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Review

Recent Advances in Flavin-Dependent Halogenase Biocatalysis: Sourcing, Engineering, and Application

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Abstract: The introduction of a halogen atom into a small molecule can effectively modulate its properties, yielding bioactive substances of agrochemical and pharmaceutical interest. Consequently, the development of selective halogenation strategies is of high technological value. Besides chemical methodologies, enzymatic halogenations have received increased interest as they allow the selective installation of halogen atoms in molecular scaffolds of varying complexity under mild reaction conditions. Today, a comprehensive library of aromatic halogenases exists, and enzyme as well as reaction engineering approaches are being explored to broaden this enzyme family's biocatalytic application range. In this review, we highlight recent developments in the sourcing, engineering, and application of flavin-dependent halogenases with a special focus on chemoenzymatic and coupled biosynthetic approaches.

Keywords: flavin-dependent halogenases; enzyme engineering; anion promiscuity; reaction engineering; co-factor recycling; biocatalysis

1. Introduction

In nature, over 5000 halogenated products have been described since their first discovery over 100 years ago including potent antimicrobial agents such as vancomycin and chloramphenicol (Figure 1) [1]. The incorporation of a halogen can significantly alter a molecule's properties and may impact its bioactivity, metabolism, and pharmacokinetic profile [2]. As a consequence, the selective halogenation of small molecules is of great interest in the pharmaceutical and agrochemical industry. Around 20% of small molecule drugs and approximately 30% of agrochemicals are halogenated [3] Examples in the pharmaceutical industry include blockbusters such as rivaroxaban and empagliflozin with annual sales revenues in 2018 of $6.6 and $2.3 billion, respectively (Figure 1) [4]. Furthermore, C-X motifs are useful synthetic handles for modification of the molecule in further chemical steps, making halogenated species common intermediates in synthetic manufacturing routes [5,6]. However, chemical halogenation, even though being a well-established technology, suffers from the use of hazardous or even highly toxic chemicals and sometimes poor atom efficiency [7,8]. In addition, chemical methods often lack selectivity or have specific demands on the substrate structure [8,9]. Consequently, the replacement of chemical halogenation by biological means may offer a more selective, greener, and less dangerous process route toward molecules of industrial interest.

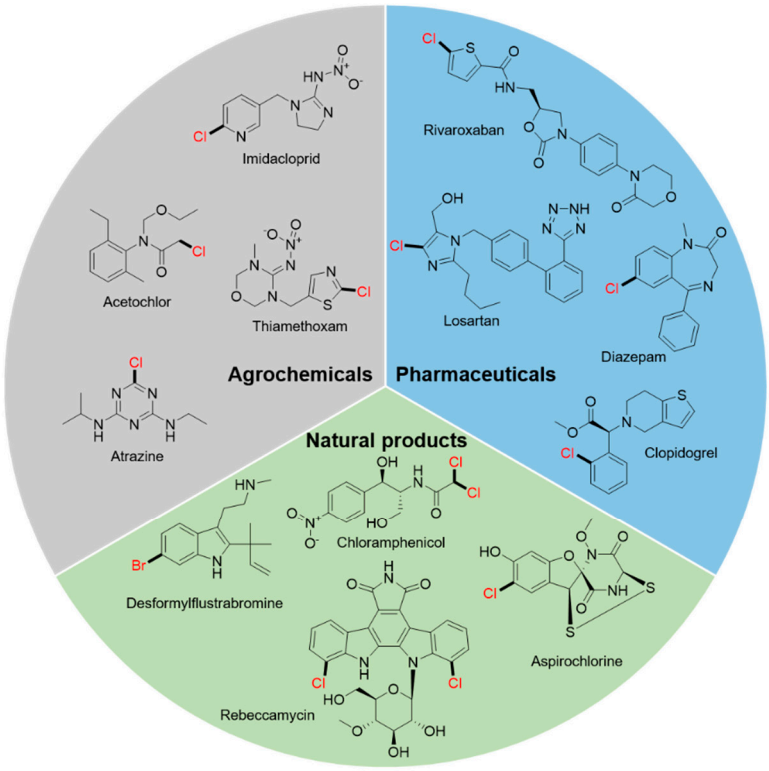

Figure 1. Selection of valuable halogenated agrochemicals, pharmaceuticals and natural products.

Biocatalytic halogenation is carried out by enzymes called halogenases or haloperoxidases, which can be classified according to their catalytic mechanism: heme, vanadium, and flavin-dependent halogenases follow an electrophilic mechanism, while non-heme iron halogenases halogenate through the formation of radical intermediates, and S-adenosyl-L-methionine (SAM) fluorinases react via a nucleophilic pathway [10]. This review will focus on work carried out on flavin-dependent halogenases (electrophilic halogenases), which in contrast to heme- and vanadium-dependent halogenases, selectively derivatize small molecules, making them especially interesting for applications.

Flavin-dependent halogenases (Fl-Hals) use reduced flavin, bound in the FAD binding site of the enzyme, to create a C4a-hydroperoxyflavin species upon reaction with oxygen [11]. The generated peroxy species cannot react directly with the substrate as the flavin binding site and the substrate binding site are separated by an approximately 10 Å long tunnel [12–15]. Instead, crystal structures of flavin-dependent halogenases show the presence of a chloride binding site near the flavin binding site [15,16], and mechanistic studies suggest that a nucleophilic attack of the chloride on the peroxyflavin may create hypohalous acid (HOX) [11]. The hypohalous acid then travels to the substrate binding site and is believed to interact with an active site lysine either by reaction or via hydrogen bonding (Figure 2) [16,17]. It is assumed that this electrophilic chlorine species is responsible for electrophilic aromatic substitution of the proximally bound substrates. Notably, an active site glutamate residue has also been found important for catalysis; however, its precise role in the reaction mechanism still remains debated [2,18].

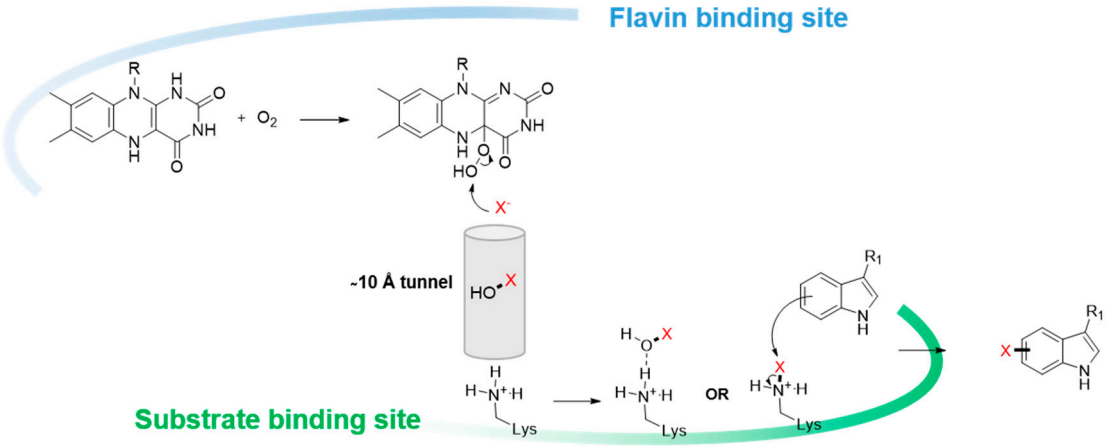

Figure 2. Reaction mechanism of flavin-dependent halogenases. Reduced flavin reacts with oxygen to form a peroxyflavin species, which in turn reacts with a halide ion, generating free hypohalous acid. The hypohalous acid travels through an approximately 10 Å tunnel before reacting with an active site lysine. Through an electrophilic aromatic substitution mechanism, the halogenated aromatic compound is formed.

2. Enzyme Diversity and Substrate Scope

To date, a plethora of flavin-dependent halogenases (Fl-Hals) has been identified. Fl-Hals accept a range of different substrates but preferentially have been found to halogenate L-tryptophan (Figure 3: red square) [19], pyrrole-like compounds (Figure 3: green triangle) [20], phenolic compounds (Figure 3: blue circle) [21], and indole (Figure 3: orange square) [22]. In this section, we will give an overview of the natural diversity of flavin-dependent halogenases emphasizing the recently discovered members of this enzyme family.

The first flavin-dependent halogenases were identified in the mid-1990s [19,23]. PrnA, discovered in *Pseudomonas fluorescens* in 1998, is for example involved in the biosynthesis of pyrrolnitrin, a secondary metabolite derived from L-tryptophan with a strong antifungal activity [19]. PrnA catalyzes the chlorination at the 7-position of the indole moiety of L-tryptophan (Product Type I, Figure 3). Following this discovery, research intensified and many additional L-tryptophan Fl-Hals (Trp-Fl-Hals) were described [19,24–27]. The widely applied 7-tryptophan halogenase RebH, for example, stems from the bacterium *Lechevalieria aerocolonigenes* and its natural function is the chlorination of precursors of the antitumor agent rebeccamycin [24]. Another Fl-Hal able to halogenate at the 7-position is KtzQ found in *Kutzneria* sp. 744 [26]. This halogenase is involved in the biosynthesis of Kutzneride, an antifungal nonribosomal hexadepsipeptide. After chlorination at the 7-position, tryptophan is further chlorinated at the 6-position by KtzR, which has a ~120-fold preference for 7-Cl-L-Trp over L-Trp [26]. Other halogenases have a natural preference to chlorinate at the 6-position of L-Tryptophan, such as Thal from *Streptomyces albogriseolus* [25] and a thermophilic tryptophan halogenase (Th-Hal) found in *Streptomyces violaceusniger* [28]. Furthermore, SttH found in *Streptomyces toxytricini* [29] and the recently identified halogenases in *Saccharomonospora* sp. CNQ-490 (Tar14) [30], as well as a halogenase found through metagenomic screening of soil samples from the Anza-Borrego desert (BorH) [31] are able to chlorinate and brominate at the 6-position of this aromatic amino acid. The 5-position of the indole moiety of tryptophan can be modified by PyrH from *Streptomyces rugosporus* [27], SpmH from *Streptomyces* sp. SCSIO 03032 [32] and XszenFHal identified in *Xenorhabdus szentirmaii* [33]. Recently, a novel set of flavin-dependent halogenases called BrvH [22], xcc-b100_1333, xcc-b100_4156, and xcc-b100_4345 [34] were described. These enzymes were found to have a high sequence identity to known Trp-Fl-Hals, but could not convert L-tryptophan. This property was ascribed to the fact that certain amino acids required to stabilize tryptophan within the active site pocket were missing [22,34]. Instead, these halogenases can accept smaller substrates such as indole (Product Type III, Figure 3), which triggered us to name these enzymes "Indole Fl-Hals" in the frame of this review, and prefer bromination over chlorination. BrvH

was found in *Brevundimonas* BAL3, whereas the three xcc-b100 enzymes stem from *Xanthomonas campestris* (*Xcc*). The plant pathogen *Xcc* belongs to a group of Proteobacteria that is known to infect over 350 plant species by secreting a characteristic yellow pigment called xanthomonadin, a mixture of brominated aryl polyene esters. Finally, in 2019, a viral halogenase, named VirX1, was discovered in a cyanophage and was shown to act on 6-azaindole (Product Type II, Figure 3) [35].

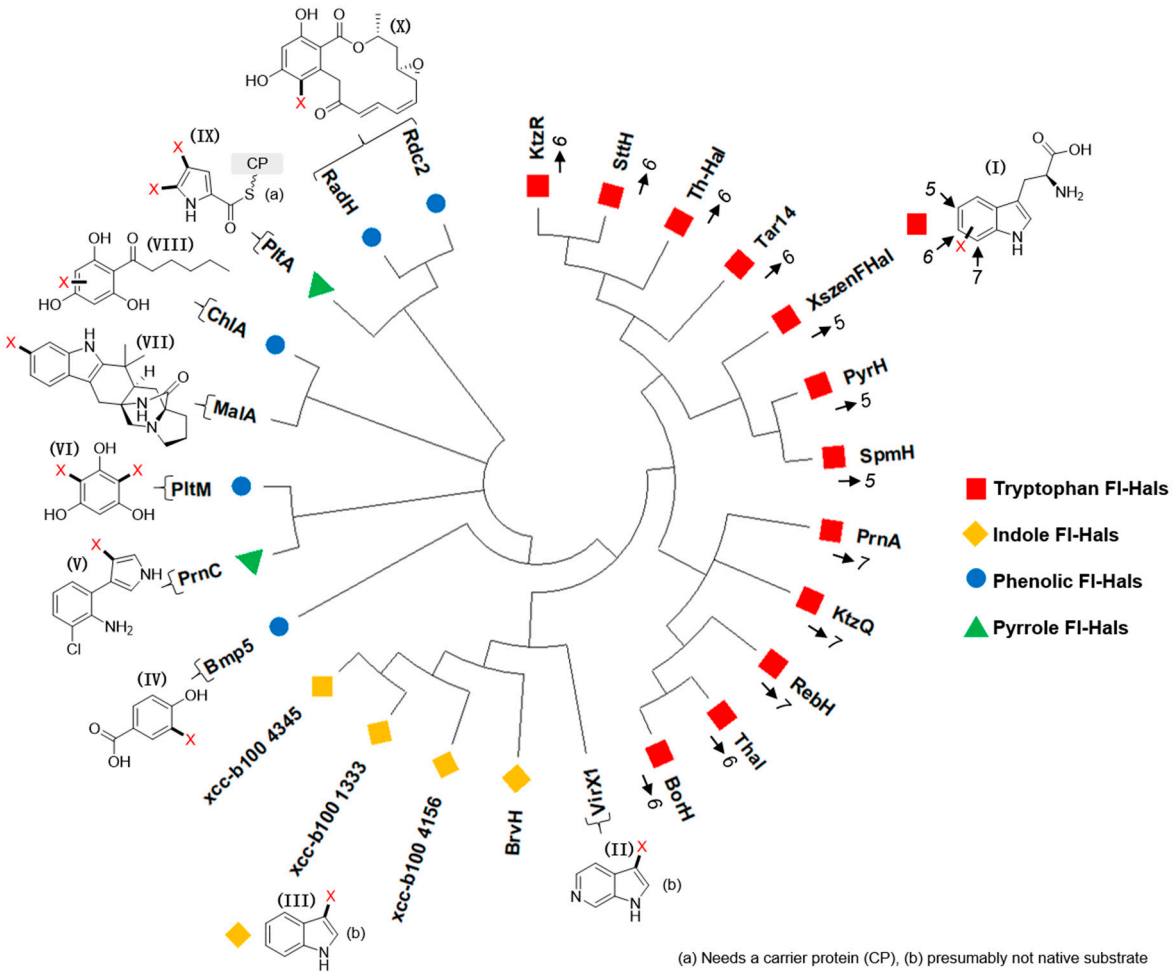

Figure 3. Phylogenetic tree depicting a selection of flavin-dependent halogenases and their native products. The tree was constructed using the maximum likelihood method, a bootstrap of 1000, with the alignment based on amino acids by MEGAX [36] and MUSCLE [37]. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed.

Today, the biocatalytic toolbox contains flavin-dependent halogenases that can convert molecules beyond L-tryptophan derivatives. PrnC and PltA found in *Pseudomonas fluorescens*, for example, convert pyrrole-like substrates [19,20]. While PltA requires its substrate to be tethered to a carrier protein [20], PrnC acts on a free-standing intermediate in the biosynthesis of pyrrolnitrin. After the PrnA catalyzed chlorination of L-tryptophan to form 7-chloro-L-tryptophan (*vide infra*), which is followed by a ring rearrangement and a decarboxylation step, PrnC chlorinates the 3-position of the pyrrole ring to form aminopyrrolnitrin (Product V, Figure 3) [19]. Phenols are an additional molecule class that is known to be converted by halogenases such as Rdc2, RadH, Bmp5, and ChlA [38–41]. Phenolic halogenases typically originate from fungi except for PltM, which was isolated from *Pseudomonas fluorescens* Pf-5 [42]. Initially, PltM was identified as one of three putative halogenases encoded in the biosynthetic gene cluster of pyoluteorin (one of the others being PltA), an antifungal compound containing a dichloropyrrole moiety [42]. Almost twenty years later, it was shown that PltM catalyzes mono- and di-chlorination of phloroglucinol (Product Type VI, Figure 3), yielding a compound that serves as a transcriptional factor of the pyoluteorin biosynthesis rather

than a biosynthetic intermediate [43]. MalA', a rather unusual halogenase from *Malbranchea graminicola*, can act on the bulky molecule premalbrancheamide (Product Type VII, Figure 3) [44].

Except for the very electronegative fluoride ion, flavin-dependent halogenases have been shown to introduce all common halides into small molecules. However, certain anion preferences exist: The well described Trp-Fl-Hals RebH, Thal and PyrH prefer chlorination over bromination [24,25,27], while BrvH and VirX1 prefer bromination over chlorination [22,35]. For the three xcc-b100 enzymes, no chlorination was observed, and iodination was only found for Bmp5, PltM, and VirX1 [34,35,40,45] (Table 1).

Looking forward, the discovery of flavin-dependent halogenases with alternative substrate preferences will be fueled by the rapidly expanding amount of genetic information available from public databases. As gene synthesis is becoming more cost effective and high throughput assays are being established, putative halogenases can be verified quickly in an experimental setup. In a recent study from Fisher et al. [46], the authors used a genome mining approach to identify 128 putative Fl-Hals. By co-expression with the chaperones GroES and GroEL, 87 halogenase candidates were obtained in sufficient soluble amounts for the later analysis via high throughput LC-MS analysis (11 s/analysis). The soluble enzymes were screened towards electron rich substrates known to be commonly converted by Fl-Hals, as well as more complex substrates. With this approach, 39 new Fl-Hals were identified, some of which showed halogenation of complex compounds, offering advantageous starting points for further evolution [46].

Table 1. Halide versatility of Fl-Hals.

Enzyme	Halogen (X)	Literature	
RebH	Cl > Br	[24]	Tryptophan-Fl-Hal ■
Thal	Cl > Br	[25]	
PyrH	Cl > Br	[27]	
KtzQ	Cl	[26]	
PrnA	Cl	[19]	
Th-Hal	Cl, Br	[47]	
KtzR	Cl	[26]	
SttH	Cl, Br	[29]	
Tar14	Cl, Br	[30]	
SpmH	Cl, Br	[32]	
XszenFHal	Cl, Br	[33]	
BorH	Cl, Br	[31]	
BrvH	Cl < Br	[22]	Indole-Fl-Hal ◆
xcc-b100-4156	Br	[34]	
xcc-b100-1333	Br	[34]	
xcc-b100-4345	Br	[34]	
VirX1	Cl < Br < I	[35]	
Bmp5	Br, I	[40]	Phenolic-Fl-Hal ●
ChlA	Cl	[41]	
PltM	Cl, Br, I	[45]	
RadH	Cl	[39]	
Rdc2	Cl, Br	[21]	
PrnC	Cl	[19]	Pyrrole-Fl-Hal ▲
PltA	Cl, Br	[20]	
MalA	Cl, Br	[44]	

3. Cofactor and Reaction Engineering

As can be deduced from the reaction mechanism (Figure 2), flavin-dependent halogenases require a constant supply of reduced $FADH_2$ to function. In nature, the oxidized flavin species is reduced by a flavin reductase (Fre). In the case of halogenase RebH, for example, the corresponding flavin reductase called RebF was identified in the native organism *Lechevalieria aerocolonigenes* [24]. Likewise, a flavin halogenase–flavin reductase pair, PrnA and PrnF, was identified in *Pseudomonas fluorescens*. For *in vitro* studies of halogenases, however, alternative flavin reductases may be used. Examples comprise a thermophilic Fre from *Bacillus subtilis* WU-S2B [47], a Fre from *Pseudomonas fluorescens* [22], or a Fre from the *Escherichia coli* strain K12 [33]. Flavin reductases employ NADH to reduce flavin; therefore, biocatalytic applications may include an additional enzyme for the reduction of NADH such as a glucose dehydrogenase (GDH) (Figure 4) or an alcohol dehydrogenase (ADH). By adding the necessary auxiliary enzymes (Fre and GDH or ADH), halogenation biocatalysis reactions can run on the inexpensive substrate glucose or isopropanol, while the cost intensive cofactors FAD and NAD^+ are recycled.

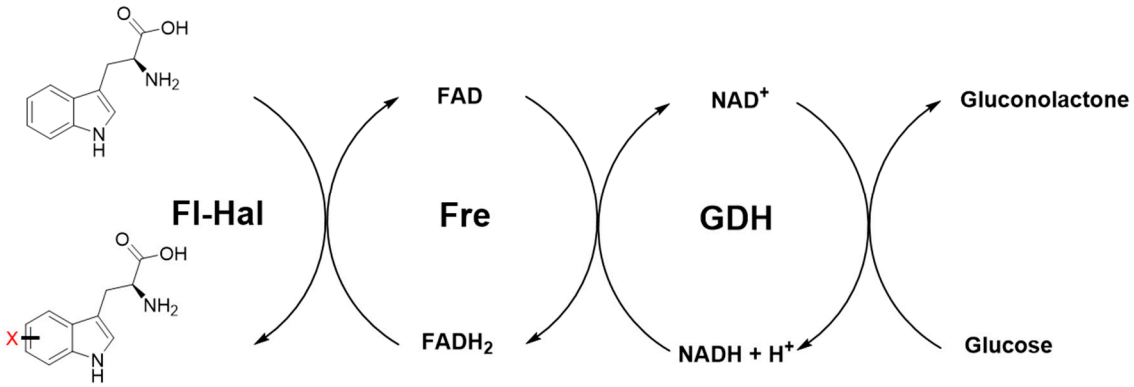

Figure 4. For the regeneration of $FADH_2$, which is needed for the production of the reactive species hypohalous acid, two auxiliary enzymes can be used: a flavin reductase (Fre) for the reduction of FAD and a glucose dehydrogenase for the reduction of NAD^+. Overall, the co-substrate glucose is used in excess, whereas the more expensive cofactors FAD and NAD^+ can be recycled.

Despite the described advantages, the introduction of a cofactor-recycling system renders the biocatalytic reaction more complicated, less robust, and harder to scale-up. Consequently, several studies addressing the drawbacks of cofactor regeneration have been carried out. Andorfer et al. constructed a fusion protein of a flavin halogenase and a reductase by linking the enzymes RebH and RebF. Interestingly, the fusion construct showed higher product titers in vivo for native and non-native substrates than the co-expression of the individual enzymes [48]. Furthermore, the authors suggested that the creation of the fusion protein could simplify evolution assays because the reductase must not be regularly prepared and purified. Following an alternative strategy, Frese and Sewald linked a halogenase and the auxiliary enzymes after translation by employing the bifunctional chemical glutaraldehyde. In their study, the authors overexpressed RebH (Fl-Hal), PrnF (Fre), and an alcohol dehydrogenase (use of isopropanol instead of glucose for the regeneration of NAD^+) individually and subsequently crosslinked the three enzymes to so-called combi cross-linked enzymes aggregates (combiCLEAs) (Figure 5) [49]. By creating combiCLEAs, the system could be reused at least ten times and stored for several months, whereas the purified free RebH showed a considerable loss in activity after being stored for 12 weeks at 4 °C [49].

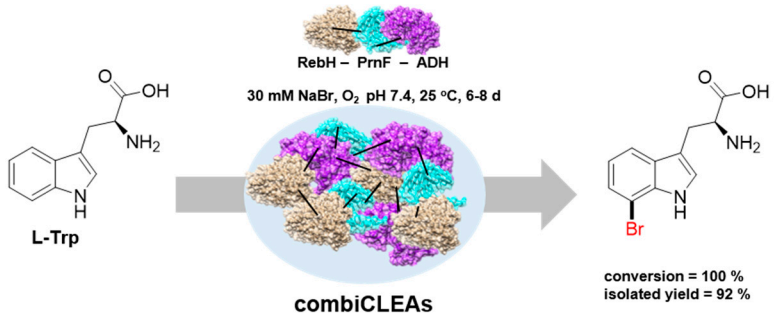

Figure 5. Bromination of L-Trp using RebH cross-linked with the auxiliary enzymes PrnF and ADH (combi cross-linked enzymes aggregates (combiCLEAs)).

Going beyond traditional co-factor recycling schemes, Schroeder et al. showed that the need for auxiliary enzymes can be entirely overcome by using EDTA and light for the regeneration of $FADH_2$ (Figure 6). In their study, the authors reduced enzyme bound FAD with EDTA as the sacrificial electron donor and light at 450 nm. In this way, 58% of a 0.5 mM L-Trp solution was chlorinated within 25 h by using the flavin halogenase PyrH. Although the light driven cofactor recycling approach had a clearly lower turnover frequency (0.72 h^{-1}) compared to the enzymatic regeneration (3.0 h^{-1}), this study described the production of chlorinated compounds by applying only light, halide salt, air, and $FADH_2$ in aqueous solution [50].

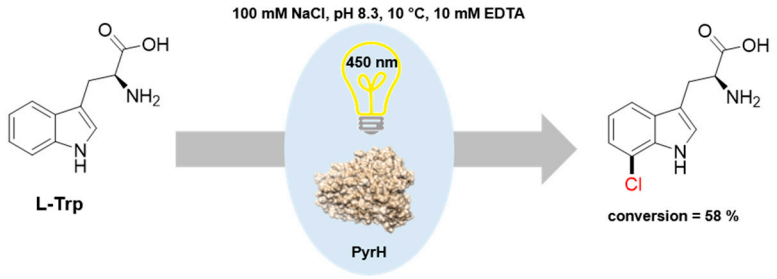

Figure 6. Chlorination of L-Trp by applying the flavin-dependent halogenase PyrH, EDTA, and light.

Another approach to circumvent the use of auxiliary enzymes was developed by Ismail et al. [51]. Employing a set of three tryptophan halogenases (PyrH, Thal, and RebH) in combination with an NADH mimic for FAD^+ reduction, L-tryptophan could be selectively halogenated at the 5-, 6-, or 7-position of the indole moiety, respectively (Figure 7). Notably, the biocatalysis reactions using the NADH mimic showed a 3.5–4.7 times faster initial rate compared to the enzymatic cofactor regeneration system [51].

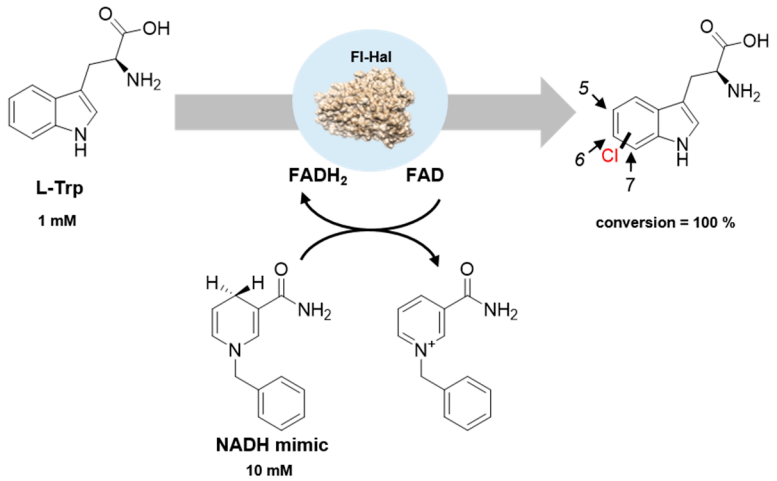

Figure 7. Chlorination of L-Trp using different Fl-Hals (PyrH, Thal, and RebH) applying an NADH mimic for the regeneration of FAD.

4. Strategies to Scale-up Biocatalytic Aromatic Halogenations

Flavin-dependent halogenases selectively install halides into small molecules under mild reaction conditions, thus providing a promising green alternative to the often toxic catalysts used in traditional organic halogenations. However, restrictions associated with the biocatalytic approach such as low expression yields and limited stability of the enzymes, as well as a narrow substrate scope currently still hinder industrial application. In order to broaden the application potential of flavin-dependent halogenases, several studies addressing the above enumerated drawbacks have been carried out in the last decade.

4.1. Protein Overexpression and Stabilization

The first attempt to upscale enzymatic halogenation was carried out by the group of Lewis [52]. In this study, the authors increased the expression level of the soluble halogenase RebH on a scale sufficient for preparative biocatalysis. Co-expression of chaperones (GroEL and GroES) in tandem with cell culture condition optimization led to a 10-fold increase of enzyme concentration in *E. coli* (111 mg L^{-1}) compared to previously reported values [24]. In the same study, a regeneration system utilizing glucose dehydrogenase (GDH) together with a fusion protein of reductase RebF and maltose binding protein (MBP) for preparative scale reactions (10 mg) was developed. High halogenation yields were observed for the transformation of several non-natural bioactive substrates including indoles and naphthalenes highlighting the synthetic utility of the enzyme (Figure 8). In order to explore the accessible synthesis scale, 100 mg of L-tryptophan were used as a substrate for chlorination by crude cell lysate of RebH. Although the substrate was not fully converted, the enzyme remained active under these conditions, and a 69% final yield could be obtained [52].

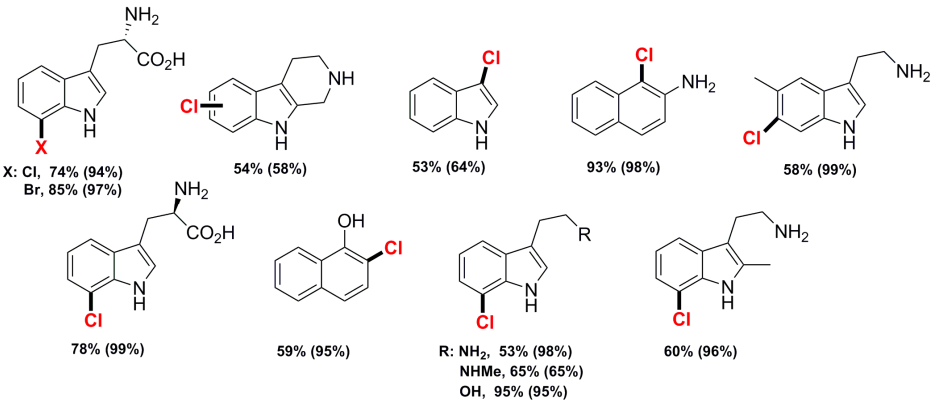

Figure 8. Product structure and isolated yields of preparative scale RebH catalyzed halogenation. HPLC conversion yields are shown in brackets.

The preparative efficiency of RebH has also been explored by Frese and Sewald who developed a multifunctional biocatalytic system exploiting the technique of cross-linked enzyme aggregates (combiCLEAs, *vide infra*). Using this enzymatic preparation, complete bromination of L-tryptophan was achieved after eight days, and 1.813 g of L-7-bromotryptophan TFA were isolated in high purity (isolated yield 92%). CombiCLEA formation was found to increase significantly the long-term stability of RebH, thus facilitating the enzymatic regioselective halogenation for gram scale synthesis [49]. In the same manner, the Micklefield group halogenated non-natural substrates such as anthranilamide and tryptophol using CLEAs of four different Fl-Hals as the first step of a chemoenzymatic reaction [5].

The instability of flavin-dependent halogenases in certain reaction conditions has also been addressed by directed evolution. The Lewis group was among the first to generate a thermostable halogenase variant by employing three rounds of error prone PCR and recombinations. The obtained RebH variant, which contained eight mutations, was characterized by an 18 °C increased melting temperature compared to the wildtype enzyme. However, the catalytic efficiency of the mutant was significantly decreased at 21 °C owing to a lower conformational flexibility of the enzyme [53]. Exploiting a high throughput assay for rapid library screening, Schnepel et al. also evolved thermostable flavin-dependent halogenases. In their study, a doubly mutated variant was found with a 10 °C increase of its T_m compared to the wildtype enzyme. Unlike the wildtype enzyme, which significantly lost its activity after 2–3 h of incubation at 40 °C, the new variant, named Thal-GR, remained active even after 6 h of incubation at this temperature. In addition to the increased catalyst stability, the specific activity of the mutant Thal-GR was also 2.5 times higher than that of the wildtype [54]. Based on these results, the same group published a comprehensive study to further improve the thermostability of the generated variant Thal-GR three years later. Combining different mutagenesis strategies, the authors identified seven variants with higher thermostability compared to the native enzyme. A variant called Thal-GLV exhibited a good stability-activity relationship as it showed a significant increase in thermostability (ΔT_{50} = 16 °C, ΔT_m = 23.5 °C) compared to the wildtype, while exhibiting a 2.4-fold increase in activity at 25 °C. The stabilizing effect of the mutations has been linked to an increased association of the halogenase monomers to form homodimers in solution. Moreover, a crucial site with a strong effect on substrate selectivity was also identified. The single mutant Thal-S359G showed a distinct preference towards L-tryptophan, and only 17% bromination of D-tryptophan was observed compared to >99% in the case of the wildtype enzyme [55]. In a complementary approach to identify thermostable halogenases, the Micklefield group identified a thermophilic tryptophan halogenase (Th-Hal) from a thermophilic and halotolerant strain of *Streptomyces* via computational genome mining. The newly discovered thermostable flavin halogenase was characterized by an approximately 10 °C higher melting temperature (T_m = 47.8 °C) than other known Fl-Hals such as RebH, SttH, PrnA, KtzR, and PyrH. Combining this thermostable enzyme with a thermophilic flavin reductase (Th-Fre) from *Bacillus subtilis* WU-S2, the authors established a more efficient and robust biocatalytic system that led to 74%

conversion of a 0.5 mM solution of tryptophan at 45 °C in contrast to other known halogenases, which gave significantly lower conversions (around 40% for PyrH) using the same reaction conditions [47].

4.2. Substrate Scope and Regioselectivity

To assess the substrate scope of flavin-dependent halogenases, Frese et al. showed that RebH can halogenate substrates beyond the native L-tryptophan such as C-5- and C-6-substituted fluoro-, amino-, chloro-, hydroxy-, and methyl-tryptophans [56]. Subsequently, Shepherd et al. successfully submitted the non-indolic substrates kynurenine, anthranilamide, anthranilic acid, and other anilines to the regiocomplementary tryptophan halogenases PyrH and PrnA highlighting that also these enzymes possess substrate promiscuity, which can be exploited in applications (Figure 9) [57].

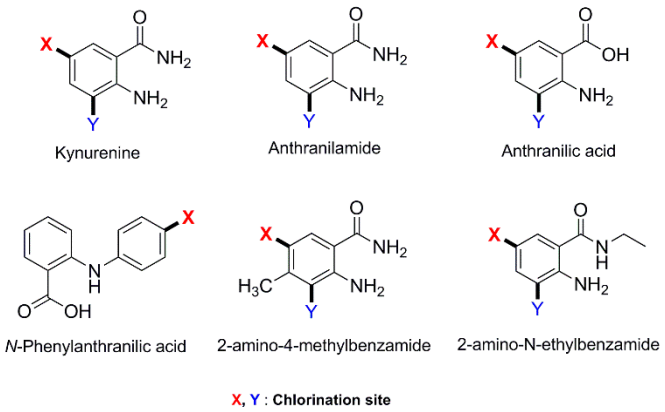

Figure 9. Non-indolic substrates accepted for chlorination. X: PyrH halogenation site, Y: PrnA halogenation site.

The Tsodikov group investigated the accepted substrate spectrum of the halide versatile phenolic halogenase PltM. The authors found that PltM can halogenate several small phenolic and aniline derivatives. Encouraged by these findings, the study was successfully continued towards more bulky compounds including FDA approved drugs and natural products such as terbutaline, fenoterol, resveratrol, and catechin (Figure 10) [45]. Notably, no halogenation activity was observed in the case of a nitrobenzene derivative due to the strongly electron withdrawing nitro group that prevented the halogenation of the substrate.

Tailoring the substrate scope of enzymes towards desired molecules has also been approached through protein engineering. Interestingly, even enzymes exhibiting very little initial substrate promiscuity could be altered in this way. Following a substrate walking approach, Payne et al., for example, extended the substrate scope of the natural tryptophan halogenase RebH towards the significantly larger biologically active compounds yohimbine and carvedilol (Figure 11). Starting with a thermostable mutant of RebH (1-PVM), the authors performed three rounds of random mutagenesis and screening and could generate variants that selectively halogenate a number of unnatural substrates with a range of sizes, substitutions, and biological activities. Although the majority of the beneficial mutations were found close to the active site, a crucial mutation (A442V) was located distal to the active center. This finding highlights the worth of random mutagenesis in directed evolution strategies as this technique may identify amino acid positions that are difficult to predict via rational design strategies [58].

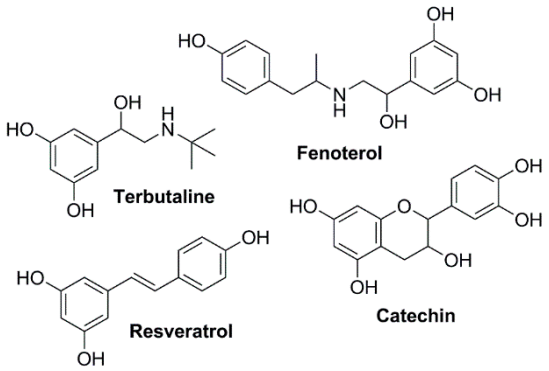

Figure 10. FDA approved drugs (terbutaline, fenoterol) and natural products (resveratrol, catechin) accepted by the phenolic halogenase PltM [45].

Enzyme engineering has also been employed to alter the regioselectivity of halogenases in order to control the site of halogenation in small molecules. In a first example of this approach, the structure of PrnA, a tryptophan 7-halogenase, was analyzed, and three aromatic residues around the enzyme's active site were mutated to alanine. One identified variant, F103A, was found to halogenate both at the C7 and C5 position of the tryptophan indole ring exhibiting a 2:1 ratio of the products. The exchange of the large amino acid phenylalanine with the smaller alanine allowed the substrate to adopt its "native" orientation but also bind in a similar mode as in the tryptophan 5-halogenase PyrH [59]. In a similar manner, structure guided mutagenesis was employed by Shepherd et al. in 2016 to alter the regioselectivity of tryptophan 6-halogenase SttH. The generated triple mutant SttH L460F/P461E/P462T transformed 75% of the supplied tryptophan into the C5-halogenated product, whereas the wildtype enzyme exhibited almost the same activity towards the C6-halogenated product [60]. In the same year, Lewis and co-workers published a thorough study in which RebH was engineered for altered regioselectivity. High throughput MALDI-MS analysis was used to screen the transformation of deuterium substituted probe substrates. Utilizing a previously evolved variant, which was able to chlorinate tryptamine with 99% selectivity towards the C7 position, the authors identified two variants, named 8F and 10S, which could selectively chlorinate tryptamine at the C6 and C5 site, respectively. Additional reverse mutations (mutations that lead to the initial starting sequence) proved beneficial for the enzymes activity while maintaining the achieved selectivity [61]. In a structure-based protein engineering approach, Moritzer et al. substituted five residues in the substrate binding site of Thal by the corresponding amino acids of the closely related tryptophan halogenase RebH to switch regioselectivity from the 6- to 7-position. The resulting variant, named Thal-RebH5, exhibited a 95% change in regioselectivity towards C7-halogenation for both chlorination and bromination of L-tryptophan, further confirming the importance of the binding pose of the substrate for the regioselectivity outcome [62]. Building on previous learnings, Payne et al. recently reported the first enantioselective desymmetrization of methylenedianinelines catalyzed by engineered RebH variants. Docking simulations and structure guided mutagenesis afforded mutants with improved activity and altered selectivity [63].

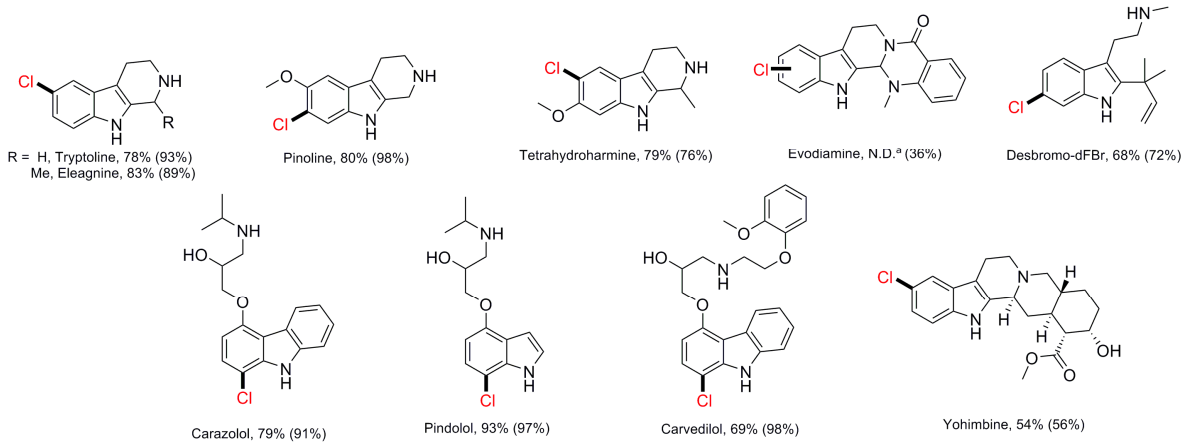

Figure 11. RebH catalyzed halogenation of various substrates indicating the isolated product yields and conversion yields (in brackets). [a] Multiple closely eluting products were observed and were not individually isolated.

4.3. Biosynthetic Pathways

Fueled by the discovery of novel halogenases with interesting properties, the incorporation of halogenases into biosynthetic pathways has become an attractive tool to increase the diversity of accessible products and to synthesize bioactive molecules or valuable intermediates.

In 2010, Roy et al. carried out the first introduction of a halogenase gene into a synthetically unrelated pathway. In their study, the authors complemented the biosynthesis of the uridyl peptide antibiotic (UPA) pacidamycin from *Streptomyces coeruleorubidus* with the *prnA* gene expressing the tryptophan 7-halogenase PrnA. The resulting strain could produce the "unnatural" chloropacidamycin in a yield of 1 mg per liter culture with a ratio to its non-chlorinated analogue as high as 4:1. The installed chlorine then served as a handle, enabling the selective chemical functionalization of the antibiotic to yield a variety of derivatives [64]. Today, halogenase genes have also been integrated into medicinal plant metabolisms in order to produce chlorinated indole alkaloids with altered and/or improved pharmacological properties. Two prokaryotic halogenase genes encoding 7-tryptophan halogenase RebH or 5-tryptophan halogenase PyrH together with RebF as the partner reductase were introduced into *Catharanthus roseus*. It was reported that both halogenases function productively in the plant cell environment leading to the formation of the corresponding halotryptophans (7- or 5-halotryptophan), which subsequently enter the metabolic pathway of the plant cells to form chlorinated indole alkaloids. However, the efficiency of tryptophan decarboxylase, an enzyme that converts tryptophan to tryptamine, was significantly lower towards the halogenated substrates. This led to an accumulation of substantial levels of a non-natural intermediate, which had an adverse effect on the morphology and the growth rate of tissues [65]. To overcome this negative outcome, Glenn et al. employed site directed mutagenesis to generate a RebH mutant, which preferentially halogenates tryptamine rather than the native substrate tryptophan. Using this approach, the accumulation of 7-chlorotryptophan and its negative effect on the cell metabolism could be bypassed [66].

The potential application of plants as platforms for the manufacture of rare and valuable halogenated products has been also studied by Fräbel et al. Tryptophan 6-halogenase from *Streptomyces toxytricini* SttH has been successfully integrated into tobacco plants allowing the formation of chlorotryptophan in the cytosol when co-expressed with the flavin reductase RebF. The introduction of an additional flavin-dependent halogenase (RebH) into the metabolic setup of the plant led to the formation of 6,7-dichlorotryptophan, while a novel precursor of monoterpenoid indole alkaloids, 6-chlorotryptamine, was observed when SttH was co-expressed with tryptophan decarboxylase (TDC). Notably, SttH and RebH remained active even in the absence of the partner reductase when localized in chloroplasts, indicating the presence of sufficient reduction equivalents in these organelles for halogenation reactions [67].

Following a combinatorial biosynthesis approach, Wang et al. combined enzymes from different sources in *Escherichia coli* to generate valuable compounds. Using a multiple vector system, the fungal Fl-Hal Rdc2 was co-expressed with tyrosine ammonia-lyase (TAL), 4-coumarate:CoA ligase (4CL), and stilbene synthase (STS), resulting in a novel chlorinated derivative in addition to resveratrol. This novel compound was identified as 2-chloro-resveratrol highlighting the broad substrate scope of Rdc2 [68].

4.4. Chemoenzymatic Synthesis

Chemoenzymatic synthesis has proven to be a powerful tool for the manufacture of complex compounds offering numerous advantages such as higher yields, decreased costs, environmental benefits, and high selectivity compared to purely chemical strategies [69]. In this context, the site-specific enzymatic halogenation of natural products can provide halogenated compounds that are of interest for the further functionalization by cross-coupling chemistry. The combination of chemo- and enzyme catalysis can thus lead to the formation of C-C, C-N, C-F, and C-O bonds from C-H positions, which were beyond the scope of chemocatalytic activation chemistry.

Chemical modification through Pd catalyzed cross-coupling of a biosynthetically halogenated natural product has been reported by the group of Goss. Employing mild reaction conditions to account for the thermal instability of the substrate chloropacidamycin, the authors obtained various 7-aryl-pacidamycin derivatives in high yields. This functionalization approach could not only be applied on purified natural compounds, but also worked when using crude extracts of the halogenated substrates [64].

In a similar manner, Pd catalyzed Suzuki–Miyaura cross-coupling (SMC) reactions have been adopted by the O'Connor group to synthesize aryl and heteroaryl analogues of monoterpene indole alkaloids. Exploiting the technique of mutasynthesis in *C. roseus* hairy root cultures, the authors introduced a chemical halogen handle into the indole moiety of the monoterpene indole alkaloids followed by the subsequent chemical derivatization using the crude cell extracts. Based on their promising results, the authors further scaled up the cross-coupling reactions using 16 g of the crude hairy root extract to obtain milligram quantities of three alkaloid analogs [70]. Durak et al. further expanded the application scope of the chemoenzymatic strategy by derivatizing non-native bioactive small aromatic molecules such as carvedilol, pindolol, thenalidine, and tryptoline and its derivatives. Two RebH variants, 3-SS and 4-V, were used for the selective halogenation of the aforementioned compounds followed by subsequent Pd catalyzed cross-coupling reactions with boronic acids, amines, and trifluoroethanol on the crude biocatalysis extracts to generate new C-C, C-N, and C-O bonds [71].

Although the advantages of chemoenzymatic reactions for the synthesis of demanding and complex compounds has been successfully demonstrated, the detrimental effects of protein contaminants on the metal catalyst require an intermediate extraction step of the halogenated precursor that limits process scale-up. To address this challenge, the group of Sewald utilized combiCLEAs of several Fl-Hals in a single pot approach [72]. In this study, a bromine substituent was introduced biocatalytically at the C5, C6, or C7 position of L-tryptophan on a preparative scale. After filtration of the biocatalyst, a SMC reaction led to an efficient modification of the halogenated compounds, and the resulting cross-coupling product could subsequently be Boc protected and purified for further applications. This three-step one pot approach is an important proof-of-concept for the development of more sustainable and consecutive biohalogenation-chemocatalytic cross-coupling processes.

Latham et al. further simplified the one pot halogenase-SMC cascade by separating the protein from the Pd catalyst using polydimethylsiloxane membranes (PDMS). Compartmentalization was achieved thanks to the hydrophobic nature of the PDMS membranes, which allowed for the diffusion of non-polar compounds such as substrates and intermediates while the diffusion of polar compounds (enzymes, Pd catalysts, and cofactors) was prevented (Figure 12). Combining this method with halogenase CLEAs led to higher concentrations of the aryl halide and reduced Pd loading (2 mol%) compared to free pure protein reactions (Pd loading: 10 mol%) [5].

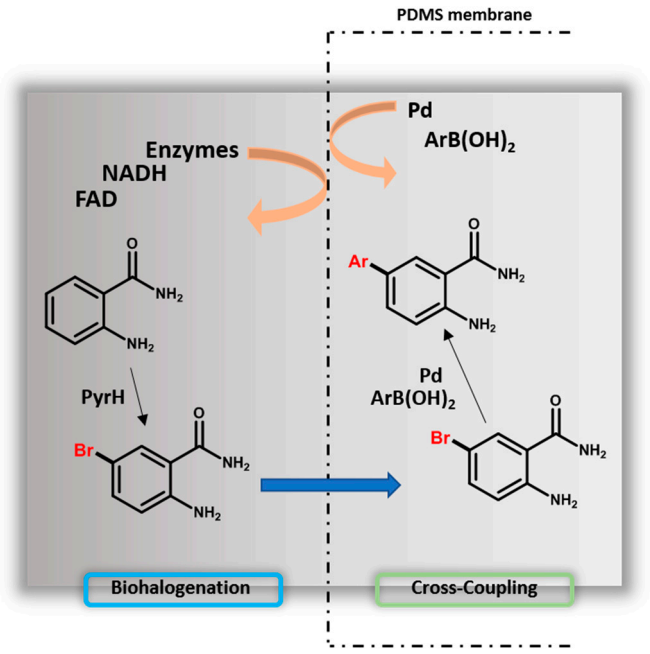

Figure 12. Polydimethylsiloxane (PDMS) compartmentalization. PDMS membrane permits diffusion of non-polar compounds (substrates, intermediates), while polar compounds such as enzymes, Pd catalysts, and cofactors are blocked. This compartmentalization overcomes the incompatibility of the enzyme and transition metal that reduces the efficiency of the chemoenzymatic process. Figure adapted from [5].

Further pioneering the one pot chemoenzymatic reactions, the Goss group combined synthetic biology with synthetic chemistry. After an optimization of the cultivation media of the engineered strains to minimize the impact of media components on the cross-coupling efficiency, the authors developed a biological system for the generation of brominated metabolites that can synchronously be modified through cross-coupling in living cultures (Figure 13). This approach provides the advantage of a simplified product isolation and a continuously producing biosynthetic system [6].

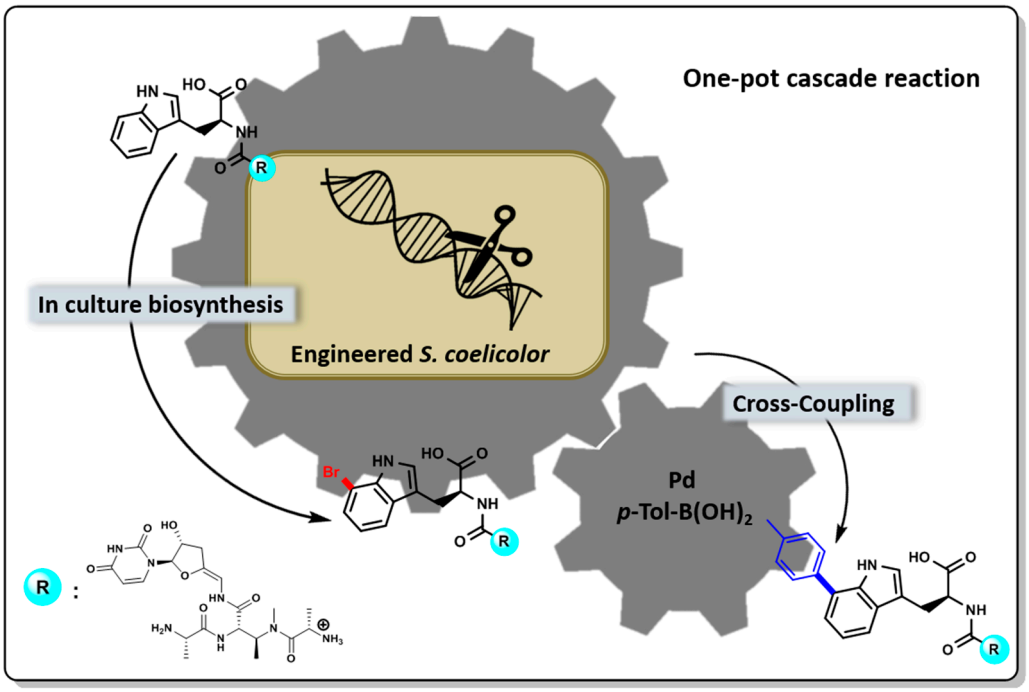

Figure 13. In culture biosynthesis and cross-coupling of Br-pacidamycin-D. The engineered strain of *S. coelicolor* RG-1104 grown in the presence of sodium bromide is able to produce Br-pacidamycin-D. The brominated compound is subsequently modified through Pd catalyzed Suzuki–Miyaura cross-coupling reactions under mild conditions.

5. Conclusion

The selective halogenation of small molecules is of high interest for many industries and is especially sought after in the pharmaceutical industry and agrochemistry [2,3]. Today, biocatalytic halogenations are promising tools for the preparation of small quantities of selectively derivatized products (Table 2), which can be used in medicinal chemistry, or serve as intermediates for the total synthesis of complex molecules (Chapter 4.4). However, to our knowledge, more than a gram quantity of halogenated target compound has not yet been prepared (Table 2), illustrating two of the current challenges in aromatic halogenase catalysis: the enzymes suffer from low productivity and limited scalability. Thus, in order to expand the application scope of flavin-dependent halogenases, further studies aimed to broaden the substrate scope and increase the robustness of these enzymes are required. These can entail bioinformatic studies to identify additional members of the flavin-dependent halogenase superfamily with an extended substrate scope [46] or higher thermostability [47]. Additionally, approaches such as the "catalophore" strategy might be successful in the identification of novel flavin-dependent halogenases [73]. In this bioinformatic method, Steinkellner et al. carried out a structure guided search for the discovery of ene-reductases by predicting enzyme activity based on three-dimensional constellations of functional groups.

Following the discovery of promising wildtype enzymes, engineering strategies that should equally consider enzyme stability and activity will, at least to some extent, inform about the structure-function relationship in flavin-dependent halogenases. In this respect, in silico design based on energy functions might be a very valuable tool, as methods such as Rosetta_ddg and FireProt have been proven to optimize protein structures successfully [74–78]. The obtained knowledge may then lead to the development of tailored enzyme variants, which through their increased stability, activity and, robustness may facilitate industrial usage. Looking forward, engineering flavin-dependent halogenases towards the acceptance of more electron poor substrates [79] through increasing the electrophilicity of the halogenating species will be a way to further enhance their applicability. As proposed by Latham et al., reaction engineering aspects, such as optimizing oxygen concentration in

the reaction medium and the management of the level of reactive oxygen species could be further levers to increase flavin-dependent halogenase productivity [2].

Table 2. Examples of preparative scale applications of flavin-dependent halogenases.

Enzyme	Scale (mg)	Substrate	Halogen Insertion	Conversion Yield %/Isolated product yield %	Ref.
PyrH (combiCLEAs)	136	Anthranilamide	Br	n.d. [a]	[5]
PyrH and SttH (combiCLEAs)	15	3-Indolepropionic acid			
RebH (combiCLEAs)	24	Tryptophol			
Th-Hal (purified)	16	L-tryptophan	Cl	-/62.5	[47]
	18	1-methyl-L-tryptophan		-/12	
	17	5-hydroxy-L-tryptophan		-/16	
	16	L-kynurenine,		-/21	
	11	Anthranilic acid		-/8	
	11	Anthranilamide		-/19	
RebH (combiCLEAs)	1000	L-tryptophan	Br	100/-	[49]
	40	D-tryptophan		57 /	
	40	L-5-hydroxytryptophan		53 /	
RebH (purified)	10	L-tryptophan	Cl	94/74	[52]
				97/85	
RebH (purified)	10	D-tryptophan	Br	99/78	
		Tryptamine	Cl	98/53	
		N-Ω-methyltryptamine		65/65	
		Tryptophol		95/95	
		2-methyltryptamine		96/60	
		5-methyltryptamine		99/58	
		2,3-disubstituted indole tryptoline		58/54	
		Indole		64/53	
		2-aminonaphthalene		98/93	
		1-naphthol		95/59	
RebH (lysate)	100	L-tryptophan	Cl	74/69	
RebH (purified)	10	L-tryptophan	Cl	-/69	[53]
		2-methyltryptamine		-/56	
		2-aminonaphthalene		-/62	
		Tryptoline		-/67	
PyrH (combiCLEAs)	50	L-tryptophan	Br	-/76	[54]
Thal (combiCLEAs)	50	L-tryptophan		-/61	
RebH (combiCLEAs)	150	L-tryptophan		-/84	
SttH (combiCLEAs)	100	Anthranilamide	Cl	-/25	[60]
RebH variants 0S/8F/10S (purified)	10	Tryptamine	Cl	98/73/78 [b]	[61]
Thal-RebH5 variant (lysate)	30	L-tryptophan	Cl	30/-	[62]
			Br	15/-	
RebH variant 4-V (purified)	10	t-Bu dianiline methane	Cl	80/-	[63]

RebH variant 3-SS (purified)	10	Tryptoline Eleagnine Pinoline Tetrahydroharmine	Cl	-/78 -/83 -/80 -/79	[58]
RebH variant 4-V (purified)	10	Debromodeformylflustrabromine Yohimbine Pindolol Carazolol Carvedilol	Cl	-/68 -/54 -/93 -/79 -/69	
RebH variant 3-SS (purified)	10	Tryptoline	Br	n.d. [a]	[71]
RebH variant 4-V (purified)	10	Carvedilol Pindolol Thenalidine	Cl Br		
RebH/ThaI/PyrH (combiCLEAs)	150	L-tryptophan	Br	100 [c]	[72]

[a] Enzymatic halogenation yields not determined for these chemoenzymatic cascade reactions. [b] Isolated yields % for the three different variants studied (0S/8F/10S). [c] Conversion yield % was 100 for all cases of immobilized Fl-Hals studied.

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