CHAPTER 2

Biosynthesis of Halogenated Alkaloids

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Contents

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I. INTRODUCTION

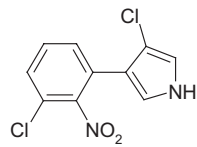
The knowledge about enzymatic halogenation has increased enormously during the last 15 years. The identification of $FADH_2$-dependent halogenases and nonheme iron, α-ketoglutarate- and O_2-dependent halogenases, the two types of halogenating enzymes shown to be involved in the biosynthesis of halogenated metabolites, has also contributed substantially to the identification of biosynthetic gene clusters of halometabolites. Whereas the cloning, sequencing, and the construction of mutants in specific genes of these gene clusters have made the characterization of the biosyntheses of halometabolites on a genetic basis much easier, biochemical characterization, such as analyses of *in vitro* activity of enzymes involved in these biosynthetic pathways, is still lacking behind. This is mostly due to the problem of identifying the natural substrates of these enzymes and the availability of these substrates.

In this review, examples for the biosynthesis of halogenated alkaloids, derived from different precursor molecules, will be shown, illustrating the various degrees of knowledge about the pathways and the enzymatic reactions involved.

II. PYRROLES

A. Pyrrolnitrin

Pyrrolnitrin (**1**) [3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole; Figure 1] was isolated in 1964[1] as an antibiotic showing activity against fungi, yeast, and Gram-positive bacteria[2] from *Pseudomonas pyrrocinia*. Later, **1** was found to be produced by a large variety of different bacteria such as *Pseudomonas* species, *Burkholderia cepacia* strains, *Myxococcus fulvus*, *Corallococcus exiguous*, *Cystobacter ferrugineus*, *Enterobacter agglomerans*, and *Serratia*.[3,4] The antibiotic activity of **1** is mainly due to inhibition of glycerol kinase leading to accumulation of glycerol and thus leaky cell membranes.[5] Early investigations into the biosynthesis of **1** by Lively et al. showed that **1** was derived from the aromatic amino acid tryptophan (**2**).[6]



Pyrrolnitrin **1**

Figure 1 Chemical structure of pyrrolnitrin (**1**).

Detailed studies using multiple-labeled **2** showed that the indole nitrogen gives rise to the nitro group nitrogen, whereas the nitrogen of the amino group was incorporated into the pyrrole ring. All carbon atoms with the exception of the carboxylic acid group were incorporated into **1**.[7] However, the question whether the D- or the L-enantiomer of **2** was the starting point for the biosynthesis of **1** could not be answered, although the D-enantiomer of **2** seemed more likely since addition of the D-enantiomer of **2** resulted in enhanced production of **1**.[8] The isolation of aminopyrrolnitrin (**3**) led to the conclusion that the oxidation of the amino to the nitro group was the last step in the biosynthesis of **1**.[9] 2-Carboxy-4-(2′-amino-3′-chlorophenyl)pyrrole was isolated by Salcher et al.[10], and monodechloroaminopyrrolnitrin (**4**) could be obtained by incubation of **1**-producing *Pseudomonas* species under chloride-limiting conditions in the presence of 7-chlorotryptophan (**5**), suggesting the chlorination of **2** to be the first step in the biosynthesis of **1**.[11] Furthermore, the isolation of carboxy-4-(2′-amino-3′-chlorophenyl)pyrrole and monodechloroaminopyrrolnitrin (**5**) suggested the ring rearrangement to occur before the second chlorination step.

First attempts to isolate the biosynthetic gene cluster of **1** from *Pseudomonas fluorescens* led to the detection of a global regulator required for the formation of chitinase, cyanide, and pyrrolnitrin by *P. fluorescens*.[12,13] Employment of this regulator to complement a wild-type *P. fluorescens* strain lacking this global regulator to induce the formation of **1** allowed the identification of the biosynthetic gene cluster of **1**.[14] The genes *prnA–D* were identified as being sufficient to allow pyrrolnitrin production in *Escherichia coli*. Detailed analysis into the function of the corresponding enzymes PrnA–D *via* the construction of individual mutants revealed that PrnA was a tryptophan 7-halogenase that showed no obvious sequence similarity to any enzyme known until that date. Detailed analysis of PrnA revealed that this enzyme is a flavin-dependent halogenase requiring $FADH_2$ and oxygen for its chlorinating activity. Isolated PrnA does not contain flavin. $FADH_2$ is produced by a flavin reductase and is then bound by the halogenase where it reacts with oxygen to form a flavin hydroperoxide in a reaction similar to flavin-dependent monooxygenases. This flavin hydroperoxide is attacked by a chloride ion bound close to the isoalloxazine ring of flavin leading to the formation of hypochlorous acid. This hypochlorous acid cannot leave the active site but diffuses toward the substrate that is bound about 10Å away from the flavin.[15] A more detailed description of the mechanism of flavin-dependent halogenases can be found below (Section VI.A.).

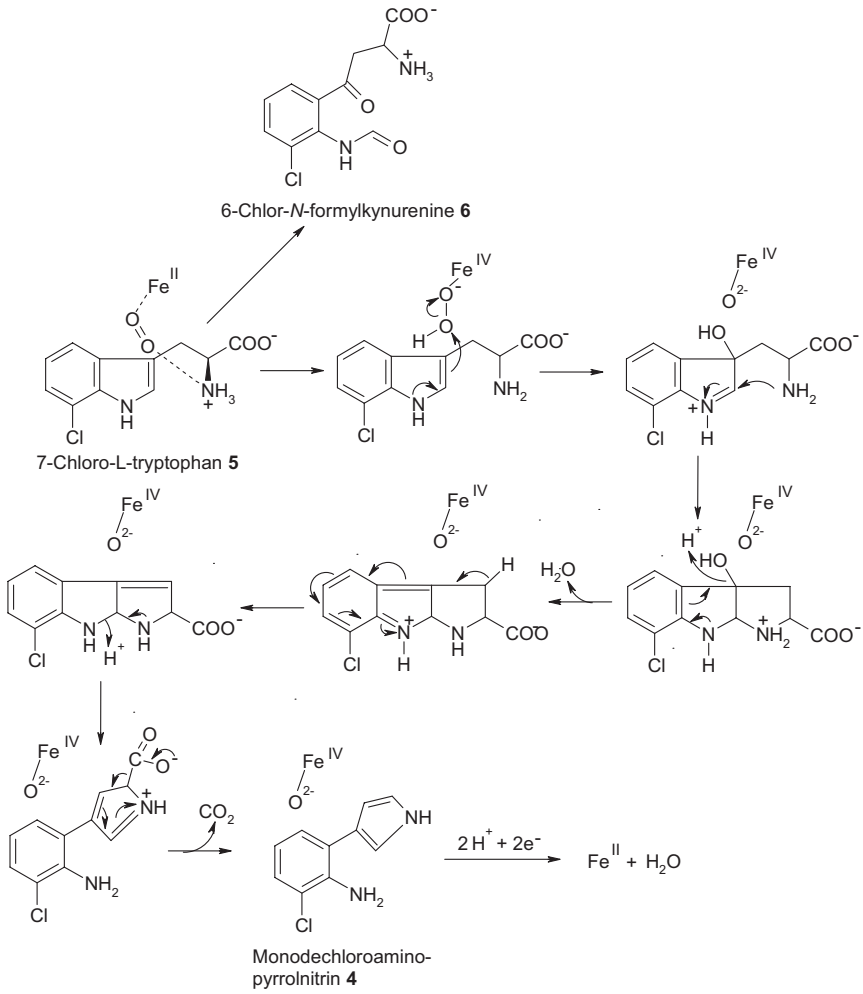
PrnB was identified as the enzyme catalyzing the rearrangement of tryptophan (**2**) into a phenylpyrrole derivative. PrnB is a heme-containing dioxygenase that shows high structural similarity to tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO).[16,17]

However, albeit the high structural similarity between the enzymes, the product of the reaction catalyzed by PrnB is quite different from that of the reaction catalyzed by TDO and IDO. TDO and IDO catalyze the formation of *N*-formylkynurenine (**6**), thus cleaving the indole ring between C-2 and C-3 by introducing two oxygen atoms (Scheme 1). PrnB cleaves the indole ring between the indole ring nitrogen and C-2 of the indole ring. Concomitant formation of a new pyrrole ring by attack of the amino group of tryptophan at C-2 of the indole ring leads to the formation of the aminophenylpyrrole derivative monodechloroaminopyrrolnitrin (**4**). It is suggested that the indole ring is opened by the incorporation of oxygen; however, the two oxygen atoms are lost during formation of the pyrrole ring as is the carboxyl group of **2**. Thus, only a very early common intermediate of the reactions catalyzed by TDO, IDO, and PrnB is possible from which the TDO/IDO-catalyzed reaction diverges from that catalyzed by PrnB (Scheme 1).[16,17] Structural analysis of PrnB in complex with the D- and the L-enantiomers of **2** and the D- and L-enantiomers of **5** have shown that only the binding of the L-enantiomers leads to productive binding of the substrate. Thus, only the L-enantiomer of **5** can be used as the starting point for the biosynthesis of **1**.

PrnC was shown to catalyze the regioselective chlorination of the phenylpyrrole derivative **4** formed by the action of PrnB. PrnC has high sequence homology to the halogenating enzyme involved in 7-chlorotetracycline biosynthesis.[14,18] The final step in the biosynthesis of **1** is catalyzed by aminopyrrolnitrin oxygenase (PrnD). PrnD was identified as the enzyme catalyzing the oxidation of the amino group of **3** to the nitro group of **1**.[19] PrnD is a Rieske *N*-oxygenase that requires reduced flavin adenine dinucleotide (FAD) formed by a flavin reductase.[20,21] PrnD has a rather broad substrate specificity. It accepts a variety of not only aminophenylpyrrole derivatives[22] but also *p*-aminobenzylamine, *p*-aminobenzyl alcohol, and *p*-aminophenylalanine.[20] The data obtained from feeding experiments, isolation of intermediates, and the molecular genetic data resulted in the biosynthetic pathway shown in Scheme 2.

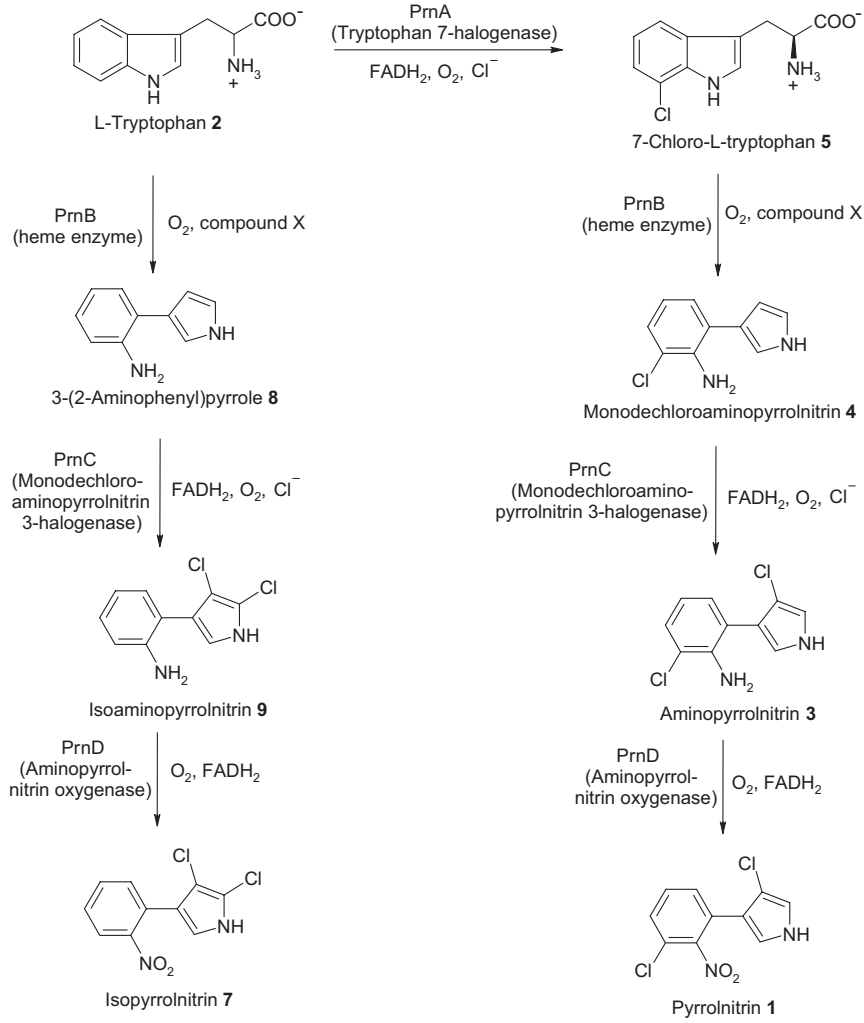
The isolation of derivatives of **1** chlorinated at different positions or lacking chlorine atoms at specific positions can be explained by the less strict substrate specificity or regioselectivity of PrnC, the second halogenase involved in pyrrolnitrin biosynthesis compared to the very high substrate specificity and regioselectivity of the tryptophan 7-halogenase PrnA. No derivative of **1** has been isolated with a chlorine atom at the phenyl ring at a position differing from the 7-position of the indole ring. However, isopyrrolnitrin (**7**) lacks the chlorine atom at the phenyl ring but contains two chlorine atoms at positions 2 and 3 of the pyrrole ring (Scheme 2).[23]

Whereas PrnA accepts the L- as well as the D-enantiomer of **2** as a substrate,[3] PrnB, the second enzyme in the biosynthetic pathway, can only use the L-enantiomer. However, PrnB accepts the L-enantiomer of tryptophan



Scheme 1 Proposed reaction mechanism for the formation of the phenylpyrrole derivative **4** from the L-enantiomer of **5** catalyzed by the heme enzyme PrnB.[7] It is not known, yet, how the reduction of the enzyme from the Fe^{IV} state to the Fe^{II} state is achieved. The heme enzymes TDO/IDO would catalyze the formation of the corresponding chloro derivative **6**.

(**2**) as well as the L-enantiomer of 7-chlorotryptophan (**5**). The formation of isopyrrolnitrin (**7**) could be explained by the reaction of PrnB on **2** followed by dichlorination of 3-(2-aminophenyl)pyrrole (**8**) to isoaminopyrrolnitrin (**9**) by the second halogenase PrnC followed by oxidation of the amino group catalyzed by PrnC (Scheme 2).



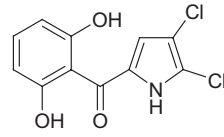
Scheme 2 Proposed pathway for the biosynthesis of **1** including by-products formed due to relaxed substrate specificity and regioselectivity of PrnB and PrnC, respectively. The main pathway is the pathway to **1** *via* the L-enantiomer of **5**. $FADH_2$, required for the reactions catalyzed by the halogenases PrnA, PrnC, and the aminopyrrolnitrin oxygenase PrnD, is provided by flavin reductases.

When chloride is substituted by bromide in the growth medium, brominated derivatives of **1** are formed. The regioselectivity of PrnC with bromide as the halide substrate is obviously more relaxed than with chloride since a number of different brominated derivatives of **3** and **1** have been isolated.[22,24]

The two halogenases, tryptophan 7-halogenase (PrnA) and monodechloroaminopyrrolnitrin-3 halogenase (MCAP-3 halogenase) (PrnC) as well as the aminopyrrolnitrin oxidase (PrnD), are two-component systems requiring a flavin reductase. Since a flavin reductase gene (*prnF*) was found very close to the four genes (*prnA–D*) considered to constitute the pyrrolnitrin biosynthetic gene cluster, *prnF* was suggested to be part of the cluster.[25] However, PrnF can be substituted by a variety of flavin reductases in the halogenase reaction[26–29] as well as in the aminopyrrolnitrin oxygenase system,[20] and no specific interaction between the halogenases and aminopyrrolnitrin oxygenase with the reductases seems to be required. A mutant lacking the flavin reductase gene *prnF* even shows increased production of **1** compared to the wild-type strain (Hatscher and van Pée, unpublished results). Additionally, *prnF* is not required as part of the cluster to obtain pyrrolnitrin production in a heterologous host.[14]

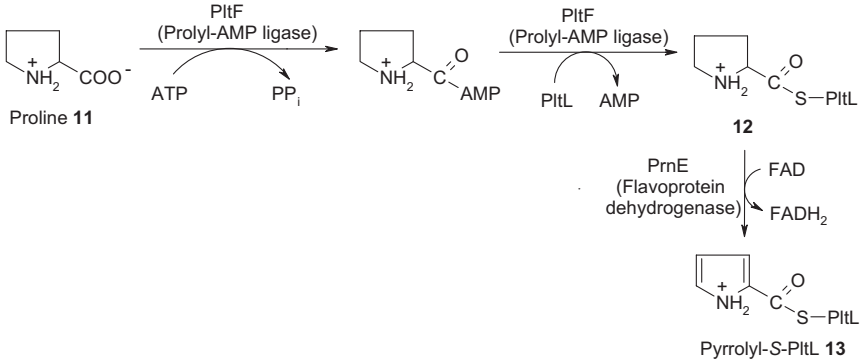
B. Pyoluteorin

Pyoluteorin (**10**, Figure 2) is an antifungal antibiotic produced by several fluorescent *Pseudomonas* spp.,[30,31] some of which also produce **1**.[32] In contrast to **1**, **10** is not derived from **2**. Feeding studies using [1,2-$^{13}C_2$]acetate showed that the resorcinol moiety of **10** is formed *via* polyketide biosynthesis,[33] whereas the pyrrole ring was found to originate from proline (**11**).[34] A genomic region required for the biosynthesis of **10** was identified in *P. fluorescens* Pf-5, the producer of **10**.[35] Nowak-Thompson et al. reported on the isolation of two polyketide synthase (PKS) genes required for the biosynthesis of **10**.[36] These PKS genes showed high similarity to type I PKS genes. In 1999, the complete biosynthetic gene cluster for the biosynthesis of **10** was characterized.[34] The cluster contains 10 genes (*pltA–G*, *pltL–M*, and *pltR*) within a 24-kb genomic region. Eight of these genes (*pltA–G* and *pltM*) were suggested to be directly involved in the biosynthesis of **10**. Individual destruction of each of these genes resulted in loss of the production of **10**. *pltR* was proposed to function as a transcriptional activator. At first, no function could be suggested for *pltL*. However, later PltL was identified as a peptidyl carrier protein (PCP) required for the binding of **11** after activation by the prolyl-adenosine monophosphate (AMP) ligase PltF.[37] *pltB* and *pltC* showed high homology to type I polyketide biosynthetic genes, whereas *pltA*, *pltD*, and *pltM* showed homology to $FADH_2$-dependent halogenases. However, in PltD, the two regions found in all flavin-dependent halogenases, the nucleotide-binding site (GxGxxG) and a motif containing two tryptophan residues (WxWxIP), were not strictly conserved, suggesting that PltD does not function as a halogenase. PrnE shows homology to acyl-CoA dehydrogenases and was suggested to catalyze the formation of pyrrole carboxylic acid from **11**. Dorrestein et al.



Pyoluteorin **10**

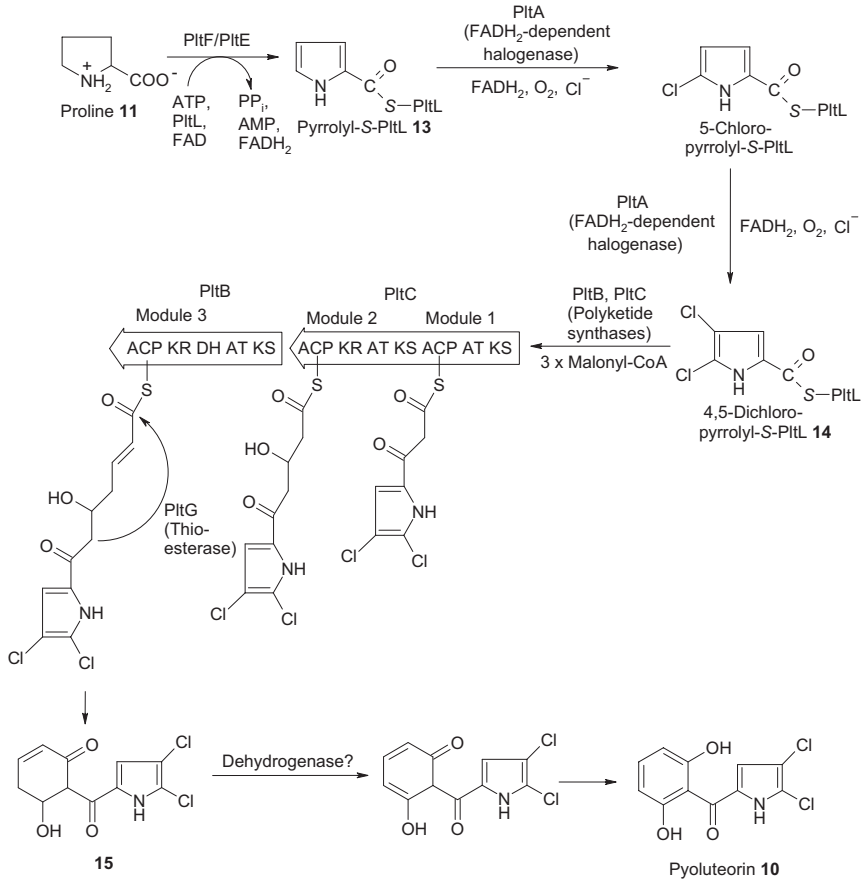
Figure 2 Chemical structure of pyoluteorin (**10**).



Scheme 3 Formation of pyrrole carboxylic acid from **11** during the biosyntheses of **10** and other pyrrole carboxylic acid-containing metabolites.[28]

demonstrated that PrnE catalyzes the dehydrogenation of **11** bound to the PCP PltL (**12**) (Scheme 3).[28]

According to these results obtained by different groups, **10** is produced *via* a mixed nonribosomal peptide synthesis (NRPs)/polyketide synthesis (PKs) pathway. The biosynthesis starts with **11** that is activated by the prolyl-AMP ligase PltF and transferred onto the PCP PltL. PltL-tethered **11** is then dehydrogenated by the flavin-dependent dehydrogenase PrnE. Whether PrnE catalyzes only the introduction of the first double bond followed by spontaneous formation of the second double bond in the presence of oxygen or whether PltE catalyzes the formation of both double bonds is not known. Dorrestein et al. showed that PltL-bound pyrrole carboxylic acid **13** can be dichlorinated by the $FADH_2$-dependent halogenase PltA.[28] Whether PltA also catalyzes the incorporation of both chlorine atoms of pyoluteorin *in vivo* or whether one is incorporated *via* the action of the second $FADH_2$-dependent halogenase PltM has not been investigated. As already mentioned above, PltD, which shows high overall similarity to flavin-dependent halogenases, is definitely not a halogenase; however, its real function is not known, yet.



Scheme 4 Hypothetical biosynthetic pathway for the biosynthesis of **10**[28,34,36] using the activity of all the gene products coded for by modules 1–3. According to Dorrestein et al.,[28] PltA catalyzes the incorporation of both chlorine atoms and thus PltM would not be required. PltD is not shown in this figure since its function is not known.

The dichlorinated pyrrole carboxylic acid moiety bound to PltL **14** could then act as the starter unit for the formation of the resorcinol moiety *via* polyketide synthesis. The polyketide synthase PltB consists of two modules and PltC consists of one. The thioesterase PltG is not part of a PKS module. Using three malonyl-CoA molecules for elongation and the activities of a ketoreductase domain and a dehydratase domain in the second polyketide synthase PltC followed by cyclization catalyzed by PltG would give the dihydroxycyclohexanone intermediate **15**. For the formation of **10** as the final product, a dehydrogenase would be required (Scheme 4). However, so far, no gene for an additional dehydrogenase has been found in the cluster.

C. Pyrrolomycins

Pyrrolomycins (**16**) (Figure 3) are structurally closely related to **10** (Figure 2). Some of the pyrrolomycins contain a nitro group (pyrrolomycin A, B, G, H and dioxapyrrolomycin). In contrast to **10**, they all contain, with the exception of pyrrolomycin A (**16A**), two chlorine atoms at the phenyl ring. Feeding experiments have shown that labeled acetate is incorporated into the phenyl ring but not into the pyrrole ring. Feeding of ^{15}N-labeled **11** resulted in **16** labeled in the pyrrole ring nitrogen only but not in the nitro group. The nitro group, however, was labeled when a $Na^{15}NO_3$-enriched medium was used.[38] This clearly points toward a biosynthetic pathway very similar to that of **10** with the phenyl ring being formed *via* polyketide biosynthesis and the pyrrole ring *via* NRPs. In contrast to the biosynthesis of **1** during which the nitro group is formed by oxidation of an amino group, biosynthesis of **16** would employ a nitration reaction as known from the formation of the 4-nitro-tryptophan moiety of the thaxomins.[39]

Zhang and Parry cloned the gene cluster for the biosynthesis of **16** from *Actinosporangium vitaminophilum*.[40] The polyketide synthase Pyr25 consists of two modules, and the polyketide synthase Pyr24 consists of one module. This is identical to the situation in the biosynthesis of **10**. Interestingly, module 2 of Pyr25 contains a ketoreductase gene that was suggested to be nonfunctional in the biosynthesis of **10**. The presence of a ketoreductase gene in a polyketide synthase in the biosynthetic gene cluster of **10** as well as in the biosynthetic gene cluster of **16** suggests that it might actually be required for the biosyntheses of **10** and **16**. However, module 3 does not contain a dehydratase gene that is present in the corresponding polyketide synthase module from the biosynthesis of **10** but was also suggested to be inactive. This supports the hypothesis that the dehydratase module might not be

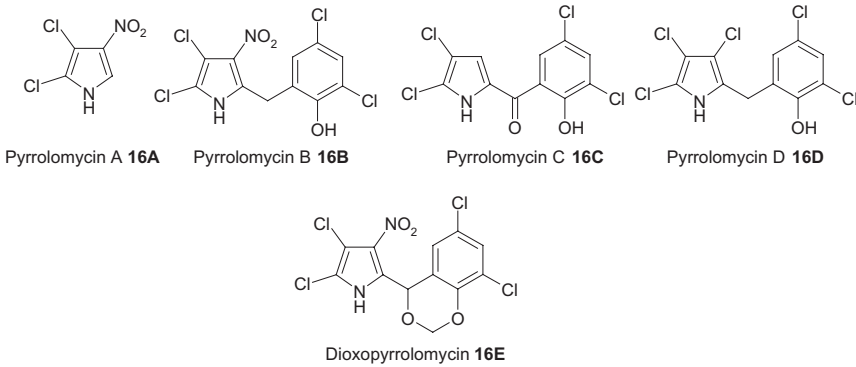


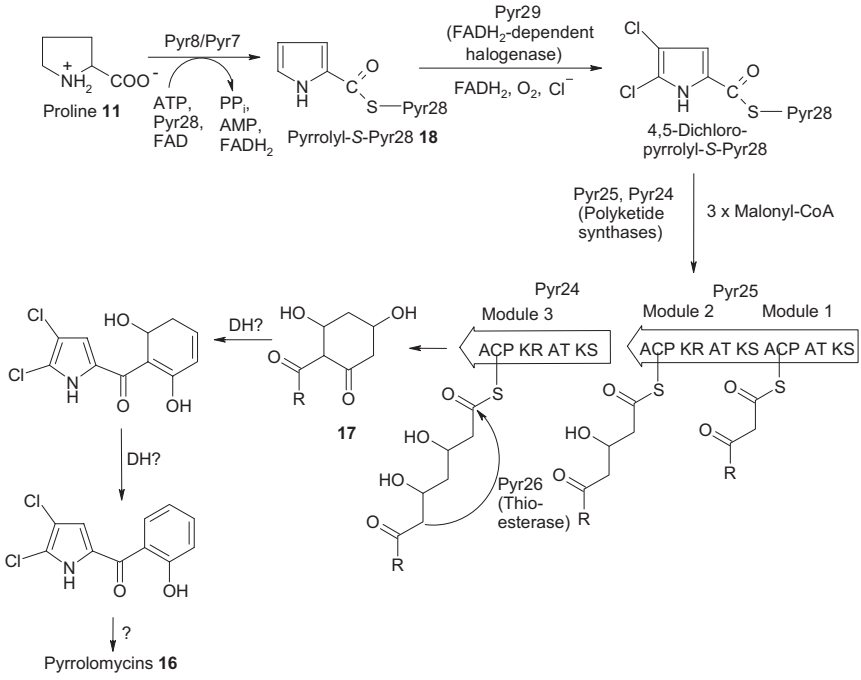
Figure 3 Chemical structures of the representative pyrrolomycins A–D (**16A–D**) and dioxapyrrolomycin A (**16E**).

functional during the biosynthesis of **10**. On the other hand, the domains contained in the three modules are not quite sufficient to fully explain the formation of the phenyl ring since the dihydroxycyclohexanone intermediate **17** formed *via* the activity of the thioesterase Pyr26 needs to be dehydrated twice to give the phenol ring of **16**. It is suggested that these dehydration reactions could occur spontaneously. There is no information concerning the reduction of the keto group to the methylene group of the pyrrolomycins.

Due to the high similarity of the cloned halogenase genes from *A. vitaminophilum*, the producer of **16**, with those from *P. fluorescens* Pf-5, the producer of **10**, it can be assumed that one $FADH_2$-dependent halogenase (Pyr29) catalyzes the chlorination of the PCP-bound pyrrole carboxylic acid **18** at positions 2 and 3; however, no experimental data to support this assumption have been presented so far. Although some of the pyrrolomycins contain five chlorine atoms, only two additional genes for $FADH_2$-dependent halogenases have been detected. Interestingly, a gene with an overall homology to genes of $FADH_2$-dependent halogenases, but lacking the conserved nucleotide-binding site motif like PltD from the biosynthesis of **10**, was found in the cluster. The function of the corresponding enzyme is not known, yet. A flavin reductase gene (*pyr9*) was also found in the cluster. The role of Pyr9 could be to produce $FADH_2$ for the $FADH_2$-dependent halogenases. No gene with similarity to a nitric oxide synthase has been detected in the gene cluster for the biosynthesis of **16**, and no experimental data concerning the mechanism of the nitration reaction have been presented, so far. A hypothetical pathway for the biosynthesis of **16** is shown in Scheme 5.

D. Pentachloropseudilin

The biosynthesis of the phenylpyrrole alkaloid pentachloropseudilin (**19**, Figure 4) by *Actinoplanes* sp. ATCC 33002 shows a number of similarities to the biosyntheses of **10** and the pyrrolomycins (**16**). It is assumed that the pyrrole ring is also derived from **11**. However, in **19** the phenyl ring is directly connected to the pyrrole ring and not *via* a keto group. Thus, the integration of the pyrrole carboxylic acid moiety into the structure of **19** must differ from that in the biosyntheses of **10** and **16**. Molecular genetic investigations into the biosynthesis of **19** started with the detection of the genes of two $FADH_2$-dependent halogenases (*halA* and *halB;* later renamed *pcpI* and *pcpK*, respectively).[41] A third gene of an $FADH_2$-dependent halogenase, *pcpE*, with rather high similarity to *pltA* from the biosynthesis of **10** and *pyr29* from the biosynthesis of **16** was detected later by Mann.[42] Due to this high similarity to halogenases known to act on pyrrole carboxylic acid derivatives as the substrate, it is suggested that PcpE also catalyzes the chlorination of a pyrrole carboxylic acid moiety as the substrate. However, in the case of PcpE, it might be that this enzyme catalyzes the incorporation of all three chlorine atoms at the pyrrole ring of **19**. **11** is suggested to be



Scheme 5 Hypothetical pathway for the biosynthesis of **12**.[40]

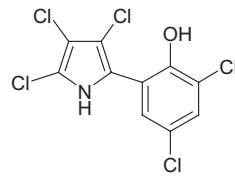
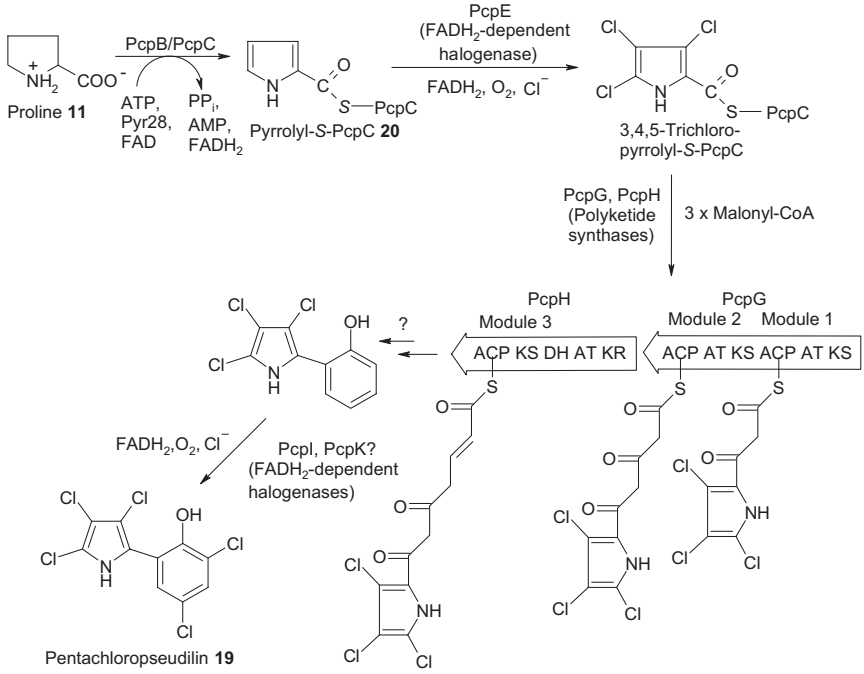


Figure 4 Chemical structure of pentachloropseudilin (**19**).

activated and transferred onto a PCP by an A-domain of a nonribosomal peptide synthetase (PcpB) where it is dehydrogenated to PCP-tethered pyrrole carboxylic acid (**20**) by a flavin-dependent dehydrogenase (PcpA) as shown for the biosynthesis of **10** (Scheme 3).[28] It is assumed that the PCP-bound pyrrole carboxylic acid moiety (**20**) serves as the starting point for the formation of the phenyl ring *via* polyketide biosynthesis. Interestingly, as in the biosynthesis of **10** as well as in the biosynthesis of **16**, a gene (*pcpL*) with high homology to $FADH_2$-dependent halogenases, but lacking a correct nucleotide-binding motif (GxGxxG) and the so-called tryptophan motif (WxWxIP), was also found in the gene cluster for the biosynthesis

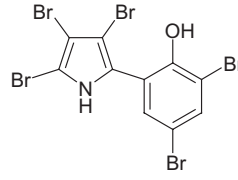


Scheme 6 Hypothetical pathway for the biosynthesis of **19** according to Mann.[42]

of **19**. The function of the corresponding enzyme in the biosynthesis of **19** is also unknown. In contrast to the gene clusters for the biosyntheses of **10** and **16**, no thioesterase was found in the biosynthetic gene cluster of **19**. A possible biosynthetic pathway according to the genes detected in the genome of *Actinoplanes* sp. ATCC 33002 is shown in Scheme 6.

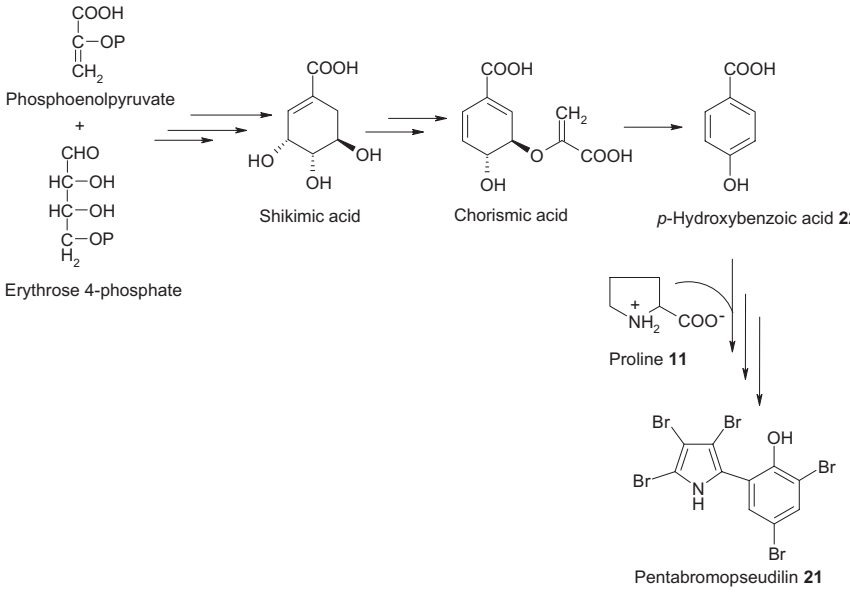
E. Pentabromopseudilin

Pentabromopseudilin (**21**, Figure 5) is produced by the marine bacterium *Alteromonas luteoviolaceus*. Its chemical structure is identical to that of **19** with the exception that it contains five bromine atoms instead of five chlorine atoms. The producer of **21** is not able to produce **19** and the producer of **19** cannot produce **21**. Obviously, the halogenases involved are highly specific concerning their halide substrate. As in the case of **10**, **16**, and **19**, the pyrrole ring is derived from **11** according to feeding studies.[43] However, the lack of incorporation of acetate into **21** suggests that, unlike in the biosynthesis of **19**, the phenyl ring is not formed *via* polyketide biosynthesis. Instead, it was shown that glucose and *p*-hydroxybenzoic acid **22** are incorporated suggesting the phenyl ring to be derived from the shikimate pathway.[43] In the case of **21**, no molecular genetic data are available, so far. But it can be



Pentabromopseudilin **21**

Figure 5 Chemical structure of pentabromopseudilin (**21**).



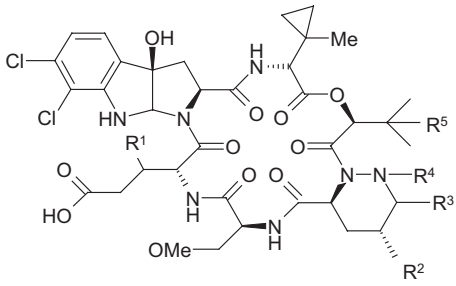
Scheme 7 Hypothetical biosynthetic pathway for the formation of **21**.[43,44]

assumed that bromination is catalyzed by $FADH_2$-dependent halogenases, although nothing is known when the bromination steps occur. There is also no information about the conversion of **11** into the pyrrole moiety and how the connection between the pyrrole and the benzene ring occurs. A hypothetical pathway, based on feeding studies, is shown in Scheme 7.

III. INDOLE-DERIVED ALKALOIDS

A. Kutznerides

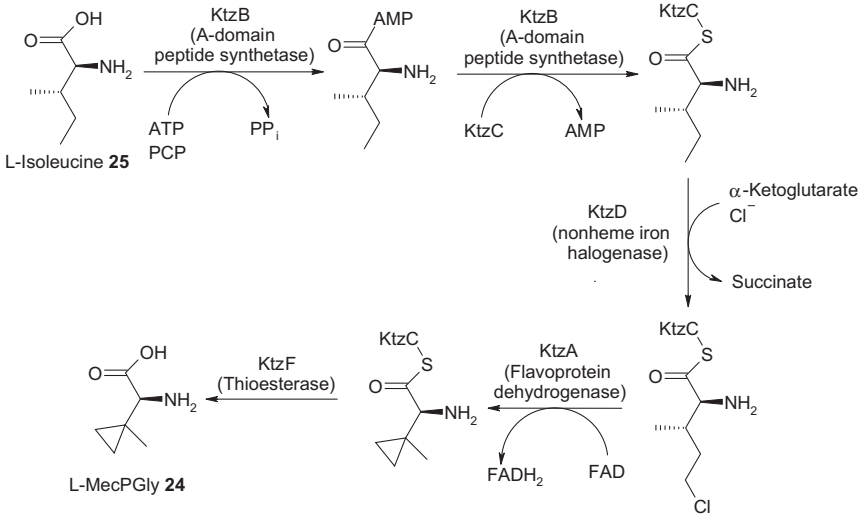
The cyclohexadepsipeptides kutznerides 1–4 (**23**, Figure 6) were isolated from the actinomycete *Kutzneria* sp. isolated from mycorrhizal roots of *Picea*



	R_1	R_2	R_3	R_4	R_5
Kutzneride 1	(S)-OH	H	H	H	Me
Kutzneride 2	(S)-OH	Cl	H	H	Me
Kutzneride 3	(R)-OH	H	H	H	Me
Kutzneride 4	(R)-OH	H	π-bond	π-bond	Me

Figure 6 Chemical structures of kutznerides 1–4 (**23**).

abies seedlings due to their antifungal activity.[45] Later, five low-abundant derivatives of **23** (kutznerides 5–9) were isolated.[46] The biosynthetic gene cluster was isolated *via* polymerase chain reaction (PCR) using degenerate primer for the genes of halogenating enzymes suggested to be involved in the biosynthesis of **23**. **23** consists of five unusual nonproteinogenic amino acids and one hydroxy acid. The various derivatives of **23** show differences in the extent of substitution and in the stereochemistry of the constituent residues. All derivatives of **23** contain a dichlorinated pyrroloindole derivative, and kutznerides 4 and 8 contain an additional chlorine atom at the 1,2-diazacyclohexane moiety of **23**. Whereas the incorporation of the two chlorine atoms into the pyrroloindole moiety was assumed to be catalyzed by $FADH_2$-dependent halogenases, incorporation of the chlorine atom into the 1,2-diazacyclohexane moiety was most likely catalyzed by a nonheme iron, O_2- and α-ketoglutarate-dependent halogenase. Such an enzyme was also hypothesized to be involved in the formation of the cyclopropyl moiety (**24**) of **23**.[47] Thus, degenerate primers for these two types of halogenases could be successfully employed in the screening of a cosmid library of the producer of **23**. The gene cluster for the biosynthesis of **23** consists of 29 open reading frames (ORFs). For 17 of these ORFs, roles in the biosynthesis of **23** have been assigned. Biosynthesis of **23** is suggested to start with L-isoleucine **25**, which is transferred onto the PCP KtzC. This KtzC-tethered **25** is then chlorinated at the terminal methyl group by KtzD, a nonheme iron halogenase. This chlorination reaction is cryptic since the chlorine atom serves as a leaving group allowing the formation of the cyclopropyl ring catalyzed by the dehydrogenase KtzA. Subsequent hydrolysis of the thioester bond catalyzed by KtzF results in the formation of free 2-(1-methylcyclopropyl)-L-glycine (L-MecPGly) **24** (Scheme 8), which is then loaded onto the nonribosomal peptide synthetase KtzE. The next NRPS (KtzE) is suggested to be responsible for the isomerization of **24** to its D-enantiomer. The next NRPS, KtzG, is suggested to be responsible for the formation of the β-branched α-hydroxy acid

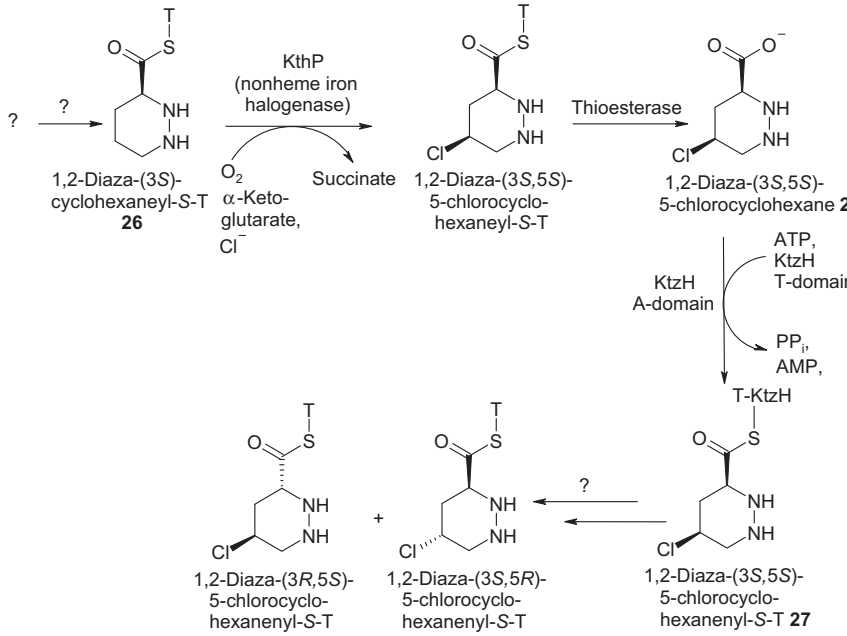


Scheme 8 Formation of 2-(1-methylcyclopropyl)-L-glycine **24** *via* cryptic chlorination.[47]

monomer from 3,3-dimethyl butyric acid involving NADH-dependent reduction of the keto group by the ketoreductase (KR) domain of KtzG. The 1,2-diazacyclohexane residue onto which the dipeptide is transferred is probably formed from glutamate and glutamine *via* the activity of the lysine/ornithine *N*-monooxygenase KtzI.

Chlorination of the 1,2-diazacyclohexane derivative was shown by Jiang et al. to be catalyzed by a second nonheme iron halogenase (KthP) whose gene was found outside of the cluster.[48] KthP only accepts the (3*S*)-1,2-diazacyclohexane residue bound to a thiolation domain (**26**) but not the (3*R*) epimer. Interestingly, only the 1,2-diaza-3*S*,5*S*-5-chlorocyclohexane derivative (**27**) is formed and not the 3*S*,5*R* stereoisomer found in the mature kutznerides suggesting further processing of **27** (Scheme 9).

KtzH, a four-module NRPS consisting of 5260 amino acids, is suggested to catalyze the incorporation of the remaining four amino acid residues (piperazate$_3$-*O*-Me-Ser$_4$-3-OH-Glu$_5$-diClPIC$_6$). The tricyclic dichlorinated pyrroloindole moiety is derived from tryptophan (**2**) that is dichlorinated as a free substrate by the tandem action of the two $FADH_2$-dependent halogenases KtzQ and KtzR. From enzyme kinetic studies of the KtzQ- and KtzR-catalyzed chlorination of tryptophan (**2**), it was concluded that KtzQ catalyzes the first chlorination at position 7 of the indole ring followed by chlorination of the 6-position catalyzed by KtzR.[49] The formation of the unusual tricyclic pyrroloindole ring that can also be found in pyrroindomycins A and B (**28**, Figure 7)[50] is suggested to be catalyzed by KtzM. KtzM is proposed to be a cytochrome P450 monooxygenase.

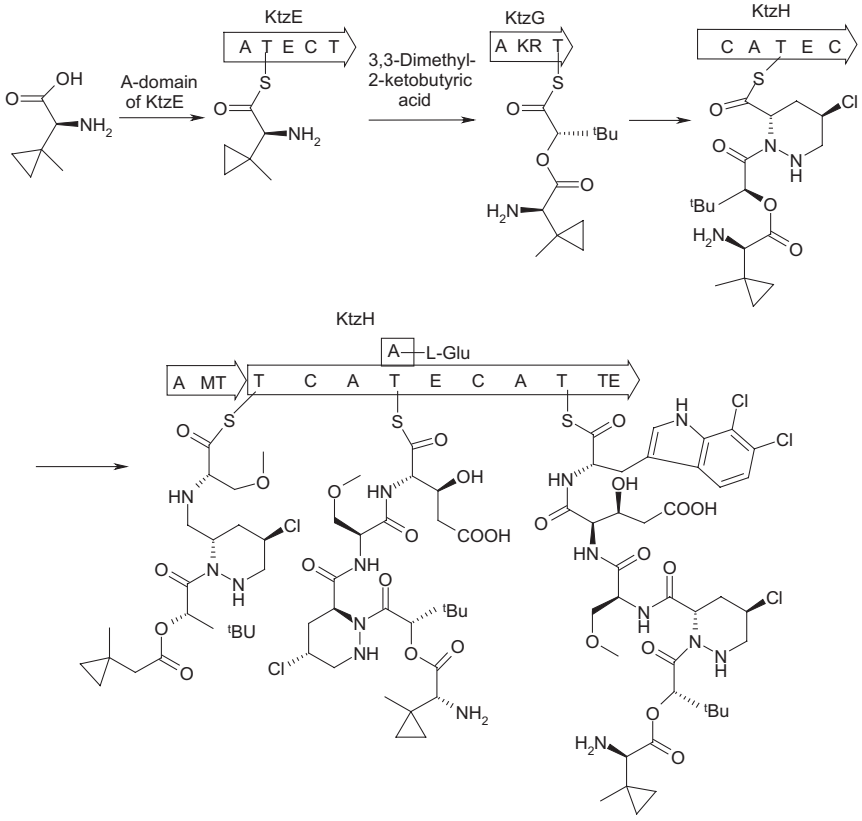


Scheme 9 Formation of the chlorinated 1,2-diazacyclohexane residue (**27**) of **23**.[48]

It is postulated that post-assembly line epoxidation of the indole ring of 6,7-dichlorotryptophan and cyclization might result in the tricyclic hexahydropyrroloindole moiety.[47] Interestingly, Zhu et al.[17] suggested the formation of a tricyclic pyrroindole ring system as an intermediate in the reaction catalyzed by the heme enzyme PrnB during a dioxygenase reaction during the rearrangement of the indole ring of tryptophan (**2**) to the phenylpyrrole ring system in the biosynthesis of **1**. However, no experimental data for KtzM supporting its suggested role in the biosynthesis of **23** are available, so far. A hypothetical pathway for the biosynthesis of the derivative 2 of **23** is shown in Scheme 10.

B. Pyrroindomycins

Pyrroindomycins A and B (**28**) (Figure 7) consist of an indolopyrrole ring connected *via* a trisaccharide to a macrocyclic polyketide moiety that contains a tetramic acid moiety (**29**) (Figure 7, Scheme 12).[50] Whereas pyrroindomycin A does not contain a chlorine atom, pyrroindomycin B is chlorinated at the 5-position of the indole ring. The indolopyrrole ring is derived from tryptophan (**2**).[51] The macrocyclic polyketide shows similarity to the polyketide moiety of chlorotrycin.[52] However, instead of the



Scheme 10 Proposed biosynthetic pathway for the derivative 2 of **23** according to Fujimori et al.,[47] Jiang et al.,[48] and Heemstra and Walsh.[49]

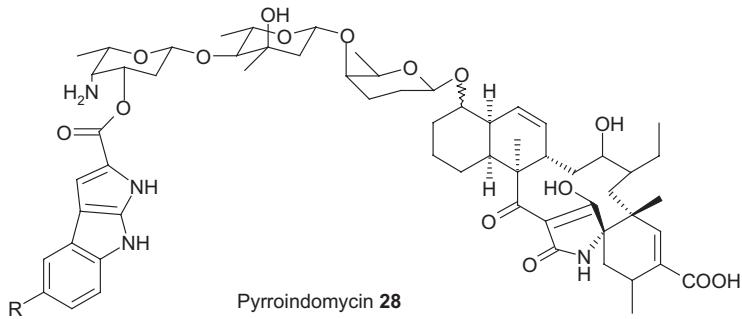
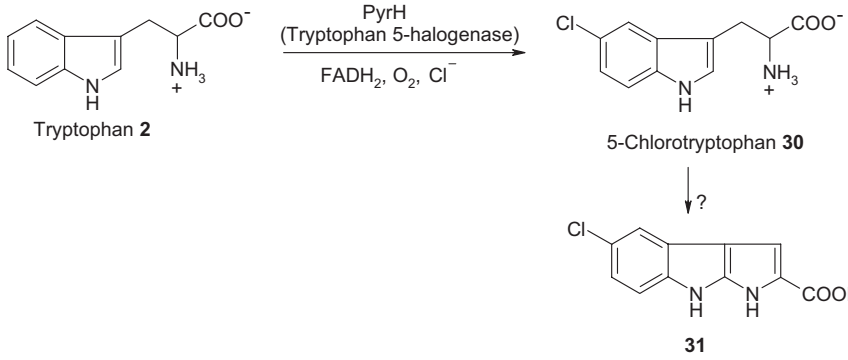


Figure 7 Chemical structure of pyrroindomycins A (R=H) and B (R=Cl) (**28**).

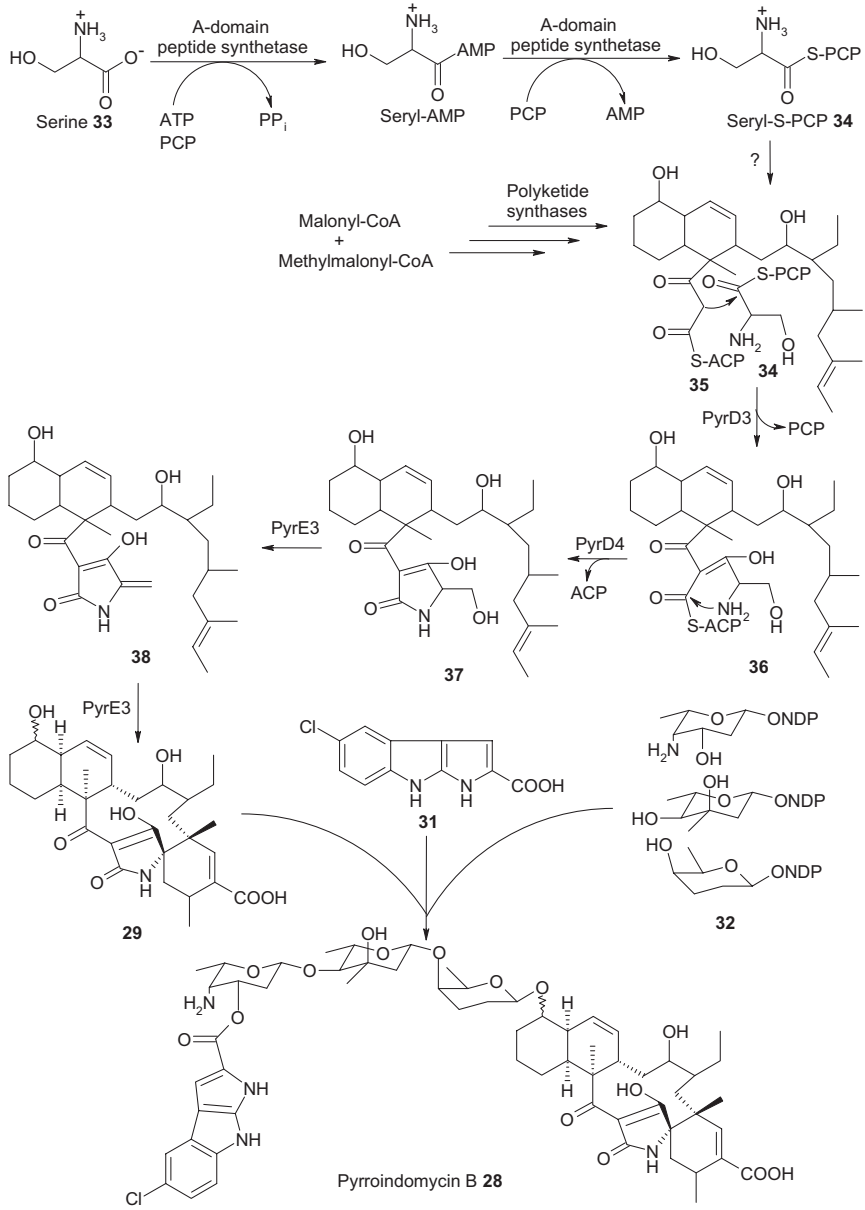


Scheme 11 Proposed formation of the chlorinated indolopyrrole moiety **31** during the biosynthesis of **28** *via* 5-chlorotryptophan (**30**) according to Zehner et al.[53] and Patallo et al. (unpublished results).

spirotetronate moiety of chlorotrycin, **28** contains a spirotetramate moiety. The gene cluster for the biosynthesis of **28** was detected by screening of a cosmid library of *Streptomyces rugosporus*, the producer of **28**, using PCR primers derived from the conserved sequences of $FADH_2$-dependent halogenases.[53] PyrH, the halogenase detected during this screening, was identified as an $FADH_2$-dependent, regioselective tryptophan 5-halogenase. Analysis of a gene disruption mutant in the tryptophan 5-halogenase gene showed that this mutant only produced the nonchlorinated derivative of **28**. Chlorination of **2** in the 5-position was thus shown to be the first step in the biosynthesis of **28** (Scheme 11).

Nothing is known yet about the formation of the tricyclic indolopyrrole moiety **31**, although a similarity to the tricyclic hexahydropyrroloindole moiety of kutznerides (**23**) and the proposed intermediate in the ring rearrangement reaction in the biosynthesis of pyrrolnitrin (**1**) seems quite likely. However, whereas during the biosynthesis of **1** the carboxylic acid group of tryptophan (**2**) is lost, in the biosyntheses of **23** and **28**, it is used to connect the tricyclic ring system *via* a peptide or an ester bond, respectively, to other parts of the molecule. In the case of **28**, the indolopyrrole moiety is connected to the polyketide moiety *via* a trisaccharide moiety. The monomers of the trisaccharide moiety are derived from glucose by derivatization of nucleoside diphosphate-tethered glucose. The NDP-activated sugars (**32**) are then transferred to the polyketide by glycosyltransferases.

The tetramic acid moiety is produced *via* polyketide biosynthesis with incorporation of an amino acid. This amino acid is suggested to be serine (**33**) that would be activated and transferred onto a PCP. The PCP-bound serine (**34**) is then suggested to react with the octahydronaphthalene derivative **35.** The formation of the octahydronaphthalene ring structure



Scheme 12 Proposal for the formation of the tetramic acid-containing polyketide moiety of **28** *via* nonribosomal peptide and polyketide synthesis and formation of **28** according to Patallo et al. (unpublished results) and Jia et al.[52]

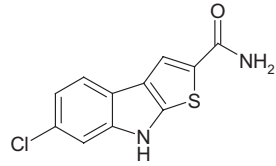
35 is suggested to proceed *via* polyketide biosynthesis in a similar way as proposed for chlorothricin and kijanimicin biosynthesis.[52,54] An anion formed at C-2 of **35** could then lead to the condensation of **35** with **34** suggested to be catalyzed by the β-ketoacyl acyl carrier protein (ACP)-synthase III analog PyrD3 leading to the formation of nitrogen containing ACP-tethered intermediate **36**. PyrD4, homolog of C-terminal domain of KijE from kijanimicin biosynthesis, could then catalyze the formation of the lactame ring leading to the free intermediate **37**. Generation of the spirotetramide ring is hypothesized to be catalyzed by PyrE3 *via* dehydratization to the intermediate **38** followed by a cyclization to give the spirotetramide ring system of the final polyketide aglycon **29** (Scheme 12).

So far, there is hardly any information available about the sequence of reactions in which the individual parts of **28** are put together. Scheme 12 shows a proposal for the formation of the tetramic acid-containing polyketide moiety of **28** and the origin of the other moieties required for formation of the final product **28** according to Patallo et al. (unpublished results).

C. Thienodolin

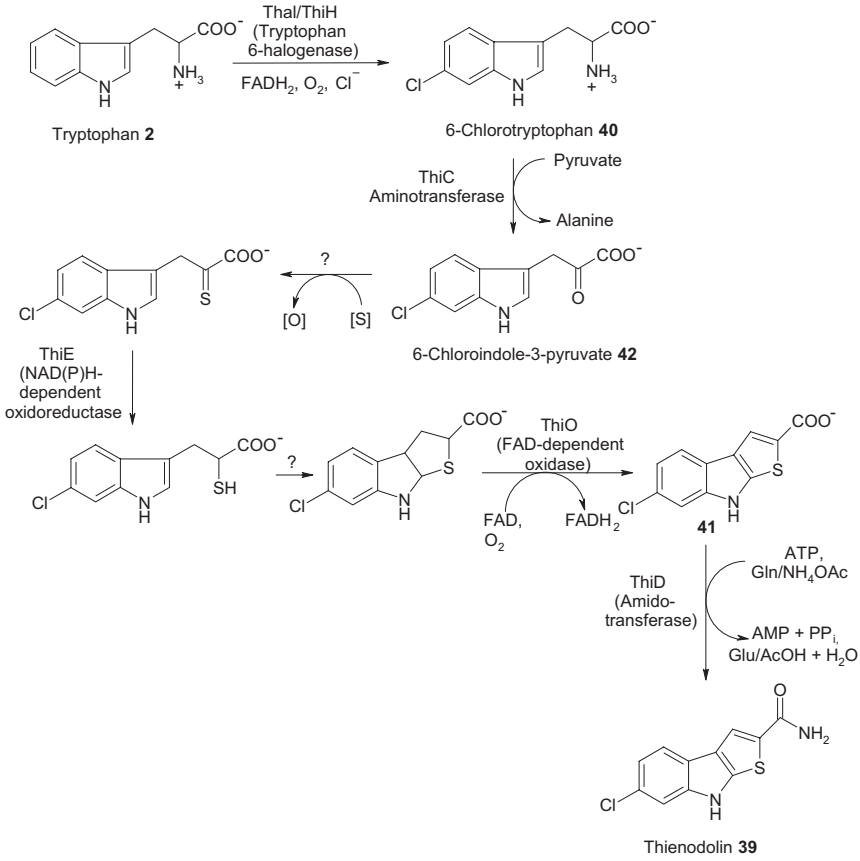
Thienodolin (6-chlorothieno[2,3-b]indole-2-carbamide; **39**, Figure 8) was isolated during a screening for compounds showing plant growth-regulating effects.[55,56] A halogenase gene (*thal or thiH*) was isolated from an enriched gene library of the producer of **39**, *Streptomyces albogriseolus*.[57] Heterologous overexpression of the halogenase gene in a *P. fluorescens* strain allowed the purification and biochemical characterization of the enzyme. It could be shown that Thal/ThiH was an $FADH_2$-dependent tryptophan halogenase that catalyzes the regioselective chlorination and bromination of the L- and D-enantiomers of tryptophan (**2**). The halogenase gene was used for the screening of a cosmid library of *S. albogriseolus* leading to the detection and isolation of the biosynthetic gene cluster of **39**. The construction of gene disruption mutants of *S. albogriseolus* showed that Thal catalyzes the first step in the biosynthesis of **39**. Without Thal, and thus no formation of 6-chlorotryptophan (**40**), biosynthesis of **39** cannot proceed. The disruption of an amido transferase gene (*thiD*) revealed that formation of the amide group of **39** was the last step in the biosynthesis of **39**. *In vitro* analysis of the amido transferase ThiD showed that it requires ATP for the transfer of an amino group onto its substrate 6-chlorothieno[2,3-b]indole-2-carboxylate (**41**).[58]

The incorporation of the sulfur atom into **39** is still a mystery. None of the genes detected in the cluster show any similarity to genes known to code for sulfur-transferring enzymes. A proposal for a biosynthetic pathway for the formation of thienodolin involving the formation of 6-chloroindole-3-pyruvate (**42**) catalyzed by the aminotransferase ThiC is shown in Scheme 13.



Thienodolin **39**

Figure 8 Chemical structure of thienodolin (**39**).

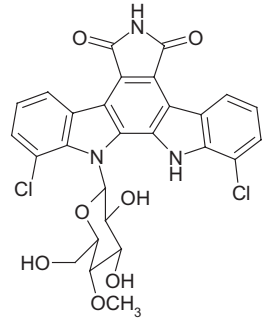


Scheme 13 Proposed biosynthetic pathway for the formation of **39** according to Milbredt.[58]

IV. INDOLOCARBAZOLES

A. Rebeccamycin

The antitumor compound rebeccamycin (**43**) was isolated from *Lechevalieria aerocolonigenes* by Nettleton et al.[59] **43** is a chlorinated symmetrical



Rebeccamycin **43**

Figure 9 Chemical structure of rebeccamycin (**43**).

indolocarbazole that is *N*-glycosylated with a 4′-*O*-methylglucose attached to one of the indolocarbazole nitrogens *via* an *N*-glycosidic linkage (Figure 9).

Biosynthetic studies showed that **43** is derived from two molecules of tryptophan (**2**), one glucose and one methionine molecule.[60] The gene cluster for the biosynthesis of **43** was detected using an internal fragment of the *N*-glycosyltransferase gene from the biosynthesis of the indolocarbazole staurosporine[61] for screening of a cosmid library of *L. aerocolonigenes*. To verify that the isolated cosmids contained genes of the gene cluster for biosynthesis of **43**, these cosmids were subjected to PCR analysis with degenerate primers for $FADH_2$-dependent halogenases.[62] Further analysis of cosmids containing the *N*-glycosyltransferase as well as the halogenase gene allowed the isolation and sequencing of the complete gene cluster for the biosynthesis of **43**.[63]

The first step in rebeccamycin biosynthesis is the regioselective chlorination of tryptophan (**2**) by the tryptophan 7-halogenase RebH. $FADH_2$ required by RebH might be provided by the flavin reductase RebF. The next step is catalyzed by the 7-chloro-L-tryptophan oxidase RebO that converts 7-chlorotryptophan (**5**) to 7-chloroindole-3-pyruvic acid (**44**). Although this L-amino oxidase also accepts **2** as a substrate, its catalytic efficiency with **5** is about 57 times higher than that with **2** as the substrate.[64] The next step is suggested to be catalyzed by the chromopyrrolic acid synthase RebD. RebD has 54% sequence identity to the analogous enzyme (StaD) from staurosporine biosynthesis. A *rebD*-disruption mutant did not produce any indolocarbazole-related compound. In the reaction catalyzed by RebD, **44** produced in the RebO reaction is suggested to react with a second molecule of **5** to form 11,11′-dichlorochromopyrrolic acid (**45**).[65] Nishizawa et al. and Howard-Jones and Walsh suggest that **44** should react with 7-chloroindole-3-pyruvate imine (7-chlorodehydrotryptophan, **46**) that is proposed to be an intermediate in the RebO-catalyzed reaction.[64,66] The mechanism for the RebD-catalyzed reaction

leading to the formation of the bis-indole core of **43** as proposed by Howard-Jones and Walsh[66] is shown in Scheme 14. The formation of the bis-indole core starts with the condensation of a 7-chloroindole-3-pyruvate imine (**46**) with its corresponding enamine tautomeric form (**47**) to yield the intermediate **48**. This intermediate is favored as a substrate for the following oxidative coupling step by Howard-Jones and Walsh[66] due to the inherent stability of its highly conjugated skeleton and its ability to participate in one-electron oxidation involving hydrogen abstraction. Abstraction of a hydrogen radical is suggested to lead to formation of **49** allowing formation of the pyrrolic C-3 to C-4 bond, and abstraction of a second hydrogen radical followed by tautomerization would yield the dicarboxypyrrole **45**.

It is proposed that **45** is oxidized by RebP followed by an oxidation at C-7 by RebC to give the rebeccamycin aglycon **50**. The final two steps would be the glycosylation at N-12 with glucose catalyzed by the *N*-glycosyltransferase RebG followed by methylation of the glucose by RebM that uses *S*-adenosylmethionine as the methyl group donor[63] (Scheme 15).

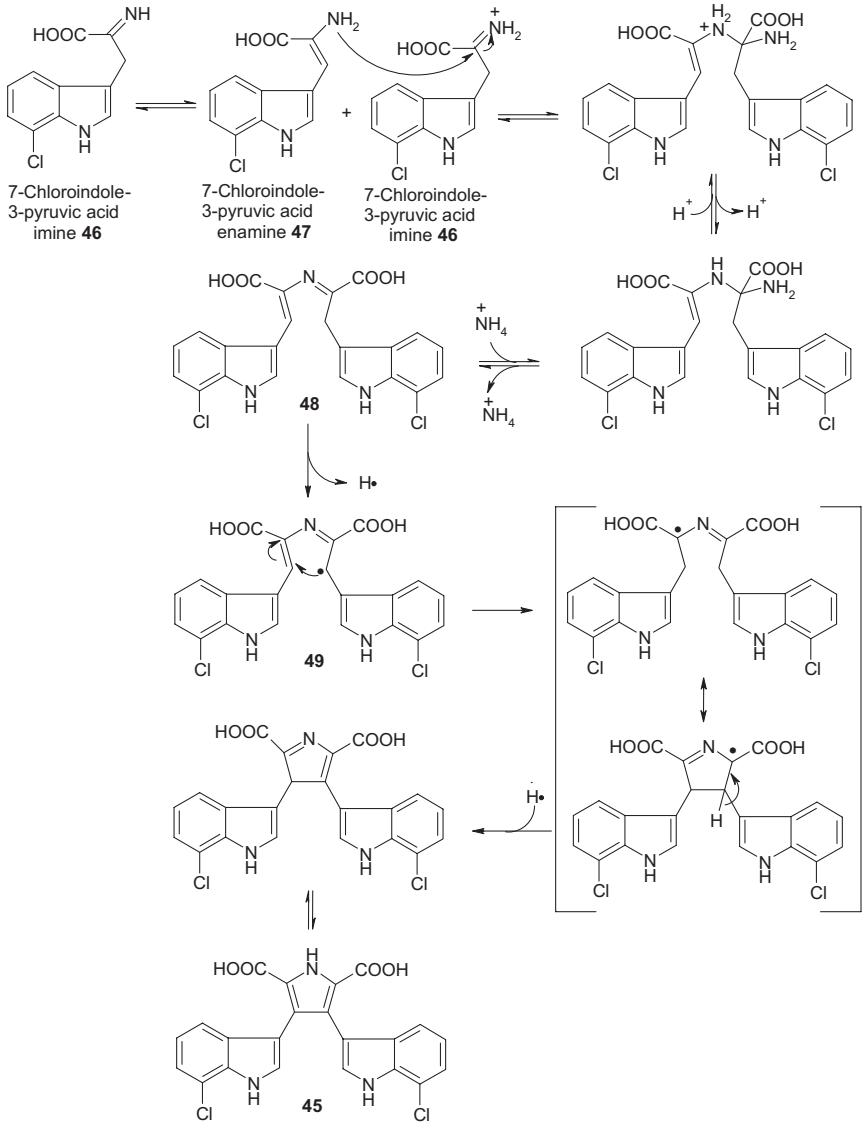
B. Cladoniamides and BE-54017

Cladoniamides (**51**–**53**) are bis-indole alkaloids with an unusual indenotryptoline structure (Figure 10) whose biosynthesis is closely related to the biosynthesis of the indolocarbazole **43**. **51**–**53** are produced by the lichen-associated actinomyces strain *Streptomyces uncialis*.[68] In contrast to **43**, **51**–**53** as well as BE-54017 (**54**),[69] are chlorinated in the position corresponding to the 5-position of the indole ring of tryptophan (**2**). However, the major difference between **43** and **51**–**53**/**54** is that in the indenotryptoline scaffold of **51**–**53**/**54**, one indole ring is "flipped" relative to the other. Albeit this structural difference, Williams et al. had suggested that **54** could be derived from an indolocarbazole intermediate by enzymatic degradation and rearrangement of this intermediate.[68]

The gene cluster for the biosyntheses of **51**–**53** was isolated by screening a cosmid library of the *S. uncialis*, the producer of **51**–**53**, by PCR using primers for the *rebC* analog in the biosyntheses of **51**–**53**.[70]

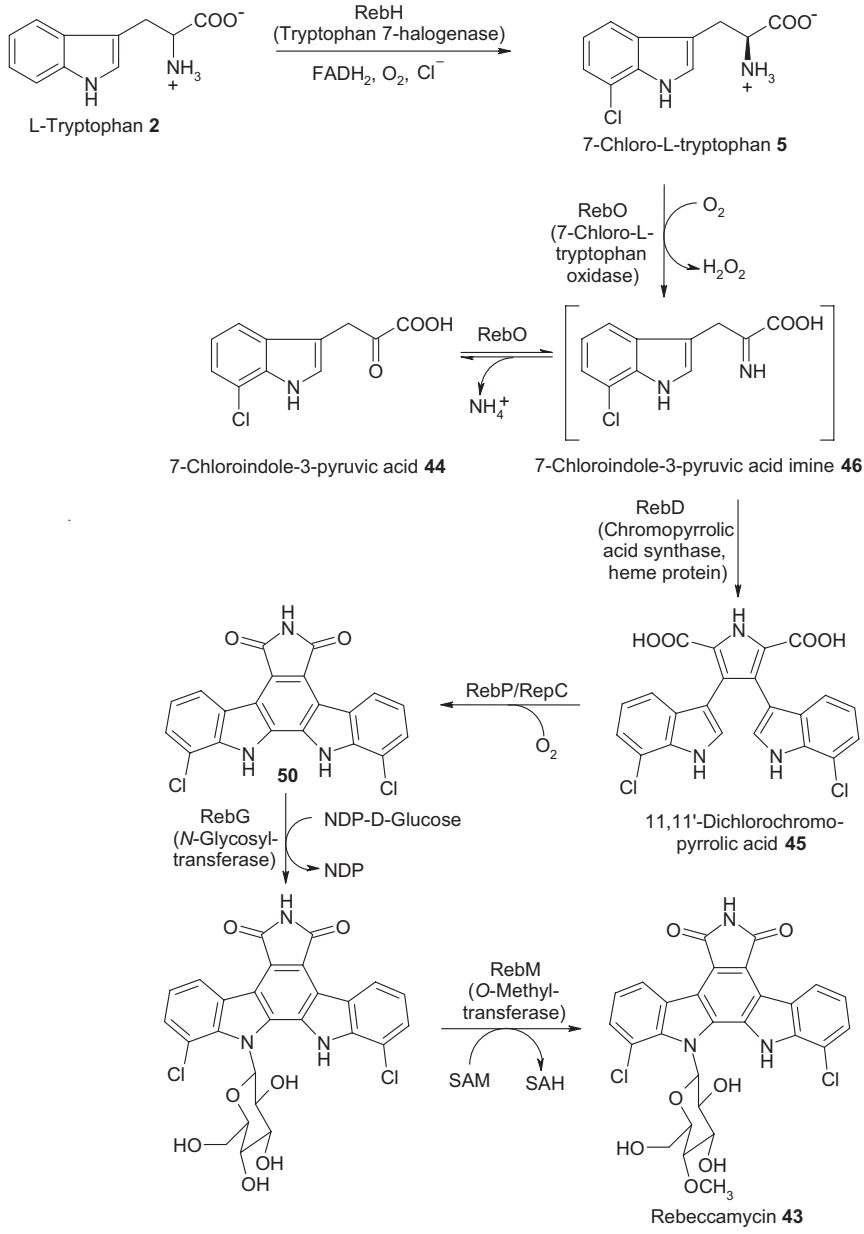
The gene cluster for the biosyntheses of **51**–**53** and its derivatives has been isolated from environmental DNA using primers designed to detect oxytryptophan dimerization genes.[71]

The first five steps in the biosyntheses of **43** and **51**–**53**/**54** are identical with the exception of the position of chlorination of the starter molecule tryptophan (**2**). In the biosyntheses of **51**–**53**/**54**, chlorination is catalyzed by an $FADH_2$-dependent tryptophan 5-halogenase (ClaH). The second step is catalyzed by the amino acid oxidase ClaO. Chromopyrrolic acid (**55**) formation is catalyzed by the chromopyrrolic acid synthase ClaD. The

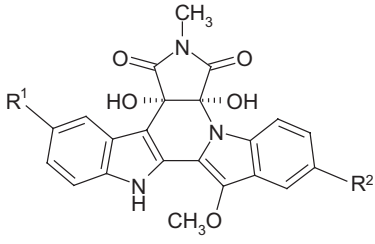


Scheme 14 Proposed mechanism for the formation of the bis-indole core of **43** catalyzed by the heme enzyme RebD.[66]

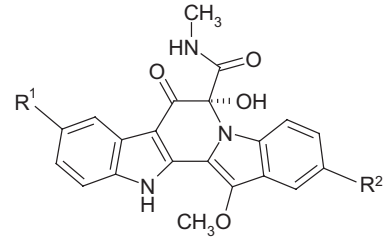
next reaction is suggested to be catalyzed in tandem by the cytochrome P450 enzyme ClaP and the flavin monooxygenase ClaC resulting in the formation of the intermediate **56** corresponding to the rebeccamycin aglycon, which only differs from this compound by the position of the chlorine



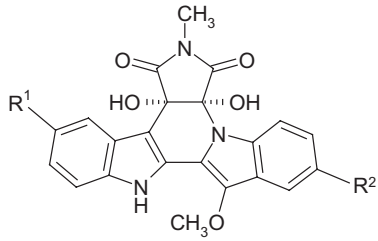
Scheme 15 Proposed biosynthetic pathway for the biosynthesis of **43**.[63,66,67]



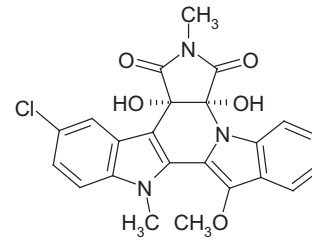
Cladoniamides A-C **51**



Cladoniamides D-E **52**



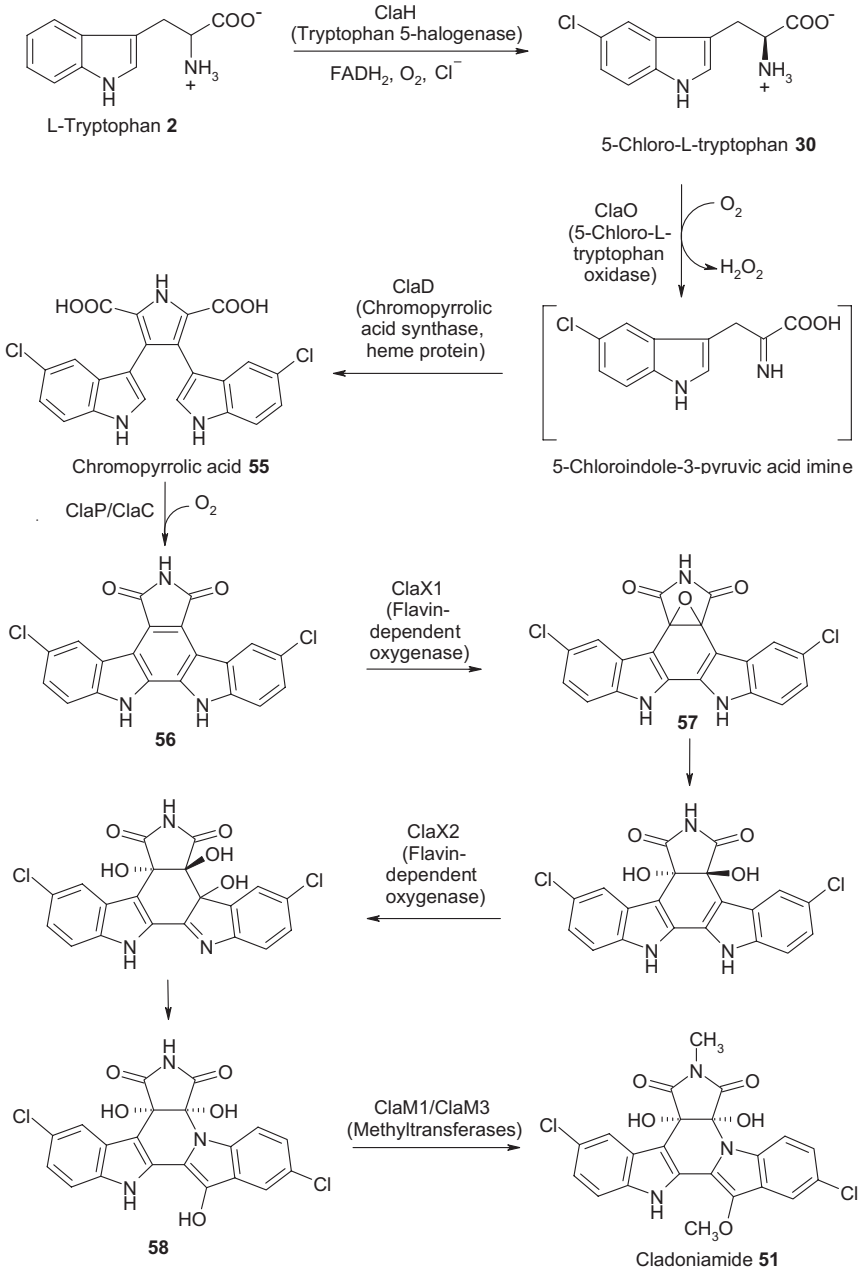
Cladoniamides F-G **53**



BE-54017 **54**

Figure 10 Chemical structures of cladoniamides. Cladoniamides A–C (**51**) (cladoniamide A: R^1=Cl, R^2=H; cladoniamide B: R^1, R^2=Cl; cladoniamide C: R^1, R^2=H); cladoniamides D–E (**52**) (cladoniamide D: R^1=Cl, R^2=H; cladoniamide E: R^1, R^2=H); cladoniamide F–G (**53**) (cladoniamide F: R^1=Cl, R^2=H; cladoniamide G: R^1, R^2=H), and BE-54017 (**54**).

atom or lack of a chlorine atom, respectively. From this "common" intermediate of the biosyntheses of **43** and **51**–**53**/**54**, the pathways diverge. ClaX1, a potential flavin-dependent oxygenase, is suggested to catalyze an epoxidation reaction of the double bond between C-4c and C-7a followed by hydrolysis of the epoxide **57**, either enzyme catalyzed or spontaneous. ClaX2, a second potential flavin-dependent oxygenase, is hypothesized to either catalyze epoxidation between C-7a and C-7b or hydroxylation at C-7b suggested to lead to opening of the carbazole scaffold. Attack of the indole nitrogen at the intermediately formed keto group would lead to formation of the indenotryptoline scaffold (**58**) of **51**–**53** and **54** (Scheme 16).[70,71] Methylation of the succinimide nitrogen is suggested to be catalyzed by the *N*-methyltransferases ClaM1 or AbeM1, respectively. *O*-Methylation is proposed to be catalyzed by the *O*-methyltransferases ClaM3 or AbeM1, respectively. AbeM2, a second *N*-methyltransferase, is only present in BE-54017 biosynthesis and is suggested to catalyze the methylation of the indole nitrogen of the tryptoline moiety.[71] Cladoniamides D–G are suggested to be formed from cladoniamide A or B, respectively, through opening of the *N*-methylsuccinimide ring and oxidative decarboxylation.[70]



Scheme 16 Proposed pathway for the biosynthesis of cladoniamide B (**51**).[70] Genes for the corresponding enzymes involved in the biosynthesis of **34** (Abe) have been identified by Chang and Brady.[71] The other cladoniamide derivatives are suggested to be formed *via* the same biosynthetic pathway.

V. FORMATION OF NEW HALOGENATED ALKALOIDS BY PATHWAY MODIFICATIONS

Sanchez et al. reported on the first use of halogenases to obtain new compounds by introducing halogenase genes into an alkaloid-producing bacterial strain.[72] The substitution of the tryptophan 7-halogenase by a tryptophan 6- and a tryptophan 5-halogenase in the biosynthesis of **43** led to formation of new bis-indole derivatives (**59**–**62**) (Figure 11). However, a new derivative of **43** containing a chloride atom at a different position could not be obtained. When RebH, the tryptophan 7-halogenase involved in the biosynthesis of **43**, was substituted by PyrH, the tryptophan 5-halogenase from the biosynthesis of **28**,[53] bis-indole formation was still possible; however, RebP/RebC, the enzymes downstream of the chromopyrrolic acid synthase, could not cope with the chlorine atoms at positions 9 and 9′ of chromopyrrolic acid (**59**–**60**). Only the monochlorinated compound 9-chlorochromopyrrolic acid (**60**) could be transformed to 3-chloroarcyriaflavin A (**61**) by RebP/RebC. When tryptophan 6-halogenase was used for the substitution of the tryptophan 7-halogenase RebH, only 10-chlorochromopyrrolic acid (**62**) was obtained indicating that the enzyme further downstream could not cope with the chlorine atom at position 10.

Seibold et al. introduced the tryptophan 6-halogenase gene (*thal*) from *S. albogriseolus*, the producer of thienodolin (**39**), into *Pseudomonas chlororaphis*, the producer of pyrrolnitrin (**1**).[57] Overexpression of *thal* was so high that although the host strain still contained a tryptophan 7-halogenase

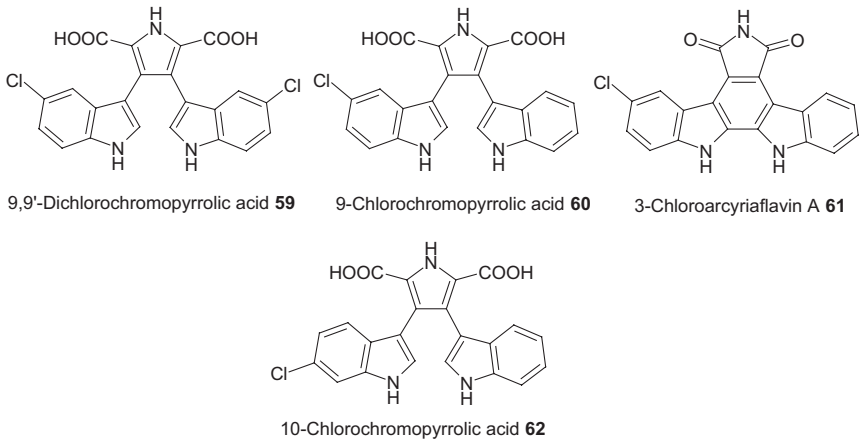
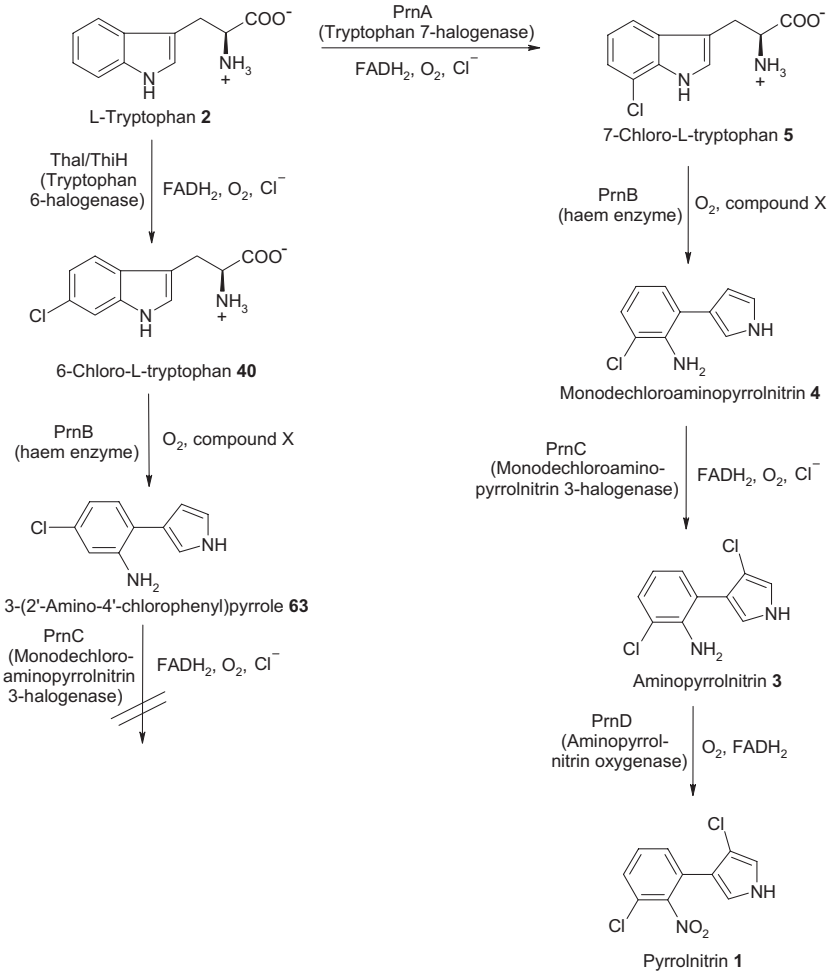


Figure 11 Compounds obtained by substituting the tryptophan 7-halogenase RebH by the tryptophan 5-halogenase PyrH or the tryptophan 6-halogenase Thal/ThiH, respectively, in rebeccamycin biosynthesis.[72]



Scheme 17 Modification of the biosynthetic pathway of **1** using the tryptophan 6-halogenase from *Streptomyces albogriseolus*.[57]

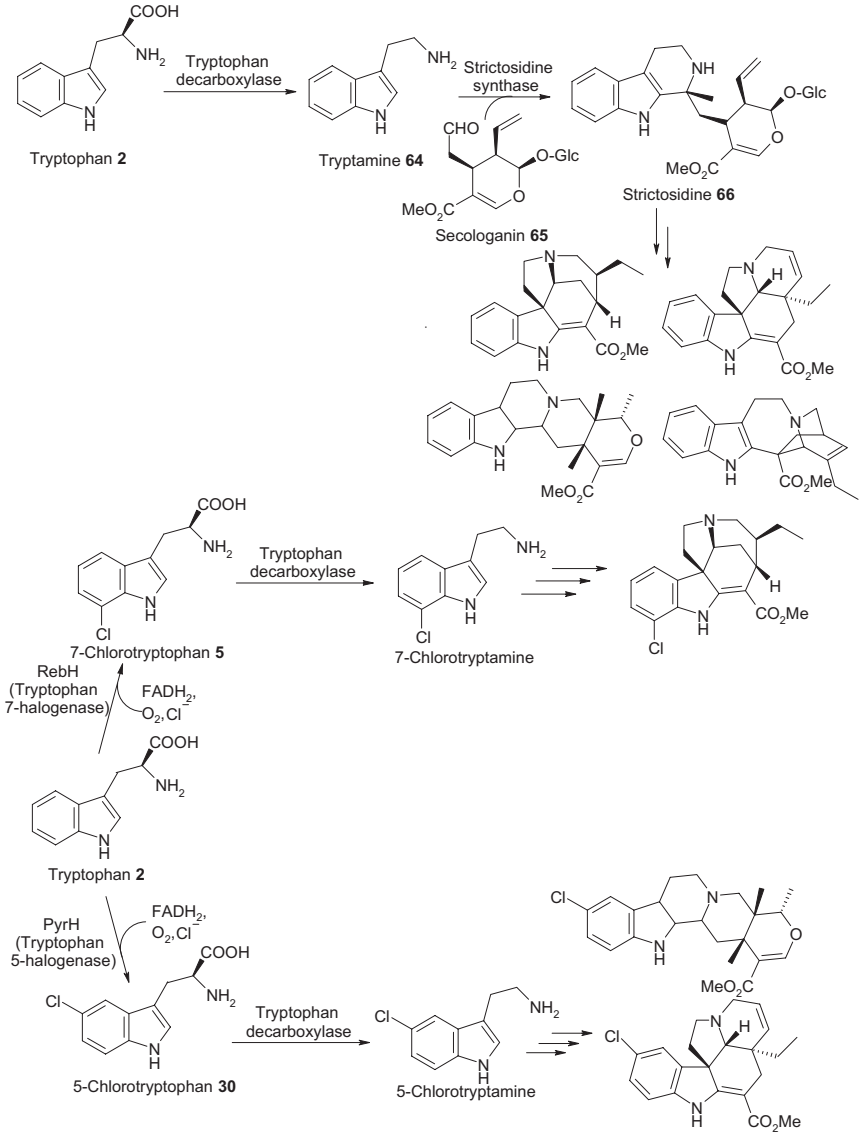
(PrnA), **1** was not formed anymore. All available tryptophan (**2**) was converted by Thal into 6-chlorotryptophan (**40**) leading to the formation of the monodechloroaminopyrrolnitrin (**4**) analog 3-(2′-amino-4′-chlorophenyl) pyrrole (**63**) catalyzed by PrnB. However, **63** was not accepted by the second halogenase, MCAP-3 halogenase, and thus, the biosynthesis of the analog of pyrrolnitrin (**1**) did not proceed further (Scheme 17).

Runguphan et al. went a step further and introduced the tryptophan 7-halogenase gene *rebH* and the tryptophan 5-halogenase gene *pyrH* into the medicinal plant *Catharanthus roseus* (Madagascar periwinkle),[73] which

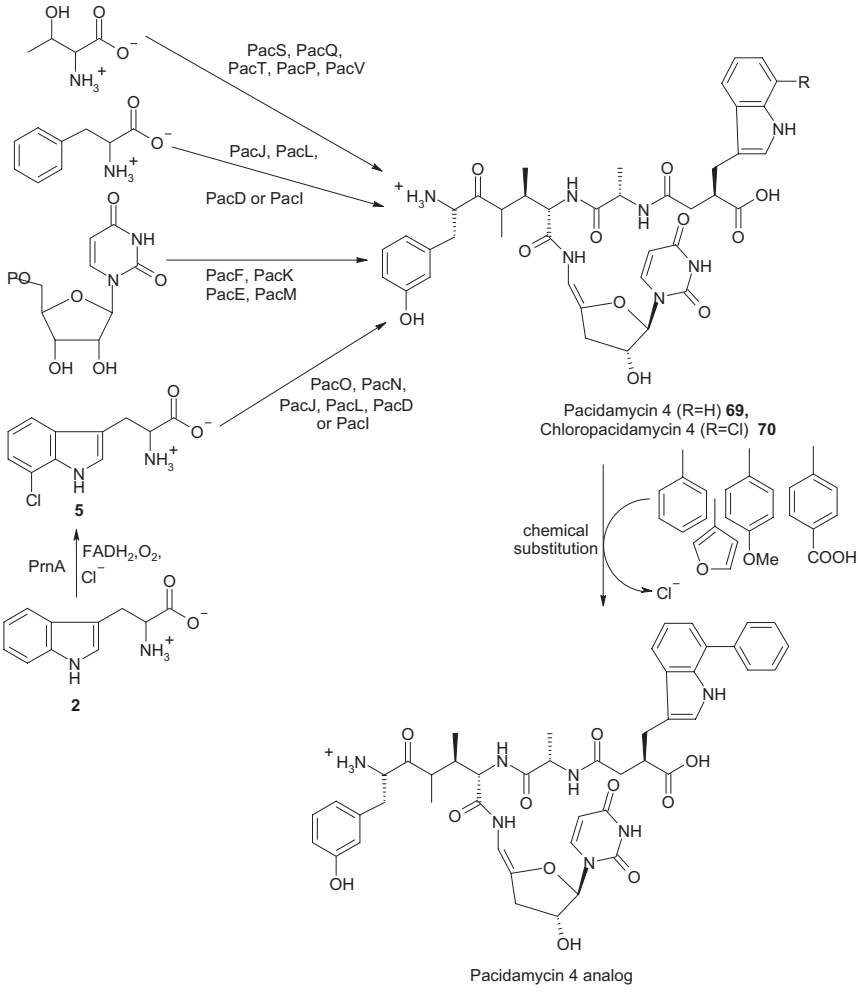
produces a wide variety of monoterpene indole alkaloids (Scheme 18).[74] Biosynthesis of these metabolites starts with tryptophan (**2**) that is decarboxylated by a tryptophan decarboxylase to tryptamine (**64**). **64** reacts with secologanin (**65**) to form strictosidine (**66**) that is further metabolized *via* several reactions to form the final products. The idea behind the work of Runguphan et al. was to start the biosynthesis with chlorotryptophan.[73] Based on the availability of tryptophan 7-halogenases and a tryptophan 5-halogenase, Runguphan et al. introduced the tryptophan 7-halogenase gene *rebH* together with the flavin reductase gene *rebF* or the tryptophan 5-halogenase gene *pyrH* together with the flavin reductase gene *rebF*, respectively.[73] The *in vivo* formed chlorotryptophans were decarboxylated to the corresponding 5- or 7-chlorotryptamines (**67**–**68**), respectively. To ensure that the chlorotryptophans were produced in the cytosol, no signal sequences were used. The chlorinated alkaloids obtained differed depending on the regioselectivity of the tryptophan halogenase present in the plant cells. This indicates that some of the enzymes downstream of strictosidine synthase differ in their substrate specificity.

The accumulation of the chlorotryptophans in the plant cells indicated that decarboxylation of the chlorotryptophans was a bottleneck in the biosynthesis of halogenated alkaloids by *C. roseus*. To overcome this bottleneck, Glenn et al. decided to reengineer RebH and thus change its substrate specificity.[75] In contrast to the tryptophan 7-halogenase PrnA from pyrrolnitrin biosynthesis that chlorinates tryptamine derivatives in the 2-position of the indole ring,[76] RebH retains its regioselectivity when chlorinating tryptamine; however, its activity with tryptamine (**64**) as a substrate is extremely low. To allow RebH to accept **64** as a substrate, it seemed logic to change those amino acids that are involved in binding tryptophan *via* its carboxylic acid and amino group. Mutant RebH Y455W showed the desired change in substrate specificity. The phenolic oxygen of Y455 was only 2.6Å away from the carboxylic acid group. By filling this space with a tryptophan residue reduces the affinity of tryptophan to the RebH Y455W mutant but still allows binding of **64**. Thus, the bottleneck due to the low activity of the tryptophan decarboxylase from *C. roseus* toward 7-chlorotryptophan (**5**) could be overcome by moving the chlorination from tryptophan (**2**) to **64**. When chloride was substituted in the cultivation medium by bromide, the corresponding brominated compounds were formed. However, iodide was not incorporated.

Using a combination of molecular genetics and chemical synthesis, Roy et al. obtained novel derivatives of the uridyl peptide antibiotic pacidamycin (**69**).[77] **69** is produced from tryptophan (**2**) by *Streptomyces coeruleorubidus*. Introduction of the tryptophan 7-halogenase gene *prnA* from the biosynthesis of pyrrolnitrin (**1**) resulted in the formation of chloropacidamycin (**70**) by *S. coeruleorubidus* due to efficient *in situ* chlorination of **2** as the starting molecule. **70** was then chemically modified by



Scheme 18 Formation of alkaloids by *Catharanthus roseus*. (a) Formation of alkaloids in wild-type *C. roseus*. (b) Formation of chlorinated alkaloids by hairy root cell cultures of *C. roseus* transformed with the tryptophan 7-halogenase gene *rebH* or the tryptophan 5-halogenase gene *pyrH*, respectively.[73]



Scheme 19 Formation of pacidamycin (R=H) (**69**). Chloropacidamycin (R=Cl (**70**) can be obtained by introducing the tryptophan 7-halogenase gene *prnA* into the pacidamycin producer *Streptomyces coeruleorubidus*. New pacidamycin derivatives can be obtained by subsequent substitution of the chlorine atom against other substituents.[77]

using the chlorine atom as a reactive site and substituting it with a phenyl, 4-methoxyphenyl, 4-carboxyphenyl, or 3-furfuryl moiety under exceptionally mild cross-coupling conditions (Scheme 19).

These results clearly show that tryptophan halogenases can be used *in vivo* to obtain new halogenated metabolites with potentially new interesting properties and which can be chemically derivatized.

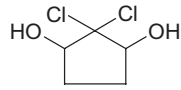
VI. HALOGENATING ENZYMES INVOLVED IN ALKALOID BIOSYNTHESIS

A. Halogenation of Aromatic Rings and Aliphatic Substrates Activated for Electrophilic Attack

The first halogenating enzyme that was described to be involved in halometabolite biosynthesis was chloroperoxidase from the caldariomycin (**71**) (Figure 12) producer *Caldariomyces fumago*.[78] This enzyme was thought to be involved in caldariomycin biosynthesis on the basis that it converts β-ketoadipic acid to δ-chlorolevulinic acid and that incorporation of the chlorine atom of δ-chlorolevulinic acid into caldariomycin was observed.[79] Cyclization of δ-chlorolevulinic acid was assumed to give rise to 2-chloro-1,3-cyclopentanedione that was considered as a possible intermediate in caldariomycin biosynthesis. It has to be noted that chloroperoxidase also catalyzes the chlorination of 1,3-cyclopentandione.[80]

Chloroperoxidase is a heme enzyme catalyzing the oxidation of the halide ions iodide, bromide, and chloride in the presence of hydrogen peroxide leading to the formation of free hypohalous acids. This free hypohalous acid then leads to nonspecific halogenation of electron-rich substrates.[81] Thus, the chloroperoxidase-initiated halogenation reactions proceed with lack of substrate specificity and regioselectivity. After the detection of chloroperoxidase from *C. fumago*, a large number of chloro- and bromoperoxidases from many different organisms have been detected and characterized. In addition to heme-containing haloperoxidases, vanadium-containing haloperoxidases were isolated from various organisms.[82] However, due to the discrepancy between the lack of regioselectivity and the chemical structure of the halometabolites isolated, there was some doubt about the involvement of haloperoxidases in halometabolite biosynthesis, especially with genetic proof such as cloning of the caldariomycin biosynthetic gene cluster and disruption of the chloroperoxidase gene in the caldariomycin producer still being missing.[83]

This situation lasted until 1995, when Dairi et al. cloned the 7-chlorotetracycline biosynthetic gene cluster and identified the gene for the halogenating enzyme.[18] This gene and the corresponding enzyme showed no sequence similarity to any of the known haloperoxidases.



Caldariomycin **71**

Figure 12 Chemical structure of caldariomycin (**71**).

However, neither expression of the gene nor any *in vitro* studies were performed. Two years later, Hammer et al. reported on the isolation and characterization of a biosynthetic gene cluster of pyrrolnitrin (**1**) where they also found a gene with high similarity to the 7-chlorotetracycline halogenase.[14] The corresponding enzyme was subsequently identified as MCAP-3 halogenase, the second halogenase in the biosynthetic pathway of **1**.[19] However, a close inspection of the amino acid sequence showed that about 100 amino acids and thus a nucleotide-binding motif (GxGxxG) were missing in the 7-chlorotetracycline halogenase.[14] Interestingly, this motif was also present in the tryptophan 7-halogenase PrnA shown to catalyze the first step in the biosynthesis of **1**, although PrnA shows no overall sequence similarity to the 7-chlorotetracycline and the MCAP-3 halogenase. During purification of PrnA and MCAP-3 halogenase, it was detected that a flavin reductase as a second enzyme component was required.[84] This flavin reductase produces $FADH_2$ from FAD and NAD(P)H. The detection of $FADH_2$-dependent halogenases had an enormous impact on the understanding of enzymatic halogenation and the detection of gene clusters for halometabolite biosynthesis. In the mean time, a large number of such gene clusters has been isolated and characterized.[85,86] All the gene clusters that are involved in the biosynthesis of halogenated phenols or pyrroles or other activated aromatic rings or electron-rich aliphatic compounds have been found to contain $FADH_2$-dependent halogenases. However, *in vitro* activity has only been demonstrated for a few of these halogenases. This is due to lack of knowledge concerning the natural substrate and/or lack of its availability.

Thus, most information exists about the tryptophan 7-halogenases PrnA and RebH. PrnA was the first $FADH_2$-dependent halogenase to be purified and characterized.[84] The elucidation of its three-dimensional structure revealed that the purified enzyme does not contain flavin but contains a single chloride ion.[15] This chloride ion is bound close to the isoalloxazine ring of FAD (Figures 13 and 14),[87] which was introduced into the crystals by soaking them in an FAD solution. Complexes of the enzyme with its substrate tryptophan (**2**) and also with its reaction product 7-chlorotryptophan (**5**) showed that they were bound at a distance of about 10Å away from the isoalloxazine ring preventing direct contact between them. Thus, a diffusible halogenating agent must be formed at the active site to bridge the distance between the isoalloxazine ring and the substrate. This diffusible compound is hypochlorous or hypobromous acid, when bromide is present.

The first step is the binding of $FADH_2$, in an *in vitro* system produced either by a flavin reductase or, chemically,[26] by the halogenase. Then, in analogy to the reaction of flavin-dependent monooxygenases, $FADH_2$ reacts with oxygen to form a flavin hydroperoxide. This flavin hydroperoxide is attacked by the nearby bound chloride or bromide ion resulting

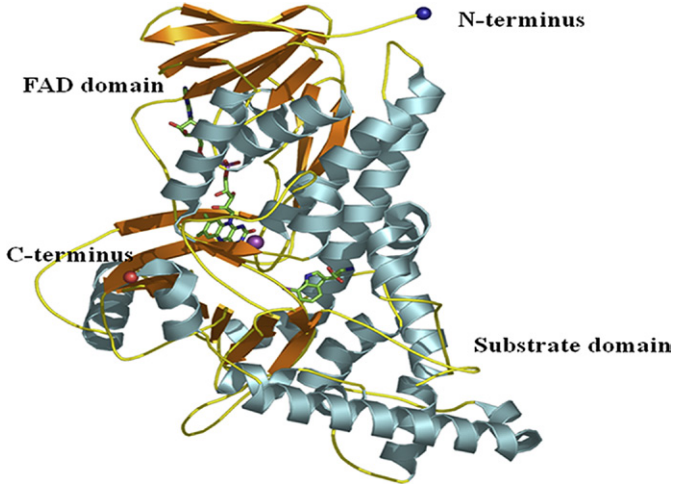


Figure 13 Ribbon model of the tryptophan 7-halogenase PrnA. The enzyme consists of two domains, the nucleotide-binding domain (top and left) and the substrate-binding domain (bottom and right). The positions of FAD and the substrate tryptophan are indicated by showing there chemical structure at the respective position. The chloride ion bound near the isoalloxazine ring of tryptophan is shown as sphere.

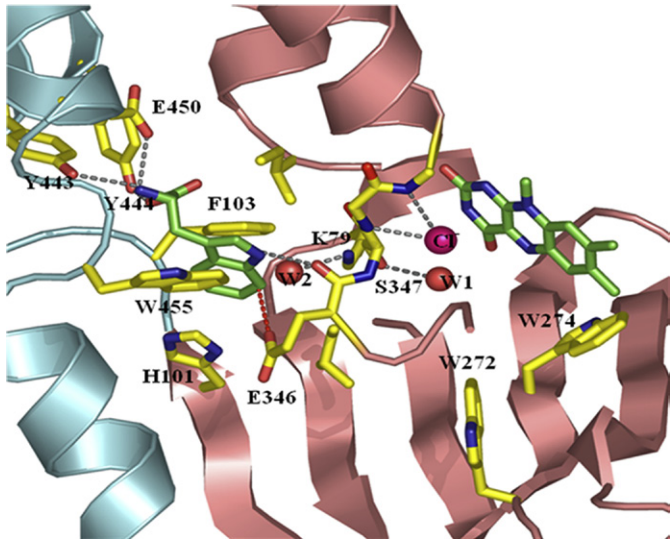
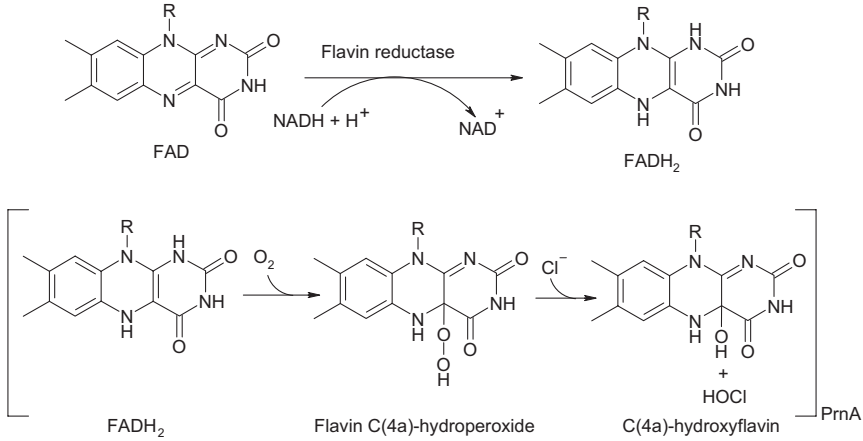


Figure 14 Active site of the tryptophan 7-halogenase PrnA. The isoalloxazine ring (right-hand site) and the substrate tryptophan (left-hand site) are shown in green. The chloride ion bound at the active site is shown as a Two water molecules in the active site (W1, W2) are shown as balls.



Scheme 20 Formation of $FADH_2$ by a flavin reductase and formation of hypochlorous acid from halogenase-bound $FADH_2$ during the reaction of the two-component halogenase system.[15]

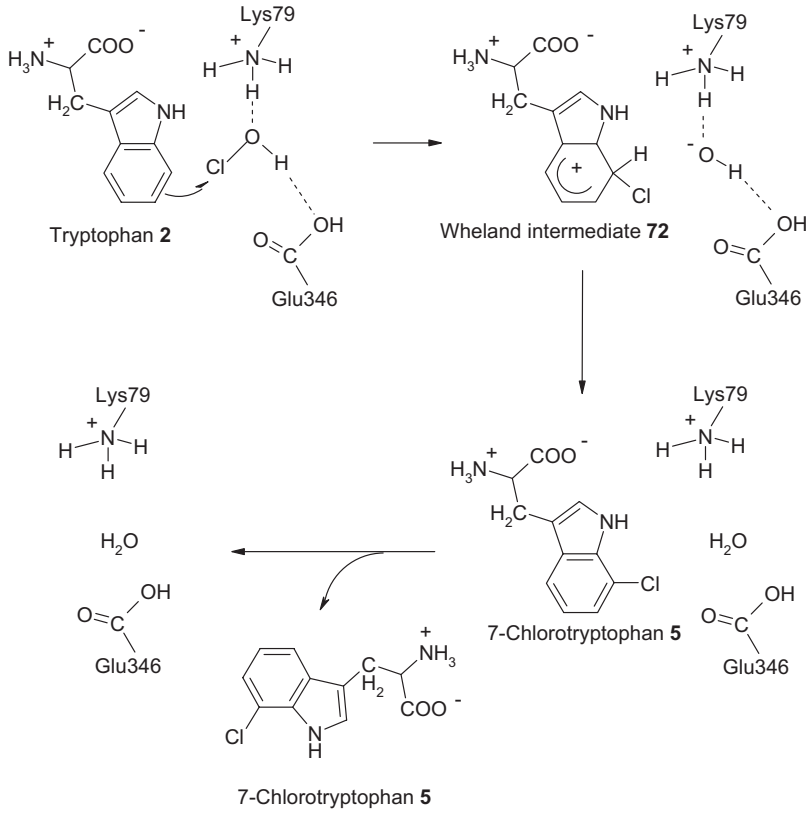
in the formation of hypohalous acid and hydroxyflavin (Scheme 20). In contrast to the situation in haloperoxidases, this hypohalous acid cannot leave the active site[15] but is guided along the 10Å tunnel toward the substrate. Interaction with a lysine and a glutamate residue that are essential for activity ensures correct positioning of the halide species to attack the aromatic ring (Figure 14). Deprotonation of the Wheland intermediate **72** leads to the chlorinated final product (Scheme 21).

The regiospecificity of the reaction is determined by the binding of the substrate.[17] While all the amino acids required for activity are in the same position in tryptophan halogenases with different regioselectivities, it is the substrate that is turned around in such a way that the position to be halogenated overlaps in halogenases with different regioselectivities (Figure 15).

Changing the regioselectivity is possible by modifying the amino acids keeping the substrate in a highly fixed position and thus creating space around the substrate that allows for some movements and the presentation of a different position toward the chlorine species interacting with the lysine and the glutamate residues (Figure 16).

B. Halogenation of Nonactivated Carbon Atoms

The detection of $FADH_2$-dependent halogenases had led to the understanding of how halogenation of substrates activated for the reaction with an electrophilic halide species proceeds but did not explain how nonactivated carbon atoms such as methyl groups can be halogenated



Scheme 21 Reaction mechanism of $FADH_2$-dependent halogenases showing the involvement of the two absolutely required amino acid residues lysine79 and glutamate346 in the formation of **6** by the tryptophan 7-halogenase PrnA.[15,87]

in an enzymatic reaction. This riddle was solved by the work of Vaillancourt et al.[89] They detected a type of halogenating enzymes showing some similarity to nonheme iron, α-ketoglutarate-dependent hydroxylases. The elucidation of the structure of the nonheme iron, α-ketoglutarate- and O_2-dependent halogenase SyrB2 that chlorinates L-threonine in syringomycin biosynthesis, indicated that the reaction proceeds *via* the formation of a substrate radical by H atom abstraction.[90] The substrate radical then reacts with the chloride ion to form the chlorinated product (Scheme 22).

Vaillancourt et al. found that such a reaction is involved in the formation of a cyclopropane ring as a cryptic chlorination reaction, where chloride serves as a leaving group.[88] Analogously, a cyclopropane ring is formed during the biosynthesis of kutznerides.[47]

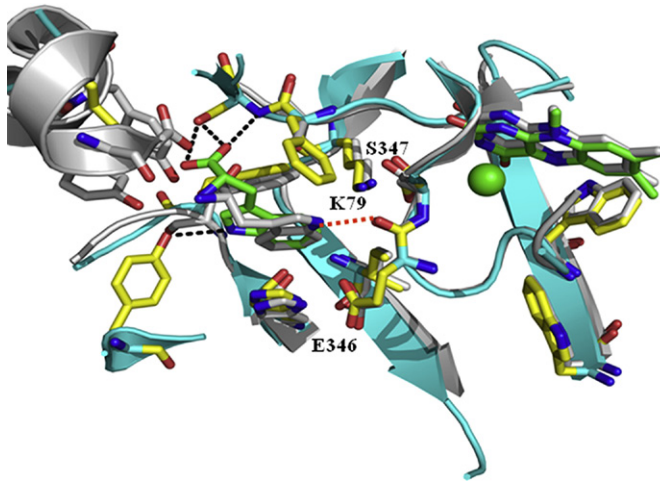


Figure 15 Overlay of the active sites of the tryptophan 7-halogenase PrnA and the tryptophan 5-halogenase PyrH showing the overlap of the amino acids involved in catalysis and the positions to be halogenated.[17] The substrate bound by PrnA is shown below of the substrate bound by PyrH. The chloride ion bound near the isoalloxazine ring is shown as a sphere.

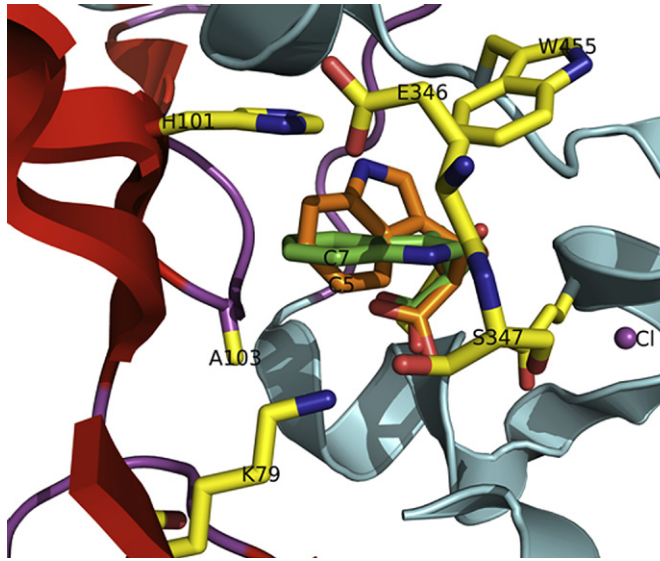
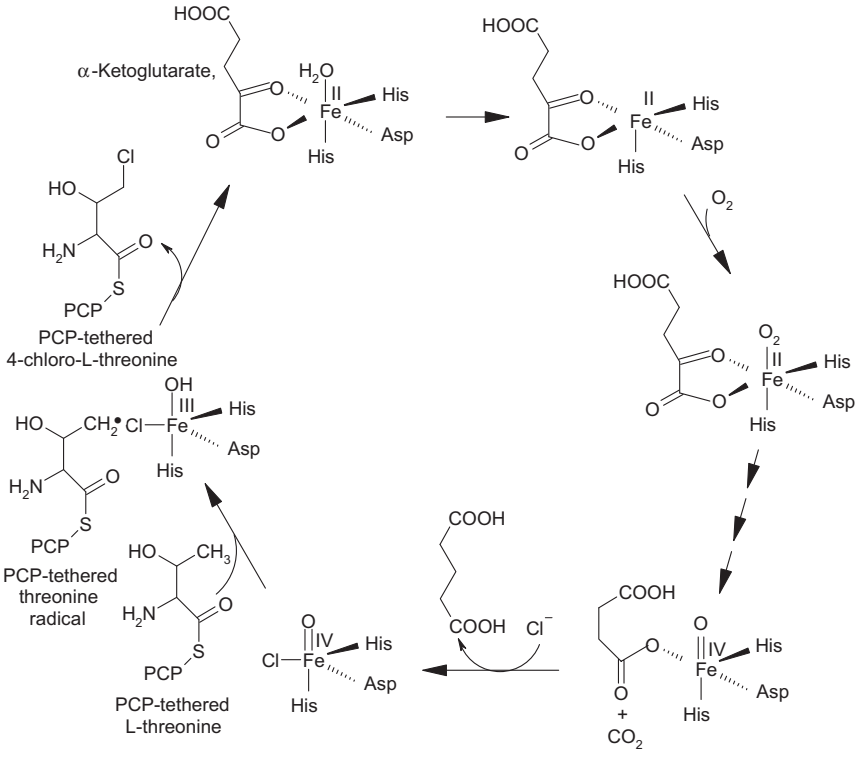


Figure 16 Model of the binding of the substrate in the PrnA F103A mutant leading to a change in regioselectivity of the halogenation reaction.[88] The 7-position (C7) of the substrate bound by native PrnA and the 5-position (C5) of the substrate bound by the F103A mutant are indicated.



Scheme 22 Proposed reaction mechanism for the halogenation of nonactivated carbon atoms by nonheme iron, α-ketoglutarate- and O_2-dependent halogenases.[88]

VII. CONCLUSIONS

From the examples shown above for the biosynthetic pathways of halogenated alkaloids, it can be seen that a lot of knowledge has been obtained by molecular genetic investigations. The cloning of gene clusters has led to the suggestion of possible pathways, but in many cases, the sequence of the individual steps is still unclear or there are still gaps in the pathways or discrepancies between the genes found and the enzymatic reactions expected to be necessary to obtain the final product. This is mostly due to the fact that biochemical investigations of the individual steps are very often lacking. Only in a few cases, individual enzymes have been identified and the activity has been investigated *in vitro*. This is mostly due to a lack of the substrates for these enzymes and knowledge about their cofactor requirements. To fill these gaps, the creation of mutants in the pathways allowing the accumulation and isolation of intermediates is required. These intermediates could then serve as substrates for enzymatic reactions

after overexpression of the genes and purification of the enzymes in active form. Overexpression is very often required since the activities of enzymes involved in secondary metabolism are often rather low making characterization of these enzymes *in vitro* very difficult. Thus, there is still a lot of work to do to fully understand the biosynthetic pathway of halogenated alkaloids.

For the application of enzymes from secondary metabolism for the production of interesting products, the biochemical characterization is a prerequisite. As an alternative, however, the enzymes can be used in *in vivo* systems by integrating them into existing biosynthetic pathways and thus modifying these pathways.

It will be interesting to see whether halogenating enzymes different from the ones known to date will be discovered and how enzymatic halogenations will be applied in *in vitro* or *in vivo* systems in the future to obtain new alkaloid derivatives and other compounds with interesting biological activities and properties.

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