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MINI-REVIEW

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Flavin-dependent halogenases involved in secondary metabolism in bacteria

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Abstract The understanding of biological halogenation has increased during the last few years. While haloperoxidases were the only halogenating enzymes known until 1997, it is now clear that haloperoxidases are hardly, if at all, involved in biosynthesis of more complex halogenated compounds in microorganisms. A novel type of halogenating enzymes, flavin-dependent halogenases, has been identified as a major player in the introduction of chloride and bromide into activated organic molecules. Flavin-dependent halogenases require the activity of a flavin reductase for the production of reduced flavin, required by the actual halogenase. A number of flavin-dependent tryptophan halogenases have been investigated in some detail, and the first three-dimensional structure of a member of this enzyme subfamily, tryptophan 7-halogenase, has been elucidated. This structure suggests a mechanism involving the formation of hypohalous acid, which is used inside the enzyme for regioselective halogenation of the respective substrate. The introduction of halogen atoms into non-activated alkyl groups is catalysed by non-heme Fe^{II} α-ketoglutarate- and O_2-dependent halogenases. Examples for the use of flavin-dependent halogenases for the formation of novel halogenated compounds in in vitro and in vivo reactions promise a bright future for the application of biological halogenation reactions.

Introduction

The number of halogenated metabolites isolated from living organisms is steadily increasing. Many of these compounds have biological activity, such as the important antibiotic vancomycin produced by soil bacteria (Williams and Bardsley 1999), the anticancer compound cryptophycin A isolated from a cyanobacterium (Trimurtulu et al. 1994), the signal molecule 3,5-dichlorohexanophenone from *Dictyostelium* involved in fruiting body formation (Morris et al. 1987) or the thyroxines found in the mammalian thyroid gland (Kendall 1919). In his review, Gribble (2003) reported the identification of more than 3,700 halometabolites. The majority of these compounds have been isolated from marine organisms where brominated metabolites play a dominant role. From terrestrial organisms, mostly chlorinated compounds are produced; however, bacteria, cultivated in the presence of bromide ions instead of chloride ions, often produce the bromoanalogues of the normally synthesised chlorometabolites (Smith 1958; Doerschuk et al. 1959; van Pée et al. 1983; Bister et al. 2003). The important factor in this context seems to be the relative concentration of bromide to chloride (Doerschuk et al. 1959).

Brominated metabolites like bromotyrosines are also produced by humans; however, this seems rather to be a side reaction and not part of a biosynthetic pathway (Wu et al. 1999). An example of an iodocompound synthesised by mammals is the thyroid hormone thyroxine. In contrast to bromo- and chlorometabolites, iodinated metabolites are rather rare, and it does not seem to be possible, like in the bromoanalogues of chlorometabolites synthesized by terrestrial organisms, to obtain the iodoanalogues by substituting chloride with iodide in the culture medium. Whereas chlorinated, brominated and iodinated metabolites are produced by many different organisms, fluorinated metabolites have only been isolated from plants and bacteria so far (Oelrichs and McEwan 1961; Morton et al. 1969). The best known fluorometabolite is fluoroacetate, produced by a number of plants and soil bacteria (Oelrichs and McEwan 1961; Sanada et al. 1986).

The detection of a halogenating enzyme, a chloroperoxidase, was already reported in 1959 (Shaw and Hager 1959). Although this enzyme was detected during the investigation of the biosynthesis of caldariomycin by the fungus *Caldariomyces fumago*, it was never shown that this enzyme is actually involved in caldariomycin biosynthesis. This also holds for the large number of other haloperoxidases detected after the isolation of the *Caldar-*

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iomyces enzyme. Haloperoxidases are enzymes that require hydrogen peroxide, halide ions (chloride, bromide, or iodide) for the halogenation of an organic substrate susceptible to attack by an electrophile. Due to their reaction mechanism, haloperoxidases produce hypohalous acids as a freely diffusible halogenating agent (Fig. 1a; Sundaramoorthy et al. 1998; Wever and Hemrika 1998) and have thus no substrate specificity or regioselectivity. This holds for every type of haloperoxidase, regardless whether it is a haem- or a vanadium-containing haloperoxidase. In addition, perhydrolases, originally detected as haloperoxidases, catalyse the formation of short-chain aliphatic peracids, which oxidise halide ions, again resulting in the formation of hypohalous acids as the halogenating agent (van Pee and Unversucht 2003; Fig. 1b). Because of this lack of substrate specificity and regioselectivity, haloperoxidases and perhydrolases are not suitable for the specific halogenation reactions required during halometabolite biosynthesis. The type of halogenating enzymes playing a major role in halometabolite biosynthesis was detected about 45 years after the detection of haloperoxidases (Dairi et al. 1995; Hammer et al. 1997; Hohaus et al. 1997). This review deals with recent gain of knowledge about this novel type of halogenating enzymes and their potential for the formation of novel compounds.

The detection of flavin-dependent halogenases

While investigating the biosynthesis of 7-chlorotetracycline on a molecular genetics basis, Dairi et al. (1995) cloned and sequenced the biosynthetic gene cluster for 7-chlorotetracycline. By complementing a mutant that only produced non-chlorinated tetracycline and not 7-chlorotetracycline with a 2.6-kb fragment from the cloned gene cluster, they identified the gene coding for the halogenating enzyme involved in 7-chlorotetracycline biosynthesis. However, neither the expression of the gene nor any in vitro activity of the enzyme was reported. The nucleotide sequence and the amino acid sequence deduced thereof did not show any similarity to haloperoxidases, peroxidases or perhydrolases. From this, it could be concluded that the halogenating enzyme involved in 7-chlorotetracycline biosynthesis must be a novel halogenase. In addition, they did not reveal any similarity to other known enzymes that might be related to this halogenase.

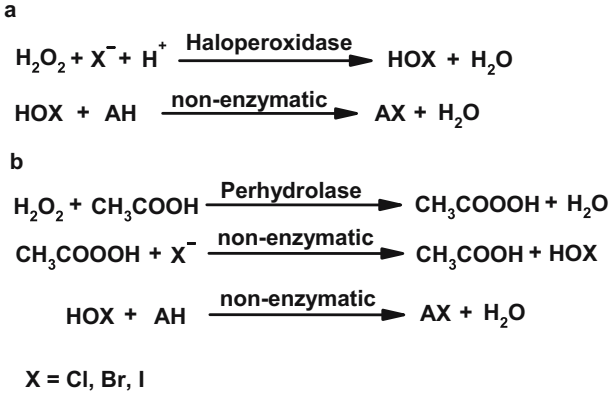


Fig. 1 Halogenation reactions catalysed by **a** haem- and vanadium-containing haloperoxidases and **b** perhydrolases

Hammer et al. (1997) reported the functional cloning of the pyrrolnitrin biosynthetic gene cluster from *Pseudomonas fluorescens* BL915. Pyrrolnitrin is an antifungal antibiotic produced by a number of *Pseudomonas* and *Burkholderia* strains and a few other soil bacteria. Pyrrolnitrin is derived from tryptophan, and it was assumed for quite some time that the first step in pyrrolnitrin biosynthesis would be the regioselective chlorination of tryptophan at the 7-position of the indole ring, followed by a second halogenation later in the biosynthesis (Fig. 2). Analysis of the cloned gene cluster showed that this assumption was correct and that chlorination of tryptophan was actually the first step in pyrrolnitrin biosynthesis (Hohaus et al. 1997). Transformation of *E. coli* with a plasmid containing the four genes of the pyrrolnitrin biosynthetic gene cluster led to the production of pyrrolnitrin by the recombinant *E. coli* strain (Hammer et al. 1997). From this, it was concluded that all the genes required for pyrrolnitrin biosynthesis were the four genes coding for two halogenases, tryptophan 7-halogenase (PrnA) and monodechloroaminopyrrolnitrin 3-halogenase (PrnC), an enzyme catalysing the ring rearrangement from the indole ring system to the phenyl pyrrole ring system (PrnB) and the enzyme oxidising the amino to the nitro group (PrnD).

When attempts were made to detect in vitro activity of PrnA and PrnC using tryptophan and monodechloroaminopyrrolnitrin as the respective substrates, it was realised that, in crude extracts, the addition of NADH was required (Hohaus et al. 1997). During early stages of the purification of PrnA, it was revealed that FAD and a second protein component, a flavin reductase, were also required for halogenating activity (Keller et al. 2000). The flavin reductase uses NADH for the reduction of FAD. The resulting $FADH_2$ is required by the halogenase for catalysis of the halogenation reaction. Reduced FMN or riboflavin are not accepted by the halogenases. Due to their strict dependence on $FADH_2$, all these halogenases contain a flavin binding site (GxGxxG) near the amino terminal end (van Pée and Zehner 2003).

$FADH_2$ as the co-substrate required by halogenases

$FADH_2$ is also required by a number of monooxygenases, and from some, it is known that they use freely diffusible $FADH_2$ and that no specificity between the flavin reductase and the halogenase is required (Galan et al. 2000; Louie et al. 2002, 2003; Kirchner et al. 2003; Otto et al. 2004). This seemed also to be the case with the flavin-dependent halogenases, since amongst the four genes of the pyrrolnitrin biosynthetic gene cluster, there was no flavin reductase gene. The finding that *E. coli* produced pyrrolnitrin when transformed with the four genes on a plasmid

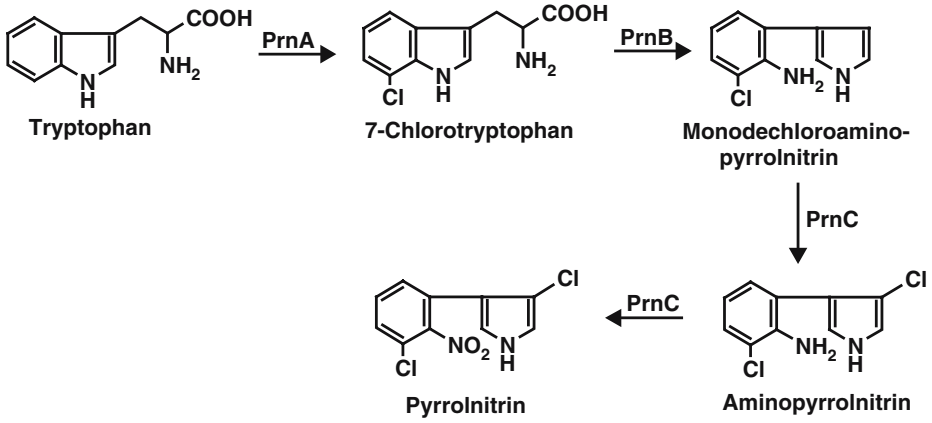


Fig. 2 Pyrrolnitrin biosynthetic pathway. The first step in pyrrolnitrin biosynthesis is the regioselective chlorination of tryptophan by PrnA to 7-chlorotryptophan

clearly showed that a non-specific *E. coli* reductase could be used for providing the halogenase with reduced flavin (Hammer et al. 1997). Thus, it was possible to substitute the flavin reductase from the pyrrolnitrin producer using reductases from several bacteria such as NADH oxidase from *Thermus thermophilus* and SsuE and Fre from *E. coli* to substitute the flavin reductase from the pyrrolnitrin producer to restore the halogenating activity of PrnA after purification (Keller et al. 2000; Unversucht et al. 2005). SsuE was also used with the tryptophan 5-halogenase involved in pyrroindomycin B biosynthesis in *Streptomyces rugosporus* (Zehner et al. 2005). Unversucht et al. (2005) went even further and used chemically reduced FAD or $FADH_2$ regenerated using the organometallic complex (pentamethylcyclopentadienyl)rhodium-bipyridine. This catalyst was employed as the redox catalyst with formate as the electron donor, substituting the flavin reductase/NADH system. However, freely diffusible $FADH_2$ very rapidly reacts with oxygen to form a flavin hydroperoxide, which spontaneously decomposes to FAD and hydrogen peroxide. Since this seems to be a waste of energy that is not very likely to occur in a living cell, the question as to whether in vivo specific reductases might form a complex with halogenases arises.

In rebeccamycin biosynthesis, like in pyrrolnitrin biosynthesis, the first step is the regioselective chlorination of tryptophan in the 7-position (Fig. 3; Kling et al. 2005). Interestingly, Yeh et al. (2005) report for the His-tagged tryptophan 7-halogenase (RebH) from rebeccamycin biosynthesis that even after purification by Ni-chelating affinity chromatography, this enzyme preparation still shows some flavin reductase activity. This suggests that a flavin reductase might bind quite tightly to the halogenase, although in this case, it would be a reductase from *E. coli* since the halogenase was purified from a recombinant *E. coli* strain. In their in vitro activity assay, Yeh et al. (2005) also used a reductase (RebF) whose gene is part of the rebeccamycin biosynthetic gene cluster (Sanchez et al. 2002).

In the case of some of the flavin-dependent monooxygenases, the genes for the monooxygenase and the flavin reductase are close together, suggesting co-regulation (Louie et al. 2003). However, a complex between the reductase and the monooxygenase could not be shown for them (Louie et al. 2002; Otto et al. 2004), with the exception of the luciferase/flavin reductase system from *Vibrio harveyi* (Low and Tu 2003).

A closer look at some of the gene clusters for halometabolite biosynthesis supports the hypothesis that there might be a specific flavin reductase acting together with the halogenase. As already mentioned, the rebeccamycin biosynthetic gene cluster contains a flavin reductase, which is rather close to the halogenase gene (Fig. 4a; Sanchez et al. 2002). A flavin reductase gene was also found in the biosynthetic gene cluster for the antitumor polyenone neocarzilin (Fig. 4b) from "*Streptomyces carzinostaticus*" with the halogenase separated from the flavin reductase by three type I polyketide synthases and a thioesterase (Otsuka et al. 2004). Recently, the whole genome of *P. fluorescens* Pf-5 has been sequenced (Paulsen et al. 2005). A reanalysis of the gene clusters for pyrrolnitrin and pyoluteorin biosyntheses contained in this strain suggests that flavin reductase genes are part of or at least are located in the close vicinity of these clusters (Fig. 4c,d). Whether these flavin reductases are specifically required for the function of the halogenases is not yet known.

Distribution and substrate specificity of flavin-dependent halogenases

Flavin-dependent halogenases seem to play a major role in catalysing the introduction of halogen atoms during the biosynthesis of organohalogen compounds. In almost every gene cluster for the biosynthesis of a halometabolite, one or several genes for flavin-dependent halogenases have been detected. All flavin-dependent halogenases consist of about 500 amino acids and have two absolutely conserved regions. One is the GxGxxG motif located near the amino terminal end, which is involved in the binding of the flavin co-substrate. However, in PltD from pyoluteorin biosynthesis, this motif is not absolutely conserved (GxSxxV). Although PltD shows a high overall similarity to those flavin-dependent halogenases that use pyrrole and phenol derivatives as their substrates, it is only a halogenase-like protein of unknown function (Paulsen et al. 2005).

A second absolutely conserved motif located near the middle of the enzymes contains two tryptophan residues

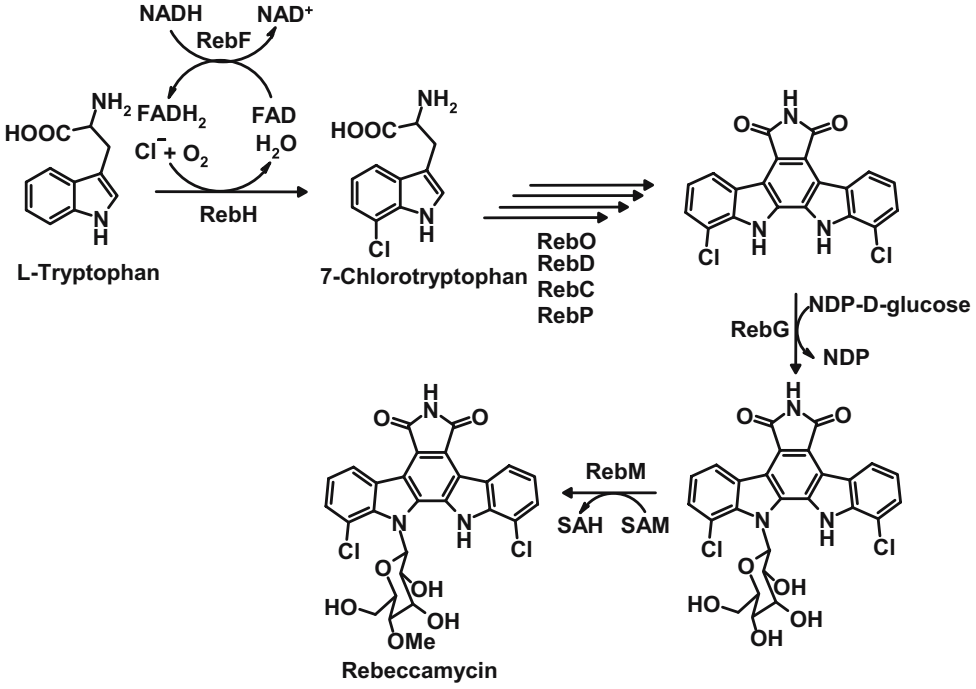


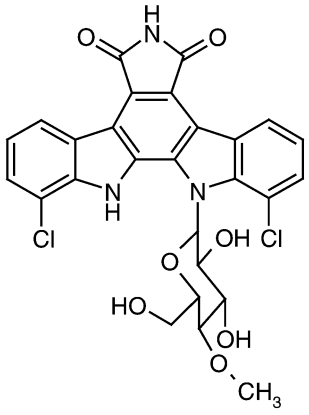
Fig. 3 Partial rebeccamycin biosynthetic pathway. The first step in rebeccamycin biosynthesis is the regioselective chlorination of tryptophan by RebH to 7-chlorotryptophan

(WxWxIP). Again, this motif is not absolutely conserved in PltD (WxGxIP), showing that this enzyme is not a halogenase. The two tryptophan residues of this motif are located near the flavin, and they are suggested to block the binding of a substrate close to the flavin and thus prevent the enzyme from catalysing a monooxygenase reaction (Dong et al. 2005). These two motifs can be found in every flavin-dependent halogenase detected so far, although it has to be noted that the huge majority of the flavin-dependent halogenases have only been identified on the basis of sequence homology and not by experimental evidence. Halogenating activity in vitro has only been shown for the tryptophan 7-halogenases from pyrrolnitrin biosynthesis (PrnA; Hohaus et al. 1997), rebeccamycin biosynthesis (RebH; Kling et al. 2005; Yeh et al. 2005), tryptophan 5-halogenase (PyrH) from pyrroindomycin biosynthesis (Zehner et al. 2005), tryptophan 6-halogenase (Thal) from the thienodolin producer *Streptomyces albogriseolus* (Kling et al. 2005), monodechloroaminopyrrolnitrin 3-halogenase (PrnC) from pyrrolnitrin biosynthesis (Hohaus et al. 1997), and HalB from the pentachloropseudilin producer *Actinoplanes* sp. ATCC 33002 (Wynands and van Pée 2004; Table 1). Proof via molecular genetic experiments was obtained for Chl from 7-chlorotetracyclin biosynthesis (Dairi et al. 1995), BhaA from balhimycin biosynthesis (Puk et al. 2002), and Clo-hal from clorobiocin biosynthesis (Eustaquio et al. 2003).

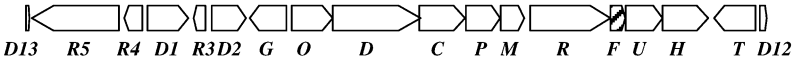
The fact that in vitro halogenase activity could only be demonstrated for a few halogenases is mostly due to the lack of knowledge about the natural substrates of these enzymes. In only very few cases is the substrate of the halogenases in the biosynthetic pathway known or available. This explains why progress on the investigation of halogenases involved in halometabolite biosynthesis has been rather slow and has only been accomplished with tryptophan halogenases so far. These halogenases accept free tryptophan, whereas in most other cases, it is suggested that the enzymes act on coenzyme A derivatives of the substrates (Piraee et al. 2004; Walsh 2003) or on substrates bound to peptidyl carrier proteins (Eustaquio et al. 2003; Puk et al. 2004).

There are only two examples where genes of flavin-dependent halogenases have been detected in gene clusters for the biosynthesis of chlorinated aliphatic compounds. One is *cmlS* from chloramphenicol biosynthesis (Piraee and Vining 2002; Piraee et al. 2004), and the other is ORF3 from the neocarzilin biosynthetic gene cluster (Otsuka et al. 2004). While chloramphenicol contains a dichloracetyl moiety of yet unknown origin, neocarzilin has a trichloromethyl group. Interestingly, there are a number of other halometabolites from various cyanobacteria that contain a trichloromethyl group (Jiménez and Scheuer 2001; Unson et al. 1993). However, in the biosynthetic gene clusters for these compounds, no genes of flavin-dependent halogenases have been found (Chang et al. 2002; Ridley et al. 2005). BarB1/BarB2 and DysB1/DysB2 have been suggested to be the halogenases catalysing the chlorinating reactions in barbamide and disidenin/dysideathiazole biosynthesis, respectively. This suggestion is supported by the finding of Vaillancourt et al. (2005a) who showed that the halogenase involved in syringomycin E biosynthesis is a different type of halogenating enzyme with similarity to BarB2 and DysB2. SyrB2 from syringomycin E biosynthesis catalyses the chlorination of the methyl group of a peptidyl carrier protein-bound L-threonine. This novel halogenase is a non-heme Fe^{II} α-ketoglutarate- and O_2-dependent enzyme (Fig. 5). This halogenase, unlike haloperoxidases and flavin-dependent halogenases, does not require a substrate with a double bond for introduction of halogen atoms and thus might be the type of halogenase

a



Rebeccamycin



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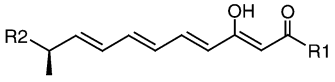


R1= CCl_3	R2= CH_2CH_3	**Neocarzilin A**
R1= CCl_3	R2= CH_3	**Neocarzilin B**
R1= $CHCl_2$	R2= CH_2CH_3	**Neocarzilin C**

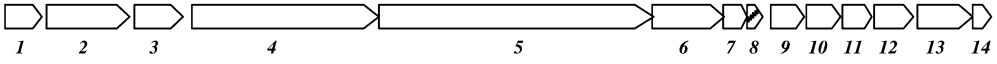
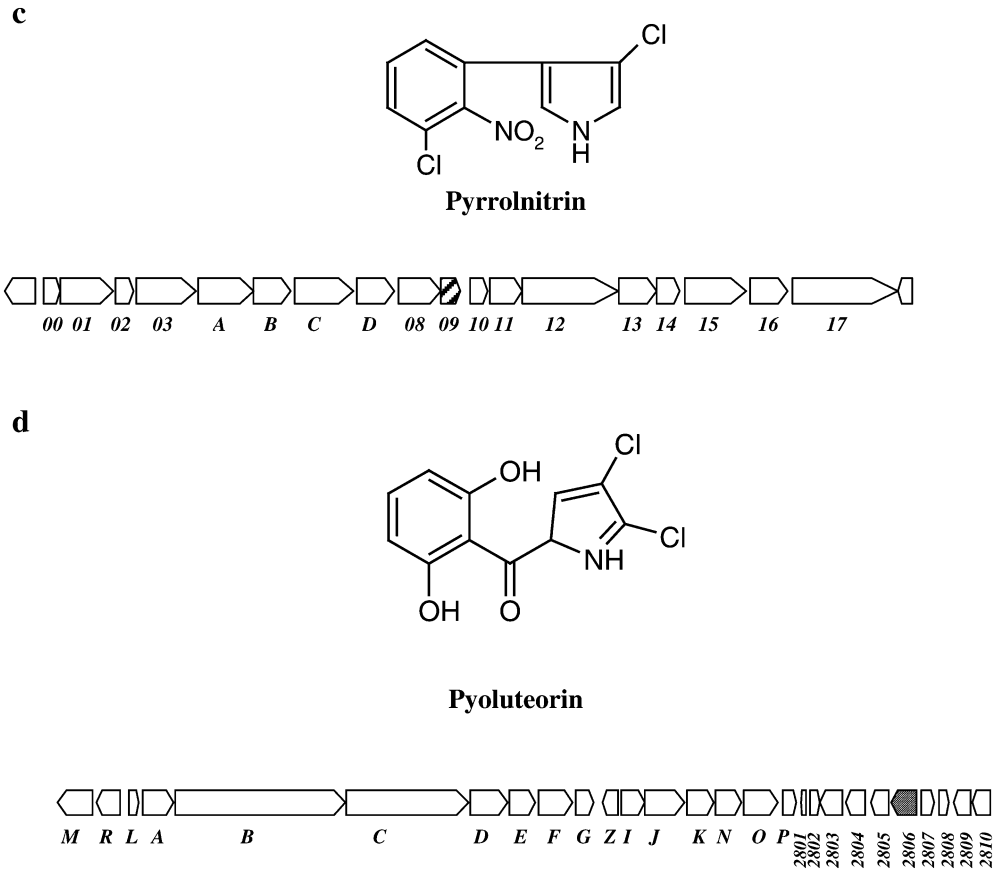


Fig. 4 Biosynthetic gene clusters for **a** rebeccamycin (Sanchez et al. 2002), **b** neocarzilin (Otsuka et al. 2004), **c** pyrrolnitrin (Paulsen et al. 2005), and **d** pyoluteorin biosynthesis (Paulsen et al. 2005) showing the location of *flavin reductase genes*. **a** ORFD13, hypothetical protein; ORFR5, putative regulatory protein; ORFR4, hypothetical protein; ORFD1, putative secreted esterase; ORFR3, hypothetical protein; ORFD2, hypothetical protein; rebG, putative glycosyltransferase; rebO, putative L-tryptophan oxidase; rebD, hypothetical protein; rebC, putative monooxygenase; rebP, putative P450 protein; rebM, putative methyltransferase; rebR, putative regulatory protein; *rebF, flavin reductase*; rebU, putative integral membrane transporter; rebH, tryptophan halogenase; rebT, putative integral membrane transporter; ORFD12, putative regulatory protein. **b** ORF1, putative antiporter; ORF2, putative activator; ORF3, putative halogenase; ORF4, polyketide synthase; ORF5, polyketide synthase; ORF6, polyketide synthase; ORF7, putative thioesterase; *ORF8, putative flavin reductase*; ORF9, putative acyl-CoA synthetase; ORF10, putative dehydrogenase α subunit; ORF11, putative dehydrogenase β subunit; ORF12, putative acyltransferase; ORF13, putative oxidoreductase; ORF14, putative oxidoreductase. **c** PFL 3600, glcG protein; PFL 3601, drug resistance transporter EmrB/QacA family; PFL 3602, oxidoreductase; FAD-binding putative; PFL 3603, poxB pyruvate dehydrogenase; PFL 3604, PrnA tryptophan 7-halogenase; PFL 3605, PrnB; PFL 3606, PrnC monodechloroaminopyrrolnitrin 3-halogenase; PFL 3607, PrnD; PFL 3608, sodium/hydrogen antiporter putative; *PFL 3609, flavin reductase domain protein*; PFL 3610, RNA polymerase sigma-70 family protein; PFL 3611, sigma factor regulatory protein; FecR/PupR family; PFL 3612, putative TonB-dependent receptor; PFL 3613, di-haem cytochrome C peroxidase family protein; PFL 3614, ptsN PTS system; nitrogen regulatory IIA component; PFL 3615, ABC-type export system; outer membrane channel protein; PFL 3616, multidrug efflux RND membrane fusion protein; PFL 3617, mtrD multidrug efflux RND transporter. **d** PFL 2784, PltM putative halogenase; PFL 2785, PltR transcriptional regulator; PFL 2786, PltL conserved hypothetical protein; PFL 2787, PltA putative halogenase; PFL 2788, PltB probable polyketide synthase type I; PFL 2789, PltC polyketide synthase type I; PFL 2790, PltD halogenase-like protein; PFL 2791, PltE acyl-CoA dehydrogenase; PFL 2792, PltF putative non-ribosomal peptide synthetase; terminal component; PFL 2793, PltG CFA synthetase; thioesterase component; PFL 2794, PltZ transcriptional regulator; PFL 2795, PltI putative membrane fusion protein; PFL 2796, PltJ ABC transporter; ATP-binding/permease protein; PFL 2797, PltK ABC transporter; ATP-binding protein/permease; PFL 2798, PltN putative ABC transporter/permease protein; PFL 2799, PltO multidrug efflux MFS outer membrane protein; PFL 2800, PltP putative cmaU protein; PFL 2801, hypothetical protein; PFL 2802, hypothetical protein; PFL 2803, zinc-binding oxidoreductase; PFL 2804, hydrolase; α/β-fold family; PFL 2805, oxidoreductase; short-chain dehydrogenase/reductase family; *PFL 2806, NADH: flavin oxidoreductase/NADH oxidase family protein*; PFL 2807, transcriptional regulator; TetR family; PFL 2808, hypothetical protein; PFL 2809, hypothetical protein; PFL 2810, transcriptional regulator; LuxR family

Fig. 4 (continued)



responsible for the halogenation of non-activated aliphatic compounds (Vaillancourt et al. 2005a,b).

Reaction mechanism of flavin-dependent halogenases

So far, the purification and partial biochemical characterization of only three of these halogenases have been reported. Their in vitro activities are rather low with k_{cat} values of 1.4 min^{-1} for tryptophan 7-halogenase RebH from rebeccamycin biosynthesis (Yeh et al. 2005), 0.1 min^{-1} for

Table 1 Some properties of flavin-dependent halogenases for which in vitro activity has been demonstrated

Producing strains	Enzyme	Molecular mass of subunits (number of subunits)	Substrates	References
Pseudomonas fluorescens Bl915	Tryptophan 7-halogenase (PrnA)	61,100 (2)	L- and D-tryptophan and some tryptophan and indole derivatives	Dong et al. 2005; Hölzer et al. 2001
Lechevalieria aerocolonigenes	Tryptophan 7-halogenase (RebH)	60,300 (?)	L- and D-tryptophan	Kling et al. 2005; Yeh et al. 2005
Streptomyces rugosporus	Tryptophan 5-halogenase (PyrH)	58,100 (?)	L- and D-tryptophan	Zehner et al. 2005
Streptomyces albogriseolus	Tryptophan 6-halogenase (Thal)	60,000 (?)	L- and D-tryptophan	Kling et al. 2005
Pseudomonas fluorescens BL915	Monodechloroamino-pyrrolnitrin 3-halogenase (PrnC-Hal)	66,000 (2)	Monodechloroamino-pyrrolnitrin	Hohaus et al. 1997
Actinoplanes sp. ATCC 33002	HalB	63,500 (?)	2-(3,5-Dibromophenyl)pyrrole	Wynands and van Pée 2004
Pseudomonas fluorescens Pf-5	PltA	50,700 (?)	Pyrrolyl-*S*-carrier protein	Dorrestein et al. 2005

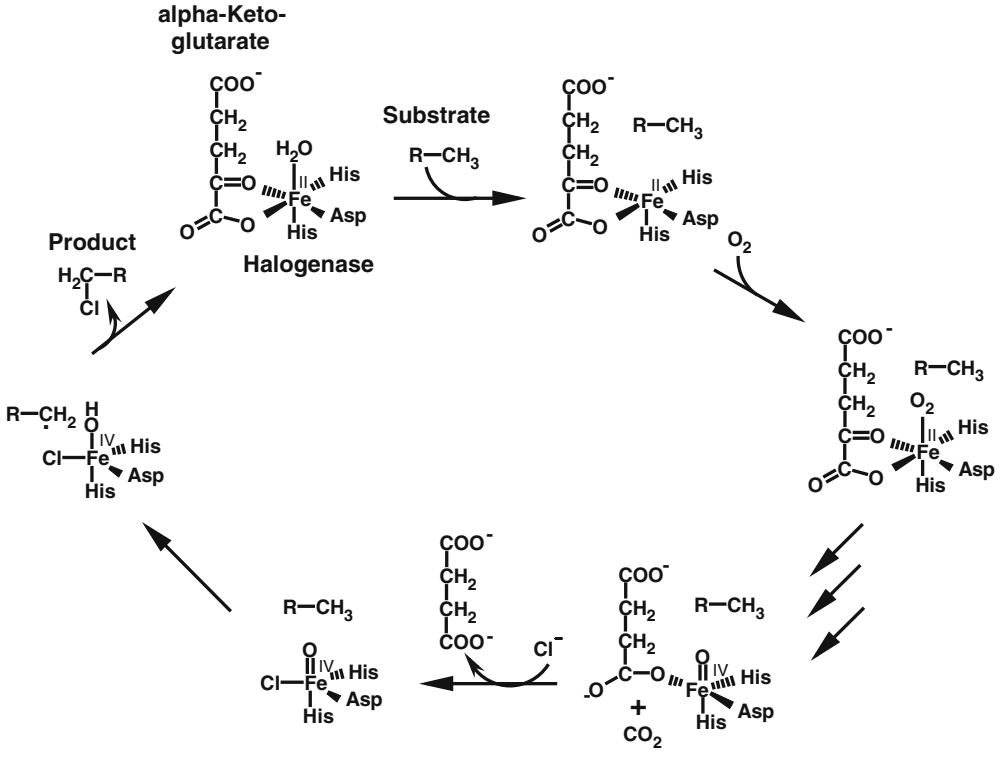


Fig. 5 Hypothetical reaction mechanism of α-ketoglutarate-dependent halogenases as suggested by Vaillancourt et al. (2005a,b)

tryptophan 7-halogenase PrnA from pyrrolnitrin biosynthesis (Keller et al. 2000), and 0.5 min^{-1} for tryptophan 5-halogenase PyrH from pyrroindomycin biosynthesis (Zehner et al. 2005). The reason for these low turnover numbers in the in vitro reaction is not yet known. However, since the activities seem to be much higher in vivo, this could be an indication that a further component might still be missing in the in vitro system or that a specific interaction between the two components of the system, the flavin reductase and the halogenase, could be necessary for higher activity.

Two reaction mechanisms that require the formation of reduced $FADH_2$ by a flavin reductase in a reaction preceding the actual halogenation reaction had been suggested. It seemed extremely unlikely that free hypochlorite could be formed by the flavin-dependent halogenases as the halogenating agent since this would not allow the reactions to proceed with the required regioselectivity and substrate specificity seen in the biosynthesis of secondary metabolites. This reasoning led to the suggestion of a nucleophilic reaction mechanism (Fig. 6a; Keller et al. 2000). In this mechanism, the flavin hydroperoxide formed by the reaction of the halogenase-bound reduced flavin with oxygen would react with a double bond of the organic substrate, resulting in the formation of an epoxide (Keller et al. 2000) or the addition of a hydroxy group (Unversucht et al. 2005). This would then be followed by the nucleophilic attack of a halide ion (chloride or bromide), leading to the formation of a halohydrin. In the last step, elimination of water would give rise to the halogenated product. In an alternative, electrophilic mechanism (Fig. 6b), flavin hydroperoxide would react with the halide ion to form a FAD–O–Cl intermediate. Attack of the aromatic π electrons on the FAD–O–Cl intermediate would lead to formation of a chlorinated substrate intermediate that, after deprotonation, would give the chlorinated product (Yeh et al. 2005).

However, the elucidation of the three-dimensional structure of PrnA, which has recently been crystallised (Dong et al. 2004a), suggests that none of the above-described mechanisms is the correct one. The structure reveals that the initially formed flavin hydroperoxide cannot interact directly with the substrate tryptophan since they are separated by about 10 Å. However, chloride bound next to the flavin could be oxidised by the flavin hydroperoxide, leading to the formation of free hypochlorite, which cannot leave the active site. To prevent the reaction of hypochlorite with anything other than the correct substrate, one has to guide it to the substrate through the active site tunnel to the substrate tryptophan, which has to be positioned in such a way that the position to be halogenated is presented to the hypochlorite (Fig. 6c; Dong et al. 2005).

A halogenating enzyme introducing a halide ion as a nucleophile is the fluorinase from the soil bacterium *Streptomyces cattleya* (O'Hagan et al. 2002), the three-dimensional structure of which has been recently reported (Dong et al. 2004b). This fluorinase catalyses the fluorination of *S*-adenosyl-L-methionine to 5'-fluoro-5'-deoxyadenosine, which is then further metabolised to fluoroacetate and 4-fluorothreonine (Fig. 7). The fluoride ion has to be dehydrated by the enzyme for it to act as a nucleophile. This desolvation seems to be achieved by the enzyme via the formation of hydrogen bonds between the fluoride ion and the OH group of a serine residue and a backbone amide of the fluoride binding pocket. Interestingly, this fluorinase shows no sequence similarity to any of the known flavin-dependent halogenases.

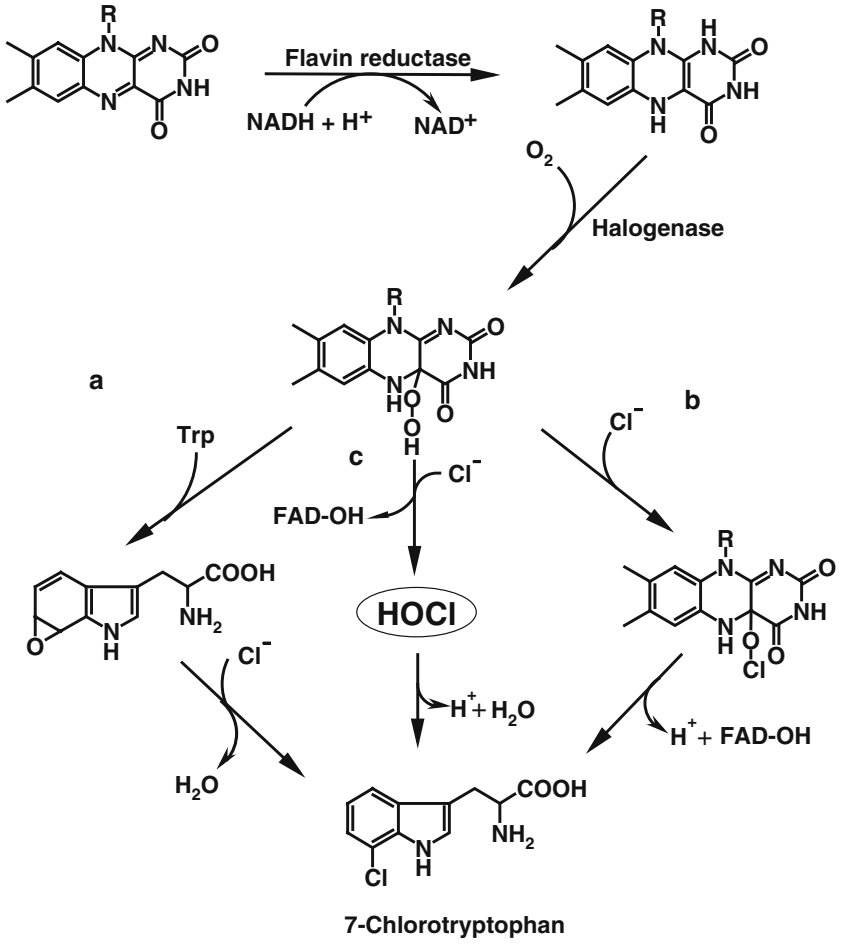


Fig. 6 Hypothetical reaction mechanisms for flavin-dependent halogenases. Tryptophan is shown as an example for a substrate. **a** Nucleophilic mechanism suggested by Keller et al. (2000), **b** electrophilic mechanism suggested by Yeh et al. (2005) and **c** mechanism suggested by Dong et al. (2005) based on the three-dimensional structure of PrnA and biochemical data. *HOCl in an oval* depicts a hypochlorite molecule that is not released from the active site of the enzyme

Application of halogenases to obtain new products

Attempts have been made to use haloperoxidases for the production of novel halogenating compounds, but they have been rather disappointing. Due to the formation of freely diffusible hypochlorite, halogenation occurs without regioselectivity and the same products are obtained as with chemical halogenation (van Pée 1996).

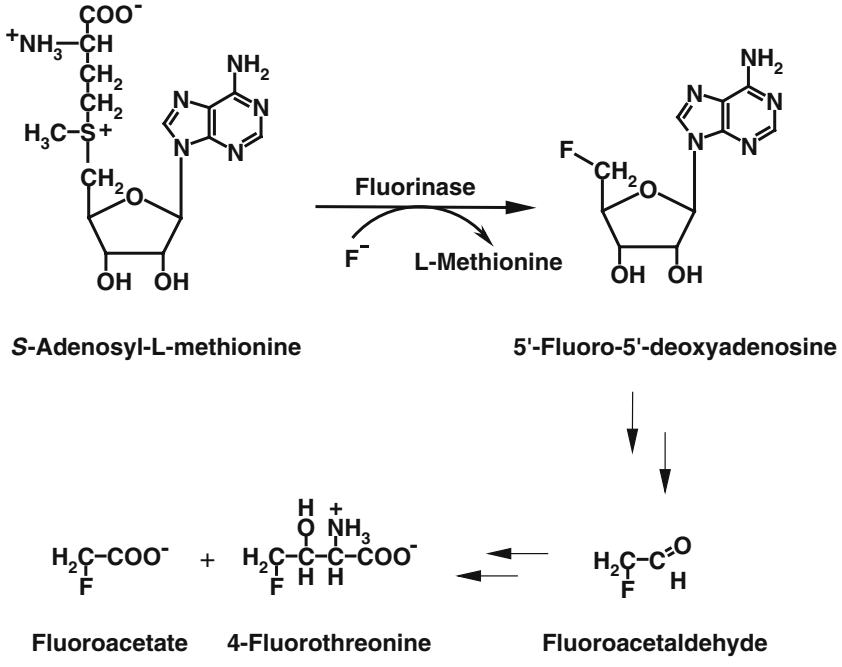


Fig. 7 Fluorination of *S*-adenosyl-L-methionine by 5'-fluoro-5'-deoxyadenosyl-adenosine fluorinase and subsequent formation of 4-fluorothreonine and fluoroacetate via fluoroacetaaldehyde

However, the use of flavin-dependent halogenases, which catalyse the regioselective halogenation of their respective substrates, should allow the formation of novel halogenated compounds. For the use of these enzymes in biotechnology, the question of their substrate specificity is of great importance. Hölzer et al. (2001) investigated the substrate specificity of tryptophan 7-halogenase from the pyrrolnitrin biosynthetic pathway. They found that although a number of tryptophan, indole and phenylpyrrole derivatives are accepted by the enzyme, only tryptophan is chlorinated regioselectively in the 7-position. All the other substrates are chlorinated at the same positions, which would be chlorinated by electrophilic chemical halogenation. A further problem besides the question of the substrate specificity is the cofactor requirement and the fact that two enzymes, a flavin reductase and a halogenase, are required. First investigations to circumvent the requirement of the reductase and NADH have shown that it is possible to use just the halogenase and regenerate the reduced flavin using an organometallic catalyst (Unversucht et al. 2005). However, in the system used, strong inhibition of the tryptophan 7-halogenase by formate, which was used as the electron donor, was observed. Whether this will also be the case with other halogenases is not yet known.

The problem of cofactor regeneration can be avoided in an in vivo system in which reduced flavin is produced by flavin reductases of the host cells. Eustaquio et al. (2004) demonstrated that the halogenase from clorobiocin biosynthesis can be used to obtain a novobiocin derivative, which contains a chlorine atom at position 8 instead of a methyl group, by coexpression of the novobiocin biosynthetic gene cluster and the halogenase Clo-hal of clorobiocin biosynthesis in *Streptomyces coelicolor*. The new compound, novclobiocin 114, in which the methyl group of novobiocin is substituted by a chlorine atom, shows 50% of the antibiotic activity of novobiocin in a bioassay with *Bacillus subtilis*. Attempts to substitute Clo-hal with BhaA, the halogenase from balhimycin biosynthesis, failed. This is probably due to the differences in substrate specificity of the two enzymes. Since neither the substrate for BhaA nor the one for Clo-hal is known, in vitro activity has not been shown for these two enzymes so far.

Sanchez et al. (2005) used genes coding for tryptophan halogenases with different regioselectivities to produce novel rebeccamycin derivatives. Coexpression of the tryptophan 5-halogenase gene from the pyrroindomycin producer *S. rugosporus* (Zehner et al. 2005) with genes from rebeccamycin and staurosporine biosynthesis and the tryptophan 6-halogenase gene from the thienodolin producer *S. albogriseolus* (Kling et al. 2005) with genes from rebeccamycin biosynthesis in *Streptomyces albus* led to the formation of novel halogenated bisindole derivatives (Fig. 8). Unfortunately, no data concerning their biological activity are available.

The genes of flavin-dependent halogenases have been identified in the biosynthetic gene clusters of structurally very different compounds. From this, it can be concluded that a large number of different organic compounds can be chlorinated by flavin-dependent halogenases. One structural feature required for the chlorination by a flavin-dependent halogenase is the existence of a double bond that is accessible to electrophilic substitution. According to amino acid similarities, flavin-dependent halogenases can be divided into two groups. One group contains the tryptophan halogenases with different regioselectivities (Zehner et al. 2005), and the other group contains the enzymes catalysing the chlorination and bromination of phenol and pyrrole derivatives. Although the chloramphenicol and the neocarzilin biosynthetic gene clusters are the only clusters for aliphatic halometabolites in which the genes for flavin-dependent halogenases have been found so far, similar enzymes acting on aliphatic substrates might form a third group. Thus, although the structural diversity of the substrates for flavin-dependent halogenases seems extremely high, in vitro halogenation of the substrates might be very problematic since it must be assumed that many substrates are not halogenated in their free form but only when they are tethered to peptidyl or acyl carrier proteins. The range of potential substrates was extremely

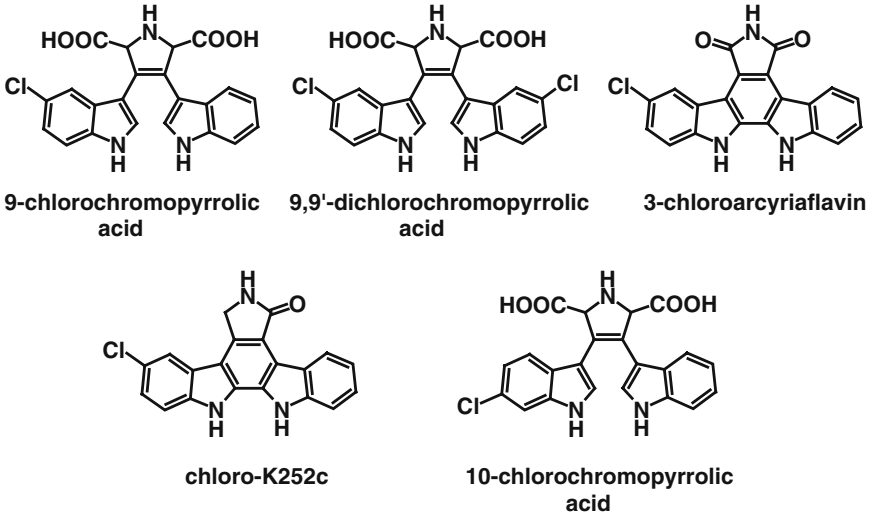


Fig. 8 Novel bisindole derivatives obtained by combinatorial biosynthesis using different tryptophan halogenase genes and genes from the rebeccamycin and staurosporine biosynthetic pathway, respectively (Sanchez et al. 2005)

extended by the detection of the α-ketoglutarate-dependent halogenases that catalyse the chlorination of a methyl group obviously accepting a large variety of substrates containing an inactivated methyl group (Vaillancourt et al. 2005a,b). Whether these enzymes accept not only substrates tethered to a peptide carrier protein but also freestanding substrates is not yet known.

The use of flavin-dependent halogenases for the in vitro as well as for the in vivo formation of novel halogenated compounds is just in its infancy; however, the very recent progress made in the whole field of biological halogenation has created an extremely good basis for further investigations into the application of these novel halogenating enzymes for the production of biologically active compounds, especially in combinatorial biosynthesis.

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