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Review

Halogenation Strategies In Natural Product Biosynthesis

Christopher S. Neumann,[1] Danica Galonić Fujimori,[1] and Christopher T. Walsh[1,*]
[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
*Correspondence: Christopher_Walsh@hms.harvard.edu
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Halogenation is a frequent modification of secondary metabolites and can play a significant role in establishing the bioactivity of a compound. Enzymatic halogenation through oxidative mechanisms is the most common route to these metabolites, though direct halogenation via halide anion incorporation is also known to proceed through both enzymatic and nonenzymatic pathways. In this article, we review the current state of knowledge regarding the mechanisms of these transformations, highlight applications of this knowledge, and propose future opportunities and challenges for the field.

Introduction

Compounds containing carbon–halogen bonds constitute a diverse group of natural products that display a wide range of biological activities, including anticancer and antibiotic properties. Over 4000 halogenated products have been isolated from natural sources (Gribble, 2004). Chlorination is the predominant modification, followed by bromination, while iodination and fluorination are rare in Nature. Examples of halogenated natural products include the remarkably potent anticancer agents β-lactone salinosporamide A, macrocyclic lactone polyether spongistatin, indolocarbazole rebeccamycin, and enediyne calicheamicin γ_1^I, and a number of antibiotics, such as vancomycin, chlortetracycline, and chloramphenicol (Fenical and Jensen, 2006; Gribble, 1998, 2004; Nicolaou et al., 1993).

The presence of halogen substituents in many natural products profoundly influences their biological activity (Figure 1). For example, two chlorine substituents in vancomycin, the antibiotic of last resort for the treatment of multiple-drug resistant *S. aureus* infections, are required to achieve the clinically active conformation of this antibiotic through the control of atropisomer distribution (Harris et al., 1985). The therapeutic efficacy of salinosporamide A, a proteasome inhibitor currently in clinical trials for multiple myeloma treatment, requires the presence of a chlorine substituent. Addition of the hydroxyl moiety of the proteasome's active site threonine into the lactone of salinosporamide leads to opening of the β-lactone and release of the free hydroxyl group in this molecule. This nucleophilic hydroxyl, capable of reforming the lactone and releasing the inhibitor, instead reacts in an intramolecular fashion to displace the primary chloride substituent and form a tetrahydrofuran ring (Groll et al., 2006). The two-reaction sequence provides for an irreversible covalent inhibition and underlies the potency of this anticancer agent. The presence of a chloride substituent is also relevant for the biological activity of rebeccamycin: in contrast to rebeccamycin, its deschloro derivatives lack antimicrobial activity (Rodrigues Pereira et al., 1996). Similarly, the halogenated sesterterpenes neomangicol A and B show in vitro cytotoxic effect toward HCT-116 human colon tumor cell line, while their nonhalogenated analog neomangicol C was inactive in this assay (Renner et al., 1998).

Given the vital role that halogens can play in determining the biological activity of secondary metabolites, their biosynthesis has been a topic of study for over 40 years. In the last decade, the cloning and sequencing of biosynthetic gene clusters has revealed new chemistries for halogen incorporation and provided a driving force for detailed mechanistic studies of these enzymes. A combination of biochemical, spectroscopic, and structural characterizations has provided ever more detailed insights into the workings of these catalysts. In return, the knowledge gained is being used to facilitate the identification of new natural products and gene clusters while enabling the metabolic engineering of novel metabolites.

Halogenation by Hypohalite, X^+

The first general strategy for enzymatic halogenation is to generate a reactive hypohalite species by two-electron oxidation of the corresponding halide. Consistent with the electronic deficient nature of the hypohalite, this strategy is used by Nature to halogenate electron-rich carbon centers in natural products. The specific oxidant distinguishes the two broad classes of halogenating enzymes. Hydrogen peroxide is used by the haloperoxidase class, whereas molecular oxygen is used by the halogenase class.

Heme-Fe Haloperoxidases

The first halogenating enzyme to be characterized, chloroperoxidase (CPO) from *Caldariomyces fumago*, serves as the prototypical heme-iron haloperoxidase. The protein was isolated based on its postulated involvement in the biosynthesis of the natural product caldariomycin. CPO was shown to dichlorinate the activated carbon of 1,3-cyclopentanedione and halogenate other electron rich carbons including the β-carbon of dimedone and the 3- and 5- positions of the amino acid tyrosine (Hager et al., 1966). CPO also catalyzes a host of related reactions, including oxidation of iodide to iodine, sulfoxidation of dialkyl sulfides, P450-like insertion of oxygen into C–H σ-bonds and C–C π-bonds, and catalase-like disproportionation of hydrogen peroxide into oxygen and water (van Rantwijk and Sheldon, 2000). This broad range of chemistry coupled with the protein's stability have made it of interest for commercial applications.

The proposed mechanism for halogenation (Figure 2A) involves the initial binding of peroxide at the axial position of

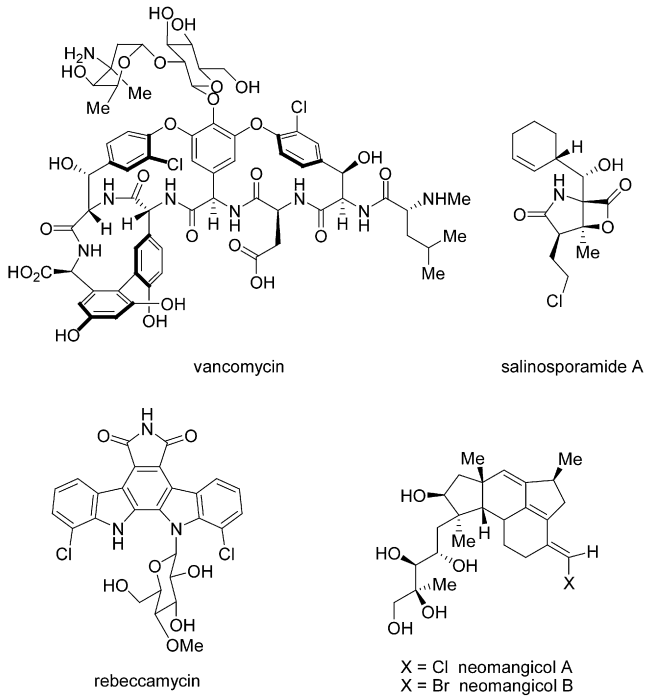

Figure 1. Representative Natural Products with Halogen-Dependent Bioactivity

the resting Fe^{III}–porphyrin complex. Water is eliminated to give an intermediate Fe^{IV}–oxo species known as compound I which is then intercepted by halide to form a Fe^{III}–hypohalite species. Halogenation of the organic substrate can proceed directly from this compound, or alternatively free hypohalous acid can be released and perform the actual halogenation more distant from the Fe center. Given the acidic conditions in which CPO works best and the general lack of substrate specificity, it is widely believed that free hypohalous acid is the dominant halogenating agent.

Structurally, CPO appears to blend characteristics of the cytochrome P450 enzymes and heme peroxidases. Spectroscopic (Dawson and Sono, 1987) and structural studies (Sundaramoorthy et al., 1995) confirmed that, as in P450s, the proximal axial ligand to the catalytic iron is thiolate derived from a cysteine side chain. The distal binding pocket is more reminiscent of heme peroxidases in that it is composed primarily of polar amino acid side chains. Partially occupied binding sites for bromide and iodide were found near a channel from the protein's surface to the Fe center, and these may provide transient sites to guide the halide ion to the catalytic center. When the substrate 1,3-cyclopentanedione was soaked in at high concentrations, the compound was observed to bind directly above the heme center. Unfortunately, the orientation of the substrate and its too-close proximity to the active site suggest that the observed interaction is not productive for catalytic purposes.

Vanadium-Dependent Haloperoxidases

Studies of halogenation in marine natural products identified a second subclass of haloperoxidases that contained vanadium as the necessary cofactor. These vanadium-dependent haloperoxidases (V-HPO) are thought to be responsible for the vast majority of halogenation events in marine natural products. Vanadium-dependent bromoperoxidases (V-BPO) are widely distributed throughout marine seaweeds (Butler and Carter-Franklin, 2004) while the vanadium-dependent chloroperoxidases

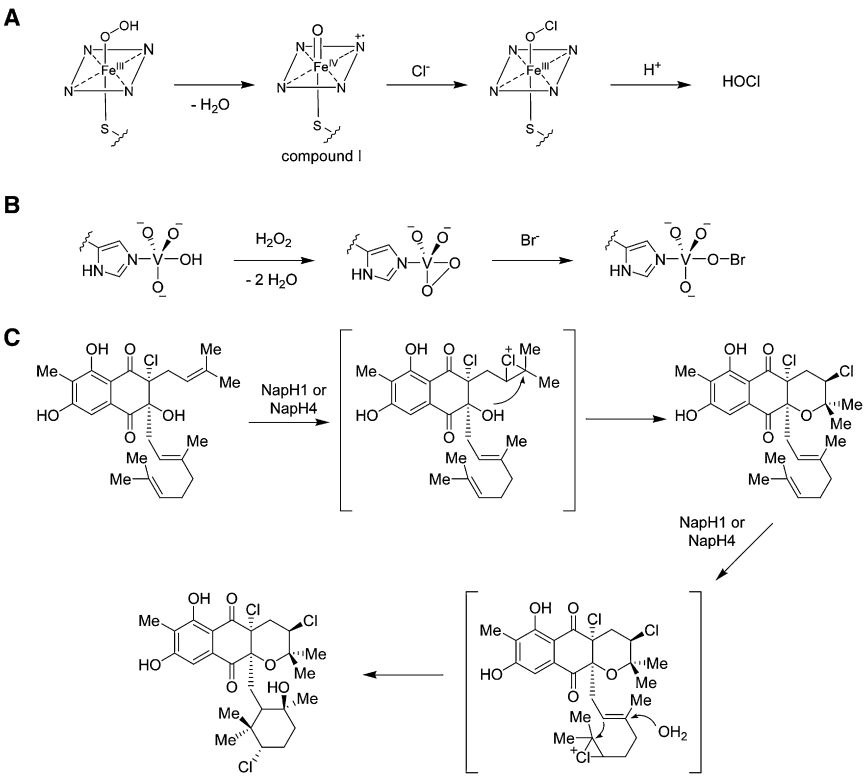

Figure 2. Oxidative Halogenation by Haloperoxidases

(A) Formation of hypochlorous acid by heme Fe-dependent chloroperoxidase.

(B) Formation of vanadium-bound hypobromite by V-BPO.

(C) Chloronium-mediated cyclizations catalyzed by V-CPOs to produce a chlorinated napyradiomycin derivative. The chlorine on the benzoquinone core is likely incorporated via a flavin-dependent halogenase.

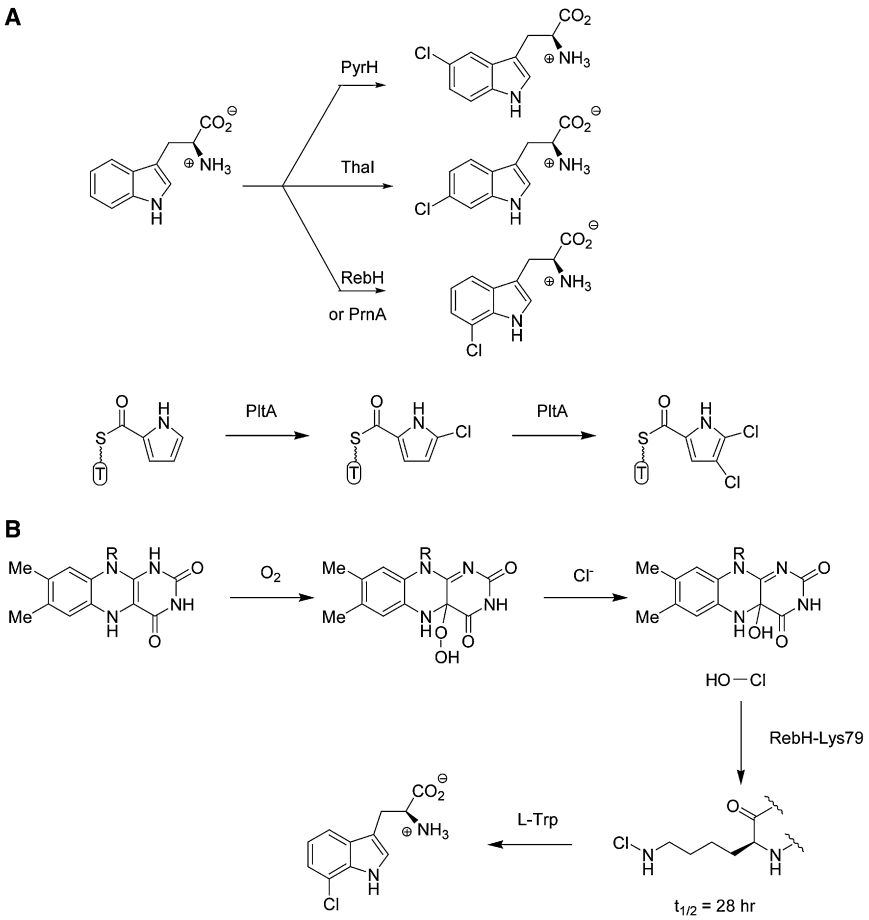

Figure 3. Chemistry of Flavin-Dependent Halogenases

(A) Regioselective chlorination of tryptophan by FAD-dependent halogenases and dichlorination of pyrrolyl-S-PltL by PltA.

(B) Mechanism of halogenation by RebH. Flavin-generated hypochlorous acid is trapped by the enzyme as lysine chloramine before tryptophan halogenation.

(V-CPO) have only been found in terrestrial fungi and in two bacterial species (Winter et al., 2007).

Like the heme-iron haloperoxidases, the metal center of V-HPO binds hydrogen peroxide and activates it for attack by halide (Figure 2B). However, the vanadium atom does not appear to be redox active, instead maintaining its V(V) oxidation state throughout the catalytic cycle. Peroxide binds to provide an activated η^2-peroxo intermediate, whereupon bromide reacts to form vanadium-bound hypobromite. Again, it is not clear if this bound intermediate is the active oxidizing agent, or if hypobromous acid is released from the metal center before bromination occurs. Structural studies of V-BPO (Isupov et al., 2000; Weyand et al., 1999) and V-CPO (Messerschmidt and Wever, 1996) show large differences in the overall structure, but a highly similar active site that supports the proposed mechanism. The vanadate ion sits at the bottom of a wide substrate funnel and is coordinated to the protein via a conserved histidine residue. When cocrystallized with azide—a known inhibitor of V-CPO— the ion displaces the water opposite the histidine suggesting this is the site of initial coordination of peroxide (Messerschmidt and Wever, 1996).

The reactions catalyzed by V-HPO generally proceed through halonium intermediates. Complex molecules can be formed when the initial halonium ion is intercepted by intramolecular nucleophiles including hydroxyl groups and π-bonds. Recently, three genes with homology to vanadium-dependent chloroperoxidases were found in the napyradiomycin gene cluster of *Streptomyces aculeolatus* NRRL 18422 and *Streptomyces* sp. CNQ-525 (Winter et al., 2007). Two of the enzymes, NapH1 and NapH4, are proposed to play a role in the oxidative cyclizations of two terpene-derived side chains on the dihydroquinone core (Figure 2C). In one case, the chloronium ion is quenched by addition of an intramolecular hydroxyl group; in the other, the chloronium ion is first intercepted by a π-bond and the resulting carbonium ion is quenched by water. The third enzyme, NapH3, is suggested to be a hydroxylase based on the absence of a key active site residue necessary for haloperoxidase activity. These genes are the first example of V-HPOs in bacterial species as well as the first to be placed in the context of a dedicated biosynthetic gene cluster. Given the highly similar reactions performed on a common molecular structure, biochemical characterization of these enzymes will provide a unique opportunity to address questions of selectivity within this class of enzymes.

Flavin-Dependent Halogenases

Enzymatic halogenation via hypohalite can also be catalyzed using molecular oxygen as the oxidant with flavin as the redox active cofactor. The first halogenase, PrnA, was described in 2000 (Keller et al., 2000) and since that time a number of related family members have been purified and characterized (Figure 3A). These genes are commonly found within microbial biosynthetic gene clusters and the enzymes catalyze specific halogenation events in the biosynthetic pathways. PrnA catalyzes the conversion of free tryptophan to 7-chlorotryptophan during the biosynthesis of pyrrolnitrin. The identical transformation to 7-chlorotryptophan is catalyzed by RebH in the biosynthesis of the indolocarbazole natural product rebeccamycin (Yeh et al., 2005). In each case, the reaction was shown to be dependent on the reduced flavin cofactor, oxygen, and chloride ion. Additionally, an NAD(P)H-dependent flavin reductase is necessary to reduce the oxidized cofactor; for PrnA a general reductase is recruited from cellular metabolism whereas for RebH a dedicated reductase (RebF) is encoded in the biosynthetic gene cluster. Halogenases for the 5- and 6-positions of tryptophan have also been described for the biosyntheses of pyrroindomycin (Zehner et al., 2005) and thienodolin (van Pee and Zehner, 2003), respectively. That these homologous enzymes each produce only a single chlorotryptophan isomer

demonstrates the exquisite selectivity characteristic of this class of halogenases.

Amino acids are also known to be halogenated when bound to the peptidyl carrier proteins found in nonribosomal peptide synthase assembly lines. In the biosynthesis of pyoluteorin, thiolation domain-bound proline (prolyl-*S*-PltL) is oxidized to pyrrolyl-*S*-PltL (Thomas et al., 2002). This electron-rich aromatic variant is then doubly chlorinated at C5 and C4 by the halogenase PltA (Dorrestein et al., 2005). SgcC3 was recently shown to catalyze the selective chlorination of the C3 position of β-tyrosyl-*S*-SgcC2 during the biosynthesis of the enediyne antibiotic C-1027 (Lin et al., 2007).

A series of structural, spectroscopic, and biochemical studies has helped to refine the proposed mechanism for flavin halogenases (Figure 3B). The crystal structures of PrnA (Dong et al., 2005) and RebH (Yeh et al., 2007) show the expected flavin binding domain with a chloride ion bound in a pocket on the solvent-protected face of the cofactor. This binding module is similar to those in known flavin monooxygenases and initial reaction of reduced flavin with molecular oxygen is thought to proceed via the same mechanism to provide a flavin hydroperoxide (FAD-C_{4a}-OOH) intermediate (Entsch and van Berkel, 1995). The structure also shows that the tryptophan binding pocket is located 10Å away from the flavin cofactor with only a narrow channel connecting the two sites, excluding direct interaction between the substrate and oxidized flavin (either FAD-C_{4a}-OOH or FAD-C_{4a}-OCl). Instead it is proposed that chloride attack on the distal oxygen of FAD-C_{4a}-OOH produces enzyme-trapped HOCl that can diffuse toward the substrate binding site. Spectroscopic and kinetic studies of RebH (Yeh et al., 2006) support this view by showing that flavin redox chemistry is complete before substrate halogenation. Furthermore, the redox chemistry can occur in the absence of substrate proving that chloride oxidation by flavin and substrate oxidation are uncoupled processes.

While free hypohalous acid would be a powerful enough oxidant to perform the observed reactions, it is also known to react with chemical functionalities within proteins. In addressing this question of how flavin-dependent halogenases control this reactive species to achieve selective substrate halogenation, it was discovered that the competent oxidizing species in the reaction has a half-life of 28 hr (Yeh et al., 2007). When $Na^{36}Cl$ was used as the chlorine source, the radiolabel was found to move sequentially from chloride to protein to product. These data suggest the formation of a covalent adduct between the protein and chlorine. Based on the known reactivity of lysine sidechains with hypochlorous acid (Nightingale et al., 2000) and the strategic location of a conserved lysine residue at the interface of the channel and substrate pocket, it is proposed that Lys79 first reacts with the flavin-generated acid to produce a lysine chloramine species. With its attenuated reactivity, the lysine chloramine is better suited to perform the regioselective halogenation of the substrate. Thus, it appears that Nature covalently captures an indiscriminate halogenating reagent (hypochlorous acid) within the protein to provide a less reactive but more selective reagent (lysine chloramine) for the halogenation of the substrate.

Halogenation via Halogen, $X^{\bullet}$

Identification of several halogenated natural products, such as the trichlorinated marine molluscicide barbamide, produced by cyanobacterium *Lyngbya majuscula* (Orjala and Gerwick, 1996), and *Streptomyces armentosus*-derived antibiotic armentomycin (Argoudelis et al., 1967), revealed chlorine incorporation at unactivated carbon centers. Such carbons are not amenable to modification by electrophilic agents, suggesting an entirely different mechanism for carbon–halogen bond formation. A radical mechanism of chlorination was proposed to account for the regio- and stereoselective conversion of pro-*R* methyl group of L-leucine to the trichloromethyl moiety of barbamide (Hartung, 1999; Sitachitta et al., 1998). Recently, in vitro reconstitution and elucidation of the mechanistic logic for halogenases capable of chlorinating unactivated carbon centers has been achieved. Mononuclear non-heme iron halogenases are imbedded in nonribosomal peptide synthetase assembly lines, and act on the methyl groups of thiolation domain-tethered amino acids (Figure 4A). Chlorination of the methyl group of SyrB1-bound L-threonine in the biosynthesis of the lipo-nonapeptidolactone syringomycin E is carried out by a member of this class of enzymes, SyrB2 (Vaillancourt et al., 2005b). Similarly, the halogenase CytC3 adds two chlorine atoms to the terminal methyl group of L-2-aminobutyric acid (Aba) tethered to thiolation domain CytC2 in biosynthesis of armentomycin by soil *Streptomyces* sp. RK95-74 (Ueki et al., 2006). In vitro reconstitution of the trichlorination event in barbamide biosynthesis requires two halogenating enzymes, BarB1 and BarB2, which act on L-Leu-*S*-BarA as a substrate (Galonic et al., 2006).

Detailed evaluation of cofactor requirements established that ferrous iron, α-ketoglutarate (αKG), chloride, and oxygen are required for enzymatic activity (Vaillancourt et al., 2005b), evoking similarity with Fe(II)- and αKG-dependent dioxygenases. To coordinate iron, dioxygenases of this class utilize αKG and three protein residues, two histidine and one carboxylate (Asp or Glu), in a characteristic His-x-Asp/Glu-x_n-His "facial triad" sequence (Costas et al., 2004; Hausinger, 2004; Que, 2000). A key catalytic intermediate in the hydroxylation reaction is an Fe(IV)–oxo species that abstracts hydrogen atom from the substrate to form a substrate radical and an Fe(III)-OH species (Bollinger et al., 2005; Hoffart et al., 2006). Subsequent transfer of the hydroxyl group to the substrate radical—"oxygen rebound"—results in the formation of an alcohol product. The X-ray crystal structure of syringomycin halogenase SyrB2 shows that, in contrast to dioxygenases, the active site iron is coordinated by two protein-derived histidine ligands (Blasiak et al., 2006). The carboxylate residue is replaced by chloride, coordinated to iron opposite to the C2 carbonyl moiety of αKG. In the primary sequence of SyrB2, the conserved carboxylate of dioxygenases is replaced by an alanine, resulting in His-x-Ala-x_n-His motif. Sequence alignment of known mononuclear non-heme iron halogenases indeed confirms the presence of a conserved, sterically undemanding residue, such as Ala or Gly. The loss of the carboxylate ligand and its replacement by exogenous halide is likely crucial to the enzyme's ability to bind and subsequently transfer halide to organic substrate.

To evaluate if substrate activation proceeds through an analogous Fe(IV)–oxo species, direct characterization of intermediates was carried out for the CytC3-catalyzed chlorination of L-Aba-*S*-CytC2 (Figure 4B; Galonic et al., 2007). When the starting CytC3-Fe(II)-αKG-Cl-L-Aba-*S*-CytC2 complex was reacted with oxygen, accumulation of an intermediate state with absorption feature characteristic of Fe(IV)–oxo intermediate

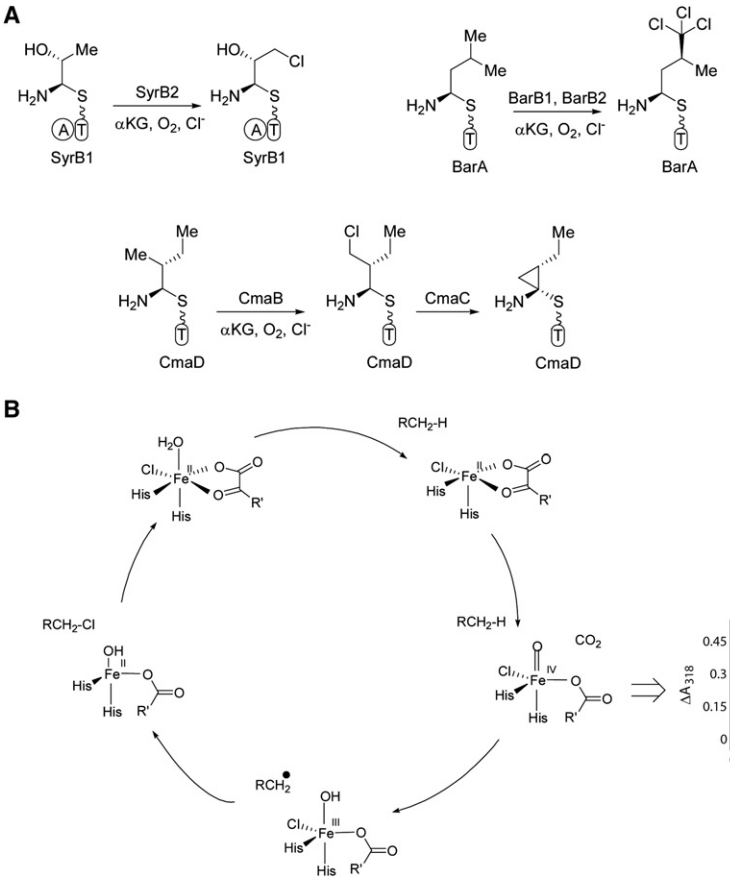

Figure 4. Chemistry of Fe(II)/αKG-Dependent Halogenases

(A) Representative biosynthetic transformations.

(B) Catalytic cycle for Fe-dependent halogenases. Inset graph shows time-dependent 318 nm absorbance of the reactive Fe(IV)–oxo species in the presence of L-Aba-*S*-CytC2 (red), with extended half-life in the presence of d_3-L-Aba-*S*-CytC2 substrate (blue).

in dioxygenases was detected. Subsequent characterization of the intermediate state using freeze-quench (FQ) Mössbauer spectroscopy revealed that it consists of two rapidly equilibrating high-spin Fe(IV)–oxo species. To determine if these species, in analogy to iron–oxo intermediates in dioxygenases, abstract hydrogen atom from the substrate, d_3-L-Aba-*S*-CytC2 was prepared and subjected to spectroscopic analysis. Observed accumulation of the intermediate state, as a result of a kinetic isotope effect, suggests that the iron–oxo complexes in halogenases catalyze C–H cleavage reaction to initiate substrate halogenation. To obtain insight into the structure of the halogenation intermediate, FQ Mössbauer and X-ray absorption studies were performed on an intermediate trapped in the reaction of CytC3-Fe(II)-αKG-Br-L-Aba-*S*-CytC2 complex with dioxygen (Fujimori et al., 2007a). Extended X-ray absorption fine structure spectroscopy (EXAFS) allowed for determination of iron–bromide bond length in both control samples (2.53 Å) and the intermediate state obtained upon mixing with oxygen (2.43 Å). In addition, the intermediate also displayed a short iron–oxo interaction (1.62 Å), assigned to Fe(IV)–oxo species. The obtained structural insights provide validation for the ligation of halide to iron at the Fe(IV) intermediate stage of the catalytic cycle and additional evidence for short iron–oxo interaction. Thus, binding of dioxygen to Fe(II) in halogenases leads to the formation of Cl-Fe(IV)–oxo species which activate the substrate for halogenation via hydrogen atom abstraction to form substrate radical and Cl-Fe(III)-OH intermediate.

Subsequent oxidative ligand transfer of the chlorine atom to the substrate radical results in the formation of a chlorinated product and reduction of the active-site iron to the Fe(II) oxidation state, ready for the next cycle of catalysis. While the substrate radical has two potential reaction pathways, oxygen rebound to form an alcohol and chlorine atom transfer to form an alkyl halide, chlorination is the only observed outcome. This is consistent with studies on alkane functionalization by inorganic $[Fe^{III}X_2L]^+$ complexes (X = Br, Cl; L = tris(2-pyridylmethyl)amine or tris(N-ethylbenzimidazol-2-ylmethyl)-amine) (Kojima et al., 1993). In the reaction with alkyl peroxides these species form $[LXFe^{V} = O]^{2+}$ intermediates, which abstract H• from the substrate and then transfer the bound halide back to the generated substrate radical. The preference for halide over oxygen transfer is rationalized by lower reduction potential of halogen atom when compared to hydroxyl radical (Kojima et al., 1993).

The conserved mechanism of hydrogen atom abstraction by mononuclear non-heme iron halogenases and dioxygenases suggests that high-valent Fe(IV)–oxo intermediates are a common strategy for modification of unactivated carbon centers. It is tempting to postulate that halogenation may have evolved from hydroxylation activity by the loss of iron-coordinating carboxylate ligand and coordination of exogenous halide. Initial efforts to engineer hydroxylase activity into halogenases by single mutations at this site have been unsuccessful, showing that in addition to changes in the iron coordination sphere, more subtle changes in the active site are also necessary to switch the reaction manifold.

In addition to their involvement in biosynthesis of halogenated metabolites, non-heme iron halogenases have also been shown to participate in the formation of cyclopropane-containing bacterial metabolites. One such transformation is the formation of coronamic acid. CmaB, a halogenase found in the biosynthetic gene cluster of this molecule in *Pseudomonas syringae* (Buell et al., 2003), chlorinates γ-methyl group of L-*allo*-Ile-*S*-CmaD thioester (Vaillancourt et al., 2005a). The product of this reaction, γ-Cl-L-*allo*-Ile-*S*-CmaD is then the substrate for the cyclopropane-forming enzyme CmaC, which catalyzes cyclopropane formation through the intramolecular displacement of chloride by the thioester enolate (Kelly et al., 2007).

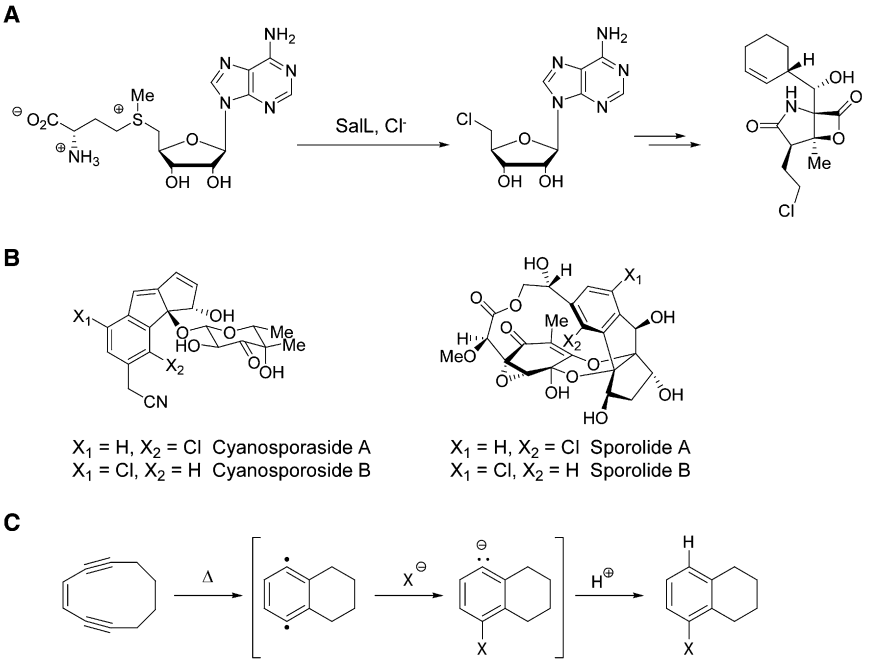

Figure 5. Halogenation of Natural Products via Halide Anion

(A) Incorporation of chloride into 5′-chloro-5′-deoxyadenosine in the biosynthesis of salinosporamide A.

(B) Monochlorinated enediyne-derived cyanosporasides and sporolides.

(C) Proposed mechanism for nonenzymatic halide incorporation into enediyne natural products.

Halogenation via Halide, X^-

The final mechanism of halogen incorporation is via the common halide oxidation state. Fluoride, chloride and bromide are widespread in the earth's crust and marine environments (Murphy, 2003; van Pee, 1996), but their direct incorporation into natural product scaffolds is rare and accounts for a small subset of halogenated metabolites. Nonetheless, two entirely different mechanisms for halide incorporation into secondary metabolites—one enzymatic, one nonenzymatic—have been described in the literature. Each system provides intriguing insights into the reactivity of halides in Nature.

A well-studied example of a halogenase that incorporates fluoride anion is the fluorinase from *Streptomyces cattleya* (O'Hagan et al., 2002). This enzyme transfers fluoride anion to the electrophilic 5′ carbon atom of *S*-adenosylmethionine (SAM) substrate to generate 5-fluoroadenosine, which is then metabolized in a series of reactions to generate fluoroacetate and 4-fluorothreonine. The crystal structure of fluorinase reveals that the enzyme is a hexamer, with the active site positioned at the interface of two monomer units (Dong et al., 2004). The extensive contacts between SAM and the protein suggest the crucial importance of substrate recognition and positioning for efficient catalysis. Interestingly, the ribose ring of SAM is bound in a high-energy conformation which is postulated to weaken the C5′–S bond and provide an optimal trajectory for the nucleophilic substitution reaction. The fluoride ion must also be carefully prepared by the enzyme for catalysis. Because fluoride's nucleophilicity is greatly diminished by hydrogen bonding to water, the enzyme must provide a binding pocket that can desolvate the ion but still provide sufficient stabilizing interactions. Structural, thermodynamic, and kinetic studies of the native and mutant proteins suggest that initial desolvation is accomplished by exchanging two water molecules for an active site serine sidechain and the backbone amide N–H of the same residue (Zhu et al., 2007). Subsequent binding of SAM provides the main energetic driving force to fully desolvate the fluoride ion, making it a potent nucleophile capable of participating in an S_N2-type reaction with the sulfonium center. It should be pointed out that, due to the prohibitively high redox potential of fluoride anion, enzymatic fluorination is limited to this nonoxidative manifold. This limitation may rationalize the scarcity of organofluorine metabolites in nature.

Recently, an analogous SAM-dependent chlorinase was identified as the enzyme responsible for the incorporation of chlorine into salinosporamide A (Figure 5A; Eustaquio et al., 2008). Earlier work on the biosynthesis of this molecule showed that the chloroethyl sidechain was derived from a sugar precursor (Beer and Moore, 2007). Analysis of the biosynthetic gene cluster identified a putative chlorinase, SalL, with 35% identity to the known fluorinase enzyme. Examination of an engineered *salL*$^-$ strain showed it to be deficient in salinasporamide A production, though this deficiency could be overcome by chemical complementation with synthetic 5′-chloro-5′-deoxyadenosine (5′-ClDA). Structurally, the protein shows features that reinforce the model of halide desolvation and nucleophilic displacement. The halide is observed to make one key H-bonding interaction with the protein to aid in initial desolvation, and the ion is properly aligned for in-line displacement of methionine to generate 5′-ClDA. Biochemical characterization of the proteins also showed the predicted synthesis of 5′-ClDA from SAM and chloride ion. Though equilibrium favors the reverse reaction in vitro, the authors reason that downstream enzymes likely act in vivo to maintain metabolic flux in the forward direction. Overall, this work describes a novel, chemically orthogonal route to chlorinated metabolites and provides a valuable tool for future efforts in metabolic engineering.

Isolation and structural characterization of polycyclic metabolites sporolides (Buchanan et al., 2005) and cyanosporasides (Oh et al., 2006) from marine actinomycete genus *Salinispora* revealed that these compounds contain chlorine in one of two positions (but never both) on an aromatic ring (Figure 5B). It has been hypothesized that the complex aromatic framework of these structures originates from nine-membered enediyne precursors, where chlorination was postulated to occur during the aromatization of the enediyne unit (Fenical and Jensen, 2006; Oh et al., 2006). Two lines of recently obtained experimental evidence strongly support this hypothesis. Sequencing of the genome of the sporolide producer *Salinispora tropica* showed the presence of a 9-membered enediyne polyketide synthase biosynthetic gene cluster (Udwary et al., 2007). Furthermore,

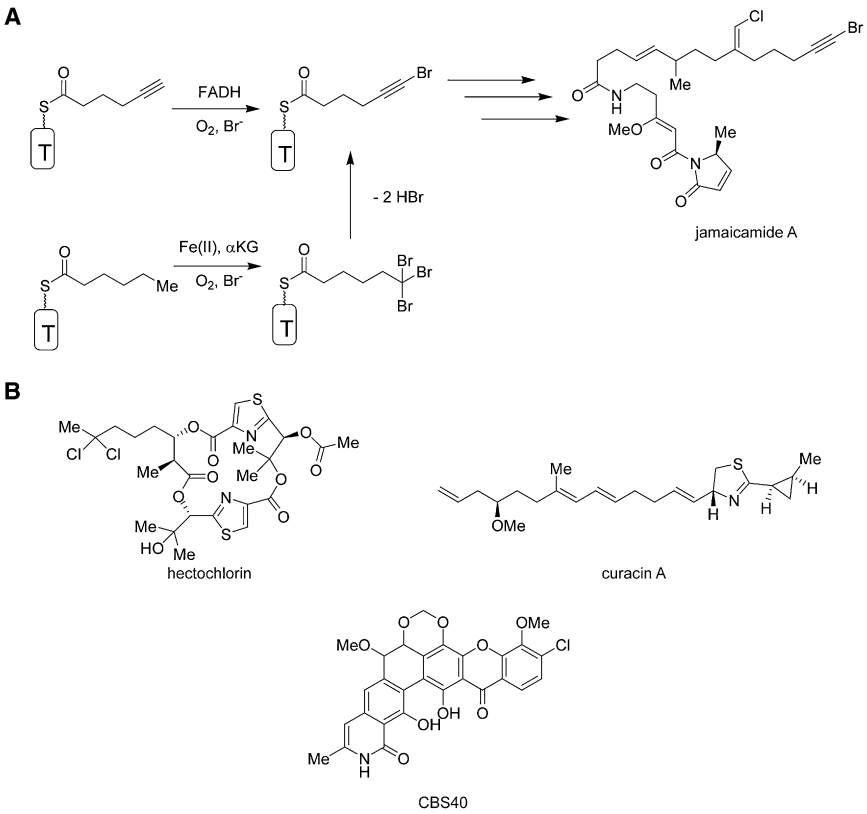

Figure 6. New Opportunities for Enzymatic Halogenation

(A) Alternative hypotheses, invoking FADH- or Fe-dependent halogenases, for the formation of the alkynyl bromide functionality in jamaicamide.

(B) Natural products with novel halogenated or halogen-derived functional groups.

model studies on halogenation of the 10-membered enediyne cyclodeca-1,5-diyn-3-ene confirmed the feasibility of the proposal (Perrin et al., 2007). Slight heating of this molecule in the presence of a lithium halide and weak acid result in the formation of 1-halotetrahydronaphthalenes. Kinetic dissection of the reaction mechanism showed that the rate-limiting step is the formation of the *p*-benzyne intermediate, which results from the Bergman cyclization of the precursor. This intermediate is proposed to undergo an unprecedented reaction in which chloride adds to *p*-benzyne to give chlorine and an electron pair in a 1,4 arrangement (Figure 5C). Subsequent protonation of the aromatic ring provides the observed monohalogenated products. This halide addition–protonation sequence invokes unusual chemistry yet provides a simple explanation for why 1,4-dihalogenated sporolides have not been isolated.

Accounting for New Functional Groups

A number of unique halogen-containing functional groups cannot be readily explained by systems already characterized. Further studies of these should shed new light on the scope of biological halogenation.

The jamaicamide family of natural products exhibits unusual alkenyl- and alkynyl-halogenations and two predicted halogenases (one FADH-dependent, one non-heme-iron dependent) have been found in the biosynthetic gene cluster (Edwards et al., 2004). The vinyl chloride functionality is present at a β-methyl branch along the backbone of the molecule. One hypothesis for its formation has been put forward (Vaillancourt et al., 2006), whereby the β-methyl branch is installed according to a known mechanism (Calderone et al., 2007) to give an α,β-unsaturated thioester. Chlorination of the methyl group followed by isomerization of the double bond would provide the vinyl chloride via an alkyl chloride intermediate. The alkynyl bromide could be formed by two alternative mechanisms (Figure 6A). Direct halogenation of the terminal alkyne could be realized by the FADH-dependent halogenase, consistent with a common synthetic method to generate bromoalkynes using hypobromous acid. Alternatively, one cannot rule out a mechanism in which tribromination of the terminal methyl group of a fully saturated starting unit is performed by the non-heme-iron halogenase. Subsequent double dehydrobromination would provide the observed alkynyl bromide functionality. Genetic and biochemical characterization of the system will no doubt provide a satisfying answer to this question.

New groups of substrates are suggested by other natural products (Figure 6B). Hectochlorin contains a dichlorinated methylene group, suggesting radical halogenation of a substrate other than methyl. The recently described gene cluster for this molecule indeed predicts a protein, HctB, whose N-terminal domain shows high homology to known non-heme-iron halogenases (Ramaswamy et al., 2007). Curacin contains a cyclopropane group whose biosynthesis could be possible via chlorination of a β-methyl branch (as suggested for jamaicamide) followed by enolate cyclization to the cyclopropane (as shown for coronamic acid). Flavin halogenases have also been implicated in the biosynthesis of napyradiomycin (Winter et al., 2007) and xanthones (Hornung et al., 2007), suggesting that the substrate scope of these enzymes encompasses much more than just amino acids and their derivatives. Continued biochemical characterization of these systems will provide additional insights into halogenation chemistry as well as new tools for ongoing efforts in metabolic engineering.

Identification of Novel Metabolites and Gene Clusters

Conserved sequence motifs in both flavin-dependant and mononuclear non-heme-iron halogenases have been exploited for identification of new halogenated metabolites, as well as cloning of a gene cluster that encodes production of halogenated metabolites. A sequence-guided genetic screening strategy was developed to profile a collection of 550 actinomycetes strain for their biosynthetic potential (Figure 7A; Hornung et al., 2007). The screening strategy is based on the assumption that similar structural elements in natural product scaffolds are produced by homologous enzymes, and therefore identification of genes that encode such enzymes can predict the biosynthetic

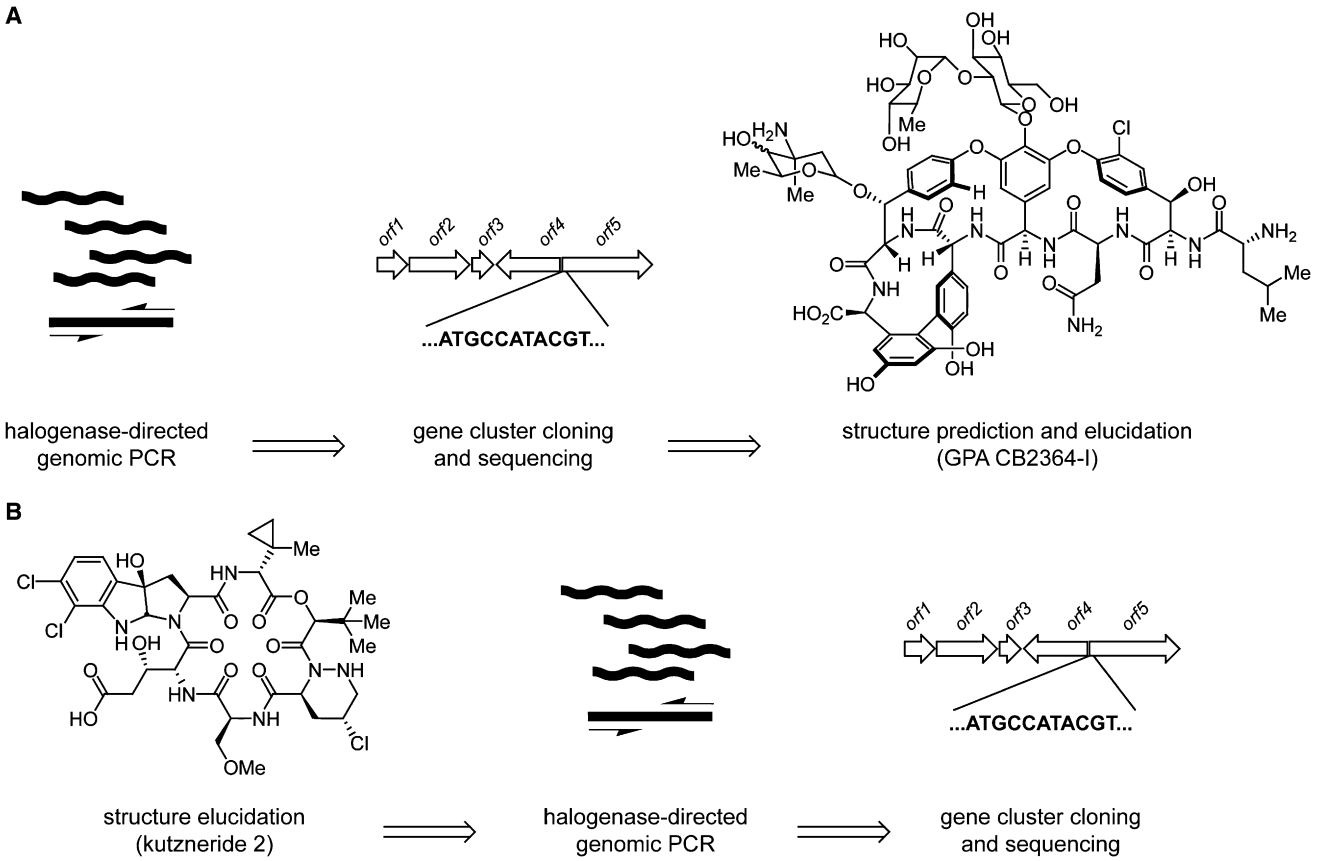

Figure 7. Alternative Strategies for Halogenase-Based Discovery
(A) Discovery of novel structures following PCR amplification of halogenases from potential producer strains.
(B) Discovery of kutzneride gene cluster following PCR amplification of predicted halogenases.

capabilities of the producer. Due to the involvement of flavin-dependent halogenases in the production of a number of compounds of therapeutic importance, genes encoding these tailoring enzymes were selected as an ideal target. PCR-based screening with a single set of degenerate primers identified the presence of flavoprotein halogenases in 20% of screened microorganisms. Among these, several novel putative halogenases showed high homology to previously identified enzymes. Interestingly, a number of newly identified enzymes showed no similarity to known halogenase subgroups, while preserving required halogenase sequence elements. To determine the genetic content surrounding the halogenase gene, further sequencing was carried out both upstream and downstream of the gene. The sequence of these adjacent genes allowed for the prediction of the natural product class to which the putative metabolite belongs. The production of halogenated metabolites was confirmed by heterologous expression of two complete gene clusters, which led to the identification of the glycopeptide antibiotic CB2364-1 and the polycyclic xanthone antibiotic CBS40. These findings confirm that the systematic genetic profiling of bacterial strain collections with flavin halogenase probes is a powerful technique for natural product discovery.

Recently, degenerate PCR probes targeting both flavin-dependant and mononuclear non-heme-iron halogenases were used in identification of the biosynthetic gene cluster encoding production of antifungal and antimicrobial secondary metabolites kutznerides (Figure 7B). Kutznerides are cyclic hexadepsipeptides isolated from soil actinomycete *Kutzneria* sp. 744 (Broberg et al., 2006; Pohanka et al., 2006). Nine structurally related structures have thus far been identified, all containing five nonproteinogenic amino acids and one hydroxy acid. The presence of two halogenated residues, 6,7-dichlorinated hexahydropyrroloindole in all known kutznerides and γ-chloropiperazic acid in kutznerides 2 and 8, suggests involvement of flavin-dependent halogenases in kutznerides biosynthesis. Furthermore, the conserved 2-(1-methylcyclopropyl)glycine residue in these molecules could be derived via a cryptic halogenation pathway, involving a non-heme-iron halogenase. We postulated that the gene cluster encoding the production of kutznerides could be identified based of the requirement for these two types of halogenating enzymes. Thus, genomic DNA was screened with degenerate primer-based PCR probes targeting highly conserved sequences of both types of halogenases, resulting in identification of two flavin-dependent and one non-heme-iron halogenase. Based on the obtained amplicons, specific primers were designed and used to screen a cosmid library of genomic DNA to identify a gene cluster consisting of 29 ORFs distributed along 56 kb region of kutzneria's genome (Fujimori et al., 2007b). Distinct roles in kutznerides production have been postulated for 17 ORFs, including all three halogenase genes. The putative

flavoprotein halogenases KtzQ and KtzR could be involved in chlorination of electron-rich indole moiety of tryptophan and γ-carbon of γ,δ-dehydropiperazate. KtzD, homologous to mononuclear non-heme-iron halogenases, could be involved in the biosynthesis of methylcyclopropyl glycine moiety. It is postulated that this putative halogenase can chlorinate δ-methyl group of KtzC-bound Ile or *allo*-Ile. The formed δ-chloro-(*allo*-)Ile-*S*-KtzC could then be a substrate for KtzA, a protein homologous to acyl-CoA dehydrogenase-like proteins. Thioester facilitated abstraction of the α-proton, followed by the removal of the β-hydride by the oxidized form of the flavin cofactor, would form an enamine species which could act as an intramolecular nucleophile to displace chloride and form the cyclopropane. The resulting imine species could be reduced in turn by the dihydroflavin cofactor to provide the tethered amino acid in the correct oxidation state. This proposed mode of enzymatic cyclopropane formation awaits biochemical characterization.

Prospects for Metabolic Engineering

An important goal for the field of biosynthesis is the rational engineering of new metabolites using combinations of enzymes from distinct biosynthetic pathways. Such efforts could lead to therapeutics with enhanced potency, optimized pharmacokinetic properties, or altogether novel mechanisms. The application of halogenating enzymes presents significant challenges in this area. The haloperoxidase enzymes generally exhibit a broad substrate scope that could potentially allow them to be shuffled between pathways. However, this could come at the expense of selectivity making it difficult to predict or control the outcome of the reactions.

The non-heme-iron and flavin halogenases offer more selectivity in their chemical transformations, but potentially at the expense of portability. An early success in their application to metabolic engineering was presented by Sanchez et al. (Sanchez et al., 2005, 2006). By enlisting tryptophan halogenases with differing regioselectivities, they were successful in engineering the biosynthesis of analogs of rebeccamycin and the related indolocarbazole staurosporine. In their work, the enzymes responsible for the indolocarbazole core structures were cloned into an actinomycete host along with combinations of tailoring enzymes. RebH, the endogenous halogenase responsible for 7-chlorotryptophan formation, was replaced with either Thal or PyrH which generate 6- and 5-chlorotryptophan, respectively. Such swaps were successful in producing novel patterns of halogenation around the indolocarbazole core. Moving forward, it will be necessary to identify new halogenases and carefully assess their substrate specificity and product selectivity to identify those best suited for combinatorial applications. For metabolites biosynthesized on assembly lines, additional understanding of the protein–protein interactions will also be needed in order to realize the full potential of metabolic engineering.

Concluding Remarks

The halogenation of natural product scaffolds allows organisms to optimize the bioactivity of small molecules for evolutionary advantage. Nature has adopted a wide variety of chemistries to perform this task, the particular mechanism being matched to the electronics of the substrate. The more common one- and two-electron oxidation of halides are targeted to unactivated and nucleophilic substrates, respectively. In special circumstances, Nature also takes advantage of direct halide incorporation to halogenate electrophilic sites. Through a variety of experimental approaches, the details of these mechanisms have become increasingly clear, and this knowledge is now being used to drive the discovery of new natural products and their gene clusters, as well as the metabolic engineering of new metabolites.

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