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Scope and potential of halogenases in biosynthetic applications

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A large and diverse series of halogenated natural products exist. In many of these compounds the halogen is important to biological activity and bioavailability. We now recognise that nature has developed many different halogenation strategies for which well-known enzyme classes such as haem oxidases or flavin-dependent oxidases have been adapted. Enzymes capable of halogenating all kinds of different chemical groups from electron-rich to electron-poor, from aromatic to aliphatic have been characterised. Given that synthetic halogenation reactions are not trivial transformations and that halogenated molecules possess pharmaceutical usefulness, it will be worth investing into further research of halogenating enzymes.

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Introduction

A large and diverse series of halogenated natural products exist, with the number identified by 2010 exceeding 4700 [1]. These compounds, initially considered nothing more than an oddity, attract interest because of their structures, their biogenesis and their biological activity [2]. In many of these compounds the halogen is important to biological activity and bioavailability. Halogens are also found in biosynthetic intermediates where they act as facilitators for chemical reactions such as the cyclopropane ring formation in the marine natural product curacin (Figure 1) [3]. The most common halogen found in natural products is chlorine followed by bromine, whilst iodine and fluorine are considerably rare [4]. The occurrence of brominated compounds is more frequently observed in marine natural products. In terms of drug discovery and development, chlorinated and brominated compounds have the merit of being amenable to specific chemical modification using the halogen as a functionalisable chemical handle [5] in addition to modulating biological activity and physical properties. Fluorination is a particularly sought-after halogenation with an excellent track record of increasing drug efficacy through numerous mechanisms [6].

For many years the only known halogenases were the haloperoxidases. We now have a much better understanding of enzymatic halogenation. In the past decade, a veritable explosion of new halogenation mechanisms and enzymes has come to light. Among them are flavin-dependent halogenases and α-ketoglutarate-dependent non-haem iron oxygenases that have modified well known reaction mechanism to perform chlorination and bromination reactions; and *S*-adenosylmethionine-utilising (AdoMet-utilising) enzymes that are capable of fluorination or chlorination through a nucleophilic mechanism [7,8].

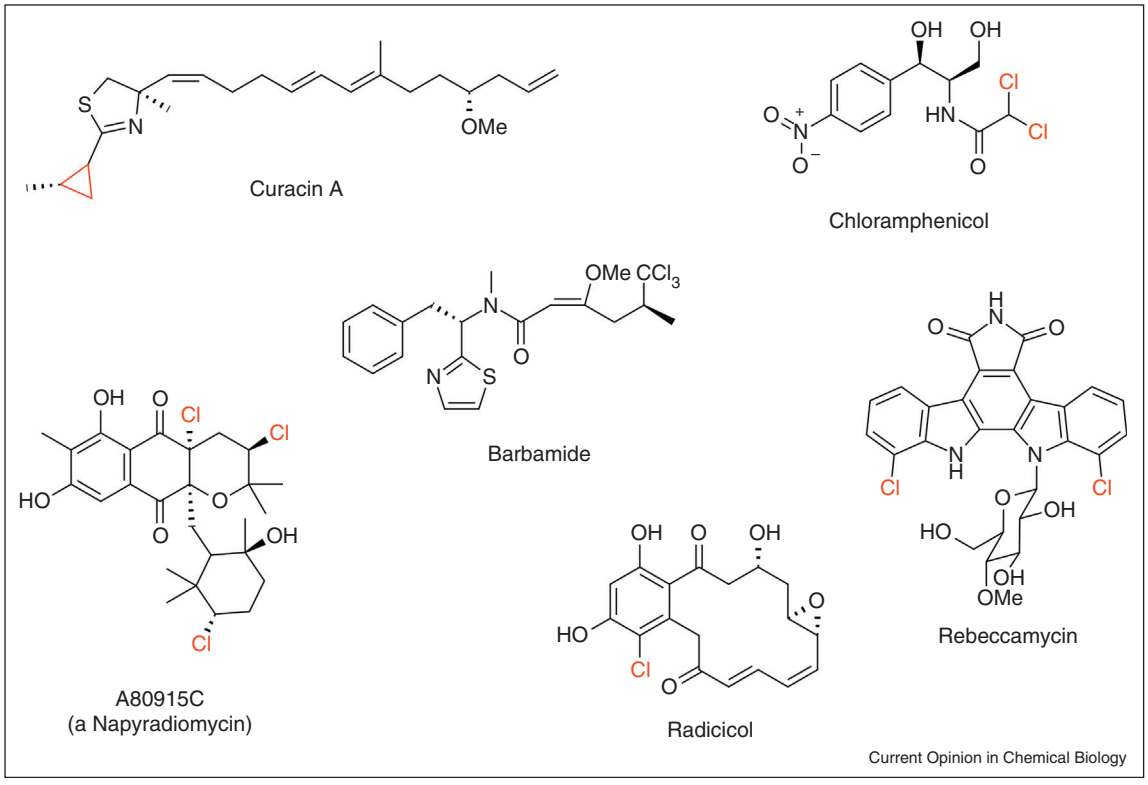
Haloperoxidases

Haloperoxidases have been known for a very long time as halogenating enzymes. One example is thyroid peroxidase which is involved in thyroxine biosynthesis [9]. The two major classes comprise haem iron peroxidases and vanadium peroxidases. The electrophilic halogenating species generated by these enzymes is likely to be hypohalous acid, a highly reactive halogenating agent. Chlorination, bromination as well as iodination are observed with this family of halogenases; however, no fluorination activity has yet been demonstrated, mostly likely due to the high electronegativity of fluorine.

The general mechanism of haloperoxidases involves the generation of the halogenating species, through oxidation of the halide, and is dependent on hydrogen peroxide (Figure 2a). The reaction mechanism of haem peroxidases is likely to parallel that of other haem enzymes [10,11]. The halide ion is oxidised in the active site to ferric hypohalite by the ferryl-oxo species. This species in turn is generated through binding of hydrogen peroxide to the ferric resting state (Figure 2c). Similarly, in vanadium-dependent halogenases hydrogen peroxide binds to the metal which is followed by halide addition and finally the release of the hypohalous acid (Figure 2b) [8,12].

Haloperoxidases often exhibit low regiospecificity due to the freely diffusing halogenating agent that is generated and due to the high reactivity of hypohalous acid; as a result a suite of various monohalogenated, dihalogenated and trihalogenated metabolites can be generated. Studies on the bromination of methyl pyrrole-2-carboxylate using a vanadium-dependent haloperoxidase from the alga

Figure 1



Examples of halogenated natural products.

Ascophyllum nodosum gave mixtures of 4-bromopyrroles and 5-bromopyrroles as well as 4,5-dibromocompounds [13]. However, successful applications with haloperoxidases have been demonstrated. For example, the bioprocess department of Merck demonstrated that indene oxide, an intermediate in the synthesis of the HIV-1 protease inhibitor Crixivan® (Indinavir) can be obtained through biotransformation [14].

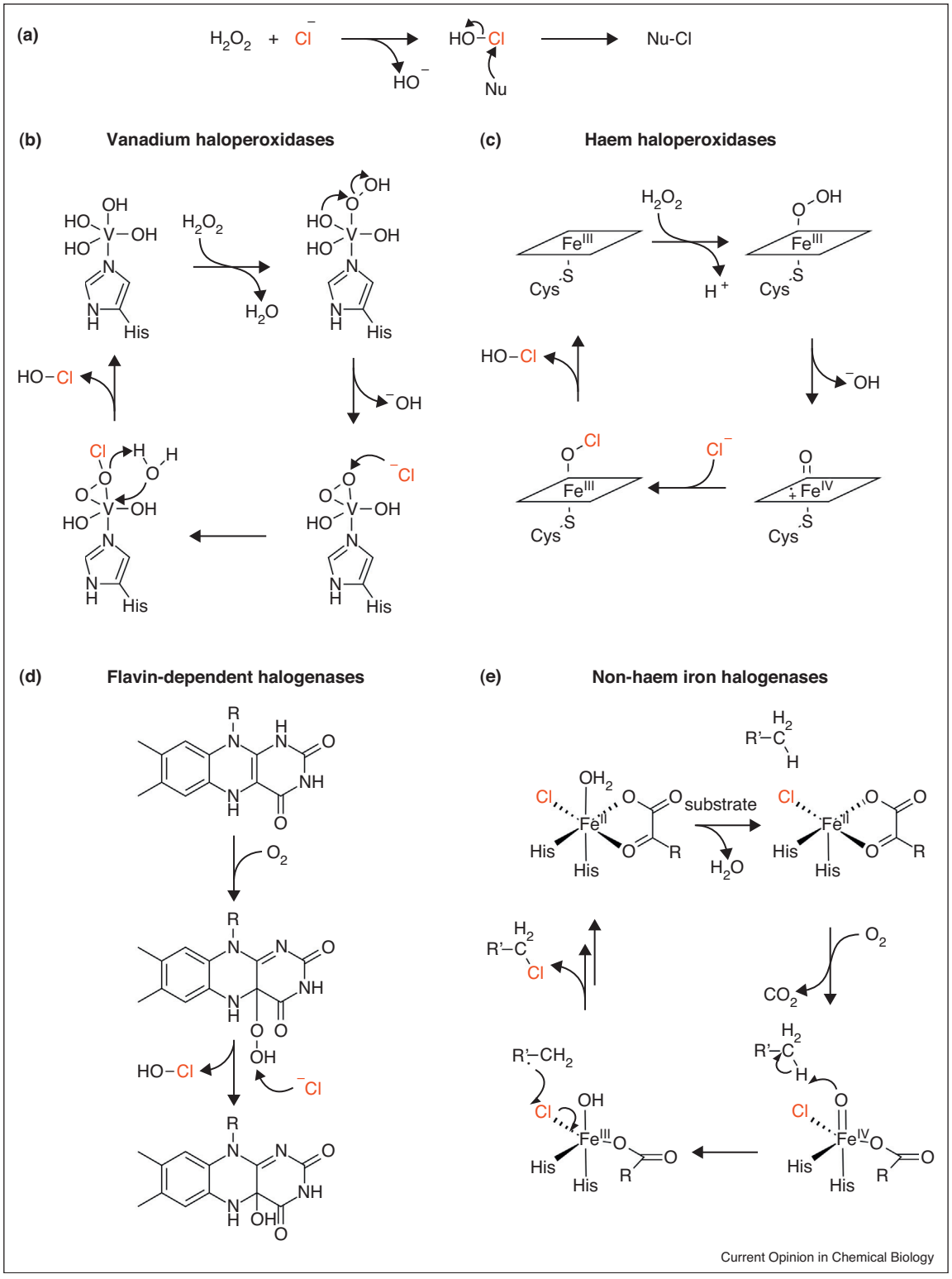
It can be argued that many of the chloroperoxidases or bromoperoxidases described in the literature are not halogenases by primary function but the observed halogenating activity stems from the general ability of peroxidases to oxidise halides when taken out of biological context. Non-native halogenase activity could be the cause of HOCl being released from the enzyme active site. This could also explain the apparent lack of stereoselectivity and regioselectivity of some haloperoxidases that seem to contradict the specific halogenation patterns in natural products.

The existence of highly regiospecific and stereospecific vanadium-dependent haloperoxidases such as Mcl24 of the merochlorin pathway (speculative) and NapHI of the napyradiomycin pathway implies that the highly reactive nature of the postulated hypohalous acid reagent can be moderated and that these enzymes do indeed bind their substrates in a highly specific fashion [15,16•,17].

Flavin-dependent halogenases

The discovery of PrnA, a flavin-dependent halogenase from the pyrrolnitrin biosynthesis, demonstrated that alternative, more specific mechanisms of halogenation existed [18]. The exact mechanism with which flavin-dependent halogenases operate is still under debate, and mechanistic details, such as which active site residues participate, may actually vary between members of the family. The reader is referred to a number of excellent reviews on the topic [8,19–21]. Halogenation with flavin-dependent enzymes involves an electrophilic species such as hypohalous acid which is generated from the reaction of reduced flavin with molecular oxygen (Figure 2d). The reduced flavin in turn is provided by an additional enzyme, flavin reductase. Most halogenases bind the flavin co-factor non-covalently; however, studies on CmlS from the chloramphenicol biosynthesis have demonstrated that the flavin co-factor can also be covalently attached to the enzyme [21]. The most notable feature in flavin-dependent halogenases is that the flavin binding site and the substrate binding site are separated by a 10 Å long tunnel through which the hypohalous acid

Figure 2



Outline of halogenase reaction mechanisms. **(a)** General scheme for reactions with hypohalous acid. **(b)** Catalytic cycle for vanadium-dependent halogenases. **(c)** Catalytic cycle for haem-dependent halogenases. **(d)** Generation of hypochlorite in flavin-dependent halogenases; **(e)** Catalytic cycle of non-haem iron-dependent halogenases; R: $–CH_2CH_2CO_2H$. Schemes have been adapted from [8,10–12].

has to migrate. Thus, the sites of halide oxidation and substrate halogenation are spatially separated.

So far, all characterised flavin-dependent halogenases catalyse electrophilic substitution at electron-rich or activated centres as found in indoles and pyrroles, in the *ortho* position of phenolic compounds or at Cα of β-ketoacids. The substrates can be free compounds as for tryptophan chlorination by PrnA, or they can be attached to carrier proteins as for the PltA-catalysed dichlorination of pyrrole-2-carboxylate [18,22]. Other research has demonstrated that flavin-dependent halogenases can be found working in parallel with NRPS/PKS enzymes, showing halogenation taking place alongside synthesis rather than only as a pre-synthetic or post-synthetic modification [23].

Non-haem iron-dependent halogenases

Whereas the above-discussed halogenases are restricted to electron-rich substrates, the non-haem iron-dependent halogenases (also referred to as α-ketoglutarate-dependent halogenases) exploit a radical halogen species to allow halogenation of unactivated, aliphatic carbon centres [24]. This can result in natural products containing halogens in unusual contexts such as the dichlorovinyl feature found in Trigocherrin A [25].

The mechanism is thought to follow closely that of other α-ketoglutarate-dependent oxygenases and has been discussed in more detail elsewhere [8,26,27]. The iron(II) in halogenases is liganded by two histidine residues, α-ketoglutarate and chloride (Figure 2e). It has been shown that binding of α-ketoglutarate and chloride induces a conformational change in the curacin halogenase domain from an open to a closed form [28••]. Upon binding of the substrate and molecular oxygen, the decarboxylation of α-ketoglutarate is triggered and the reactive oxo-ferryl intermediate is formed. Hydrogen abstraction from the substrate is followed by transfer of the halide to the substrate radical.

There are two issues that might affect the potential of non-haem iron-dependent halogenases as general biocatalysts. To date, all substrates for this class of enzyme are molecules tethered to the phosphopantetheinyl arm of carrier proteins [26]. Should this trend prove consistent, the formation of carrier protein-bound unnatural substrates would be required before halogenation and the analysis of reaction products is somewhat less straightforward compared to diffusible products. Furthermore, it has been shown for SyrB2 that slight repositioning of the substrate can alter the reaction outcome to favour hydroxylation over chlorination [29]. It remains to be seen to what extent this will impede the halogenation of alternative substrates.

An interesting aspect of α-ketoglutarate-dependent halogenases is the number of halogen transfers that can be carried out by one active site. In the biosynthesis of barbamide, for example, BarB2 is capable of oxidising leucyl-*S*-carrier protein up to the δ-dichlorinated product but an additional halogenase, BarB1, is required to obtain the final δ-trichlorinated valine derivative [30,31]. In contrast, the aforementioned SyrB2 halogenase transfers only a single chlorine to Cδ of threonyl-*S*-carrier protein [32].

Halogenases using *S*-adenosylmethionine

The only fluorinating enzyme discovered so far is FDAS from *Streptomyces cattleya* [33]. A fluoride anion acts as the nucleophile attacking the C5′ of AdoMet liberating methionine in an S_N2 fashion [34,35]. In *S. cattleya* 5′-fluoro-5′-deoxyadenosine is subsequently catabolised to give fluoroacetaldehyde as a branch point metabolite. Fluoroacetaldehyde can either be oxidised to fluoroacetic acid or undergo a transaldol reaction to give fluorothreonine [36,37]. In addition to fluoride, FDAS was also able to utilise chloride but not bromide or iodide [38]. Native chlorination using the AdoMet-dependent mechanism was reported for salinosporamide biosynthesis [39]. The halogenase SalL is not capable of utilising fluoride whereas bromide and iodide are accepted in this case. The chlorinase SalL could be substituted with the fluorinase FDAS to produce fluorosalinosporamide [40•] (Figure 3).

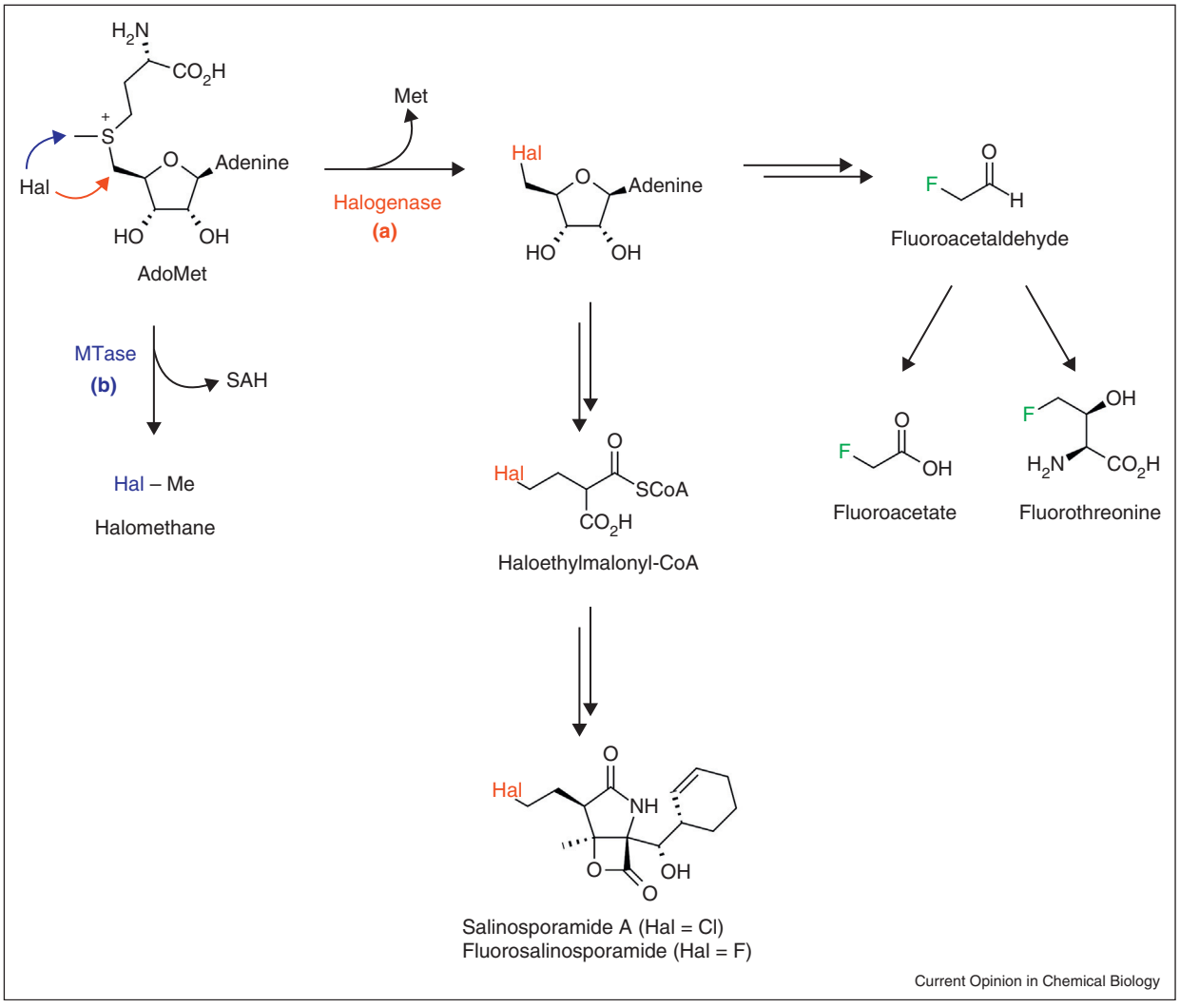
The only other example of a nucleophilic halogenation is found in the biosynthesis of halomethanes (MeCl, MeBr, MeI). The two substrates are the same as for the above mentioned FDAS and SalL enzymes, a halide ion and AdoMet, but rather than substitution at C5′ of AdoMet the nucleophile attacks at the *S*-methyl as in all other methyltransferases. The similarity in mechanism to methyltransferases is reflected in the structure of the halomethane synthase from *Arabidopsis thaliana* [41].

Halogenases in synthetic biology applications

There now exist a number of examples where halogenases have been used to manipulate biosynthetic pathways. Tryptophan halogenases have proven to be of particular usefulness, as many biologically active alkaloids are derived from tryptophan, and hence, halogenation of the amino acid precursor can potentially lead to the engineered biosynthesis of the correspondingly halogenated alkaloid [42–44].

One of the earliest examples is the inclusion of different tryptophan halogenases in hybrid rebeccamycin–staurosporin systems [45]. A heterologous host, *Streptomyces albus*, was employed to reconstitute the indolocarbazole pathways from *Lechevalieria aerocolonigenes* (rebeccamycin) and *Streptomyces longisporoflavus* (staurosporin). This facilitated the recombination of pathway genes and the inclusion of halogenases in a new context. The rebeccamycin tryptophan 7-halogenase RebH [46] was capable of

Figure 3



Reactions of AdoMet-dependent halogenases. Pathway **(a)** yields the intermediate 5′-halo-5′-deoxyadenosine that is further metabolised to a variety of products. Pathway **(b)** follows a methyltransferase (MTase) mechanism to give halomethanes. SAH: *S*-adenosylhomocysteine.

promoting production of chlorinated indolocarbazole compounds similar to staurosporin. This finding is perhaps unsurprising as both pathways are evolutionarily related. Interestingly, when the tryptophan 5-halogenase PyrH [47] was co-expressed, the resulting indolocarbazoles were mono-chlorinated indicating that the substrate tolerance of the downstream pathway enzymes was reaching a limit. The same was observed when the tryptophan 6-halogenase Thal [48] was co-expressed. In the latter case, the genes for the conversion of chromopyrrolic acid to the indolocarbazole aglycone had not been included.

Early studies targeting the biosynthetic pathways of aminocoumarin antibiotics showed that replacement of the novobiocin 8′-methyltransferase with the clorobiocin 8′-halogenase yielded 8′-chlorinated novobiocin derivatives [49]. However, it was noted that introduction of the balhimycin halogenase BlhA did not lead to chlorinated novobiocin derivatives. As both halogenases are thought to halogenate the same substrate, a carrier protein-bound β-hydroxytyrosine, the failure with the evolutionarily more distant *blhA* gene in the novobiocin context could be due to the specificity of the halogenase for its cognate carrier protein highlighting an additional level of complexity with respect to substrate specificity. It has to be noted though that the actual substrate for Clo-Hal is not known and halogenation may occur at a later stage. In contrast, the hormaomycin halogenase HrmQ, that is thought to use carrier protein-bound pyrrole-2-carboxylate as substrate, was able to produce chloropyrrole-containing clorobiocin derivatives demonstrating that carrier protein specificity can be relaxed [50].

The O'Connor group have been highlighting the versatility of halogenases by incorporating PrnA into the biosynthesis of monoterpene indole alkaloid natural products produced by the Madagascar periwinkle plant *Catharanthus roseus* [51•]. Plants are known for their capacity to produce a large and diverse range of natural products, but biosynthetic work in plants is often hampered by the fact that the genes involved in plant secondary metabolite biosynthesis tend to be scattered around the genome, rather than being found in clusters as in bacteria. The O'Connor group felt that this made plants an attractive target for biosynthetic modification using halogenases. In 2010 the group published a paper that demonstrated the successful incorporation of chlorine into tryptophan-derived alkaloids derived from strictosidine *in planta* by introducing the tryptophan 5-halogenase PyrH or the tryptophan 7-halogenase RebH [51•]. However, the group noted the accumulation of chloro-tryptophan in the plant tissues that was due to the substrate specificity of tryptophan decarboxylase. This bottleneck was eliminated by reengineering RebH to act preferentially on tryptamine, a substrate that is not accepted by the native halogenase, thus opening up the potential scope of flavin-dependent halogenases [52••]. In the same year, van Pée and co-workers demonstrated that the regioselectivity of flavin-dependent halogenases could be altered through site-directed mutagenesis in the active site. A single amino acid change converted a tryptophan 7-halogenase into an enzyme that produced the native 7-halotryptophan alongside the new product 5-halotryptophan [53].

The flavin-dependent tryptophan 7-halogenase PrnA was also employed to produce chlorinated pacidamycin derivatives [5]. Pacidamycin is a uridyl peptide antibiotic noted for its activity against *Pseudomonas aeruginosa*. To avoid potential bottlenecks, precursor-directed biosynthesis with tryptophan derivatives was conducted to test for the scope and limitations of the natural pathway enzymes to cope with substituted indole rings [54]. As substitution at the 7-position of the indole ring was very well tolerated, *prnA* was introduced into the genome of the producing organism, *Streptomyces coeruleorubidus*, resulting in production of the halogenated metabolite. It was not necessary to include the concomitant flavin reductase or to target the halogenase gene into the pacidamycin gene cluster. The group's work went on to highlight that natural product halogenation is not necessarily the end of the line synthetically, going on to use mild, aqueous, palladium catalysed Suzuki–Miyaura coupling techniques that act on the installed chlorine handle to produce a range of chemically diversified products [5], highlighting the potential that this approach can have as a true collaboration between biosynthetic and chemical approaches.

Problems with intermediate accumulation have also been encountered when attempting to incorporate a 6-chlorinase from the thienodolin biosynthesis into the pyrrolnitrin pathway by Siebold *et al.*, leading to the production of a new 6-chlorinated pyrrolnitrin precursor that was not accepted downstream, and consequently not to the targeted compound [48].

The future challenges of halogenases in biotechnology and synthetic biology

Major challenges will lie in modulating and adapting substrate specificities. When working with halogenases that require carrier protein-bound substrates it will have to be determined how much protein-protein interactions contribute to the substrate selectivity of the halogenase. Very little data exist to address this question directly. A notable exception is a recent study by Busche *et al.* that indicated that the curacin halogenase domain CurA Hal requires its cognate acyl carrier protein partner [55••]. Another issue lies in the timing of the halogenation: for many if not most of the characterised halogenases to date the halogenation occurs very early on. Taking these halogenases into a new biosynthetic context entails that all downstream enzymes will have to cope with the halogenation. Abrogated biosynthesis or production of shunt products, possibly even mediated through the presence of a relatively reactive bromine or chlorine, are likely to occur and some examples have been listed above. Fluorination and its equivalent mechanism in chlorination are taking this to the extreme as only a single reaction is known, that of the nucleophilic substitution of methionine from *S*-adenosylmethionine with fluoride or chloride. Diversification then occurs through modification or breakdown of 5′-halo-5′-deoxyadenosine. Taking into account how useful fluorination is in pharmacology, the discovery and development of further fluorinases will be extremely important.

But not all is doom and gloom. Halogenases can be isolated from a wide variety of organisms and habitats, and we have probably only found the tip of the iceberg of the catalytic potential that exists. For all classes of halogenases more examples of late-stage halogenations are emerging. The concomitant larger active site that is required to accommodate highly elaborate substrates may be a good starting point for further enzyme engineering. Examples include Rdc2 from radicicol biosynthesis or the ansamycin halogenases Asm12 and Nat1 [56,57]. The discovery of a flavin-dependent halogenase in lantibiotic biosynthesis further opens up the possibility of chlorinating specific residues in peptide substrates [58]. And lastly, even though haloperoxidases have fallen out of favour in recent years they may yet celebrate a comeback. In particular the recently discovered highly specific vanadium-dependent peroxidases from the merochlorin and napyradiomycin pathways may bring about a renewed interest in the catalytic potential of this class of enzymes.

Currently, synthetic routes win out (with a few exceptions) with respect to versatility and efficiency in the preparation of halogenated compounds. However, enzymes capable of halogenating all kinds of different chemical groups from electron-rich to electron-poor, from aromatic to aliphatic have been characterised. Given that synthetic halogenation reactions are not particularly easy and trivial transformations in combination with the pharmaceutical usefulness of halogenated molecules, it will be worth investing into the research of halogenating enzymes.

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