CHAPTER FOUR

Application and Modification of Flavin-Dependent Halogenases

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Abstract

The application of flavin-dependent halogenases is hampered by their lack of stability under reaction conditions. However, first attempts to improve halogenase stability by error-prone PCR have resulted in mutants with higher temperature stability. To facilitate the screening for mutants with higher activity, a high-throughput assay was developed. Formation of cross-linked enzyme aggregates (CLEAs) of halogenases has increased halogenase lifetime by a factor of about 10, and CLEAs have been used to produce halogenated tryptophan in gram scale. Analyses of the substrate specificity of tryptophan halogenases have shown that they accept a much broader range of substrates than previously thought. The introduction of tryptophan halogenase genes into bacteria and plants led to the in vivo formation of peptides containing halogenated tryptophan

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or novel tryptophan-derived alkaloids, respectively. The halogen atoms in these compounds could be chemically exchanged against other substituents by cross-coupling reactions leading to novel compounds. Site-directed mutageneses have been used to modify the substrate specificity and the regioselectivity of flavin-dependent tryptophan halogenases. Since many flavin-dependent halogenases only accept protein-bound substrates, enzymatic and chemoenzymatic syntheses for protein-tethered substrates were developed, and the synthesized substrates were used in enzymatic halogenation reactions.

1. INTRODUCTION

Flavin-dependent halogenases are a promising group of halogenating enzymes. They catalyze halogenation reactions with high regioselectivity and without the formation of by-products except water and thus provide the opportunity for industrial-scale production of chlorinated pharmaceuticals and organic compounds. However, the low stability of halogenases under reaction conditions, leading to fast inactivation, hampers their application in industry. Thus, it is of great importance to increase their stability under reaction conditions. While their high substrate specificity is of advantage in their natural environment, it is a disadvantage for their extensive application and therefore needs to be broadened. Furthermore, all so far known flavin-dependent halogenases have a rather low reactivity with k_{cat}-values around 1–7 min^{-1} which also needs considerable improvement. Flavin-dependent halogenases are a two-component system consisting of a flavin reductase providing the actual halogenase with $FADH_2$ produced from NADH and FAD. For industrial application of the two-component system, effective regeneration of the reducing agents is also required. The best-investigated flavin-dependent halogenases are the tryptophan 7-halogenases PrnA from pyrrolnitrin biosynthesis (Keller et al., 2000) and RebH from rebeccamycin biosynthesis (Sanchez et al., 2002), the tryptophan 5-halogenase PyrH from pyrroindomycin B biosynthesis (Zehner et al., 2005), and the tryptophan 6-halogenase Thal/ThdH from thienodolin biosynthesis (Milbredt, Patallo, & van Pée, 2014; Seibold et al., 2006) which all accept L- and D-tryptophan as their natural substrates. Additionally, work on halogenases catalyzing the halogenation of peptidyl carrier protein (PCP)-bound pyrrole carboxylic acid or PCP-tethered peptides showed how complex natural substrates of halogenases can be produced by enzymatic or chemical synthesis. The last 10 years have seen considerable progress toward the understanding and improvement of the properties of halogenases which will be described later.

2. INACTIVATION OF HALOGENASES UNDER REACTION CONDITIONS

2.1 Improvement of Halogenase Stability by Error-Prone PCR

Flavin-dependent halogenases were found to completely lose their activity already 2–3 h after the start of the reaction (Fig. 1). One possible reason for this instability could be a simple temperature sensitivity, but there is also the possibility of an altered protein structure due to reaction with the halogenating intermediate or other side products formed at the active site. The halogenating agent produced by the enzymes is hypohalous acid which is directed to the substrate through a 10-Å long tunnel (Dong et al., 2005). This hypohalous acid might not only react with the substrate but also with amino acids located along the tunnel. For example, the reaction of amino acids with hypochlorous acid can lead to formation of chloramines, 3-chlorotyrosines or 3-hydroxytryptophans (Stadtman & Levine, 2006). On the other hand, the reaction of $FADH_2$ with oxygen can lead to the formation of hydrogen peroxide or even superoxide anion radical. These could in turn react with the halogenase and damage the protein structure and

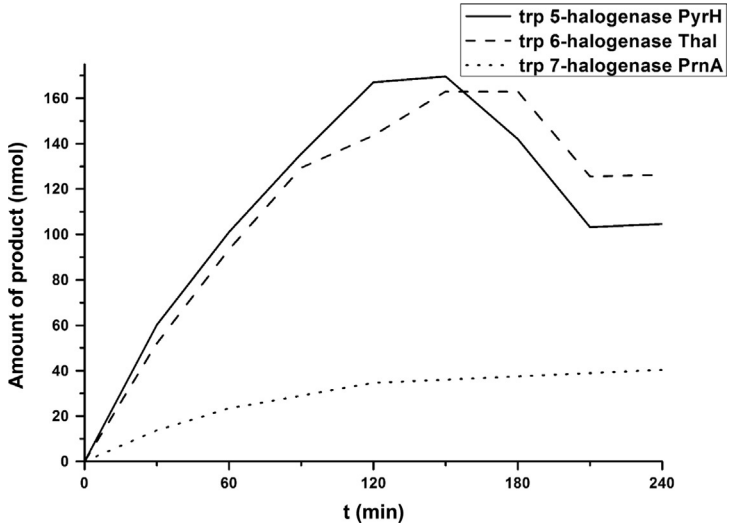

Fig. 1 Time course of conversion of tryptophan to 5-chlorotryptophan, 6-chlorotryptophan, and 7-chlorotryptophan by PyrH, Thal/ThdH, and PrnA, respectively. The amount of tryptophan at the start of the reaction was 400 nmol for PyrH and Thal/ThdH and 50 nmol for PrnA. The reason for the observed decrease in formed product by PyrH and Thal/ThdH after 2.5–3 h is not known.

thereby result in its loss of activity. Product inhibition was also considered as a possible reason for the loss of activity during incubation under reaction conditions. Up to now, there are no studies dealing with the mechanisms of inactivation of flavin-dependent halogenases. Nevertheless, the last few years have seen investigations dealing with ways to increase the stability of flavin-dependent halogenases.

Muffler, Kuetchou Ngnigha, and Ulber (2010) characterized the activity of the tryptophan-5-halogenase PyrH from *Streptomyces rugosporus* LL-42D005 which is involved in the biosynthesis of the antibiotic pyrroindomycin B (Zehner et al., 2005). Checking the deactivation constants of PyrH, they recognized the instability of PyrH at 30°C. After 3 h at this temperature, the enzyme showed nearly no activity, anymore. PyrH was longer active at lower temperatures (after 6 h of incubation, 70% activity at 18°C and 90% activity at 0°C were left). For this reason, they recommended a lower temperature for the enzyme activity assay than the normally used 30°C. However, this results in a decrease of the activity of the halogenases, since most of them have their temperature optimum around 30°C, eg, PrnA at 30°C (Flecks et al., 2008) and Thal/ThdH at 32°C (Ernyei, 2008). The addition of compounds known to stabilize enzymes such as glycerol, glucose, or sucrose had no stabilizing effect under reaction conditions (Fig. 2).

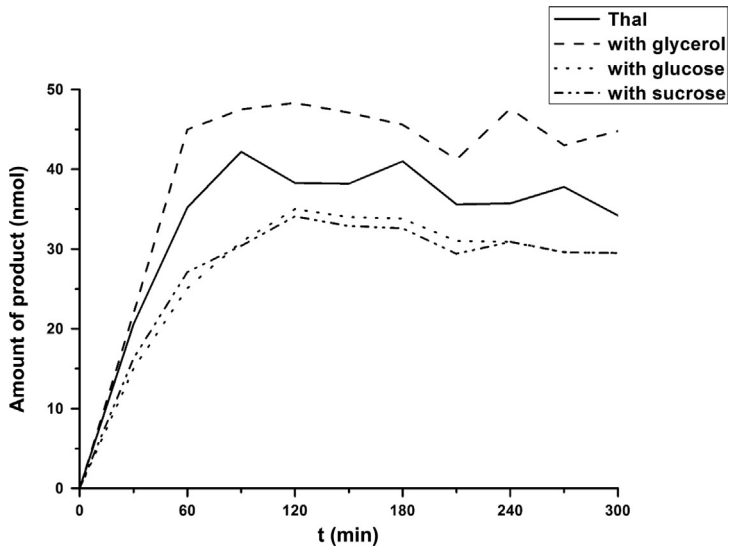

Fig. 2 Time course of conversion of tryptophan to 6-chlorotryptophan by Thal/ThdH in comparison with reactions containing glycerol (2.2%), glucose (250 mg/mL), and sucrose (250 mg/mL), respectively. The amount of tryptophan at the start of reaction was 60 nmol.

Investigations into the linearity of the reaction of the three tryptophan halogenases PrnA, PyrH, and Thal/ThdH showed that the reaction velocities of these enzymes are only linear for a short period of time of about 30 min and then start to decline. This is of high importance for the determination of kinetic data (Fig. 1). Therefore, kinetic data should always be taken in the first minutes of the reaction to be sure to stay in the linear region. However, since activity measurements have to be done by HPLC analysis, rather high amounts of the enzymes are required to make sure that the product peaks are high enough for quantification.

The first problem to solve when working with flavin-dependent halogenases is the issue of overexpression of the halogenase gene. Until now, nobody has achieved to show halogenating activity in a wild-type strain producing a halogenase. Only after high overexpression of the halogenase gene and production of soluble halogenase, halogenating activity can be detected. Overexpression of halogenase genes in soluble form in *E. coli* seems to be quite problematic and forced the group of Jared Lewis to construct a maltose-binding protein halogenase fusion protein to obtain enough of the tryptophan 7-halogenase RebH and the flavin reductase RebF from rebeccamycin biosynthesis in *Lechevalieria aerocolonigenes* for their work (Payne, Andorfer, & Lewis, 2013; Sanchez et al., 2002). In our hands, expression of halogenase genes from different sources in *Pseudomonas* strains works excellent with extremely high overexpression (Keller et al., 2000; Seibold et al., 2006; Zehner et al., 2005), although sometimes the clones completely lose their ability to express the genes. In these cases, fresh transformation of *Pseudomonas* with the plasmids isolated from *E. coli*, not from *Pseudomonas*, will solve the problem.

During their further work with RebH, Poor, Andorfer, and Lewis (2014) also encountered the problem of instability. During the enzyme reaction in a preparative scale, an extensive precipitation of RebH occurred. In their opinion, an increase of thermostability could lead to a longer lifetime of RebH, an increase of stress tolerance and might also enable higher reaction temperatures. Due to the lack of a high-throughput assay, every single *E. coli* clone obtained by error-prone PCR had to be grown separately, lysed, and was then incubated at elevated temperatures, followed by incubation under reaction conditions for 16 and up to 48 h, and analyzed for overall product formation by HPLC. The mutations of variants showing higher product formation after this treatment were combined using overlap extension PCR. This procedure was repeated twice, and the most promising variants of RebH were characterized in detail.

Table 1 Kinetic Data for the Halogenation of 2-Methyltryptamine by the Wild-Type Tryptophan 7-Halogenase RebH and Its 3-LSR Variant at Different Temperatures (Poor et al., 2014)

Enzyme	T (°C)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
RebH wild type	21	16.8 ± 3.8	0.060 ± 0.0057	3.6×10^{-3}
3-LSR variant	21	40.2 ± 3.7	0.013 ± 0.00036	3.3×10^{-4}
RebH wild type	40	280.1 ± 18.4	0.25 ± 0.0095	8.8×10^{-4}
3-LSR variant	40	202.5 ± 12.7	0.15 ± 0.0093	7.9×10^{-4}

The two RebH variants 3-LR and 3-LSR showed the best conversion and were further characterized. The mutant RebH variant 3-LR showed a 5°C higher optimal temperature and 100% more product than wild-type RebH at its optimal temperature. Additionally, the yield of product for the conversion of several other substrates was higher, compared to the wild-type enzyme. An examination of the reaction profiles showed an about threefold longer lifetime of 3-LSR than the wild-type enzyme. Furthermore, steady-state kinetic data were collected at 21°C and 40°C for wild-type RebH and the mutant 3-LSR. Both enzymes show a similar k_{cat}/K_m at 40°C, whereas the wild type has a significantly higher k_{cat}/K_m at 21°C. This is due to a significantly lower catalytic efficiency of the 3-LSR mutant at 21°C (Table 1; Poor et al., 2014). Thus, the higher product yield of the RebH variant is due to a longer lifetime, compensating a lower catalytic activity. This shows that directed evolution is one way to create better halogenases but seems not to be the most efficient.

2.2 Stabilization of Halogenases by Formation of Cross-Linked Enzyme Aggregates (CLEAs)

Characterization of kinetic parameters such as K_m, k_{cat} and total turnover numbers of different tryptophan halogenases showed that in vitro enzymatic halogenation is an inefficient reaction (Table 2) which is in a large part due to the instability of the halogenases (Dong et al., 2005; Frese, Guzowska, Voß, & Sewald, 2014; Seibold et al., 2006; Yeh, Garneau, & Walsh, 2005; Zehner et al., 2005). Thus, the biotechnological application of these enzymes is still restricted. Enzyme immobilization has been proven as an efficient method to deal with enzyme stability problems. In contrast to soluble enzymes, immobilized enzymes are more stable, easy to handle, and facilitate efficient recovery and reuse. Cross-linked enzyme aggregates (CLEAs) are biocatalysts prepared via physical enzyme aggregation followed

Table 2 k_{cat} and K_m Values of Different Tryptophan Halogenases for the Chlorination of L-Tryptophan

Halogenase	k_{cat} (min^{-1})	K_m (μM)	References
PrnA	6.8	50	Lang et al. (2011)
Thal/ThdH	2.8	110	Seibold et al. (2006)
PyrH	0.5	150	Zehner et al. (2005)
RebH	1.4	2	Yeh et al. (2005)

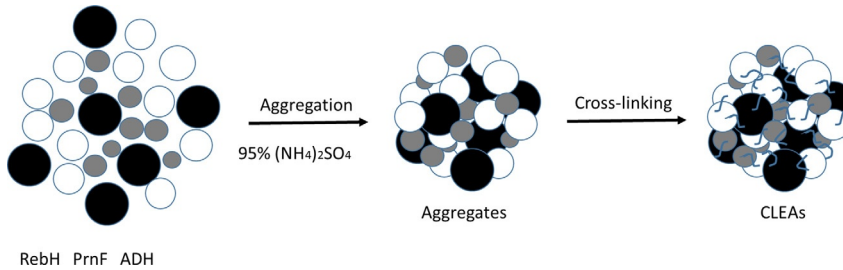

Fig. 3 Scheme for the preparation of CLEAs containing the tryptophan 7-halogenase RebH, the flavin reductase PrnF, and the alcohol dehydrogenase ADH (Frese & Sewald, 2014).

by treatment with a cross-linking agent, most commonly glutaraldehyde (Sheldon, 2011) to covalently link the proteins. Preparation of CLEAs includes two simple steps: precipitation of native enzymes with ammonium sulfate or polyethylene glycol and a cross-linking reaction of free amino groups of lysine residues with neighboring enzyme molecules by addition of glutaraldehyde.

Recently, Frese and Sewald (2014) described the formation of CLEAs for halogenases. These CLEAs contained the tryptophan 7-halogenase RebH from *L. aerocolonigenes* and enzymes required for cofactor regeneration, a flavin reductase from *Pseudomonas fluorescens* BL915 (PrnF; K.-H. van Pée, unpublished) and an alcohol dehydrogenase from *Rhodococcus* sp. (ADH; Fig. 3). Using these CLEAs, halogenation of the natural substrate L-tryptophan as well as D-tryptophan and L-5-hydroxytryptophan was achieved in a gram scale, showing that the long-term stability and the preparative application of tryptophan halogenases can be achieved.

2.2.1 Preparation of CLEAs

1. Inoculate 1.5 L LB medium with an overnight culture of *E. coli* BL21 (DE3) pGro7 containing the plasmid pET28aRebH and the appropriate

antibiotics kanamycin and chloramphenicol. Incubate at 37°C and 150 rpm shaking until OD_{600} reaches 0.4–0.6.

2. Induce the expression of the halogenase gene by addition of 100 μ*M* IPTG and 2 mg/mL arabinose and incubate for further 20 h at 25°C.
3. Harvest cells by centrifugation (4000 × *g*, 30 min, 4°C).
4. Resuspend cells in 25 mL of 100 m*M* Na_2HPO_4, pH 7.4, lyse cells twice by French Press, and centrifuge (10,000 × *g*, 30 min, 4°C).
5. Add 2.5 U/mL flavin reductase PrnF and 1 U/mL alcohol dehydrogenase to the cleared lysate and mix thoroughly.
6. Add 16.2 g of ammonium sulfate (~95% saturation) at 4°C in a tube rotator for 1 h.
7. Add glutaraldehyde to a final concentration of 0.5% (w/v) and incubate for additional 2 h at 4°C in a tube rotator.
8. Centrifuge the resulting CLEAs (10,000 × *g*, 30 min, 4°C) and wash three times with 50 mL of 100 m*M* Na_2HPO_4, pH 7.4.
9. Resuspend the solid biocatalyst in 50 mL of 100 m*M* Na_2HPO_4, pH 7.4, and store overnight at 4°C prior to use.

2.2.2 Halogenation Using CLEAs

CLEAs obtained by the procedure described earlier can be used for batch-wise halogenation of tryptophan (Frese & Sewald, 2014). For this purpose, 30 mL of 15 m*M* Na_2HPO_4, pH 7.4, containing 3 m*M* tryptophan, 100 μ*M* NAD, 10 μ*M* FAD, 5% (v/v) isopropanol, and 30 m*M* NaCl or NaBr (for chlorination or bromination, respectively) are used to resuspend CLEAs in a 50-mL Erlenmeyer flask and incubated under slow stirring at 25°C. After 24 h, the reaction solution is centrifuged for 20 min at 10,000 × *g*, and the supernatant is analyzed by RP-HPLC.

CLEAs can be reused after a washing step with 20 mL of 10 m*M* Na_2HPO_4 buffer, pH 7.4. It has been demonstrated that CLEAs containing RebH can be recycled at least 10 times with no significant loss of activity.

In our hands, the life time of PyrH under reaction conditions was improved up to about 20 h compared to about 2 h for the free enzyme (Fig. 4). However, after about 20 h, the enzyme became inactive. Obviously, the halogenase inside the CLEAs was stabilized by a yet unknown mechanism.

2.2.3 Gram-Scale Halogenation Reaction Using CLEAs

A serious disadvantage of halogenation reactions using tryptophan halogenases is their low overall turnover number that strongly reduces their

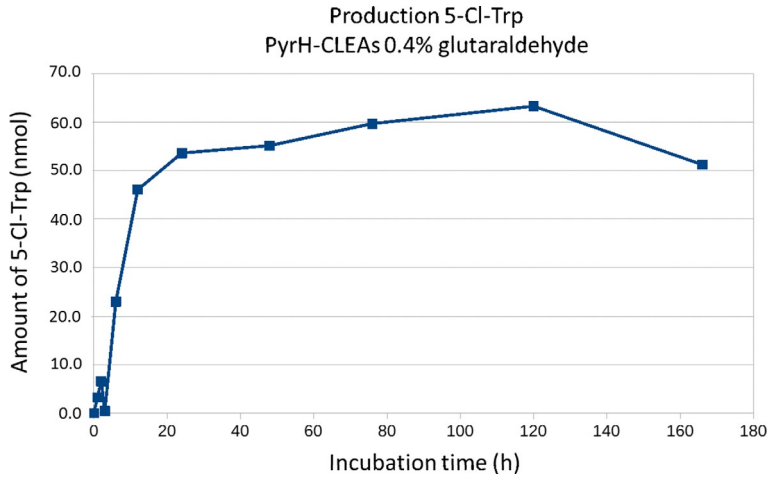

Fig. 4 Plot of the conversion of tryptophan to 5-chlorotryptophan by CLEAs containing the tryptophan 5-halogenase PyrH and the flavin reductase PrnF against time.

industrial application. The CLEA methodology is suitable to overcome stability and enzyme recovery problems. Payne et al. (2013) used 10 L *E. coli* culture overexpressing *rebH* to obtain around 100 mg chlorinated product. Frese and Sewald (2014) achieved gram-scale reaction using CLEAs containing the overproduced halogenase RebH from 6 L *E. coli* culture. The reaction mixture consisted of 15 m*M* Na_2HPO_4, pH 7.4, 1 m*M* tryptophan, 100 μ*M* NAD, 1 μ*M* FAD, 5% (v/v) isopropanol, and 30 m*M* NaBr in a final volume of 5 L at 25°C and 150 rpm. After 8 days, complete conversion of L-tryptophan was observed, yielding 1.8 g of L-Br-tryptophan.

The conversion of tryptophan to 5-chlorotryptophan by CLEAs of the tryptophan 5-halogenase PyrH and the flavin reductase PrnF is shown in Fig. 4.

3. THE BIOCATALYTIC SCOPE OF FLAVIN-DEPENDENT TRYPTOPHAN HALOGENASES

3.1 Substrate Specificity of Tryptophan Halogenases

The first findings about the substrate specificity of flavin-dependent tryptophan halogenases were published in 2001 for the tryptophan 7-halogenase PrnA from *P. fluorescens* BL915 (Hölzer, Burd, Reißig, & van Pée, 2001). It was shown that this enzyme is able to chlorinate a number of tryptophan, indole, and phenylpyrrole derivatives, but only with its natural substrate tryptophan regioselective chlorination in the position 7 of the indole ring

was achieved. With all the compounds different from tryptophan, halogenation proceeded with a relaxed regioselectivity and chlorination mostly occurred at the electronically most activated 2-position of the indole ring. In some cases, even a mixture of different chlorinated products or dichlorination was observed. These findings led to the conclusion that only the natural substrate tryptophan can be bound correctly in the active site of the enzyme PrnA to allow regioselective chlorination and that this might also be the case for other flavin-dependent tryptophan halogenases from other secondary metabolite biosynthetic pathways. During the following years, a multitude of new halogenase genes, among them the genes of several flavin-dependent tryptophan halogenases, was isolated from secondary metabolite gene clusters. In a few cases, the genes were heterologously expressed, the enzymes characterized in regard to their natural substrates and the kinetic parameters of the reaction were determined (Weichold, Milbredt, & van Pée, 2016). In some cases also the 3D structures of the enzymes were solved (Bitto et al., 2008; Dong et al., 2005; Yeh, Blasiak, Koglin, Drennan, & Walsh, 2007; Zhu et al., 2009). No further investigations were published on the substrate specificity of tryptophan halogenases until more than one decade later. The next data on this issue were published by Payne et al. (2013) who described the chlorination and also the bromination of indole, several tryptophan derivatives and also less structurally related compounds like tryptoline and the two differently monosubstituted naphthalenes, 2-aminonaphthalene and 1-hydroxynaphthalene. In 2014, Frese et al. (2014) described the conversion of several fluoro-, methyl-, amino-, and hydroxy-substituted tryptophan derivatives by RebH. Extending the biocatalytic scope of tryptophan halogenases even further, Shepherd et al. (2015) showed that the tryptophan 5-halogenase PyrH and the tryptophan 7-halogenase PrnA are not only able to convert tryptophan derivatives to their respective halogenated analogs but also accept nonindolic substrates such as the aniline derivatives kynurenine and anthranilamide. All the mentioned groups working on the issue of the conversion of compounds others than tryptophan by tryptophan halogenases described different experiments to obtain new halogenated compounds. The protocols differ in expression hosts and fusion tags for protein purification. The reaction conditions during the large scale conversions as well as the methods used for quenching of the reactions and product purification are different which makes it quite difficult to compare the existing protocols. A short overview summarizing the reaction conditions applied for the large-scale conversion of different compounds is given in Table 3.

Table 3 Overview of the Conditions Used for Large Scale Conversions of Different Substrates by Tryptophan Halogenases

Hölzer et al. (2001)	Payne et al. (2013)	Frese et al. (2014)	Shepherd et al. (2015)
Halogenase(s)			
PrnA (produced in *Pseudomonas fluorescens* BL915)	His-RebH (produced in *E. coli* BL21 (DE3))	His-RebH (produced in *E. coli* BL21 (DE3))	His-PrnA and different mutants His-PyrH (produced in *E. coli* Arctic Express)
Substrates			
Indole derivatives, phenylpyrrole derivatives	Indole, tryptophan derivatives, tryptoline, substituted naphthalenes	Hydroxy-, amino-, fluoro-, methyl-substituted tryptophans	Kynurenine, anthranilic acid, anthranilamide
Assay mixtures for the enzymatic halogenation reactions for large scale bioconversions			
4.45 mU PrnA 550 μU Fre 0.1–0.6 m*M* substrate 50 m*M* NADH 10 μ*M* FAD 100 m*M* NaCl → total volume of 64 mL for tryptophan and indole derivatives and 200 mL for phenylpyrrole derivatives → incubation for 16 h at 30°C	25 μ*M* His-RebH 2.5 μ*M* MBP-RebF 0.5 m*M* substrate 100 μ*M* NAD 100 μ*M* FAD 10 m*M* NaCl 50 U/mL glucose dehydrogenase 20 m*M* glucose → in crystallization dishes (125 × 65 or 100 × 50 mm)	His-RebH in cell lysate of 1.5 L culture 2.5 U/mL His-PrnF 2–6 m*M* substrate 1 m*M* NADH 10 μ*M* FAD 30 m*M* NaCl 1 U/mL alcohol dehydrogenase 5% isopropanol (v/v) 20 m*M* DTT	25 μ*M* His-halogenase 2.5 μ*M* His-Fre 3 m*M* substrate 10 μ*M* NADH 10 μ*M* FAD 100 m*M* $MgCl_2$ 12.5 μ*M* glucose dehydrogenase 2 200 m*M* glucose

Continued

Table 3 Overview of the Conditions Used for Large Scale Conversions of Different Substrates by Tryptophan Halogenases—cont'd

Hölzer et al. (2001)	Payne et al. (2013)	Frese et al. (2014)	Shepherd et al. (2015)
	covered with perforated aluminum foil → stirring of reaction mixture with magnetic bar at 60 rpm	→ total volume of 30 mL in Erlenmeyer flask (50 mL) → incubation for 16–24 h at 25°C in orbital shaker at 100 rpm	→ total volume of 10 mL → incubation at 30°C in orbital shaker
Quenching method and product purification			
→ extraction with *tert*-butyl methyl ether → evaporation to dryness under vacuum → purification by HPLC	→ addition of 5 *M* HCl until pH < 2 → addition of NaCl to saturation → filtration of precipitated protein → extraction with $CHCl_2$ → strong cation exchange chromatography → purification by chromatography	→ dialysis of reaction mixture against 5 × 500 mL Millipore water + 0.1% TFA → evaporation of solvent under reduced pressure → preparative HPLC	→ incubation at 95°C for 5 min → centrifugation for 10 min at 4°C and 12,000 × *g* → semipreparative HPLC

3.1.1 A High-Throughput Assay for the Detection of Halogenase Activity

It has been shown that the substrate specificity of tryptophan halogenases is not as high as originally assumed and that it is even possible to create variants of the native enzymes with modified substrate specificity. Unfortunately, the construction of such improved halogenases is severely hampered by the issue of mutant screening. Every single clone of a mutant library needs to be grown, followed by cell lysis and incubation of the cell-free extracts with potential substrates and finally the enzyme assays need to be analyzed for possible conversion products by HPLC or other methods. This makes the screening of mutant libraries for halogenases accepting new substrates a really tough challenge. To overcome this problem, it is necessary to develop high-throughput techniques using simple detection methods. A publication by Hoshford, Shepherd, Micklefield, and Wong (2014) describes such a high-throughput assay for arylamine halogenation in aqueous solutions. This colorimetric assay is based on a peroxidase (HRP)-catalyzed in situ oxidation of 4-methyl-catechol and the following addition of chlorinated or nonhalogenated arylamines (benzoquinone–amine-coupling reaction) which can be analyzed spectrophotometrically due to a shift of the absorbance maxima of the halogenated compounds in comparison to their nonhalogenated analogs (Fig. 5). Unfortunately, this reaction requires the presence of an arylamine functionality in the molecule which makes this assay suitable for only a limited number of potential halogenase substrates.

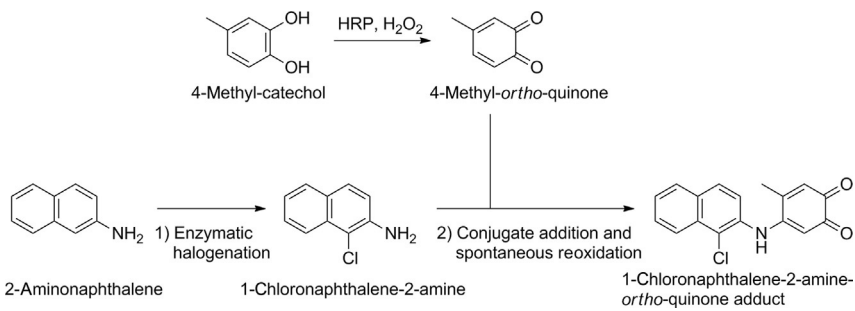

Fig. 5 Reaction sequence for RebH halogenation of 2-naphthylamine followed by in situ oxidation of 4-methyl-catechol and formation of the chlorinated 2-naphthylamine-*ortho*-quinone adduct.

Quantification of the halogenated arylamines can be done according to the method described by Hoshford et al. (2014) for the halogenation of 2-naphthylamine by the tryptophan 7-halogenase RebH:

1. Halogenation reaction
   - The halogenation reaction is carried out in 50 m*M* K_2HPO_4 (pH 7.4) with 5% isopropanol (v/v).
   - Add 2-naphthylamine (0.6 m*M*), NAD (100 μ*M*), FAD (100 μ*M*), NaCl (10 m*M*), Fre (2.5 μ*M*), glucose dehydrogenase 2 (5 μ*M*), and glucose (20 m*M*) to make a final volume of 300 μL.
   - Start the reaction by adding RebH to a final concentration of 25 μ*M*.
   - Incubate the reaction at 21°C under constant shaking.
   - Stop the reaction by heating at 95°C for 5 min and remove precipitated protein by centrifugation.
2. Conjugate addition and quantification of halogenated arylamine
   - To 125 μL of the quenched halogenation reaction, add 4-methylcatechol to a final concentration of 0.5 m*M* (stock solution of 200 m*M* in 50 m*M* K_2HPO_4, pH 7.4) and hydrogen peroxide to a final concentration of 3 m*M*.
   - To start the reaction, add 1 μL horseradish peroxidase (0.1 mg/mL stock solution in 50 m*M* K_2HPO_4, pH 7.4)
   - Incubate under shaking for 5 min.
   - Measure the absorption of the 2-naphthylamine adduct at 516 nm in a spectrophotometer.
   - Quantify product formation by fitting to a calibration curve prepared from known concentrations of 2-naphthylamine and the chlorinated reaction product in reaction buffer containing all the components of the halogenation reaction except the RebH solution.

3.2 Modification of Biosynthetic Pathways Using Tryptophan Halogenases

All the before-mentioned publications dealt with the substrate specificity of mostly native tryptophan halogenase enzymes in in vitro reactions, but during the last few years, a lot of effort was placed into the construction of enzyme variants with modified regioselectivity or substrate specificity obtained by error-prone or site-directed mutagenesis and the use of native halogenases and respective variants in metabolic engineering approaches. Introducing genes coding for tryptophan halogenases with different

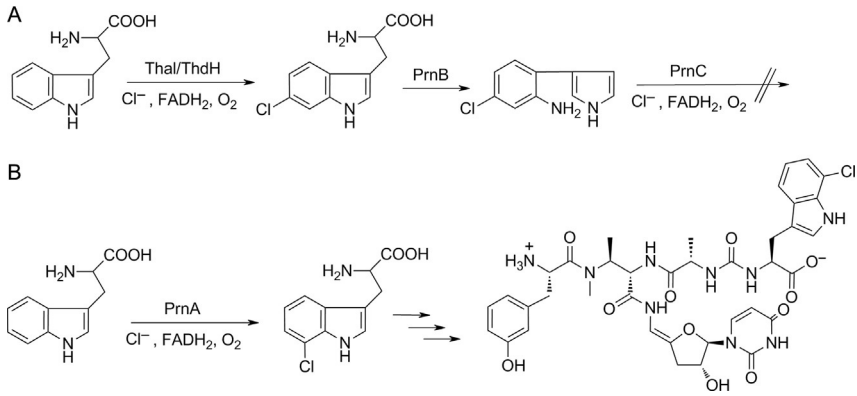

Fig. 6 Effect of the introduction of (A) the tryptophan 6-halogenase gene *thal/thdH* into the pyrrolnitrin producer *Pseudomonas chlororaphis* on pyrrolnitrin biosynthesis (Seibold et al., 2006) and (B) the tryptophan 7-halogenase gene *prnA* into the pacidamycin producer *Streptomyces coeruleorubidus* on pacidamycin biosynthesis (Roy, Grüschow, Cairns, & Goss, 2010).

regioselectivities into bacteria or plants producing halogenated secondary metabolites resulted in the production of variants of those halogenated compounds. Seibold et al. (2006) introduced the tryptophan 6-halogenases gene (*thal/thdH*) from the biosynthesis of thienodolin in *Streptomyces albogriseolus* (Milbredt et al., 2014) into the pyrrolnitrin producer *Pseudomonas chlororaphis* ACN. Although this strain still contained the tryptophan 7-halogenase gene *prnA*, overexpression of the tryptophan 6-halogenase gene *thal/thdH* was so high that all available tryptophan was converted to 6-chlorotryptophan thus no 7-chlorotryptophan was available for pyrrolnitrin biosynthesis. However, 6-chlorotryptophan formed by the reaction of Thal/ThdH was accepted as a substrate by the second enzyme in the pyrrolnitrin biosynthetic pathway, PrnB, resulting in the formation of the phenylpyrrole derivative 3-(2′-amino-4′-chlorophenyl)pyrrole. But the next enzyme, the monodechloroaminopyrrolnitrin 3-halogenase (PrnC; Hohaus et al., 1997; Kirner et al., 1998) did not accept this compound as a substrate which was thus not further metabolized and accumulated. This example shows the importance of the substrate specificity of the downstream enzymes in the biosynthetic pathway (Fig. 6A). The gene for the tryptophan 6-halogenase Thal/ThdH was introduced into *P. chlororaphis* by biparental conjugation as described later.

3.2.1 *Introduction of* thal/thdH *into* P. chlororaphis *and Isolation of 3-(2′-Amino-4′-Chlorophenyl)Pyrrole*

The donor strain *E. coli* S17-1 containing the plasmid pCIBhisthal (Seibold et al., 2006) and the recipient strain *P. chlororaphis* ACN were precultured in 5 mL of HNB medium at 37°C and 30°C, respectively, overnight. 100 μL of each culture were pipetted onto the center of an agar plate containing *Pseudomonas* minimal medium (PMM; Kirner et al., 1996) and the antibiotic tetracycline (20 μg/mL). After incubation at 30°C for 8 h the bacteria were scratched from the plate, diluted, and plated onto PMM agar plates containing tetracyclin for selection of *Pseudomonas* clones containing the plasmid pCIBhisthal, since under these conditions, only recombinant *Pseudomonas* clones were able to grow. The obtained clones could be used for overproduction of the tryptophan 6-halogenase Thal/ThdH or for the isolation of 3-(2′-amino-4′-chlorophenyl)pyrrole. For this purpose, *P. chlororaphis* ACN containing pCIBhisthal was grown in 6 x 1 L of HNB medium at 30°C for 96 h. After removal of the cells by centrifugation, the medium was extracted twice with 1.5 L of methyl-*tert*-butyl ether. The organic extracts were combined and evaporated to dryness in vacuo. The residue was dissolved in 2 mL of methanol and loaded onto a LH-20 column and eluted with methanol. Elution of metabolites was monitored at 254 nm. Fractions showing an UV spectrum with a maximum at 303 nm were combined and purified further by preparative HPLC using an Eurosphere-100 RP-18 column with water/methanol (35:65) as the eluent. The structure of the purified product was elucidated by HPLC-MS and ^{1}H-NMR.

3.2.2 *In Vivo Modification of Biosynthetic Pathways Using Halogenase Genes*

Roy et al. (2010) used the introduction of the gene of the tryptophan 7-halogenase PrnA from pyrrolnitrin biosynthesis (Kirner et al., 1998) into the pacidamycin producer *Streptomyces coeruleorubidus* for the modification of the peptide antibiotic pacidamycin. Due to the activity of PrnA, tryptophan was chlorinated to 7-chlorotryptophan and used as a substrate by the non-ribosomal peptide synthetase involved in pacidamycin biosynthesis resulting in the production of chloropacidamycin (Fig. 6B). Runguphan, Qu, and O'Connor (2010) were able to introduce the gene of the tryptophan 7-halogenase RebH into the medicinal plant *Catharanthus roseus*. They also introduced the flavin reductase gene *rebF* into the plant which is not necessary in the bacterial systems, because there are several flavin reductases in the bacteria which can provide the halogenase with reduced flavin. It is not

clear, whether transformation of plants with a flavin reductase gene is absolutely necessary. Runguphan et al. (2010) obtained halogenated tryptophan-derived alkaloids, some of which were identified as halogenated analogs of known alkaloids produced by the medicinal plant, but due to the substrate specificity of downstream enzymes present in the plant, also new chlorinated tryptophan-based compounds were formed. It was observed that the indole alkaloid formation from 7-chlorotryptophan in *C. roseus* is limited by the activity of the tryptophan decarboxylase converting tryptophan to tryptamine. Based on the crystal structure of RebH and thus the knowledge of the exact coordination of its natural substrate tryptophan in the active site of the enzyme, a tyrosine residue interacting with the carboxylic acid group of tryptophan was exchanged by a sterically more demanding tryptophan residue. This mutation resulted in the preferential halogenation of tryptamine compared to tryptophan. This clearly demonstrates that it is possible to change the substrate specificity of flavin-dependent tryptophan halogenases by site-specific mutagenesis.

3.3 Chemical Substitution of Enzymatically Introduced Halogen Atoms

Halogen atoms can have a profound influence on the biological activity of compounds and in addition they can serve as a useful handle for further chemical derivatization. Bromine and chlorine atoms can be substituted to afford aryl and heteroaryl analogs. Roy, Goss, Wagner, and Winn (2008) developed a method for Suzuki-Miyaura cross-coupling of halogenated tryptophans obtained by enzymatic synthesis from the corresponding haloindoles and serine by a simple one-pot reaction (Goss & Newill, 2006). They used the water-soluble phosphine ligand *tris*(4,6-dimethyl-3-sulfonatophenyl) phosphine trisodium salt (TXPTS) and various arylboronic acids and achieved yields of up to 90%. Since the reaction also worked with small peptides, they adapted the reaction to chlorinated pacidamycin. They achieved the modification of not only purified chloropacidamycin but also the modification of chloropacidamycin in crude extracts (Roy et al., 2010).

Runguphan and O'Connor (2013) described modification of chlorinated alkaloids obtained by in vivo chlorination of the precursor tryptophan by the tryptophan 7-halogenase RebH in crude extracts by Pd-catalyzed Suzuki-Miyaura cross-coupling. Chlorinated alkaloids obtained via the exogenous addition of 6-chlorotrytophan to cultures of the plant *C. roseus* could also be modified by cross-coupling.

3.4 Modification of the Regioselectivity of Tryptophan Halogenases

The regioselectivity of the tryptophan 7-halogenase PrnA could be modified by site-directed mutagenesis (Lang et al., 2011). Based on the three-dimensional structure of PrnA and the binding of the substrate tryptophan seen in this structure, amino acid residues involved in the binding of the substrate via ionic interactions with the amino and carboxylic acid group, and/or with the indole ring were exchanged. The exchange of a phenylalanine residue shielding the pyrrole ring from attack by the halogenating agent against a much smaller alanine residue led to a modification of the regioselectivity. Obviously, this mutation allowed the substrate a higher degree of flexibility in the active site and resulted in the possibility of an attack of the substrate also in the 5-position in addition to halogenation at the 7-position. The procedure for the construction of the PrnAF103A variant with modified regioselectivity and the use of this mutant for the formation of 7-chloro- and 5-chlorotryptophan is described below.

1. The primers used for the construction of the His-tagged PrnAF103A variant by overlap extension PCR were:
   primer a: 5′-GACTCTAGAGG**GGATCC**CATGAACAAGCC GATCAAGAAT-3′ (sense)
   primer b: 5′-GTTCGGCACGTTGCCGGCCAAATGGTAGAA GT-3′ (antisense)
   primer c: 5′-ACTTCTACCATTTGGCCGGCAACGTGCC GAAC-3′ (sense)
   primer d: 5′-ATC**AAGCTT**CTACAGGCTTTCCTGCGCTGC GAGCTT-3′ (antisense).
   Mutagenic sites are underlined, and restriction sites are in bold. The template used was pUC-*prnA* (Dong et al., 2005).
2. The fusion fragment obtained by overlap extension PCR was ligated into pBluescript II SK (+) and introduced into *E. coli* TG1 by electroporation.
3. For expression of the mutated halogenase gene, the gene was ligated into the *E. coli-Pseudomonas* shuttle vector pCIBhis (Wynands & van Pée, 2004) and introduced into *P. fluorescens* BL915 ΔORF 1–4 (Hammer, Hill, Lam, van Pée, & Ligon, 1997) by triparental conjugation (Ditta, Stanfield, Corbin, & Helinski, 1980).
4. Purification of His-tagged enzyme was performed by Ni-chelating affinity chromatography using a sepharose FF column.

5. Halogenating activity was analyzed using the following reaction mixture: 50 μL enzyme-containing protein solution, 10 mU flavin reductase, 1 μ*M* FAD, 2.4 m*M* NADH, 12.5 m*M* $MgCl_2$, 100 U catalase, and 0.25 m*M* L-tryptophan in a total volume of 200 μL in 10 m*M* potassium phosphate buffer, pH 7.2. After incubation at 30°C for 30 min, the reaction was stopped by incubation at 95°C for 5 min. Denatured proteins were removed by centrifugation, and the solution was analyzed by HPLC. One unit of halogenating activity is defined as the formation of 1 μmol product per min.
6. For identification of the reaction products formed by the PrnAF103A variant, a large scale preparation was performed. The assay mixture consisted of 850 μL enzyme solution in 10 m*M* potassium phosphate buffer, pH 7.2, 50 mU flavin reductase, 10 μ*M* FAD, 2.4 m*M* NADH, 12.5 m*M* NaBr, and 0.25 m*M* L-tryptophan in a total volume of 1000 μL.
7. After incubation at 30°C for 4 h, the reaction was stopped by boiling in a water bath for 5 min. Denatured protein was removed by centrifugation, and the supernatant was loaded onto a solid phase extraction column, equilibrated with methanol and water. After washing with 10% methanol, the brominated products were eluted with 100% methanol. The eluates of 10 large scale preparations were concentrated in vacuo.
8. The two halogenated products were separated and purified by HPLC using a RP18 column with methanol:water (40:60), 0.1% TFA (v/v) as the eluent.
9. The reaction products were identified by ^{1}H-NMR and ESI-MS.

4. SYNTHESIS OF PCP-BOUND HALOGENASE SUBSTRATES

The so far characterized tryptophan halogenases all accept a free substrate. However, most of the flavin-dependent halogenases and also most of the nonhaem iron-dependent halogenases require a substrate bound to a peptidyl carrier or acyl carrier protein (Weichold et al., 2016). The preparation of these substrates is highly challenging. Attempts to circumvent the synthetic problems by using the much easier to synthesize SNAC-derivatives of the substrates with the small *N*-acetylcysteamine moiety mimicking the phosphopantetheinyl arm of holo-carrier proteins in the case of thioesterases and NRPS condensation domains (Ehmann, Trauger, Stachelhaus, & Walsh, 2000; Yeh, Kohli, Bruner, & Walsh, 2004) failed,

probably due to the substrate specificity of the halogenases and a proposed conformational change upon interaction with the protein-coupled substrate (Pang, Garneau-Tsodikova, & Tsodikov, 2015). Instead, chemoenzymatically or purely enzymatically synthesized protein-bound substrates were used (Agarwal et al., 2014; Dorrestein, Yeh, Garneau-Tsodikova, Kelleher, & Walsh, 2005; Lin, Van Lanen, & Shen, 2007; Schmartz, Zerbe, Abou-Hadeed, & Robinson, 2014).

So far, there are only a few examples for synthesis and use of protein-bound substrates to show halogenase activity. One method has previously been described by Li et al. (2009) for the synthesis of a vancomycin precursor hexapeptide. Only the hexapeptide precursor with a β-hydroxytyrosine residue at the C-terminus and not the corresponding dipeptide with a β-hydroxytyrosine residue at the C-terminus was accepted by the $FADH_2$-dependent halogenase VhaA (Schmartz et al., 2014).

Synthesis of a PCP-bound β-tyrosine and halogenation by SgcC3 from C-1027 biosynthesis was shown by Lin, Huang, and Shen (2012).

For some nonhaem iron, α-ketoglutarate- and O_2-dependent halogenases, activity has been shown by providing them with enzymatically derived carrier protein-tethered amino acids. Exemplarily, carrier protein-tethered L-threonine is used by SyrB2 from syringomycin E biosynthesis and peptide-bound L-*allo*-isoleucine is accepted by CmaB from coronatine biosynthesis yielding the 4-chloro-derivatives (Vaillancourt, Yeh, Vosburg, O'Connor, & Walsh, 2005; Vaillancourt, Yin, & Walsh, 2005). Trichlorination in position 5 of peptide-bound leucine can be achieved by BarB1 and BarB2 from barbamide biosynthesis (Galonic, Vaillancourt, & Walsh, 2006).

For the formation of PCP-tethered pyrrole-2-carboxylic acid substrates, two different main strategies have been used. The first one relies on a fully enzymatic catalysis based on the natural production of pyrrole residues from proline (Fig. 7) (Dorrestein et al., 2005). However, the enzymatic synthesis of PCP-bound pyrrole-2-carboxylic acid requires the heterologous expression of a number of genes, and the purification of the corresponding proteins and enzymes can therefore be rather complicated. The second strategy uses organic synthesis to create a coenzyme A-coupled pyrrolyl residue that is then enzymatically transferred to an apo-PCP by a nonspecific phosphopantetheinyl transferase (Garneau-Tsodikova, Stapon, Kahne, & Walsh, 2006).

4.1 Enzymatic Synthesis of Pyrrolyl-*S*-PCPs

For enzymatic synthesis of pyrrolyl-*S*-PCPs, the PCPs are required in their holo-form, but expression of PCP genes in *E. coli* yields predominantly the

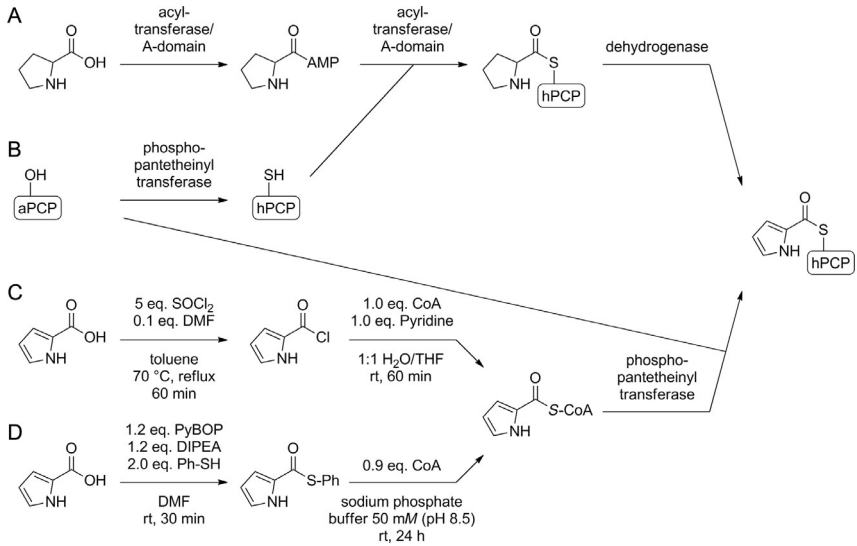

Fig. 7 Synthetic strategies for generation of pyrrolyl-*S*-PCPs. (A) The natural biosynthetic pathway that can also be reproduced in vitro. (B) Completion of apo-PCP (aPCP) by transferring a phosphopantetheine structure to it forms holo-PCP (hPCP) in vivo and in vitro. (C) and (D) Synthesis of pyrrolyl-*S*-CoA via pyrrole-2-carboxylic acid chloride or *S*-phenylpyrrole-2-carbothioate which can be transferred to apo-PCP by a nonspecific phosphopantetheinyl transferase (for conditions of the enzymatic in vitro reactions see text).

apo-PCPs (Gocht & Marahiel, 1994). Thus, it is necessary to attach a phosphopantetheinyl moiety which can be done by treatment with a phosphopantetheinyl transferase and coenzyme A or coexpression with the transferase gene. For example, Thomas, Burkart, and Walsh (2002) obtained more than 95% holo-PCP by coexpressing *sfp*, the gene of an unspecific phosphopantetheinyl transferase from *Bacillus subtilis*, together with the gene of the PCP, compared to only 25% without coexpression.

Enzymatic synthesis of PCP-pyrrole-2-carbothioates was demonstrated for pyoluteorin biosynthesis by Dorrestein et al. (2005) using previously published protocols (Thomas et al., 2002).

To generate holo-PCP, apo-PCP (52 μ*M*) was treated with Sfp (1.1 μ*M*) and coenzyme A (295 μ*M*) supplied with $MgCl_2$ (7.1 μ*M*) in 300 μL reaction volume and was incubated for 1 h.

For adenylation of proline and transfer to the PCP, the enzyme CouN4 from coumermycin biosynthesis was used, and for oxidation to form the pyrrolyl-residue CloN3 from clorobiocin biosynthesis was employed.

The previously provided reaction mixture was used without further purification and supplied with CouN4 (84 μg/mL), L-proline (4.2 m*M*), ATP (3.3 m*M*) followed by CloN3 (2.7 μ*M*), and FAD (150 μ*M*) in a total volume of 720 μL in 50 m*M* Tris/HCl (pH 7.4) buffer containing 1 m*M* TCEP.

Incubation at room temperature for 3 h led to the formation of pyrrolyl-*S*-PCP which could be detected by LC/MS/MS and could be used as the substrate for the halogenase PltA from pyoluteorin biosynthesis.

Another protocol for the one-step preparation of pyrrolyl-*S*-PCP with proteins from the biosynthesis of nargenicin A_1 was described by Maharjan et al. (2012).

4.2 Chemoenzymatic Synthesis of Pyrrolyl-S-PCPs

4.2.1 Synthesis of Pyrrolyl-S-CoA via Acid Chloride

Garneau-Tsodikova et al. (2006) described a method for activation of pyrrole carboxylic acid via the acid chloride. Pyrrole-2-carboxylic acid (100 mg) was stirred in dried toluene (5 mL) with a catalytic amount of DMF (7 μL, 0.1 eq.) which is meant to form the actually chlorinating (chloromethylene)dimethyliminium later on (Fritz & Oehl, 1971; Jugie, Martin, & Smith, 1975). Then thionyl chloride (328 μL, 5 eq.) was added, and the mixture was heated at 70°C for 1 h using a reflux condenser. Afterwards, the reaction mixture was concentrated by a rotary evaporator, 5 mL of dry toluene were added and concentrated again. The residue was then dried under vacuum for at least 30 min. It can also be stored at −20°C under dry conditions for several days.

To coenzyme A trilithium salt (20 mg, 1 eq.) in a 1:1 mixture of THF/H_2O with pyridine (1.9 μL, 1 eq.), pyrrole-2-carbonyl chloride (3.3 mg, 1 eq.) was added. The mixture was stirred for 1 h at room temperature and then purified by semipreparative HPLC in 50 μL portions diluted by H_2O to 1 mL. For this purpose a 60 min gradient from H_2O with 0.1% TFA to 50% MeCN/H_2O with 0.1% TFA was used on an Agilent Zorbax 5 μm 300SB-C18 semiprep HPLC column with a flow rate of 5 mL/min and detection of CoA thioesters at 310 nm.

4.2.2 Synthesis of Pyrrolyl-S-CoA via S-Phenyl Thioates

Agarwal et al. (2014) used a traditional peptide-coupling reaction as described by Li et al. (2009) to obtain *S*-phenyl-pyrrole-2-carbothioate that could be transferred to coenzyme A via a transesterification reaction. To a solution of pyrrole-2-carboxylic acid (20 mg) in DMF (500 μL) PyBOP

(112 mg, 1.2 eq.), DIPEA (38 μL, 1.2 eq.) and thiophenol (37 μL, 2 eq.) were added. The mixture was stirred at room temperature for 30 min, quenched by saturated brine, and extracted with ethyl acetate. After drying over $MgSO_4$, the organic phases were concentrated in vacuo and chromatographed on a silica column using a 40:1 mixture of hexane/ethyl acetate which yielded 27% of *S*-phenyl-pyrrole-2-carbothioate.

4 mg of the *S*-phenyl-pyrrole-2-carbothioate were dissolved in 200 μL THF and added to 300 μL of sodium phosphate buffer (50 m*M*, pH 8.5) containing coenzyme A sodium salt (14 mg, 0.9 eq.). The mixture was stirred for 24 h at room temperature and subsequently chromatographed on a reverse-phase C18 semipreparative HPLC column using a gradient from 5% to 100% MeCN with 0.1% TFA against H_2O with 0.1% TFA over 30 min. Lyophilization of product-containing fractions yielded 7% (2% overall) of the pyrrolyl-CoA thioester.

4.3 Transfer of Pyrrolyl-*S*-CoA Thioesters to Carrier Proteins and Halogenation of the Substrate

A procedure for the transfer of synthesized CoA thioesters to apo-PCP was previously described by Li et al. (2009) for a hexapeptidyl-CoA thioester which works also well for the pyrrolyl-CoA thioester. All components were dissolved in 50 m*M* Tris/HCl (pH 7.5) reaction buffer and added as follows. The purified apo-PCP (120 μ*M*) was supplied with the pyrrolyl-CoA thioester (100 μ*M*), the unspecific phosphopantetheinyl transferase Sfp (5 μ*M*) and $MgCl_2$ (50 m*M*). The mixture was incubated for 30 min at 37°C and then purified by HPLC.

Agarwal et al. (2014) did not purify the product but directly used the reaction mixture for enzymatic halogenation. To 100 μL of the PCP-pyrrole-2-carbothioate-containing mixture 5 m*M* NADPH, 100 μ*M* FAD, 100 m*M* KBr for bromination, 20 μ*M* halogenase, and 6 μ*M* SsuE flavin reductase were added in 50 m*M* Tris buffer (pH 8.0). The reaction was maintained by periodic addition of additional NADPH (5 m*M* every hour).

So far, no kinetic data for the halogenation of PCP-tethered pyrrole-2-carboxylic acid could be obtained due to the very low activity of these reactions.

4.4 Release of Halogenated Pyrrole-2-Carboxylic Acid from PCPs

Although PCP-coupled substrates and products can be analyzed by MALDI-TOF (Garneau-Tsodikova et al., 2006; Thomas et al., 2002) or

LC/MS/MS after proteolytic digestion (Agarwal et al., 2014; Dorrestein et al., 2006), in many cases a release from the PCP is desired.

Thomas et al. (2002) described a method to release the products from the PCP hydrolytically by treatment with KOH. For this purpose the proteins from a sample (500 μL) were precipitated by addition of trichloroacetic acid up to 5–10% (w/v) and pelleted by centrifugation. The pellet was then washed twice with H_2O and resuspended in 200 μL of 0.1 *M* KOH. Incubation at 65°C for 15 min, addition of TFA for neutralization, and removal of precipitated proteins by centrifugation or filtration lead to halogenated pyrrole-2-carboxylic acid. However, in our hands release by KOH treatment did not work satisfactorily; we could hardly detect any released pyrrol-2-carboxylic acid.

Another strategy inspired by Li et al. (2009) was employed by Schmartz et al. (2014) who treated the vancomycin precursor hexapeptide-PCP conjugate with 1/10 volume of an aqueous hydrazine solution (25% v/v) at 30°C for 30 min to lyse the thioester bond freeing the peptide hydrazide. However, in our hands this did not lead to detectable amounts of released pyrrole carbonyl hydrazide.

Dorrestein et al. (2005) used the type II thioesterase TycF from tyrocidine biosynthesis (Mootz & Marahiel, 1997) for the enzymatic release of pyrrole-2-carboxylic acid from the PCP according to a protocol from Yeh et al. (2004), by incubating the PCP-pyrrole-2-carbothioate in 50 m*M* Hepes pH 7.5 with 5 m*M* $MgCl_2$ and 1 m*M* TCEP with 10 μ*M* TycF to release the substrate.

5. CONCLUSIONS

The lack of stability of flavin-dependent halogenases under reaction conditions poses a serious problem for their use in industrial applications. To improve enzyme stability, the construction of mutants is required. However, for screening of mutants, the methods available for high-throughput screening have to be improved considerably, especially in their general applicability. Elucidation of the reason for the loss of activity should allow the modification of the enzymes in such a way that they cannot be inactivated anymore. The substrate scope of tryptophan halogenases has been broadened substantially. Error-prone PCR and site-directed mutagenesis in combination with high-throughput assays should allow the improvement of halogenating activity toward substrates, now only halogenated with a very low activity and should thus tremendously increase the suitability

of halogenases for industrial application. The in vivo use of halogenases will lead to the production of novel compounds with new or improved biological activities and the possibility to exchange the halogen substituents via cross-coupling reactions increases the possibility for the formation of novel compounds tremendously. The requirement of protein-bound substrates hampers the use of many halogenases in in vitro reactions enormously, since the amount of substrate obtained by these syntheses is rather low as well as the activities of the enzymes. Here, the in vivo use seems to be a good alternative. The future for enzymatic halogenation reactions looks very promising, especially with a steadily increasing number of potent groups working on this subject.

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REFERENCES

Agarwal, V., El Gamal, A. A., Yamanaka, K., Poth, D., Kersten, R. D., Schorn, M., et al. (2014). Biosynthesis of polybrominated aromatic organic compounds by marine bacteria. *Nature Chemical Biology*, *10*, 640–647.

Bitto, E., Huang, Y., Bingman, C. A., Singh, S., Thorson, J. S., & Phillips, G. N., Jr. (2008). The structure of flavin-dependent tryptophan 7-halogenase RebH. *Proteins*, *70*, 289–293.

Ditta, G., Stanfield, S., Corbin, D., & Helinski, D. R. (1980). Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proceedings of the National Academy of Sciences of the United States of America*, 77, 7347–7351.

Dong, C., Flecks, S., Unversucht, S., Haupt, C., van Pée, K.-H., & Naismith, J. H. (2005). Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination. *Science*, *309*, 2216–2219.

Dorrestein, P. C., Bumpus, S. B., Calderone, C. T., Garneau-Tsodikova, S., Aron, Z. D., Straight, P. D., et al. (2006). Facile detection of acyl- and peptidyl-intermediates on thiotemplate carrier domains via phosphopantetheinyl elimination reactions during tandem mass spectrometry. *Biochemistry*, *45*, 12756–12766.

Dorrestein, P. C., Yeh, E., Garneau-Tsodikova, S., Kelleher, N. L., & Walsh, C. T. (2005). Dichlorination of a pyrrolyl-S-carrier protein by $FADH_2$-dependent halogenase PltA during pyoluteorin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 13843–13848.

Ehmann, D. E., Trauger, J. W., Stachelhaus, T., & Walsh, C. T. (2000). Aminoacyl-SNACs as small-molecule substrates for the condensation domains of nonribosomal peptide synthetases. *Chemistry & Biology*, 7, 765–772.

Ernyei, A. J. (2008). *Untersuchungen zur Substratspezifität der Tryptophan-5- und der Tryptophan-6-Halogenase und Optimierung der Reaktionen*. Doctoral thesis, Fachrichtung Chemie und Lebensmittelchemie, Technische Universität Dresden.

Flecks, S., Patallo, E. P., Zhu, X., Ernyei, A. J., Seifert, G., Schneider, A., et al. (2008). New insights into the mechanism of enzymatic chlorination of tryptophan. *Angewandte Chemie International Edition*, *47*, 9533–9536.

Frese, M., Guzowska, P. H., Voß, H., & Sewald, N. (2014). Regioselective enzymatic halogenation of substituted tryptophan derivatives using the FAD-dependent halogenase RebH. *ChemCatChem*, *6*, 1270–1276.

Frese, M., & Sewald, N. (2014). Enzymatic halogenation of tryptophan on a gram scale. *Angewandte Chemie International Edition*, *54*, 298–301.

Fritz, H., & Oehl, R. (1971). Halogen-Sauerstoff-Austausch zwischen Formamidchloriden und Formamiden, nachgewiesen bei der Einfuhrung deuterierter Formylgruppen nach Vilsmeier. *Liebigs Annalen der Chemie*, *749*, 159–167.

Galonic, D. P., Vaillancourt, F. H., & Walsh, C. T. (2006). Halogenation of unactivated carbon centers in natural product biosynthesis: Trichlorination of leucine during barbamide biosynthesis. *Journal of the American Chemical Society*, *128*, 3900–3901.

Garneau-Tsodikova, S., Stapon, A., Kahne, D., & Walsh, C. T. (2006). Installation of the pyrrolyl-2-carboxyl pharmacophore by CouN1 and CouN7 in the late biosynthetic steps of the aminocoumarin antibiotics clorobiocin and coumermycin A1. *Biochemistry*, *45*, 8568–8578.

Gocht, M., & Marahiel, M. A. (1994). Analysis of core sequences in the D-Phe activating domain of the multifunctional peptide synthetase TycA by site-directed mutagenesis. *Journal of Bacteriology*, *176*, 2654–2662.

Goss, R. J. M., & Newill, P. L. A. (2006). A convenient enzymatic synthesis of L-halotryptophans. *Chemical Communications*, *2006*, 4924–4925.

Hammer, P. E., Hill, D. S., Lam, S. T., van Pée, K.-H., & Ligon, J. M. (1997). Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Applied and Environmental Microbiology*, *63*, 2147–2154.

Hohaus, K., Altmann, A., Burd, W., Fischer, I., Hammer, P. E., Hill, D. S., et al. (1997). NADH-dependent halogenases are more likely to be involved in halometabolite biosynthesis than haloperoxidases. *Angewandte Chemie International Edition in English*, *36*, 2012–2013.

Hölzer, M., Burd, W., Reißig, H.-U., & van Pée, K.-H. (2001). Substrate specificity and regioselectivity of tryptophan 7-halogenase from *Pseudomonas fluorescens* BL915. *Advanced Synthesis and Catalysis*, *343*, 591–595.

Hoshford, J., Shepherd, S. A., Micklefield, J., & Wong, L. S. (2014). A high-throughput assay for arylamine halogenation based on a peroxidase-mediated-quinone amine coupling with applications in the screening of enzymatic halogenations. *Chemistry - A European Journal*, *20*, 16759–16763.

Jugie, G., Martin, G., & Smith, J. A. S. (1975). Nuclear magnetic resonance investigations of carbonium ion intermediates. Part III. A chlorine-35 quadrupole resonance study of several (R-chloromethylene)dimethylammonium salts (Vilsmeier-Haack and Viehe reagents). *Journal of the Chemical Society. Perkin Transactions II*, *516*, 925–927.

Keller, S., Wage, T., Hohaus, K., Hölzer, M., Eichhorn, E., & van Pée, K.-H. (2000). Purification and partial characterization of tryptophan 7-halogenase (PrnA) from *Pseudomonas fluorescens*. *Angewandte Chemie International Edition*, *39*, 2300–2302.

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J., et al. (1998). Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. *Journal of Bacteriology*, *180*, 1939–1943.

Kirner, S., Krauss, S., Sury, G., Lam, S. T., Ligon, J. M., & van Pée, K.-H. (1996). The non-haem chloroperoxidase from *Pseudomonas fluorescens* and its relationship to pyrrolnitrin biosynthesis. *Microbiology*, *142*, 2129–2135.

Lang, A., Polnick, S., Nicke, T., William, P., Patallo, E. P., Naismith, J. H., et al. (2011). Changing the regioselectivity of the tryptophan 7-halogenase PrnA by site-directed mutagenesis. *Angewandte Chemie International Edition*, *50*, 2951–2953.

Li, D. B., Woithe, K., Geib, N., Abou-Hadeed, K., Zerbe, K., & Robinson, J. A. (2009). In vitro studies of phenol coupling enzymes involved in vancomycin biosynthesis. *Methods in Enzymology*, *458*, 487–509.

Lin, S., Huang, T., & Shen, B. (2012). Tailoring enzymes acting on carrier protein-tethered substrates in natural product biosynthesis. *Methods in Enzymology*, *516*, 321–343.

Lin, S., Van Lanen, S. G., & Shen, B. (2007). Regiospecific chlorination of (*S*)-*β*-tyrosyl-*S*-carrier protein catalyzed by SgcC3 in the biosynthesis of the enediyne antitumor antibiotic C-1027. *Journal of the American Chemical Society*, *129*, 12432–12438.

Maharjan, S., Aryal, N., Bhattarai, S., Koju, D., Lamichhane, J., & Sohng, J. K. (2012). Biosynthesis of the nargenicin A1 pyrrole moiety from *Nocardia* sp. CS682. *Applied Microbiology and Biotechnology*, *93*, 687–696.

Milbredt, D., Patallo, E. P., & van Pée, K.-H. (2014). A tryptophan 6-halogenase and an amidotransferase are involved in thienodolin biosynthesis. *ChemBioChem*, *15*, 1011–1020.

Mootz, H. D., & Marahiel, M. A. (1997). The tyrocidine biosynthesis operon of *Bacillus brevis*: Complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. *Journal of Bacteriology*, *179*, 6843–6850.

Muffler, K., Kuetchou Ngnigha, A. R., & Ulber, R. (2010). Bestimmung kinetischer Parameter der $FADH_2$-abhängigen Tryptophan-5-Halogenase aus *Streptomyces rugosporus*. *Chemie Ingenieur Technik*, *82*, 121–127.

Pang, A. H., Garneau-Tsodikova, S., & Tsodikov, O. V. (2015). Crystal structure of halogenase PltA from the pyoluteorin biosynthetic pathway. *Journal of Structural Biology*, *192*, 349–357.

Payne, J. T., Andorfer, M. C., & Lewis, J. C. (2013). Regioselective arene halogenation using the FAD-dependent halogenase RebH. *Angewandte Chemie International Edition*, *52*, 5271–5274.

Poor, C. B., Andorfer, M. C., & Lewis, J. C. (2014). Improving the stability and catalyst lifetime of the halogenase RebH by directed evolution. *ChemBioChem*, *15*, 1286–1289.

Roy, A. R., Goss, R. J. M., Wagner, G. K., & Winn, M. (2008). Development of fluorescent aryltryptophans by Pd mediated cross-coupling of unprotected halotryptophans in water. *Chemical Communications*, 4831–4833.

Roy, A. D., Grüschow, S., Cairns, N., & Goss, R. J. M. (2010). Gene expression enabling synthetic diversification of natural products: Chemogenetic generation of pacidamycin analogs. *Journal of the American Chemical Society*, *132*, 12243–12245.

Runguphan, W., & O'Connor, S. E. (2013). Diversification of monoterpene indole alkaloid analogs through cross-coupling. *Organic Letters*, *15*, 2850–2853.

Runguphan, W., Qu, X., & O'Connor, S. E. (2010). Integrating carbon-halogen bond formation into medicinal plant metabolism. *Nature*, *468*, 461–464.

Sanchez, C., Butovich, I. A., Brana, A. F., Rohr, J., Mendez, C., & Salas, J. A. (2002). The biosynthetic gene cluster for the antitumor rebeccamycin. Characterization and generation of indolocarbazole derivatives. *Chemistry & Biology*, *9*, 519–531.

Schmartz, P. C., Zerbe, K., Abou-Hadeed, K., & Robinson, J. A. (2014). Bis-chlorination of a hexapeptide-PCP conjugate by the halogenase involved in vancomycin biosynthesis. *Organic & Biomolecular Chemistry*, *12*, 5574–5577.

Seibold, C., Schnerr, H., Rumpf, J., Kunzendorf, A., Hatscher, C., Wage, T., et al. (2006). A flavin-dependent tryptophan 6-halogenase and its use in modification of pyrrolnitrin biosynthesis. *Biocatalysis and Biotransformation*, *24*, 401–408.

Sheldon, R. A. (2011). Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). *Applied Microbiology and Biotechnology*, *92*, 467–477.

Shepherd, S. A., Karthikeyan, C., Latham, J., Struck, A.-W., Thompson, M. L., Menon, B. R. K., et al. (2015). Extending the biocatalytic scope of regiocomplementary flavin-dependent halogenase enzymes. *Chemical Science*, *6*, 3454–3460.

Stadtman, E. R., & Levine, R. L. (2006). Chemical modification of proteins by reactive oxygen species. In I. Dalle-Donne, A. Scaloni, & D. A. Butterfield (Eds.), *Redox proteomics: From protein modifications to cellular dysfunction and disease* (pp. 3–23). Hoboken: John Wiley & Sons, Inc.

Thomas, M. G., Burkart, M. D., & Walsh, C. T. (2002). Conversion of L-proline to pyrrolyl-2-carboxyl-S-PCP during undecylprodigiosin and pyoluteorin biosynthesis. *Chemistry & Biology*, *9*, 171–184.

Vaillancourt, F. H., Yeh, E., Vosburg, D. A., O'Connor, S. E., & Walsh, C. T. (2005). Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis. *Nature*, *436*, 1191–1194.

Vaillancourt, F. H., Yin, J., & Walsh, C. T. (2005). SyrB2 in syringomycin E biosynthesis is a nonheme Fe^{II} α-ketoglutarate- and O_2-dependent halogenase. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 10111–10116.

Weichold, V., Milbredt, D., & van Pée, K.-H. (2016). Specific enzymatic halogenation has come of age. *Angewandte Chemie International Edition*. http://dx.doi.org/10.1002/anie.201509573.

Wynands, I., & van Pée, K.-H. (2004). A novel halogenase gene from the pentachloropseudilin producer *Actinoplanes* sp. ATCC 33002 and detection of *in vitro* halogenase activity. *FEMS Microbiology Letters*, *237*, 363–367.

Yeh, E., Blasiak, L. C., Koglin, A., Drennan, C. L., & Walsh, C. T. (2007). Chlorination by a long-lived intermediate in the mechanism of flavin-dependent halogenases. *Biochemistry*, *46*, 1284–1292.

Yeh, E., Garneau, S., & Walsh, C. T. (2005). Robust *in vitro* activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 3960–3965.

Yeh, E., Kohli, R. M., Bruner, S. D., & Walsh, C. T. (2004). Type II thioesterase restores activity of a NRPS module stalled with an aminoacyl-S-enzyme that cannot be elongated. *ChemBioChem*, *5*, 1290–1293.

Zehner, S., Kotzsch, A., Bister, B., Süssmuth, R. D., Méndez, C., Salas, J. A., et al. (2005). A regioselective tryptophan 5-halogenase is involved in pyrroindomycin biosynthesis in *Streptomyces rugosporus* LL-42D005. *Chemistry & Biology*, *12*, 445–452.

Zhu, X., De Laurentis, W., Leang, K., Herrmann, J., Ihlefeld, J., van Pée, K.-H., et al. (2009). Structural insights into regioselectivity in the enzymatic chlorination of tryptophan. *Journal of Molecular Biology*, *391*, 74–85.