Nature's Inventory of Halogenation Catalysts: Oxidative Strategies Predominate

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1. Introduction

Halogenated molecules are widely distributed in the biosphere. A variety of halogenated aromatic and aliphatic compounds are man-made and can be harmful to the environment due to their slow rates of biodegradation.[1] Halogenated organic molecules can also arise as natural products in which the carbon−halogen bonds are generated enzymatically. To date, more than 4500 halogenated natural products have been discovered,[2] though it is likely to be a substantially incomplete inventory. Enzymatic incorporation of halogens during natural product assembly alters physical properties, including electronic and steric effects that can be consequential for determining the affinity and selectivity of interactions with biological targets. Many of the genes encoding such halogen-incorporating enzymes are embedded in specific biosynthetic gene clusters, enabling coordinate regulation to activate these secondary metabolite pathways.

Biological halogenation occurs on a diverse array of organic scaffolds, from terpenes to polyketides to nonribosomal peptides. Within these scaffolds, halogen atoms are incorporated on aliphatic carbons, olefinic centers, and a wide variety of aromatic and heterocyclic rings. A recent inventory of halogen-containing natural products indicated 2300 organochlorines, 2100 organobromines, 120 organoiodines, and 30 organofluorines.[2] The larger representation of chlorinated and brominated metabolites probably reflects the abundance of chloride and bromide ions in microenvironments of terrestrial and marine producer organisms. A brief list of some chlorinated, brominated, and iodinated metabolites is noted below.

Among the chlorinated natural products of therapeutic interest are vancomycin (**1**), rebeccamycin (**2**), chlortetracycline (**3**), and chloramphenicol (**4**; Figure 1A).[3−6] The natural products **1**−**3** carry chlorine groups on aromatic rings while chloramphenicol has a 2,2-dichloroacetyl group. As shown in Figure 1B, the cyanobacterial metabolite barbamide (**5**) has a trichloromethyl substituent and syringomycin E (**6**), produced by the phytotoxic *Pseudomonas syringae*, is monochlorinated on the terminal methyl of a threonyl residue.[7,8] In the biocontrol agents pyrrolnitrin (**7**) and pyoluteorin (**8**; Figure 1C), also produced by *Pseudomonas* strains, pyrrole rings are mono- and dichlorinated, respectively.[9,10]

Brominated metabolites are typically produced by marine microorganisms, and three such molecules are shown in Figure 2. The bromoterpene snyderol comes in three isomeric forms (**9**−**11**; α, *β*, and *γ*), reflecting three modes of quenching an intermediate carbocation.[11,12] Convolutamine A (**12**) has a tribromoanisole ring presumably reflecting three tandem bromination steps during maturation.[13] The terpenoid product laurallene (**13**) has two C−Br bonds, most notably the terminal bromoallene group.[14]

Among iodinated natural products, calicheamicin (**14**), produced by a soil actinomycete, and tetraiodothyronine (**15**; thyroxine; T_4), synthesized in humans (Figure 3), show

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different contexts for aryl iodides in biology. Calicheamicin is one of a family of enediyne natural products with potent cytotoxicity due to radical-initiated cleavage of DNA on reduction-induced Bergman cyclization of the enediyne.[15−17] Tetraiodothyronine (T_4) and the triiodo version (T_3) are the major circulating forms of mammalian thyroid hormone,[18] required for metabolic control and homeostasis.

2. Fluorinating Enzymes: Nonoxidative Construction of the C–F Bond

Despite the relatively high abundance of fluoride in the earth's crust, fluorinated metabolites are relatively rare and enzymatic construction of the C−F bond appears to proceed via a distinct route from formation of C−Cl, C−Br, and C−I bonds. The paucity of organofluorine metabolites in nature may reflect restricted access to fluoride ions in cells or, perhaps more importantly, the difficulty in desolvating the highly electronegative F^- in aqueous environments to generate the naked nucleophilic fluoride ion.[19] The potential toxicity to producer organisms from fluorinated metabolites that can be processed to fluoroacetate may also limit biodistribution.[20]

The structure of one fluorinating enzyme from the thienamycin-producing *Streptomyces cattleya*, generating 5-fluorodeoxyadenosine from *S*-adenosylmethionine (SAM) and F^-, has been solved. The energy cost of desolvation of fluoride ion in the active site appears to be compensated by

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pairing with a side chain hydroxyl of a serine residue in that active site (Scheme 1A).[21] The reaction is formulated with the fluoride ion attacking the polarized carbon−sulfur bond at the sulfonium cation site of SAM, taking advantage of that favorable leaving group to drive C−F bond formation in a simple nucleophilic substitution (Scheme 1B).[22] Interestingly, the enzyme will also catalyze the reverse reaction to break the C−F bond. In addition, chloride ion is also accepted by the enzyme to generate 5-chlorodeoxyadenosine in a rare example of nucleophilic substitution during biological chlorination, although the equilibrium of this reaction lies heavily in favor of the starting substrates.[23]

As noted, this anticipated mode of reactivity of the highly nucleophilic fluoride ion is distinct from the enzymatic strategies typically used for biological chlorination, bromination, and iodination that are the main focus of this review. The inventory of enzymatic machinery that carries out these latter types of reactions predominantly employs oxidative strategies, converting the abundant ionic species Cl^-, Br^-, and I^- to oxidized states that react not as nucleophiles but as electrophilic or radical species. The biological strategy of oxidizing a halide anion to create a reactive species would be forbiddingly high in energy for fluoride ion and has not been detected. The lack of an oxidative route for C−F bond formation and consequent incompatibility with the biological redox strategy for halogenation may be the major reason natural organofluorine compounds are rare in biology.

3. Oxidative Logic in Chlorinating, Brominating, and Iodinating Enzymes

Since the initial characterization of a fungal chlorinating enzyme in 1966 as a heme-dependent chloroperoxidase,[24−26] the discovery of other classes of halogenating enzymes in the ensuing 40 years has turned up three additional redox cofactors, vanadium, the dihydro form of FAD ($FADH_2$), and a two-His, non-heme Fe^{2+} center (Figure 4), as obligate cofactors in different types of halogenating enzymes.[27−31]

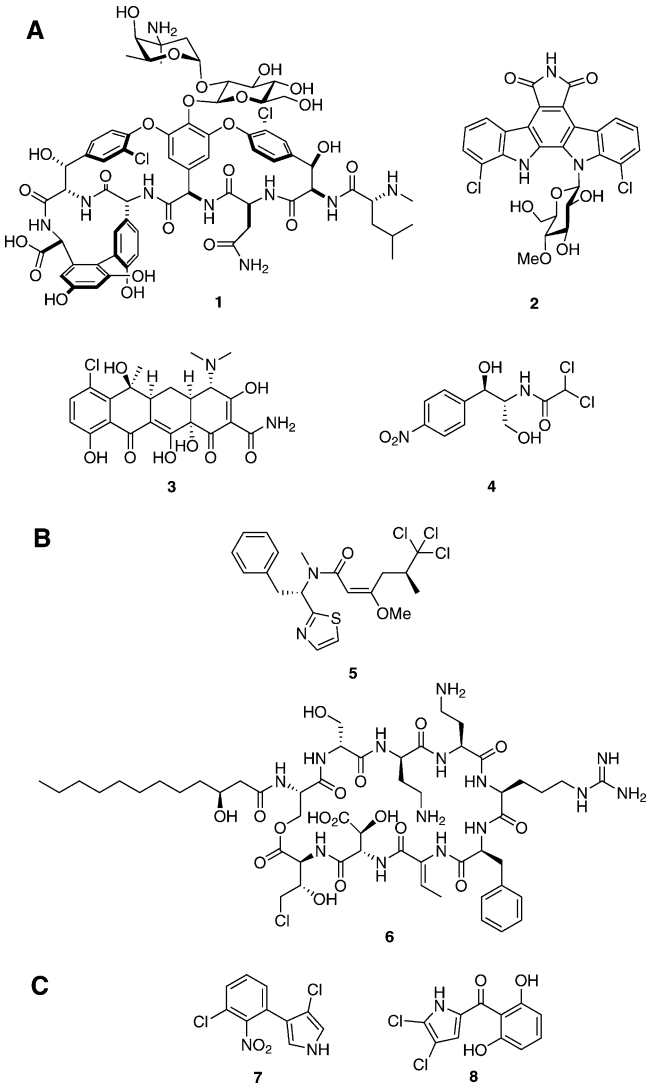


Figure 1. Chlorinated natural products. (A) Natural organohalogens of therapeutic interest: vancomycin (**1**), rebeccamycin (**2**), chlortetracycline (**3**), and chloramphenicol (**4**). (B) Aliphatic natural organohalogens: barbamide (**5**) and syringomycin E (**6**). (C) Aromatic and heteroaromatic natural organohalogens: pyrrolnitrin (**7**) and pyoluteorin (**8**).

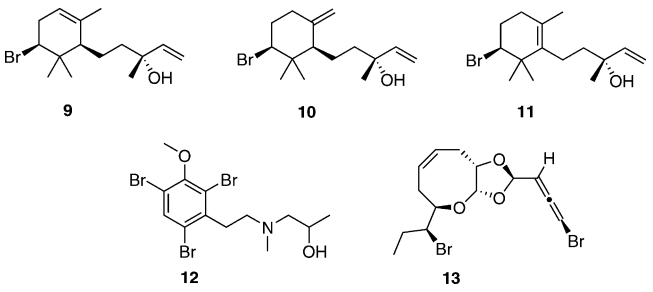


Figure 2. Brominated natural products: α-snyderol (**9**), *β*-snyderol (**10**), *γ*-snyderol (**11**), convolutamine A (**12**), and laurallene (**13**).

These four redox cofactors distribute into two pairs based on the requirement for an oxygen-based oxidant as cosubstrate. The heme- and vanadium-dependent enzymes use H_2O_2 as a cosubstrate and so are termed haloperoxidases (Table 1). By contrast, the flavin-dependent and non-heme, Fe^{2+}-dependent enzymes require dioxygen (O_2) as a reducible cosubstrate and will not work with hydrogen peroxide. This latter set of oxygen-requiring catalysts has been termed O_2-dependent halogenases (Table 1).

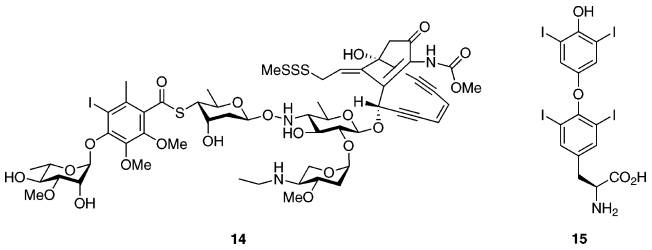
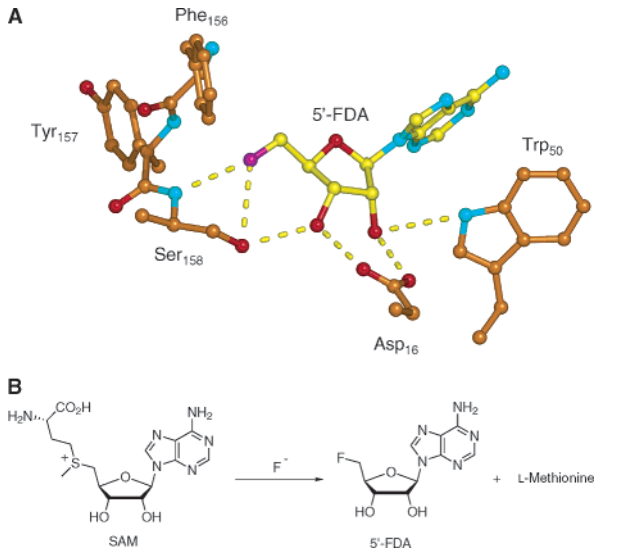


Figure 3. Iodinated natural products: calicheamicin (**14**) and tetraiodothyronine (**15**; thyroxine; T_4).

Scheme 1. Structure and Reaction Catalyzed by the Fluorinase Enzyme[a]



[a] (A) Crystal structure of the active site of fluorinase in complex with 5′-fluoro-5′-deoxyadenosine (5′-FDA) (PDB 1RQR).[21] The 5′-FDA carbons are yellow. Other carbon, oxygen, nitrogen, and fluorine atoms are orange, red, cyan, and purple, respectively. The yellow dotted lines indicate hydrogen bonds. Figure made using Pymol.[113] (B) Reaction catalyzed by fluorinase.

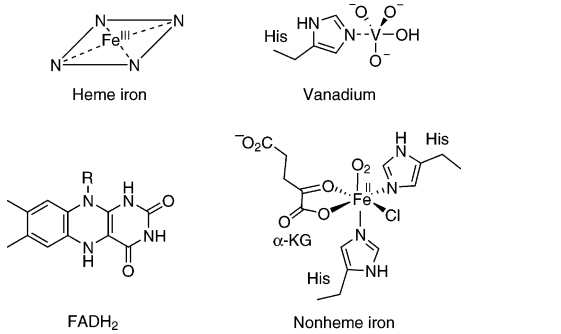
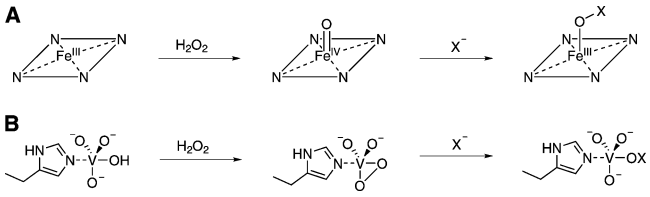


Figure 4. Redox active centers present in four different types of haloperoxidases and halogenases.

Although oxygen is employed in two different oxidation states as O_2 or as H_2O_2, there are close parallels in the logic of chloride, bromide, and iodide ion activation for C−Cl, C−Br, or C−I bond formation by the haloperoxidases and O_2-dependent halogenases. As suggested in Scheme 2, both heme- and vanadium-dependent haloperoxidases are thought

Scheme 2. Reactive Intermediates Generated by the Heme- and Vanadium-Dependent Haloperoxidases[a]



[a] (A) Intermediates in the mechanism of heme-dependent haloperoxidases. (B) Intermediates in the mechanism of vanadium-dependent haloperoxidases.

to generate bound hypohalite (−OX) intermediates. These can react as "X^+" equivalents with electron-rich substrates. Analogously, dioxygen is likely reduced in the active site of the $FADH_2$-dependent and the non-heme, Fe^{2+}-dependent halogenases to generate reactive species for halogenation (Scheme 3). Oxidation of ground-state X^- ions to generate the proximal halogenating species for C−X bond formation is the unifying theme for each of the four categories of enzymes discussed in the next sections.

4. Haloperoxidases

4.1. Heme-Dependent Haloperoxidases

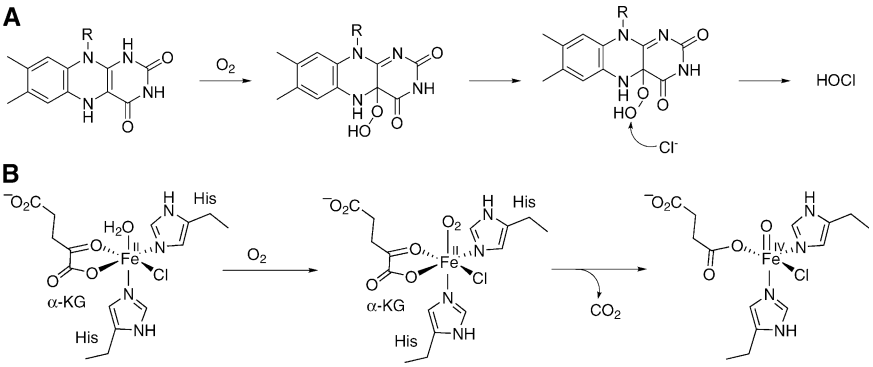
The prototypical heme-dependent haloperoxidase is the fungal enzyme from *Caldariomyces fumago* that was extensively characterized by Hager and colleagues.[24−26] A typical reaction is shown in Scheme 4, in which tandem chlorination of the enol form of the dione substrate produces the gem-dichloro metabolite caldariomycin (**16**). The substrate clearly reacts via a stabilized carbanion. Therefore, the polarity of halogen transfer should correspond to a "Cl^+" equivalent, as in an axially coordinated heme−OCl. The Fe^{III}−OCl species appears to arise by the capture of an Fe^{IV}=O/porphyrin-thiolate radical (compound I) by Cl^-.[32,33] Chloroperoxidase will also generate −OBr ("Br^+" equivalents) for substrate bromination reactions.[34]

Mammalian heme-dependent haloperoxidases are also known. In particular, a haloperoxidase in thyroid epithelial cells is responsible for a remarkable series of posttranslational oxidative modifications of tyrosyl residues in the protein thyroglobulin.[18] For example, Tyr_5 and Tyr_{130} in the 1000-residue thyroglobulin can be iodinated in the *ortho* positons of the phenolic side chains to generate monoiodo- and diiodo-Tyr residues (Scheme 5). The C−I bond formation chemistry is presumed to mirror that of the fungal chloroperoxidase described above. The regiochemistry of iodination at the *ortho* positions is consistent with nucleophilic attack by the C_3 and C_5 carbons of the electron-rich phenolic side chain on a heme−OI species in the active site for net I^+ transfer.[35] Subsequently, the diiodo ring from one residue must be transferred to the phenolate of the second diiodo-Tyr residue, which could occur by one-electron processes. The net result is C−C fragmentation of one Tyr residue (e.g., Tyr_{130}), producing $dehydroAla_{130}$, with concomitant O−C bond

Table 1. Redox Cofactors and Cosubstrates Used by Different Classes of Halogenases

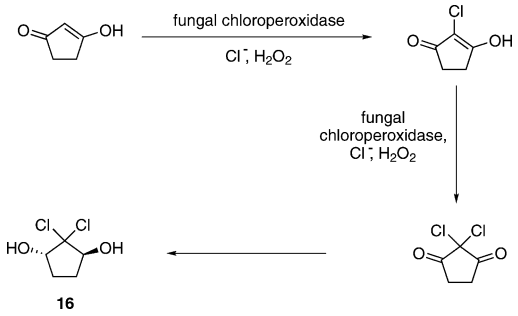
enzyme type	redox cofactor	oxidant	other cosubstrates
haloperoxidase	heme iron	H_2O_2	halide
haloperoxidase	vanadium	H_2O_2	halide
O_2-dependent halogenase	$FADH_2$	O_2	halide
O_2-dependent halogenase	non-heme iron	O_2	halide, α-ketoglutarate

Scheme 3. Reactive Intermediates Generated by the $FADH_2$- and Non-heme Fe^{2+}-Dependent Halogenases[a]



[a] (A) Intermediates in the mechanism of $FADH_2$-dependent halogenases. (B) Intermediates in the mechanism of Fe^{2+}-dependent halogenases.

Scheme 4. Formation of Caldariomycin (16) by the Prototypic Heme-Dependent Chloroperoxidase



formation at Tyr_5 producing tetraiodothyronine (**15**; T_4) at residue 5 (Scheme 5). To release the free T_4 amino acid, the iodinated, rearranged thyroglobulin is degraded by thyrocyte proteases to ultimately release the free amino acids. About one to two T_3/T_4 equivalents are generated for each thyroglobulin molecule.[18] The yield of one T_4 per thousand amino acid residues emphasizes the importance of this hormone to mammalian endocrinology.

4.2. Vanadium-Dependent Haloperoxidases

Surprisingly, when haloperoxidases isolated from red and brown algae were initially characterized, they contained stoichiometric amounts of vanadium, instead of the anticipated heme cofactor required for bromination and chlorination catalysis.[27,36,37] The resting state of vanadium-dependent

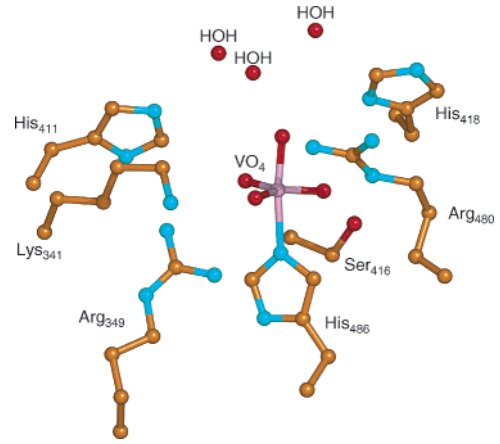
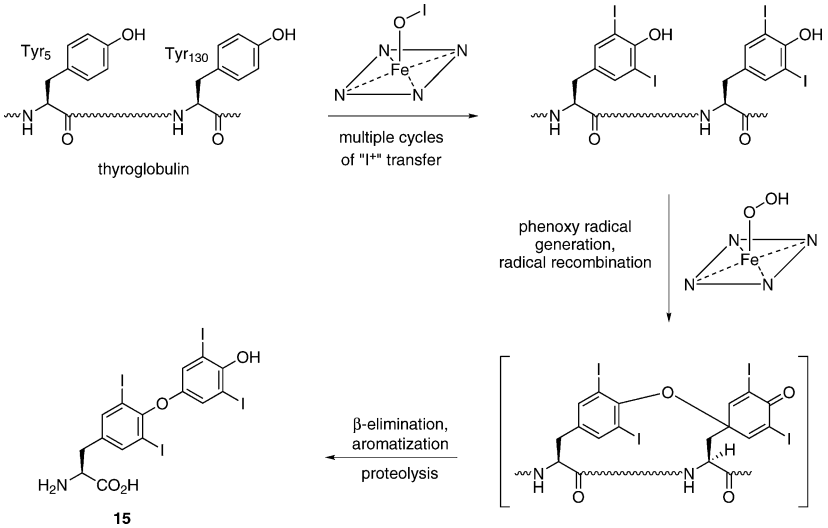


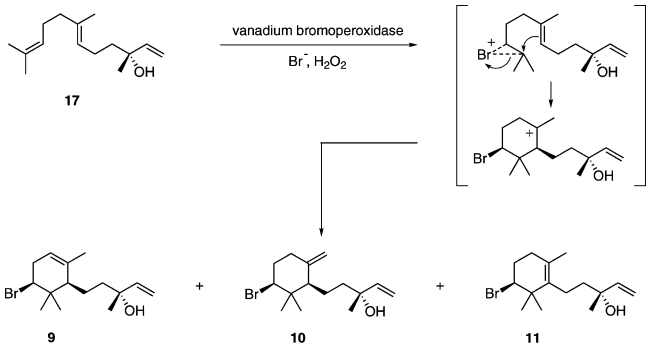
Figure 5. Structure of the active site of a vanadium-dependent haloperoxidase. The carbon, oxygen, nitrogen, and vanadium atoms are orange, red, cyan, and pink, respectively (PDB 1QI9).[39] Figure made using Pymol.[113]

haloperoxidases contains a vanadate ion ligated to one protein ligand, the imidazole ring of a histidine residue, to anchor the redox cofactor in the active site (Figure 5) at the bottom of a long channel.[38−40] The vanadate ion coordinates the incoming hydrogen peroxide. This complex is thought to oxidize the halide ion, creating a bound hypochlorite or hypobromite species as the electrophilic halogenating agent. Notably, unlike the case of heme-dependent haloperoxidases, there is no change in the oxidation state at the metal center

Scheme 5. Formation of Tetraiodothyronine (15; Thyroxine; T_4) by a Mammalian Heme-Dependent Haloperoxidase



Scheme 6. Formation of α-, β-, and γ-Snyderol (9−11) by a Vanadium-Dependent Bromoperoxidase Utilizing the Sesquiterpene Nerolidol (17)



during the generation of the halogenating species. The reaction scheme of a vanadium-dependent bromoperoxidase on the sesquiterpene nerolidol (**17**) is shown in Scheme 6, featuring a bromonium ion as an early intermediate in the haloperoxidase active site.[12] Simultaneous C−Br bond formation and C−C bond formation to close the six-membered ring generates a tertiary carbocation. Deprotonation at three possible positions can quench the carbocation and generate the monobrominated isomers of α-, β-, and γ-snyderol (**9**−**11**) as shown.[11,12]

The cumulative evidence from both heme- and vanadium-dependent haloperoxidases is that these enzymes utilize H_2O_2 to generate high-valent metal-oxo species that can react with Cl^-, Br^-, and I^- to create electrophilic −OCl ("Cl^+"), −OBr ("Br^+"), and −OI ("I^+") equivalents in their active sites. In the presence of electron-rich substrates, electrophilic halogens are delivered regiospecifically and with varying degrees of stereoselectivity.

4.3. Possible Non-heme Iron Haloperoxidases

Several studies on the isolation and initial characterization of haloperoxidases that seem to depend on non-heme ferric iron have been described.[41−44] These enzymes were isolated from algae or *Pseudomonas* species. The mechanism seems to be similar to the one described for the heme-dependent haloperoxidases except that the ligands of the iron are different. The reaction depends on the presence of halides, H_2O_2, and electron-rich aromatic substrates. Further characterization of this class of enzymes should elucidate if they indeed contribute to the formation of halogenated natural products.

5. O_2-Dependent Halogenases

In recent years, two new classes of enzymes with halogenase activity have been discovered, driven in large part by the sequencing and annotation of many microbial biosynthetic gene clusters. The first class consists of halogenases that are flavoproteins. The original member of this class is the enzyme responsible for inserting chlorine at C_7 of the tetracycline nucleus during chlortetracycline biosynthesis.[5,45] Homologues of this halogenase were subsequently found in dozens of other clusters, including those in which electron-rich aromatic side chains of natural products become chlorinated (Table 2).[4,6,10,17,45−63] A gene product resembling a flavin halogenase has even been detected in the genome of a eukaryote, *Dictyostelium discoideum*, in proximity to another gene with homology to type III polyketide synthases.[54] Given the prevalence of these halogenase-encoding genes in biosynthetic clusters of halogenated products, their presence could be useful in indicating new gene clusters that produce organohalogen compounds.

The second class of O_2-dependent halogenases turns out to be non-heme Fe^{2+} enzymes which are homologous to the

Table 2. Proven and Predicted $FADH_2$ O_2-Dependent Halogenases Encountered in Biosynthetic Gene Clusters for Halogenated Natural Products

gene name	organism	final compound produced	accession number	ref
adpC	*Anabaena circinalis* 90	anabaenopeptilides 90B	CAC01605	46
Asm12	*Actinosynnema pretiosum* subsp. auranticum	ansamitocin	AAM54090	47
aviH	*Streptomyces viridochromogenes* Tu57	avilamycin A	not deposited	48
bhaA	*Amycolatopsis mediterranei* DSM5908	balhymicin	CAA76550	49
calO3	*Micromonospora echinospora*	calicheamicin	AAM70353	17
cepH	*Amycolatopsis orientalis*	chloroeremomycin	CAA11780	50
clohal	*Streptomyces roseochromogenes* subsp. oscitans	chlorobiocin	AAN65237	51
cmlS	*Streptomyces venezuelae* ISP5230	chloramphenicol	AAK08979	6
comH	*Streptomyces lavendulae*	complestatin	AAK81830	52
Cts4	*Streptomyces aureofaciens* NRRL3203	chlortetracycline	BAA07389	45
Dbv10	*Nonomuraea* sp. ATCC 39727	A40926	CAD91205	53
DDB0189108	*Dictyostelium discoideum*	unknown	EAL62046	54
halA	*Actinoplanes* sp. ATCC 33002	pentachloropseudilin	AAQ04684	55
halB	*Actinoplanes* sp. ATCC 33002	pentachloropseudilin	AAQ04685	55
Orf3	*Streptomyces carzinostaticus*	neocarzilin	BAD38872	56
pltA	*Pseudomonas fluorescens* Pf-5	pyoluteorin	AAD24884	10
pltD	*Pseudomonas fluorescens* Pf-5	pyoluteorin	YP_259896	10
pltM	*Pseudomonas fluorescens* Pf-5	pyoluteorin	AAD24882	10
prnA	*Pseudomonas fluorescens* Pf-5	pyrrolnitrin	YP_260706	57
prnC	*Pseudomonas fluorescens* Pf-5	pyrrolnitrin	YP_260708	57
prnA	*Pseudomonas fluorescens* BL915	pyrrolnitrin	AAB97504	58
prnC	*Pseudomonas fluorescens* BL915	pyrrolnitrin	AAB97506	58
pyrH	*Streptomyces rugosporus* LL-42D005	pyrroindomycin	AAU95674	59
rebH	*Lechevalieria aerocolonigenes* ATCC 39243	rebeccamycin	CAC93722	4
sgcC3	*Streptomyces globisporus*	C-1027	AAL06656	60
staI	*Streptomyces toyocaensis* NRRL 15009	A47934	AAM80532	61
staK	*Streptomyces toyocaensis* NRRL 15009	A47934	AAM80530	61
tcp21	*Actinoplanes teichomyceticus*	teicoplanin	CAE53362	62
tha1	*Streptomyces albogriseolus*	thienodolin	not deposited	63

Table 3. Proven and Predicted Non-heme Iron O_2-Dependent Halogenases Encountered in Biosynthetic Gene Clusters for Halogenated or Cyclopropane-Containing Natural Products

gene name	organism	final compound produced	accession number	ref
barB1	*Lyngbya majuscula* 19L	barbamide	AAN32975	7
barB2	*Lyngbya majuscula* 19L	barbamide	AAN32976	7
BambDRAFT_2739	*Burkholderia ambifaria* AMMD	unknown	ZP_00688041	database
Bcen2424DRAFT_6712	*Burkholderia cenocepacia* HI2424	unknown	ZP_00458753	database
Bcep1808DRAFT_5329	*Burkholderia vietnamiensis* G4	unknown	ZP_00422612	database
BpseP_01002553	*Burkholderia pseudomallei* Pasteur	unknown	ZP_00494070	database
BpseS_01000177	*Burkholderia pseudomallei* S13	unknown	ZP_00502117	database
BpseS_01002934	*Burkholderia pseudomallei* S13	unknown	ZP_00499433	database
BPSS1183	*Burkholderia pseudomallei* K96243	unknown	CAH38651	64
BPSS1813	*Burkholderia pseudomallei* K96243	unknown	CAH39289	64
cmaB	*Pseudomonas syringae* pv. tomato DC3000	coronatine	NP_794454	65
cmaB	*Pseudomonas syringae* pv. glycinea PG4180	coronatine	AAQ93485	66
cytC3	*Streptomyces* sp. RK-95−74	cytotrienin, diCl L-Aba, diCl L-Val	not deposited	67
dysB1	*Oscillatoria spongeliae* 35P1	dysideathiazole	AAT46557	68
dysB2	*Oscillatoria spongeliae* 35P1	dysideathiazole	AAT46558	68
dysB1	*Oscillatoria spongeliae* 39P1	dysidenin	AAT46559	68
dysB2	*Oscillatoria spongeliae* 39P1	dysidenin	AAT46560	68
dysB1	*Oscillatoria spongeliae* 02155a	chlorination of leucine intermediate	AAT68250	69
herB1	*Lyngbya majuscula* (Panama)	chlorination of leucine intermediate	AAT68251	69
herB2	*Lyngbya majuscula* (Panama)	chlorination of leucine intermediate	AAT68252	69
Psyr_2610	*Pseudomonas syringae* pv. syringae B728a	phytotoxin	YP_235687	70
syrB2	*Pseudomonas syringae* pv. syringae B301D	syringomycin E	AAD50521	8
curA	*Lyngbya majuscula* (Curacao)	curacin A	AAT70096	71
jamE	*Lyngbya majuscula* JHB	jamaicamides	AAS98777	72

two-His, one-carboxylate family of non-heme Fe^{2+} oxygenases that require α-ketoglutarate as a cosubstrate.[7,8,64−72] Several of these orfs also map to biosynthetic gene clusters for halogenated or cyclopropane-containing (see section 7) natural products (Table 3).

Each of these two classes of halogenases requires O_2 as a cosubstrate that is reduced during the catalytic cycle. We shall note that the choice of flavin or non-heme Fe^{2+} correlates with the oxidative potential of the carbon site to be halogenated, such that the redox power of the flavin- or the stronger iron-based oxo reagent is titrated to the substrate reactivity.

5.1. Two-Component $FADH_2$-Dependent Halogenases

The seminal work of van Pée and colleagues established key requirements for the class of flavin-dependent halogenases. They successfully reconstituted *in vitro* the activity of PrnA, a tryptophan-7-halogenase, as the first step of pyrrolnitrin biosynthesis.[28] Both O_2 and FAD were required for activity. Furthermore, they determined that FAD was presented as $FADH_2$ and that the dihydro $FADH_2$ form was provided by a second enzyme, an NAD(P)H-dependent flavin reductase. The two-component flavin reductase/halogenase utilizes the redox cofactor $FADH_2$ as a diffusible intermediate between the two enzyme components to catalyze chlorination of the substrate tryptophan in the following reactions:

[Reductase]

$$\mathrm{FAD} + \mathrm{NADH} + \mathrm{H^+} \rightarrow \mathbf{FADH_2}\ (\text{diffusible}) + \mathrm{NAD^+}$$

[Halogenase]

$$\mathbf{FADH_2} + \mathrm{O_2} + \mathrm{Cl^-} + \text{L-Trp} + \mathrm{H^+} \rightarrow \mathrm{FAD} + \text{7-Cl-L-Trp} + 2\mathrm{H_2O}$$

Similarly, the two enzymes RebF, a flavin reductase, and RebH, a flavin halogenase, have also been demonstrated to catalyze 7-chlorotryptophan formation at the start of the rebeccamycin pathway.[73]

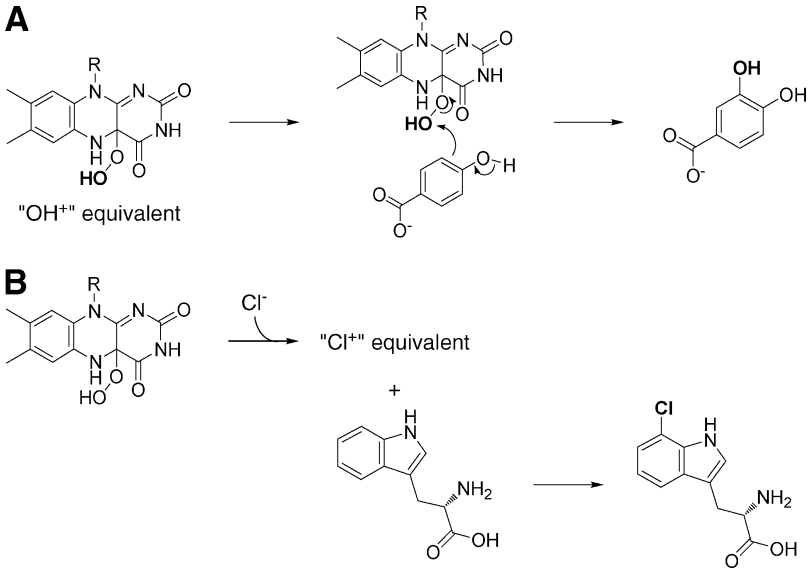
Flavin-dependent halogenases share high sequence homology with flavoprotein monooxygenases. Such flavoproteins can also be found as two-component systems with separate reductase and oxygenase partners,[74−77] but much more often the two activities are fused in one protein. The reactions catalyzed by several flavoprotein monooxygenases have been extensively studied and provide an important basis for investigation of the flavin-dependent halogenase mechanism. First, NAD(P)H reduces bound FAD to $FADH_2$, which is now an O_2-reactive form of the cofactor (in contrast to air-stable FAD). A facile reaction with O_2 proceeds by single-electron pathways through the flavin semiquinone and $O_2^{\bullet-}$, followed by regiospecific radical recombination to the FAD-C_{4a}-OOH hydroperoxyflavin adduct. Among hydroxylases, this versatile peroxide species can deliver an electrophilic hydroxyl group "OH^+" to nucleophilic substrates bound in the enzyme active sites.[78] The prototypical reaction is shown for *ortho* hydroxylation of 4-hydroxybenzoate in Scheme 7A, wherein the electron-rich phenol ring allows transfer of "OH^+" from the FAD-OOH in the enzyme active site.[78−80]

5.1.1. Electrophilic Chlorine at the Active Site of Flavoprotein Halogenases

For the flavoprotein halogenases, we intuit similar logic. The requirement for O_2 and $FADH_2$ is to generate FAD-C_{4a}-OOH as a common intermediate in the enzyme active site.[81] Now Cl^- could react either at the distal oxygen or the proximal oxygen of the peroxide and produce an HOCl equivalent in the active site or an FAD-OCl, both with presumably comparable reactivity as sources of "Cl^+" (Scheme 7B).

To resolve this dichotomy requires more detailed mechanistic studies as well as structural insight into flavin-dependent halogenases. Such insight has recently been provided by the X-ray structure of PrnA, which shows the substrate tryptophan 10 Å away from the bound FAD, strongly suggesting a diffusible OCl^- within a tunnel in the active site.[82] The OCl^- would move from its point of generation at the FAD site to its point of consumption by the bound tryptophan substrate (Figure 6). From the structure,

Scheme 7. Analogy between Reactions Catalyzed by a Flavin-Dependent Hydroxylase and a Halogenase[a]



[a] (A) In *p*-hydroxybenzoate hydroxylase, a representative flavoprotein monooxygenase, an FAD(C4a)-OOH intermediate acts as an "OH^+" equivalent for electrophilic aromatic substitution of position 3 of *p*-hydroxybenzoate. (B) In the active site of a flavin-dependent halogenase, an FAD-OOH intermediate likely oxidizes Cl^- to form a "Cl^+" equivalent for halogenation. Chlorination by such an oxidized chlorinating agent is shown for the formation of 7-chlorotryptophan by PrnA.

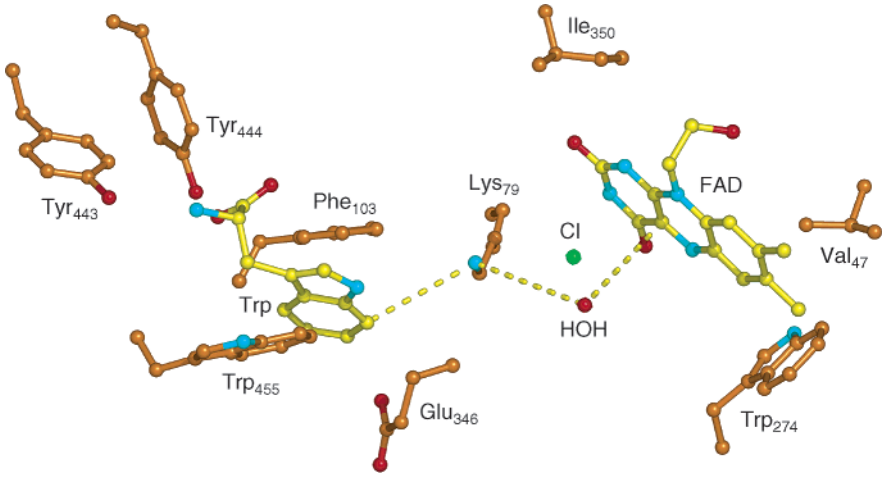


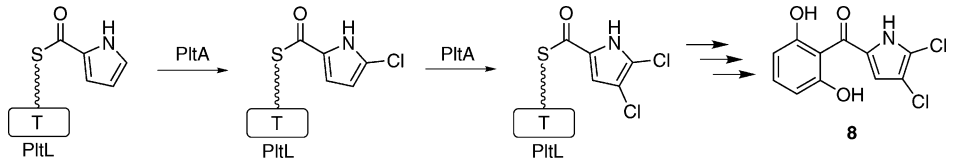
Figure 6. Structure of tryptophan 7-halogenase (PrnA) with FAD, tryptophan, and chloride bound. The yellow dotted lines indicate the most probable path for HOCl from FAD to chlorination of position 7 of the tryptophan substrate. Only a truncated FAD molecule is shown for clarity. The FAD and tryptophan substrate carbons are yellow. Other carbon, oxygen, nitrogen, and chloride atoms are orange, red, cyan, and green, respectively (PDB 2AQJ).[82] Figure made using Pymol.[113]

lysine 79 is proposed to make a critical hydrogen bond with the diffusing HOCl to orient it for reaction with tryptophan. Potentially, the ϵ-NH_2 of Lys_{79} could itself react with HOCl to form a chloramine intermediate, and the Lys-NH-Cl could then react with tryptophan to specifically chlorinate position 7 of the indole ring. The intermediacy of a chloramine adduct has been suggested for chlorination of protein residues by myeloperoxidase-generated HOCl.[83,84] Supporting the initial formation of HOCl, stopped-flow spectroscopy studies of the RebH reaction have detected multiple flavin intermediates during the oxidation of $FADH_2$ to FAD, including likely FAD-OOH and FAD-OH intermediates. The product formation rate determined by rapid quench indicates that C−Cl bond formation is rate determining in the overall reaction and slow compared to rates for formation/decay of flavin intermediates, consistent with formation of an OCl^- equivalent before chlorination of tryptophan.[81]

An alternative mechanism has been proposed, in which tryptophan and FAD-OOH react to generate an arene oxide form of tryptophan. The C−Cl bond would be formed upon nucleophilic addition of chloride ion at C_7, followed by dehydration to rearomatize the product.[85] This mechanism seems less likely, given the general oxidative strategy employed by biological halogenation catalysts. In addition, this mechanism is unlikely to account for the tandem 4,5-dichlorination of the pyrrole ring in pyoluteorin biosynthesis, where chlorination by OCl^- equivalents seems chemically preferable (discussed in section 5.1.2).

The specificity of a free hypochlorite-based chlorination must be stringently controlled by the orientation and accessibility of substrate to diffusing OCl^- within the halogenase active site. Given the challenge of maintaining specificity in the reaction, this class of enzyme displays an impressive versatility since other flavin-dependent halogenases that chlorinate tryptophan with distinct regiochemistries, chlorinating specifically at C_5 or C_6 rather than at C_7, have been described.[59,63] The availability of these alternative tryptophan halogenases has been useful for *in vivo* reprogramming and

Scheme 8. Timing of Dichlorination by PltA during Biosynthesis of Pyoluteorin (8)



generation of rebeccamycin analogues with altered chlorination patterns.[86]

5.1.2. Timing of Chlorination during Assembly Line Catalysis

While chlorination of the indole ring in pyrrolnitrin and also rebeccamycin biosynthesis occurs on free tryptophan as the first step in these pathways, determination of the timing of halogenation during the construction of nonribosomal peptides or polyketides has been more difficult. These natural products are constructed on large nonribosomal peptide synthetases (NRPSs) or polyketide synthases (PKSs), multimodular enzymatic assembly lines in which intermediates are tethered on carrier proteins.[87] For example, in the vancomycin family of glycopeptides or the aromatic polyketide chlortetracycline, there are indications that halogenation could occur while the elongating acyl chain is still tethered to carrier proteins within these assembly lines.[3,5]

It has been possible to address this question in the biosynthesis of pyoluteorin, an antifungal biocontrol agent produced by *Pseudomonas fluorescens*.[10] This hybrid polyketide−nonribosomal peptide is constructed from proline and three malonyl-CoA units. The proline is activated as prolyl-AMP and then covalently tethered as a thioester to the phosphopantetheinyl prosthetic group of a nonribosomal peptide synthetase thiolation (T) domain.[88] While tethered, the prolyl ring undergoes two tandem two-electron oxidations to yield the pyrrolyl-*S*-T domain that turns out to be the substrate for PltA,[88] an O_2- and $FADH_2$-requiring halogenase.[89]

PltA first chlorinates at C_5 of the pyrrolyl-*S*-T domain and then installs a second chlorine at C_4 to generate the 4,5-dichloropyrrolyl moiety of pyoluteorin covalently attached to the carrier protein PltL (Scheme 8).[89] This will become the acyl donor for the polyketide synthase modules to create the resorcinol ring and finish the assembly of pyoluteorin before it is released from the most downstream thiolation domain of the assembly line. It is likely that tailoring of acyl-*S*-protein intermediates during NRPS and PKS assembly by dedicated halogenases will be the rule, including the *ortho* chlorination of tyrosyl residues during assembly of vancomycin family glycopeptide antibiotics.[3]

An interesting variation on halogenation by aromatic substitution is predicted to be mediated by the flavoprotein halogenase CmlS during formation of the dichloroacetyl moiety of the antibiotic chloramphenicol.[6] Given the likelihood that "Cl^+" equivalents are generated in this halogenase class, it is probable that an acetyl thioester, either acetyl-CoA or an acetyl-*S*-carrier protein, will be the substrate, since such thioesters enable low-energy carbanion formation at C_2 of the acetyl moiety as the thioester enolate to attack an OCl^-. The monochloroacetyl thioester as a nascent product could undergo a second, tandem chlorination by the same mechanism to yield the dichloroacetyl thioester that would be the substrate for acylation of the *para*-nitrophenylalanine scaffold. Alternatively, a malonyl thioester could also undergo dichlorination followed by decarboxylation to yield the substrate for acylation. A similar strategy is likely employed in the biosynthesis of the di- and trichloro moieties of neocarzilins.[56]

5.2. Non-heme Iron Halogenases for Unactivated Carbon Sites in Substrates

Several considerations suggested that there may be an additional class of halogenases beyond the flavoproteins. First, sequencing of several biosynthetic clusters which produced halogenated natural products did not identify flavoprotein halogenases among the annotated orfs. Second, the halogenated molecules in these instances contained C−Cl bonds on *aliphatic carbons*, often remote from activating groups (e.g., barbamide).[7] The low chemical reactivity of these substrate aliphatic carbons, compared to the electron-rich aromatic rings that are the typical substrates of flavin-dependent halogenases, suggested that a different class of enzymatic catalysts would be required for these unactivated substrates.

Extending the analogy between biological hydroxylation and halogenation discussed in the context of flavoprotein catalysts, when nature carries out hydroxylation at unactivated carbon sites (e.g., octane to 1-octanol), it turns to iron enzymes and generates high-valent oxoiron species as powerful oxidants.[90] Iron in two microenvironments can serve this catalytic oxygenation purpose in biology. The most familiar are heme-dependent monooxygenases exemplified by cytochrome P450s,[91] but non-heme iron enzymes which decarboxylate the cosubstrate α-ketoglutarate are also frequent microbial oxygenases.[92] Enzymes in the latter class contain a two-His, one-carboxylate (Asp/Glu) structural motif.[92,93]

It turns out that the non-heme iron enzyme precedent is relevant to the second class of O_2-dependent halogenases. The first example is found in the *Pseudomonas syringae* biosynthetic gene cluster for the lipo-nonapeptidolactone syringomycin E (**6**; Figure 7).[8,94] Residue nine in this nonribosomal peptide is a 4-Cl-Thr. Chlorination of the CH_3 group of Thr occurs after the NRPS module SyrB1 activates threonine as the Thr-AMP and then installs it on the

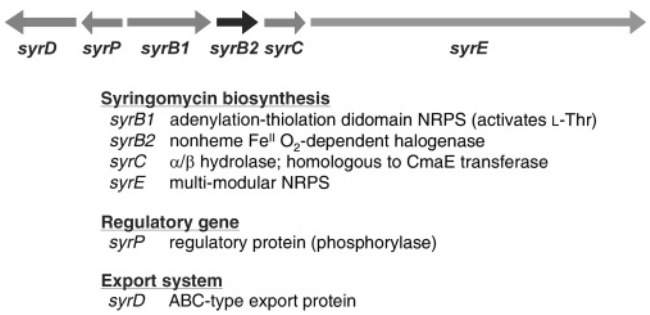
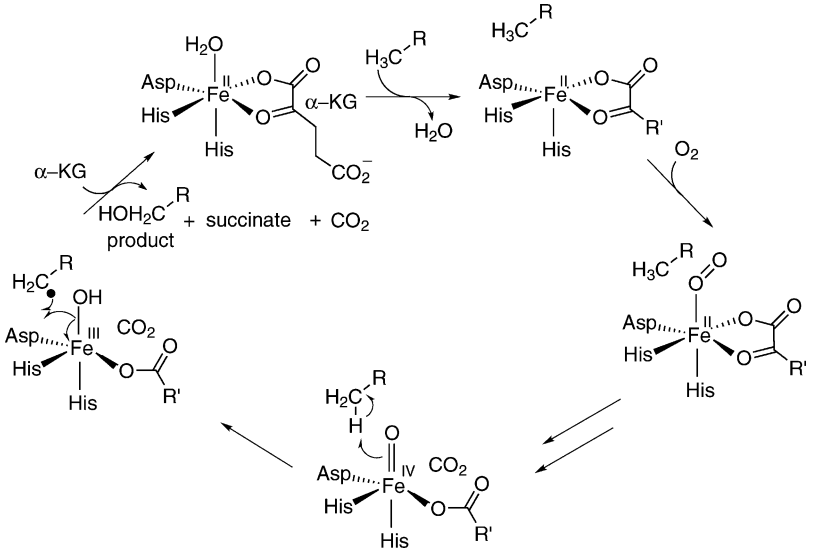
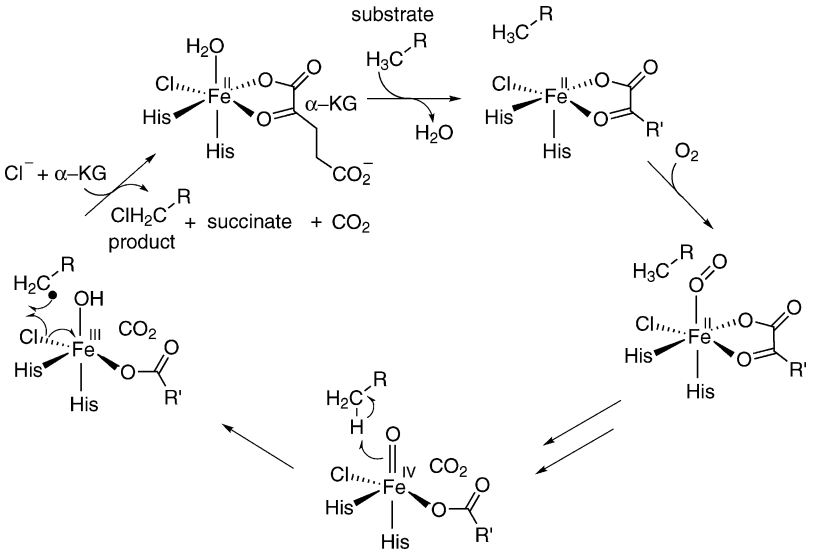


Figure 7. Biosynthetic gene cluster for the lipo-nonapeptidolactone syringomycin E (**6**) produced by *Pseudomonas syringae* pv. *syringae* B301D.

Scheme 9. Catalytic Mechanism of Non-heme Iron O_2- and α-Ketoglutarate-Dependent Hydroxylases



Scheme 10. Catalytic Mechanism of Non-heme Iron O_2- and α-Ketoglutarate-Dependent Halogenases



phosphopantetheinyl arm of its thiolation (T) domain in typical NRPS logic.[29] The Thr-*S*-SyrB1 protein is the substrate for SyrB2, a novel halogenase that requires Fe^{2+}, O_2, α-ketoglutarate, and chloride ions.[29] The requirement for Fe^{2+}, O_2, and α-ketoglutarate is the hallmark of the two-His, one-carboxylate α-ketoglutarate-dependent oxygenase reactions, and SyrB2 falls into that protein superfamily. However, it shows chlorinating activity, and no hydroxylation of the threonyl moiety presented by SyrB1 is observed. The 4-Cl-Thr-*S*-SyrB1 product is then used by the NRPS assembly line enzyme SyrE to complete syringomycin E elongation and cyclization.[8,95] In the presence of bromide and the absence of chloride, the SyrB2 enzyme is also able to brominate its Thr-*S*-SyrB1 substrate to yield 4-Br-Thr-*S*-SyrB1.[96]

The α-ketoglutarate-requiring Fe^{2+} oxygenases decarboxylate the keto acid during the catalytic cycle to create a potent Fe^{IV}═O species which can abstract H• from substrate-CH_3 groups to yield Fe^{III}—OH and the substrate CH_2• radical in proximity to each other in the active site. Radical rebound involves OH• transfer to the methylene radical, yielding the observed alcohol product (Scheme 9) and regenerating the starting Fe^{2+} oxidation state. The key to the catalytic strategy is generation of the high-valent oxoiron (Fe^{IV}═O) species that removes a hydrogen atom from even the most unactivated carbon sites of a bound substrate.[92,97]

It is reasonable to expect that SyrB2 and other non-heme Fe^{2+} halogenases use the same chemical logic to generate the Fe^{IV}═O oxidant, but do so with a chloride (or bromide) ligand in the coordination sphere of the catalytic iron. Then, after H• abstraction, the halogenase would contain an Fe^{3+} with both an −OH and a −Cl ligand (Scheme 10) as potential sources of OH• or Cl• transfer to the substrate CH_2•. In SyrB2, catalytic chlorination, not hydroxylation, is the observed outcome, suggesting a specific orientation of substrate-derived radical and the chlorine on the active site iron. Competition between OH• and Cl• transfer to a cyclohexane-derived radical is observed in model reactions catalyzed by chromyl chloride, while a model iron compound that likely forms an oxoiron species reacts with cyclohexane to specifically yield the chlorinated product.[98−100]

Insight into how SyrB2 acts as a halogenase rather than a hydroxylase is obtained from the X-ray structure of SyrB2 with α-ketoglutarate and bound chloride or bromide ion.[31] Most surprising but satisfying is the finding that, while SyrB2 has the anticipated two His ligands, the third protein ligand,

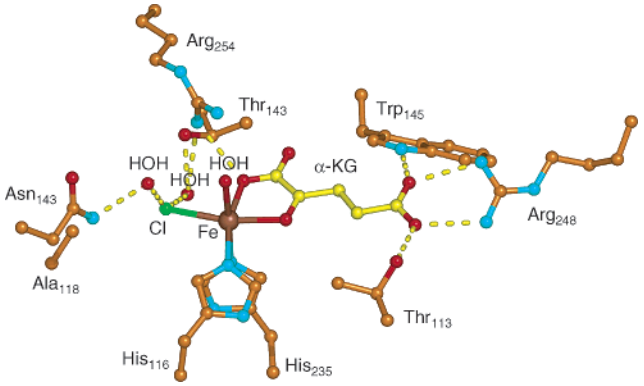


Figure 8. Structure of the SyrB2 halogenase with α-ketoglutarate and chloride bound. The α-ketoglutarate carbons are yellow. Other carbon, oxygen, nitrogen, chloride, and iron atoms are orange, red, cyan, green, and brown, respectively (PDB 2FCU).[31] The yellow dotted lines indicate hydrogen bonds. Figure made using Pymol.[113]

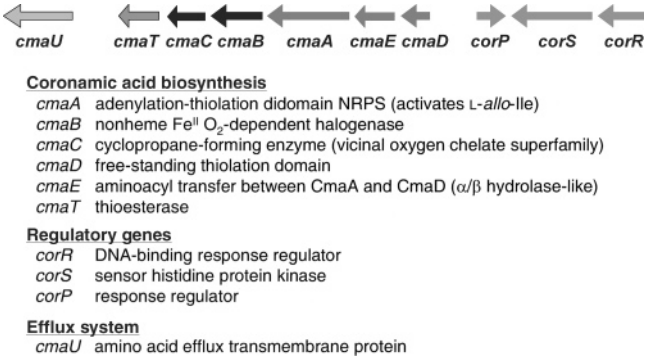


Figure 9. Biosynthetic gene cluster for coronamic acid produced by *Pseudomonas syringae* pv. *tomato* DC3000.

a side chain Asp or Glu carboxylate in the hydroxylases, is not present. Rather, the residue in the location normally occupied by Asp or Glu is Ala. The absence of the side chain $R-CO_2^-$ creates an opportunity for chloride or bromide to substitute for the missing protein ligand (Figure 8).[31] Indeed, Fe^{2+} is bound to apo-SyrB2 almost 3 orders of magnitude more tightly in the presence of chloride,[101] consistent with completion of the ligand set. As anticipated, α-ketoglutarate binds in bidentate fashion to occupy the fourth and fifth coordination sites on Fe^{2+} in the resting state of the enzyme.[31]

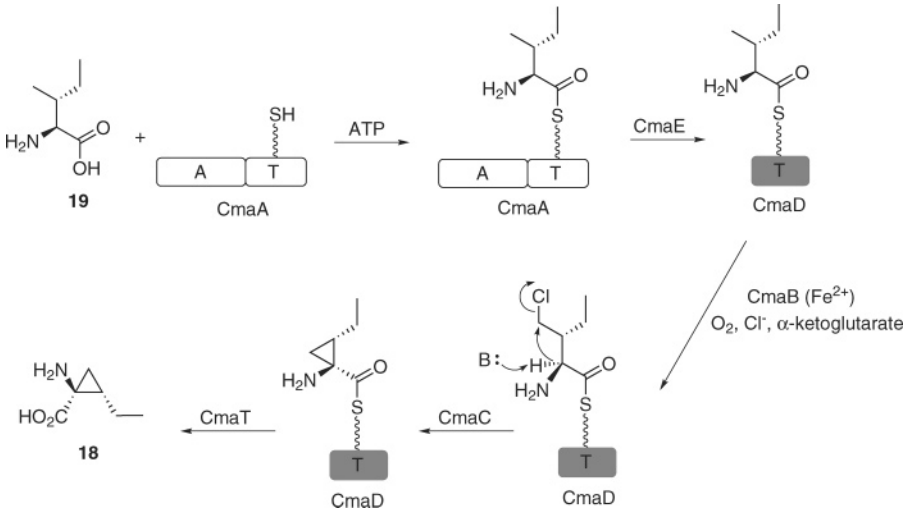
During a catalytic cycle when $Fe^{IV}=O$ is generated and presumably abstracts $H^\bullet$ to generate $-CH_2^\bullet$, the Fe^{3+} species has the requisite chlorine ligand as a source of $Cl^\bullet$ for the radical rebound chlorination mechanism. As a structural corollary, it is likely that the halogenase activity evolved from hydroxylase activity by mutation of Asp or Glu to Ala to convert enzymes from Fe−His_2Asp to Fe−His_2 to provide the vacant coordination site for halide binding. It will be of interest to see if one can swap hydroxylase and halogenase activities in other members of the α-ketoglutarate and O_2-requiring iron enzymes by such directed Asp/Glu ⇔ Ala mutations.

6. Cryptic Chlorination during Cyclopropyl Ring Construction at Unactivated Carbons

Bioinformatic analysis of homologues to SyrB2 in other microbial genomes should provide leads to additional members of the non-heme iron, O_2-utilizing halogenases, and more than two dozen are found in microbial genome databases (Table 3), including the barbamide and curacin biosynthetic gene clusters.[7,71] Most intriguing is a homologue CmaB in the coronamic acid biosynthetic gene cluster from a strain of *Pseudomonas syringae* (Figure 9).[65,66] Inspection of the structure of coronamic acid (**18**; 1-amino-1-carboxy-2-ethylcyclopropane) and its biosynthetic precursor, L-*allo*-Ile (**19**), does not give any hint of chlorination chemistry.[102,103] However, there is a significant chemical problem in conversion of the unactivated γ-position CH_3 of the L-*allo*-Ile skeleton to the α,γ-cyclopropane ring in coronamic acid.

In fact, nature uses an NRPS halogenation strategy much like that just noted for syringomycin E.[29] L-*allo*-Ile is activated by a comparable A−T didomain NRPS protein (CmaA) to the L-*allo*-Ile-*S*-phosphopantetheinyl thioester (Scheme 11).[66] The L-*allo*-Ile-*S*-CmaA is not a substrate for CmaB. The amino acid is effectively shuttled from CmaA to the free-standing thiolation domain CmaD by the CmaE acyltransferase.[30] The L-*allo*-Ile-*S*-CmaD is the substrate for CmaB that is also a chloride-, α-ketoglutarate-, and O_2-requiring, non-heme iron halogenase.[30] The product from CmaB action is the γ-Cl-L-*allo*-Ile-*S*-CmaD thioester. This substrate is then transformed by the enzyme CmaC, which catalyzes the intramolecular displacement of chloride, most likely via the thioester enolate.[30] The 1-amino-2-ethyl-cyclopropyl-*S*-CmaD is then likely hydrolyzed by the specific

Scheme 11. Biosynthesis of Coronamic Acid (18) from L-*allo*-Ile (19) by CmaA, CmaB, CmaC, CmaD, and CmaE



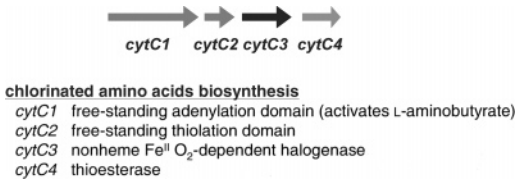


Figure 10. Biosynthetic gene cluster for chlorinated amino acids and the precursor of the 1-aminocyclopropane carboxylic acid (ACC) moiety of cytotrienin produced by *Streptomyces* sp. RK-95-74.

thioesterase CmaT to yield free coronamic acid. In this system, an elegant piece of activation chemistry is accomplished by a cryptic biological chlorination strategy.

We anticipated this route to convert unactivated methyl groups to cyclopropane rings would be utilized in the biosynthesis of other natural products and have cloned a set of four contiguous genes from the cytotrienin-producing *Streptomycete* (Figure 10) that converts L-aminobutyrate (**20**) to γ-chloroaminobutyrate by the same strategy, with CytC3 as the non-heme, Fe^{2+}-, O_2-dependent halogenase to make the γ-chloroaminobutyryl-*S*-CytC2 protein intermediate.[67] This species can be subject to the same kind of intramolecular displacement by CmaC (the CmaC homologue has yet to be found in *Streptomyces* sp. RK-95-74) to yield the aminocyclopropyl thioester that can be hydrolyzed by CytC4.[67]

7. Multiple Chlorinations, Vinyl Halides, and Cyclopropanes

If the CytC1−C2−C3 system is allowed to proceed through multiple turnovers, CytC3 will chlorinate sequentially to yield γ,γ-dichloroaminobutyryl-*S*-CytC2 (Scheme 12A).[67] The free dichloroamino acid, which would then arise by hydrolytic release of the thioester, is a known antibacterial natural product (armentomycin), and this path is likely its biosynthetic origin.[104] By analogy, the trichloromethyl group of barbamide (**5**) is installed by tandem trichlorination of a L-Leu-*S*-phosphopantetheinyl protein in the barbamide pathway by two SyrB2 homologues, BarB1 and BarB2.[7,105] BarB2 was shown to efficiently catalyze the dichlorination of L-Leu-*S*-BarA, and BarB1 was shown to catalyze the addition of the third chlorine atom (Scheme 12B).[105] In addition, when the reaction catalyzed by SyrB2 is performed with an excess of SyrB2, the formation of diCl-L-Thr-*S*-SyrB1 is observed.[96] Investigation of the rates and mechanism for a second and third halogenation at a single carbon center should further understanding of the difficult chemistry carried out by non-heme, Fe^{2+}-, O_2-dependent halogenases.

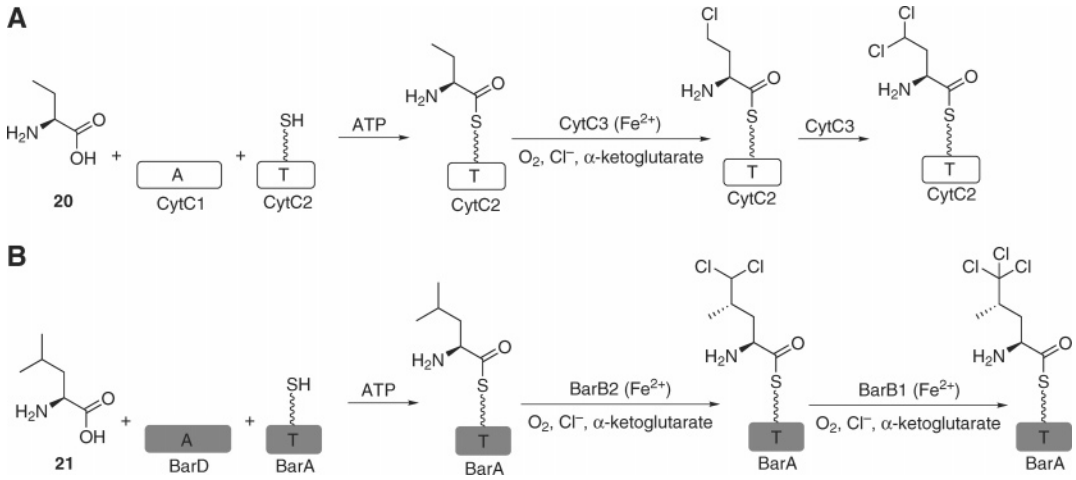
The catalytic capability of the non-heme iron halogenases is impressive, and many diverse examples of this biological chemistry are starting to be uncovered from PCR cloning of known halogenase homologues.[69] For example, the cyanobacterial metabolite jamaicamide contains a vinyl chloride moiety that is part of a branched chain that is thought to be introduced by HMG-CoA synthase type activity on a β-keto acyl-*S*-ACP intermediate.[72] Thioester hydrolysis, decarboxylation, and dehydration could yield a Δ^3-isopententenyl-*S*-ACP (Scheme 13).[72] Isomerization to the conjugated Δ^2-isopentenyl-*S*-ACP could proceed by a facile α-carbanion-mediated enzymatic process. Inspection of the jamaicamide protein JamE indeed suggests a non-heme Fe^{2+} halogenase domain.[72] Chlorination of the Δ^2-isopentenyl-*S*-ACP, as shown, could yield the vinyl chloride as a branch point substituent. In the curacin biosynthetic cluster, a similar isopentenyl-*S*-ACP intermediate can be formulated; a common intermediate could yield the divergent vinyl chloride and chloride-free methylcyclopropane substituents (Scheme 13).[71] For curacin, the 4-Cl-Δ^2-isopentenyl-*S*-ACP, if reduced by a flavoprotein reductase to a 4-Cl-3-methyl saturated chain, could yield the thioester enolate and cyclize to give the methyl cyclopropyl substituent at the start of the curacin chain. This biosynthetic logic would tie isopentenyl metabolism to halogenation and cyclopropane ring formation.

8. Other Halogenation Reactions Observed in Different Classes of Enzymes

Other types of halogenation reactions have been reported in different classes of enzymes. Whether these reactions contribute to the production of halogenated natural products remains to be shown.

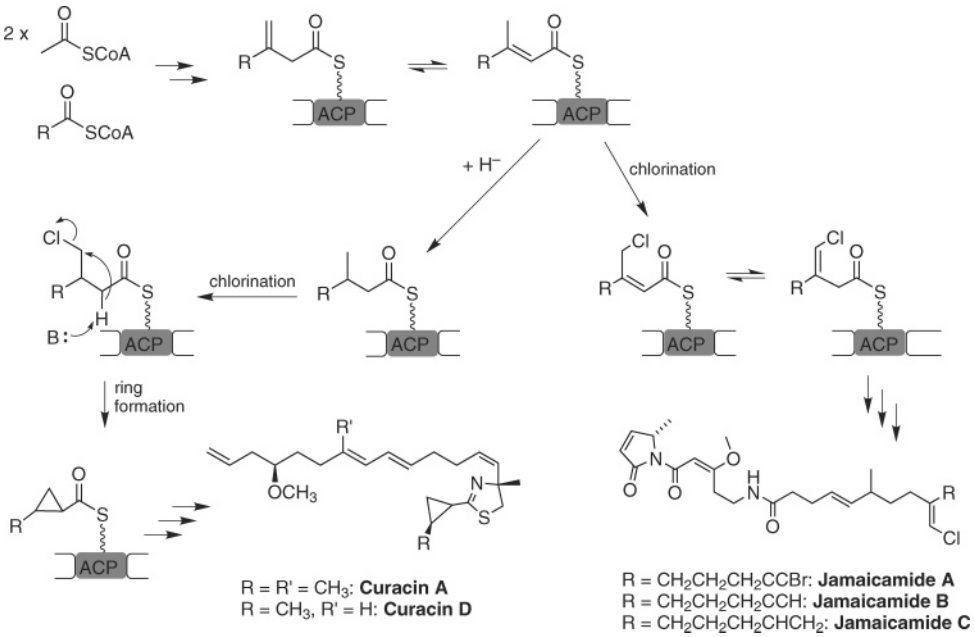
One of these reactions is catalyzed by a class of enzymes termed perhydrolases.[106−109] The reaction depends on the presence of H_2O_2, halide ions, and carboxylic acids. This class of enzymes contains a catalytic triad (Ser-His-Asp) in which the formation of an acyl intermediate on a Ser residue is thought to be a key step in the formation of peracids.[106] Interestingly, one perhydrolase was shown to be activated

Scheme 12. Multiple Chlorinations Catalyzed by Non-heme Iron O_2-Dependent Halogenases[a]

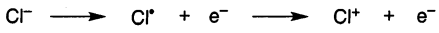


[a] (A) Mono- and dichlorinations of L-aminobutyrate (**20**) catalyzed by CytC3. (B) Formation of the trichloromethyl group of barbamide (**5**) by tandem trichlorination of L-leucine (**21**) with BarB1 and BarB2.

Scheme 13. Proposed Biosynthesis of Curacin and Jamaicamides: Formation of a Cyclopropyl Ring and a Vinyl Halide[a]



[a] Isomerization of the Δ^3-isopentenyl-*S*-ACP to the conjugated Δ^2-isopentenyl intermediate generates the terminal CH_3 group that, in the case of jamaicamide (right), can be halogenated by an iron halogenase domain found in the NRPS synthetase JamE. Conversion back to the Δ^3-isomer would yield the vinyl halide found in the final jamaicamide structure. In curacin biosynthesis (left), the Δ^2-isopentenyl-*S*-ACP can be diverted by reduction to the saturated aliphatic acyl chain. Following halogenation by an iron halogenase domain present in the NRPS synthetase CurA, the chlorinated intermediate could cyclize to form the cyclopropyl ring found in the final compound.



Cl species:	Reacts with:	Biological examples:
Cl^- (most abundant)	Carbon electrophiles	(Methyl chloride transferase)
$Cl^•$	Carbon radicals	Nonheme iron halogenase
Cl^+ (as hypochlorite)	Carbon nucleophiles	Heme-dependent haloperoxidase Vanadium-dependent haloperoxidase Flavin-dependent halogenase (Nonheme-dependent haloperoxidase) (Perhydrolase)

Figure 11. Oxidation states of chlorine for halogen transfer.

by Co^{2+} ions, but the mechanism is unknown.[109] The perhydrolase reaction was recently shown to be due to an extra hydrogen bond present in certain enzymes that stabilized the H_2O_2 in the active site.[108] It is likely that this reaction is not of quantitative significance to biosynthetic halogenations and is simply a slow side reaction of hydrolytic enzymes. In the past, this type of enzyme was thought to be responsible for the halogenation of several natural products (e.g., balhimycin). However, it was later shown that an $FADH_2$-dependent halogenase was actually responsible for catalyzing the reaction in the biosynthesis of balhimycin.[107]

Another reaction has also been described in the formation of methyl halides catalyzed by methyl halide transferase. In this reaction mechanism, the halide reacts as a nucleophile with a SAM cofactor to displace *S*-adenosylhomocysteine.[110−112] Whether this reaction is biologically relevant in the formation of methyl halides also remains to be shown.

9. Unifying Perspective for Oxidative Catalysis during Biological Halogenations

This inventory of biological halogenation catalysts suggests that nature employs a general oxidative strategy. At the outset, fluoride ions are likely to segregate as a separate reactivity class from chloride, bromide, and iodide ions. The high nucleophilicity of fluoride and low reactivity toward biological oxidants distinguishes it from these more polarizable halide ions and suggests that a direct nucleophilic attack by fluoride ion is likely to be the only route for rare C−F bond formation in biology.

Figure 11 notes that the abundant, stable ground-state halide ions (e.g., chloride ion) can react as nucleophiles; but as there are few carbon electrophiles in biological metabolites, nucleophilic halogenation in biological systems seems rare for Cl^-, Br^-, and I^- (an exception is the reaction catalyzed by methyl halide transferases; see section 9). One-electron oxidation of chloride ion yields a chlorine atom while two-electron oxidation yields Cl^+. We have noted that $Cl^•$ (and $Br^•$ and presumably $I^•$) equivalents can be generated from Fe−Cl ligands in enzyme active sites. The two-electron-oxidized forms of halide ("X^+") are generated as hypohalite forms by heme-, vanadium-, and $FADH_2$-dependent halogen-incorporating enzymes.

The enzymatic logic and catalytic machinery for halogenation appears to have been borrowed from hydroxylation logic. Flavoprotein oxygenases generate FAD-C_{4a}-OOH as a source of "OH^+" to transfer to electron-rich aromatic and heteroaromatic rings.[78] The FAD-C_{4a}-OOH in halogenase active sites

may yield OCl^- for the corresponding delivery of "Cl^+" to the same kinds of electron-rich cosubstrates.

Even more striking is the redirecting of non-heme Fe^{2+}-, α-ketoglutarate-dependent oxygenases for tailoring of aliphatic carbon sites. In hydroxylases, the Fe^{IV}═O species generates a substrate radical and then delivers $OH^•$ for radical recombination. In halogenases, the Fe^{IV}═O is generated with chloride (bromide) already bound to iron such that $Cl^•$ ($Br^•$) can be delivered to the substrate radical instead.

Cloning of flavoprotein halogenase homologues and non-heme iron halogenase homologues from microbial genomes should aid identification of biosynthetic gene clusters of halogenated natural products and speed discovery of novel organohalogen metabolites that may have useful activities in biological systems. An added bonus may be detection of biosynthetic clusters for known and unknown cyclopropanoid and branched-chain olefinic natural products.

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