Enzymatic Halogenation

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Specific Enzymatic Halogenation—From the Discovery of Halogenated Enzymes to Their Applications In Vitro and In Vivo

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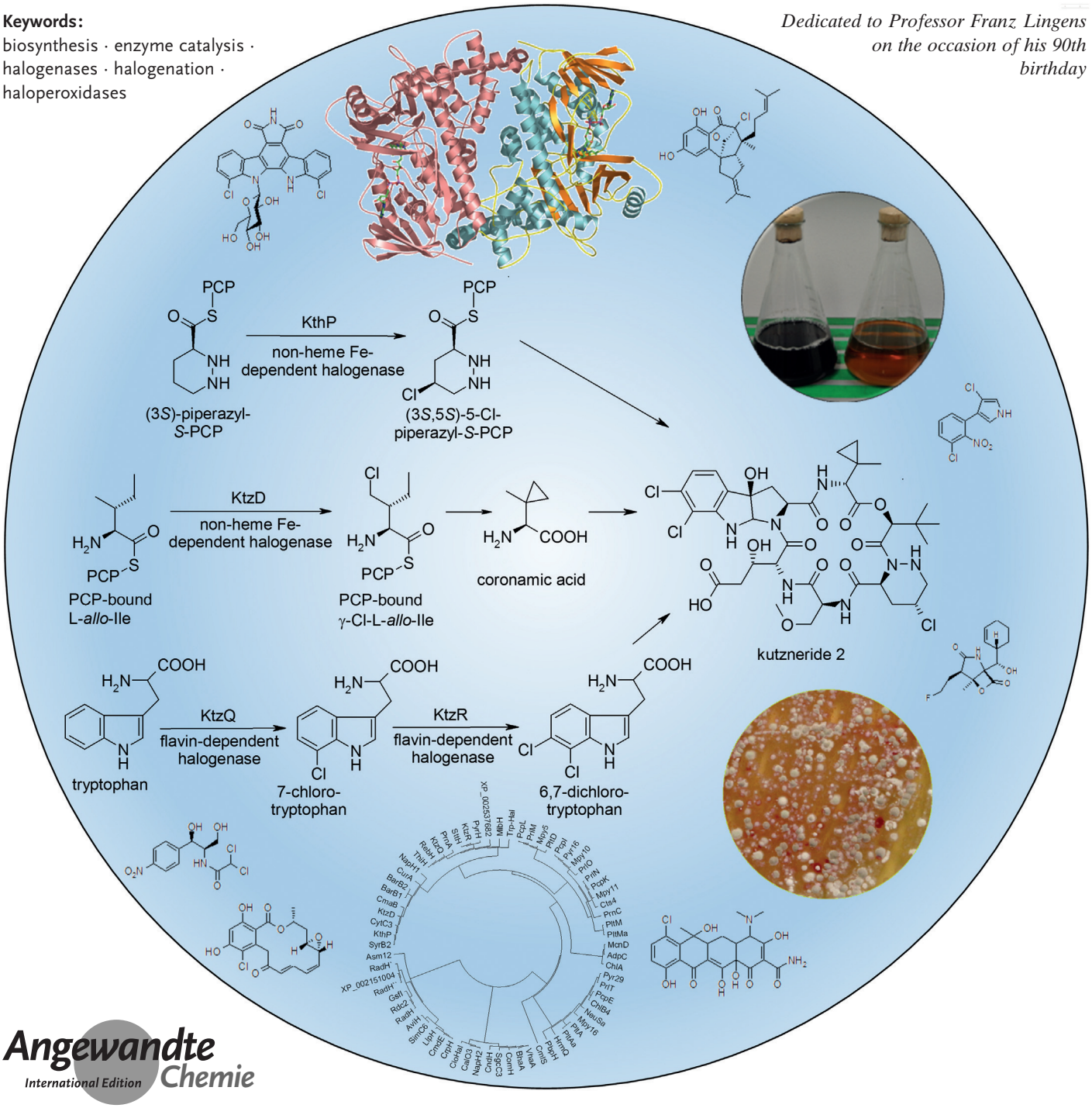
Dedicated to Professor Franz Lingens on the occasion of his 90th birthday



During the last 20 years, focus has shifted from haloperoxidases to flavin-dependent and non-heme-iron halogenases because of their proven involvement in the biosynthesis of halogenated metabolites in different organisms and the regioselectivity of their reactions. During the first 10–12 years, the main research topics were the detection of halogenases as well as the elucidation of three-dimensional structures and reaction mechanisms. This Review mainly deals with studies on halogenating enzymes published between 2010 and 2015. It focusses on the elucidation of the involvement of halogenating enzymes in halometabolite biosynthesis, application of halogenases in in vivo and in vitro systems, in vivo modification of biosynthetic pathways in bacteria and plants, improvement of enzyme stability, broadening of substrate specificity, and the combination of biocatalysis with chemical synthesis to produce new compounds.

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1. Introduction—The Detection of Halogenating Enzymes

1.1. Haloperoxidases

The detection of the first haloperoxidase, chloroperoxidase (CPO), from the fungus *Caldariomyces fumago* in 1959 was the starting point for studies on biological halogenation.[1] For about 35 years, haloperoxidases were the only halogenating enzymes known. In addition to heme-containing haloperoxidases such as CPO, vanadium-containing haloperoxidases were also detected.[2] However, the involvement of halogenated metabolites in the biosynthesis could not be demonstrated unequivocally for any of these haloperoxidases. The elucidation of the three-dimensional structures of CPO[3] and of vanadium-containing haloperoxidases[4] as well as the reaction mechanisms of these enzymes showed that they produce hypohalous acid as the halogenating agent, which diffuses out of the active site and then reacts with electron-rich compounds in a purely chemical halogenation reaction (Scheme 1, top). No specific halide binding site nor a substrate binding site could be detected in haloperoxidases during these studies.[3–5] Bacterial enzymes showing halogenating activity in the monochlorodimedone assay, the standard enzyme assay for the detection of halogenating enzymes at that time,[6] and which neither contained heme nor vanadium turned out to be perhydrolases and not peroxidases. These enzymes also produce hypohalous acid as the actual halogenating species, although via the intermediate formation of short-chain aliphatic peracids (Scheme 1).[7] As a consequence of the production of free hypohalous acids as the halogenating agent, haloperoxidases and perhydrolases lack substrate specificity, regioselectivity, and stereospecificity.

1.2. Flavin-Dependent Halogenases

Until 1995 it was unclear what the halogenating enzymes involved in halometabolite biosynthesis would look like. During their studies on 7-chlortetracycline biosynthesis, Dairi et al. cloned the biosynthetic gene cluster for the biosynthesis of 7-chlortetracycline.[8] Construction of a gene destruction mutant led to the identification of a gene, *chl*, required for the chlorination step. Unfortunately, the published gene and the deduced enzyme were truncated and 100 amino acids were missing from the amino terminal end.[9] Thus, it was not immediately recognized that this enzyme has a nucleotide binding site.

The detection of two halogenase genes in the biosynthetic gene cluster of the antifungal antibiotic pyrrolnitrin (**1**) from *Pseudomonas fluorescens* revealed that the halogenase catalyzing the chlorination of the pyrrole ring of monodechloroaminopyrrolnitrin (Scheme 2), PrnC, showed high similarity to Chl, whereas the tryptophan 7-halogenase, PrnA, hardly showed any similarity (Figure 1).[9] However, both halogenases, PrnA and PrnC, contained a nucleotide binding site motif, GxGxxG, which was missing from the published Chl sequence. Whereas no activity for Chl could be demonstrated by Dairi et al.,[8] in vitro activity for PrnA and PrnC could be shown using their natural substrates, tryptophan (**2**) and monodechloroaminopyrrolnitrin, respectively (Scheme 2).[10] NADH had to be added for activity in crude extracts, and it was assumed that nicotinamide adenine dinucleotide (NADH) was bound through the nucleotide binding site, although the requirement of NADH for the halogenation reaction could not be explained. During attempts to purify PrnC, it was realized that flavin adenine dinucleotide (FAD) was somehow involved in the reaction. More detailed studies with PrnA showed that halogenation of **2** by PrnA requires the activity of a second enzyme, a flavin reductase that reduces FAD to $FADH_2$ by using NADH (or nicotinamide adenine dinucleotide phosphate, NADPH) as the reductant.[11] $FADH_2$ is then used by the halogenase for the

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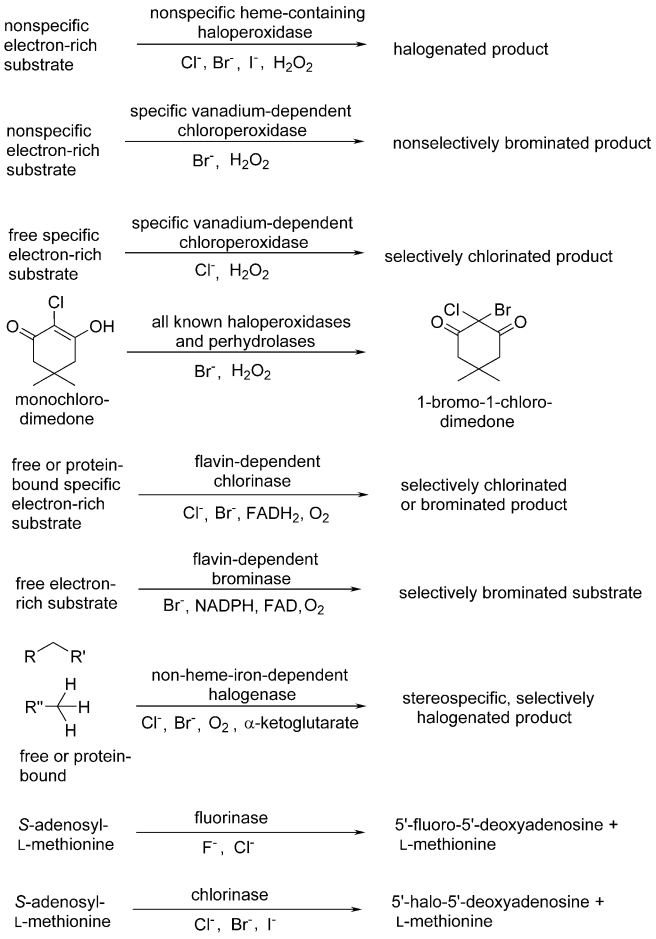

Scheme 1. General reactions of the different types of halogenating enzymes. Chlorination and bromination of monochlorodimedone was generally used as the assay for the detection of halogenating enzymes from 1966 until 1997.[6]

formation of hypochlorous acid via flavin hydroperoxide (Scheme 3).

In contrast to haloperoxidases and perhydrolases, this hypochlorous acid does not leave the active site of the enzyme.[12] No specific flavin reductase is required as part of the two-component system. Even chemically synthesized $FADH_2$ can be used.[13] Thus, the stage was set for further investigations on flavin-dependent halogenases. During the following years, a large number of genes coding for potential flavin-dependent halogenases were detected, although only in very few cases could in vitro activity of the corresponding enzymes be shown, since flavin-dependent halogenases are highly substrate-specific and thus knowledge about the identity of the natural substrate was required. Furthermore, even in cases where the natural substrate was known, it was often not available, since substrates bound to peptide or acyl carrier protein are involved in the biosynthesis of nonribosomally produced peptides or polyketide biosynthesis, and this also holds for many of the halogenase substrates.

The first three-dimensional structure of a flavin-dependent halogenase to be elucidated was that of the tryptophan 7-halogenase PrnA from the biosynthesis of pyrrolnitrin (**1**).[12] Surprisingly, the structure showed that direct interaction between the isoalloxazine ring of the flavin and the substrate tryptophan was not possible. Thus, a diffusible halogenating intermediate, hypohalous acid, must be involved. This hypohalous acid is produced by the reaction of flavin hydroperoxide with a halide ion (chloride or bromide). The flavin hydroperoxide is formed from $FADH_2$ and oxygen, as in a monooxygenase reaction. The hypohalous acid is then guided along a 10 Å tunnel towards the substrate. A lysine residue was found to be absolutely necessary for halogenating activity. Interestingly, a single chloride was detected in the crystal structure close to the isoalloxazine ring, thus showing that the enzyme molecules were saturated with chloride in the resting state.[12,14]

1.3. Non-Heme-Iron, α-Ketoglutarate, and O_2-Dependent Halogenases

Haloperoxidases and flavin-dependent halogenases require electron-rich substrates with activated carbon atoms in the form of aromatic rings or double bonds in aliphatic structures.[15–18] These enzymes cannot halogenate at non-activated carbon atoms, such as methyl groups. Interestingly, there are quite a few halogen-containing metabolites with halogenated methyl groups or halogen atoms at non-activated carbon atoms. One example of such a compound is barbamide (**3**, Scheme 4) produced by the marine cyanobacterium *Lyngbya majuscula*. Early investigations on the biosynthesis of **3** revealed that the trichlorinated methyl group of **3** originates from a leucine residue without formation of an

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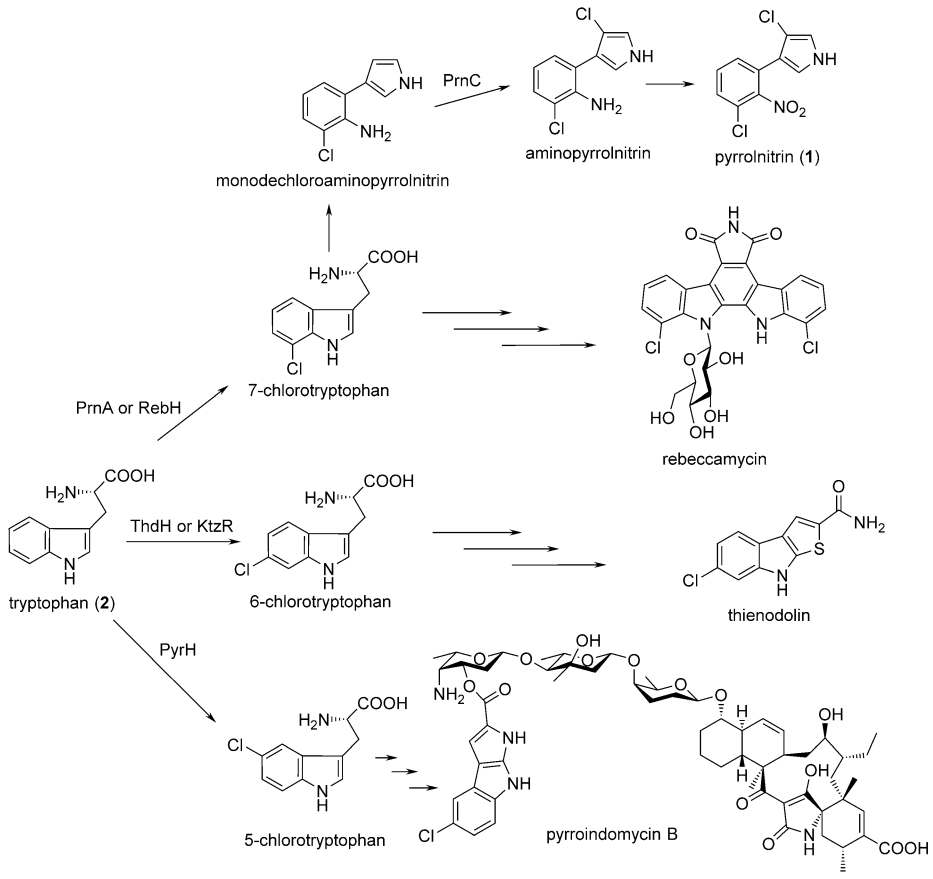

Scheme 2. Examples of the chlorination of tryptophan by flavin-dependent tryptophan halogenases with different regioselectivities during the biosyntheses of pyrrolnitrin (**1**), rebeccamycin, thienodolin, and pyrroindomycin.[15–17]

intermediate double bond.[19] Sitachitta et al. concluded from these findings that the chlorination of the methyl group of the leucine residue during the biosynthesis of **3** might proceed through a radical mechanism.[20] Comparison of the amino acid sequences of BarB1/BarB2 and BarC from the barbamide biosynthetic gene cluster[21] revealed high similarities with the potential enzymes SyrB2 and SyrC from syringomycin (Scheme 4) biosynthesis[22] and CmaA and CmaB from coronatine (Scheme 4) biosynthesis.[23] Vaillancourt et al. realized that SyrB2 shows homology to non-heme-iron- and α-ketoglutarate-dependent enzymes.[24] These enzymes use Asp/Glu and two His residues as ligands for the non-heme iron, and typically perform oxygenation reactions.[25,26] By using purified SyrB2, it could be shown that this enzyme catalyzes the chlorination of L-threonine bound to a peptidyl carrier protein (PCP; Scheme 4).[24] Subsequently, monochlorination by CmaB of L-*allo*-isoleucine tethered to a peptidyl carrier protein could be demonstrated in vitro, and Galonic et al. achieved trichlorination of L-leucine bound to a carrier protein by BarB1/BarB2 in vitro (Scheme 4).[27,28]

Based on the reaction mechanism of non-heme-iron and α-ketoglutarate-dependent hydroxylases, a reaction mechanism was proposed involving the formation of a substrate radical which abstracts a chlorine atom from the high-energy ferryl-oxo intermediate (Scheme 5). This mechanism was supported by the elucidation of the three-dimensional structure of SyrB2, which showed that in the resting state the iron is coordinated by two histidine residues, chloride, water, and α-ketoglutarate, but no aspartate (Scheme 5).[29] Although some competition between chlorine radicals and hydroxyl radicals would be expected, no hydroxylating activity was observed for SyrB2 (Scheme 1).

1.4. Fluorination

Since the oxidation of fluoride ions cannot be achieved with oxygen or hydrogen peroxide, a different type of halogenating enzyme must be involved in the formation of the carbon–fluorine bond. However, since fluoride is highly hydrated in aqueous solution, an enzyme that incorporates fluoride directly needs to remove the water molecules from the fluoride ion. Such an enzyme was detected in *Streptomyces cattleya*, the producer of fluoroacetate.[30,31] This fluorinase (FlA) has very high substrate specificity and only accepts *S*-adenosyl-L-methionine as the substrate. The reaction products of this reaction are L-methionine and 5′-fluoro-5′-desoxyadenosine (Schemes 1 and 13). Elucidation of the three-dimensional structure, site-directed mutagenesis, and kinetic studies showed that a serine and a threonine residue substitute the water molecules surrounding the fluoride ion and, thus, a naked fluoride ion is formed which can attack the substrate and lead to substitution of L-methionine.[32] Fluorinase can also react as a chlorinase in the presence of

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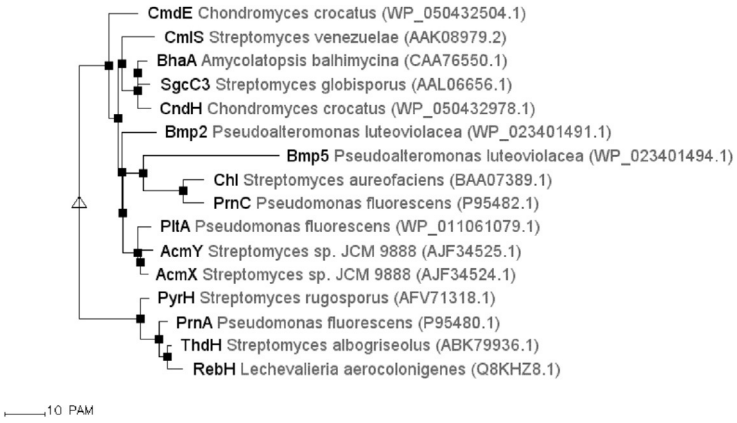
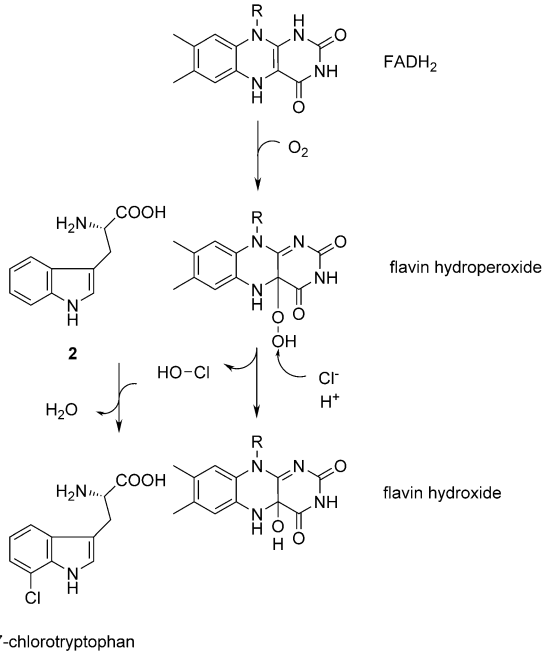


Figure 1. Phylogenetic tree of some flavin-dependent halogenases. The clustering of the tryptophan halogenases with different regioselectivities, PyrH, PrnA, ThdH, and RebH, is apparent. Pyrrole carboxylic acid halogenases using a PCP-bound substrate (PltA, AcmY, and AcmX) are separated from the pyrrole halogenase PrnC, which accepts a free substrate. Interestingly, PrnC seems to be closely related to the halogenase from the biosynthesis of 7-chlortetracycline Chl. Halogenases using a PCP-bound substrate, other than pyrrole carboxylic acid, also cluster together. 10 PAM = point accepted mutation.



Scheme 3. Reaction mechanism of flavin-dependent halogenases showing the chlorination of tryptophan to form 7-chlorotryptophan. The reduced flavin is provided by a nonspecific flavin reductase. In vitro, the halogenase halogenates both enantiomers of tryptophan. The produced HOCl cannot leave the active site. Regioselectivity is achieved by specific orientation of the substrate in the active site.[12,14]

chloride, but not as a brominase.[33] A similar enzyme with chlorinase, brominase, and iodinase activity, but no fluorinase activity, was found in the marine bacterium *Salinispora tropica* (Scheme 1).[34]

2. New Developments

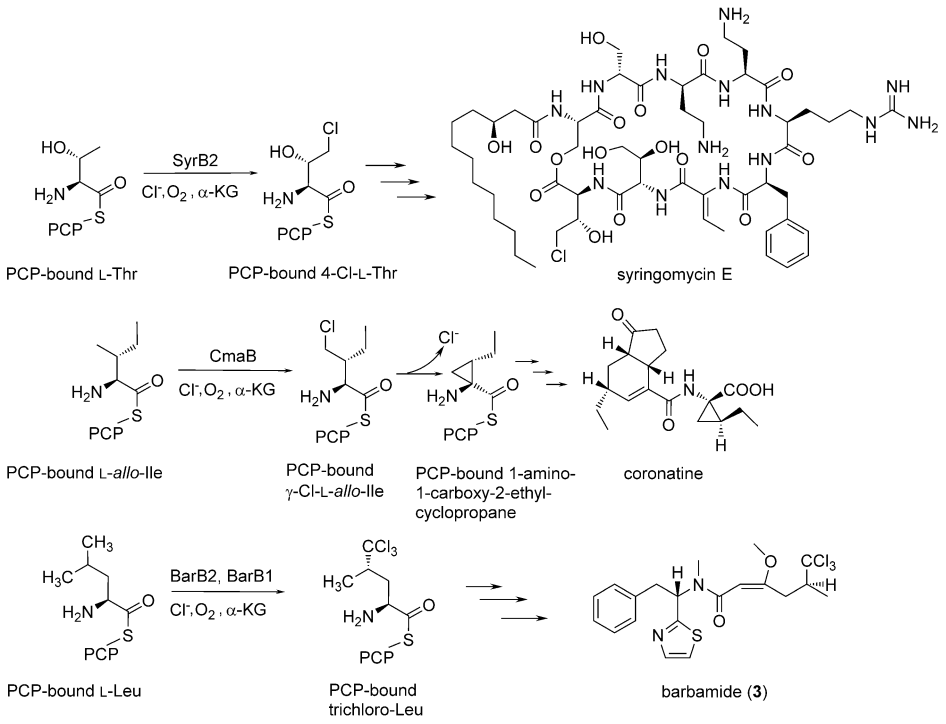
2.1. Halogenating Enzymes and Their Involvement in Halometabolite Biosynthesis

After the detection of *chl*, the gene of the flavin-dependent halogenase from 7-chlortetracycline biosynthesis and the two halogenase genes *prnA* and *prnC* from the biosynthesis of **1**, a large number of genes of other potential flavin-dependent halogenases were detected, based on sequence homologies. In most cases, the genes were found during the cloning and characterization of biosynthetic gene clusters of secondary metabolites in bacteria,[35] but there are also a few examples from fungi[36,37] and slime molds.[38,39] Unfortunately, a large number of these halogenase genes were incorrectly annotated. Many of these genes were annotated as tryptophan halogenase genes, although **2** was not used in the biosynthetic pathway.[39] The reason for this is probably that tryptophan halogenases were the first flavin-dependent halogenases investigated. Thus, at an early stage almost all the genes of flavin-dependent halogenase detected were annotated as tryptophan halogenase genes. Since only genes were found and annotated without any demonstration of in vitro activity, these errors remained largely undetected.

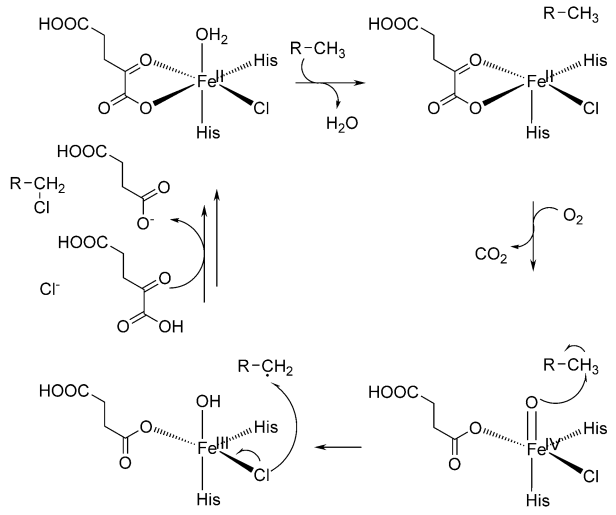
Expression of the genes of flavin-dependent halogenases and demonstration of in vitro activity was, and still is, an exception. The reason for this is twofold: Most of the halogenase genes originate from actinomycetes and are difficult to express in *E. coli* in a soluble form. However, good overexpression of the halogenase genes is a prerequisite for the detection of halogenating activity in vitro. Up to now, nobody succeeded in showing the activity of a flavin-dependent or of a non-heme-iron halogenase in extracts of a wild-type organism; activity can only be shown after overexpression of the genes. The second reason why in vitro activity has been demonstrated for only very few halogenases is the substrate problem: These halogenases have very high substrate specificity and, thus, either their natural substrate or a structurally highly related compound is required as the substrate. However, in most cases, the chemical structure of the natural substrate is not known, since it is usually not clear at which step in the biosynthetic pathway halogenation occurs. There are examples where halogenation is the first step and biosynthesis cannot continue if the starting compound is not halogenated.[15] In other examples, halogenation is the first step, but biosynthesis is unperturbed if halogenation is omitted and the non-halogenated end product is formed.[18] Thus, even deletion of the halogenase gene in the producing strain will not help to identify the natural substrate of the enzyme.

2.1.1. Halometabolites Derived from Halogenated Tryptophan Groups or Containing a Halogenated Tryptophan Moiety

In the case of the tryptophan 7-halogenase PrnA and the monodechloroaminopyrrolnitrin 3-halogenase PrnC, the first



Scheme 4. Top: Chlorination of PCP-bound L-threonine by the non-heme-iron, α-KG, and O_2-dependent halogenase SyrB2 involved in syringomycin E biosynthesis. Middle: Cryptic chlorination of PCP-bound L-*allo*-isoleucine by the non-heme-iron, α-KG, and O_2-dependent halogenase CmaB involved in coronatine biosynthesis. Bottom: Chlorination of PCP-bound L-leucine by the non-heme-iron, α-KG, and O_2-dependent halogenases BarB2 and BarB1 involved in barbamide (**3**) biosynthesis.[24,27,28]



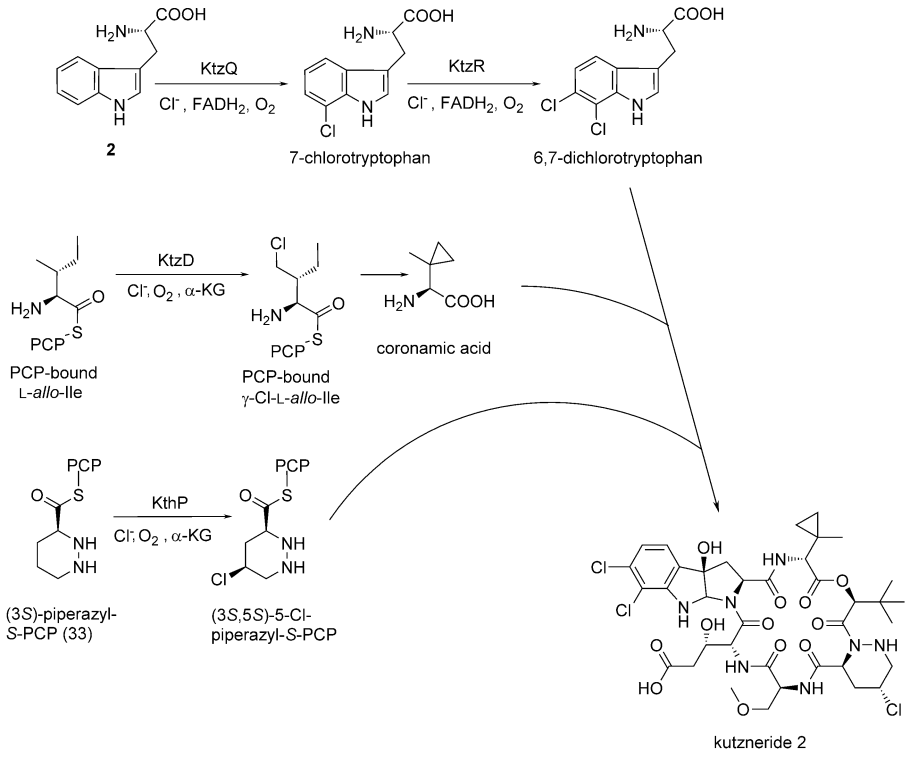
Scheme 5. Reaction mechanism of non-heme-iron, α-KG, and O_2-dependent halogenases.[29]

flavin-dependent halogenases for which in vitro activity could be shown, free **2** and free monodechloroaminopyrrolnitrin, respectively, are the natural substrates.[10] This is also the case for the tryptophan 6-halogenase (ThdH or Thal) from *Streptomyces albogriseolus*, involved in thienodolin biosynthesis,[17,40] and the tryptophan 5-halogenase from *Streptomyces rugosporus*, involved in pyrroindomycin B biosynthesis (Scheme 2).[18] These tryptophan halogenases catalyze the chlorination of both enantiomers of **2** in vitro, however, the L enantiomer of **2** is the better substrate.[92]

Cloning and characterization of the biosynthetic gene cluster for kutzneride 2, an antimicrobial cyclic hexadepsipeptide, revealed the presence of the genes of two flavin-dependent tryptophan halogenases, KtzQ and KtzR, and of one non-heme-iron halogenase, KtzD.[41] KtzQ, a tryptophan 7-halogenase, catalyzes the regioselective chlorination of free **2** to 7-chlorotryptophan, which is further chlorinated by KtzR to produce free 6,7-dichlorotryptophan.[42] This product is suggested to be incorporated into the growing kutzneride assembly line by the KtzH adenylation domain.[42] The non-heme-iron halogenase KtzD catalyzes the cryptic chlorination of L-*allo*-isoleucine bound to a peptidyl carrier protein (Schemes 4 and 6).[41] The gene of a second non-heme-iron halogenase, KthP, was later detected separated from the kutzneride biosynthetic gene cluster. This halogenase was shown to catalyze the stereospecific chlorination of a PCP-bound piperazate residue[43] that was suggested to be synthesized from glutamate/glutamine (Scheme 6).[41] From this example, it can be clearly seen that halogenation of a free substrate can occur before this compound is incorporated into the biosynthetic pathway, but chlorination occurs at PCP-tethered substrates during nonribosomal peptide synthesis.

2.1.2. *Halometabolites Containing Halogenated Pyrrole Residues*

During the cloning and characterization of the biosynthetic gene cluster of the antibiotic pyoluteorin, Nowak-Thompson et al. detected the genes of three potential flavin-dependent halogenases, *pltA*, *pltD*, and *pltM*.[44] However, pyoluteorin only contains two chlorine atoms. One of the deduced halogenases, PltD, does not contain the correct motif of the nucleotide binding site (GxSxxV instead of GxGxxG) and a second conserved motif, found in Chl, PrnC, and the tryptophan halogenases, the so-called tryptophan motif, was also aberrant (GWxGxI instead of GWxWxI). Therefore, it was concluded that PltD is not functional or not a halogenase at all.[44,45] Thus, PltA and PltM remained as candidates for the introduction of the two chlorine atoms into the pyrrole moiety

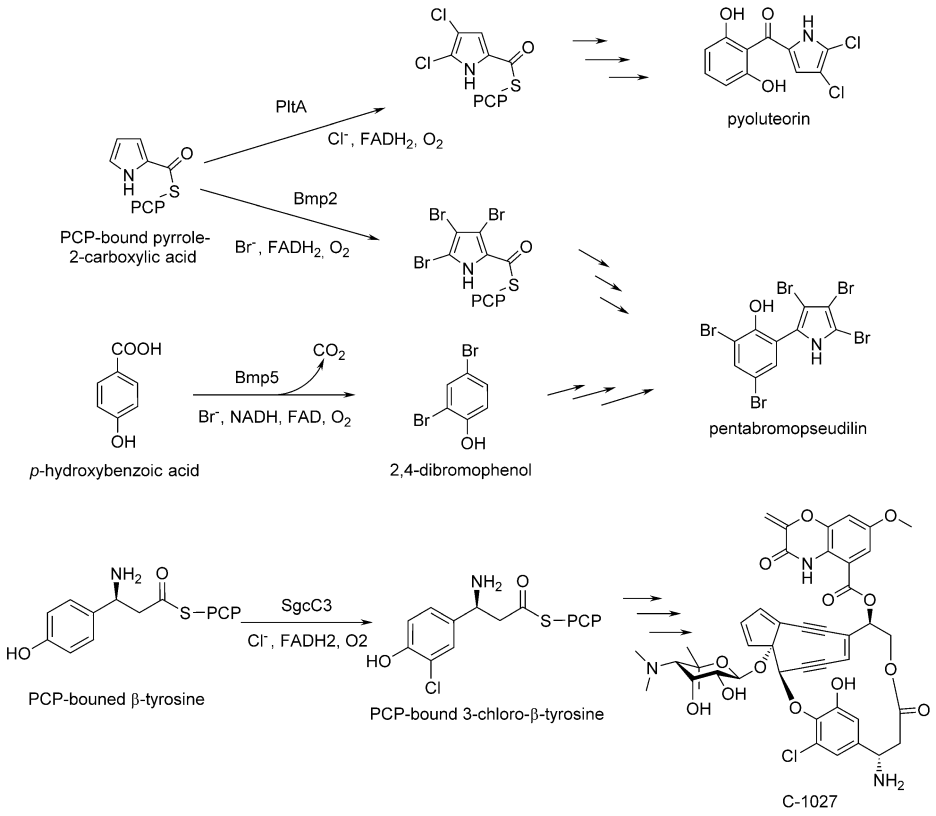


Scheme 6. Chlorination reactions occurring during the biosynthesis of kutzneride 2. Dichlorination of tryptophan (**2**) to 6,7-dichlorotryptophan by the flavin-dependent halogenases KtzQ and KtzR, chlorination of PCP-bound L-*allo*-isoleucine by the non-heme-iron, α-KG, and O_2-dependent halogenase KtzD, and chlorination of (3*S*)-piperazyl-S-PCP by the non-heme-iron, α-KG, and O_2-dependent halogenase KthP. The chlorinated building blocks are then incorporated into the biosynthesis of kutznetide 2.[41–43]

of pyoluteorin, which was known to be derived from proline. Formation of the pyrrole moiety was shown to require the activity of a nonribosomal peptide synthetase that results in a PCP-bound proline. This is the substrate for a flavin-dependent dehydrogenase that catalyzes the formation of PCP-tethered pyrrole carboxylic acid, which is the natural substrate for the halogenase (Scheme 7).[46] Surprisingly, the dichlorination of this compound was catalyzed in vitro by PltA alone, whereas no chlorinated product could be detected when PltM was used. To show PltA had in vitro activity, a rather complex enzyme system was required that consisted of a peptidyl carrier protein (PltL), to which a 4′-phosphopantetheinyl prosthetic group had to be attached by the nonspecific 4′-phosphopantetheinyltransferase Sfp. Proline was then loaded onto PltL by using the L-prolyl-AMP-ligase PltF. The oxidation of the prolyl-*S*-carrier protein was catalyzed by PltE. For the chlorination of the resulting pyrrolyl-*S*-carrier protein, the flavin-reductase SsuE from *E. coli* was added for the formation of $FADH_2$, which was required by the halogenase PltA. Electrospray ionization/Fourier transform MS and L-[^{14}C]proline had to be used for analysis of the reaction products, because the overall activity of this complex enzyme system was very low.[45] Agarwal et al. encountered the same problem when they analyzed the activity of the brominase Bmp2 from the biosynthesis of pentabromopseudilin (**4**). Bmp2 catalyzes the tribromination of pyrrolyl-*S*-acyl carrier protein (ACP; Scheme 7).[47] Although they attached pyrrole carboxylic acid to holo-ACP by chemical synthesis, LC/MS/MS was necessary to detect the brominated products. This clearly shows how difficult it is to detect the in vitro activity of halogenases, even when the natural substrate is used.

Very recently, the three-dimensional structure of PltA was elucidated.[48] The flavin-binding fold is very similar to those of other flavin-dependent halogenases, with a chloride ion bound closely to the isoalloxazine ring, as found for PrnA and PyrH.[12,14] Furthermore, the catalytically active lysine residue is also at a similar position as in PrnA, RebH, and PyrH, but the glutamate residue required for activity of PrnA and PyrH is missing. Unfortunately, no complex with PCP-bound pyrrole carboxylic acid could be obtained. The distance between the substrate and the isoalloxazine ring is assumed to be only 6 Å instead of 10 Å, as in the case of PrnA and PyrH. One important difference is an additional helical region at the C-terminus which blocks a putative substrate-binding cleft, thus suggesting that a conformational change involving repositioning of this region might be necessary to allow binding of the substrate.[48]

With the exceptions of Bmp2 and Bmp5,[47] all flavin-dependent and non-heme-iron halogenases detected so far catalyze chlorination and bromination reactions. However, in contrast to haloperoxidases, chlorinating activity is higher than brominating activity. This is probably due to better binding of the chloride than of the bromide ion in the active site, where the halides are bound in the vicinity of the isoalloxazine ring.[12,14,48] Feeding studies have shown that substitution of chloride in the growth medium by bromide results in the brominated metabolite.[49] Interestingly, the producer of pentachloropseudilin, *Actinoplanes* sp. ATCC 33002, can only synthesize pentachloropseudilin and not the brominated analogue **4**. This is produced by the marine bacteria *Pseudoalteromonas luteoviolacea* and *P. phenolica*, which cannot, on the other hand, produce the chlorinated analogue. From feeding studies, it was known that the pyrrole ring of **4** is derived from proline, as in the case of pyoluteorin,[44,50] and that the phenol ring originates from the shikimate pathway via *p*-hydroxybenzoic acid,[51] whereas the phenolic ring of pentachloropseudilin is synthesized by polyketide biosynthesis.[52] During their investigations on the biosynthesis of **4**, Agarwal et al. cloned and characterized the biosynthetic gene cluster of **4**.[47] The cluster contains the gene



Scheme 7. Halogenation of PCP-tethered substrates by flavin-dependent halogenases. Top: PCP-bound pyrrole-2-carboxylic acid is halogenated during the biosyntheses of pyoluteorin and pentabromopseudilin (**4**) by PltA and Bmp2, respectively.[45,47] Middle: Formation of 2,4-dibromophenol from *p*-hydroxybenzoic acid is catalyzed by Bmp5 during the biosynthesis of **4**.[47] Bottom: (*S*)-β-Tyrosyl-*S*-carrier protein is chlorinated by SgcC3 during the biosynthesis of the enediyne antitumor antibiotic C-1027.[53]

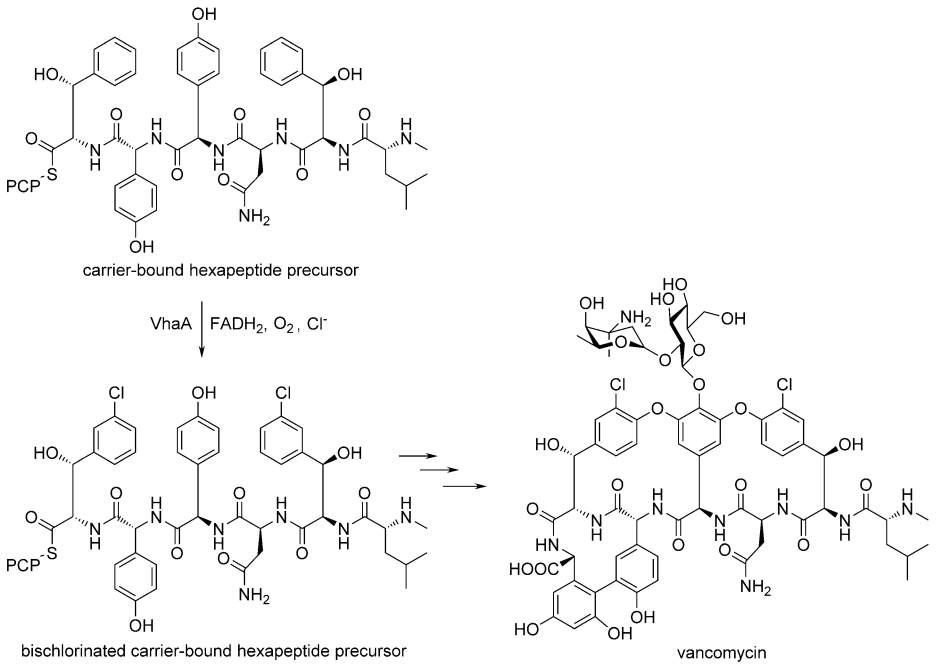
of the flavin-dependent halogenase Bmp2, whose deduced amino acid sequence is similar to PltA and PltM from pyoluteorin biosynthesis (Figure 1).[44] Bmp2 could be shown to catalyze mono-, di-, and tribromination, but not the chlorination of pyrrolyl-*S*-Bmp1 (Scheme 7).

2.1.3. *Halometabolites Containing Halogenated Phenol Moieties*

The gene of a second flavin-dependent halogenase involved in pentabromopseudilin biosynthesis, *bmp5*, was not easily detected, since its sequence homology to any known flavin-dependent halogenase is rather low (Figure 1). Bmp5 contains the nucleotide binding site. The catalytically active lysine residue is, as in PrnC and in other flavin-dependent phenylpyrrole halogenases, shifted about 12 amino acid residues towards the C-terminal end. Bmp5 catalyzes the monobromination of *p*-hydroxybenzoic acid, and after decarboxylation of the 3-bromo-4-hydroxybenzoic acid intermediate, a second bromine is incorporated, thus leading to formation of the 2,4-dibromophenol moiety of **4** (Scheme 7). Sufficient product was formed by Bmp5 to be analyzed by HPLC. No addition of a flavin reductase was required, since Bmp5 also serves as a flavin reductase. This is reflected in the sequence homology of Bmp5 with single-component flavin-dependent oxygenases and the presence of a NADPH binding site (GLGES-SAD). Bmp5 is, thus, the first single-component flavin-dependent halogenase with additional decarboxylase activity. Similar to Bmp2, Bmp5 does not catalyze the incorporation of chlorine.[47]

Regiospecific chlorination of (*S*)-β-tyrosyl-carrier protein was achieved with the flavin-dependent halogenase SgcC3 from the biosynthesis of the enediyne antitumor antibiotic C-1027 (Scheme 7). As in the case of the chlorination of PCP-bound pyrrole-2-carboxylic acid by PltA, the substrate was synthesized enzymatically. The activity obtained was rather high compared to the activity of the pyrrolyl-*S* carrier protein halogenases, thus allowing for kinetic characterization of the enzyme. The k_{cat} value for the chlorination reaction was much lower than those found for the tryptophan halogenases (1.1 h^{-1} compared to 1–7 min^{-1}). Interestingly, brominating activity was about twofold lower than chlorinating activity, which is in good agreement with findings for tryptophan halogenases.[53]

The glycopeptides vancomycin, balhimycin, and chloroeremomycin all contain chlorinated β-hydroxytyrosine residues. Since the feeding of 3-chloro-β-hydroxytyrosine did not lead to the formation of balhimycin, it could be ruled out that, during balhimycin biosynthesis, the precursor compound β-hydroxytyrosine is chlorinated before its incorporation into the growing peptide chain.[54] Analysis of the balhimycin biosynthetic gene cluster revealed the presence of two potential halogenating enzymes, Bhp and BhaA. Whereas Bhp, a "cofactor-free haloperoxidase/perhydrolase" could be identified as the enzyme hydrolyzing the β-hydroxytyrosine-*S*-carrier protein,[55,56] BhaA was identified by gene inactivation as the flavin-dependent halogenase involved in balhimycin biosynthesis.[54] BhaA was assumed to catalyze the incorporation of the chlorine atoms into both β-hydroxytyrosine residues of balhimycin. It took a further four years until Schmartz et al. showed that VhaA, the analogous halogenase to BhaA from vancomycin biosynthesis, catalyzes bis-chlorination of the hexapeptide-peptidyl carrier protein conjugate intermediate of vancomycin biosynthesis (Scheme 8).[57] Thus, regioselective chlorination of both β-hydroxytyrosine residues occurs directly after incorporation of the second β-hydroxytyrosine residue into the growing peptide chain and before incorporation of the last amino acid residue. VhaA is highly substrate specific and does not accept the dipeptide, which also has a carboxy terminal β-

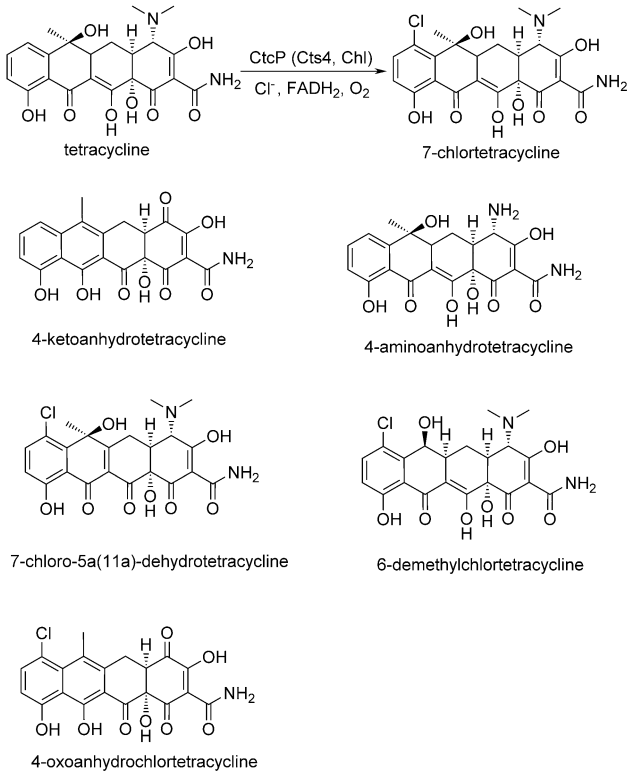


Scheme 8. Chlorination of the PCP-bound hexapeptide precursor by the flavin-dependent halogenase VhaA during vancomycin biosynthesis.[57]

hydroxytyrosine residue. Clearly, a larger part of the peptide is recognized by VhaA and required for binding of the substrate.

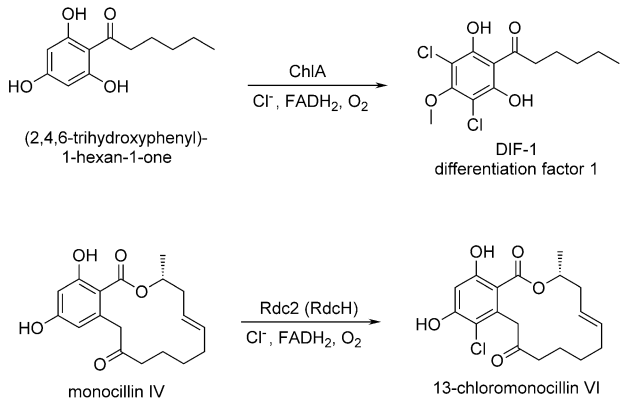
Feeding studies for the elucidation of the biosynthesis of 7-chlortetracycline have shown that the chlorinating enzyme involved in the biosynthesis of 7-chlortetracycline has a high substrate specificity and also regioselectivity. It was shown that 4-ketoanhydrotetracycline must be the substrate for the halogenating enzyme, and that chlorination is no longer possible after transamination to 4-aminoanhydrotetracyclin (Scheme 9).[58] Thus, it came as a big surprise when Zhu et al. published that CtcP, formerly known as Chl or Cts4, catalyzes the chlorination of tetracycline as the last step in the biosynthesis of 7-chlortetracycline (Scheme 9).[8,59] This finding is in absolute disagreement with the isolation of 7-chloro-5a(11a)-dehydrotetracycline, 6-demethylchlortetracycline, and especially with 4-oxoanhydrochlortetracycline (Scheme 9).[60–62] If chlorination is the last step in the biosynthesis of 7-chlortetracycline, the existence of these compounds cannot be explained.

Neumann et al. demonstrated chlorinating activity for ChlA, a flavin-dependent halogenase from the social amoeba *Dictyostelium discoideum*.[38] ChlA was shown to catalyze both chlorinations in the biosynthesis of the differentiation-factor 1 in vivo. (2,4,6-Trihydroxyphenyl)-1-hexan-1-one (THPH) was used as the substrate for the in vitro analysis of ChlA (Scheme 10). The detected chlorinating activity was very low, especially for the incorporation of the second chlorine atom. THPH is produced through polyketide synthesis and, thus, it should be considered whether the natural substrate might be an intermediate still bound to the acyl carrier protein StlB.



Scheme 9. Chlorination of tetracycline to form 7-chlortetracycline by the flavin-dependent halogenase CtcP (also named Cts4 or Chl) and a few chlorinated 7-chlortetracycline derivatives, whose existence cannot be explained if chlorination is the last step in the biosynthesis of 7-chlortetracycline.[8,58–62]

Radicicol is a chlorine-containing polyketide produced by the fungus *Pochonia chlamydosporia*. Its biosynthetic gene cluster harbors the gene of a flavin-dependent halogenase, Rdc2.[36,63] Zeng and Zhan analyzed the halogenating activity of Rdc2 in vitro using monocillin I, the non-halogenated derivative of radicicol.[37] However, many peaks appeared in the HPLC trace. Thus, substrates with fewer double bonds, such as monocillin IV, were used, and mono- and dichlorination and bromination were found to occur at the phenolic ring (Scheme 10). The formation of dichlorinated products was unexpected, since dichlorinated monocillin I has never been found in nature. Monocillin IV is likely not the natural substrate of Rdc2. In experiments using 6-hydroxyisoquinoline as a sub-



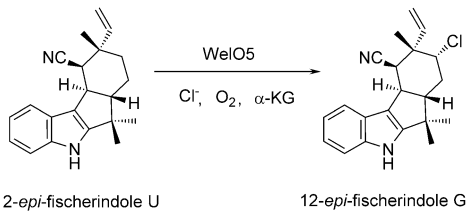
Scheme 10. Chlorination of (presumably) non-natural substrates by various flavin-dependent halogenases.[37,38]

strate for Rdc2, mono- and dichlorinated products were again obtained.[64]

2.1.4. *Metabolites Halogenated at Aliphatic Moieties or Non-Aromatic Rings*

In biosynthetic pathways where the moiety to be halogenated is not part of a nonribosomal peptide or polyketide synthesis, such as in the biosyntheses of **1**, rebeccamycin, thienodolin, pyrroindomycin B (Scheme 2), or kutzneride 2 (Scheme 6), a free halogenase substrate is used.[10,16,18,40,42,45] If the halogenase is involved in nonribosomal peptide or polyketide biosynthesis, it is extremely unlikely that the halogenase substrate is a free molecule. This would only be the case if halogenation were the first step before activation of the amino acid for peptide synthesis or the starter unit for polyketide synthesis. Thus, it is not surprising that in a biosynthetic pathway, such as the one for welwitindolinone, which does not involve any peptidyl or acyl carrier proteins,[65] halogenation occurs at a free substrate. 12-*epi*-Fischerindole U (Scheme 11) and 12-*epi*-hapalindole C are chlorinated by the non-heme-iron halogenase WelO5.[66]

The conversion of biosynthetic intermediates of snyderol biosynthesis into brominated products by a vanadium-containing haloperoxidase resulted in formation of a number of by-products and only a small percentage of the brominated biosynthetic intermediate.[67] Molecular genetic evidence for



Scheme 11. Chlorination of free 12-*epi*-fischerindole U by the non-heme-iron, α-KG, and O_2-dependent halogenase WelO5 from the biosynthesis of welwitindolinone.[65,66]

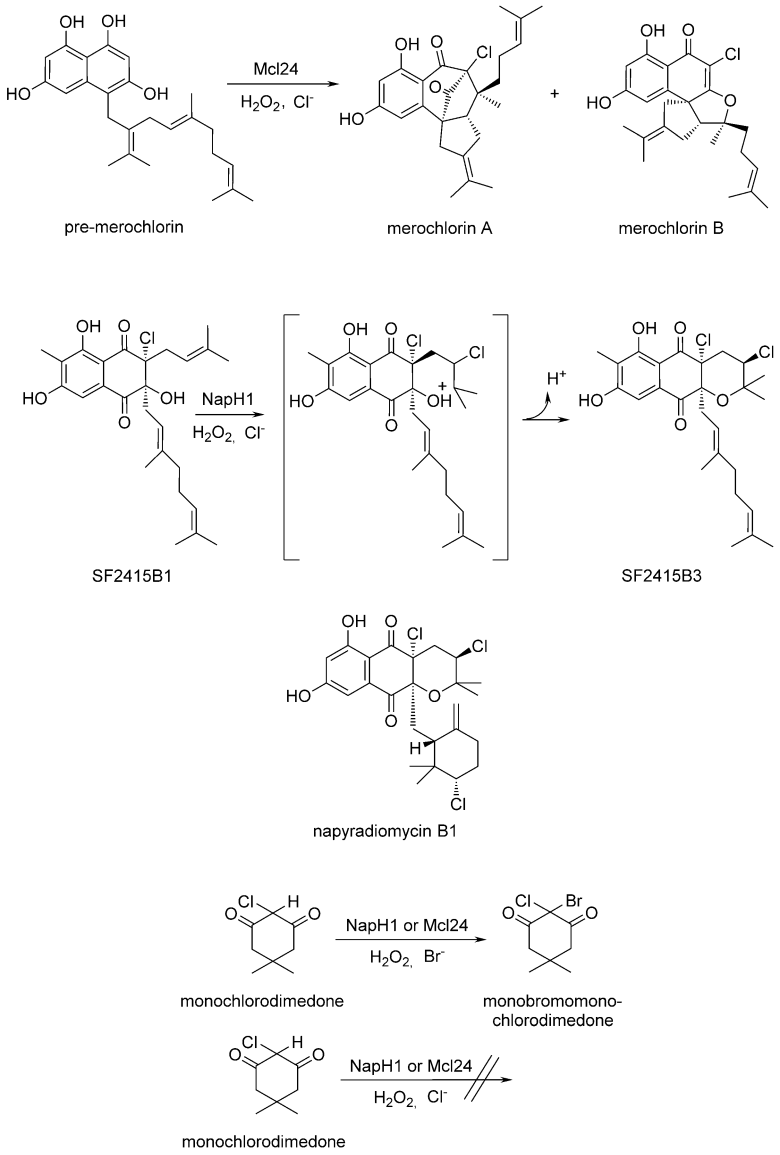
the involvement of the used vanadium haloperoxidase in snyderol biosynthesis has so far not been found.

However, Kaysser et al. recently cloned the biosynthetic gene cluster for merochlorin biosynthesis from the marine *Streptomyces* sp. CNH-189, in which they detected two genes encoding vanadium-dependent haloperoxidases. They expressed two different fosmid clones which differed by the presence or absence of the gene *mcl40*, which codes for one of the two vanadium-dependent halogenases. The clone lacking *mcl40* was not able to produce merochlorin C, thereby suggesting that Mcl40 was involved in the final chlorination and cyclization step.[68] It was later realized that it is actually Mcl24 and not Mcl40, as previously thought, that not only catalyzes the site-specific chlorination of the naphthol moiety of pre-merochlorin, but additionally initiates a series of cyclization reactions leading to dearomatization/terpene cyclization to build up the complex carbon framework of merochlorin A and merochlorin B. Mcl24 does not lead to a decrease in the absorbance of monochlorodimedone in the presence of chloride, only in the presence of bromide (Scheme 12).[69,70]

Analysis of the napyradiomycin (Scheme 12) biosynthetic gene cluster from *Streptomyces* sp. CNQ-525 revealed the presence of the gene of a vanadium-dependent chloroperoxidase, *napH1*. Purified NapH1 catalyzes the stereoselective chlorination and bromination of an intermediate (SF2415B1) to SF2415B3 in the biosynthesis of the trichlorinated meroterpenoid A80915 (Scheme 12). The NapH1-catalyzed halogenation reaction in addition to chlorination also leads to a cyclization reaction. However, in the presence of bromide, NapH1 carries out a nonspecific bromination of the substrate that leads to two new brominated compounds. It is assumed that NapH1 interacts intimately with its substrate and is highly substrate specific in the presence of chloride. When the unnatural substrate monochlorodimedone was used, NapH1 only catalyzed bromination, not the chlorination of monochlorodimedone (Scheme 12), as already observed for Mcl24 in the merochlorin biosynthesis.[69,70] NapH1 as well as Mcl24 behave like nonspecific bromoperoxidases in the presence of bromide, and form hypobromous acid which is released from the active site and leads to bromination of the unnatural substrate monochlorodimedone.

2.1.5. *Halogenases with a Known Three-Dimensional Structure but an Unknown Substrate*

The three-dimensional structures of some halogenases have been elucidated, but demonstration of in vitro activity is still missing. One of these halogenases is CndH, a flavin-dependent halogenase which was shown to be involved in chondrochloren A (Figure 2) biosynthesis.[71] CndH is assumed to catalyze the regioselective monochlorination of a PCP-bound tyrosine residue in the 3-position. Although in vitro activity of CndH could not be demonstrated, Buedenbender et al. solved the three-dimensional structure of the enzyme and found a very similar architecture of the active site to that of PrnA.[72] The catalytically active lysine residue was present;[12] however, the glutamate residue, found to be necessary for activity in PrnA,[73] is missing. The glutamate



Scheme 12. Top: Chlorination of pre-merochlorin by the vanadium-dependent chloroperoxidase Mcl24 and of the intermediate SF2415B1 of the biosynthesis of the meroterpenoid A80915C by the vanadium-dependent chloroperoxidase NapH1. Middle: Chemical structure of napyradiomycin B1. Bottom: Both chloroperoxidases, Mcl24 and NapH1, catalyze the bromination but not the chlorination of monochlorodimedone.[69,70]

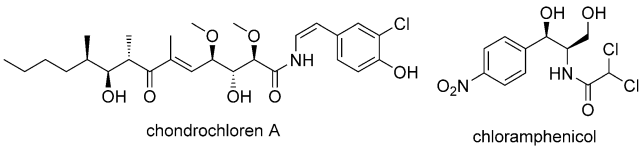


Figure 2. Halogenated metabolites for the biosyntheses of potential halogenases which have been identified and the three-dimensional structures of the enzymes have been elucidated, but in vitro activities of the enzymes have not been shown, so far.[71,74]

residue seems only to be necessary in halogenases that catalyze the halogenation of less-reactive substrates such as tryptophan. In the case of more-reactive substrates, such as a phenol or pyrrole derivatives, the glutamate residue does not seem to be required for activity. Buedenbender et al. compared the amino acid sequences of flavin-dependent halogenases that accept free substrates with those that accept carrier-bound substrates, and found that they clearly split into two amino acid sequence variants. However, they only used four halogenases for their comparison: the two tryptophan 7-halogenases PrnA and RebH and the two tyrosine halogenases CndH and SgcC3.[72] This is quite problematic, since the tryptophan halogenases show a rather high sequence homology with each other and will thus always cluster, but have rather low sequence homology with other halogenases (Figure 1). On the other hand, it is correct that some halogenases that accept carrier protein bound substrates can be distinguished from those using free-standing substrates by comparing their amino acid sequences. In the case of the pyrrolyl-*S*-carrier protein-halogenating enzymes, they are about 70 amino acids shorter than the halogenases that accept other PCP-bound or free substrates. In the phylogenetic tree in Figure 1, it can be seen that the monodechloroaminopyrrolnitrin 3-halogenase PrnC is well separated from the pyrrole halogenases that use bound substrates; PrnC uses free monodechloroaminopyrrolnitrin, which it halogenates regioselectively at the 3-position of the pyrrole moiety (Scheme 2).[10]

A second example of a halogenase with known three-dimensional structure but so far no in vitro activity is that of the flavin-dependent halogenase CmlS involved in the biosynthesis of chloramphenicol (Figure 2).[74] Activity for CmlS could not be shown because of the fact that its natural substrate is not known. This is in agreement with feeding studies which did not succeed in solving the riddle of the chlorination step in vivo, either.[75] It is suggested that the halogenase substrate is a CoA derivative. CmlS is particularly unusual, since elucidation of its three-dimensional structure revealed that it contains FAD covalently bound to the protein through an aspartate residue.[76] Interestingly, the structure of CmlS is used quite frequently for modeling of other halogenase structures.[64]

2.2. Application of Halogenases

2.2.1. In Vivo Application

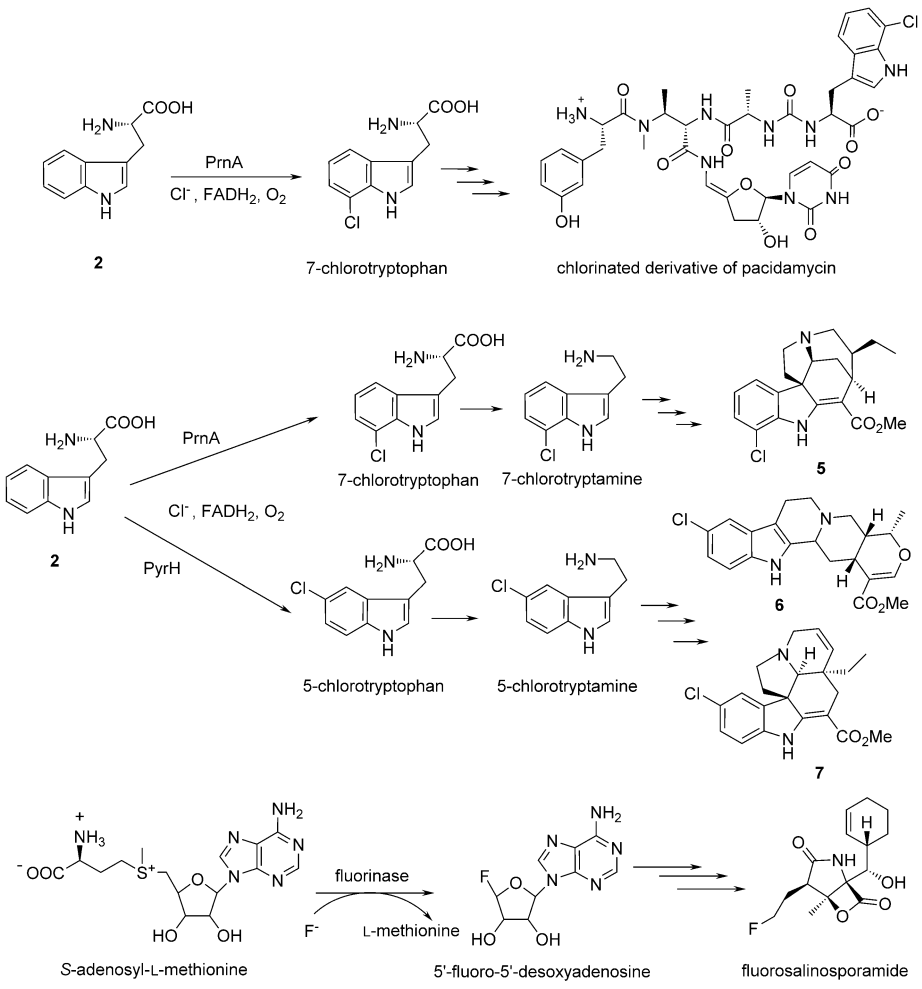
Since the use of flavin-dependent halogenases in in vitro reactions was very much hampered by the lack of knowledge of and/or the availability of their substrates, investigations into the use of these enzymes for the formation of novel

compounds in vivo were carried out. In these investigations, genes of tryptophan halogenase with different regioselectivities were introduced into bacteria which originally already produced halogenated metabolites. Thus, variants of the originally produced halogenated metabolites were obtained. In some cases, biosynthesis stopped or was diverted because an upstream enzyme did not accept the intermediate halogenated at a different position.[52,77]

Roy et al. introduced the tryptophan 7-halogenase gene *prnA* from the biosynthesis of **1** into the pacidamycin producer *Streptomyces coeroleorubidus* and obtained pacidamycin with a tryptophan residue chlorinated in the 7-position (Scheme 13). This chlorine atom could then be substituted chemically by other substituents, thus allowing the introduction of other functional groups.[78]

Runguphan et al. introduced the tryptophan 7-halogenase gene *rebH* from rebeccamycin biosynthesis[16] and the tryptophan 5-halogenase gene *pyrH* from the biosynthesis of pyrroindomycin,[18] together with the flavin reductase gene *rebF* from the rebeccamycin producer *Lechevalieria aerocolonigenes*[16] into the medicinal plant *Catharanthus roseus*.[77] Expression of the halogenase genes and the flavin reductase gene resulted in the formation of chlorinated and brominated (in the presence of bromide) tryptophan-derived alkaloids. Some were the halogenated analogues of alkaloids normally produced by *C. roseus*, but due to the substrate specificities of enzymes present in the plant, new chlorinated tryptophan-derived alkaloids were also formed (**5**—**7**; Scheme 13).[77] Some of the halogenated compounds could also be chemically derivatized by Suzuki–Miyaura cross-coupling.[79]

Eustáquio et al. introduced the fluorinase gene *flA* from *S. cattleya* into the salinosporamide producer *Salinispora tropica*.[80] Since *S. tropica* harbors a chlorinase gene (*salL*), this gene was destroyed and the fluorinase gene (*flA*) was introduced into the genome of *S. tropica*. However, production of fluorosalinosporamide was not straightforward. To circumvent the fluoride toxicity issue, fluoride was not added until the cultures reached an early to middle exponential phase. In this way, the *S. tropica* clone harboring the fluorinase gene was able to produce fluorosalinosporamide (Scheme 13), In addition, other fluorinated compounds of unknown structures were detected. Thus, it could be shown that fluorinase can also be used in vivo for the production of novel fluorometabolites.



Scheme 13. Manipulation of biosynthetic pathways to obtain novel halogenated metabolites by introduction of halogenase genes. Top: In vivo formation of chlorinated pacidamycin by the chlorination of tryptophan (**2**) to form 7-chlorotryptophan by the flavin-dependent tryptophan halogenase PrnA prior to incorporation into pacidamycin biosynthesis. Middle: Transformation of the medicinal plant *Catharanthus roseus* with tryptophan halogenase genes leads to the formation of halogenated alkaloids such as **5**–**7**. Bottom: Introduction of the fluorinase gene from *Streptomyces cattleya* into the salinosporamide producer *Salinispora tropica* leads to the formation of fluorosalinosporamide.[77,78,80]

2.2.2. *Production of Halogenated Compounds In Vitro*

Halogenases are of high interest for use in "green chemistry". They catalyze highly selective and specific halogenation reactions without the formation of any by-products except water, and in the case of flavin-dependent halogenases, hydrogen peroxide, formed by the reaction of $FADH_2$ with oxygen. Hydrogen peroxide, however, can be conveniently decomposed in situ by catalase to oxygen and water. Fluorinase has extremely high substrate specificity and so far *S*-adenosyl-L-methionine is the only known substrate of this enzyme and of the related chlorinase. However, fluorinase can be used to produce ^{18}F-labeled molecules for use in diagnostics.[81,82] Non-heme-iron halogenases have to be purified under anaerobic conditions and

they are very sensitive to oxygen. Flavin-dependent halogenases are highly stable and can be stored in the presence of oxygen for months. Unfortunately, flavin-dependent halogenases show very low activity ($k_{cat} = 1–7\ min^{-1}$). There has been some confusion about the stability of tryptophan halogenases under reaction conditions, but in the meantime it is agreed that they are already inactivated after about two hours of incubation.[73,83–86]

Lang et al. showed in 2011 that the regioselectivity of halogenases can be modified by site-directed mutagenesis. When a large phenylalanine residue close to the bound substrate tryptophan in the tryptophan 7-halogenase PrnA was exchanged against a small alanine residue, the regioselectivity of PrnA was affected and PrnA showed additional tryptophan 5-halogenase activity.[87] Runguphan et al. had observed that the formation of indole alkaloids from 7-chlorotryptophan was limited by the activity of the tryptamine-forming tryptophan decarboxylase in the plant *C. roseus*.[77] Thus, it was decided to enhance the acceptance of tryptamine as a substrate by the tryptophan 7-halogenase RebH. Based on rational design, a tyrosine residue in the region where the carboxylic acid group of the substrate tryptophan interacts with the enzyme was exchanged against a bulkier tryptophan residue. This mutation led to preferential halogenation of tryptamine over **2**, thus demonstrating that changing the substrate specificity of flavin-dependent halogenases by site-directed mutagenesis is also possible.[88]

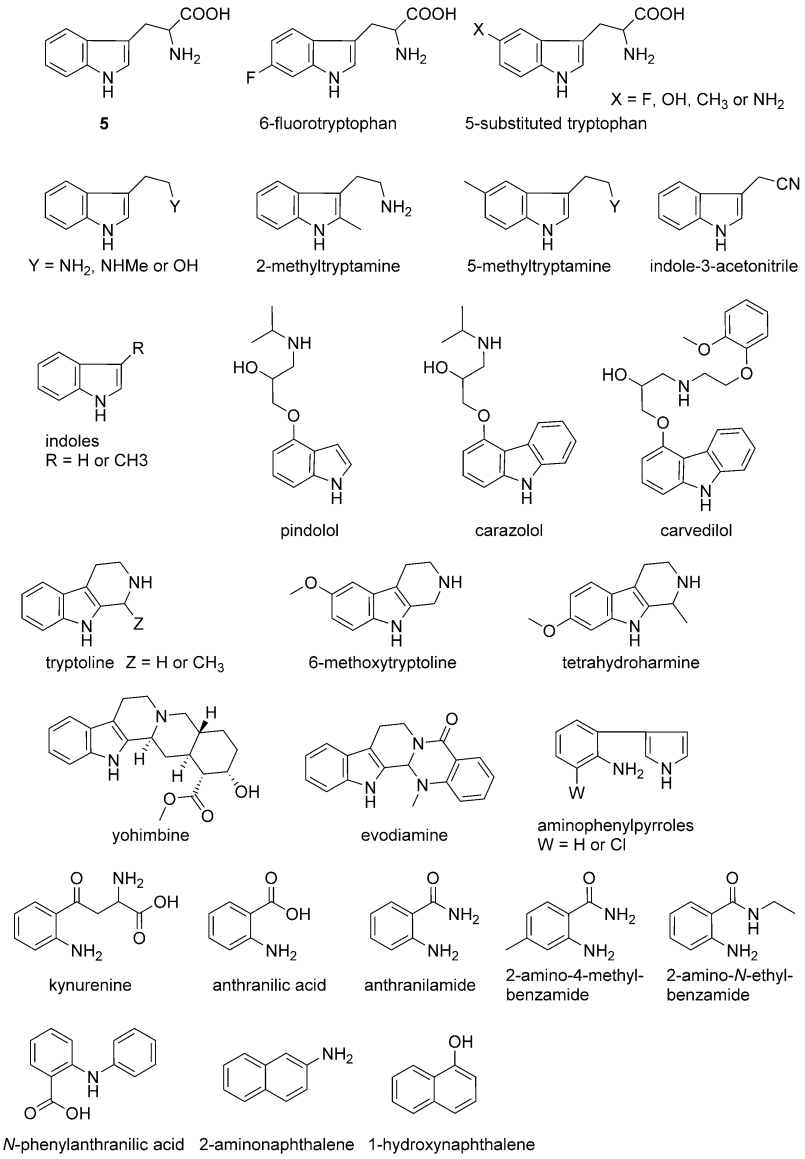
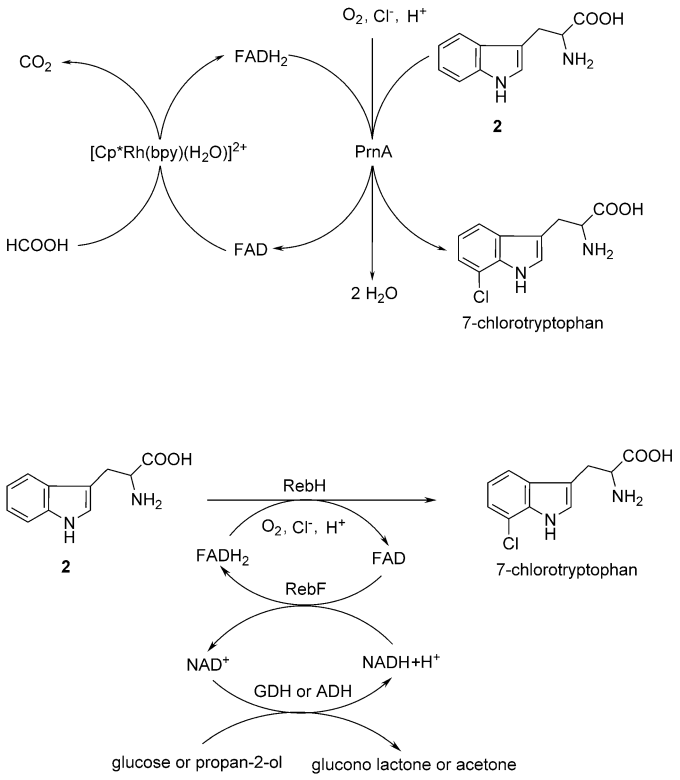


Figure 3. Substrates accepted by tryptophan halogenases.[83,85,89,90]

After the first publication on the substrate specificity of the flavin-dependent tryptophan 7-halogenase PrnA in 2001,[89] no further investigations on the substrate specificity of flavin-dependent halogenases were published for the following 12 years. Payne et al. used the tryptophan 7-halogenase RebH and achieved chlorination and bromination of indole as well as a number of substituted tryptophan derivatives, tryptoline, and 2-aminonaphthalene (Figure 3).[83] Frese et al. showed that RebH also accepts substituted tryptophan derivatives such as 5-fluoro-, 5-hydroxy-, 5-methyl-, 5-amino-, and 6-fluorotryptophan (Figure 3).[85] Shepherd et al. extended the biocatalytic scope of PrnA and PyrH even further by not only using substituted tryptophans, but also aniline derivatives, such as anthranilic acid and kynurenine (Figure 3), thereby showing that the substrate specificity of tryptophan halogenases is clearly not as high as originally assumed and that their substrate scope is not restricted to indole derivatives.[90]

It had been shown that flavin-dependent halogenases do not require a specific flavin reductase, just free $FADH_2$.[13] Thus, a number of different flavin reductases can be used in vitro or $FADH_2$ can be formed in situ by using a metal catalyst. However, halogenase activity is highly decreased in this system.[13] If $FADH_2$ is to be formed enzymatically, NADH regeneration is required. To solve the issue of NADH regeneration, Payne et al. and Shepherd et al. used glucose dehydrogenase for NADH regeneration (Scheme 14).[83,90] RebH and RebF were employed after purification from *E. coli* extracts by affinity chromatography. Frese et al.[85] used a similar system, but glucose dehydrogenase was substituted by alcohol dehydrogenase from *Rhodococcus* sp. produced in *E. coli* with 2-propanol as the substrate. RebH and the flavin reductase PrnF[93] were used as purified enzymes, whereas alcohol dehydrogenase was used after heat precipitation. The introduction of a NADH regeneration system into the tryptophan halogenase reaction is an important step towards the large-scale production of halogenated compounds. Frese and Sewald[86] achieved the enzymatic



Scheme 14. Regeneration systems used for flavin-dependent halogenases: Top: Direct regeneration of $FADH_2$ can be achieved using an organometallic complex; bpy = 2,2'-bipyridyl, Cp* = pentamethylcyclopentadienyl. Bottom: For the enzymatic formation of $FADH_2$, NADH is required, which can be regenerated using either glucose and glucose dehydrogenase or 2-propanol and alcohol dehydrogenase.[13,84,91]

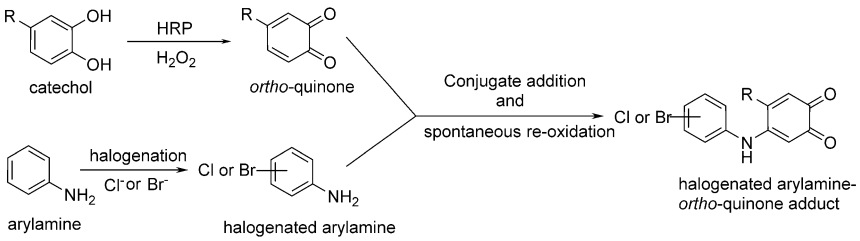
halogenation of **2** on a gram scale by preparing cross-linked enzyme aggregates (CLEAs) from crude *E. coli* extracts. The flavin reductase PrnF and alcohol dehydrogenase were added to the crude extract, and enzymes were cross-linked with glutaraldehyde after precipitation with ammonium sulfate. It was found that CLEA formation considerably extended the lifetime of RebH and that the CLEAs could be recycled at least 10 times.[86] However, crude extracts of *E. coli* were used and the halogenase substrates tryptophan and tryptophan derivatives are also substrates for tryptophanase from *E. coli*. Thus, a mixture of products is produced, which reduces the efficiency of the reaction and also requires additional purification steps. This could be avoided if tryptophanase-free hosts, such as *Pseudomonas* strains, were used, which are very suitable for expression of halogenase genes from various sources.[11,17,18]

The thermal stability and the catalytic lifetime of RebH were improved by directed evolution. Poor et al. obtained a RebH mutant containing eight mutations that had an increased lifetime at elevated temperature compared to the wild-type enzyme, but also with a significantly reduced turnover number.[84]

The construction of improved halogenases with higher activities, reduced substrate specificity, and even longer catalytic lifetimes is severely hampered by the issue of mutant screening. So far, libraries have to be screened in rather cumbersome procedures by growing the clones, lysing them, followed by incubation with substrate and HPLC analysis. Thus, an assay with a simple detection method would be very welcome and helpful in the construction of improved halogenase variants. Hosford et al. developed a high-throughput assay for arylamine halogenation based on horseradish peroxidase (HRP) mediated quinone-amine coupling (Scheme 15) which is suitable for spectrophotometric analysis of enzymatic halogenation reactions as a result of a shift in the absorbance maxima of the halogenated compounds compared to the non-halogenated ones.[91] Unfortunately, however, the requirement of an arylamine functionality very much restricts this assay to a rather small group of halogenase substrates.

3. Outlook

The last few years have seen highly promising achievements in the field of enzymatic halogenation. After the detection of flavin- and non-heme-iron-dependent halogenases and the elucidation of their basic reaction mechanisms, focus of the research has shifted towards the application of halogenases for the by-product-free production of halogenated compounds in vivo and in vitro. First successes in the improvement of halogenase stability,



Scheme 15. High-throughput assay for the detection of halogenated arylamines.[91]

increase in lifetime of the enzymes under reaction conditions, modification of substrate specificity, and regioselectivity by error-prone or site-specific mutagenesis have been described. It has also been shown that the substrate specificity, at least of tryptophan halogenases, is not as high as originally assumed. The development of high-throughput assays is in progress, which will allow the simple screening of mutant libraries. One important issue, however, is the low activity of halogenases, which so far has not been improved to the necessary level for the industrial in vitro application of the enzymes. However, with the development of new, easy, and fast activity assays, this will hopefully also be achieved in the near future, thereby paving the way for the intensive use of halogenating enzymes in industrial productions. The use of in vivo systems already

allows the improved production of important halogenated compounds such as vancomycin and 7-chlortetracycline by increasing the halogenase content in the producing organisms.

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[1] P. D. Shaw, L. P. Hager, *J. Am. Chem. Soc.* **1959**, *81*, 1011 – 1012.
[2] H. Vilter, *Phytochemistry* **1984**, *23*, 1387 – 1390.
[3] M. Sundaramoorthy, J. Taylor, T. L. Poulos, *Chem. Biol.* **1998**, *5*, 461 – 473.
[4] A. Messerschmidt, L. Prade, R. Wever, *Chem. Biol.* **1997**, *378*, 309 – 315.
[5] K. Kühnel, W. Blankenfeld, J. Terner, I. Schlichting, *J. Biol. Chem.* **2006**, *281*, 23990 – 23998.
[6] D. R. Morris, L. P. Hager, *J. Biol. Chem.* **1966**, *241*, 1763 – 1768.
[7] B. Hofmann, S. Tölzer, I. Pelletier, J. Altenbuchner, K.-H. van Pée, H. J. Hecht, *J. Mol. Biol.* **1998**, *279*, 889 – 900.
[8] T. Dairi, T. Nakano, K. Aisaka, R. Katsumata, M. Hasegawa, *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1099 – 1106.
[9] P. S. Hammer, D. S. Hill, S. T. Lam, K.-H. van Pée, J. M. Ligon, *Appl. Environ. Microbiol.* **1997**, *63*, 2147 – 2154.
[10] K. Hohaus, A. Altmann, W. Burd, I. Fischer, P. E. Hammer, D. S. Hill, J. M. Ligon, K.-H. van Pée, *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2012 – 2013; *Angew. Chem.* **1997**, *109*, 2102 – 2104.
[11] S. Keller, K. Hohaus, M. Hölzer, E. Eichhorn, K.-H. van Pée, *Angew. Chem. Int. Ed.* **2000**, *39*, 2300 – 2302; *Angew. Chem.* **2000**, *112*, 2380 – 2382.
[12] C. Dong, S. Flecks, S. Unversucht, C. Haupt, K.-H. van Pée, J. H. Naismith, *Science* **2005**, *309*, 2216 – 2219.
[13] S. Unversucht, F. Hollmann, A. Schmid, K.-H. van Pée, *Adv. Synth. Catal.* **2005**, *7 – 8*, 1163 – 1167.
[14] X. Zhu, W. De Laurentis, K. Leang, J. Herrmann, K. Ihlefeld, K.-H. van Pée, J. H. Naismith, *J. Mol. Biol.* **2009**, *391*, 74 – 85.
[15] S. Kirner, P. E. Hammer, S. D. Hill, A. Altmann, I. Fischer, L. J. Weislo, M. Lanahan, K.-H. van Pée, J. M. Ligon, *J. Bacteriol.* **1998**, *180*, 1939 – 1943.
[16] C. Sánchez, I. A. Butovich, A. F. Brana, J. Rohr, C. Mendez, J. A. Salas, *Chem. Biol.* **2002**, *9*, 519 – 531.
[17] C. Seibold, H. Schnerr, J. Rumpf, A. Kunzendorf, C. Hatscher, T. Wage, A. J. Ernyei, C. Dong, J. H. Naismith, K.-H. van Pée, *Biocatal. Biotransform.* **2006**, *24*, 401 – 408.
[18] S. Zehner, A. Kotzsch, B. Bister, R. D. Süssmuth, C. Méndez, J. A. Salas, K.-H. van Pée, *Chem. Biol.* **2005**, *12*, 445 – 452.
[19] N. Sitachitta, J. Rossi, M. A. Roberts, W. H. Gerwick, M. D. Fletcher, C. L. Willis, *J. Am. Chem. Soc.* **1998**, *120*, 7131 – 7132.
[20] N. Sitachitta, B. L. Marquez, R. T. Williamson, J. Rossi, M. A. Roberts, W. H. Gerwick, V. A. Nguyen, C. L. Willis, *Tetrahedron* **2000**, *56*, 9103 – 9113.
[21] Z. Chang, P. Flat, W. H. Gerwick, V. A. Nguyen, C. L. Willis, D. H. Sherman, *Gene* **2002**, *296*, 235 – 247.
[22] J. H. Zhang, N. B. Quigley, D. C. Gross, *J. Bacteriol.* **1995**, *177*, 4009 – 4020.
[23] M. Ullrich, C. L. Bender, *J. Bacteriol.* **1994**, *176*, 7574 – 7586.
[24] F. H. Vaillancourt, J. Yin, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10111 – 10116.
[25] E. L. Hegg, L. Que, Jr., *Eur. J. Biochem.* **1997**, *250*, 625 – 629.
[26] K. D. Koehntop, J. P. Emerson, L. Que, *J. Biol. Inorg. Chem.* **2005**, *10*, 87 – 93.
[27] F. H. Vaillancourt, E. Yeh, D. A. Vosburg, S. E. O'Connor, C. T. Walsh, *Nature* **2005**, *436*, 1191 – 1194.
[28] D. P. Galonic, F. H. Vaillancourt, C. T. Walsh, *J. Am. Chem. Soc.* **2006**, *128*, 3900 – 3901.
[29] L. C. Blasiak, F. H. Vaillancourt, C. T. Walsh, C. L. Drennan, *Nature* **2006**, *440*, 368 – 371.
[30] D. O'Hagan, C. Schaffrath, S. Cobb, J. T. G. Hamilton, C. D. Murphy, *Nature* **2002**, *416*, 279.
[31] M. Sanada, T. Miyano, S. Iwadare, J. M. Williamson, B. H. Arison, J. L. Smith, A. W. Douglas, J. M. Liesch, W. Inamine, *J. Antibiot.* **1986**, *39*, 259 – 265.
[32] X. Zhu, D. A. Robinson, A. R. McEwan, D. O'Hagan, J. H. Naismith, *J. Am. Chem. Soc.* **2007**, *129*, 14597 – 14604.
[33] H. Deng, S. L. Cobb, A. R. McEwan, R. P. McGlinchey, J. H. Naismith, D. O'Hagan, D. A. Robinson, J. B. Spencer, *Angew. Chem. Int. Ed.* **2006**, *45*, 759 – 762; *Angew. Chem.* **2006**, *118*, 773 – 776.
[34] A. S. Eustáquio, F. Pojer, J. P. Noel, B. S. Moore, *Nat. Chem. Biol.* **2008**, *4*, 69 – 74.
[35] K.-H. van Pée, E. P. Patallo, *Appl. Microbiol. Biotechnol.* **2006**, *70*, 631 – 641.
[36] S. Wang, Y. Xu, E. A. Maine, E. M. K. Wijeratne, P. Espinosa-Artiles, A. A. L. Gunatilaka, I. Molnár, *Chem. Biol.* **2008**, *15*, 1326 – 1338.
[37] J. Zeng, J. Zhan, *ChemBioChem* **2010**, *11*, 2119 – 2123.
[38] C. S. Neumann, C. T. Walsh, R. R. Kay, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 5798 – 5803.
[39] K. Mohri, T. Hata, H. Kikuchi, Y. Oshima, H. Urushihara, *Biol. Open* **2014**, *3*, 553 – 560.
[40] D. Milbredt, E. P. Patallo, K.-H. van Pée, *ChemBioChem* **2014**, *15*, 1011 – 1020.
[41] D. G. Fujimori, S. Hrvatin, C. S. Neumann, M. Strieker, M. A. Marahiel, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16498 – 16503.
[42] J. R. Heemstra, Jr., C. T. Walsh, *J. Am. Chem. Soc.* **2008**, *130*, 14024 – 14025.
[43] W. Jiang, J. R. Heemstra, Jr., R. R. Forseth, C. S. Neumann, S. Manaviazar, F. C. Schroeder, K. J. Hale, C. T. Walsh, *Biochemistry* **2011**, *50*, 6063 – 6072.
[44] B. Nowak-Thompson, N. Chaney, J. S. Wing, S. J. Gould, J. Loper, *J. Bacteriol.* **1999**, *181*, 2166 – 2174.
[45] P. C. Dorrestein, E. Yeh, S. Garneau-Tsodikova, N. L. Kelleher, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 13843 – 13848.
[46] M. G. Thomas, M. D. Burkart, C. T. Walsh, *Chem. Biol.* **2002**, *9*, 171 – 184.
[47] V. Agarwal, A. A. El Gamal, K. Yamanaka, D. Poth, R. D. Kersten, M. Schorn, E. E. Allen, B. S. Moore, *Nat. Chem. Biol.* **2014**, *10*, 640 – 647.
[48] A. H. Pang, S. Garneau-Tsodikova, O. V. Tsodikov, *J. Struct. Biol.* **2015**, *192*, 349 – 357.
[49] K.-H. van Pée, *Annu. Rev. Microbiol.* **1996**, *50*, 375 – 399.
[50] J. D. Peschke, U. Hanefeld, H. Laatsch, *Biosci. Biotechnol. Biochem.* **2005**, *69*, 628 – 630.
[51] U. Hanefeld, H. G. Floss, H. Laatsch, *J. Org. Chem.* **1994**, *59*, 3604 – 3608.
[52] K.-H. van Pée, *Alkaloids* **2012**, *71*, 167 – 210.
[53] S. Lin, S. G. Van Lanen, B. Shen, *J. Am. Chem. Soc.* **2007**, *129*, 12432 – 13438.
[54] O. Puk, P. Huber, D. Bishoff, J. Recktenwald, G. Jung, R. D. Süßmuth, K.-H. van Pée, W. Wohlleben, S. Pelzer, *Chem. Biol.* **2002**, *9*, 225 – 235.
[55] S. Pelzer, R. Süßmuth, D. Heckmann, J. Recktenwald, P. Huber, G. Jung, W. Wohlleben, *Antimicrob. Agents Chemother.* **1999**, *43*, 1565 – 1573.
[56] S. Mulyani, E. Egel, C. Kittel, S. Turkanovic, W. Wohlleben, R. D. Süssmuth, K.-H. van Pée, *ChemBioChem* **2010**, *11*, 266 – 271.
[57] P. C. Schmartz, K. Zerbe, K. Abou-Hadeed, J. A. Robinson, *Org. Biomol. Chem.* **2014**, *12*, 5574 – 5577.
[58] J. R. D. McCormick, "Tetracyclines", in *Antibiotics II* (Eds.: D. Gottlieb, P. D. Shaw), Springer, Berlin and New York, **1967**.
[59] T. Zhu, X. Cheng, Y. Liu, Z. Deng, D. You, *Metab. Eng.* **2013**, *19*, 69 – 78.

[60] J. R. D. McCormick, N. O. Sjolander, P. A. Miller, U. Hirsch, N. H. Arnold, A. P. Doerschuk, *J. Am. Chem. Soc.* **1958**, *80*, 6460 – 6461.
[61] D. Perlman, L. J. Heuser, J. B. Semar, W. R. Frazier, J. A. Boska, *J. Am. Chem. Soc.* **1961**, *83*, 4481 – 4481.
[62] Z. Host'álek, Z. Vanek, *Biosynth. Tetracyclines* in *Tetracyclines* **1985**, 137 – 178.
[63] C. D. Reeves, Z. Hu, R. Reid, J. T. Kealey, *Appl. Environ. Microbiol.* **2008**, *74*, 5121 – 5129.
[64] J. Zeng, A. K. Lytle, D. Gage, S. J. Johnson, J. Zhan, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1001 – 1003.
[65] M. L. Hillwig, H. A. Fuhrmann, K. Ittiamornkul, T. S. Sevco, D. H. Kwak, X. Liu, *ChemBioChem* **2014**, *15*, 665 – 669.
[66] M. L. Hillwig, X. Liu, *Nat. Chem. Biol.* **2014**, *10*, 921 – 923.
[67] J. N. Carter-Franklin, A. Buttler, *J. Am. Chem. Soc.* **2004**, *126*, 15060 – 15066.
[68] L. Kaysser, P. Bernhardt, S.-J. Nam, S. Loesgen, G. Ruby, P. Skewes-Cox, P. R. Jensen, W. Fenical, B. S. Moore, *J. Am. Chem. Soc.* **2012**, *134*, 11988 – 11991.
[69] S. Diethelm, R. Teufel, L. P. Kaysser, B. S. Moore, *Angew. Chem. Int. Ed.* **2014**, *53*, 11023 – 11026; *Angew. Chem.* **2014**, *126*, 11203 – 11206.
[70] P. Bernhardt, T. Okino, J. M. Winter, A. Miyanaga, B. S. Moore, *J. Am. Chem. Soc.* **2011**, *133*, 4268 – 4270.
[71] S. Rachid, M. Scharfe, H. Blöcker, K. J. Weissman, R. Müller, *Chem. Biol.* **2009**, *16*, 70 – 81.
[72] S. Buedenbender, S. Rachid, R. Müller, G. E. Schulz, *J. Mol. Biol.* **2009**, *385*, 520 – 530.
[73] S. Flecks, E. P. Patallo, X. Zhu, A. J. Ernyei, G. Seifert, A. Schneider, C. Dong, J. H. Naismith, K.-H. van Pée, *Angew. Chem. Int. Ed.* **2008**, *47*, 9533 – 9536; *Angew. Chem.* **2008**, *120*, 9676 – 9679.
[74] M. Piraee, R. L. White, L. C. Vining, *Microbiology* **2004**, *150*, 85 – 94.
[75] J. Doull, Z. Ahmed, C. Stuttard, L. C. Vining, *Microbiology* **1985**, *131*, 97 – 104.
[76] K. Podzelinska, R. Latimer, A. Bhattacharya, L. C. Vining, D. L. Zechel, Z. Jia, *J. Mol. Biol.* **2010**, *397*, 316 – 331.
[77] W. Runguphan, X. Qu, S. E. O'Connor, *Nature* **2010**, *468*, 461 – 464.
[78] A. D. Roy, S. Grüschow, N. Cairns, R. J. M. Goss, *J. Am. Chem. Soc.* **2010**, *132*, 12243 – 12245.
[79] W. Runguphan, S. E. O'Connor, *Org. Lett.* **2013**, *15*, 2850 – 2853.
[80] A. S. Eustáquio, D. O'Hagan, B. S. Moore, *J. Nat. Prod.* **2010**, *73*, 378 – 382.
[81] S. Dall'Angelo, N. Bandaranayaka, A. D. Windhorst, D. J. Vugts, D. van der Born, M. Onega, L. F. Schweiger, M. Zanada, D. O'Hagan, *Nucl. Med. Biol.* **2013**, *40*, 464 – 470.
[82] S. Thompson, Q. Zhang, M. Onega, S. McMahon, I. Fleming, S. Ashworth, J. H. Naismith, J. Passchier, D. O'Hagan, *Angew. Chem. Int. Ed.* **2014**, *53*, 8913 – 8918; *Angew. Chem.* **2014**, *126*, 9059 – 9064.
[83] J. T. Payne, M. C. Andorfer, J. C. Lewis, *Angew. Chem. Int. Ed.* **2013**, *52*, 5271 – 5274; *Angew. Chem.* **2013**, *125*, 5379 – 5382.
[84] C. B. Poor, M. C. Andorfer, J. C. Lewis, *ChemBioChem* **2014**, *15*, 1286 – 1289.
[85] M. Frese, P. H. Guzowska, H. Voß, N. Sewald, *ChemCatChem* **2014**, *6*, 1270 – 1276.
[86] M. Frese, N. Sewald, *Angew. Chem. Int. Ed.* **2015**, *54*, 298 – 301; *Angew. Chem.* **2015**, *127*, 302 – 305.
[87] A. Lang, S. Polnick, T. Nicke, P. William, E. P. Patallo, J. H. Naismith, K.-H. van Pée, *Angew. Chem. Int. Ed.* **2011**, *50*, 2951 – 2953; *Angew. Chem.* **2011**, *123*, 3007 – 3010.
[88] W. S. Glenn, E. Nims, S. E. O'Connor, *J. Am. Chem. Soc.* **2011**, *133*, 19346 – 19349.
[89] M. Hölzer, W. Burd, H.-U. Reißig, K.-H. van Pée, *Adv. Synth. Catal.* **2001**, *343*, 591 – 595.
[90] A. A. Shepherd, C. Karthikeyan, J. Latham, A.-W. Struck, M. L. Thompson, B. R. K. Meno, M. Q. Styles, C. Levy, J. Micklefield, *Chem. Sci.* **2015**, *6*, 3454 – 3460.
[91] J. Hosford, S. Shepherd, J. Micklefield, L. S. Wong, *Chem. Eur. J.* **2014**, *20*, 16759 – 16763.
[92] K. H. van Pée, unpublished results.
[93] K. H. van Pée, unpublished results.

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