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Biosynthesis**

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Cryptic Halogenation Reactions in Natural Product Biosynthesis

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Abstract

Enzymatic halogenation reactions are essential for the production of thousands of halogenated natural products. However, in recent years, scientists discovered several halogenases that transiently incorporate halogen atoms in intermediate biosynthetic molecules to activate them for further chemical reactions such as cyclopropanation, terminal alkyne formation, C/O alkylation, biaryl coupling, and C–C rearrangements. In each case, the halogen atom is lost in the course of biosynthesis to the final product and is hence termed “cryptic”. In this review, we provide an overview of our current knowledge of cryptic halogenation reactions in natural product biosynthesis.

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1. Introduction

Halogenation reactions in a test tube or in a living cell share a lot in common. Generally, the introduction of a halogen atom to an organic substrate occurs by an addition or a substitution reaction. Many reaction strategies have either been developed by chemists or evolved in nature from microbes to humans to control halide selectivity, the regio- and stereochemistry of the reaction, as well as its stoichiometry. Synthetic and enzymatic halogenation reactions, however, do not always share common mechanisms, which is due to their very different reaction environments. Nonetheless, the introduction of halogens to organic molecules, whether in the laboratory or in nature, imparts a myriad of changes on its physical, chemical, biological and medicinal properties.¹⁻⁴

In nature, over 4,500 organohalogens have been documented from microbes, algae, plants, invertebrates and even mammals.⁵ Notable examples include ozone-depleting bromoform from algae,⁶⁻⁸ the clinical antibiotic vancomycin from bacteria,⁹ and the thyroid hormone triiodothyronine from humans.^{10, 11} The preponderance of enzymatic strategies to install inorganic halide anions into organic products involves oxidative reactions in which halides are converted to halonium ions or halogen radicals to combine with electron-rich or electron-poor natural organic substrates, respectively.¹²⁻¹⁶ Few cases in comparison involve the direct halide addition to electrophilic substrates by enzymes, most notably the *S*-adenosyl-L-methionine (SAM)-dependent halogenases.¹⁷⁻¹⁹

For the most part, biohalogenation reactions are metabolic endpoints in nature. That is, however, often not the case in synthetic organic chemistry. The installment of a halogen can be a very useful strategy to guide further chemical reactions such as substitutions, eliminations, additions, and rearrangements.²⁰ Quite a few named organic reactions involve alkyl or aryl halide reactants, such as in the Grignard reaction, the Suzuki coupling, the Favorskii rearrangement, and the Darzens condensation.

Only recently have natural product biochemists discovered that nature too takes advantage of the reactivity of C–X bonds to guide subsequent biochemical reactions that result in the removal of the newly introduced halogen. While the list is presently small, it has grown in recent years to

include reactions that support the formation of cyclopropyl rings, terminal alkynes, ether and biaryl connections, and other types of C–C rearrangements. In most cases, distinct enzymes catalyze the installation of the halogen and the subsequent chemistry concomitant with the release of the halide. In rare cases, single enzymes have dual functions to both install the halogen and its removal in order to initiate biochemical cascade reactions. Herein, we highlight the known enzymatic pathways that involve *cryptic* halogenation strategies in natural product biosynthesis.

2. Cyclopropanation reactions

Nature has evolved multiple strategies to install cyclopropyl rings in natural products. Most cyclopropane-containing natural products are terpenoids and are created via carbocation rearrangements.^{21, 22} In cyclopropane fatty acids, SAM formally incorporates a methylene group across the double bond of an unsaturated acyl chain to form the cyclopropyl ring.²³ A similar role of SAM has been proposed for the biosynthesis of cyclopropane-containing sterols by marine microbes.²⁴ There has also been a report of cyclopropanation via carbene transfer using an engineered cytochrome P450 enzyme²⁵ amongst other recently discovered strategies.^{26, 27} Interestingly, there are three bacterial natural products where chlorination has been utilized as an activation strategy to form the cyclopropyl ring. These include the phytotoxin coronatine (**1**) from *Pseudomonas syringae*,²⁸ the antimicrobial agent kutzneride (**2**) from soil actinomycete *Kutzneria* sp.,²⁹ and the antimetabolic agent curacin (**3**) from the marine cyanobacterium *Moorea producens* (formerly *Lyngbya majuscula*)³⁰ (Figure 1). In all three cases, halogenation occurs on biosynthetic substrates while they are tethered to carrier proteins by α -ketoglutarate (α -KG)-dependent non-heme iron enzymes.

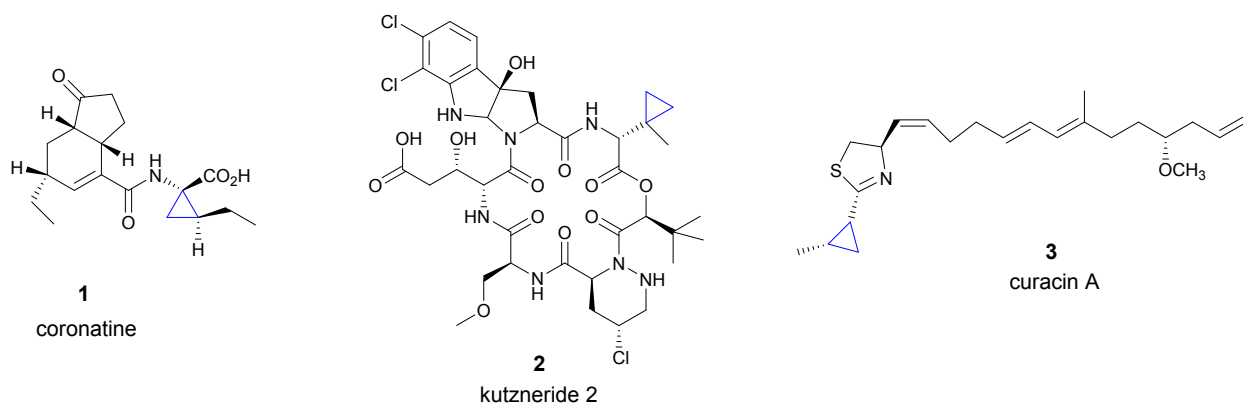


Figure 1: Structure of natural products with a cyclopropyl moiety originating from activation via chlorination.

The cyclopropane-containing amino acid coronamic acid is a distinctive constituent of coronatine (**1**) and was the first example of a biosynthetic product shown to be constructed through a cryptic halogenation mechanism.²⁸ The biohalogenation reaction is considered *cryptic* in the sense that although a halogenated pathway intermediate is indeed formed, the halogen is subsequently lost *en route* to the final pathway product. Early studies revealed that the biosynthetic gene cluster for coronamic acid contained five genes homologous to amino acid-activating enzymes.³¹ Extensive biochemical studies established the precise function of each gene-encoded protein.³² Starting with the activation of L-*allo*-isoleucine as an AMP ester by the adenylation (A) domain of the didomained CmaA protein and transfer to the attached peptidyl carrier protein (PCP) domain, the amino acid is shuttled from one PCP domain (CmaA) to another on the free-standing protein CmaD (Figure 2). The non-heme iron dependent halogenase CmaB then catalyzes the formation of γ -chloro-L-aminobutyryl-S-CmaD from L-aminobutyryl-S-CmaD. Significantly, CmaB was the first α -ketoglutarate (α -KG)-dependent non-heme iron enzyme characterized to halogenate an inactivated aliphatic group via a radical mechanism.²⁸ Halogenation sets the stage for CmaC, a Zn-dependent enzyme belonging to the vicinal oxygen chelate family of enzymes, to catalyze the intramolecular displacement of the chlorine atom to form the cyclopropyl ring.³³

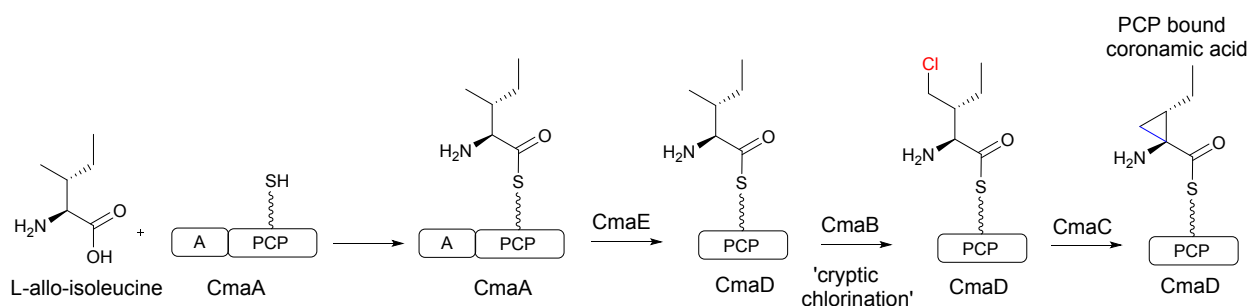


Figure 2: Reaction scheme for the biosynthesis of coronamic acid tethered to the CmaD PCP. CmaB catalyzes the halogenation reaction, while CmaC executes the intramolecular elimination for cyclopropyl ring formation.

An analogous strategy was observed in the *in vitro* characterization of enzymes involved in the biosynthesis of cyclic depsipeptide kutzneride (**2**).²⁹ By interrogating a cassette of enzymes, KtzABCD, the novel cyclopropane-containing metabolite allocornamic acid (alloCMA) was generated. In this system, α -KG-dependent non-heme iron enzyme KtzD catalyzes chlorination on L-Ile-S-KtzC to yield the γ -chlorinated pathway intermediate (Figure 3). Interestingly, KtzD does not accept tethered L-*allo*-Ile as substrate *in vitro* as observed in the biosynthesis of **1**. Subsequent nucleophilic displacement reaction is catalyzed by acyl-CoA dehydrogenase type flavoenzyme KtzA. The bound flavin cofactor in KtzA does not participate in a redox reaction; instead, the flavin is proposed to be essential to maintain the tertiary structure of KtzA. It is also possible that the bound flavin cofactor lowers the pKa of the α -proton through charge transfer interaction between enolate and flavin.²² Thus, this study adds KtzA to the small list of redox-inactive flavoenzymes.³⁴ The formation of the enolate is hypothesized to drive the intermolecular cyclization and displacement of chloride to yield the observed tethered product alloCMA. This alloCMA product represents an unforeseen potential biosynthetic intermediate to kutzneride (**2**). Relative to kutzneride (**2**), the observed alloCMA product has improper placement and stereochemistry of the cyclopropane ring. If alloCMA is a genuine biosynthetic intermediate toward kutzneride (**2**), additional enzymes would be necessary to rearrange the position of the cyclopropane ring in the final product. Furthermore, it should also be considered that alloCMA may not be a true biosynthetic intermediate of the 2-(1-methylcyclopropyl)-D-glycine (MecPGly) amino acid residue, but rather, an artifact of *in vitro* enzymatic reaction conditions. Regardless, the cryptic halogenation manipulations mediated by KtzD and KtzA represent an effective strategy to synthesize cyclopropyl containing unnatural amino acids.

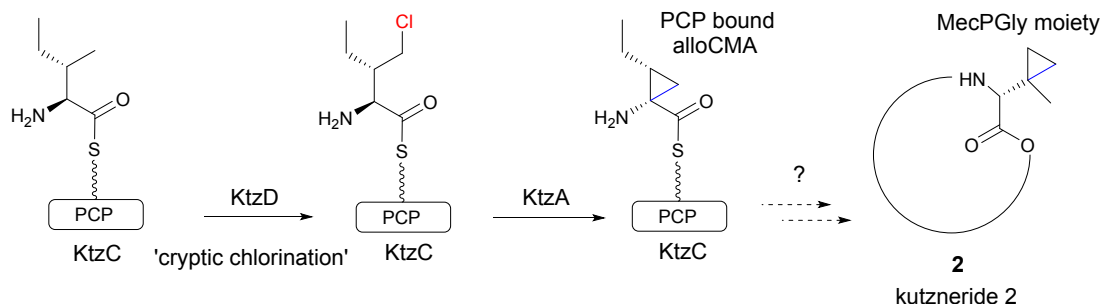


Figure 3: Reaction scheme for the construction of allocoronamic acid (alloCMA) bound to the KtzC PCP and its hypothesized conversion to MecPGly during kutzneride (**2**) biosynthesis. KtzD catalyzes the cryptic halogenation reaction.

The third halogen-assisted cyclopropanation example presented here with the antiproliferative curacin (**3**) rather involves a polyketide synthase (PKS)-derived substrate instead of isoleucine as shown in the two above examples.³⁵ Polyketide assembly line construction of (*S*)-3-hydroxy-3-methylglutaryl (HMG)-*S*-ACP from three malonyl-CoA substrate molecules initiates a four-step conversion to the cyclopropyl moiety of **3** (Figure 4). Biosynthetic investigations with the marine cyanobacterium *M. producens* revealed that the α -KG-dependent non-heme iron enzyme Cur Hal catalyzes chlorination at the aliphatic carbon of the HMG-ACP substrate. The chlorinated product is then processed by CurE ECH1 dehydratase and CurF ECH2 decarboxylase to yield the 4-chloro-3-methylcrotonyl-*S*-ACP intermediate. Subsequently, the CurF enoyl reductase (ER) catalyzes cyclopropane ring formation by a nucleophilic displacement reaction. A homologous non-heme iron enzyme with 92% sequence identity with Cur Hal is present in the jamaicamide (**4**) biosynthetic pathway.³⁶ However, the two pathways diverge from a structurally similar intermediate in which the Jam ECH2 decarboxylase enzyme rather installs jamaicamide's distinctive vinyl chloride functional group (Figure 4). Thus, the biosynthesis of curacin and

jamaicamide exemplifies the evolutionary interplay to generate molecular diversity in specialized metabolites.

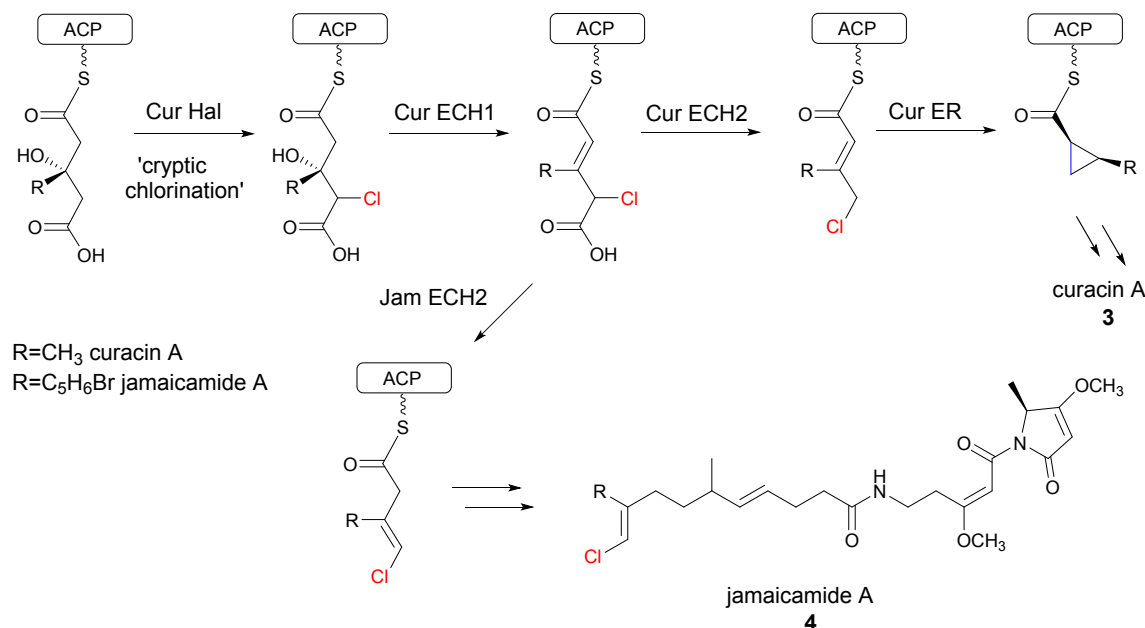


Figure 4: Formation of the cyclopropane ring during the biosynthesis of curacin. Cur Hal catalyzes the cryptic halogenation reaction. While the chlorine atom is lost in route to curacin A, it remains as a vinyl halide in the case of jamaicamide A.

From the above discussion, it is clear that nature utilizes a common strategy for cyclopropane ring formation in coronatine, kutzneride, and curacin. In each case, cryptic chlorination triggers cyclopropane ring formation by an intramolecular displacement reaction with chloride being a good leaving group for S_N2-type chemistry. Interestingly, while nature employs the same enzymatic strategy in each case to install a chlorine atom at an inactivated carbon via α -KG-dependent non-heme iron chlorinases, enzymes from three different protein superfamilies have been evolved to catalyze the similar intramolecular displacement reactions: a Zn-dependent enzyme in coronatine biosynthesis, a FAD-dependent enzyme in kutzneride biosynthesis, and a nicotinamide-dependent enzyme in curacin biosynthesis (Figure 5). Cyclopropanation in these three examples is yet another fascinating example of convergence in evolution by bacteria belonging to different phyla.

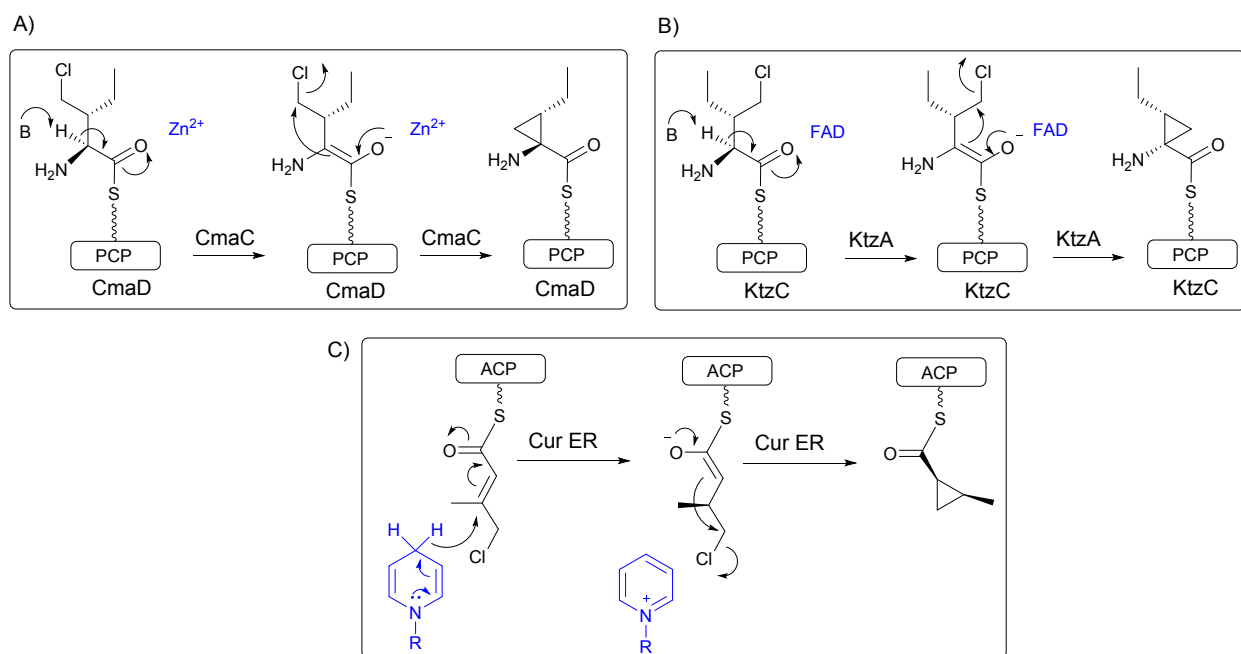


Figure 5: Intramolecular displacement reactions in coronatine (**1**), kutzneride (**2**), and curacin (**3**) biosyntheses (panels A–C, respectively) are catalyzed by enzymes from three different enzyme superfamilies.

3. Terminal alkyne formation

Terminal alkyne-containing natural products are also uncommon, and, until only recently, they too were not known to involve halogenation biochemistry for their construction.^{37–39} The majority of natural terminal alkyne-containing compounds are derived from marine organisms, including jamaicamide B⁴⁰, carmabin A⁴¹, guineamide G⁴², georgamide⁴³, vatiamides⁴⁴, dragomabin⁴⁵, and prymnesin.⁴⁶ Fatty acid desaturases are generally responsible for the formation of alkynes such as in polyacetylenes^{47, 48} and enediynes^{49, 50} as well as in the terminal alkyne functional group in jamaicamide B and carmabin A.^{40, 51} This mechanism was considered to be a widespread biosynthetic strategy until the terminal alkyne-containing amino acids L-propargylglycine (**5**) and L-β-ethynylserine (**6**) were shown to be derived from desaturase-independent pathway involving a halogenated intermediate.⁵²

L-Propargylglycine (**5**) and L- β -ethynylserine (**6**) are unusual nonproteinogenic amino acids with a terminal alkyne functional group produced by the soil bacterium *Streptomyces cattleya*.⁵³ Because the terminal alkyne functional group has broad application in azide-alkyne click chemistry,⁵⁴ the biosynthesis of **5** and **6** was evaluated as the first step toward incorporating these natural, alkynyl amino acid as chemical biology probes into proteins. When *S. cattleya* mutants maintained their ability to produce β -ethynylserine with genetically inactivated, nonessential fatty acid desaturases, a comparative genomic approach led to the discovery and characterization of a six-gene cluster associated with a cryptic chlorination mediated terminal alkyne formation.⁵² In this biosynthetic pathway, an α -KG-dependent non-heme iron halogenase BesD catalyzes the cryptic halogenation reaction on lysine yielding 4-Cl-lysine (Figure 6). Unlike the halogenases involved in coronatine and kutzneride biosynthesis that require their amino acid substrates bound to a carrier protein, BesD was the first α -KG-dependent non-heme iron halogenase observed to act on a free amino acid substrate. Although BesD contains the characteristic HXG/A motif of halogenases, it shows low sequence identity to other characterized halogenases such as the carrier protein-dependent SyrB2⁵⁵ (7% identity) and -independent WelO5⁵⁶ (11% identity). Rather it shows greater sequence identity to α -KG-dependent non-heme iron hydroxylases.

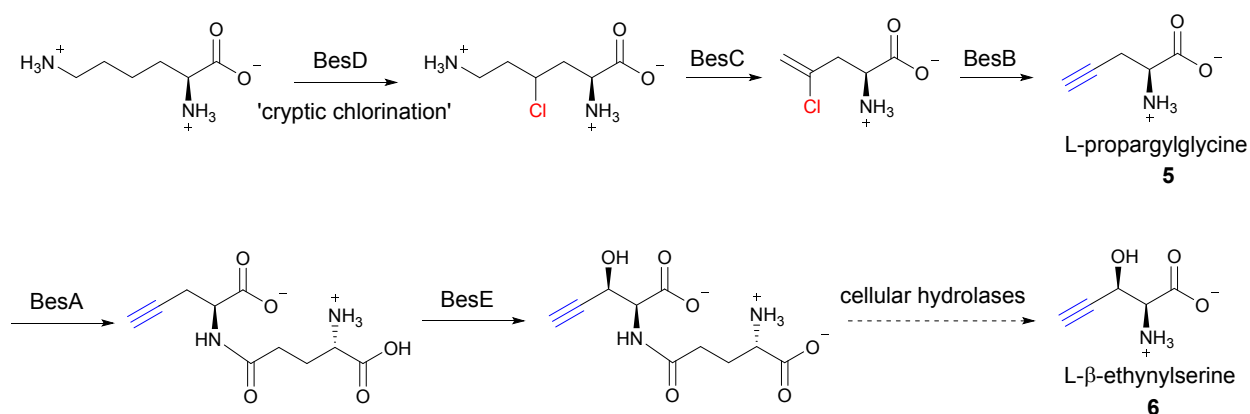


Figure 6: Biosynthesis of L-propargylglycine and L- β -ethynylserine in *Streptomyces cattleya*. BesD catalyzes the cryptic chlorination step.

The second step in the biosynthetic pathway involves the non-heme dinuclear Fe enzyme BesC that catalyzes an unusual C–C bond cleaving reaction to give 4-Cl-allyl-glycine (Figure 6). The construction of the vinyl chloride group in this case is very much unlike that for the jamaicamides introduced earlier in Figure 4. The next step in the pathway catalyzed by the PLP-dependent enzyme BesB results in the loss of the chloride via a presumed allene intermediate (Figure 7) to produce the terminal alkyne moiety in L-propargylglycine (**5**). When the BesB catalyzed reaction was performed in the presence of D₂O, two deuterium atoms get incorporated in the final product, suggesting at least two deprotonation–protonation events occur during the course of BesB catalysis. Both BesC- and BesD-catalyzed reactions have little precedence in nature. Surprisingly, amino acid ligase BesA attaches glutamyl moiety on L-propargylglycine to give a dipeptide which in turn is the substrate for the α -KG-dependent non-heme iron hydroxylase BesE. It has been proposed that cellular hydrolases are responsible for the last hydrolysis step releasing free L- β -ethynylserine (**6**).⁵²

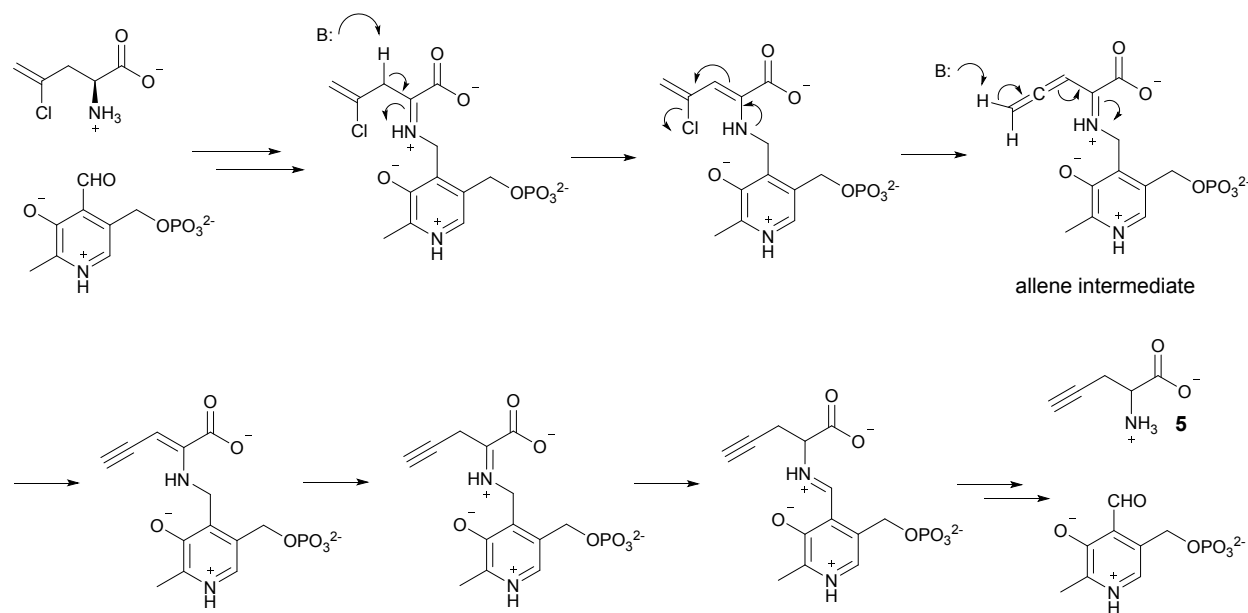


Figure 7: Proposed mechanism for BesB catalyzed formation of the alkyne functional group in L-propargylglycine (**5**) involves the displacement of the chloride from the vinyl chloride substrate.

4. C-/O-alkylation reactions

The next halogenation-assisted pathway examples also involve a radical C–H activation mechanism, yet via a different class of radical halogenase enzymes, to support C- and O-alkylation reactions. Enzyme-catalyzed alkylation reactions are well documented in the literature,⁵⁷ often involving common metabolic enzymes such as SAM-dependent methyltransferases^{58, 59} and prenyltransferases.⁶⁰ In methyltransferases, the electrophilic methyl group bound to the sulfonium center of SAM gets transferred to the nucleophilic substrates via an S_N2 reaction. Alkylation reactions catalyzed by prenyltransferases proceed via an S_N1 mechanism where an allyl carbocation generated through the loss of diphosphate reacts with nucleophilic substrates. In both cases, their alkylating substrates are electron-donating and are matched with electrophilic substrates such as SAM or isoprene diphosphates. Recently, two examples of chlorination-assisted alkylation reactions were reported in cyanobacteria for the biosynthesis of cyclophanes and fatty acid esters that require C–H activation for alkylation.

Cylindrocyclophanes are cytotoxic natural products⁶¹ that contain an unusual, all-carbon [7.7]paracyclophane framework.⁶² While isotope labeling experiments established the polyketide origin of the resorcinol monomer in the cylindrocyclophanes,⁶³ the mechanism for the activation and dimerization of the two monomers remained an enigma until its biosynthetic gene cluster was identified.⁶⁴ Its biosynthesis starts with the tethering of decanoic acid via thioester linkage to a pathway-specific carrier protein (Figure 8). Then CylC catalyzes a regio- and stereospecific chlorination of the unactivated methylene group at C6 of the 10-carbon acyl chain to yield the 6*R*-chloro-decanoyl-*S*-ACP. Next, it is elongated by a type I PKS assembly line, followed by off-loading by a type III PKS to produce an alkyl resorcinol intermediate. Finally, the saturated chloroalkyl chain undergoes stepwise Friedel-Crafts alkylation reactions catalyzed by CylK to construct the unique paracyclophane framework.⁶⁵ Thus, the CylC-catalyzed chlorination reaction is cryptic as it sets the stage for the CylK-catalyzed Friedel-Crafts reaction towards the end of the biosynthetic pathway that results in new C–C bonds at the expense of the alkylhalide. The predicted secondary structure of CylC shows similarity to diiron-carboxylate enzyme family, and its activity has been shown to be metal dependent. However, the active site architecture of

the metal cofactor is yet to be established for this first in-class halogenase. Interestingly, the CylK-catalyzed reaction involves inversion of stereochemistry, suggesting an S_N2 mechanism contrary to the classic Friedel-Crafts reaction that proceeds via a carbocation intermediate. CylK is annotated as hemolysin-type calcium-binding protein, and its activity is calcium dependent; however, the exact role of calcium is currently unknown.

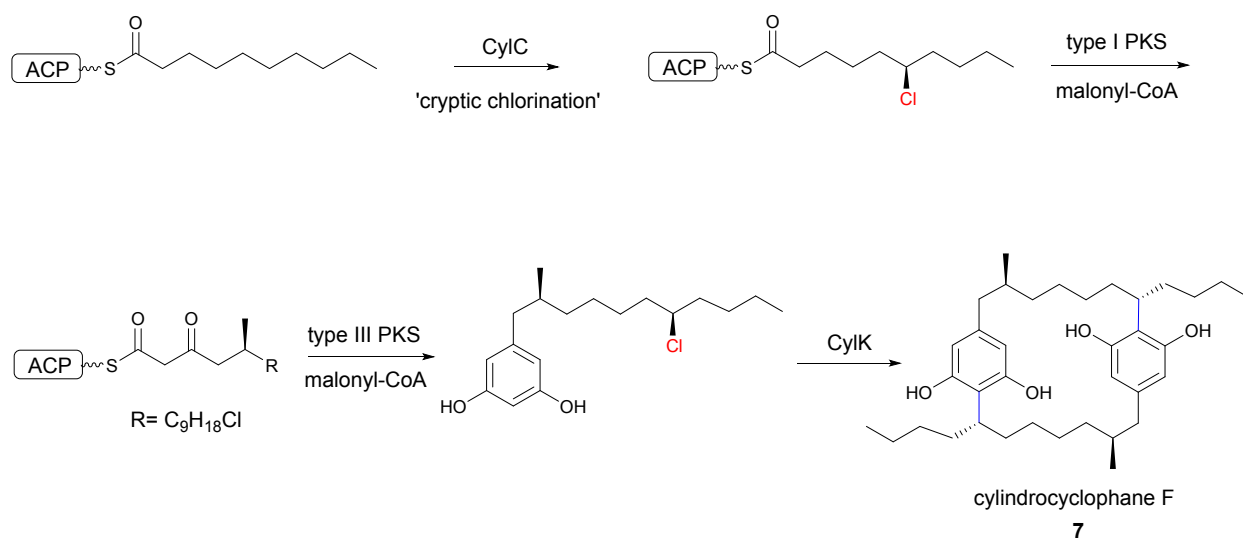


Figure 8: Biosynthetic scheme for cylindrocyclophane F (**7**). The diiron-carboxylate enzyme CylC catalyzes the cryptic chlorination reaction.

The [7.7]paracyclophane skeleton is present in many other cyanobacterial compounds,⁶⁶ including the carbamidocyclophanes,⁶⁷ merocyclophanes,⁶⁸ and nostocyclophanes.⁶⁹ Recently, the linear, dimeric and chlorinated product carbamidocylindrofridin A was isolated from the carbamidocyclophane-producing *Cylindrospermum stagnale*,⁷⁰ providing *in vivo* support to the *in vitro* enzymatic reaction sequence involving the CylC/CylK cryptic chlorination/Friedel-Crafts alkylation reaction (Figure 9).

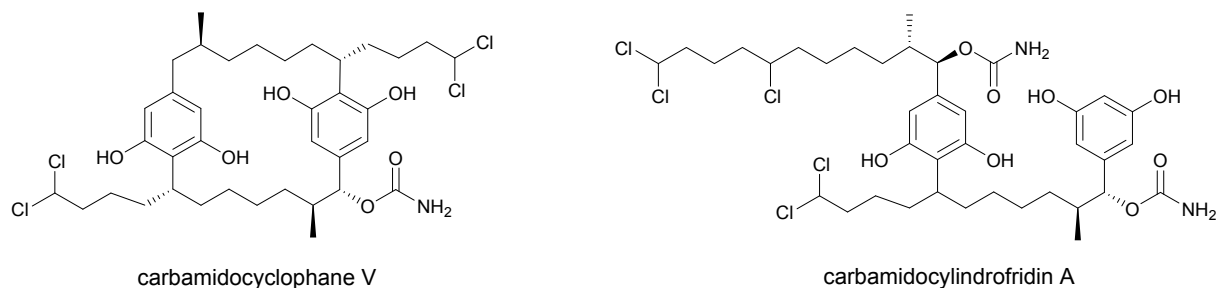


Figure 9: The chemical structures of carbamidocyclophane V and carbamidocylindrofridin A.

CylC and CylK homolog pairs have been identified bioinformatically in other cyanobacteria, suggesting that halogenation-assisted alkylation reactions may be more widespread in nature. One pair of homologs led to the discovery of the bartoloside aromatic glycolipids from the cyanobacterium *Synechocystis salina* LEGE 06099.⁷¹ Biochemical evidence showed that BrtB (CylK homolog) catalyzes ester bond formation using fatty acids with varying chain lengths (C2 to C16) as nucleophiles to displace the alkyl halide⁷² (Figure 10). Thus, unlike the C-alkylating enzyme CylK in cylindrocyclophane biosynthesis, BrtB is an O-alkylating enzyme. Although the activity of BrtJ (CylC homolog) is yet to be established experimentally, it likely catalyzes the cryptic dichlorination on the alkyl chain of bartoloside A. Despite showing amino acid sequence homology, CylK and BrtB (31% identity) catalyze completely different reactions: CylK catalyzes a Friedel-Crafts reaction, whereas BrtB catalyzes ester bond formation using a fatty acid carboxylate as a nucleophile and alkyl halide as electrophile. Since CylK homologs are widespread in cyanobacteria,^{65, 66} the diversity of enzymatic reactions catalyzed by this class of enzymes remains to be fully appreciated.

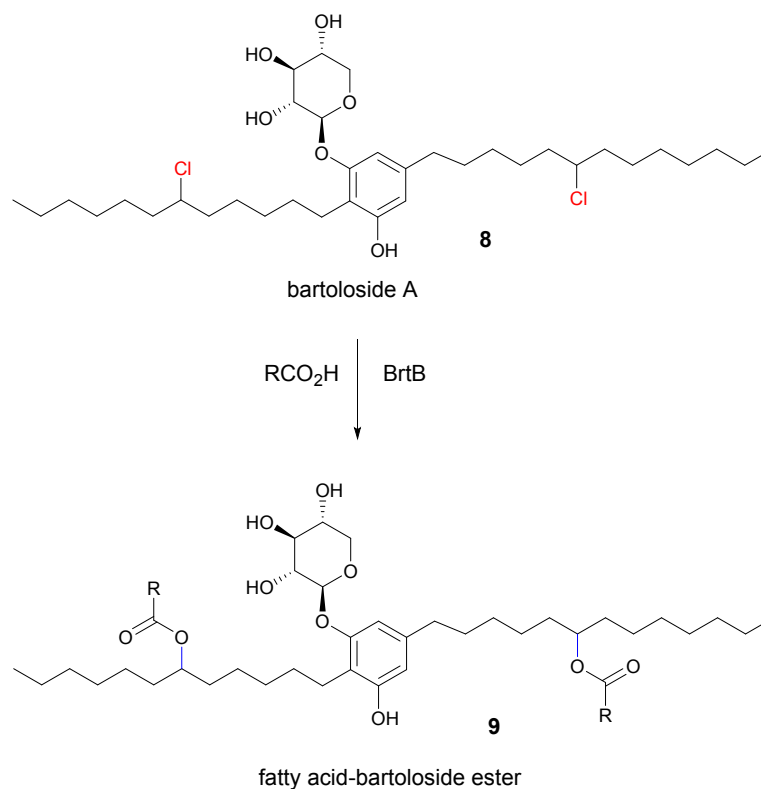


Figure 10: BrtB-catalyzed ester bond formation during the biosynthesis of fatty acid-bartoloside esters.

5. Biaryl coupling

In contrast to the myriad of synthetic methods available for the construction of axially chiral biaryl compounds, there are relatively few reports of similar enzyme-catalyzed reactions.⁷³ The most common class of enzymes to carry out phenol coupling reactions belong to the cytochrome P450 superfamily as exemplified in the biosynthesis of the glycopeptide antibiotic vancomycin.^{74, 75} Apart from vancomycin, cytochrome P450-dependent enzymes are involved in biaryl coupling reactions during the biosynthesis of alkaloids,⁷⁶ phenols,^{77, 78} lipopeptides,⁷⁹ and plant-derived aromatics.⁸⁰ Flavoenzyme-mediated N–N and N–C aryl coupling has been reported in the biosynthesis of indoloterpenes natural products,⁸¹ however recent *in vitro* experiments⁸² contradict the previous results and reopen the mechanism of the biaryl coupling in those bacterial metabolites. Although the majority of biaryl coupling reactions reported in the literature have been proposed to proceed via radical chemistry, there are two recent reports of

halogenation-mediated biaryl coupling reactions during the biosynthesis of marinopyrrole (**10**) and pentabromopseudilin (**11**) (Figure 11). In both cases, flavin-dependent halogenases catalyze the cryptic halogenation reactions.

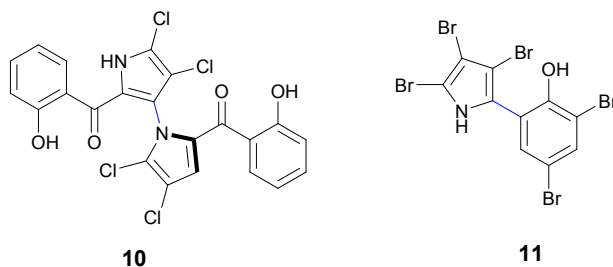


Figure 11: Structure of marinopyrrole A (**10**) and pentabromopseudilin (**11**). Both the natural products are biosynthesized via cryptic halogenation-mediated biaryl coupling.

Marinopyrrole A (**10**) is a halogenated bipyrrole natural product isolated from marine bacterium *Streptomyces* sp. CNQ-418.^{83, 84} It is one of the few naturally occurring atropoisomers⁸⁵ (exist in M configuration) and shows antibiotic activity against methicillin-resistant *Staphylococcus aureus*.⁸⁶ Detection of monomeric monodeoxyoluteorin (**12**) in the culture broth of CNQ-418 suggested the intermediacy of this compound in the production of the marinopyrrole.⁸³ Indeed, detailed genetic studies using heterologous host *Streptomyces coelicolor* M512 revealed that the atroposelective product is formed via an unprecedented bipyrrole homocoupling reaction.⁸⁷ Two flavin dependent halogenases (Mpy10 and Mpy11) in the presence of a flavin reductase (Mpy1) catalyze this unique *N,C*-biaryl homocoupling reaction. Figure 12 depicts the two possible mechanisms for this transformation that has yet to be confirmed biochemically. In one of the proposed mechanisms (Figure 12, route A), flavin dependent halogenases catalyze C-3 halogenation of compound **12**, thereby setting the stage for nucleophilic addition by nitrogen atom of a second molecule of **12**. Loss of the halide completes the reaction. In the alternate proposal (Figure 12, route B), halogenation takes place at nitrogen atom and subsequent nucleophilic displacement of halide by a second molecule of **12** renders the final product **10**. One of the synthetic methods to make marinopyrrole involves an intermolecular Ullman coupling reaction,⁸⁸ and it is fascinating that nature appears to capitalize a similar strategy using cryptic halogenation. At this point, it is not clear why nature utilizes two halogenases instead of one

enzyme: one possibility is that two halogenases may operate as a functional complex. *In vitro* reconstitution of this reaction is yet to be achieved and will lay the foundation for further mechanistic investigation on this unusual dimerization reaction.

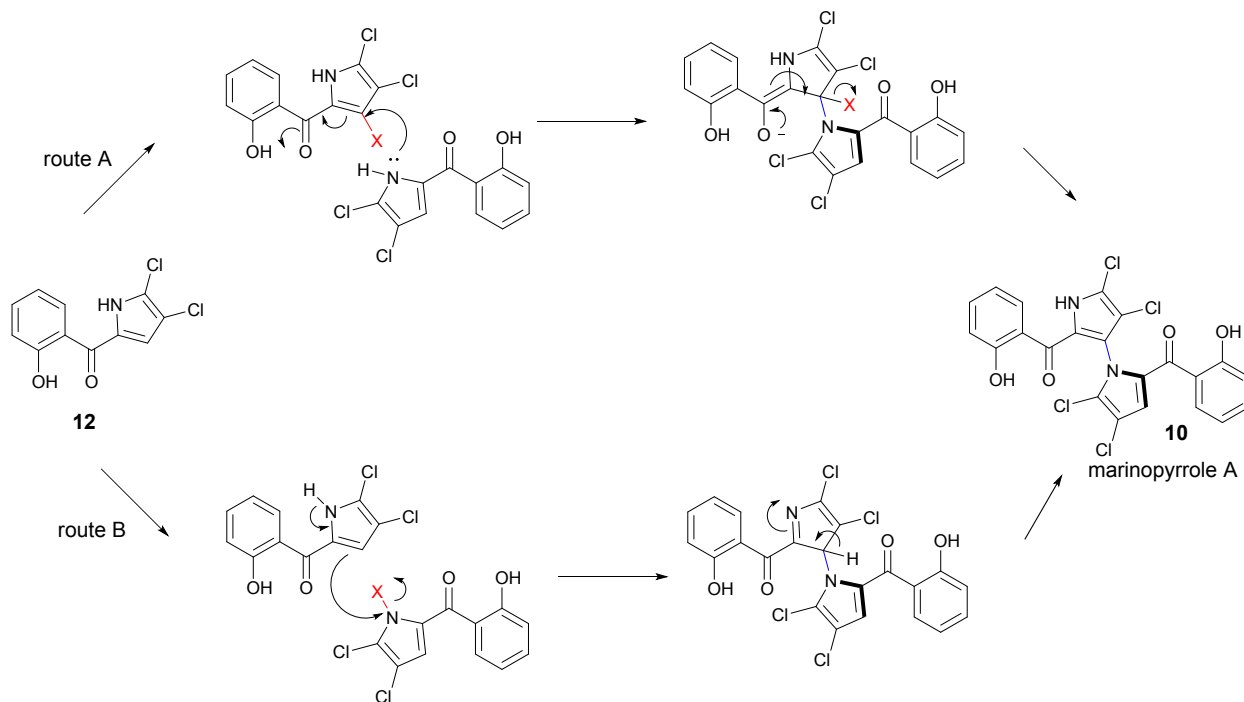


Figure 12: Proposed mechanisms for the atroposelective *N,C*-biaryl homocoupling reaction during biosynthesis of marinopyrrole A.

The biosynthesis of pentabromopseudilin (**11**) features the second example of cryptic halogenation-mediated biaryl coupling, albeit with a mechanistic twist on the halogenation-assisted biosynthetic theme. Pentabromopseudilin is a polybrominated antibacterial agent produced by marine *Pseudoalteromonas* gammaproteobacteria.^{78, 89} Its biosynthesis follows a convergent route in which cytochrome P450 (Bmp7) coupling of 2,3,4-tribromopyrrole (**14**) to 2,4-dibromophenol (**15**) leads to the final product (Figure 13).⁹⁰ During the biosynthesis of 2,3,4-tribromopyrrole (**14**), a single flavin-dependent halogenase, Bmp2, catalyzes the incorporation of not three, but four, bromine atoms on the ACP-bound pyrrole substrate (Figure 14). The incorporation of four halogens by a single enzyme is unprecedented in nature. The fourth bromination by Bmp2 has two implications: it triggers the release of the sequestered pyrrole

moiety from ACP by presumably making the thioester susceptible to hydrolysis; secondly, it activates the substrate for the elimination of the L-proline-derived carboxyl group. Interestingly, tetrabromopyrrole undergoes debromination before the coupling reaction with 2,4-dibromophenol (**15**).^{91, 92} This observation is in stark contrast with all other examples of cryptic halogenation reactions discussed in the review, where a halogen is rather used to activate a carbon for subsequent chemistry.

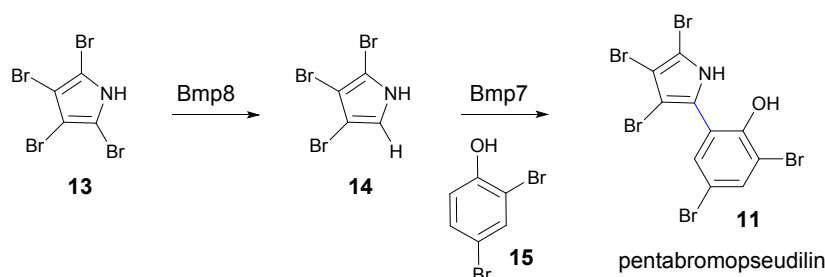


Figure 13: The convergent biosynthesis of pentabromopseudilin requires the Bmp8-catalyzed debromination step before Bmp7-assisted biaryl coupling. The detailed debromination mechanism is shown in Figure 14.

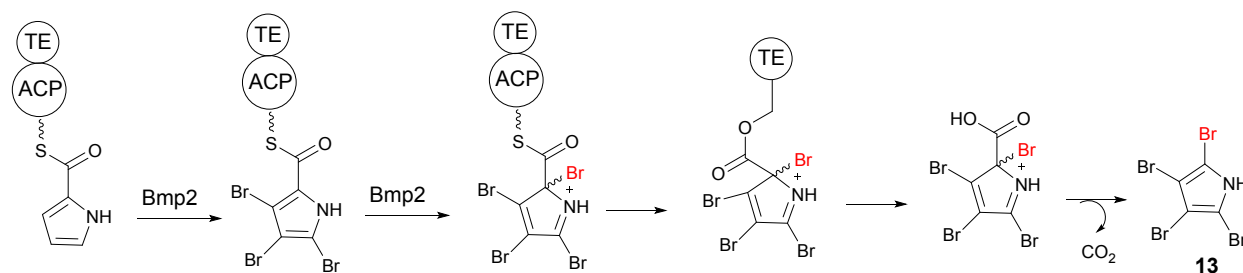


Figure 14: Bmp2 catalyzes the tetrabromination on ACP-bound pyrrole whereupon the fourth bromine atom added is ultimately removed in the formation of pentabromopseudilin.

The debromination reaction is catalyzed by Bmp8 (Figure 13) utilizing a pair of catalytic cysteine residues. Based on structural, mutagenesis, and computational modeling experiments, a mechanism has been proposed⁹³ (Figure 15). According to this proposal, active site His₈₈ deprotonates the tetrabromopyrrole (**13**) to give tetrahedral intermediate **16** followed by

nucleophilic addition of the Cys₈₅ thiolate on the bromine atom. Next, rearomatization forms the tribromo pyrrole product **14** and Cys₈₂ involved in disulfide bond formation to release bromide. Finally, the disulfide bond can be reduced by a small molecule reducing agent or disulfide reductase to complete the catalytic cycle.

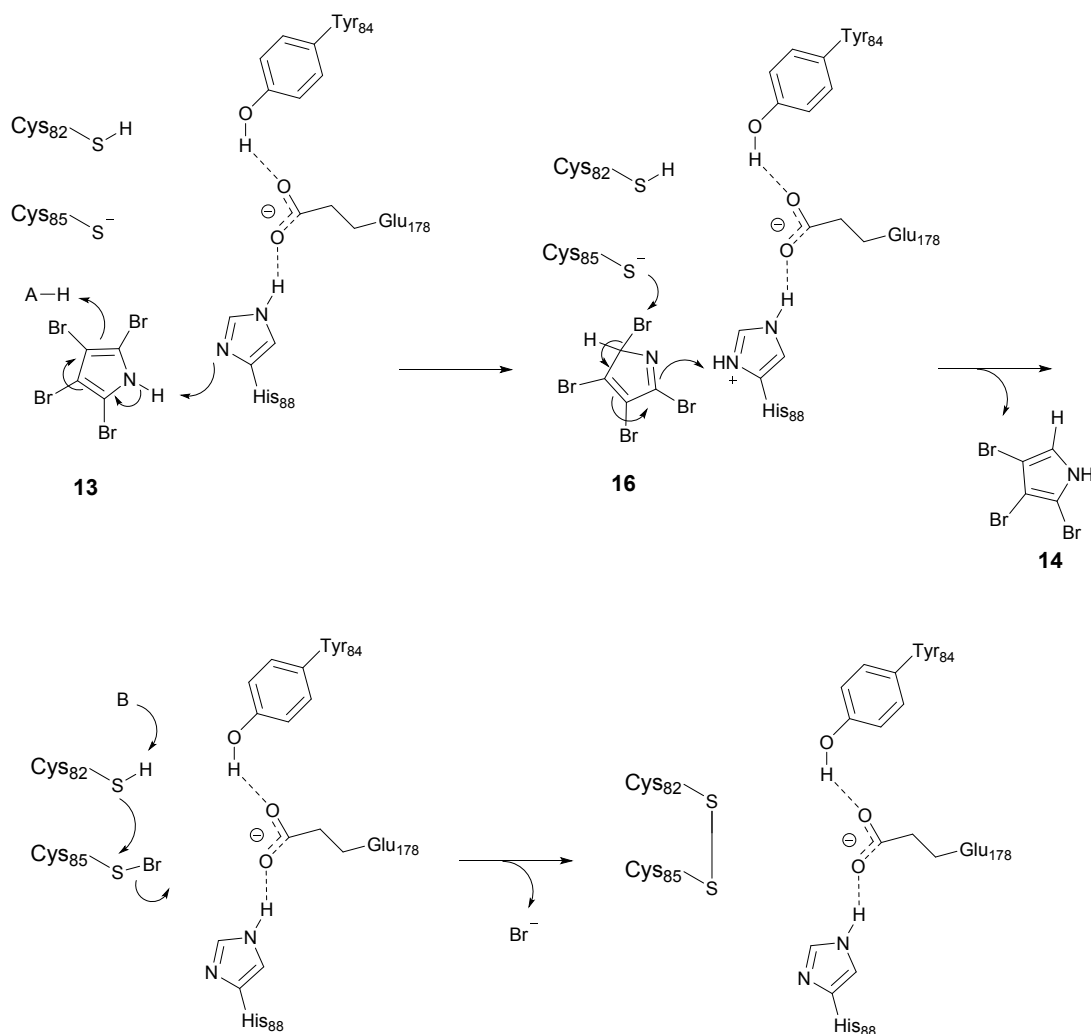


Figure 15: Mechanistic proposal for the Bmp8-catalyzed debromination reaction.

6. C-C rearrangements

Up to this point, all examples of halogenation-assisted biosynthesis involved enzymes pairs: one to install the halogen and the second to remove it in the course of a biosynthetic transformation.

Here, those two actions are catalyzed by a single halogenating enzyme, which creates a reactive intermediate that initiates a reaction cascade to support C–C rearrangement reactions. Enzyme-catalyzed C–C rearrangements are essential for generating complexity in natural product molecules.⁹⁴ Although nature has evolved several strategies for C–C rearrangements, there are two instances where cryptic chlorination is central in biosynthetic complexity generation. These cases involve streptomycete meroterpenoids belonging to the merochlorin structural family⁹⁵ and marinone.⁹⁶

Merochlorins are structurally unique chlorinated meroterpenoid secondary metabolites from marine *Streptomyces* sp. CNH-189 (Figure 16).^{97,98} Merochlorin A (**17**) contains an unusual bicyclo [3.2.1]octane core with four stereocenters, while merochlorin B (**18**) and C (**19**) contain distinctive carbon skeletons not present in known natural products. Both merochlorin A and B are highly active against methicillin-resistant *Staphylococcus aureus* strains with MICs in the range 2-4 $\mu\text{g}/\text{mL}$. Early studies based on gene knockout, genome mining, and expression in a heterologous host identified the biosynthetic gene cluster.⁹⁷ Subsequent detailed biochemical studies revealed that four enzymes (Mcl17, Mcl22-24) are sufficient to yield merochlorin A and B from biosynthetic precursors malonyl-CoA, dimethylallyl pyrophosphate (DMAPP), and geranyl pyrophosphate (GPP).⁹⁹ Prenyl diphosphate synthase Mcl22 catalyzes the coupling of DMAPP and GPP to form a unique sesquiterpene isosesquilavandulyl pyrophosphate **21** (Figure 17).¹⁰⁰ Mcl23 then attaches the isosesquilavandulyl moiety to the aromatic tetrahydroxynaphthalene **22** (THN) scaffold, which is generated through the activity of the type III polyketide synthase Mcl17.

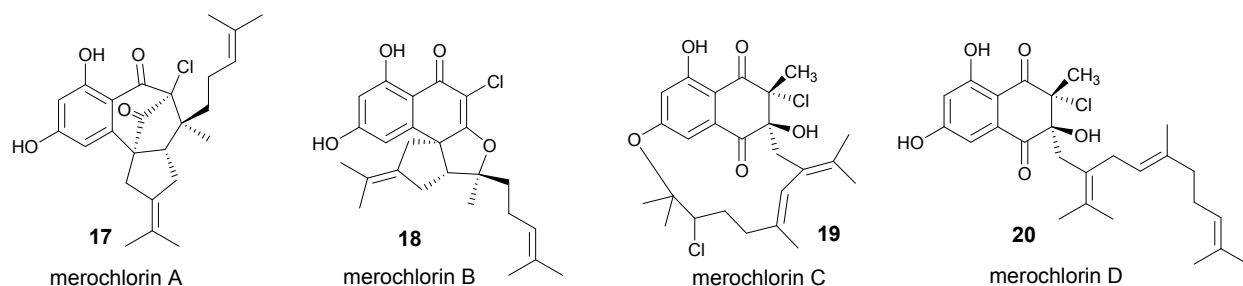


Figure 16: Structures of the merochlorin natural products.

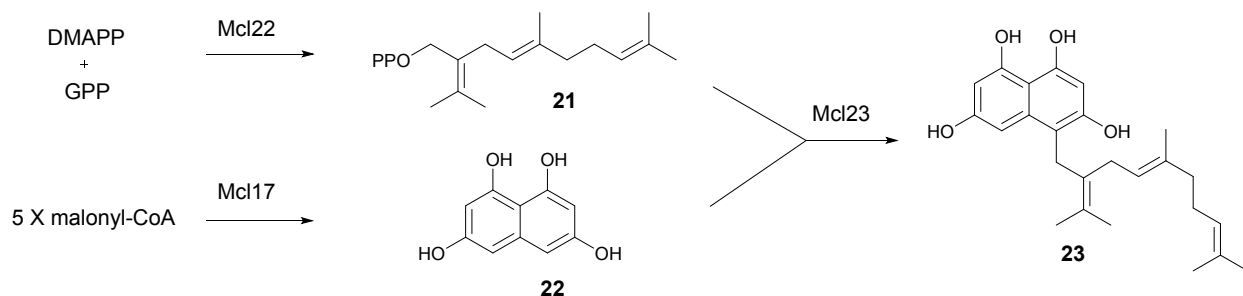


Figure 17: Biosynthetic scheme for the formation of premerochlorin **23**.

The key reaction in the biosynthetic pathway is catalyzed by the vanadium-dependent chloroperoxidase (VCPO) Mcl24, which performs up to three halogenation reactions, including a cryptic halogenation-mediated α -hydroxyketone rearrangement, to account for the structural complexity in the merochlorin molecules.⁹⁵ Figure 18 depicts the current mechanistic understanding of the Mcl24-catalyzed reaction. C-2 monochlorination of premerochlorin (**23**) to compound **24** is followed by an additional *O*-chlorination reaction yielding an aromatic hypochlorite intermediate **25**. The loss of the reactive, oxygen-linked chloride forms the benzylic carbocation intermediate **26** that sets up differential carbocation-induced terpene cyclizations to give either merochlorin A (**17**) or B (**18**). Thus, the Mcl24-catalyzed second chlorination is cryptic as it triggers the formation of a benzylic carbocation and subsequent rearrangement reactions. Unlike the halogenases involved in all of the above examples, Mcl24 singlehandedly catalyzes both halogenation and subsequent rearrangement chemistry. Interestingly, under mild basic conditions such as at pH 8.0, the benzylic carbocation intermediate **26** is preferentially hydrated at C-4 to give tertiary alcohol **28**. Compound **28** then undergoes a third Mcl24-catalyzed chlorination followed by an unusual α -hydroxyketone rearrangement to give **30**.¹⁰¹ The chemical logic established within the pH dependence of Mcl24 catalyzed reaction provides insight into the biosynthesis of merochlorin C (**19**) and D (**20**). It is likely that the Mcl24 catalyzed reaction product **28** would be stereoselectively methylated at C-2 by a SAM-dependent methyltransferase to give compound **31**. Di-substitution at C-2 would then enable Mcl24 to carry out an analogous α -hydroxyketone rearrangement to generate merochlorin D (**20**). A second VCPO, Mcl40, is suspected to be involved in macrocyclic chloroether ring formation in the biosynthesis of merochlorin C (**19**) based on heterologous expression studies.⁹⁷

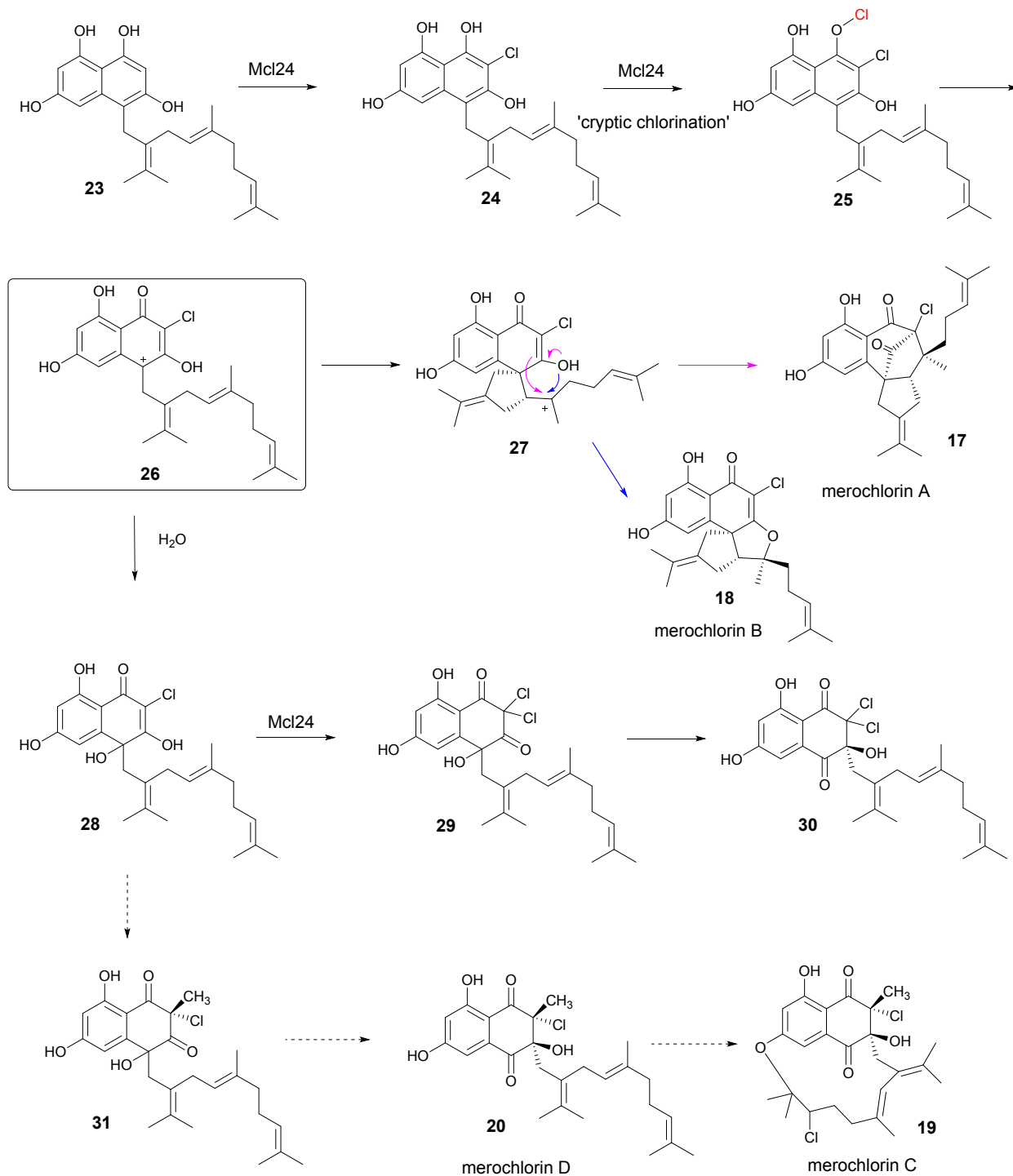


Figure 18: Mcl24-catalyzed cryptic chlorination-mediated C–C rearrangements during merochlorin biosynthesis. The boxed carbocation intermediate **26** is the branching point in the biosynthesis of different merochlorin natural products.

The second example of cryptic halogenation-mediated α -hydroxyketone rearrangement is reported in the biosynthesis of another class of naphthoquinone-based meroterpenoid antibiotics named marinone (Figure 19). Debromomarinone (**32**) and marinone (**33**) were first isolated from marine actinomycete *Streptomyces* sp. CNB-632, and both compounds showed *in vitro* antibacterial activity against Gram-positive bacteria.¹⁰² Isomarinone (**34**) was isolated from marine actinomycete CNH-099 and showed *in vitro* cytotoxicity against the HCT-116 colon carcinoma cell line.¹⁰³ Early feeding experiments with stable isotopes provided evidence regarding the origin of naphthoquinone and isoprene units.¹⁰⁴ Similar to the merochlorins, marinones contain terpene substituents at the C-3 position, despite THN being nucleophilic only at C-2 and C-4. Although there is no chlorine atom in these natural products, it was hypothesized that cryptic chlorination would play a key role in selectively oxidizing the THN ring and facilitating the predicted α -hydroxyketone rearrangement. Indeed, two VCPOs have been characterized *in vitro* in the putative marinone biosynthetic pathway.⁹⁶ MarH1 catalyzes oxidative dearomatization and subsequent C-2 chlorination on compound **35**, while MarH3 catalyzes additional chlorination at C-2 and an α -hydroxyketone rearrangement to yield geminal dihalide **38** (Figure 20). Postulated later steps in the biosynthetic pathway are as follows: (1) formation of chloroepoxide **39**, (2) reductive dehalogenation to give hydroxynaphthoquinone **40**, (3) oxidation and alkene isomerization to yield **41**, (4) intramolecular hetero-Diels–Alder reaction to construct the tetracyclic scaffold of debromomarinone **32**, and (5) late-stage vanadium-dependent bromoperoxidase (VBPO)-catalyzed bromination at C-5 or C-7 to deliver marinone (**33**) or isomarinone (**34**), respectively. While this biosynthetic pathway has yet to be confirmed, the chemical synthesis of marinone was achieved via a biomimetic route based on this biosynthetic hypothesis, thereby providing the chemical underpinnings of this strategy.⁹⁶

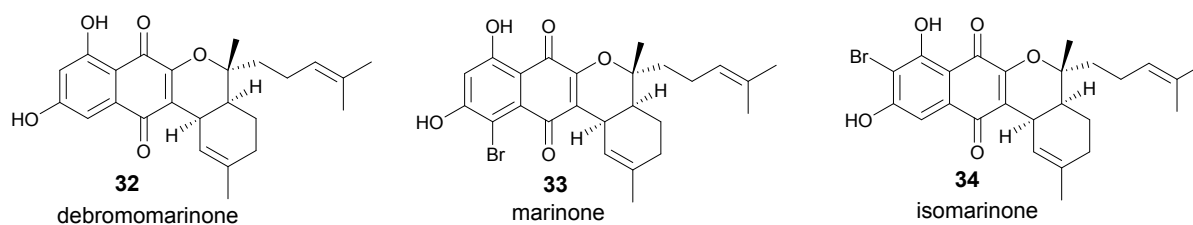


Figure 19: Structures of marinone natural products.

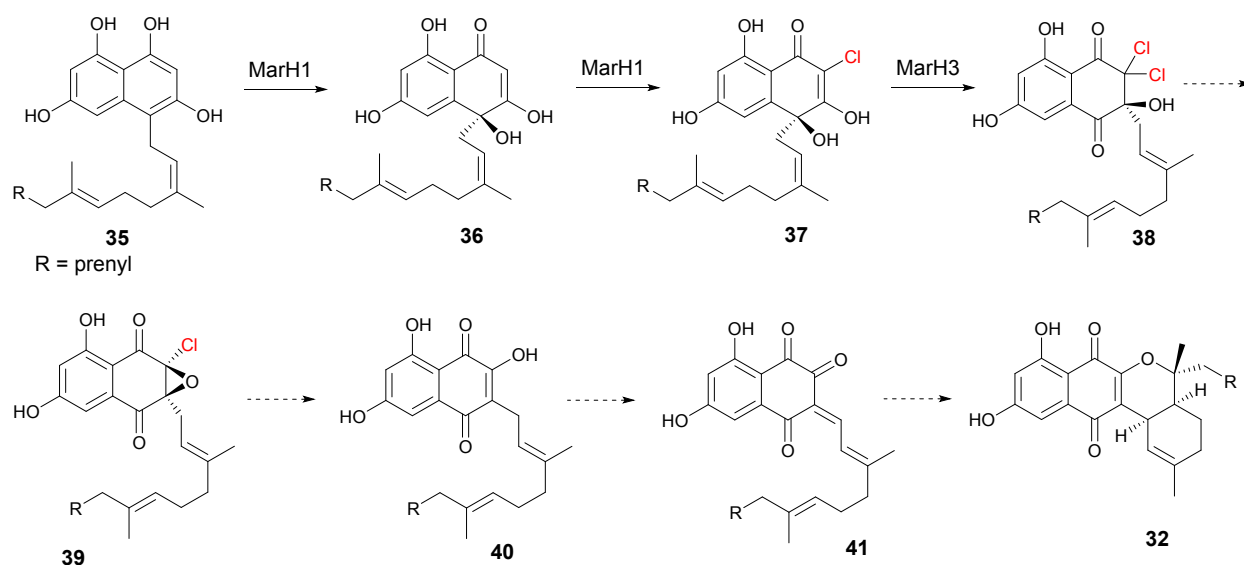


Figure 20: Proposed marinone biosynthetic pathway. MarH1 and MarH3 are involved in the cryptic halogenation reactions. A biomimetic synthesis of marinone has been achieved that follows this chemical logic.⁹⁶

7. Conclusion

The first natural product halogenating enzyme was reported back in mid 1960s.¹⁰⁵ Since then numerous halogenases have been discovered and characterized to install halogen substituents that are often crucial for the bioactivity and function of the halogenated molecule. However, it is only recently appreciated that enzymatic halogenation is a metabolic strategy to install non-halogenated, structural scaffolds in natural product molecules. While the number of established cryptic halogenation examples is relatively small, it is possible that cryptic halogenation may be underappreciated in natural product biosynthesis due to the difficulty in recognizing when such reactions may have occurred based on the chemical structure of the natural product alone. This is particularly challenging when nature has evolved one or more strategies for functional group installation, such as in cyclopropanation and terminal-alkyne formation, whereupon cryptic halogenation pathways are just one of many possible mechanisms.

The cryptic halogenation-catalyzing enzymes discussed in this review are not restricted to a particular enzyme superfamily; instead, they are distributed amongst most of the known

halogenase superfamilies, including α -KG-dependent non-heme iron halogenases, vanadium-dependent haloperoxidases, flavin dependent halogenases, and diiron-carboxylate halogenases. Thus, it is clear that nature utilizes its enzyme toolkit based on matching reactivities between substrates and halogenating species. Although bromide and iodide are better leaving groups than chloride, nature still chose chlorine in most cryptic halogenation reactions. This could well be due to the higher natural abundance of chloride compared to the other halogens.

Except for CylK,¹⁰⁶ the biocatalytic potential of the remaining enzymes discussed here is untapped and thus presents a future research area. Although as of yet there is no general algorithm to predict cryptic halogenation in a biosynthetic pathway, the presence of a putative halogenase in the biosynthetic gene cluster of a non-halogenated natural product likely provides an indication of cryptic halogenation. Given more and more halogenases will be functionally characterized in coming years we are expecting to discover many more examples of cryptic halogenation reactions in natural product biosynthesis.

8. Conflicts of interest

There are no conflicts to declare.

9. Acknowledgements

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