

Chlorinated Natural Products and Related Halogenases

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Abstract: Halogenated compounds are well-known for their diverse bioactivities and wide applications in pharmaceutical industry, chemical engineering and other fields. Novel halometabolites isolated from nature inspire scientists to synthesize these “chance compounds”, which were, however, usually hampered by the extreme catalytic conditions and severe selectivity challenges. In the past several decades, enzymatic reactions have been brought into attention for their efficiency, selectivity, and mild reaction conditions. Synthetic biology which is in compliance with the laws of nature to a large extent shed light on the rational biosynthesis of halogenated compounds. This article summarizes several representative categories of halogenated natural products and halogenases to seek clues to the relationship

between the structures and halogenase mechanisms. Some types of halogenase convert the basic building blocks to diverse early intermediates during natural product biosynthetic processes, while others are involved in the late tailoring steps to afford the final structures of halogenated molecules. The broad substrate specificity and strong regioselectivity exhibited in some halogenases are irreplaceable advantages for biosynthesis. In addition, tremendous efforts and encouraging results enable us to engineer the enzymes or techniques to synthesize “unnatural” halogenated natural products. More reaction mechanisms and structural data are expected to be revealed exponentially in this rapid growing realm.

Keywords: Bioactivity · biosynthesis · biotransformation · catalytic reactions · halogenated compounds

Halogenated compounds play a crucial role in modern pharmaceutical industry and other realms such as pesticides, dyes, detergents, etc., with significant contribution of halogen atoms to the bioactivities or reactivity. Approximately 25% of drugs on the market contributing revenue are by halogenated compounds,^[1] including some well-known halogenated medicines such as chloramphenicol, vancomycin, and rebeccamycin. Half of the molecules in high-throughput screening contain halogen atoms. During the last 50 years of rapid development, the number of natural halogenated compounds has grown from 30 in 1968 to over 5000 in 2015.^[2] The number is expected to increase exponentially with the help of genome mining and synthetic biology.

One reason for the late initiation of natural halometabolites discovery is that halogenated compounds were usually found in the extreme environment and considered as the “chance products of nature”. With the discovery of more and more halogenated compounds from living organisms, scientists started to realize the roles those halometabolites played in living systems. Antibiotics with remarkable bioactivities were identified. During the process of synthesizing these antibiotics by organic total synthesis, chemists found that halogenated compounds can serve as outstanding intermediates in various types of reaction. Prior to the 1990s, most of the halogenated compounds were chemically synthesized, which often leads to severe pollution, undesired by-products, and low efficiency and poor selectivity. Therefore, new approaches of synthesizing halogenated compounds are desired.

Nature is infinitely better in chemistry. Halogenated compounds in living organisms are synthesized by corresponding enzymes. Since the first halogenase was identified in

Caldariomyces fumago, more halogenases have been discovered and classified into four major types: haloperoxidases (HPO), nonheme Fe(II) α -ketoglutarate-dependent halogenases, S-adenosyl-L-methionine (SAM)-dependent halogenases and flavin-dependent halogenases (FDHs). Though halogen atoms include fluorine, chlorine, bromine, and iodine, their differences in atomic sizes and electronegativity lead to different enzyme capabilities. Most reported halogenases catalyze reactions with chlorine and bromine donors, while the latter is commonly observed in marine living organisms. The goal of this review is not to comprehensively summarize all chlorinated natural products, but to focus on representative halometabolites that are synthesized with the involvement of various types of halogenase.

1. Aliphatic Chlorinated Compounds

Caldariomycin is a natural product that was found to be synthesized by a halogenating enzyme in *Caldariomyces fumago*.^[3] Since then, the biosynthesis of halogenated compounds was brought to attention. Caldariomycin contains aliphatic carbon-halogen (C–X) bonds that are ubiquitous in

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medicinal drugs and bioactive metabolites. The majority of halogenases involved in aliphatic C–X bond formation fall into three categories: haloperoxidases providing electrophilic activation of an alkene or its equivalent by a halonium ion (X^+) donor; S-adenosyl-L-methionine (SAM)-dependent halogenases providing nucleophilic environment at an electrophilic carbon center with a halide anion (X^-); and nonheme iron- α -ketoglutarate-dependent halogenases providing unactivated aliphatic C–H bond with a halogen radical ($X\cdot$) equivalent.^[4] Several representative aliphatic chlorinated compounds are listed in Figure 1.

Formation of the C–X bond in caldariomycin is catalyzed by a chloroperoxidase. Chloroperoxidases were considered as the only type of halogenase for a long time. It catalyzes the oxidation of the halide ions in the presence of hydrogen peroxide, resulting in the formation of free hypohalous acids. No substrate-binding pocket was observed in the structural studies on chloroperoxidases, which implies that the electrophile, (i.e., OCl^-), is released into bulk solution and reacts with substrates directly. Therefore, the halogenation catalyzed by chloroperoxidase lacks substrate specificity and regioselectivity, similar to chemical halogenation in organic synthesis.

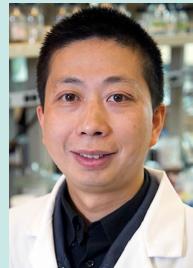
In addition to caldariomycin which is catalyzed by heme-Fe haloperoxidases, vanadium-dependent haloperoxidases (V-HPOs) belong to another group of hypohalite providers. Most V-HPOs were found in marine seaweeds and use bromide as the halogen donor. The family of napyradiomycin compounds are unusual chlorinated dihydroquinones reported from *Streptomyces aculeolatus*. For instance, as shown in Figure 1, napyradiomycin A1 showed antiangiogenic activity and inhibited HUVEC tube formation in a concentration-dependent manner. It inhibited endothelial cell proliferation and suppressed the migration and invasion of vascular endothelial cells but did not affect human dermal fibroblast proliferation. It also modulated cell permeability.^[5] Three enzymes in the napyradiomycin biosynthetic pathway, including NapH1, NapH3 and NapH4, are proposed to catalyze the chlorination. The biosynthetic cyclization of the terpenoid subunits is initiated through a chloronium ion. The *in vivo* experiments revealed the molecular basis of V-HPOs.^[6]



Dr. Jia Zeng obtained his PhD degree in metabolic engineering at Utah State University and acquired his postdoctoral training in biochemistry and crystallography at the University of Texas at Austin. His current research interests include tailoring enzymes in biosynthetic pathways of natural products including halogenases, and applying synthetic biology tools and metabolic engineering approaches to alternatively solve the current limitation of chemical synthesis in pharmaceutical applications.

Electrophilic carbon center with a halide anion is usually related to the SAM-dependent halogenases. Salinosporamide A (marizomib), an anticancer agent currently in clinical trials for the treatment of multiple-myeloma, was discovered from the marine bacterium *Salinispora tropica*.^[7] Parental cells displayed sensitivity to salinosporamide A ($IC_{50}=5.1\text{ nM}$), whereas their bortezomib-resistant sublines were 9- and 17-fold cross-resistant to salinosporamide A, respectively.^[8] Salinosporamide A also exhibited impaired capacity to inhibit β 5-associated catalytic activity. A chlorinase SalL was characterized and it chlorinates SAM to generate 5'-chloro-5'-deoxyadenosine. The chlorination showed an unusual nucleophilic substitution that is similar to a fluorinase in *Streptomyces cattleya*.^[9] SalL also accepts bromide and iodide as substrates, but not fluoride.^[10]

Radical reaction catalyzed by nonheme iron- α -ketoglutarate-dependent halogenases was exemplified by barbamide. Barbamide was isolated from the marine cyanobacterium *Lyngbya majuscula* with molluscicidal activity. ($LC_{100}=10\text{ }\mu\text{g/ml}$).^[11] The structure of barbamide is comprised of a trichloromethyl group and the methyl enol ether of a β -keto amide (Figure 1). Two halogenating enzymes, BarB1 and BarB2, are involved in barbamide biosynthesis, with L-Leu-S-BarA as the substrate. The *in vitro* reconstitutions revealed that the trichlorination of unactivated carbon was carried out by these two halogenases tandemly.^[12] Another iron- α -ketoglutarate-dependent halogenases SyrB2 was found in the syringomycin (Figure 1) biosynthetic pathway in *Pseudomonas syringae* pv. *syringae*.^[13] Syringomycin belongs to the family of antimicrobial and phytotoxic lipopeptide compounds.^[14] Its prominent fungicidal activity is strongly dependent on the presence of chlorine in the C-terminal residue.^[15] Syringomycin forms pores on membranes to act as non-selective ion channels, resulting in cell death via the membrane depolarization and non-controlled ion flux. SyrB2 catalyzes both mono-halogenation and di-halogenation reactions with threonyl-S-carrier protein as substrate.^[16] A radical mechanism was proposed for SyrB2 and its crystal structure was reported.^[13,17] Similarly, formation of hapalindole-type alkaloids was catalyzed by two nonheme iron-dependent



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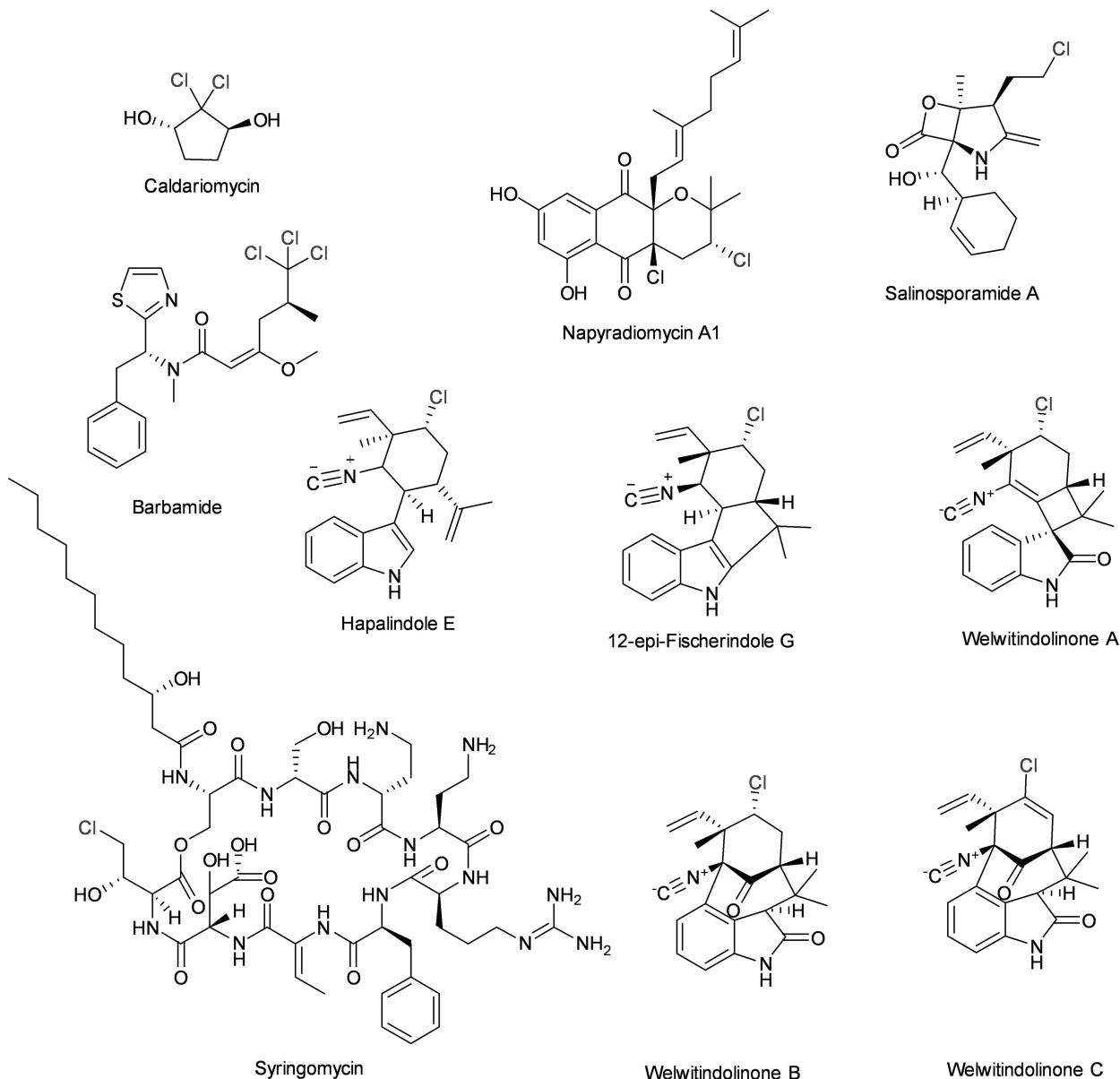


Figure 1. Representative aliphatic chlorinated compounds.

halogenases, WelO5 and AmbO5.^[18] 12-epi-Hapalindole E, 12-epi-fischerindole G, and welwitindolinones A–C (Figure 1) are all halogenated compounds catalyzed by iron- α -ketoglutarate-dependent halogenases, though the radical mechanism has not been reported.

2. Aromatic Chlorinated Compounds

In 1995, Dairi et al. discovered a novel halogenase in the 7-chlortetracycline biosynthetic pathway, which showed a different catalytic mechanism from the previously reported haloperoxidases.^[19] The function of this enzyme was proved by knockout experiment and later characterized by *in vitro*

reconstitution.^[20] Tetracycline antibiotics are bacteriostatic agents which act to inhibit bacterial growth and reproduction, and chlortetracycline is usually used as a veterinary drug. The enzyme involved in the chlorination of tetracycline belongs to the FDH category. Apart from the differences in the primary amino acid sequence, FDHs possess stronger regioselectivity than haloperoxidases. After the first characterized FDH PrnA was reported in 1997, this class of enzymes is drawing increasing attentions in the last two decades.^[21]

2.1 Early-Stage Halogenation

Unlike the incorporation of the chlorine atom by an FDH in a late biosynthetic step of chlortetracycline, the majority of reported FDHs use tryptophan or other early biosynthetic precursors as starter units. Deletion of these genes resulted in the complete shutdown of the biosynthetic pathways, and thus no unchlorinated intermediates are present. Therefore, it is more favorable to classify these halometabolites as the result of early-stage halogenation. Most of these compounds were isolated from prokaryotic strains.

2.1.1 Tryptophan-Derived Chlorinated Compounds

Tryptophan-derived halometabolites are well studied. They are biosynthesized with tryptophan as the starting precursor, with

chlorine atoms on different positions on the indole ring. Based on the substitution positions, we can separate these chlorinated compounds into different groups (Figure 2).

2.1.1.1 Trp-2 Chlorinated Compounds

Chondramides are a group of depsipeptides comprised of three amino acids and a polyketide chain. The tryptophan residue can be modified by chlorination to form chondramide B and D. Chondramides were isolated from the myxobacterium *Chondromyces crocatus* Cm c5, which showed a high cytostatic activity in mammalian cell cultures. They also react against yeasts but not on filamentous fungi or bacteria.^[22] Knock-out experiment of *cmdE* yielded chondramide A and chondramide C that lack chlorine atoms, which indicated that CmdE is a putative tryptophan 2-halogenase.^[23] Due to the

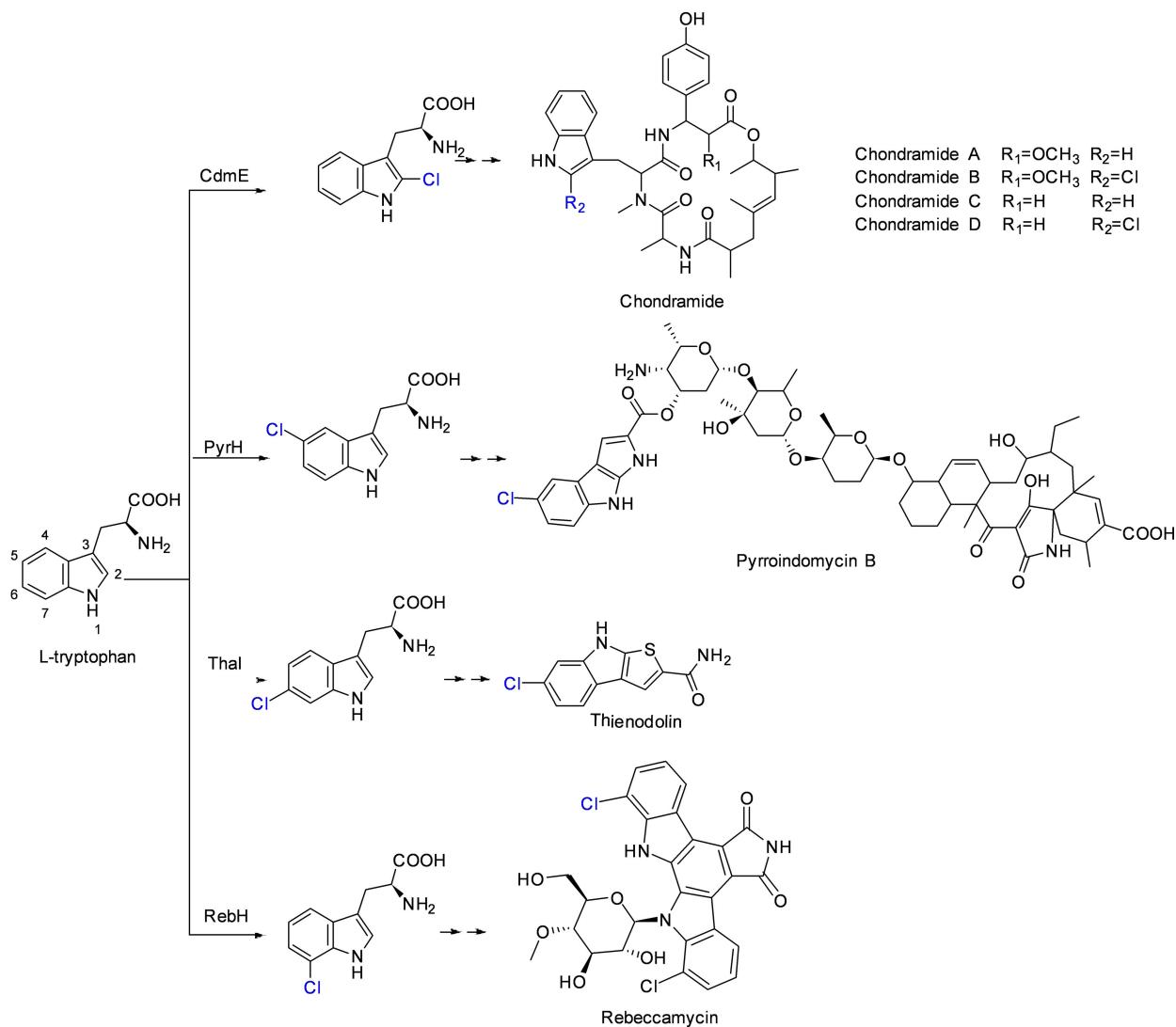


Figure 2. Flavin-dependent tryptophan halogenases and related natural products.

instability of free 2-chlorotryptophan, the *in vitro* results were not acquired. Rachid et al. deduced that CmdE function on nonribosomal peptide synthetase (NRPS) bound species or the nascent product of the NRPS. The chondramide biosynthetic enzymes can also accept 5-fluorotryptophan as the extender unit, but the chlorination was abolished due to the influence of fluorine on the indole ring.^[23]

2.1.1.2 Trp-5 Chlorinated Compounds

Pyrroindomycin B is an antibiotic against gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) strains. It was isolated from *Streptomyces rugosporus*. Its structure contains a polyketide macro-ring and trisaccharide linkage to a pyrroloindole moiety that is derived from chlorinated tryptophan. Chlorination occurs at 5-position

on the indole ring. PyrH can catalyze 5-halogenation of free tryptophan *in vitro*^[24] (Figure 2).

Similarly, ulleungmycin, a cyclic hexapeptide isolated from *Streptomyces* sp. KCB13F003 with 5-chlorotryptophan, exhibits moderate bioactivities against gram-positive bacteria including MRSA and quinolone-resistant *Staphylococcus aureus*^[25] (Figure 3). The functional halogenase has not been characterized, but it is very likely that it accepts tryptophan as substrate to generate 5-chlorotryptophan, which serves as a building block in the subsequent biosynthetic process.

Not limited to hexapeptides, larger polypeptides may also contain chlorinated tryptophan as a moiety. NAI-107 from *Microbispora corallina*, also called microbisporicin A1, is a ribosomally synthesized post-translationally modified peptides (RiPPs). This type of lanthipeptide exhibits antimicrobial activity and are known as lantibiotics. The structure of NAI-107 is comprised of 24 core amino acid residues, and the fourth amino acid is a 5-chlorinated tryptophan (Figure 3).

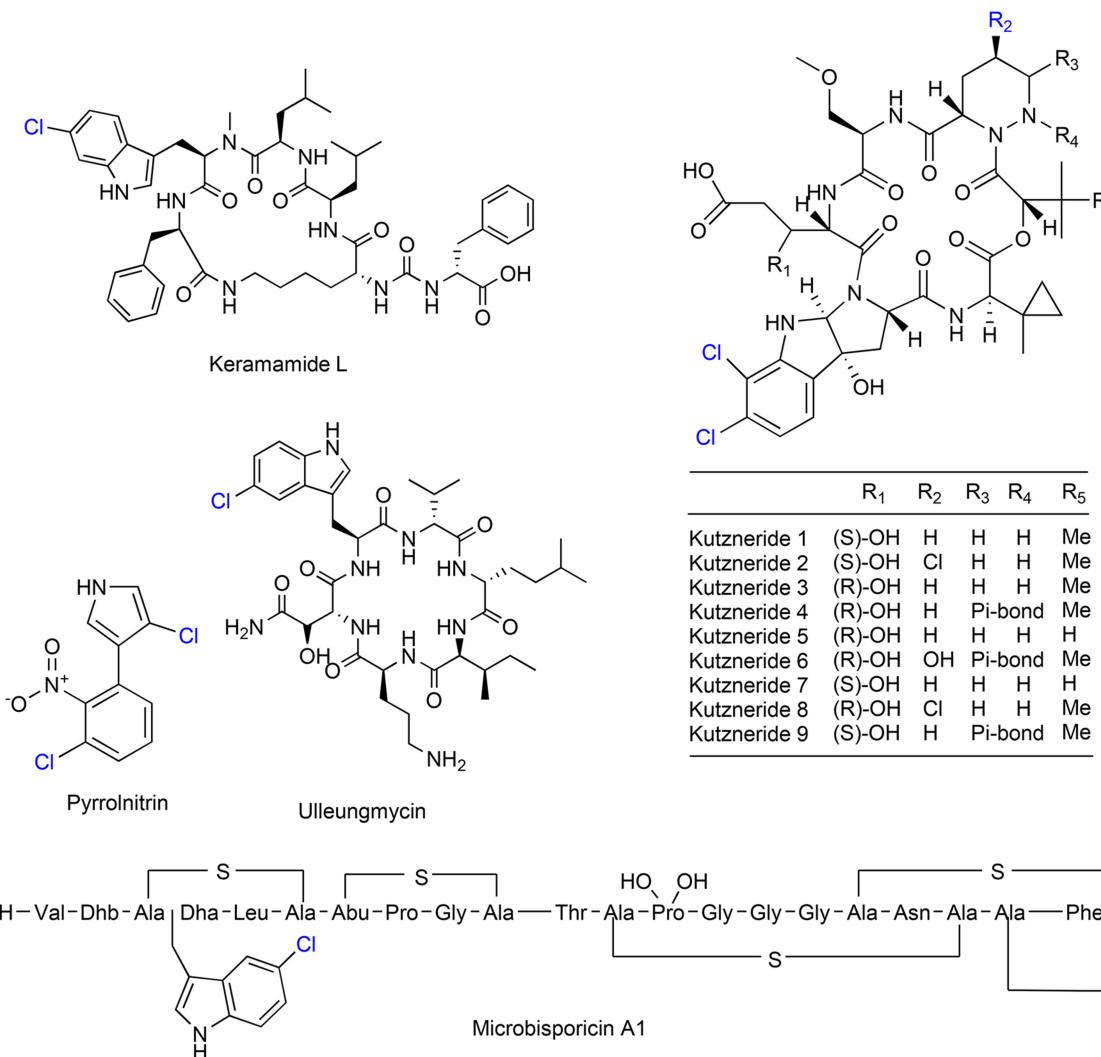


Figure 3. Other halogenated compounds with tryptophan as starting precursor.

The gene *mibH* was later identified as an FDH reacting in the early stage of NAI-107 biosynthesis. Different from other reported tryptophan halogenases, MibH cannot work on free tryptophan.^[26] Tests on the other lanthipeptides analogues showed negative results, indicating the strict substrate specificity of MibH. The structural analysis of MibH claimed that the substrate specificities for FDHs are related to the substrate-binding site rather than the active halide producing sites. This provides a clue to explain why different classes of FDH take different types of substrate.

2.1.1.3 Trp-6 Chlorinated Compounds

Thienodolin, an indolethiophen alkaloid isolated from *Streptomyces albogriseolus*, inhibits nitric oxide synthase in LPS-stimulated RAW 264.7 cells at both transcriptional and translational levels. Therefore, thienodolin can be developed into anti-inflammation and cancer chemoprevention drugs.^[27] The structure of thienodolin contains a 6-chlorotryptophan moiety, which is catalyzed by the FDH ThaL. The chlorination is the first step in thienodolin biosynthesis^[28] (Figure 2). Another Trp-6 halogenase SttH from *Streptomyces toxotricini* was reported in 2011. It may participate in the biosynthesis of a nonribosomal peptide.^[29] Besides, cyclic peptides keramides were isolated from marine sponges (Figure 3). Keramide L showed cytotoxicity against L1210 murine leukemia cells ($IC_{50}=0.46\text{ }\mu\text{g/ml}$) and KB epidermoid carcinoma cells ($IC_{50}=0.9\sim 1\text{ }\mu\text{g/ml}$) *in vitro*.^[30] Keramide L obtains a 6-chloro-5-hydroxytryptophan (5-HTP) structure which was catalyzed by KrmI. It exhibits a broad substrate specificity as it can halogenate both 5-HTP and free tryptophan.^[31]

Kutznerides are another group of natural products containing the 6-chlorotryptophan moiety, but they have chlorine at both 6- and 7-positions. They are cyclic depsipeptides comprised of six nonproteinogenic residues with antifungal and antimicrobial activities, and were isolated from the soil actinomycete *kutzneria* sp. 744^[32] (Figure 3). Two enzymes are involved in the dichlorination, including KtzQ and KtzR. KtzQ is a Trp-7 chlorinase while KtzR is a Trp-6 chlorinase. Both of them are capable of accepting free tryptophan as substrate. However, 7-chlorotryptophan is a preferred substrate for KtzR. It suggests that the dichlorination in kutznerides biosynthesis is in tandem. 7-Chlorotryptophan is first synthesized and then further halogenated by KtzR to generate 6,7-dichlorotryptophan, which serves as a building block in the subsequent processing.^[33]

2.1.1.4 Trp-7 Chlorinated Compounds

In addition to the kutznerides, other Trp-7 chlorinated compounds were reported as well. Rebeccamycin was isolated from *Lechevalieria aerocolonigenes* and known as an anti-tumor compound which binds to DNA-topoisomerase I complex to prevent the replication of DNA^[34] (Figure 2).

Analogues lacking chlorine substitution have weaker antimicrobial activity, due to the lack of cell membrane permeability.^[35] Two indole rings containing 7-chlorine on each side suggest that 7-tryptophan are the starter units for rebeccamycin biosynthesis. The Trp-7 halogenase RebH was proved to be an FDH and worked together with a partner enzyme RebF (flavin reductase) with a ratio of 1:3 to reach the highest turnover rate.^[36] Free tryptophan is the substrate for RebH, and both chlorination and bromination can occur based on the presence of different halogen donors. The broad-spectrum antifungal compound pyrrolnitrin was first isolated in 1964 from *Pseudomonas pyrrocinia*. 7-Chlorotryptophan is the starter unit which is catalyzed by PrnA, the first characterized FDH in 1997.^[21] PrnA can halogenate free tryptophan on the C-7 position. On the other hand, the chlorination on the pyrrole ring was catalyzed by PrnC (Figure 3). However, the mechanism of the latter chlorination step is still unclear.

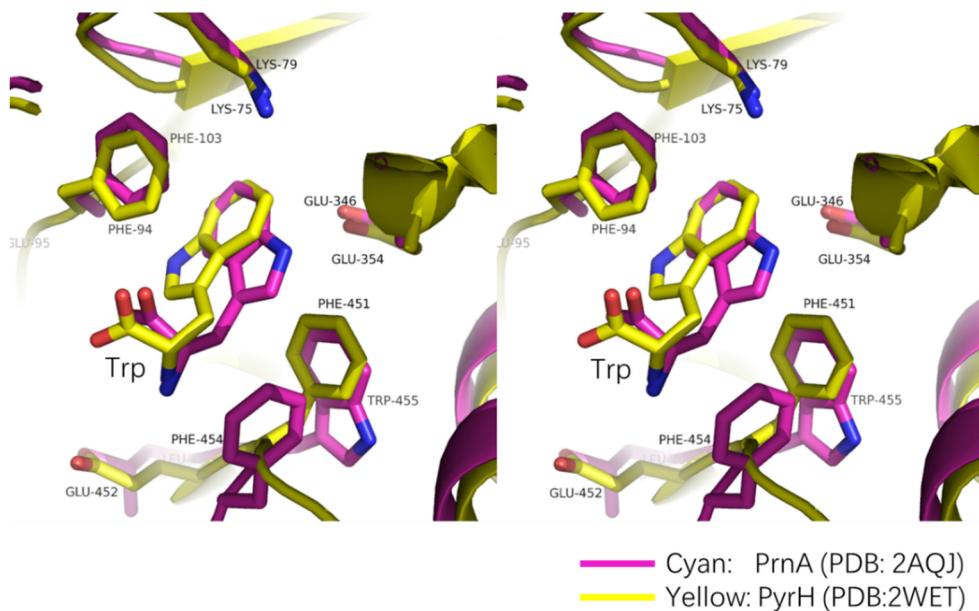
2.1.1.5 Engineered Tryptophan-Derived Chlorinated Compounds

Compared to HPOs that generate hydrogen peroxide as an intermediate, tryptophan halogenases are more regioselective. The catalytic positions of halogenation are strictly controlled. With more and more structural biology data released, researchers found out that the substrate pockets are crucial for the regioselectivity. Therefore, several attempts have been carried out to alter the regioselectivity through rational mutagenesis. Lang et al. constructed a F103A mutation of PrnA for an expanded active site, which made this Trp-7 halogenase capable for Trp-5 halogenation^[37] (Figure 4A). Similarly, based on the crystal structure of SttH, Shepherd et al. made 3 rational mutations (L460F/P461E/P462T) to switch this Trp-6 halogenase to Trp-5 halogenase^[38] (Figure 4B). These findings enlarged the pool of tryptophan halogenases as synthetic tools. Target metabolites with certain stereochemistry could be acquired by rational design of the active sites. Other efforts such as directed evolutions, thermo-stabilizing and enzyme fusions were carried out to seek better efficiency of these enzymes.^[39]

2.1.2 Natural Products with Other Chlorinated Moieties

Besides tryptophan, other chlorinated intermediates as building blocks in natural product biosynthetic pathways have been revealed. Different from tryptophan halogenases, most of this type of halogenase reacted on the substrates bonded to certain carrier proteins. As shown in the phylogenetic tree in Figure 5, tryptophan halogenases are grouped together as variant A, while CndH, PltA and SgcC3 are grouped as variant B. The FDHs and their Genebank accession numbers are listed in Table 1. Variant B shares the similar function of halogenating carrier protein-tethered substrates. The chlorinated intermediates are used as the building blocks for the following biosynthesis steps.

A



B

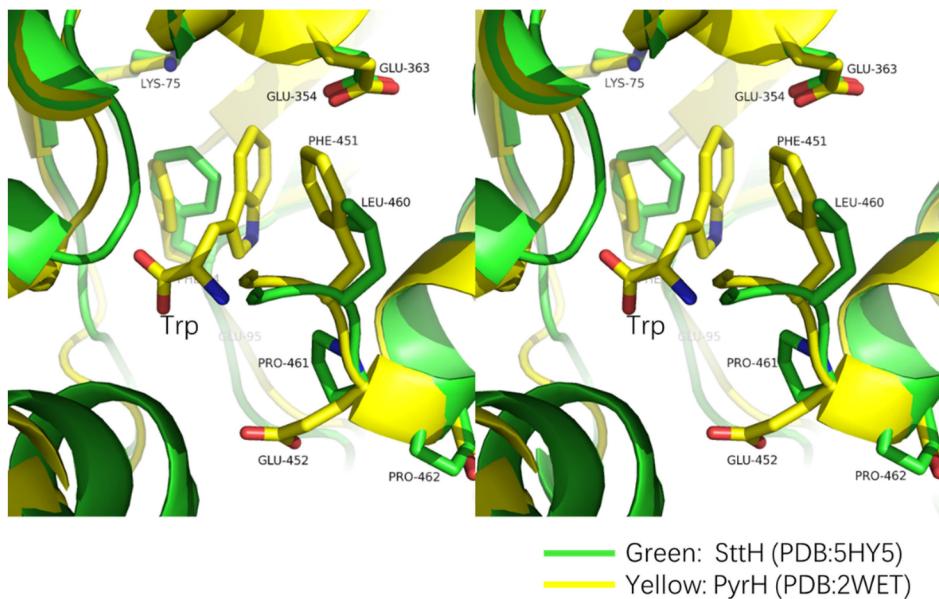


Figure 4. The active pocket of tryptophan halogenases. (A) Comparison of Trp-5 and Trp-7 halogenases. The regioselectivity is influenced by surrounding side chains. The F103 A mutation of PrnA creates an expanded pocket. (B) A comparison of the mutation of SttH (L460F/P461E/P462T) with the Trp-5 halogenase PyrH.

Pyoluteorin is an antifungal product containing a 4,5-dichloropyrrole moiety, which is isolated from *Pseudomonas fluorescens*.^[40] The 5-position on the pyrrolyl ring was attacked by a FAD-4a-OCl intermediate first to form a 5-chloropyrrolyl intermediate and then the 4,5-dichloropyrrolyl product. The reaction was catalyzed by the FDH PltA, with

the substrates covalently bound to the carrier protein. The chlorinated intermediate was further catalyzed to form pyoluteorin^[41] (Figure 6).

The structure of the famous antitumor antibiotic C-1027 also contains halogen atoms. C-1027 is a nine-membered enediynes from *Srptomycetes globisporus*.^[42] Deschloro-C-1027

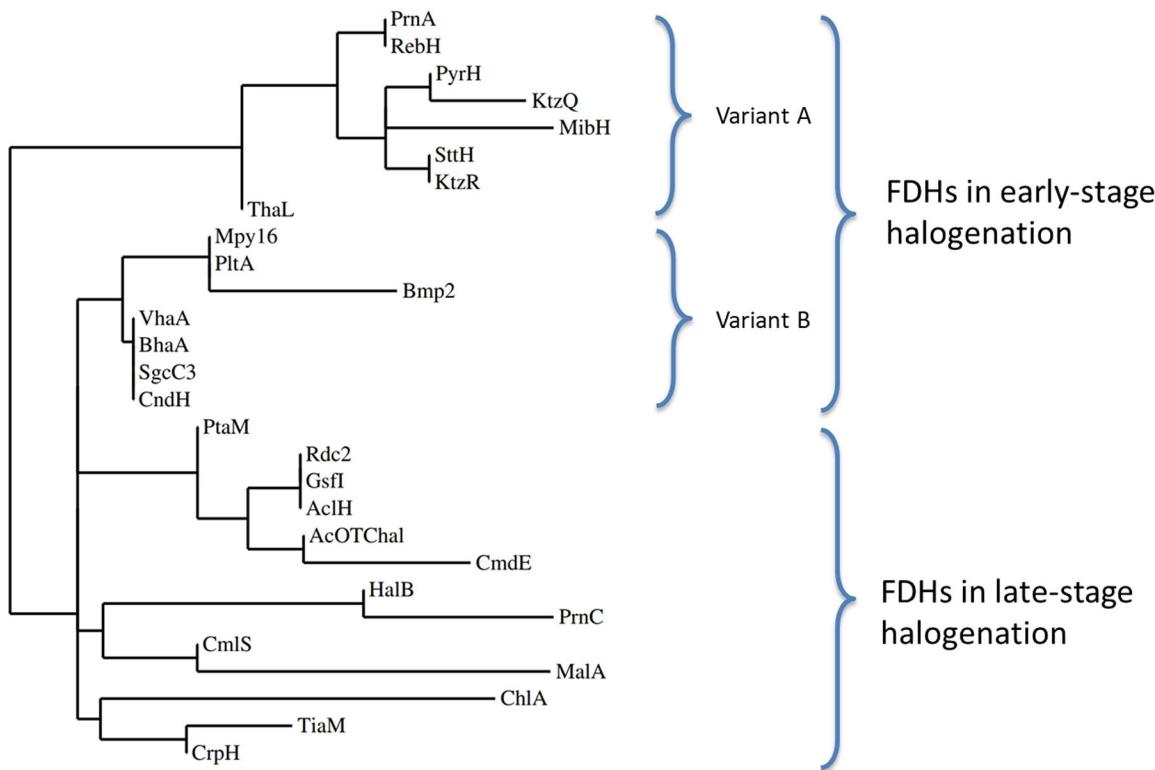


Figure 5. Phylogenetic tree of FDHs from various sources.

Table 1. FDHs used in phylogenetic analysis and their GenBank accession numbers.

Name	GenBank Accession Number	Name	GenBank Accession Number
Rdc2	ADM86580.1	KtzR	ABV56598.1
RebH	AAN01216.1	ThaL	ABK79936.1
PrnA	AAB97504.1	Mpy16	AFP87533.1
ChlA	BAP16678.1	BhaA	CAA76550.1
SttH	ADW94630.1	Bmp2	AME30285.1
PltA	AAD24884.1	MibH	ADK32563.1
PrnC	AAY92872.1	MalA	AGA37261.1
CmlS	AOR34513.1	TiaM	ADU85999.1
PyrH	AAU95674.1	VhaA	CCD33142.1
CndH	CAQ43074.1	CrpH	ABM21576.1
SgcC3	ANY94426.1	Gsfl	ADI24948.1
CmdE	CAJ46693.1	AcOTAhal	ANY27070.1
HalB	AAQ04685.1	AclH	BAE56588.1
KtzQ	ABV56597.1	PtaM	AGO59046.1

was shown to be less proficient at inducing DNA double-stranded breaks and less cytotoxic, and has altered action mechanisms.^[43] The 3-chloro-4,5-dihydroxy-β-phenylalanine moiety is crucial for the stability and reactivity of the enediyne core. It was synthesized by SgcC3 reacted on a carrier protein-tethered substrate. SgcC3 does not accept free amino acid substrates, and its function has been characterized both *in vitro* and *in vivo*^[44] (Figure 6).

The third example is chondrochlorens. Chondrochlorens A and B are weak antibiotics against *Micrococcus luteus*, *Bacillus subtilis* and *Staphylococcus aureus*. They were isolated from *Chondromyces crocatus*, containing a chlorohydroxy-styryl group. The halogenase CndH reacts on the tyrosinyl group tethered on a carrier protein. Similar to other variant B FDHs, CndH cannot work on free amino acids. Their substrate specificity is determined by the active sites which were further illuminated by crystal structural data^[45] (Figure 6).

As one of the most famous antibiotics vancomycin has been studied for a long time, and the halogenase in its biosynthetic pathway was reported recently. Vancomycin is a branched tricyclic glycosylated nonribosomal peptide, produced by the *Actinobacteria* species. Vancomycin has been shown to be active against most bacterial strains, both *in vitro* and in clinical infections. The halogenated heptapeptide backbone is crucial for the activity, which improves binding affinity to the D-Ala-D-Ala target against *Staphylococcus aureus*.^[46] The chlorinase VhaA is responsible for the dichlorination in the assembly process. The *in vitro* assay revealed that VhaA used peptide carrier protein-tethered hexapeptide as the substrate and introduced a chlorine atom into each aromatic ring of both β-hydroxytyrosine residues^[47] (Figure 6).

Some other compounds shown in Figure 7 are proposed to be catalyzed by variant B halogenases, though their functions

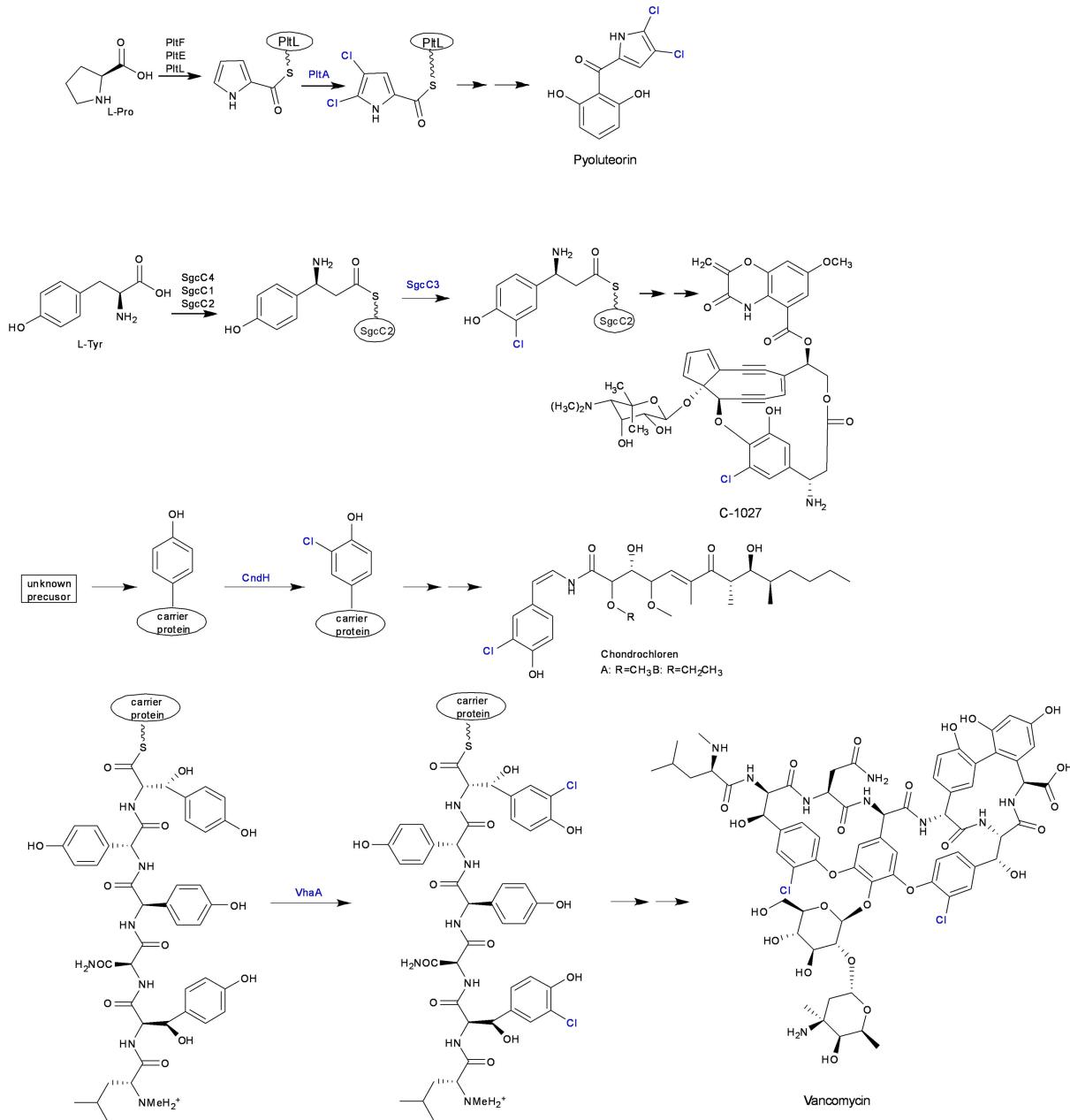


Figure 6. Natural product biosynthetic pathways involving halogenation of carrier protein-tethered substrates.

were not characterized. Balhimycin, a glycopeptide antibiotic, was isolated from the fermentation broth of *Amycolatopsis sp.* Balhimycin showed bactericidal activity against both aerobes and anaerobes, including MRSA (MIC 0.39 µg/ml) and *Clostridium* strains (MIC 0.39 µg/ml). Post-antibiotic effect (PAE) was observed as well.^[48] A halogenase gene *bhaA* was revealed in the biosynthesis of balhimycin.^[49]

Another anti-MRSA drug marinopyrrole A was from marine actinomycete CNQ-418. The research showed that the MIC of marinopyrrole A against the panel of *Staphylococcus aureus* strains is 0.188 to 1.5 µg/ml, while the measured MICs

against vancomycin-resistant *Enterococcus faecalis* and *Haemophilus influenzae* are 1 µg/ml and 2 µg/ml, respectively. A calculated PAE is between 4 and 6 h. Marinopyrrole A also exhibited a favorable therapeutic index, with IC₅₀ about 32 to 64 µg/ml against HeLa cells and 8 to 32 µg/ml against L929 cells.^[50] Deletion of the *mfp16* gene completely abolished the production of marinopyrroles, so Mfp16 was proposed to catalyze the initiating dichlorination of pyrrolyl-S-carrier protein.^[51]

The brominase Bmp2 is of high similarity of Mfp16. It converts pyrrolyl-S-Bmp1(ACP) to tribromopyrrolyl-S-Bmp1

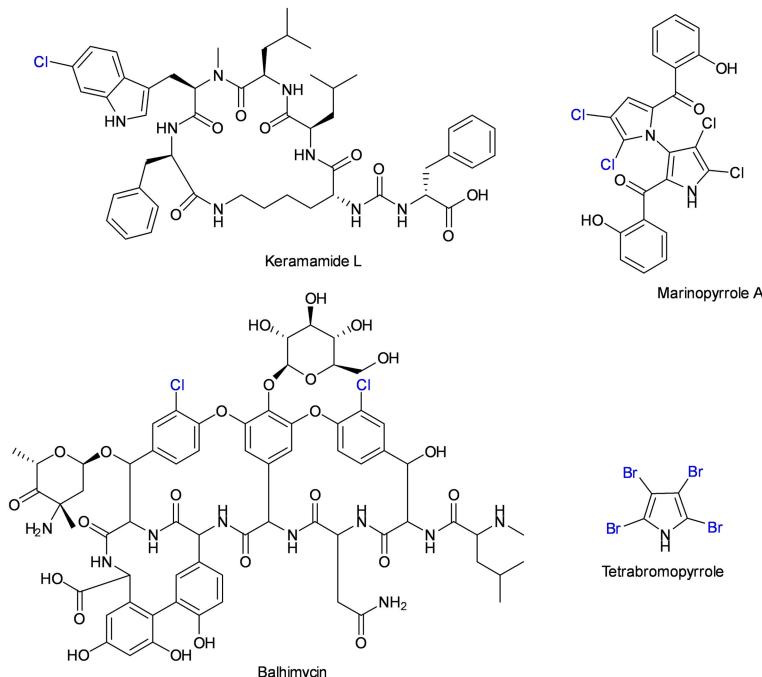


Figure 7. Additional halogenated compounds whose biosynthetic processes involve halogenations of carrier protein-tethered substrates.

(ACP), which is a key intermediate in tetrabromopyrrole biosynthesis.^[52] Tetrabromopyrrole was isolated from *Pseudoalteromonas spp.* It shows antibiotic activity against human and marine bacteria and may influence the bacterial community composition within marine biofilms.^[53] The structure of pyrrole ring and increased bromination were most lethal to the coccolithophore, *Emiliania huxleyi*. Tetrabromopyrrole induced the production of reactive oxygen species and the release of intracellular calcium stores, to activate the cellular death pathways.^[54]

2.2 Late-Stage Modification

Besides the halogenated compounds from bacteria, more and more halometabolites have been discovered from eukaryotic organisms. Different from bacterial halogenation, aromatic compounds in fungi usually serve as substrates and the halogenation steps commonly occur in a late biosynthetic step. The involved halogenases have a relatively larger pocket in their active sites and may have broader substrate specificities.

The first reported compound catalyzed by eukaryotic FDHs is differentiation-inducing factor 1 (DIF-1), a polyketide derived morphogen, was identified in *Dictyostelium*.^[55] It induces prestalk-specific gene transcription in responsive cells and plays a critical role in the proper development program of the hosts. Other bioactivities in mammalian cells were also reported.^[56] Lacking the chlorine atoms on DIF-1 resulted in the fragile slug phenotype. Mono-chlorination and di-chlorination were observed when the halogenase ChlA used (2,4,6-

trihydroxyphenyl)-1-hexan-1-one (THPH) as the substrate.^[57] The chlorination happens after the formed polyketide backbones are released from the carrier proteins (Figure 8).

The biosynthetic pathway of radicicol also contains a tailoring FDH. Radicicol is a potent heat shock protein 90 inhibitor isolated from various fungi.^[58] The structure is a resorcyclic acid lactone containing a chlorine atom on the aromatic ring. The radicicol biosynthetic pathway was identified in two different fungi, and two different FDH-encoding genes were revealed. One is Rdc2 from *Pochonia Chlamydosporia*, and the other one is RadH from *Chaetomium chiversii*.^[59] Rdc2 was first biochemically characterized both *in vitro* and *in vivo*.^[60] Monocillin IV, a stable substituent for the precursor of radicicol was tested and radicicol was synthesized (Figure 8). Changing the ratio of Rdc2 and flavin reductase led to the formation of dichlorination products. However, the dechlorinated metabolites were not detected *in vivo*, probably due to the high toxicity of dechlorinated compounds to the hosts. Rdc2 exhibits a broad substrate specificity. Similar aromatic compounds were tested, including dihydroresorcylide, zearalenone, curvularin and curcumin. The chlorination usually took place on the ortho-position of the phenol groups. RadH, on the other hand, showed similar catalytic properties, but cannot halogenate zearalenone or curvularin.^[61] It implies that these two FDH may have some interesting differences in their active sites.

In 2016, two other FDHs were characterized in chaetoviridins and griseofulvin biosynthetic pathways, respectively. Chaetoviridins are a large family of fungal secondary metabolites isolated from *Chaetomium*. They are assembled

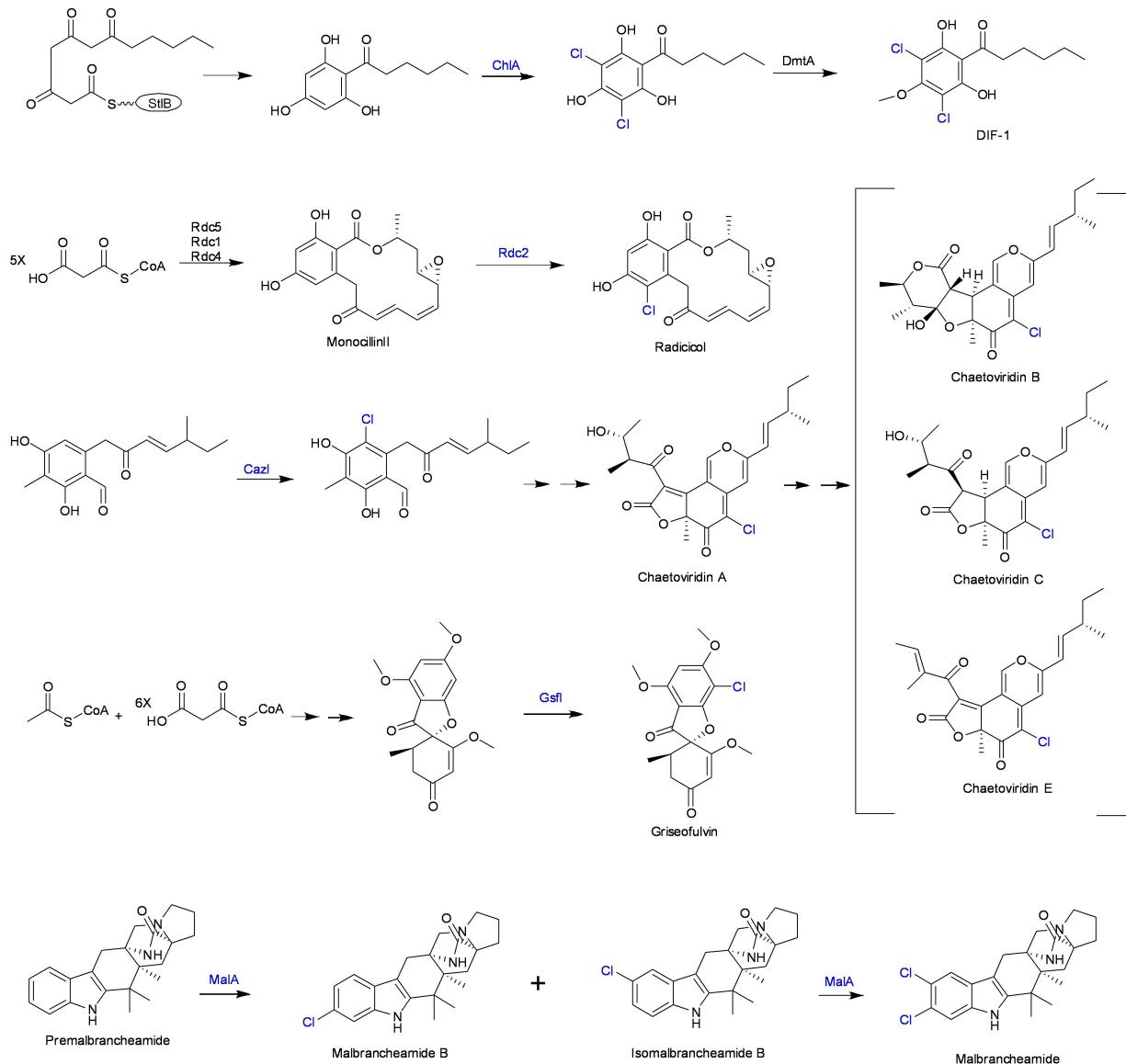


Figure 8. Chlorinated natural products whose biosynthetic pathways involve late chlorination step catalyzed by FDHs.

by an iterative polyketide synthase (PKS) and exhibit antifungal and antibiotic activities.^[62] Intermediate cazaldehyde A was chlorinated by CazI on the *ortho* position of the phenol group. Chaetoviridins A, B, C and E were further synthesized. Post-PKS modification provides chances for chemical diversity in natural product biosynthesis.^[63] On the other hand, griseofulvin, isolated from *Penicillium aethiopum*, is an antifungal drug and has been in use for many years in medical and veterinary applications due to its influence on the mitotic spindle microtubules in mitosis.^[64] The chlorination was proposed to occur in the last biosynthetic step. Similar to RadH (60% identity), GsfI was another late stage halogenase whose function has been proved by knockout experiments. This enzyme is responsible for the regiospecific chlorination

at the C-13 position, which is *ortho* to the phenol groups^[65] (Figure 8).

However, the structural information remained mysterious until the crystal structure of MalA shed a light on the actual shapes of active sites for this type of FDH. Since the eukaryotic FDHs commonly react at the late stage of biosynthesis, a large pocket compatible for mature intermediate is necessary. *malA* is an FDH-encoding gene in the malbrancheamide biosynthetic pathway. Malbrancheamide is well-known for its bicyclo[2.2.2]diazaoctane core. This calmodulin antagonist was isolated from *Malbranchea aurantiaca*, with significant vasorelaxant effect.^[66] After the proposed [4 + 2] Diels-Alder cycloaddition, premalbrancheamide was further chlorinated to malbrancheamide B, isomalbrancheamide B, and malbrancheamide, respectively. The conversion rates of

these three chlorinations are similar, which means that the C-8 and C-9 positions are of equal reactivity. Further modification on the regioselectivity and substrate scope can be accomplished based on the structural information^[67] (Figure 8).

Recently, many other promising aromatic halometabolites have been reported and are shown in Figure 9, though the related FDHs are less characterized. Ochratoxin A is a polyketide compound isolated from *Aspergillus carbonarius*. Ochratoxin A is harmful to grapes and derived products, and may cause nephrotoxic, teratogenic, immunotoxic, neurotoxic, hepatotoxic and carcinogenic effects to human.^[68] The encod-

ing gene of the halogenase (AcOTAHal) shows greatest similarity to RadH, and the deletion of *AcOTAHal* gene results in the accumulation of Ochratoxin B. The chlorination occurs at the *para* position of phenol group, and AcOTAHal is highly possible to be a late stage halogenase, which remains to be further characterized in the future.^[69]

Dichlororugulovasines A and B were isolated from the fungus *Talaromyces wortmannii*. It is a rare example for eukaryotic halogenase reacting on tryptophan moieties. Both 2- and 7- positions were chlorinated. However, the correspond-

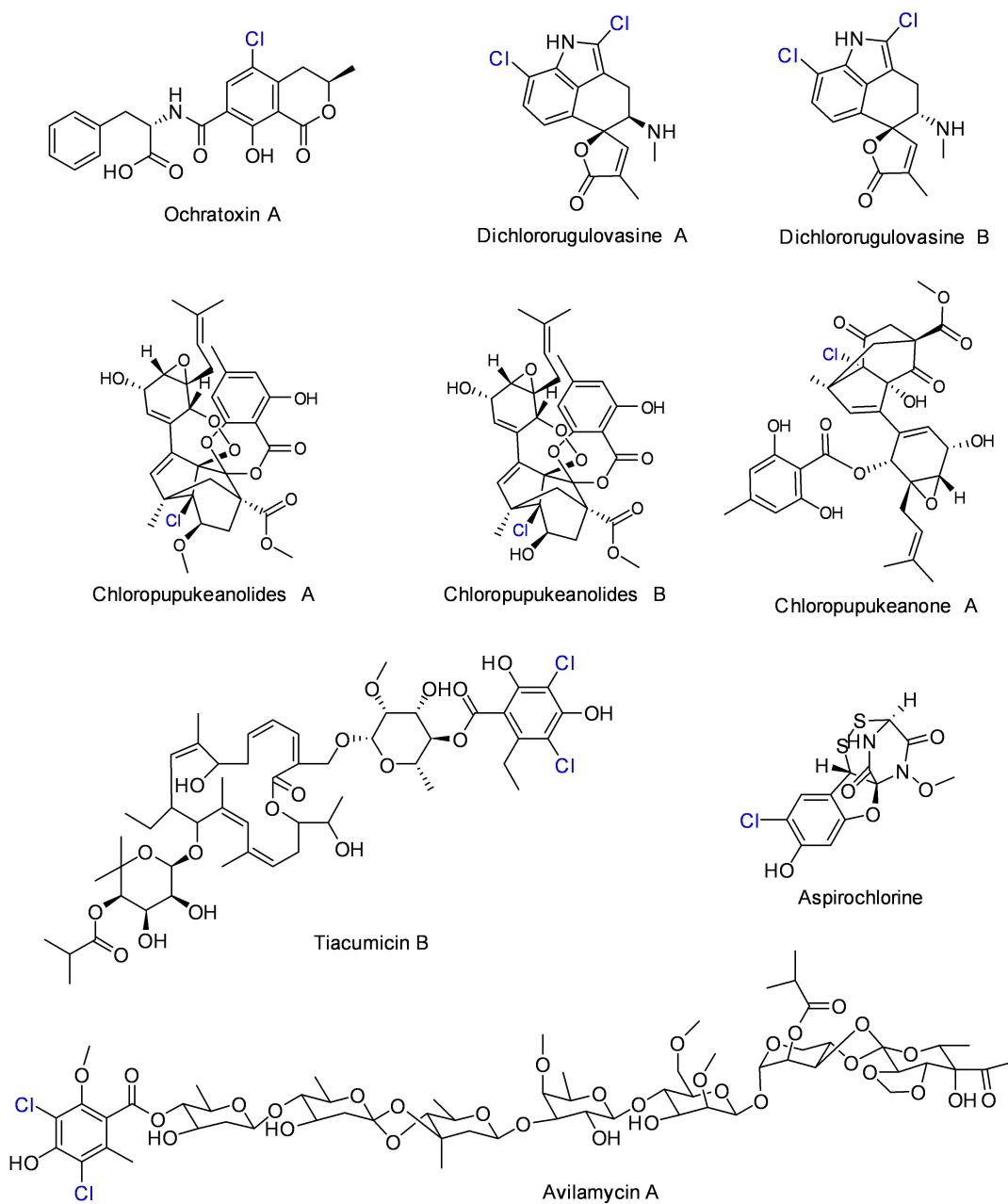


Figure 9. Other halogenated compounds with late-stage halogenation.

ing enzymes were not identified, and the characterization results would be promising if the genes are targeted.^[70]

Chloropupukeanolides A and B, and chloropupukeanone A are three chlorinated metabolites with pupukeanane core. They were isolated from an endophytic fungus *Pestalotiopsis fici*, with significant antimicrobial, antitumor, and anti-HIV activities.^[71] Pestheic acid was considered as the precursor of chloropupukeanane compounds, and the biosynthetic pathway of pestheic acid was revealed. PtaM was proved to be an FDH that catalyzes the late chlorination step in the pathway.^[72]

Tiacumicin B was isolated from various hosts such as *Dactylosporangium aurantiacum* subsp. *Hamdenensis*, *Micro-monospora echinospora*, *Catellatospora*, *Actinoplanes deccanensis*, etc.^[73] It exhibits strong activity against *Clostridium difficile*-associated diarrhoea at low concentrations.^[74] The dichlorinated homo-orsellinic acid moiety is a key part for the activities. Interestingly, the involved halogenase TiaM is from prokaryotic hosts but catalyzes a late-stage halogenation step. Two chlorine atoms were reported to be added on the aromatic ring in sequence.^[75] Another antimicrobial compound avilamycin A was isolated from *Streptomyces viridochromogenes* TÜ57, and it shares a similar di-chloro moiety. Knockout of AviH led to the formation of a dechlorinated product. Therefore, this chlorination is also considered as a late-stage modification.^[76]

Aspirochlorine, isolated from koji mold (*Aspergillus oryzae*), is an epidithiodiketopiperazine (ETP) toxin. It shows antifungal and antibacterial activity. Monti et. al. showed that it specifically inhibits fungal protein synthesis but does not inhibit protein synthesis in bacteria or higher eukaryotes.^[77] Inhibition of fungal protein synthesis by aspirochlorine is dose-dependent with a potent IC₅₀ of about 10 nM. Inhibition of *Bacillus subtilis* cells is mainly on RNA synthesis. Since the koji mold was widely used in Asian countries from ancient times, inadequate research on the potent toxic metabolites brought uneasiness to the public. The chlorine in aspirochlorine plays a key role in the bioactivities. The dechlorinated derivative was of substantially less antifungal activities and radically reduced antiproliferative and cytotoxic activities.^[78] Chlorination is the last step in the aspirochlorine biosynthetic pathway and is catalyzed by AclH. Therefore, AclH is considered another late-stage halogenase. The functions of AclH was proved by knockout and feeding experiments.

3. “Unnatural” Natural Halogenated Compounds

Remarkable progress has been made in the past two decades towards discovering and characterizing various types of halogenase. Not satisfied with the current passive observation

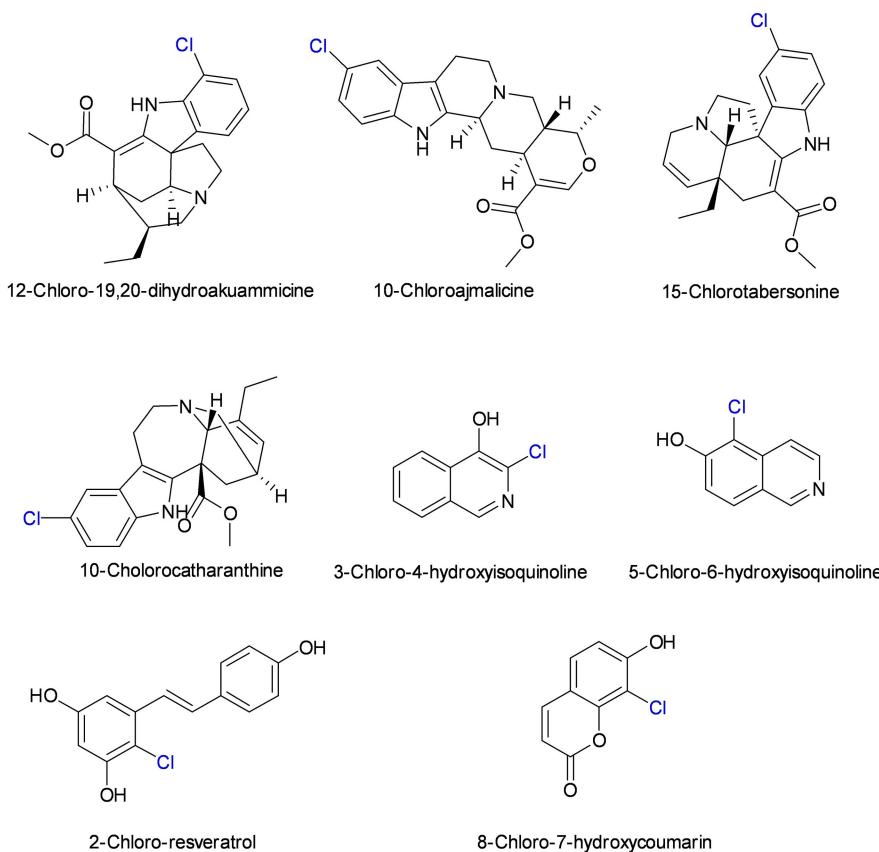


Figure 10. Representative “unnatural” halogenated natural products.

of these natural reactions, researchers attempted to manipulate the catalytic reactions for the creation of novel “unnatural” natural products. Since halogen atoms can enhance the bioactivities of many natural products, introducing the halide into the metabolic pathways may provide the chances to obtain new drugs with improved pharmaceutical properties. Runguphan et al. introduced PyrH and RebH into the medicinal plant *Catharanthus roseus* and isolated a series of chlorinated monoterpene indole alkaloids with predicted regioselectivity.^[79] These original compounds include 19,20-dihydroakuamycin, ajmalicine, tabersonine and catharanthine, which showed various bioactivities, such as antiplasmoidal, bronchodilator, and acceleration of neurological function recovery, etc.^[80] They share tryptophan starter units and the introduction of halogen atoms strongly proved the potential of using plants or microorganisms as synthetic biology platform (Figure 10).

In addition to creating novel metabolites *in vivo* using halogenases, several *in vitro* experiments were carried out to prove the viability for assembling enzymatic tools as kits to synthesize intermediates for industrial production. The late-stage halogenase Rdc2 and RadH were tested on isoquinolines, resveratrol, coumarins, and flavonoids^[61,81] (Figure 10). Halogenation is highly regioselective that it occurred at the position ortho to the phenolic hydroxyl group. Another high-throughput screening of FDHs with docking substrates revealed that FDHs could catalyze a far greater range of substrates than previously recognized.^[82] These studies inspired the chemists and biochemists to explore the potentials for halogenation in organic synthesis and synthetic biology.

The studies of halogenases and their biosynthetic pathway is just at the very early stage. However, the recent progress gradually depicts a draft picture of the relationship between the structures of halogenated compounds and the pattern of the catalytic enzymes. With the development of genomic mining and synthetic biology, it is believed that the bioinformation extracted from the biosynthetic pathways and their derived macromolecules will facilitate the development of useful halogenating tools for biochemical and medicinal applications.

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