenases with regard to the flavin-binding site and active site residues, differing amino acids surrounding the halide binding site were proposed to influence halide preference. Even though these *Xcc* halogenases were originally annotated as Trp halogenases, they do not accept Trp and exclusively brominate substrates such as indole, 7-azaindole, 5-hydroxytryptophan and various heterocyclic derivatives. Docking and molecular dynamics simulations indicated that Trp – in contrast to 5-hydroxytryptohan – is not stabilized by hydrogen bonding or other interactions, thereby impeding the correct positioning of Trp required for substrate binding.[81]

5.3. Identification of an Iodinating FDH

Goss and coworkers recently reported on the identification and characterization of a remarkable marine viral FDH catalyzing the iodination of diverse substrates *in vitro*.[47] Relying on the novel motif Fx.Px.Sx.G derived from sequence alignment that was previously overlooked, enabled the identification of VirX1 from the cyanophage Syn10 previously known for infection of *Prochlorococcus* and *Synechococcus*.[92] The wild type enzyme exhibits a broad substrate flexibility, accepting a diverse array of sterically and electronically different substrates ranging from low to high conversions. A considerable novelty is that VirX1 shows a high and surprising preference for iodination over bromination and chlorination, thereby being the first characterized FDH with a clear natural preference for iodination *in vitro*. Though iodination in most cases prevailed at the chemically most activated position, some substrates were halogenated at different positions than the correlates prepared by chemical electrophilic iodination, albeit without clarifying substitution patterns of few examples. With the presence of the box-shaped flavin binding module and the key catalytic residues K79 and E358 being in place, the structure of VirX1 shows many similarities to known FDHs such as PrnA and BrvH. Still, comparison to the homodimer structure observed for Trp halogenases, X-ray structural analysis of VirX1 revealed six monomers in the asymmetric unit predicted to form two stable trimeric assemblies. Furthermore, the wider opening of its substrate binding site and the absence of the α-helical lid to close off the active site, contrasts previous observations made for FDHs and might explain that VirX1 accepts a broad range of substrates and facilitates the accommodation of an iodide ion with a larger van der Waals radius compared to bromide or chloride ($F^- = 1.47$ Å; $Cl^- = 1.75$ Å; $Br^- = 1.83$ Å; $I^- = 1.98$ Å).[93] The higher oxidation potential of iodide compared to bromide or chloride favours formation of the hypohalous acid, but further investigations concerning halide selectivity are still required. Although the authors report higher turnover numbers of Virx1 for iodination of an indole derivative compared to efficiency of Trp 7-halogenase PrnA towards its natural substrate, for VirX1 higher K_M values were observed for the majority of substrates tested that indicates a lower affinity. In addition, it was previously shown that halogenase RebH is even a more efficient biocatalyst with non-natural substrates compared to VirX1.[94] This discrepancy is probably due to a lack of knowledge about the native substrate of VirX1. Thus, it will be interesting to resolve this question in future. Although the physiological function of VirX1 within the virus as well as the native substrate are still unknown, the new iodinase provides a versatile future tool to be used in halogenation chemistry.

5.4. FDHs Acting on Peptidic Substrates

FAD-dependent MibH unveiled a novel type of halogenase as it was shown to catalyze chlorination of Trp incorporated in a lanthipeptide precursor synthesized ribosomally. MibH is inactive towards free Trp. Interestingly, halogenation assays with a panel of different NAI-107 substrate analogues revealed MibH's unusually narrow substrate specificity, as it was inactive towards all tested substrates and did not tolerate even minor modifications of the peptide (Scheme 2a). Closer investigation on MibH's crystal structure showed structural relationships to FDHs, containing the conserved structural motifs WxWxIP, GxGxxG as well as catalytically important lysine (K102) and glutamate (E355) residues. However, MibH's larger accessible surface area and an expended more hydrophobic substrate grove are likely to accommodate larger peptidic substrates.[95]



Scheme 2. a) Structure of lantibiotic NAI-107 after post-translational modifications of the precursor protein MibA. FAD-dependent MibH is only active when Trp is embedded within the peptide substrate deschloro NAI-107. Dha = 2,3-dehydroalanine; Dhb = (*Z*)-2,3-dehydrobutyrine. b) Section of malbrancheamide biosynthetic pathway catalyzed by MalA/MalA'. Premalbrancheamide is proposed to be dichlorinated through an iterative mechanism.

In addition to a broad substrate scope reported for the FDH KrmI, this enzyme is also proposed to be involved in halogenation of peptides. Closer investigation of KrmI from the marine sponge *Theonella swinhoei WA* was recently reported by Smith *et al.* *In vitro* assays revealed that full length KrmI preferentially chlorinates and brominates 5-hydroxytryptophan over Trp, suggesting that this halogenase participates in generation of halogenated keramamides. Interestingly, the C-terminus of the protein resembles FDHs whereas the truncated *N*-terminal region shows distant homology to the ThiF enzyme family that is reported to be involved in specific binding of peptide substrates in ribosomal peptide synthesis. Therefore, the *N*-terminus may be responsible for binding of such substrates, which might open a new field for potential halogenase engineering.[96,97]

Recently a unique subclass of FDHs, catalyzing the iterative late-stage halogenation of complex substrates independent of carrier proteins was identified by Fraley *et al.* The fungal halogenases MalA/MalA' share 99% sequence identity and are postulated to perform dichlorination and monobromination of the free premalbrancheamide within the malbrancheamide biosynthesis (Scheme 2b). Even though MalA' has a similar overall structure to bacterial FAD-dependent halogenases it exhibits a unique Zn^{2+} binding C-terminus and an expansive active site enabling the accommodation of complex substrates.[98]

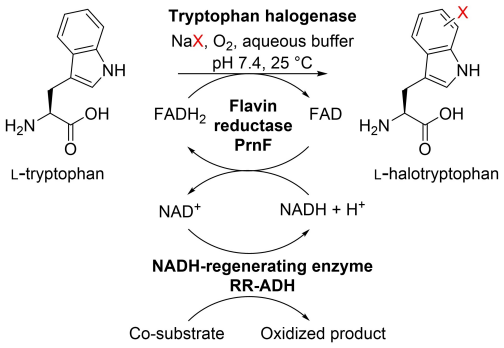
6. Strategies for the Regeneration of the Flavin Cofactor

First investigations on PrnA revealed the necessity of a flavin reductase activity responsible for *in situ* regeneration of $FADH_2$ to enable enzymatic halogenation. A flavin reductase, e.g. PrnF from *Pseudomonas fluorescens*,[17,99] RebF from *Lechevalieria aerocolonigenes*[35,94] or SsuE from *Thermus thermophilus*[17] catalyze reduction of FAD to $FADH_2$ by concomitant oxidation of NAD(P)H. Conversely, NADH regeneration can be achieved with either an alcohol dehydrogenase (ADH),[99] phosphite dehydrogenase (PtdH),[46,100,101] formate dehydrogenase (FDH),[102] or glucose dehydrogenase (Scheme 3).[94,99,103]

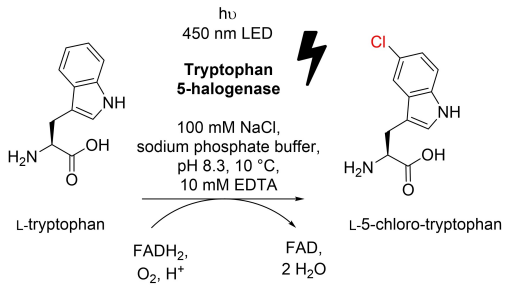
The Lewis group demonstrated that the setup of biocatalytic halogenation may be simplified using a bifunctional fusion protein consisting of FDH and flavin reductase. Here, RebH WT or engineered variants were genetically fused by different sized linkers, consisting of 10, 16 and 22 amino acid residues to flavin reductases RebF or thermostable Fre from *Bacillus subtilis*. Besides eliminating the need for addition of a separate flavin reductase, this set-up should increase the cofactor regeneration efficiency due to the close proximity of the flavin reductase and the halogenase.

Theoretically, the reduced cofactor should easily diffuse to the halogenase, while avoiding any undesired side reactions of $FADH_2$. Surprisingly, activity of the bifunctional system was lower compared to their two-component system *in vitro*. Still, higher product titers were observed for selected fusion proteins *in vivo*, which shows promise for large-scale halogenation in *E. coli*.[104]

Furthermore, photochemically induced regeneration of enzyme-bound $FADH_2$ in PyrH was reported recently.[105] Light-driven reduction of FAD employing ethylenediaminetetraacetic acid (EDTA) as sacrificial reductant enables the regioselective halogenation of Trp to 5-Cl-Trp reaching an average conversion of 58% (Scheme 4). It is arguable if light-driven regeneration of $FADH_2$ occurs in an enzyme-bound state which might contribute to lower futile cycles caused by the undesired coupling



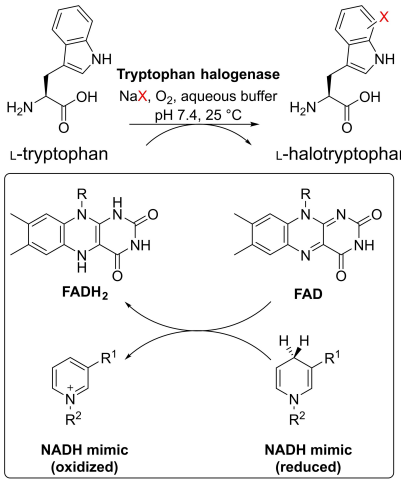
Scheme 3. Enzymatic cofactor regeneration system enabling halogenation of L-Trp leading to chlorination or bromination at the C5, C6 or C7-position of the indole moiety. The cofactor $FADH_2$ is regenerated by the flavin reductase PrnF and NADH is provided by an alcohol dehydrogenase (RR-ADH) or any other enzyme component capable of NADH formation (X = Cl, Br).



Scheme 4. PyrH-catalyzed chlorination of L-Trp employing photochemical regeneration of $FADH_2$. Enzyme bound FAD is directly regenerated by illumination for 2 min at 450 nm and usage of EDTA as a sacrificial electron donor.

reaction of $FADH_2$ and O_2 under formation of hydrogen peroxide.[106] Apart from lowering the productivity of the biocatalytic reaction, the latter product is also harmful to the protein components. As demonstrated by Ismail *et al.*, NADH mimics can be utilized to replace enzymatic cofactor regeneration in biocatalytic halogenation (Scheme 5).

Here, synthetic NADH mimics, easily obtained from inexpensive starting materials, were stoichiometrically employed for $FADH_2$ regeneration and facilitated reaction set up by circumventing the implementation of two auxiliary enzymes. Remarkably, by employing NADH mimics under similar reaction conditions and enzyme concentrations compared to enzymatic cofactor regeneration the initial reaction rates of RebH and PyrH were 1.3–1.9 times higher. However, reduced flavin also reacts with oxygen in solution, thereby producing hydrogen peroxide, compromising enzyme stability and consuming NADH mimic in a nonproductive manner. Therefore, an excess of NADH mimics providing superfluous $FADH_2$ has to be avoided. Even though NADH mimics proved their usability for scale up reactions due to their robustness and higher stability, this set-up is a promising future alternative for enzymatic cofactor regeneration, which needs to be adapted to the specific application.[107]



Scheme 5. Enzymatic halogenation of Trp employing NADH mimic 1-benzyl-1,4-dihydronicotinamide (BNAH) for $FADH_2$ regeneration. $R^1 = CONH_2$, R^2 = benzyl.

7. Preparative Scale Halogenation and Application of FDHs in Enzyme Cascades

7.1. Metabolic Synthesis of Halotryptophans

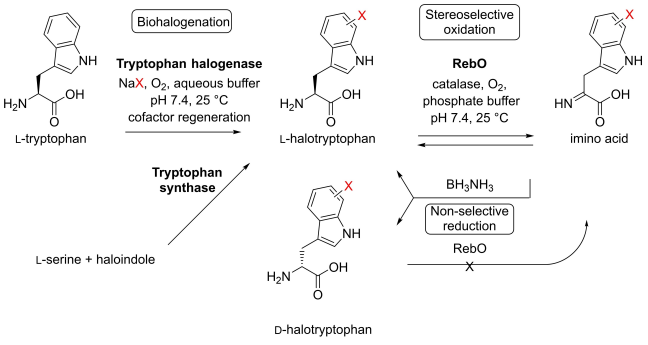
While the fermentative production of canonical amino acids such as L-glutamate and L-lysine is operated at a million-ton scale with *Corynebacterium glutamicum*,[108] the fermentative synthesis of halogenated amino acids has been reported the first time by the group of Wendisch.[109] Metabolic engineering of *C. glutamicum* resulted in a recombinant strain suitable for the production of L-7-Cl-Trp from sugars, ammonium and chloride salts. *C. glutamicum* is commonly employed for fermentative amino acid production.[110] Its inability to use Trp as nitrogen or carbon source or to metabolize the desired halogenated 7-Cl-Trp, as well as the lack of Trp degrading enzymes proved advantageous in comparison to *E. coli* that degrades Trp by tryptophanase as an undesired metabolic side pathway. After optimization of the ribosome-binding site (RBS) of *rebH*, the FDH RebH was coexpressed with flavin reductase RebF in a Trp overproducing *C. glutamicum* strain, finally enabling *in vivo* synthesis of 7-Cl-Trp at a titer of 0.1 g L^{-1}. Though production was limited by product inhibition and chlorination efficiency, strains are able to produce 7-Cl-Trp utilizing alternative carbon sources such as arabinose and glucosamine, that do not serve as feedstocks in human or animal nutrition, respectively.[109] Based on these results, Veldmann *et al.* subsequently addressed the fermentative production of 7-Br-Trp.[111] Utilizing the engineered *C. glutamicum* strain enabled upscaling to a reaction volume of 2 L in a batch and fed-batch mode resulting in significantly higher product titers of 7-Br-Trp compared to 7-Cl-Trp (1.2 g L^{-1} vs. 0.1 g L^{-1}). Though RebH is known to prefer chloride over bromide, the inhibitory effect of 7-Br-Trp on the specific growth rate was threefold lower than previously observed for 7-Cl-Trp. The smaller hydration shell of 7-Cl-Trp compared to 7-Br-Trp might facilitate its access to the active site, thus exhibiting more pronounced inhibitory effects which finally lowers the overall product titers.

7.2. Enzyme Immobilization and Enzyme Cascade Processes

The O'Connor group showcased that the applicability of FDHs can be extended towards diversification of natural products in medicinally relevant biosynthetic pathways. The integration of a bacterial halogenase gene into a plant host was based on preceding results indicating that *Catharanthus roseus* cultures can be supplemented with halogenated tryptamines which results in the production of haloalkaloid analogues.[112] In this context, reengineering of strictosidine synthase (STS) catalyzing

the initial step of monoterpene indole alkaloid biosynthesis resulted in generation of STS mutants with relaxed tryptamine specificity.[113] Thereupon, *in planta* production of chlorinated alkaloids by integration of Trp-FDH into the plant metabolism of *C. roseus* was reported: A suitable expression construct containing RebH and the required reductase was transformed into *C. roseus*, enabling halogenation of Trp in the plant cell. Subsequent decarboxylation of halo-Trp by Trp decarboxylase yielded halotryptamine which was then further processed in the metabolic pathway to generate several novel chlorinated alkaloids.[114]

The Sewald group successfully employed cross-linked enzyme aggregates (CLEAs) to improve the stability of Trp halogenases enabling regioselective bromination scalable up to the gram scale. Ammonium sulfate precipitation of RebH together with the auxiliary enzymes PrnF and ADH followed by glutaraldehyde cross-linking resulted in a solid biocatalyst performing selective halogenation with a prolonged catalyst lifetime. L-Trp is brominated up to substrate concentrations of 200 mg L^{-1} with full conversion and perfect regioselectivity.[39] The broad applicability of this methodology was subsequently demonstrated by the Micklefield group, who also applied this technique for other Trp halogenases such as the phenolic halogenase RadH or the Trp 6-halogenase SttH to increase biocatalyst efficiency in the halogenation reaction.[115] Noteworthy, immobilization offers a convenient and straightforward approach to obtain halogenated amino acids owing to its facile scalability and simple downstream processing albeit achieving low product titers compared to fermentative production of other amino acids. Yet significant efforts are required to make large-scale biotechnological halogenation processes feasible. In a cascade process FDHs along with the L-specific amino acid oxidase RebO from *L. aerocolonigenes* provided access to noncanonical halogenated D-Trp analogues by dynamic stereoinversion (Scheme 6). D-Halotryptophans were obtained from L-Trp by combining biocatalytic halogenation or Trp synthase with dynamic stereoinversion in a one-pot process: Selective oxidation of the L-enantiomer catalyzed by RebO gives rise to the α-imino acid. Combined with an *in situ* reduction using a hydride transfer agent leads to a racemic amino acid mixture. Due to the strict L-selectivity of RebO, only the L-enantiomer is re-oxidized, whereas the D-enantiomer gradually accumulates. In addition to D-configured 5- or 7-bromo-/chlorotryptophans this enzyme cascade enables the synthesis of D-7-fluoro- or 7-iodotryptophan from substituted indoles and serine using Trp synthase and stereoinversion, which are not accessible by Trp halogenases.[116]

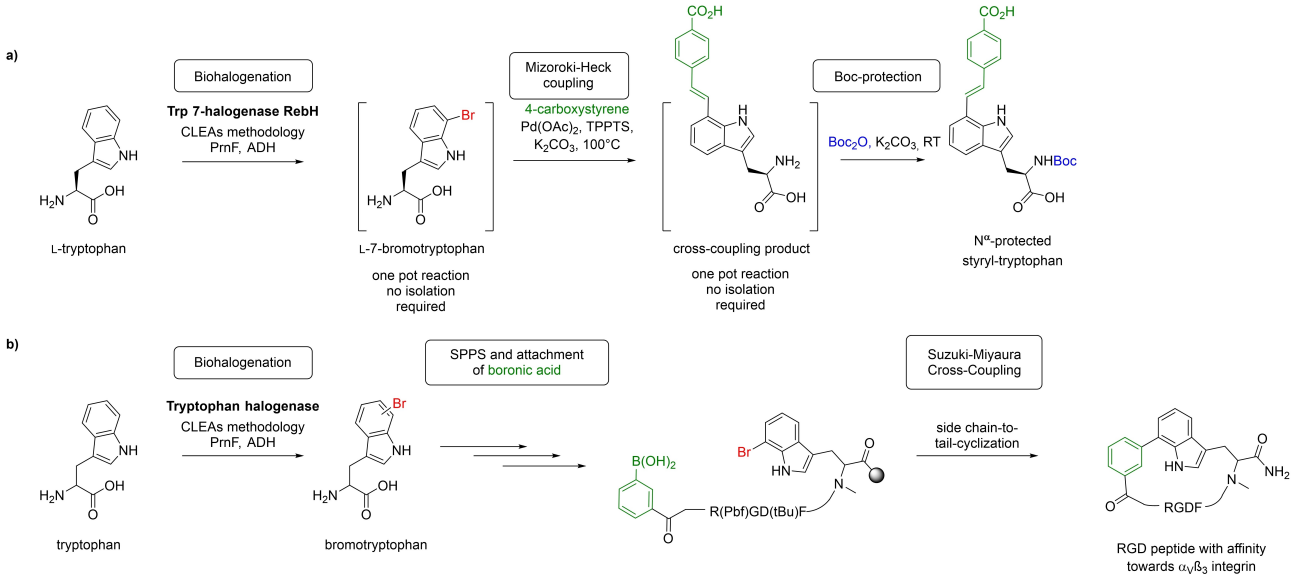


Scheme 6. L-Amino acid oxidase RebO can be employed to access enantiopure halogenated D-amino acids. Biocatalytic halogenation is followed by RebO catalyzed oxidation of the L-enantiomer to the α-imino acid. Non-selective chemical reduction leads to the racemic amino acid, but only the L-enantiomer is re-oxidized by RebO, leading to gradual accumulation of the D-enantiomer.

7.3. Cross-Coupling Reactions

Enzymatic halogenation introduces a handle allowing further aryl functionalization to be performed in a one-pot manner. Therefore, several groups sought to couple biotransformation such as enzymatic halogenation with Suzuki-Miyaura cross-coupling (SMC).[117–120] Pd-catalyzed cross-coupling reactions tolerate a variety of functional groups and represent a selective methodology to modify halogenated compounds. Especially the formation of carbon-carbon bonds is a useful approach to be addressed by an array of cross-coupling reactions.

Groundbreaking investigations combining Pd- and enzyme catalysis were performed by Sato *et al.*, demonstrating one-pot feasibility of a Pd/Cu-catalyzed Wacker oxidation and an enzymatic ketone reduction via compartmentalization of the reactions employing a polydimethylsiloxane (PDMS) thimble.[121] Building on these achievements, Latham *et al.* extended the approach to a combination of enzymatic halogenation and chemocatalysis into a sequential one-pot process. Utilizing membrane compartmentalization enabled the combination of FDHs with palladium-catalyzed cross-coupling in a one-pot manner.[115] Notably, both reactions need to be conducted in a sequential mode due to different temperature requirements. Sharma *et al.* reported an *in vivo* approach for the generation of a brominated natural product analogue and its subsequent cross-coupling for diversification of halogenated natural products in a one-pot fashion.[122] 7-Br-Trp was generated *in vivo* by engineered *E. coli* RG-1500 producing Trp 7-halogenase PrnA followed by in-culture Suzuki-Miyaura cross-coupling. Furthermore, they could also demonstrate the in-culture biosynthesis and cross-coupling of antibiotic Br-pacidamycin-D utilizing an engineered streptomyces *S. coelicolor* M1154 strain. As recently demonstrated by Gruß *et al.*, Mizoroki-Heck reaction afforded styryltryptophans from bromotryptophan (Scheme 7a). Biocatalytic halogenation of L-Trp yielded L-7-Br-Trp by making use of the CLEA methodology. Subsequent Mizoroki-Heck coupling employing an array of substituted styrenes and $Pd(OAc)_2$ in the presence of phosphine ligand trisodium 3,3′,3″-phosphinetriyl-tribenzenesulfonate (TPPTS) allows coupling of different alkenes to the indole moiety via the halogen substituent. The applicability of this cross-coupling reaction was further corroborated in a sequential, three step one-pot reaction comprising enzymatic halogenation, Mizoroki-Heck cross-coupling to water soluble 4-carboxystyrene, followed by final N^{α}-Boc protection in good overall yields up to 61%. Like the aryl substituted tryptophans,[123] styryltryptophans exhibit a bathochromically



Scheme 7. a) Derivatization of Br-Trp by Mizoroki-Heck coupling by combining bio- and chemocatalysis that is feasible in a one-pot mode. b) Stepwise synthesis of cyclic RGD peptides. Bromination of Trp is followed by solid phase peptide synthesis (SPPS) and final cyclization via SMC carried out either in solution or on-resin.

shifted absorbance and fluorescence emission compared to Trp, thereby revealing interesting potential to serve as fluorescence labels.[118,124] Haloarene containing amino acids (e.g. bromotryptophan) constitute a versatile handle for labeling, bioorthogonal reactions, site-selective and late-stage modification. The resulting biaryls obtained by SMC have been shown to possess significant potential for studies on biological activity and drug development.[125,126] Furthermore, Goss and coworkers reported about a method for aqueous Heck diversification also applicable to halotryptophans. Moreover, the transfer to a peptide based natural metabolite derivative was successfully demonstrated.[127] In this context Kemker *et al.* introduced peptide cyclization via Suzuki-Miyaura cross-coupling (Scheme 7b). This approach was exemplified for side chain-to-tail cyclization of RGD peptides by applying SMC.

The halogenated amino acid was synthesized via the CLEA methodology and introduced at the C-terminus. After synthesis of the linear precursor peptide by solid-phase synthesis, cyclization was carried out either on resin or in solution between an *N*-terminal boronic acid and the indole ring of Br-Trp. The resulting panel of cyclic RGD peptides, differing in size of macrocycle, connectivity at the indole moiety (directed by the halogen position and the boronic acid), as well as the influence of D-amino acids and *N*-methylation, exhibited a strong impact on the affinity and selectivity towards integrins.[128] Further discussion on cascades enabling indole functionalization can be found in a recently published review article.[129]

8. Exploiting the Synthetic Utility of Halogenases

8.1. Substrate Scope of WT Halogenases

Despite substantial efforts to improve *in vitro* compatibility of halogenases, their large-scale application still suffers from severe shortcomings such as enzyme stability, low activity and their limitation to act on relatively electron rich aromatic compounds. Initial studies on the substrate tolerance and regioselectivity of PrnA, carried out by Hölzer *et al.*, revealed that strict C7-selectivity exclusively occurs for Trp.[130] Though PrnA accepts a series of heterocyclic substrates, its regioselectivity appeared more relaxed for non-native substrates. Not surprisingly, electrophilic substitution in these cases occurred at the electronically favored indole 3- or 2- position, sometimes resulting in a mixture of mono and dichlorinated products. However, Patallo *et al.* recently demonstrated that PrnA regioselectively chlorinates indole-3-acetic acid at position 7, indicating the relevance of C3 side chain for selectivity and substrate fixation.[131] Apart from L-Trp, most of the Trp halogenases have also been shown to catalyze chlorination of D-Trp, albeit with preference for the native L-enantiomer.[94,132]

In contrast to the limited substrate scope of PrnA, closer investigations on the related Trp 7-halogenase RebH indicated a more relaxed substrate scope. RebH catalyzes halogenation of a range of 3-substituted indoles even at electronically disfavored sites and its synthetic utility was demonstrated in preparative halogenation reactions, revealing conversion of non-natural substrates like indole or gramine that are not accepted by PrnA.[94] Though both halogenases exhibit high sequence homology (55%) and nearly identical active sites,

RebH is more promiscuous towards other substrates rather than Trp and retains original C7-selectivity in most cases.

A likely explanation for this altered substrate acceptance was given by Payne *et al.* by aligning sites of RebH and PrnA within 5 Å of the substrate Trp. Surprisingly, only two of the 24 analyzed residues in RebH differ in identity (N467 and L456) or display a notable conformational change (N464 and N453) compared to PrnA. In RebH N467 forms a water-mediated hydrogen bond to the substrate Trp that cannot be formed by the corresponding residue L456 in PrnA. These minor differences in the active site of RebH might be decisive to position other substrates and to permit halogenation at electronically disfavored sites. Furthermore, RebH displays significantly improved kinetics compared to PrnA with k_{cat} values on Trp of 1.4 min^{-1} vs. 0.1 min^{-1}. Because of the low substrate conversion by PrnA potential halogenated products might have been missed.[94]

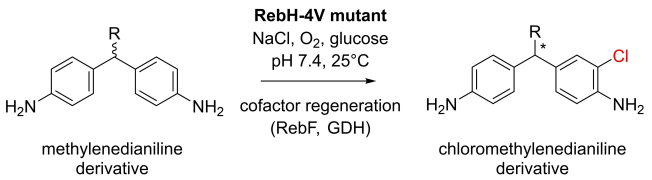
8.2. Influence of Directing Effects on Regioselectivity

Frese *et al.* further studied the influence of electron-withdrawing and -donating groups at the indole ring on regioselective halogenation employing RebH.[99] An array of C5- and C6-substituted L-Trp derivatives was obtained from L-serine and substituted indole derivatives using Trp synthase according to a procedure developed by Goss *et al.*[133] Even in presence of deactivating and *ortho/para*-directing groups, in many cases the substrate is still halogenated at the electronically less favored C7 position. Despite the presence of a deactivating 5-fluoro substituent with an *ortho-/para*-directing effect, RebH catalyzes halogenation at C7 position. Investigation of regioselectivity suggested that selectivity is governed by both electronic and steric effects. However, the binding pose of the substrate in the active site often prevails over *ortho-/para*-directing effects exerted by e.g. C5-substituents. Substrates exhibiting an electronic preference for halogenation in *ortho*-position such as 5-methyl-L-Trp gave a 1:1 mixture of derivatives chlorinated at C6 and C7. Furthermore, bromination even dominated in C6 position and dihalogenation was also found. Docking analysis pointed towards a slight rotation of the 5-methyl-derivative compared to L-Trp. This suggests a binding mode in which C6 position of 5-methyl-L-Trp is directed towards the tunnel hence overlapping with C7 from the natural substrate L-Trp. Accordingly, total turnover numbers (TTN) for the chlorination of Trp derivatives strongly depend on the steric and electronic influence of the substrate and dropped significantly from 102 for Trp over 55 for 5-methyl-L-Trp to 7 for 5-fluoro-L-Trp, respectively. These findings made by us and by other groups indicate that FDHs provide a valuable platform for regioselective halogenation hardly achievable by conventional electrophilic aromatic substitution.

8.3. Closer Investigation of Substrate Scope of FDHs

A comprehensive survey by Andorfer *et al.* further focused on profiling of the substrate spectrum of several FDH's and evolved variants towards a broad set of arenes with different steric and functional groups. A panel of 93 compounds was employed to analyze FDH activity on analytical scale in a high-throughput fashion employing LC-MS analysis. Preparative scale reactions employing up to 10 mg substrate were purified and the regioselectivity determined via NMR spectroscopy. Examination of their substrate-activity profiles revealed that the substrate scope of FDHs is less strict than initially assumed, though even minor changes in halogenase sequence can significantly affect specificity and scope. Both RebH and Thal exhibited activity on a wide range of substituted anilines and indoles and were shown to tolerate a remarkable variety of functional groups such as for instance amines, alcohols, nitriles, esters or sulfonamides. Moreover, the study unveiled that notable differences of the substrate profile exist among these enzymes despite high homology, as some substrates were exclusively and quantitatively halogenated by Thal, whereas no activity was observed for RebH.[134] Remarkably, a RebH-4V mutant, engineered for accepting larger indole containing compounds, showed the broadest scope towards the tested indoles, pyrroles, azoles, anilines and anilides. It exhibited a wider substrate scope than the WT enzyme which demonstrated the value of directed evolution for halogenase engineering. Furthermore, RebH-4 V mutant also enabled desymmetrization of prochiral methylene dianilines, thereby providing an example of engineering the enantioselectivity of a FDH (Scheme 8).[135]

Recently, the Lewis group expanded this study to a FDH family-wide profiling to gain deeper insight into sequence-function information, also shedding light on enzyme activity, halide specificity and substrate preference.[136] A BLAST search of the UniProt sequence database identified 3975 putative halogenase genes derived from the FDH family, covering a wide range of sequence and host diversity, including archaeal, eukaryotic, bacterial and viral proteins. In their survey sequence-similarity networks were generated to visualize functional relationships among putative FDH sequences. Three subnetworks emerged mainly differing in host domain and compound class. FDHs natively halogenating Trp or indole *in vitro* constituted the largest subnetwork, comprising 2270 sequences. The second largest subgroup (438 sequences) contained most known phenol halogenases such as fungal



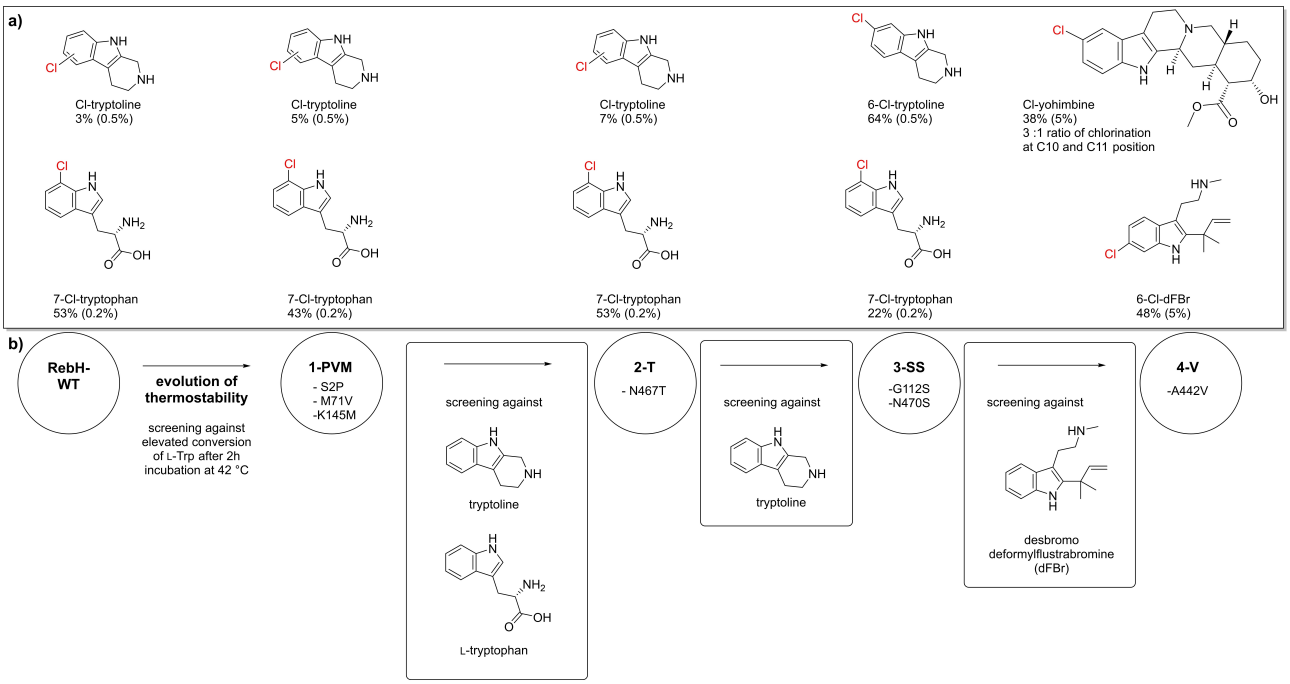
Scheme 8. Enantioselective desymmetrization of methylenedianilines. RebH-4 V mutant catalyzes chlorination of alkyl-substituted 4,4′-methylene-dianilines that constitutes the first example of asymmetric catalysis by FDHs giving chiral monochlorinated product in an enantioselectivity of 99:1.

Rdc2 or RadH. FDHs connected to chlorination of pyrrole form the smallest subnetwork, comprising only 212 sequences. For functional characterization, 128 putative halogenase sequences were codon-optimized and expressed in *E. coli*, resulting in a total of 87 new enzymes obtained in a sufficient quantity of soluble protein suitable for the following high-throughput activity screening. Therefore, a set of 12 differing substrates, including indoles, anilines and phenols possessing high inherent reactivity were selected. Analysis of activity profiles resulted in identification of 39 new enzymes capable of halogenating at least one of the tested compounds, while bromination activity prevailed over chlorination. Although the majority of FDHs reported to date is involved in the biosynthesis of chlorinated natural products, this survey indicates that bromination is much more widespread in FDHs than previously assumed. Furthermore, novel and diverse FDHs capable of halogenating complex, previously inaccessible substrates were identified. Halogenase 1-H11, classified as an indole halogenase and belonging to the BrvH subnetwork, is worth mentioning in this context. In addition to improved thermal stability compared to RebH it possesses a natural bromination activity on yohimbine, a pharmaceutically relevant substrate that served as target substrate in a previous substrate-walking approach of RebH (Scheme 9).[103] The presented genome mining approach is therefore a promising platform for future engineering approaches and might circumvent evolution of a single enzyme by identification of related enzymes initially suitable to catalyze a certain transformation of interest.

8.4. Halogenases Addressing Phenol-Based Substrates

Interestingly, Rdc2, a fungal phenolic FDH from Pochonia chlamydosporia,[70] halogenates a panel of substrates poorly accepted by Thal, RebH or evolved variants. Hydroxyisoquinolines and macrocyclic lactones were in most cases halogenated in *ortho*-position to the hydroxy group due to its strong directing influence. Sequence comparison of RebH and Rdc2 revealed a significant difference in primary sequence (43% identity, 48% query coverage). Hence it is not surprising that there is a strong difference of substrate scope between the halogenases with Rdc2 mainly acting on phenol based derivatives.[134,137]

RadH, another phenolic halogenase with close homology to Rdc2 (87%), was shown by Micklefield and coworkers to catalyze halogenation of an array of natural and synthetic phenolic compounds. The hydroxyl functionality turned out to be an essential motif whilst being inactive towards other electron-rich arenes lacking the OH group. Still, RadH appeared rather promiscuous with regard to several natural and synthetic phenolic compounds as well as flavonoids being accepted. Its ability to act on common pharmacophores served as a starting point for further engineering platform for high-throughput screening to engineer RadH variants with elevated activity for a range of substrates which are part and contribute to antimicrobial activity of antibiotics as clorobiocin. Different fluorescence properties of 7-hydroxycoumarin and its chlorinated analogue enabled identification of a RadH double mutant exhibiting significantly elevated activity on a range of substrates. This evolved RadH variant was finally integrated into coumarin



Scheme 9. Directed evolution of RebH via a stepwise substrate walking approach achieved halogenation of steric demanding indole and carbazole derivatives. a) Conversion of selected substrates determined via UPLC and mol% enzyme loading in brackets refer to the enzyme mentioned below. b) Additional mutations identified via epPCR relative to the previous parent.

biosynthetic pathway within recombinant *E. coli*, resulting in a non-native halogenated natural product derivative produced *in vivo*.[62]

9. Directed Evolution of FDHs

9.1. Expanding the Substrate Scope of FDHs by Directed Evolution

In this context, the Lewis group pioneered in applying directed evolution for widening the substrate scope of a Trp halogenase. A thermostable RebH triple mutant (1-PVM), derived from a previous evolution campaign, revealed elevated conversion of substrates such as tryptamine and tryptoline.[103,138] Therefore, 1PVM served as a template for further rounds of random mutagenesis to screen towards progressively larger substrates starting from Trp to substantially larger indole-derived compounds like carazolol or carvedilol. Even sterically demanding compounds of biological interest such as the 6-chloro analogue of deformylflustrabromine (dFBr) or α_2-blocker yohimbine (Scheme 9) could be formed from their non-halogenated precursors. Three rounds of evolution resulted in promising variants finally accepting a broad range of non-natural, challenging substrates as large indoles and carbazoles that differ significantly in terms of functionality and size to the former native substrate Trp.[103] Furthermore, a significant improvement of the regioselective halogenation of tryptoline was achieved, e.g. using variant 3-SS, exhibiting 93% conversion to the C6-chlorinated product in comparison to a 1:1 mixture of 6- and 7-chlorotryptoline observed for the WT.

9.2. Switching Regioselectivity by Random and Structure-Guided Mutagenesis

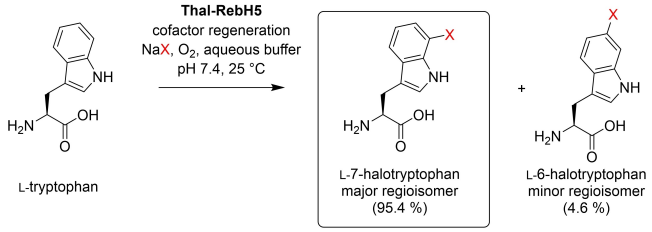
Lang *et al.* pioneered in changing the regioselectivity of Trp 7-halogenase PrnA by structure-guided mutagenesis. The X-ray single crystal structures of PrnA and PyrH in complex with Trp corroborate the position of halogenation to be merely controlled by the spatial orientation of the substrate relative to the halogenating agent.

Other reactive positions of the indole moiety are shielded by the surrounding aromatic amino acids, e.g. H101, F103 and W455 in PrnA (Figure 4). To reduce steric demands Ala mutants of residues located in the substrate-binding sites were generated. Among these mutants only PrnA-F103A impacted regioselectivity leading to a 1:2 mixture of C5- and C7-brominated product. The remaining preference for halogenation at C7 is suggested to result from the different orientation of the substrate that does not comply with the ideal geometry of the active site as observed in PyrH. Presumably the mutation allows Trp to adopt an orientation observed in a C5-halogenase and indicates that regioselectivity is guided by surrounding residues in the first sphere of bound Trp.[139]

A comparable approach was pursued by Shepherd *et al.* who intended to switch the regioselectivity of the Trp 6-halogenase SttH to position 5.[53] Based on structural differences to the 5-halogenase PyrH regarding crucial amino acid residues of the active site, point mutations were introduced replacing specific amino acid residues of SttH by the corresponding counterparts of PyrH. This approach resulted in less active biocatalysts without considerable impact on regioselectivity. However, the combination to the triple mutant SttH L460F/P461E/P462T showed similar activity as SttH WT with relaxed regioselectivity leading to 32% 5-Cl-Trp, whereas the wild-type enzyme produced 6-Cl-Trp only. Noteworthy, a significant switch in regioselectivity was observed for the more flexible substrate 3-indolepropionic acid, yielding 75% 5-chlorinated product in comparison to 90% 6-chlorination observed with SttH WT.

In a detailed survey Andorfer *et al.* focused on random as well as structure-guided selection of active site residues to modify the regioselectivity of RebH.[140] Their ambition to evolve RebH into a C5- or C6-regioselective biocatalyst led to two new variants, 10S and 8F, capable of chlorinating tryptamine with up to 95% regioselectivity for position 5 (variant 10S) and 90% regioselectivity for position 6 (variant 8F). Identification of beneficial mutations was based on up to ten generations of mutagenesis and high-throughput screening including random, site-directed as well as site-saturation mutagenesis. Therefore, the development of a high-throughput screening assay was initially necessary to identify halogenase variants with altered regioselectivity. Deuterated tryptamine that is more easily accessible than the corresponding Trp was synthesized and served as a probe to identify a switch of regioselectivity by MS-based screening. Mutants with altered selectivity towards deuterotryptamine could be identified owing to the mass shift of $\Delta m/z = 1$ caused by the deuterium in the molecule once a position other than C–D site is being halogenated. As the MALDI-TOF MS assay employed was based on deuterated tryptamine, the results were valid for tryptamine only, but not for the native substrate Trp. While recognizing this remarkable achievement, it should be pointed out that tryptamine is a less bulky substrate than Trp due to the lack of the carboxy-group. This leads to higher flexibility in the active site upon binding to the halogenase, which might facilitate the substrate to adopt a different binding pose which is beneficial to swap the regioselectivity.

Recently Moritzer *et al.* demonstrated the generation of a non-natural Trp 7-halogenase (Thal-RebH5) by mutating five active-site residues of Thal to the corresponding counterparts of RebH.[57] An impressive shift of 95% in regioselectivity towards C7-halogenation for both chlorination and bromination of Trp was achieved by site-directed mutagenesis simply by altering five amino acid residues based on rational design (Scheme 10). The resulting quintuple mutant exhibited a nearly quantitative switch in regioselectivity for halogenation of its native substrate Trp that has not been reported for another Trp halogenase so far.



Scheme 10. Change of halogenase selectivity by structure-guided mutagenesis. Thal-RebH5-catalyzed halogenation yields L-7-halotryptophan as the dominant regioisomer, thus introducing strict C7-selectivity into Thal. X = Cl, Br.

10. Engineering of Thermostability and Increased Activity

As for many other enzymes from secondary metabolism, FDH's are not essential for any metabolic functions related to survival or growth of its main host. Therefore, higher affinities and turnover rates did not evolve due to lower evolutionary pressure compared to enzymes involved in central metabolism and the host's own requirement of Trp for protein biosynthesis.[141]

Characterization of FDH kinetic parameters revealed that *in vitro* halogenation is a rather inefficient reaction and their low activity and instability currently exclude their employment *in vitro* for large scale synthetic applications (Table 3). Although enzyme immobilization as cross-linked enzyme aggregates increases productivity owing to a higher lifetime and simplified biocatalyst recycling and removal, biotechnological application of FDH is still limited to analytical and semipreparative scale.

10.1. High-Throughput Assays to Evaluate Halogenase Activity in Directed Evolution

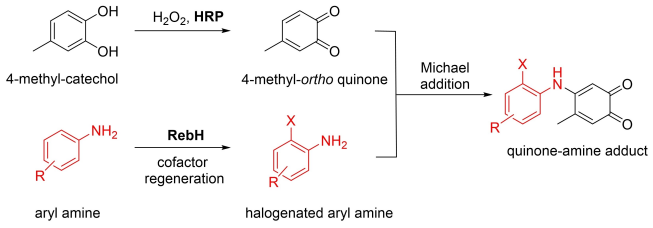
Any attempt for discovering or engineering of halogenases requires the capacity to detect and quantify enzyme activity in a facile and rapid manner. Regarding directed evolution, one of the most important and at the same time limiting factors is a reliable and rapid high-throughput screening assay to identify the desired mutants. High-performance liquid chromatography (HPLC) is considered as a standard to assay halogenase activity in kinetic analysis as well as in directed evolution. Despite being a universal technique with few limitations, such assays of biocatalytic reactions are usually very time-consuming and rely on specialized equipment such as ultra-high-performance liquid chromatography (uHPLC) instruments. Distinct differences of UV-vis absorbance spectra between halogenated anilines and their non-halogenated precursor molecules provided the basis for the development of a colorimetric assay capable of monitoring halogenation of different arylamines in a microtiter plate (Scheme 11). Hosford *et al.* described a rapid high-throughput assay in aqueous solution based on the *in situ* oxidation of 4-methylcatechol by horseradish peroxidase (HRP) to the corresponding *ortho*-benzoquinone. Subsequent Michael addition of the quinone to either the halogenated or non-halogenated arylamine leads to the formation of the desired *ortho*-benzoquinone-amine adduct.[143] The assay was coupled to halogenase RebH and confirmed reliable discrimination between the substrate and the halogenated product for several arylamines even in presence of further functionalities. Yet, this one-pot workflow needs to be quenched by heat-precipitation before HRP, H_2O_2 and 4-methylcatechol could be added to enable formation of the probe of interest. Unfortunately, the required presence of an arylamine functionality restricts the assay to a limited number of halogenase substrates and its application has not yet been demonstrated for directed evolution.

Another colorimetric high-throughput assay to detect halogenase activity was based on the specific L-amino acid oxidase RebO owing to the high specificity for halotryptophan compared to Trp. Release of H_2O_2 per equivalent of amino acid oxidized allows for quantification via coupling to HRP leading to concentration dependent dye formation. Yet the applicability of this assay was not demonstrated in a directed evolution campaign.[116]

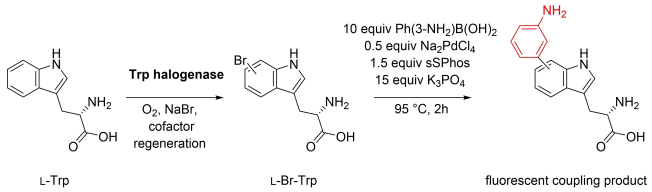
In this context, Schnepel *et al.* developed a fluorescence assay based on Suzuki-Miyaura cross-coupling appropriate for high-throughput screening to quantify the halogenation activity of Trp 5-, 6- and 7-halogenases.[123] Coupling of 3-aminophenylboronic acid and the brominated amino acid provided the basis to monitor quantitative formation of Br-Trp due to superior fluorescence properties of the coupling product compared to Trp and Br-Trp (Scheme 12). This robust activity assay applicable in microtiter plates is directly performed in crude *E. coli* lysate and enables high-throughput screening of large mutant libraries without requiring purification steps or a

Table 3. Kinetic parameters of Trp halogenases with L-Trp as substrate. Reaction temperature is given in brackets if specified.

Trp halogenase	k_{cat} [min^{-1}]	K_M [μM]	Reference
PrnA	0.1 (30 °C)	160	Dong *et al.*, 2005[51]
PyrH	0.5 (30 °C)	150	Zehner *et al.*, 2005[36]
RebH	1.4	2.0	Yeh *et al.*, 2005[35]
Thal	2.8	110	Seibold *et al.*, 2006[142]
Th-Hal	4.3 (30 °C)	12.2	Menon *et al.*, 2016[41]
BorH	4.4 (45 °C)	9.8	Lingkon *et al.*, 2019[43]



Scheme 11. UV-Vis based high-throughput screening for detection of halogenase activity. RebH-catalyzed halogenation of an aryl amine is followed by *in situ* oxidation of 4-methylcatechol and formation of the halogenated quinone-amine adduct that can be traced spectrophotometrically due to a shift of the absorbance maxima.



Scheme 12. Formation of a fluorescent biaryl resulting from derivatization of halogenated Trp by Suzuki-Miyaura reaction provides the basis for a high-throughput assay to monitor activity of Trp halogenases.

specialized laboratory equipment. Though this assay was optimized for coupling of 5-, 6-, and 7-bromotryptophan to 3-aminophenylboronic acid, the high specificity also limits the simple transfer to other substrates and requires previous calibration with a suitable standard. Still, this activity assay enabled identification of a thermostable Thal variant (Thal-GR) comprising mutations S359G/K374R, which exhibits a significantly increased thermostability and 2.5-fold improved activity compared to the WT enzyme.[123]

Based on these achievements obtained from the first round of Thal evolution Minges *et al.* focused on a more comprehensive evolution campaign to further improve its thermostability and catalytic performance.[132] Fluorogenic high-throughput screening enabled identification of significantly more thermostable Thal variants harbouring three to five mutations, mainly being located at the interface to the second Thal monomer. The individual mutation's impact on thermostability and activity was analyzed in detail, revealing that mutation S359G tremendously increases thermostability and activity although being located in the active site. Noteworthy, this Ser residue, conserved among Trp-FDHs, also increases enantiomer selectivity giving significantly reduced conversion of D-Trp. In addition to an unexpected role of this residue in substrate acceptance, activity and thermostability, native ESI-MS analysis also revealed an enhanced tendency for dimer formation of selected Thal variants compared to the WT enzyme suggesting that dimerization favors halogenase stabilization. Finally, combination of different evolution strategies including directed evolution, rational design as well as site-saturation mutagenesis led to a Thal triple mutant (Thal-GLV) exhibiting significantly increased thermostability compared to Thal WT ($\triangle T_{50} = 16.0$ K; $\triangle T_M =$ 23.5 K) and strongly elevated enzyme activity at 25 °C.

10.2. Increasing Halogenase Stability

Menon *et al.*, aimed at identifying and employing a thermophilic Trp halogenase as a productive and robust biocatalyst exhibiting improved catalytic activity and stability compared to other FDHs.[41] Th-Hal from *Streptomyces violaceusniger* strain SPC6 along with a thermophilic flavin reductase Th-Fre from *Bacillus subtilis* WU-S2B provided a thermostable halogenation system and enabled quantitative halogenation of Trp at C6 position. At 30 °C Th-Hal was more active towards Trp than any other halogenase tested and determination of melting temperature by CD spectroscopy revealed a 10 K elevated T_M compared to other mesophilic Trp halogenases. Comparison of Th-Hal with other Trp halogenases in combination with thermostable reductase also showed that combination of Th-Hal/Th-Fre afforded significantly higher conversion of Trp at 40 °C. In addition, substrate specificity and regioselectivity resembles that of the Trp 6-halogenase SttH sharing 76.2% sequence identity to each other.[53] Interestingly, a closer look into amino acid variations between Th-Hal and SttH revealed most variations occurring on the protein surface. Mainly polar residues were present that impact hydrogen bonds on the surface and probably prevent deleterious protein aggregation. Moreover, retention of activity and more robustness in polar organic solvents compared to SttH underline that thermophilic enzymes provide a promising and robust halogenating system for future engineering approaches. These findings made for Th-Hal may provide a promising starting point to modify or mutate a protein's surface to retain catalytic activity and elevate enzyme stability.

Likewise, Lingkon *et al.* closely characterized and crystallized Trp 6-halogenase BorH and its accompanying flavin reductase BorF derived from a desert soil sample.[43] BorH exhibits slightly higher thermostability than the majority of Trp 6-halogenases, possessing a T_M of 48 °C and operating at a T_{opt} of 45 °C compared to a T_{opt} of 25–30 °C for Thal ($T_M = 47.7$ °C),[132] T_{opt} of 40 °C for SttH[40] or a T_M of 47.8 °C for Th-Hal.[41] First preparative scale chlorination of 100 mg Trp employing 0.2 mol% BorH along with BorF yielded 52% chlorinated product, rendering both enzymes interesting for industrial scale bioconversion. BorH's capability of halogenating a broad range of aromatic compounds and exhibiting altered activities on substrates previously observed for other Trp-6 halogenases underline its value as promising biocatalyst or template for further evolution studies.

Poor *et al.* also focused on improving the thermostability of mesophilic Trp halogenases. Three rounds of mutagenesis resulted in a thermostable RebH variant (3-LSR) exhibiting a significant increase in enzyme stability as evident by a rise of the melting temperature (T_M) of 18 K compared to the WT enzyme. Furthermore, the optimum reaction temperature (T_{opt}) increased from 30–35 °C to 40 °C for selected mutants (3-LR). In addition to an increased conversion of Trp, improved conversion was also reported for unnatural substrates such as tryptoline or 2-methyltryptamine. However, despite increased temperature tolerance and higher lifetime its catalytic efficiency at 21 °C was reduced whereas a higher product yield resulted from a longer lifetime thus compensating its lower catalytic activity.[138]

11. Summary and Outlook

Despite recent achievements to broaden the application scope of FDH by means of protein engineering, further efforts are required to provide robust enzymes capable of large-scale C-H-functionalization that can be accomplished in multistep synthesis and reaction cascades. However, promising results have

been achieved by means of directed evolution and enzyme discovery. Protein engineering especially succeeded in expanding the substrate scope as well as improving stability and activity. As the frequent lack of compatibility of bio- and chemocatalysis cannot be neglected, further efforts are required to overcome this drawback. In light of developing novel approaches for enzymatic halogenation many steps have to be undertaken to replace conventional chemistry still being superior in terms of reaction scale and application range. Thus, the low productivity of halogenating enzymes has to be overcome for obtaining higher product titers. Presumably, future developments in reaction engineering, flow catalysis, and enzyme engineering will foster progress on large-scale enzymatic halogenation. FDHs seem to prefer electron-rich aromatics such as indoles and phenols. Halogenation of more electron-deficient substrates will make an important contribution. In particular, the discovery of novel halogenases applying bioinformatic tools along with rational protein design and broad substrate scoping will considerably streamline the progress towards more generic tools for selective C–H activation.

Owing to enormous genome data availability, ongoing progress in computer-based engineering and the growing demand for sustainable and environmentally friendly fine-chemical synthesis this versatile enzyme class should not be neglected as it expands the horizon towards possible future applications.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · C–H functionalization · directed evolution · halogenase · protein engineering

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