Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

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

Danai S Gkotsi[1], Jag Dhaliwal[1], Matthew MW McLachlan[2,3], Keith R Mulholand[4] and Rebecca JM Goss[1]

Addresses
[1] School of Chemistry, University of St Andrews, North Haugh, St Andrews, Fife KY16 9ST, UK
[2] Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK
[3] QEDDI, Staff House Road, The University of Queensland, Brisbane, QLD 4072, Australia
[4] Chemical Development, AstraZeneca, Silk Rd Business Park, Macclesfield SK10 2NA, UK

Corresponding author: Goss, Rebecca JM (rjmg@st-andrews.ac.uk)

Current Opinion in Chemical Biology 2018, **43**:119–126

This review comes from a themed issue on **Biocatalysis and biotransformation**

Edited by **Nicholas J Turner** and **Rajesh Kumar**

For a complete overview see the Issue and the Editorial

Available online 2nd February 2018

https://doi.org/10.1016/j.cbpa.2018.01.002

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.

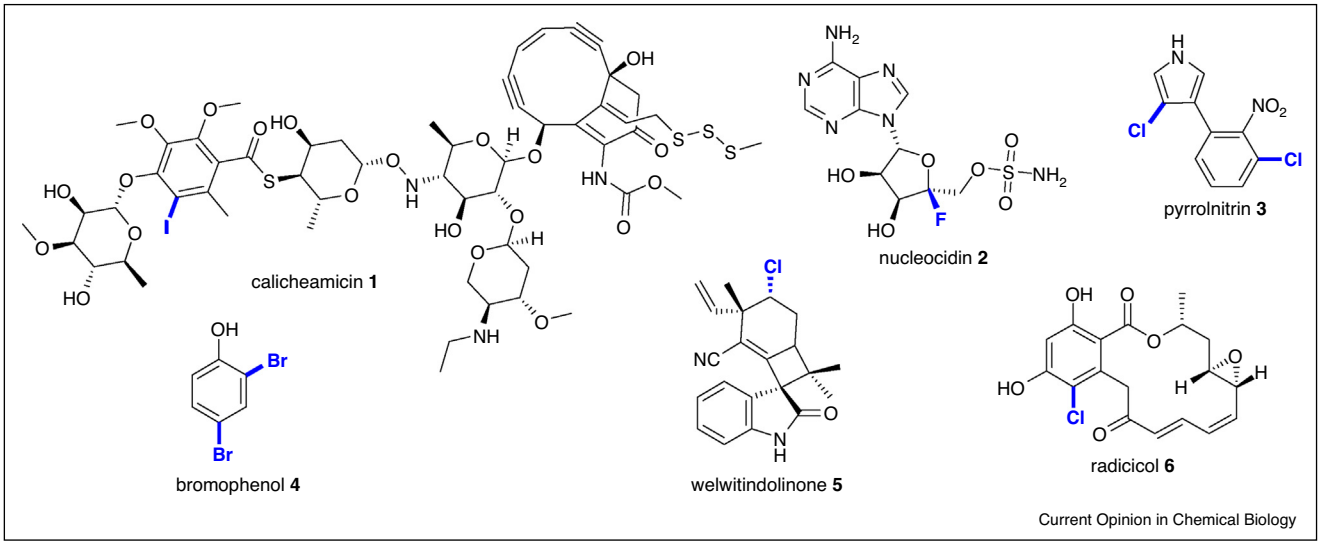
Electrophilic halogenation

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 dependent halogenases exist, such as 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 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)

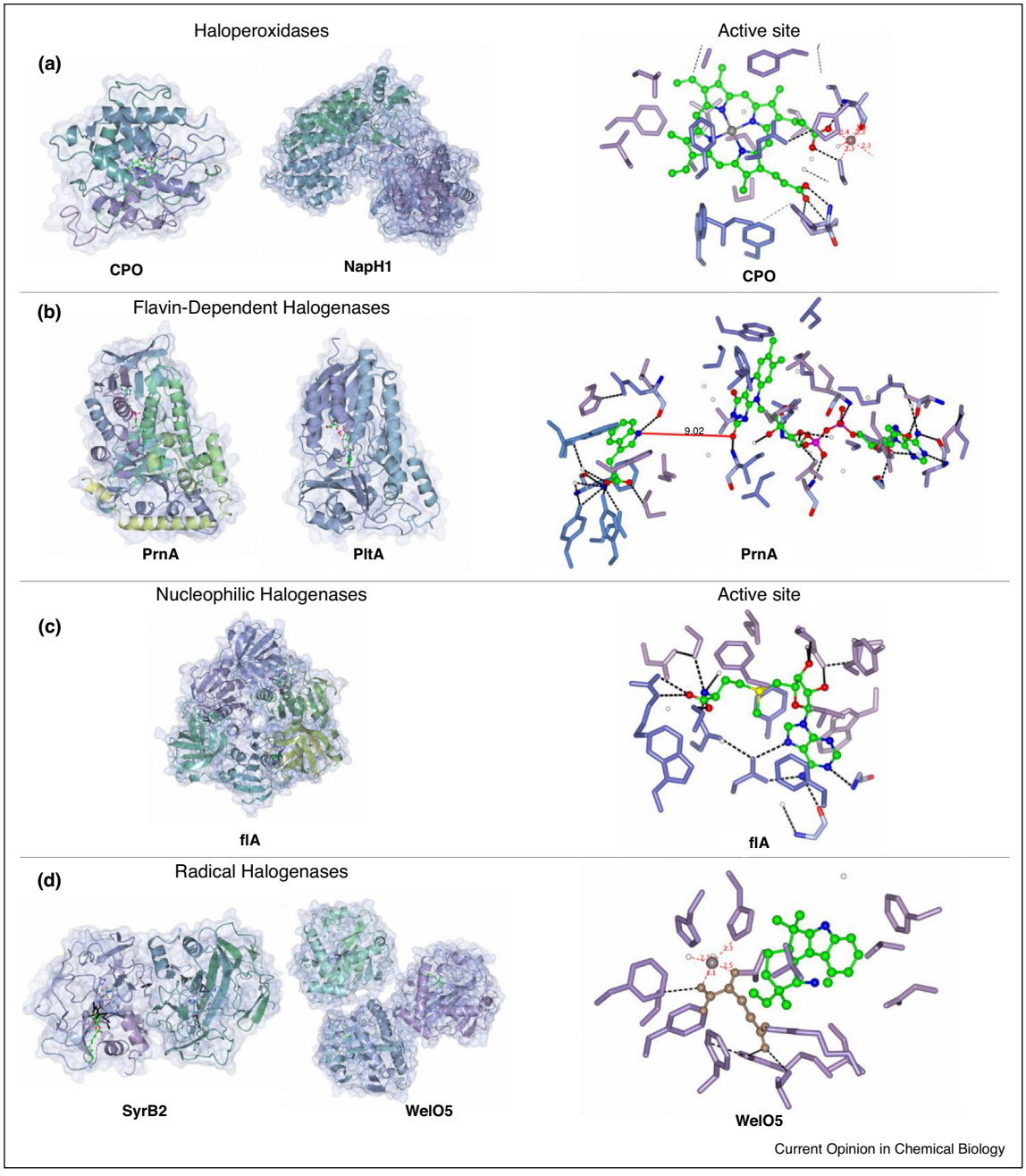
Haloperoxidases remained the only known biocatalysts enabling C–X bond formation until 1995, when Dairi *et al.* identified the first flavin dependent halogenase, *chl*. This was determined through gene inactivation studies within the biosynthetic cluster encoding 7-chlorotetracycline formation [33]. 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 in the generation of series of toxic polybrominated diphenyl

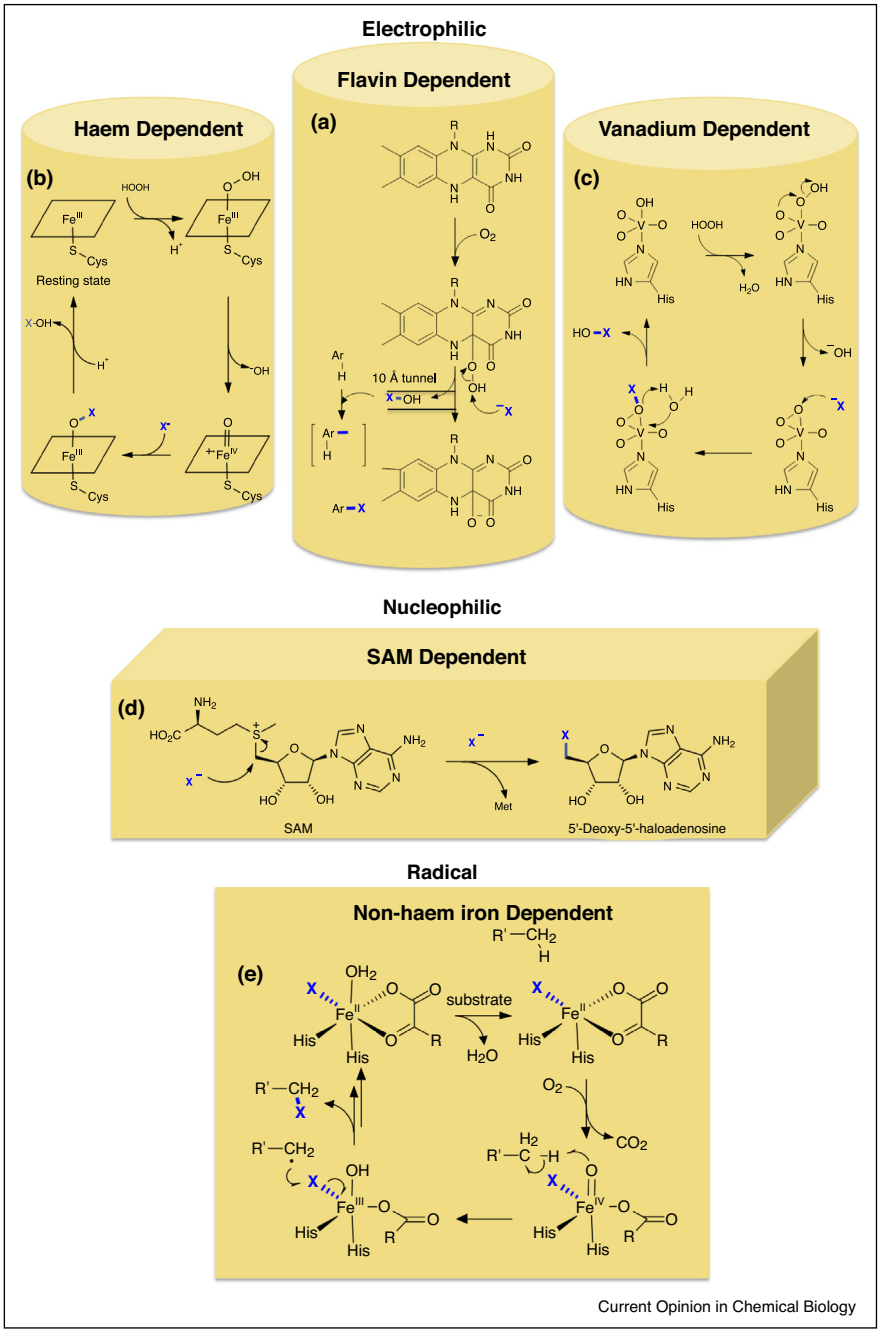
Figure 2



X-ray crystal structures of halogenases and their corresponding active sites. (A) Solved structure apo-type vanadium dependent chloroperoxidase NapH1 [31] and the heme-dependent chloroperoxidase CPO [78] complexed with protoporphyrin (green) containing Fe (grey) at the centre and the residues participating in the formation of the active site. (B) Crystal structures of the variant B flavin-dependent halogenase PltA [20••] and the variant A PrnA [18••], complexed with tryptophan and FAD (green). The distance between the tryptophan and the cofactor is also shown. (C) Solved structure of the SAM-dependent fluorinase flA [51••], complexed with SAM (green) and the amino acids participating in the active site. (D) Crystal structures of two variants of radical halogenases SyrB2 [44] and WelO5. WelO5 was complexed with 12-epifischerindole U (green), α-ketoglutarate (brown) and iron (grey) coordinated by the active site histidines [24•].

ethers and polybrominated bipyrroles that are found to persist in the food chain [21••,22]. Analysis of the coral associated bacteria *Pseudoalteromonas luteoviolacea* and planktonic *Pseudoalteromonas phenolica* O-BC30 revealed the organisms' ability to produce series of brominated compounds by utilizing the phenol brominase flavoenzyme Bmp5, with homology to flavin dependent oxygenases rather than FDHs. Flavin dependent oxygenases

Scheme 1



Outline of reaction mechanisms for electrophilic, nucleophilic and radical halogenases. **(a–c)** General scheme for reactions with hypoalous acid employed by electrophilic halogenases. **(d)** General scheme for SAM-dependent halogenases. **(e)** Catalytic cycle of non-haem iron-dependent halogenases.

that proceed via a decarboxylative mechanism are known [42]; Bmp5 is the first halogenase observed to employ such a reaction and a two-step mechanism is proposed in which a carboxylate directs bromination to the *ortho* and *para* positions followed by enzyme mediated decarboxylation [21••].

Radical halogenases

Enzymatic radical halogenation has also been reported in the biosynthesis of natural products. The non-heme-iron α-ketoglutarate(KG)-dependent enzymes are the only known enzymes that have been found so far to catalyze halogenation reactions with radical intermediates

(Scheme 1e). This class can selectively insert a halogen into a non-activated, aliphatic C–H bond, a transformation which is energetically challenging [43••,44,45•,46••]. However, these halogenases are very difficult to handle *in vitro*, due to their oxygen sensitivity and their requirement for substrate carrier proteins. Recently Hillwig and Liu identified a new member of this family of halogenases (WelO5) from welwitindolinone (**5**) biosynthesis that can regio- and stereo-selectively chlorinate the unactivated carbon centre of 12-epi-fischerindole, without the need for the substrate to be protein bound [23••,24•,43••, 44,45•,46••,47–49]. This is a breakthrough in the field of biohalogenation.

Nucleophilic halogenases

So far two families of nucleophilic halogenases are known. Both families, the halide methyltransferases and SAM halogenases utilize *S*-adenosylmethione (SAM) as a co-factor or as a co-substrate [11,12,50••,51••,52]. Such enzymes provide the only biogenesis of fluorinated natural products, (including fluoroacetate and fluorothreonine) by the impressive feat of generating fluoride anions in the absence of its hydration sheath. Other chlorinated compounds including salinosporamide are also generated by nucleophilic halogenases [53].

Applications of halogenases

The future for the industrial utilization of enzymatic halogenation shows much promise. The development and utilization of fluorinase in ^{18}F labelling of [^{18}F]-5-fluororibose for use in Positron Emission Tomography (PET) imaging is exciting, enabling quick and simple two-step synthesis of the radioisotope. Incredibly, the fluorinase has also shown the ability to process tethered substrates [54•,55,56].

The applicability of halogenases to synthesis has previously been limited by low levels of enzyme production and stability as well as the lack of sufficiently substrate-diverse halogenases. Recent progress towards addressing these defects has been manifold, for example the Lewis group have demonstrated that the simple co-expression of RebH with the chaperones GroEL and GroES can lead to up to 105 mg L^{-1} of protein [57]. Utilization of cross-linked enzyme aggregates (CLEAs) by treating crude *E. coli* lysates with glutaraldehyde has been demonstrated by Sewald and Frese to successfully stabilize the halogenase RebH, this approach has also been successfully adopted and applied by Micklefield [59••,60•]. In a complementary manner to address stability, Sewald has also explored the utilization of an engineered thermostable halogenase mutant [61•,62]. In order to enable the directed evolution of the tryptophan 6-halogenase ThaI from *Streptomyces albogriseolus* Sewald used a high-throughput screen utilizing cross-coupling conditions that the Goss group had developed and demonstrated to introduce a shift in the fluorescence of tryptophan [63•]. In parallel the Lewis group have developed a clever "combinatorial codon mutagenesis" approach and applied this to the flavin dependent tryptophan halogenase RebH extending its substrate specificity [64••,65]. The discovery of new enzymes with broader substrate specificity, notably RadH from the fungus *Chaetomium chiversii* and KermI from the metagenomic library of a marine organism, will offer further opportunities to accelerate this field [66,67•].

The building of new biosynthetic pathways into which halogenation is engineered is an exciting and growing area. Using such a strategy Eustáquio et al. have demonstrated the inter-conversion of the clorobiosin and novobiocin antibiotic pathways, and Salas has elegantly demonstrated the combinatorial use of halogenases to generate analogues of staurosporin and rebeccamycin, metabolites with chemotherapeutic properties [68•,69•]. The engineering of a new fluorinated polyketide fluorosalinosporamide, as well as fluoromalonyl-coA has also been achieved [70,71••].

The utilization of the powerful combination of halogenation and cross-coupling to effect C–H activation, first demonstrated by the GenoChemetic generation of derivatives of pacidamycin, is an emerging and exciting area [72••]. O'Connor was able to harness the bacterial halogenase RebH and engineer the production of chlorinated alkaloids within the plant system, *Catheranthus roseus* and also effect selective derivatization of the resultant arylhalide [73••,74,75]. Moving away from natural products, Lewis has demonstrated late stage diversification of biologically active small molecules using a two-step enzymatic bromination and Pd mediated cross-coupling [76]. More recently Micklefield has shown that both halogenation and cross-coupling of indole may be carried out in one pot with the halogenase stabilized as CLEAS and separated from the palladium catalyst by a permeable membrane [60•]. By developing very careful reaction conditions, even halotryptophans (which can chelate to and poison palladium), may be cross coupled at room temperature and in the presence of oxygen. This finding has enabled the Goss group to utilize the GenoChemetic approach to natural product generation in a living system, with cells engineered to produce brominated metabolites and their synchronous cross-coupling *in situ* [77••]. This approach affords the benefit of easing compound purification and drawing metabolic flux through the system.

Concluding thoughts

The past decade has brought many exciting advances in biocatalytic halogenation. There is considerable promise for application of halogenases in large scale synthesis, reducing the need for toxic reagents, overcoming issues of regioselectivity, and reducing toxic waste; however, several limitations remain: the substrate scope for halogenases is still narrow and current examples have been performed under very dilute conditions. Immobilization

and stabilization using CLEAs and the exploration of co-factor regeneration systems have laid the foundation required for efficient and scalable biocatalytic halogenation. In the context of the Pharma and Agrochemical sectors, many of the desired substrates have low solubility in aqueous media. In order to accommodate such substrates, halogenases will have to be evolved that can tolerate added organic solvents, or perhaps explored within a system that confers enhanced solvent tolerance such as within a catalytic biofilm. The promise of selective halogenation under mild conditions with renewable catalysts is a very attractive prospect and is ripe for exploitation and application.

Acknowledgements

We thank Syngenta for a CASE studentship (DSG), Astra Zeneca and EPSRC CRITICAT EP/L016419/1 for studentship support (JD), ERAIB (Grant no. 031A338A RJMG) and ERC GenoChemetics (FP7/2007-2013/ ERC consolidator grant GCGXC grant agreement no 614779 RJMG) for funding.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Kirk KL, Filler R: **Recent advances in the biomedicinal chemistry of fluorine containing compounds**. In *Biomedical Frontiers of Fluorine Chemistry.* Edited by Ojima I, McCarthy JR, Welch JT. American Chemical Society; 1996:1-24.

2. Herrera-Rodriguez LN, Khan F, Robins KT, Meyer H-P: **Perspectives on biotechnological halogenation. Part I: Halogenated products and enzymatic halogenation**. *Chim Oggi-Chem Today* 2011, **29**:31-33.

3. Xu Z, Yang Z, Liu Y, Lu Y, Chen K, Zhu W: **Halogen bond: its role beyond drug–target binding affinity for drug discovery and development**. *J Chem Inf Model* 2014, **54**:69-78.

4. Jeschke P: **The unique role of halogen substituents in the design of modern agrochemicals**. *Pest Manag Sci* 2010, **66**:10-27.

5. Mahoney KPP, Smith DRM, Bogosyan EJA, Goss RJM: **Access to high value natural and unnatural products through hyphenating chemical synthesis and biosynthesis**. *Synthesis* 2014, **46**:2122-2132.

6. Schmidt R, Stolle A, Ondruschka B: **Aromatic substitution in ball mills: formation of aryl chlorides and bromides using potassium peroxomonosulfate and NaX**. *Green Chem* 2012, **14**:1673-1679.

7. Eissen M, Strudthoff M, Backhaus S, Eismann C, Oatken G: **Oxidation numbers, oxidants, and redox reactions: variants of the electrophilic bromination of alkenes and variants of the application of oxone**. *Chem Educ* 2011, **88**:284-291.

8. Eissen M, Lenoir D: **Electrophilic bromination of alkenes: environmental, health and safety aspects of new alternative methods**. *Chemistry* 2008, **14**:9830-9841.

9. Gribble G: **Biological activity of recently discovered halogenated marine natural products**. *Mar Drugs* 2015, **13**:4044-4136.

10. Gribble GW: **Naturally occurring organohalogen compounds**. *Acc Chem Res* 1998, **31**:141-152.

11. O'Hagan DB, Harper D: **Fluorine-containing natural products**. *J Fluor Chem* 1999, **100**:127-133.

12. Deng H, O'Hagan D: **Enzymatic fluorination and biotechnological developments of the fluorinase**. *Chem Rev* 2015, **115**:634-649.

13. Nicolaou KC, Dai W-M: **Chemistry and biology of the enediyne anticancer antibiotics**. *Angew Chem Int Ed Engl* 1991, **30**:1387-1416.

14. Lee MD, Manning JK, Williams DR, Kuck NA, Testa RT, Borders DB: **Calicheamicins, a novel family of antitumor antibiotics, 3. Isolation, purification and characterization of calicheamicins beta 1Br, gamma 1Br, alpha 2I, alpha 3I, beta 1I, gamma 1I and delta 1I**. *J Antibiot (Tokyo)* 1989, **42**:1070-1087.

15. Arima K, Imanaka H, Kousaka M, Fukuta A, Tamura G: **Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas***. *Agric Biol Chem* 1964, **28**:575-576.

16. • Hammer PE, Hill DS, Lam ST, Van Pée K-H, Ligon JM: **Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin**. *Appl Environ Microbiol* 1997, **63**:2147-2154.
This benchmark paper reports the initial discovery of the flavin dependent halogenases from pyrrolnitrin biosynthesis.

17. •• Keller S, Wage T, Hohaus K, Holzer M, Eichhorn E, Van Pée K-H: **Purication and partial characterisation of tryptophan 7-halogenase (PrnA) from *Pseudomonas fluorescens***. *Angew Chemie* 2000, **39**:2300-2302.

18. •• Dong C, Flecks S, Unversucht S, Van Pée K-H, Naismith JH: **Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination**. *Science* 2005, **309**:2216-2219.
This important paper provides structural insight into the pyrrolnitrin flavin dependent halogenase and represents the first structural characterization of a flavin dependent halogenase.

19. Zhu X, De Laurentis W, Leang K, Herrmann J, Ihlefeld K, Van Pée K-H: **Structural insights into regioselectivity in the enzymatic chlorination of tryptophan**. *J Mol Biol* 2009, **391**:78-85.

20. •• Pang AH, Garneau-Tsodikova S, Tsodikov OV: **Crystal structure of halogenase PltA from the pyoluteorin biosynthetic pathway**. *J Struct Biol* 2015, **193**:349-357.
This notable paper provides structural insight into a variant B member of FDHs, the activity of which has been previously determined *in vitro*.

21. •• Agarwal V, El Gamal AA, Yamanaka K, Poth D, Kersten RD, Schorn M, Allen EE, Moore BS: **Biosynthesis of polybrominated aromatic organic compounds by marine bacteria**. *Nat Chem Biol* 2014, **10**:640-647.
The unusual enzymology underlying the biosynthesis of series of widespread marine toxins is revealed in this significant paper.

22. El Gamal AA, Agarwal V, Diethelm S, Rahman I, Schorn MA, Sneed JM, Louie GV, Whale KE, Mincer TJ, Noel JP, Paul VJ, Moore BS: **Biosynthesis of coral settlement cue tetrabromopyrrole in marine bacteria by a uniquely adapted brominase-thioesterase enzyme pair**. *Proc Natl Acad Sci U S A* 2016, **113**:3797-3802.

23. •• Hillwig ML, Liu X: **A new family of iron-dependent halogenases acts on freestanding substrates**. *Nat Chem Biol* 2014, **10**:921-923.
The first example of a radical halogenase mediating late stage C–H activation of an advanced metabolite that is not covalently linked to a carrier enzyme is reported, this is particularly noteworthy conferring such enzymes with great biotechnological potential.

24. • Mitchell AJ, Zhu Q, Maggiolo AO, Ananth NR, Hillwig ML, Liu X, Boal AK: **Structural basis for halogenation by iron- and 2-oxo-glutarate-dependent enzyme WelO5**. *Nat Chem Biol* 2016, **12**:636-640.
The first structural elucidation of a radical halogenase acting on a free-standing substrate.

25. • Zeng J, Zhan J: **A novel fungal flavin-dependent halogenase for natural product biosynthesis**. *ChemBioChem* 2010, **11**:2119-2123.
The first flavin dependent halogenase characterized from a fungus is reported here.

26. Shaw PD, Hager LP: **An enzymatic chlorination reaction**. *JACS* 1959, **81**:1011-1012.

27. Ruf J, Carayon P: **Structural and functional aspects of thyroid peroxidase**. *Arch Biochem Biophys* 2006, **445**:269-277.

28. Sundaramoorthy M, Terner J, Poulos TL: **Stereochemistry of the chloroperoxidase active site: crystallographic and molecular-modeling studies**. *Chem Biol* 1998, **5**:461-473.

29. Messerschmidt A, Prade L, Wever R: **Implications for the catalytic mechanism of the vanadium-containing enzyme chloroperoxidase from the fungus *Curvularia inaequalis* by X-ray structures of the native and peroxide form**. *Biol Chem* 1997, **378**:309-315.

30. •• Bernhardt P, Okino T, Winter JM, Miyanaga A, Moore BS: **A stereoselective vanadium-dependent chloroperoxidase in bacterial antibiotic biosynthesis**. *JACS* 2011, **133**:4268-4270.
Vanadium dependent halogenases had previously been believed to be unselective, simply generating a pool of hypohalous acid that would react with the most electron rich species. In this game-changing paper a selective halogenation and insight into enzymatic control is reported.

31. Liscombe DK, Miyunaga A, Fielding E, Bernhardt P, Li A, Winter JM, Gilson MK, Noel JP, Moore BS: **Crystal structure of apo-type bacterial vanadium-dependent chloroperoxidase**. RCSB Protein Data Bank.

32. Poulos TL: **Heme enzyme structure and function**. *Chem Rev* 2014, **113**:3919-3962.

33. Dairi T, Nakano T, Aisaka K, Katsumata R, Hasegawa M: **Cloning and nucleotide sequence of the gene responsible for chlorination of tetracycline**. *Biosci Biotechnol Biochem* 1995, **59**:1099-1106.

34. Flecks S, Patallo EP, Zhu X, Erneyi AJ, Seifert G, Schneider A, Dong C, Naismith JH, van Pée K-H: **New insights into the mechanism of enzymatic chlorination of tryptophan**. *Angew Chem* 2008, **47**:9533-9536.

35. Blasiak LC, Drennan CL: **Structural perspective on enzymatic halogenation**. *Acc Chem Res* 2009, **42**:147-155.

36. Neumann CS, Fujimori DG, Walsh CT: **Halogenation strategies in natural product biosynthesis**. *Chem Biol* 2008, **15**:99-109.

37. Podzelinska K, Latimer R, Bhattacharya A, Vining LC, Zechel DL, Jia Z: **Chloramphenicol biosynthesis: the structure of CmlS, a flavin-dependent halogenase showing a covalent flavin aspartate bond**. *J Mol Biol* 2010, **397**:316-331.

38. Zehner S, Kotzsch A, Bister B, Süssmuth RD, Mendez C, van Pée K-H: **A regioselective tryptophan 5-halogenase is involved in pyrroindomycin biosynthesis in *Streptomyces rugosporus* LL-42D005**. *Chem Biol* 2005, **12**:445-452.

39. • Zeng J, Lytle AK, Gage D, Johnson SJ, Zhan J: **Specific chlorination of isoquinolines by a fungal flavin-dependent halogenase**. *Bioorg Med Chem Lett* 2010, **11**:2119-2123.
This report demonstrated that the fungal halogenase Rdc2, has an impressively broad natural substrate flexibility ranging from larger substrates such as monocillin II to smaller isoquinolines.

40. Buedenbender S, Rachid S, Müller R, Schulz GE: **Structure and action of the myxobacterial chondrochloren halogenase CndH: a new variant of FAD-dependent halogenases**. *J Mol Biol* 2009, **385**:520-530.

41. Weichold V, Milbredt D, van Pée K-H: **Specific enzymatic halogenation – from the discovery of halogenated enzymes to their application in vitro and vivo**. *Angew Chem Int Ed Engl* 2016, **55**:6374-6389.

42. Ballou DP, Entsch B, Cole LJ: **Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases**. *Biochem Biophys Res Commun* 2005, **338**:590-598.

43. •• Vaillancourt FH, Yin J, Walsh CT: **SyrB2 in syringomycin E biosynthesis is a nonheme Fe^{II} α-ketoglutarate- and O_2-dependent halogenase**. *Proc Natl Acad Sci U S A* 2005, **102**:10111-10116.
The first biochemical characterization of a radical halogenase.

44. Blasiak LC, Vaillancourt FH, Walsh CT, Drennan CL: **Crystal structure of the non-haem iron halogenase SyrB2 in syringomycin biosynthesis**. *Nature* 2005, **440**:368-371.

45. • Chang Z, Flatt P, Gerwick WH, Nquyen VA, Willis CL, Sherman DH: **The barbamide biosynthetic gene cluster: a novel marine cyanobacterial system of mixed polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit**. *Gene* 2002, **296**:235-247.
This paper provides early insight into the operation of a new class of halogenase, later demonstrated to be radical halogenases [43••], in the generation of a complex marine natural product.

46. •• Matthews ML, Neumann CS, Miles LA, Grove TL, Booker SJ, Krebs C, Walsh CT, Bollinger JM: **Substrate positioning controls the partition between halogenation and hydroxylation in the aliphatic halogenase, SyrB2**. *Proc Natl Acad Sci U S A* 2009, **106**:17723-17728.
A crucial paper highlighting the two closely linked enzymatic reactivities of halogenation and hydroxylation and insight into how an enzyme might mediate halogenation over hydroxylation.

47. Hillwig ML, Zhu Q, Ittiamornkul K, Liu X: **Discovery of a promiscuous non-heme iron halogenase in ambiguine alkaloid biogenesis: implication for an evolvable enzyme family for late-stage halogenation of aliphatic carbons in small molecules**. *Angew Chem Int Ed Engl* 2016, **55**:5780-5784.

48. Zhu Q, Hillwig ML, Doi Y, Liu X: **Aliphatic halogenase enables late-stage C–H functionalization: selective synthesis of a brominated fischerindole alkaloid with enhanced antibacterial activity**. *Chembiochem* 2016, **17**:466-470.

49. Zhu Q, Liu X: **Characterisation of non-heme iron aliphatic halogenase WelO5* from *Hapalosiphon welwitschii* IC52-3: identification of a minimal protein sequence motif that confers enzymatic chlorination specificity in the biosynthesis of welwitindolelinones**. *Beilstein J Org Chem* 2017, **16**:1168-1173.

50. •• O'Hagan D, Schaffrath C, Cobb SL, Hamilton JT, Murphy CD: **Biochemistry: biosynthesis of an organofluorine molecule**. *Nature* 2002, **416**:279.
A highly significant publication detailing the first identification of an enzyme capable of biosynthetic fluorination.

51. •• Dong C, Huang F, Schaffrath C, Spencer JB, O'Hagan D, Naismith JH: **Crystal structure and mechanism of a bacterial fluorinating enzyme**. *Nature* 2004, **427**:561-565.
A noteworthy publication providing the first structural insight into the fluorinase enzyme.

52. Zhu X, Robinson DA, McEwan AR, O'Hagan D, Naismith JH: **Mechanism of enzymatic fluorination in *Streptomyces cattleya***. *JACS* 2007, **129**:14597-14604.

53. Eustáquio AS, Pojer F, Noel JP, Moore BS: **Discovery and characterisation of a marine bacterial SAM-dependent chlorinase**. *Nat Chem Biol* 2008, **4**:69-74.

54. • Dall'Angelo S, Bandaranayaka N, Windhorst AD, Vugts DJ, van der Born D, Onega M, Schweiger LF, Zanda M, O'Hagan D: **Tumour imaging by Positron Emission Tomography using fluorinase generated 5-[18F]fluoro-5-deoxyribose as a novel tracer**. *Nucl Med Biol* 2013, **40**:464-470.
A noteworthy demonstration of the utility of the fluorinase for medical imaging.

55. Zhang Q, Dall'Angelo S, Fleming IN, Schweiger LF, Zanda M, O'Hagan D: **Last-step enzymatic [18F]-fluorination of cysteine-tethered RGD peptides using modified Barbas linkers**. *Chem Eur J* 2016, **22**:10998-11004.

56. Thompson S, Zhang Q, Onega Q, McMahon S, Fleming I, Ashworth S, Naismith JH, Passchier J, O'Hagan D: **A localised tolerance in the substrate specificity of the fluorinase enzyme enables "late-step" ^{18}F fluorination of a RGD peptide under ambient conditions**. *Angew Chemie* 2014, **126**:9059-9064.

57. Payne JT, Andorfer MC, Lewis JC: **Regio-selective arene halogenation using the FAD-dependent halogenase RebH**. *Angew Chem Int Ed Engl* 2014, **52**.

59. •• Frese M, Sewald N: **Enzymatic halogenation of tryptophan on a gram scale**. *Angew Chem Int Ed Engl* 2015, **54**:298-301.
This particularly noteworthy publication provides a practical demonstration of halogenase stabilization and reaction upscaling.

60. • Latham J, Henry J-M, Sharif HH, Menon BRK, Shepherd SA, Greaney MF, Micklefield J: **Integrated catalysis opens new**

arylation pathways via regiodivergent enzymatic C–H activation. *Nat Commun* 2016, **7**:11873.
Demonstration of utilization of one-pot palladium and halogenase catalysis enabled by a membrane partitioned system.

61. Schnepel C, Mignes H, Frese M, Sewald N: **A high-throughput**
• **fluorescence assay to determine the activity of tryptophan halogenases**. *Angew Chem* 2016, **55**:14159-14163.
Building upon Ref. [63•], the utilization of fluorescence perturbation as a readout for the directed evolution of halogenases is exemplified.

62. Menon BRK, Lathan J, Dunstan MS, Brandenburger E, Klemstein U, Leys D, Karthikeyan C, Greaney MF, Shepherd SA, Micklefield J: **Structure and biocatalytic scope of thermophilic flavin-dependent halogenase and flavin reductase enzymes**. *Org Biomol Chem* 2016, **14**:9354.

63. Roy AD, Goss RJM, Wagner GK, Winn M: **Development of**
• **fluorescent aryltryptophans by Pd mediated cross-coupling of unprotected halotryptophans in water**. *Chem Commun* 2008, **39**:4831-4833.
First demonstration that cross-coupling of halotryptophans may be utilized to modulate fluorescence properties.

64. Payne JT, Poor CB, Lewis JC: **Directed evolution of RebH for**
•• **site selective halogenation of large biologically active molecules**. *Angew Commun* 2015, **54**:4226-4230.
Demonstration of the utilization of directed evolution to modify halogenase substrate scope.

65. Andorfer MC, Park HJ, Vergara-Coll J, Lewis JC: **Directed evolution of RebH for catalyst-controlled halogenation of indole C–H bonds**. *Chem Sci* 2016, **7**:3720.

66. Menon BRK, Brandenburger E, Sharif HH, Klemstein U, Shepherd SA, Greaney MF, Micklefield J: **RadH: a versatile halogenase for the integration into biosynthetic pathways**. *Angew Chem Int Ed Engl* 2017, **56**:11841-11845.

67. Smith DRM, Uria AR, Helfrich EJN, Milbredt D, van Pée K-H, Piel J,
• Goss RJM: **An unusual flavin-dependent halogenase from the metagenome of the marine sponge *Theonella swinhoei* WA**. *ACS Chem Biol* 2017, **12**:1281-1287.
The first reported discovery of a halogenase from a metagenome and the first tryptophan halogenase demonstrating a natural preference for modified tryptophan.

68. Eustáquio AS, Gust B, Luft T, Li SM, Chater KF, Heide L:
• **Clorobiocin biosynthesis: identification of the halogenase and generation of structural analogs**. *Chem Biol* 2003, **10**:279-288.
The first example of a halogenase being moved from one biosynthetic pathway and installed to work in concert with another biosynthetic gene cluster. The two metabolites and gene clusters are very similar, however this work exemplified the potential for utilizing halogenases in combinatorial biosynthesis/the mix and match synthetic biology of natural products.

69. Sánchez C, Zhu L, Brana AF, Salas AP, Rohr J, Mendez C,
• Salas JA: **Combinatorial biosynthesis of antitumor indolocarbazole compounds**. *Proc Natl Acad Sci U S A* 2005, **102**:461-466.
Powerful demonstration of the combinatorial introduction of halogenases from a series of different pathways to modulate antitumor agents.

70. Eustáquio AS, O'Hagan D, Moore BS: **Engineering fluorometabolite production: fluorinase expression in *Salinospora tropica* yields fluorosalinosporamide**. *J Nat Prod* 2010, **73**:378-382.

71. Walker MC, Thuronyi BW, Charkoudian LK, Lowry B, Khosla C,
•• Chang MCY: **Expanding the fluorine chemistry of living systems using engineered polyketide synthase pathways**. *Science* 2013, **341**:1089-1094.
The first integration of the fluorinase into an engineered antibiotic biosynthetic gene cluster enabling the biosynthetic generation of new to nature fluorometabolites.

72. Deb Roy A, Grüschow S, Cairns N, Goss RJM: **Gene expression**
•• **enabling synthetic diversification of natural products: chemogenetic generation of pacidamycin analogs**. *JACS* 2010, **132**:12243-12245.
The first out of context introduction of a halogenase to act in concert with a dramatically different biosynthetic gene cluster, and first example of using a halogenase: cross coupling strategy to enable selective C–H activation. The first example of GenoChemetics.

73. Runguphan W, Xudong Q, O'Connor S: **Integrating carbon-**
•• **halogen bond formation into medicinal plant metabolism**. *Nature* 2010, **468**:461-464.
The first example of engineering a halogenase utilized to complement a plant biosynthetic gene cluster.

74. Glenn WS, Nims E, O'Connor S: **Reengineering a tryptophan halogenase to preferentially chlorinate a direct alkaloid precursor**. *JACS* 2011, **133**:19348-19349.

75. Runguphan W, O'Connor S: **Diversification of monoterpene indole alkaloid analogs through cross-coupling**. *Org Lett* 2013, **15**:2850-2853.

76. Durak LJ, Payne JT, Lewis JC: **Late-stage diversification of biologically active molecules via chemoenzymatic C–H functionalisation**. *ACS Catal* 2016, **6**:1451-1454.

77. Sharma SV, Tong X, Pubill-Ulldemolins C, Cartmell C,
•• Bogosyan EJA, Rackham EJ, Marelli E, Hamed RB, Goss RJM: **Living GenoChemetics by hyphenating synthetic biology and synthetic chemistry *in vivo***. *Nat Commun* 2017, **8**:229.
First example of enabling halogenation and Pd mediated cross-coupling in the presence of living cells, this approach enables metabolite modulation in the presence of a living system, metabolic flux to be drawn through the system.

78. Sundaramoorthy M, Terner J, Poulos TL: **The crystal structure of chloroperoxidase: a heme peroxidase-cytochrome P450 functional hybrid**. *Structure* 1995, **3**:1367-1378.