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Halogenases: powerful tools for biocatalysis (mechanisms applications and scope)

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Introduction

The incorporation of a halogen into a molecule can have a striking impact on its properties [1,2]. Around 20% of small molecule drugs and over 80% of marketed agrochemicals are halogenated, including leading compounds such as sitagliptin and aripiprazole with annual sales revenues of $3.6 and $7.9 billion respectively [3,4]. These statistics are perhaps not surprising as incorporation of a halogen can significantly impact a molecule's bioactivity and bioavailability. Furthermore, the incorporation of a Cl or Br can provide a chemically reactive and orthogonal handle for selective modification through cross-coupling chemistry [5]. Synthetic halogenation ordinarily utilizes harsh conditions, noxious reagents, generates harmful by-products and often lacks regioselectivity [6–8]. The fine-chemical, pharmaceutical and agrochemical industries have an increasing interest in utilizing bio-catalysts in process, as a route to more selective, greener, and cost-effective synthesis, and it is imperative that new enzymes are discovered and developed for process. In contrast to synthetic chemical alternatives, halogenating enzymes afford the highly regiospecific incorporation of a halogen into an organic molecule. The mild reaction conditions (physiological pH and temperature), aqueous solvents and the biodegradable catalyst, also provide environmental and operational benefits.

Over 5000 halogenated natural products have now been reported, these are predominantly chlorinated and brominated metabolites, with only about 100 iodinated and 5 fluorinated metabolites having been isolated (for examples of the breadth of structural diversity, see Figure 1) [9–12]. For many years the only known halogenases were the haloperoxidases, however over the past 20 years, investigation of the biosynthetic pathways mediating the construction of diverse series of halometabolites, predominantly from actinomycetes, has revealed a diverse series of halogenases. The halogenases discovered can be broadly classified as employing electrophilic, nucleophilic or radical halogenation mechanisms (Figure 2). Electrophilic processes dominate for the installation of C–I, C–Br and C–Cl bonds, however due to the fluorine's high electronegativity, the biogenesis of C–F bonds is likely to only occur via nucleophilic processes.

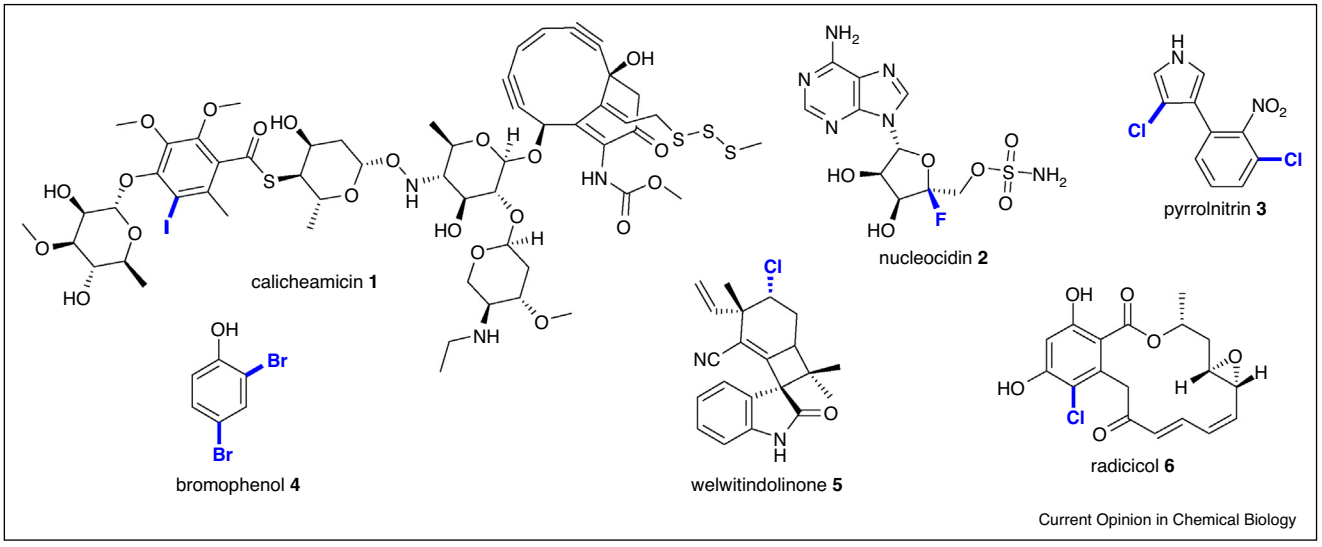
Electrophilic halogenation

Haloperoxidases (haem iron and vanadium dependent)

The earliest known enzymes involved in halogenation were the haloperoxidases with chloroperoxidase (CPO) from the fungus *Caldariomyces fumago* being discovered in 1958 [26]. For the next 35 years haloperoxidases were the only known halogenating enzymes.

The haloperoxidases may be divided into two major classes, the haem iron peroxidases and the vanadium dependent halogenases. Thyroid peroxidase (TPO) is a particularly notable example of a haem iron peroxidase, this membrane associated enzyme is responsible for the iodination event in the biosynthesis of thyroxine [27]. Haloperoxidases are believed to produce free hypohalous acids (HOI, HOBr and HOCl) According to the most electronegative halogen they can oxidize, they can be sub-classified as iodo-, bromo- or chloroperoxidases. Hypohalous acid generation occurs by the reaction of hydrogen peroxide with the ferric or vanadate resting state of the peroxidase, followed by halide addition, forming the ferric or vanadate hypohalite. Finally, the highly reactive hypohalous acid is released (see Scheme 1b,c), as it is not bound and directed by the enzyme, it is thought to diffuse freely. It reacts in an electrophilic fashion with electron rich compounds [28,29]. As a result, the haloperoxidases tend to show a very low level of regiospecificity, and oftentimes a suite of mono, di and tri-halogenated products are generated, depending upon the reactivity of the substrate. Nevertheless, a small group of highly regio- and stereo-specific vanadium

Figure 1



A glimpse of the structural and biological diversity shown by halogenated compounds: the enediyne antitumour antibiotic calicheamicin **1** [13,14], nucleosidin 2, one of only 5 naturally occurring fluorinated metabolites to be isolated to date [11,12], the antifungal antibiotic pyrrolnitrin **3** involving two flavin dependent halogenases in its assembly [15,16•,17••]. One of a series of marine bromophenols **4**, generated by flavin dependent halogenases [21••,22], as well as the cyanobacterial metabolite welwitindolinone **5**, chlorinated by the first non-haem iron halogenase shown to accept a non phosphopantetheine tethered substrate [23••,24•], the fungal natural product radicicol **6** chlorinated by a broad substrate specificity flavin dependent halogenase [25•].

dependent halogenases exist, such as vanadium-dependent NapHI involved in alkene chlorination within the napyradiomycin pathway, indicating that it is possible for such systems to evolve to bind their substrates in a highly specific manner [30••,31]. Many haloperoxidases noted within the literature may have a primary function as peroxidases, and this non-native function could result in the ready release of the hypohalous acid from the enzyme's active site. At a structural level, one significant difference between peroxidases and haloperoxidases is that the distal metal coordination site is typically occupied by a histidine residue in peroxidases, but by a cysteine in haloperoxidases [32].

Flavin dependent halogenases (FDHs)

Haloperoxidases remained the only known biocatalysts enabling C–X bond formation until 1995, when Dairi *et al.* identified the first flavin dependent halogenase, *chl*. This was determined through gene inactivation studies within the biosynthetic cluster encoding 7-chlorotetracycline formation [33]. Soon after this discovery, two further new halogenase genes, *prnA* and *prnC* from pyrrolnitrin (**3**) biosynthesis in *Pseudomonas fluorescens* were identified by Hammer et al. [16•]. There is debate as to the exact way in which FDHs function and details on which residues within the active site participate may vary from one enzyme to another [34–36].

Flavin dependent halogenases, in contrast to the vanadium and haem haloperoxidases, are highly substrate specific and regioselective catalysts. The majority of this class of halogenases utilize free reduced flavin ($FADH_2$), however in other enzymes the flavin is covalently bound to the enzyme, as in the case for CmlS from the chloramphenicol biosynthetic pathway [37]. Within natural systems $FADH_2$ is generated from FAD by a halogenase-specific flavin reductase, though notably in reconstituted systems, there is no requirement for a specific flavin reductase to be utilized. The $FADH_2$ is then used to generate hypohalous acid (Scheme 1a). In flavin dependent halogenases the flavin binding site, where the HOX is generated, and the substrate binding site, where halogenation occurs, are separated by a 10 Å tunnel. The substrate can be free as in the case of variant A FDHs such as PyrH, PrnA or Rdc2 [17••,18••,19,38,39•] or bound to a carrier protein, as occurs in the case of variant B FDHs such as PltA and Bmp2 [20••,21••,22,40]. In the last decade a large number of genes with sequence similarity to known FDHs have been detected, but only a very small fraction of these have been confirmed as having *in vitro* halogenase activity [41]. One of the reasons for this is that these enzymes have a very narrow substrate specificity, and therefore the endogenous substrate must be known, accessed, and presented in its appropriate free or bound form as required by the enzyme in order to confirm the halogenase activity.

Flavoenzyme Bmp5, a phenol brominase

An electrophilic halogenase with a subtly different mechanism has been recently identified, implicated