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Halogenases for the synthesis of small molecules

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Abstract

Enzymatic halogenation is a rapidly developing tool in the synthetic chemist's toolbox. Utilizing oxygen, halide salts and operating at ambient temperatures in aqueous media, halogenating enzymes, particularly flavin- or Fe(II)/α-ketoglutarate dependent halogenases, allow the regio- and stereoselective installation of halogen atoms to yield valuable building blocks and uniquely derivatized complex molecules, including natural products. Apart from modulating the physico-chemical properties of molecules and, in consequence, their biological activity, halogen atoms can also serve as chemical linchpins for further derivatization of the molecular scaffold, for example in chemo-enzymatic cascades. Thanks to rapid advances in bioinformatic enzyme sourcing, biosynthetic pathway elucidation and enzyme engineering strategies, the palette of enzymatically produced halogenated structures is constantly growing. In addition, successful studies to improve catalytic performance of the halogenation biocatalysts are boding well for industrial applications.

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Introduction

Halogens are privileged substituents in the synthesis of biologically active ingredients [1,2]. Beyond the ability of halogen atoms to tune the hydrophobicity of small molecules, compounds containing chlorine, bromine, iodine and—in some cases—fluorine [3] can form directed close contacts with electron donor moieties, the so-called halogen bond. Thus, the incorporation of a halogen can significantly alter a molecule's property and may impact its bioactivity, metabolism, and pharmacokinetic profile [4]. Perhaps unsurprisingly, the list of the 200 best-selling small molecule drugs of 2021 thus contains a remarkable 87 compounds that carry a halogen atom [5]. Examples include blockbusters such as the anticoagulant rivaroxaban and the antidiabetic medicine empagliflozin with annual sales revenues of $7.7 and $5.8 billion in 2021, respectively. This trend is similarly reflected in agriculture: A startling 96% of all pesticides launched since 2010 contain a halogen atom [6]. Furthermore, carbon-halogen motifs are useful handles for chemical modification, explaining why halogenated species represent common intermediates in synthetic manufacturing routes [7,8].

Even though chemical halogenation is a well-established technology, it suffers from the use of hazardous or even toxic chemicals, including halogen gas and Lewis catalysts, and sometimes poor atom efficiency [9,10]. In addition, chemical methods often lack selectivity or have specific demands on the substrate structure [9]. Consequently, development of alternative methods for the selective halogenation of small molecules is of great interest for the pharmaceutical and agrochemical industry. Within this context, Nature has developed a multitude of halogenating enzymes that combine oxidative power with selectivity. The diverse molecular scaffolds of the more than 5000 halogenated natural compounds that have been discovered to date demonstrate the catalytic power of these enzymes [11]. Hence, biocatalytic approaches to obtain halogenated small molecules appear particularly desirable as the use of enzymes is typically associated with high regio- and stereocontrol, while at the same time, the biological catalysts operate under mild reaction conditions.

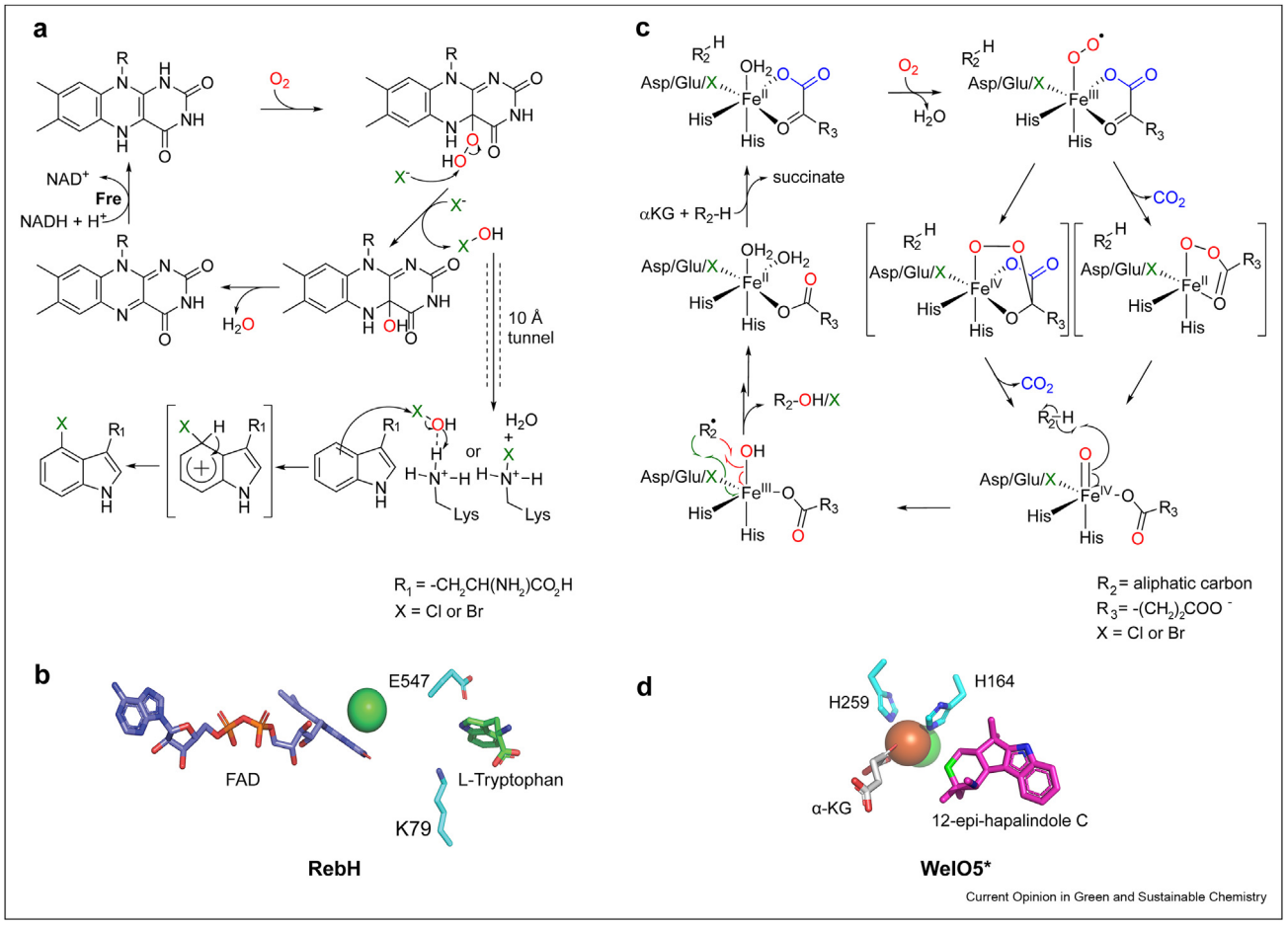
Halogenating enzymes are often classified according to the catalytic mechanism they employ [12,13]. While the rare S-adenosyl-L-methionine (SAM) fluorinases react via a nucleophilic pathway to generate a small set of fluorinated metabolites [14–16], heme-, vanadium- and flavin-dependent halogenases (FDH) follow an electrophilic mechanism in which hypohalous acid is generated and ultimately reacts with electron-rich substrates. Notably, while heme- and vanadium dependent enzymes typically do not achieve regio- or stereospecificity because the reactive acid can diffuse out of the enzyme active site, FDH architecture has been found to allow for site directed halogenation [17–19]. Mechanistically, flavin-dependent halogenases use reduced flavin and oxygen

to form a peroxyflavin species, which in turn reacts with a halide ion, generating the reactive hypohalous acid [20–22]. After traversing an approximately 10 Å long tunnel within the enzyme [22–25], the hypohalous acid then reacts with an active site lysine—either covalently or through hydrogen bonding [21,26,27]—before the selective electrophilic aromatic substitution of an aromatic compound occurs (Figure 1a, b).

Mechanistically distinct from the above-mentioned FDHs, Fe(II)/α-ketoglutarate dependent halogenases (αKGHs) exploit a radical mechanism for halogenation (Figure 1c). Within their jelly-roll fold, αKGHs harbor an active site in which an Fe^{II} is coordinated by two histidines, α-ketoglutarate and a halogen ligand (Figure 1d). Upon reaction with oxygen and subsequent oxidative decarboxylation of the α-ketoglutarate ligand, an Fe^{IV}=O species is generated which can abstract a H-atom from a properly positioned sp^3-carbon of the substrate molecule [28–30]. Radical recombination of the substrate with the halogen ligand leads to the desired halogenated product whereas a hydroxyl rebound, which is equally possible, generates the corresponding hydroxylated side product. The halogenation versus hydroxylation selectivity is considered to depend on the positioning of the substrate in the active site relative to the iron complex [31–36].

In recent years, halogenating enzymes have become a profitable target for the expansion of the biocatalytic toolbox. Within this review, we highlight recent examples of halogenase biocatalysts strategically employed for the modification of building blocks, the late-stage functionalization of complex molecules and in combination with chemocatalysts. By delineating success stories of enzymatic halogenation published primarily in the last two years, we hope to give a helpful overview of

Figure 1



Catalytic machinery of selected halogenase families. **a**) Proposed reaction mechanism of flavin dependent halogenases [21–27]. **b**) Snapshot of the geometric arrangement of active site residues (blue) and halogenide (green ball) derived from the crystal structure of the tryptophan 7-halogenase RebH in complex with substrate L-tryptophan (green) and FAD (purple) (PDB: 2OA1) [22] **c**) Proposed reaction mechanism of α-ketoglutarate dependent halogenases adapted from Mitchell et al. [28,29] and Galonić et al. [30] **d**) Snapshot of the geometric arrangement of the Fe(II) ion coordinated by active site residues (blue), α-ketoglutarate (light grey) and chloride (green ball) derived from the crystal structure of α-ketoglutarate dependent halogenase WelO5 in complex with its substrate 12-epi-hapalindole C (pink) (PDB: 5IQT) [28].

the current state-of-the-art and aim to inspire novel synthesis routes that rely on powerful enzymatic halogen installation.

Building block derivatization by wildtype and engineered halogenases

In medicinal and agricultural chemistry, indoles are among the most widespread nitrogen-containing heterocycles [37]. Consequently, methods enabling their chemical diversification are highly desirable. To date, several FDHs have been identified that allow for indole halogenation [38,39], however, the described enzymes were often found to exhibit a narrow substrate scope. Thus, the discovery of novel halogenases capable of accepting a wider chemical substrate space remains at the forefront of biocatalytic halogenation research.

To expand the current toolbox of FDHs, several research groups have employed bioinformatic search algorithms. In this spirit, Lee et al. successfully identified a novel tryptophan 6-halogenase (SatH) from *Streptomyces albus* [40]. SatH was characterized in an *Escherichia coli* whole—cell reaction system supplemented with 1.5 mM of L-tryptophan. The enzyme showed high regiospecific halogenation at the C6-position of the indole ring of L-tryptophan and a product yield of 0.86 mM. Notably, tryptophan halogenases (Trp halogenases) are classified depending on the position of halogenation and are accordingly defined as Trp 5-, 6-, and 7-halogenases. However, while some determinants for the enzymes' regiospecificity are well understood, for example structural elements defining Trp 7-halogenases [23], the factors causing the differing regioselectivity between 5- and 6-halogenases have remained mostly elusive: only mutations L456F/P357E/P458T were previously reported to have an influence on regioselectivity by affecting interactions with the amino acid backbone of tryptophan [41]. Informed by comparing substrate-docked homology models of Trp 5-halogenase PyrH from *Streptomyces rugosporus* LL-42D005 [24,42] and the newly discovered Trp 6-halogenase SatH, the authors identified two residues, A78/V79 in SatH and G77/I78 in PyrH, as further determinants for regioselectivity. Accordingly, when substituting the "AV" with "GI" residues in SatH, the authors could switch the regioselectivity of bromination reactions to also yield 5-Br-Trp (63%), whereas in chlorination reactions the "GI" motif had to be supplemented with mutations L456F/P357E/P458T to modulate the product pattern. The resulting quintuple mutant, dubbed SatH-GI-FET, produced 50% 5-Cl-Trp and 50% 6-Cl-Trp (compounds **1**—**2**, Table 1).

Similarly looking to find FDHs with a broader substrate scope in nature, the Sewald group recently developed a hidden Markov model based on the PFAM tryptophan halogenase model [43] and used it to screen the bacterial associates of the *Botryococcus braunii* consortia (PRJEB21978) leading to the identification of several putative, flavin-dependent halogenase genes [44]. After heterologously expressing two of these proteins (named SpH1 and SpH2) stemming from one gene cluster of the *B. braunii* symbiont *Sphingomonas* sp, *in vitro* activity tests (substrate load of 1 mM) revealed that both enzymes were able to halogenate indole and indole derivatives (compounds **3**—**8**, Table 1). Interestingly, SpH1 was found to exclusively catalyze monohalogenation, whereas SpH2 yielded both mono- and dihalogenation for many of the selected indole derivatives.

Biosynthetic pathways can be another rich source of halogenation biocatalysts. When investigating the mechanistic basis of aetokthonotoxin biosynthesis, Breinliger et al. [45] and Adak et al. [46] recently identified and characterized two flavin-dependent halogenases (AetF and AetA) capable of synthesizing brominated tryptophan building blocks, which are subsequently enzymatically coupled to yield the mature toxin. While purified AetF was shown to accept tryptophan as the substrate leading to the 5,7-dibrominated product (compound **9**, Table 1) in the presence of NADPH, the other investigated enzyme, AetA, which bioinformatically clustered as a pyrrole halogenase, was shown to accept 5-bromoindole, yielding 3,5-bromoindole and 2,3,5-tribromoindole (compounds **10**—**11**, Table 1) in the presence of FAD, NADPH and flavin reductase SsuE [46]. Simplifying synthesis, AetF is the first example of a naturally occurring single-component, flavin-dependent halogenase which does not require a separate flavin reductase partner protein to exhibit activity.

Besides enzyme sourcing, creative engineering approaches have also led to the construction of more powerful halogenase variants to be used for the synthesis of halogenated small molecules. Building on a thermostable RebH variant, Sana et al. used a structure guided semi-rational mutagenesis approach to engineer RebH variants that—while becoming completely inactive for their native substrate tryptophan—were capable to halogenate a broad spectrum of formerly not accepted indoles (compounds **12**—**14,** Table 1). The highest conversion was reported for 5-nitroindole using the RebH-M1 variant (S130L/N166S/Y455C/F465K/Q494R), which could achieve 50% chloination and 40% bromination on a 2.5 mM scale. Notably, the enzymatic synthesis of 3-chloro-5-nitroindole had never been reported before. In addition, computational modeling and molecular dynamics simulations provided structural insights into the guiding role of key residues lining the substrate binding site and their importance in defining substrate specificity [47].

Focusing on molecular scaffolds beyond indoles, Lewis and co-workers elegantly engineered RebH to accept 4-methoxyphenyl-4-pentenoic acid, a substrate selected for its intrinsic ability to undergo halocyclization, a key

Table 1

Overview of enzymatically halogenated compounds.

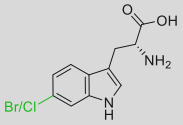
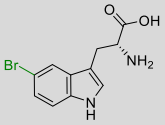
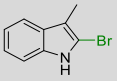
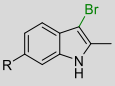
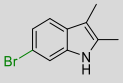
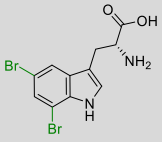
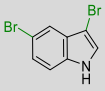
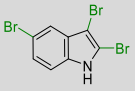
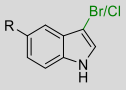
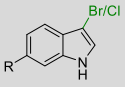
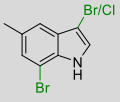
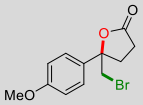
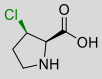
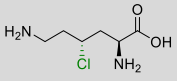
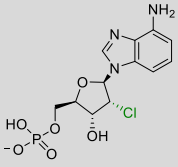
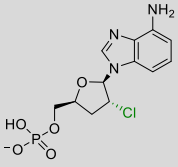
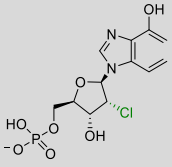
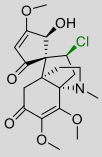
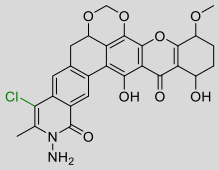
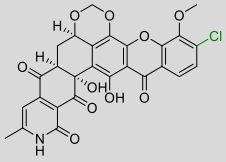
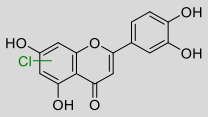
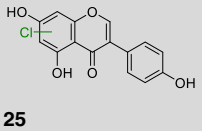
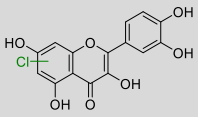
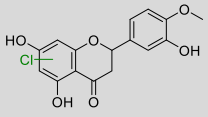
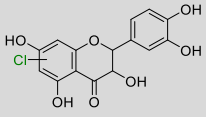
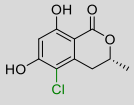
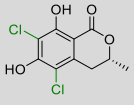
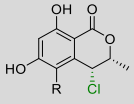
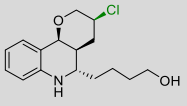
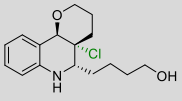
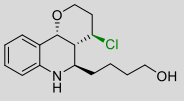
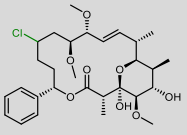
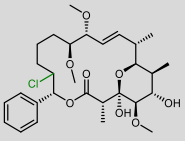
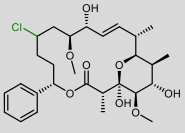
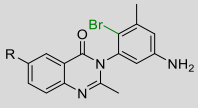
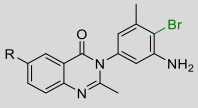
Halogenase	Halogenated compounds			References
SatH	 **1**	 **2**		[40]
SpH1/SpH2	 **3**	 **4**	 **5**	[44]
	 **6**	 **7**	 **8**	
AetF/AerA	 **9**	 **10**	 **11**	[45,46]
RebH M1/RebH M2	 **12**	 **13**	 **14**	[47]
RebH 4V + S	 **15**			[48]
SmP4H	 **16**			[50]
Hydrox	 **17**			[36]
AdeV	 **18**	 **19**	 **20**	[51]
SaDAH	 **21**			[53]

Table 1 (*continued*)

Halogenase	Halogenated compounds			References
OrfA	 **22**			[56]
XanH	 **23**			[57]
DklH	 **24**	 **25**	 **26**	[59]
	 **27**	 **28**		
ChmK/ChmN	 **29**	 **30**		[60]
PloK	 **31**			[60]
WelO5* variants	 **32**	 **33**	 **34**	[61,62]
	 **35**	 **36**	 **37**	[63]
RebH 3-T	 **38**	 **39**		[65]

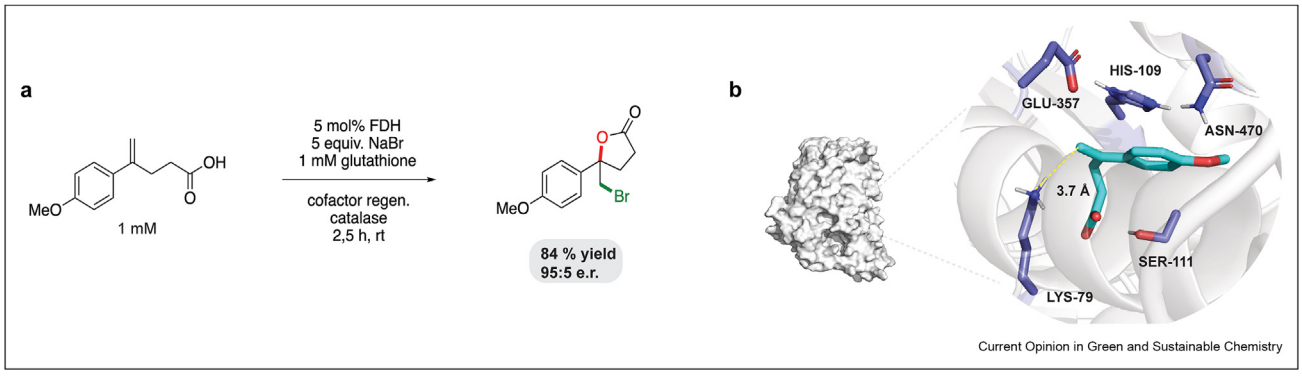
Blue sphere = wildtype halogenase, Green sphere = engineered halogenase; R = CN, NO_2, CO_2H, CO_2Me, OMe, Me, Br, Cl, F, CO_2H, F [44]; R = CN, NO_2, Cl, Br, F [47]; R = H, Cl [60]; R = H, CH_3 [65].

step in the biosynthesis of several natural products [48]. Screening a panel of 45 wildtype FDHs and 99 previously engineered RebH variants, the authors identified several variants capable of carrying out the desired non-native bromolactonization reaction yielding the cyclized product (compound **15**, Table 1). Reaction parameter optimization targeting to disarm any prematurely released HOBr enabled an increase in enantioselectivity, while evaluation of the substrate scope highlighted the influence of electron-rich, electron-neutral and electron-poor substrates on yield. This broadening of the catalytic repertoire of RebH revealed that the active sites of FDHs can tolerate intermediate and transition state structures that are distinct from those associated with electrophilic aromatic substitution (Figure 2).

With the goal to limit the detrimental HOX leakage from FDH active sites, the Chaiyen group embarked on a mechanism-guided enzyme engineering campaign of tryptophan 6-halogenase Thal [49]. Bioinformatically analyzing residues lining the tunnel connecting the two FDH active sites, where one is dedicated to HOX generation and the other enables HOX-mediated halogenation of the substrate, the researchers interrogated three amino acid positions by full randomization (NNK libraries). Remarkably, the best variant, Thal-V82I, showed reduced HOX leakage and multiple catalytic improvements such as faster halogenation, a broader substrate scope and improved thermostability and pH tolerance with respect to the wild-type enzyme. Transient kinetics and molecular dynamics simulations revealed that the improvements exemplified by Thal-V82I resulted from changes in the hydrophobic interactions in the tunnel which regulate tunnel dynamics.

Complementing FDH catalysis, α-ketoglutarate-dependent dioxygenases permit the delivery of halogenated building blocks derived from more electron-poor substrates. In this context, Papadopoulou et al. have explored the possibility of reprogramming an α-ketoglutarate-dependent hydroxylase into a halogenase with the goal to deliver halogenated L-proline (compound **16**, Table 1) [50]. Following bioinformatic screening to identify a suitable starting scaffold, the L-proline cis-4-hydroxylase from *Sinorhizobium meliloti* was selected and successfully converted into a halogenase by introducing a single point mutation (D108G) into the enzyme's active site. The reprogrammed halogenase displayed a striking regiodivergent reaction chemistry incorporating the halogen at the C3 position of the pyrrolidine ring of L-proline instead of the C4 position, where the native hydroxylation occurs. Several rounds of directed evolution delivered an optimized halogenase variant with 98-fold improved apparent k_{cat}/K_m for the chlorination of L-proline with respect to the parent enzyme SmP4H (D108G). Similarly relying on hydroxylase re-programming to supplement the biocatalytic halogenation toolbox, the Chang group bioinformatically identified a L-lysine hydroxylase (Hydrox) with 71% amino acid sequence identity to the putative halogenase from *Actinoplanes teichomyceticus* [36]. The high degree of sequence similarity between these two enzymes enabled the researchers to carry out DNA shuffling and generate a library of chimeric enzymes. The library was screened for variants with retained halogenation activity, which facilitated the identification of key residues that control reaction selectivity. Building on these learnings, the chemoselectivity of Hydrox could be switched from hydroxylation to halogenation (compound **17**, Table 1). The reprogrammed enzyme differed from the parent by a total of 14 residues, many of which were located on two β-strands adjacent to the α-ketoglutarate binding pocket. Together, these examples highlight the potential of altering the pathway selectivity of α-

Figure 2



FDH catalyzed halocyclization. a) RebH variant 4V + S (A442V/F111S) was found to be the most active and enantioselective variant for the bromolactonization of 4-methoxyphenyl-4-pentenocic acid. Optimized reaction conditions permitted the halocyclization of 4-methoxyphenyl-4-pentenoic acid on a 15 mg scale to provide compound **15** in 84% yield and 95:5 e.r. **b)** 4-methoxyphenyl-4-pentenoic acid docked in the structure of RebH 3-LSR F111S (S130L/N166S/Q494R/F111S) in which the carboxylate moiety of the substrate is suitably arranged for halocyclization. Key active site residues, including K79, H109, S111, Q357 and N470, are shown in violet. The critical F111S mutation is presumed to lead to a closer substrate positioning to the catalytic residue K79 compared to the wildtype.

ketoglutarate-dependent hydroxylases as a strategy to increase the substrate scope of enzymes for radical halogenation.

Functionalization of complex molecules by wildtype and engineered halogenases

Biosynthetic pathways also prove to be a valuable source for the identification of halogenases, whose substrate scope goes beyond amino acids, indole, and phenol derivatives. For example, an αKGH known as AdeV, capable of halogenating nucleosides, has been identified in the biosynthesis of the chlorinated natural product adechlorin in *Actinomadura* sp. ATCC 39365 [51]. AdeV represents the first halogenase that can selectively install a chlorine atom at the C2′ position of 2′-deoxyadenosine-5′-monophosphate (2'dAMP) to afford 2′-chloro-2′-deoxyadenosine monophosphate (compound **18**, Table 1). *In vitro* assays revealed that 2′ deoxyadenosine, structurally identical to 2'dAMP apart from the 5′-phosphate moiety, was not accepted as a substrate, indicating that the presence of this phosphate is essential for substrate binding and halogenation activity. In keeping with this theory, 2′,3′-dideoxyadenosine-5′-monophosphate (2′-ddAMP) and 2′-deoxyinosine-5′-monophosphate (2′-dMP) (compounds **19**—**20**, Table 1) were also halogenated. However, in a competitive experiment the authors showed that the conversion of 2′-ddAMP was 20 times lower with respect to the conversion of 2′-dAMP. Similarly, the conversion of 2′-dMP was 2 times lower, demonstrating that 2′-dAMP is the preferred substrate of AdeV. Adding to the biochemical characterization, the recently solved crystal structure of AdeV gives further insights in the structural elements governing catalysis [52]. Extending the search for novel αKGHs to the plant kingdom, the Weng group reported the discovery of a novel αKGH, SaDAH, from Menispermaceae plants that performs the terminal chlorination reaction in (−)-acutumine (compound **21**, Table 1) biosynthesis [53]. SaDAH represents the first example of an αKGH found in plants and phylogenetic analysis indicates that the enzyme evolved independently from other αKGHs previously identified from bacteria [51,54,55]. *In vitro* assays against a wide range of alkaloids illustrated that SaDAH was highly selective towards its natural substrate, with no other small molecules being accepted for halogenation. Despite its narrow substrate scope SaDAH exhibited promiscuous azide activity and was able to convert (−)-dechloroacutumine to 11-azido-dechloroacutumine.

Complementing α-ketoglutarate-dependent halogenases responsible for natural product modification, FDHs have similarly been described as suitable instruments for late-stage functionalization of complex molecules. In this context, FDH OrfA was shown to halogenate albofungin, a hexacyclic aromatic natural product with broad-spectrum antimicrobial activity (compound **22**, Table 1) [56], while XanH, a bifunctional protein capable of flavin reduction and chlorination, has recently been shown capable of regioselectively chlorinating a complex late stage xanthone intermediate in the biosynthesis of the polycyclic antibiotic xantholipin (compound **23**, Table 1) [57]. Despite XanH's bifunctional nature, the authors found that the halogenation activity of the FDH could be boosted in the presence of an external flavin reductase. Thus, a self-sufficient FDR-XanH fusion protein was constructed, which, however, did not lead to additional activity benefit. It should be noted, though, that in other cases the genetic fusion of flavin reductase to FDHs can indeed improve halogenation yields, at least *in vivo*, suggesting that increased local concentrations of $FADH_2$ can positively affect the efficiency of halogenation biocatalysis in some settings [58].

In the same spirit, the identification and characterization of the promiscuous FDH, DklH, from *Frankia alni* ACN14a has yielded a useful biosynthetic tool for the selective derivatization of flavonoids [59]. Notably, a variety of flavonoid subclasses, such as flavones (compound **24**, Table 1), isoflavones (compound **25**, Table 1), flavonols (compound **26**, Table 1), flavanones (compound **27**, Table 1) and flavanonols (compound **28**, Table 1), were accepted as substrates. While capable of utilizing bromide, this enzyme showed a strong preference for chlorination. Finally, the Oikawa group investigated oxygenated cyclopentene systems, unique structural motifs found in fungal polyketides [60]. When investigating the biosynthetic machinery responsible for transforming 6-hydroxymellein derivatives into cyclohelminthols and palmaenones, the researchers identified and characterized two FDHs, ChmK and ChmN, from *Helminthosporium velutinum* capable of installing chlorine at the 5- and 7-position of 6-hydroymellein, respectively (compound **29**—**30**, Table 1). In addition, halogenase PloK from *Lachnum Palmae* was found to derivatize the 3-position of the substrate (compound **31**, Table 1), putatively through a mechanism via the enolate.

Going beyond bioinformatic sourcing, halogenases can also be tailored for the late-stage functionalization of non-native substrates by means of directed evolution. Presented with the challenge to create halogenation catalysts for the derivatization of a martinelline-derived fragment (compounds **32**—**33**, Table 1) with reported anti-cancer activity, our group recently engineered WelO5* variants using rational protein design [61]. In this work, key amino acid positions (V81/A88/I161) were identified which play an important role in modulating substrate acceptance of WelO5* towards non-natural substrates in addition to serving as regioselectivity switches. Preparative scale experiments were performed with the best performing variants, leading to isolated product yields of 6% (7.7 mg) for compound **32** and 30% (14.4 mg) for compound **33**. Building on this work, Voss et al. highlight that the modification of the key active site

residues (V81/A88/I161) can also modulate the substrate stereopreference of WelO5*. For example, the screening of a 3-site combinatorial library led to the identification of two variants, WelO5* CB2 (V81R/I161S) and WelO5* MGA (V81M/A88G/I161A), that are capable of selectively chlorinating stereo-complementary martinelline-derived fragments (compounds **33**—**34**), directly from a racemic mixture [62]. In the quest to further expand the biocatalytic toolbox for the halogenation of more complex molecules, Büchler et al. demonstrated that through the application of algorithm-assisted enzyme evolution, WelO5* could be tailored for the selective halogenation of the bulky non-natural substrates soraphen A and soraphen C (compounds **35**—**37**, Table 1) [63]. Notably, the most active engineered variant, WelO5* VLA, catalyzes the halogenation of soraphen A to yield the chlorinated product (compound **35**, Table 1) with an apparent k_{cat} value and a total turnover number which mirror the activity of the wildtype enzyme for its natural substrate (WelO5* VLA (soraphen A): $k_{cat} = 1.96 \pm 0.51$ min^{-1}; TTN = 92 ± 22; WelO5* wildtype (12-*epi*-fischerindole U): $k_{obs} = 1.8$—1.9 min^{-1}; WelO5 wildtype (12-*epi*-fischerindole U): $k_{cat} = 1.8$ min^{-1}; TTN = 75) [54,63,64]. Furthermore, the use of machine-learning guided engineering facilitated the reliable prediction of functional properties such as improved activity and regioselectivity ultimately leading to enzyme variants enabling the biocatalytic production of the derivatized macrolides for application in structure—function activity studies.

Similarly applying the principles of directed evolution to flavin dependent halogenases, the Lewis group engineered RebH for the site- and atroposelective bromination of 3-aryl-4(3*H*)-quinazolinones via kinetic or dynamic kinetic resolution [65]. The final variant 3-T, which harboured 14 mutations relative to RebH, showed > 99:1 e.r. for the (*M*)-atropisomer of the major brominated product, 25-fold improved conversion, and a 91-fold improved site selectivity compared to the parent enzyme. Similar activities and selectivities were also obtained for the halogenation of a range of structurally diverse quinazolinone substrates (compound **38**—**39**, Table 1). In addition, the authors demonstrated that compound **38**, prepared via upscaled reactions in 58% yield (40 mg), could be further elaborated, for example through Suzuki couplings or Paal—Knorr reactions, with no significant loss of enantiomeric purity.

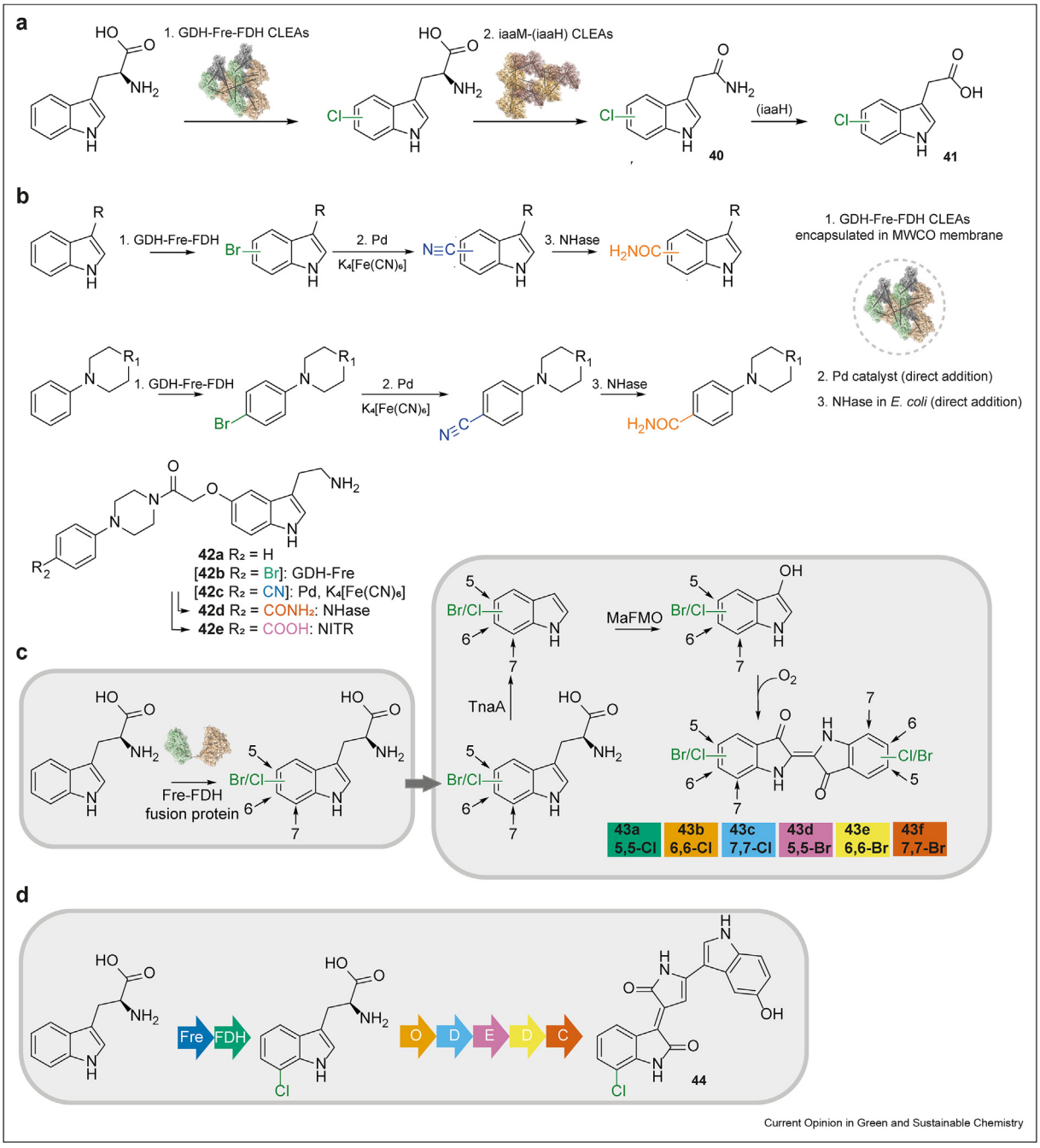
Bio- and chemocatalytic cascades centered around halogenases

Halogenases are not only used in a stand-alone fashion but can be strategically combined with other enzymes or with chemocatalysts. Menon et al. showed the production of halogenated indole-3-acetamide and indole-3-acetic acid, molecular scaffolds present in many therapeutic drugs, using a panel of regioselective halogenases in combination with tryptophan-2-monooxygenase (iaaM) and indole-3-acetamide hydrolase (iaaH, Figure 3a) [66]. In a first attempt, the flavin dependent halogenases, iaaM and iaaH were produced individually and cell lysates were mixed in the presence of L-tryptophan and all required cofactors leading to 0.73—0.76 mg of the amide and 0.2—0.4 mg of the carboxylic acid. The authors further improved the enzyme cascade by creating cross linked enzymes aggregates (CLEAs) of the regioselective FDHs (PyrH for indole position C5, SttH for position C6 and PrnA for position C7), flavin reductase (Fre) and glucose dehydrogenase (GDH, for the regeneration of NAD^+). Subsequent catalysis with CLEAs of iaaM with or without iaaH afforded halogenated indole-3-acetamide or halogenated indole-3-acetic acid, respectively (compounds **40**—**41**, Figure 3a). This setup yielded 10 mg of the amide while the carboxylic acid production was also boosted yielding 4.2 mg of the desired product [66]. Adding complexity to the FDH-containing enzymatic cascades, Lee et al. [67] showed that FDHs can be used to produce indigoid dyes from L-tryptophan. Using a consecutive two-cell reaction system, L-tryptophan was first regioselectively halogenated at the C5, C6 or C7 position of the indole moiety. Afterwards, the supernatant of the first step was added to cells expressing the tryptophanase TnaA from *E.coli* and the flavin-containing monooxygenase MaFMO from *Methylophaga aminisulfidivorans* to produce the indigo dye. Depending on the position of halogenation and the halogen used, a different colored dye was obtained (compounds **43a-43f**, Figure 3c).

Going beyond purely enzymatic cascades, halogen functionalities can be effectively used as chemical linchpins for further chemocatalytic modification. Such a combination of bio- and chemocatalysis allows the construction of diverse molecular scaffolds as exemplified by the work of Craven et al. [68]. In this work, CLEAs of FDHs (RebH, SttH, PyrH, PrnA, RebH variants and SttH variants) with GDH and Fre were encapsulated in a molecular weight cut off (MWCO) membrane allowing for a parallel palladium-catalysed cyanation of the product without damaging the biocatalysts (Figure 3b). Subsequent incorporation of nitrile hydratase or nitrilase enzymes afforded the amide or the carboxylic acid, respectively. Following this strategy allowed the authors to selectively introduce amides or carboxylic acid whereby the chemo-and regioselectivity was determined by the FDH in the first reaction step (Figure 3b). For example, by using the FDH SttH on the drug precursor **42a,** the selective installation of a cyanide was possible even though similarly activated C—H positions were present in the starting scaffold, giving the active pharmaceutical ingredient donitriptan (compound **42c**, Figure 3b) [68].

Finally, with the goal of integrating an enzymatic halogenation step into biosynthetic pathways, Lai et al. [69] expressed Fre (RebF) and FDH (RebH) together with the violacein pathway in *E. coli*. Employing this strategy,

Figure 3



Utilization of FDHs in bio- and chemocatalytic cascades. a) Single integrated one-pot process using CLEAs of glucose dehydrogenase (GDH), flavin reductase (Fre), flavin dependent halogenase (FDH) and tryptophan-2-monooxygenase (iaaM) as well as indole-3-acetamide hydroxylase (iaaH) to produce modified indole-3-acetamide and indole-3-acetic acid. **b**) Single integrated one-pot process using CLEAs of GDH-Fre-FDH encapsulated in a MWCO membrane in combination with a Pd catalyst for regioselective cyanation at the halogenation site. Subsequent treatment with a nitrile hydratase (NHase) or a nitrile reductase (NITR) affords the amide or the carboxylic acid (R_2 = CH_3–CH_2–OH/COOH/NH_2) in the context of diverse heterocycle containing substrates (R_1 = O, S, NH, CH_2–NH, CH_2–N–CH_3) [68]. **c**) Two cell reaction system producing different indigo dyes whose color depends on the site of modification and type of halogen introduced. d) FDH co-expressed in *E. coli* together with the violacein pathway to produce chlorinated violacein.

six halogenated analogues of violacein or deoxyviolacein were generated (compound **44**, Figure 3d), including a 5-brominated derivative which was directly processed via the Suzuki–Miyaura cross coupling.

Conclusion and perspective

Halogen atoms are integral components of many commercial small molecule drugs. Nevertheless, chemical methods for the direct regio- and stereospecific introduction of halogens remain underdeveloped. Before this backdrop, selective enzymatic halogenation offers an attractive alternative to established chemical strategies: 1) enzymatic halogenations rely on sodium salts as the halide source and oxygen as the terminal oxidant and allow to reduce the use of energy, organic solvents, protecting groups and expensive metals, which have become scarcer and (geopolitically) more difficult to source; 2) halogenases can act on elaborate structures allowing for late-stage diversification of small molecules, particularly attractive for medicinal chemistry campaigns and 3) if integrated in biosynthetic pathways or chemoenzymatic cascades, halogenases lead to diverse portfolios of biologically active compounds which may accelerate the discovery of new therapeutics.

Encouragingly, elaborate bioinformatic searches and powerful enzyme engineering campaigns have substantially increased the number and diversity of available halogenating enzymes during the last decade. In this context, a recent highlight is the discovery and characterization of AetF [45], a single-component flavin dependent halogenase, which operates without the need of a supplemented flavin reductase (see section 1.2). Notably, AetF's intrinsic versatility has already triggered follow-up studies including its application for the halogenation of relatively electron-poor heterocycles and aromatic compounds, such as furan, thiophene and pyrazoles lacking additional electron donating groups, as well as the enzyme's application in asymmetric catalysis. Excitingly, AetF was additionally reported to catalyze aromatic iodination and cycloiodoetherification [70]. In the case of Fe(II)/α-ketoglutarate dependent halogenases, for which enzyme discovery rates tend to be much lower [35], enzyme engineering has proven an indispensable tool to broaden substrate scope and tailor reaction scope: excitingly, novel α-ketoglutarate dependent halogenases were designed starting from related hydroxylases [36,50], while the substrate scope of a native indole-alkaloid halogenase could be broadened to comprise molecules as large as the macrolide soraphen A [63].

Nevertheless, several challenges, including improved productivity and scalability, need to be addressed before the full biocatalytic potential of halogenating enzymes can be unlocked. Most important will be the development of universal mechanism-based methods that target the improvement of enzymatic performance, which can suffer from the applied enzyme's low total turnover number: For example, Fe(II)/α-ketoglutarate dependent halogenases typically display a total turnover number of below 100 even for native substrates [13]. In the context of FDH catalysis, escaping ("free") hypohalous acid was recognized as detrimental for biotransformations. Successful reaction condition engineering (high pH, addition of GSH as HOX scavenger) [48] and enzyme tunnel reshaping to confine the hypohalous acid to the protein environment [49] are exciting concepts that have broadened selected FDH's application scope (RebH, ThaI). In addition, optimizing the concentration of oxygen in the reaction medium has been proposed as a further lever which could increase the productivity of FDHs [18]. Looking forward, analyzing the effect of these strategies in additional FDH systems will be an important endeavor. Similarly, improved mechanistic understanding of α-ketoglutarate dependent halogenases might be profitably employed to achieve higher total turnover numbers when targeting C(sp^3) centers.

Overall, the efforts described above demonstrate the strides that have already been made within the field in terms of process development and in using rational protein engineering or directed evolution toward the development of performant halogenating enzymes. The substrate and reaction scope of halogenases reported to date is remarkable and thanks to the ingenuity of the many involved research groups is constantly growing. Complementary to chemocatalysis, biocatalytic halogenation approaches have already found their way into medicinal chemistry routes and, as more robust catalysts become available, will contribute to the more sustainable production of halogenated molecules.

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Author contributions

Eimear Hegarty: Conceptualization, Writing- Original draft preparation, Visualization; **Johannes Büchler**: Conceptualization, Writing- Original draft preparation, Visualization; **Rebecca Buller:** Supervision, Funding acquisition, Conceptualization, Writing- Original draft preparation, Writing – review & editing.

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Data availability

No data were used for the research described in the article.

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* of special interest
** of outstanding interest

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