Flavin-Dependent Halogenases Catalyze Enantioselective Olefin Halocyclization

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Abstract

Catalytic enantioselective halocyclization of alkenes is a powerful bond forming tool in synthetic organic chemistry and a key step in the biosynthesis of several natural products. To date, however, no examples of enantioselective halocyclization of simple achiral olefins catalyzed by enzymes have been reported. Herein, we report that flavin-dependent halogenases (FDHs) previously engineered to catalyze site-selective aromatic halogenation can also catalyze bromolactonization of olefins with high enantioselectivity and near-native catalytic proficiency. Analysis of the selectivity of FDH variants along the lineage for the most selective enzymes reveals mutations responsible for the emergence of halocyclase activity, and docking simulations provide insight into the origins of improvements imparted by these mutations. High selectivity was achieved by characterizing and mitigating the release of HOBr from FDH variants using a combination of protein engineering and reaction optimization. Given the range of different halocyclization reactions and other organic transformations that proceed via oxidative halogenation, this expansion of FDH catalytic activity bodes well for the development of a wide range of biocatalytic halogenation reactions.

Text

Halogen substituents can profoundly influence the biological activity of natural products, pharmaceuticals, and other organic compounds.[1-4] In nature, several classes of enzymes catalyze oxidative halogenation reactions that install halogen substituents (X=Cl, Br, I) into diverse structures starting from distinct precursors.[5] The high selectivity, mild reaction conditions, and environmentally benign oxidants and halogen sources used by these enzymes have motivated efforts to employ and engineer them for biocatalytic halogenation.[6,7] Flavin-dependent halogenases (FDHs)[8] have been particularly well-studied in this regard due to their ability to site-selectively halogenate electron rich aromatic or enolate groups in a wide range of organic structures (Fig. 1a). These enzymes oxidize halide ions using O_2 as a terminal oxidant to generate a persistent lysine-derived haloamine that is believed to either directly halogenate substrates[9] or to serve as a source of HOX within the enzyme that can react with bound substrates[10]. Because many other oxidative halogenation reactions are initiated by similar electrophilic halogen species,[11] we wondered whether FDHs might possess reactivity beyond aromatic halogenation, including enantioselective olefin halocyclization.[12] This reaction involves formal addition of a halenium ion (X^+) and a nucleophile (Nu^-) to an olefin, leading to the formation of two new bonds, adjacent stereogenic centers, and a halogen substituent that can be used for further structural elaboration (Fig. 1b). Relative to other FDH-catalyzed halogenation reactions, which involve only substitution of a C-H bond with a C-X bond,[6,7] halocyclization is therefore notable for its ability to significantly increase molecular complexity.

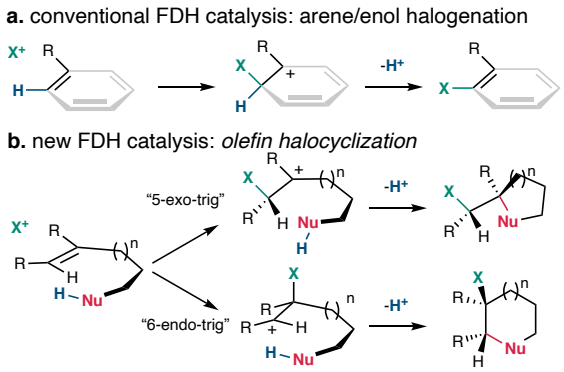


Figure 1. a) Arene/enol (R = OH) halogenation representative of reactions catalyzed by FDHs. b) Simplified scheme (omitting potential halirenium intermediates and concerted pathways)[13] for olefin halocyclization catalyzed by FDHs.

Notably, some vanadium haloperoxidases (VHPOs) that catalyze diastereoselective halocyclization have been characterized, but only native or putative substrates containing stereogenic centers have been reported to date.[14-16] Other VHPOs[17,18] and heme haloperoxidases (HHPOs)[19] can catalyze halocyclization of simple achiral substrates, but racemic products are obtained, likely due to their formation via free HOX generated by the enzyme. No examples of FDH-catalyzed halocyclization or enantioselective halocyclization involving non-native substrates using any enzyme have been reported. We reasoned, however, that FDHs could address several major challenges to enantioselective halocyclization by catalytically activating an inert stoichiometric halogen source[12] in a chiral environment[20] and prohibiting halenium ion transfer between olefins, which leads to racemization[21]. The chemical feasibility of halocyclization by a variety of *N*-halogenated amine species[12] and the broad substrate scope[6,7] and evolvability[22] of FDHs further warranted exploration of this reaction manifold, which would significantly expand the catalytic repertoire of FDHs.

A panel of FDHs comprising 45 wild-type enzymes from a recently reported genome mining effort[23] and 99 RebH variants from previous directed evolution campaigns[24-27] was assembled (See supporting sequence data file). Collectively, these enzymes halogenate a wide range of aromatic substrates, indicating that they possess structurally diverse active sites. While halocyclization requires suitable orientation of an electrophilic halogen source, an olefin, and a pendent nucleophile and involves substrate deprotonation distal to rather than *ipso* to the site of halogenation as in aromatic halogenation (Fig. 1a/b), we anticipated that the diversity of enzymes evaluated would include examples that could accommodate these differences. FDH halocyclase activity was evaluated on 4-methoxyphenyl-4-pentenoic acid (**1**).[13] We hypothesized that the styrene core of **1** could mimic the planar aromatic substrates accepted by many of the FDHs evaluated to orient the olefin for electrophilic attack within the FDH active site, and docking studies indicated that this orientation was indeed feasible (*vide infra*). FDH-catalyzed aromatic halogenation typically requires greater electronic activation than that imparted by a single methoxy substituent,[28] so this reaction was not expected to occur to a significant extent for **1**.

Analysis of FDH activity on **1** using enzyme in cell lysate led to the identification of 50 variants that provided significant yields of bromolactonization product **1a**. These enzymes were purified,

and reaction conditions were optimized to enable reliable detection of halocyclase activity and selectivity using chiral LC/MS. All purified enzymes provided measurable levels of **1a** using 5 mol% enzyme loading, and up to 94% yield was observed (Fig. 2, S1). Enantioselectivity as high as 84:16 e.r. was obtained (variant 4V+S[24]), and in general, previously engineered variants of RebH displayed the highest selectivity. Interestingly, several genome-mined FDHs provided a significant yield the opposite product enantiomer (up to 36:64 e.r. with variant 1-F08[23]). In all cases, 5-exo-trig cyclization was observed, consistent with the strong electronic bias in **1** that favors this mode of cyclization (Fig. 1b)[13]. Chlorolactonization was not observed in the presence of chloride, which parallels our previous observation[23] that FDHs catalyze selective bromination of a broader range of substrates than they can chlorinate. We speculate that the increased reactivity of heteroatom-substituted bromine species (e.g. bromamine or HOBr)[29] obviates the need for substrate activation or precise orientation with an FDH active site to enable a broader range of reactivity.

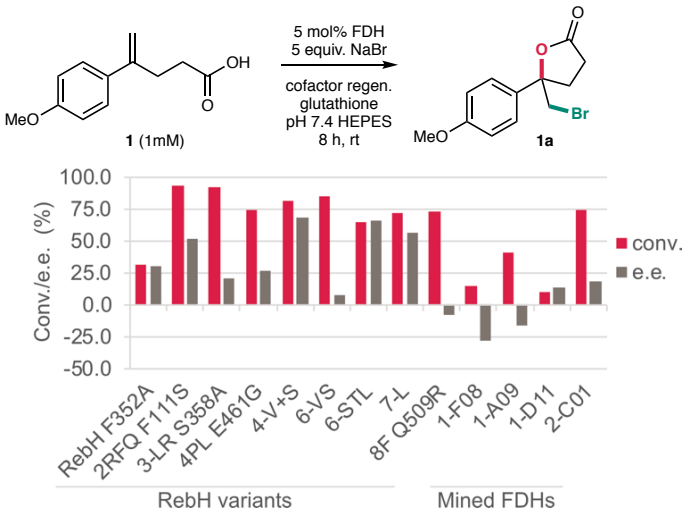


Figure 2. Selected conversion and enantioselectivity data for halocyclization of substrate **1**. Complete data are provided in Fig. S1.

The reaction parameters were next modified to improve product yield and enantioselectivity. Early studies revealed that increasing substrate concentration substantially improved the enantioselectivity of some FDH variants (Fig. S2). We hypothesized that a competing racemic halocyclization reaction involving HOBr generated under the reaction conditions was occurring outside of the FDH active site in analogy to HHPOs and some VHPOs.[17,19] We further speculated that this reaction was suppressed at high substrate concentration to ensure active site saturation and thus reaction with the desired halogenating species prior to diffusion of HOBr from the active site. Because the electrophilicity of HOBr decreases substantially above its pKa (8.7),[30] we reasoned that increased buffer pH would mitigate the reactivity of this species if it was released into solution during FDH catalysis. Indeed, the enantiomeric ratio of **1a** produced by variant 4V+S increased from 86:14 (84% yield) to 96:4 (47% yield) as a result of using Tricine buffer in place of HEPES buffer and increasing pH from 7.4 to 9 (Table 1, entries 1-4), albeit at the cost of product yield. Moreover, adding 1 mM glutathione, a known HOBr scavenger,[31] improved enantioselectivity even at pH 7.5, providing **1a** in 91% yield with 96:4 e.r. (Table 1, entry 6). These results both strongly suggest that free HOBr was being produced by 4V+S and provide a means to

eliminate the unwanted reactivity of this species to enable bromolactonization with high yield and selectivity.

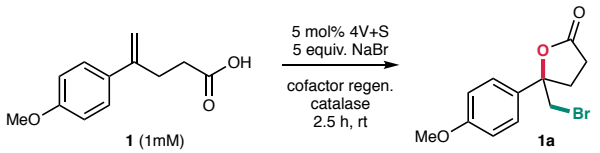


Table 1. Optimization of halocyclization reaction conditions

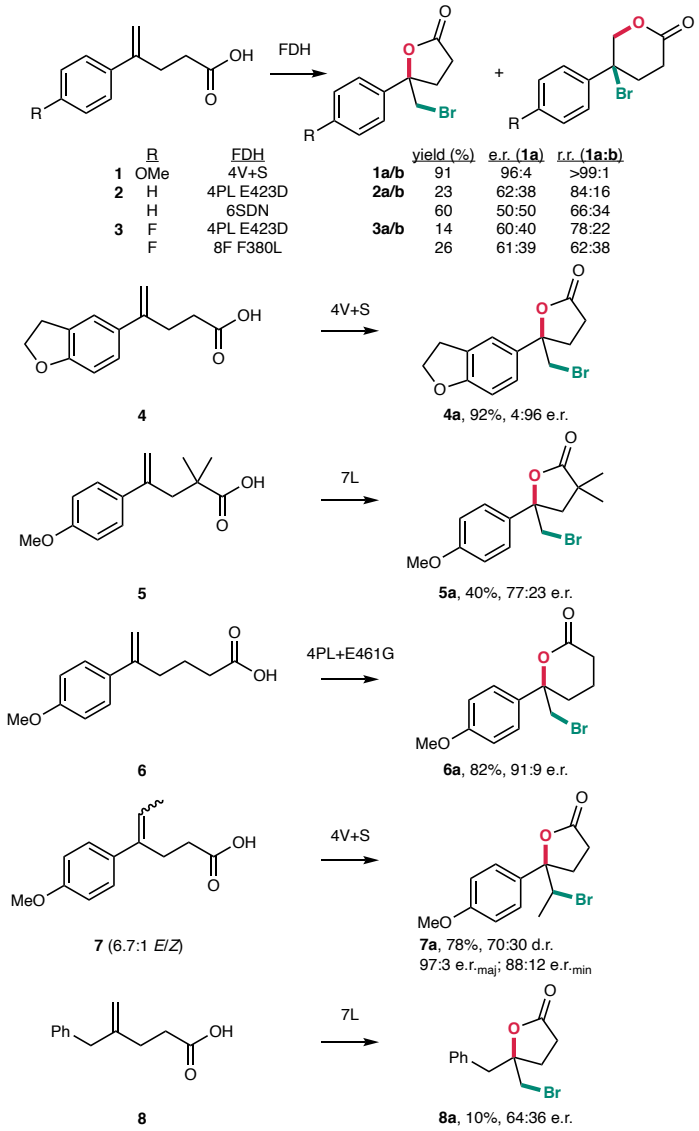
Entry	pH	1 mM glutathione	yield (%)[a]	e.r.[b]
1	7.4[c]	-	84	86:14
2	7.6[d]	-	83	90:10
3	8.5[d]	-	77	92:8
4	9[d]	-	47	96:4
5	7.5[d]	+	91	96:4

[a]Determined by LC/MS using *p*-bromoanisole internal standard. [b]Determined by chiral LC/MS. Reported yields and e.r. values are the average of triplicate measurements. [c]25 mM HEPES buffer used. [d]200 mM Tricine buffer used.

The optimized reaction conditions were then used to evaluate FDH-catalyzed bromolactonization of six additional substrates (**2**-**8**). A subset of purified variants from the initial screen using substrate **1** was examined to identify the best enzyme for each substrate (Fig. S3). Good yields and high enantioselectivities were observed for halocyclization of substrates **4**-**7**, which, like **1**, contain an electron-rich aromatic moiety that would be expected to favor formation of the observed 5-exo bromolactonization products. Substrates **4** and **5** possess additional bulk in their aromatic and aliphatic portions, respectively, while hexenoic acid derivative **6** shows that the FDH active site can accommodate formation of a 6-membered lactone. Finally, trisubstituted olefin substrate **7** (6.7:1 *E*:*Z*) underwent halocyclization in high yield to provide **7a** with modest diastereoselectivity (70:30) but high enantioselectivity (97:3 e.r. and 88:12 e.r., for the major and minor diastereomers, respectively, Fig. S4), comparing favorably to the only report of a small molecule catalyst for this reaction[32].

Significantly lower yields and enantioselectivities were observed for electron-neutral (**2**) and electron-poor (**3**) substrates. This observation is consistent with a proposed difference in mechanism for reaction of these substrates relative to **1**. Specifically, experimental and computational evidence suggests that bromolactonization of **2** proceeds via a concerted mechanism involving nucleophile-assisted alkene activation.[13] In the context of the FDH active site, this would require proper *syn* or *anti* orientation of the putative chloramine halogenating agent and the pendent carboxylate nucleophile relative to the olefin in a cleft that natively binds planar aromatic substrates (Fig. 1a/b). On the other hand, bromolactonization of **1** appears to proceed via a stepwise mechanism,[13] so precise arrangement of the nucleophile is not required. Electrophilic attack of the olefin by a bromamine species could generate a stabilized benzylic carbocation (Fig. 1b/c) or halirenium intermediate that could be intercepted by the carboxylate following movement to a more accommodating orientation within the active site. It is also notable, however, that a mixture of regioisomers resulting from 5-exo-trig and 6-endo-trig bromolactonization (up to 84:16 r.r.), were obtained for the reaction of substrates **2** and **3**. While high regioselectivity was not obtained using the variants examined to date, significant variation between variants was observed (66:34-84:16 r.r.), suggesting that the regioselectivity for halocyclization of electron neutral or electron

deficient substrates could be controlled by a suitably engineered enzyme. This finding is particularly notable given that bromolactonization of **2** using NBS gives exclusively the 5-exo-trig product, and selective formation of the 6-endo-trig product appears to have only been accomplished otherwise via photoredox catalysis[33]. Finally, aliphatic substitution was also tolerated as evidenced by the reaction of benzyl-substituted substrate **8**, and the relatively low yield for this substrate likely results from the same problems that faced unactivated aromatic substituents.



	R	FDH		yield (%)	e.r. (**1a**)	r.r. (**1a:b**)
1	OMe	4V+S	**1a/b**	91	96:4	>99:1
2	H	4PL E423D	**2a/b**	23	62:38	84:16
	H	6SDN		60	50:50	66:34
3	F	4PL E423D	**3a/b**	14	60:40	78:22
	F	8F F380L		26	61:39	62:38

Figure 3. Bromolactonization substrate scope of evolved RebH variants. All reaction mixtures contained 1 mM substrate, 5 equiv. NaBr, 5 mol% FDH, and a cofactor regeneration system comprising a flavin reductase, a glucose dehydrogenase, and glucose. Glutathione (1 mM), catalase, and optimized buffers were also added as described in the supporting information. Yields were determined by LC/MS using *p*-bromoanisole internal standard, enantioselectivities were determined by chiral LC/MS, and the reported values are the average of triplicate measurements.

The above results show that FDHs can accommodate the significant differences between the substrates and intermediates involved in aromatic halogenation and olefin halocyclization (Fig.

1b/c). Notably, however, all of the FDH variants that catalyzed bromolactonization with high yields and selectivities (4V+S, 4PL E461G, and 7L) descend from RebH, which itself provided negligible yield in the initial high throughput screen (See supporting sequence data file). The halocyclase activity of this enzyme and three other widely studied FDHs (PrnC, PltM, and ThHal)[6] was examined under optimized conditions using substrate **1**, but none of these provided >2% yield of **1a** at 5 mol% enzyme loading. The activity of variants along the lineages of the evolved enzymes was therefore examined to reveal mutations that improved halocyclase activity. In the case of 4V+S,[24] for example, some of the variants that provided improved yields for different aromatic halogenation reactions as a result of non-active site mutations also provide improved halocyclization yields (Table 2, entries 1-3). Significant improvements in both yield and enantioselectivity result from active site mutation N470S, and further improvement in enantioselectivity results from the non-active site mutation A442V (Table 2, entries 4 and 5). The largest improvement in both yield and enantioselectivity comes from mutation F111S (Table 2, entry 6). Notably, variants 4V+S, 4PL E461G, and 7L possess in common only two active site mutations, F111S/L and N470S, suggesting that these residues play particularly important roles in halocyclization catalysis.

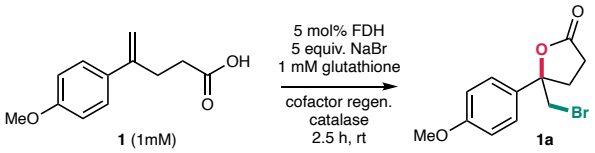


Table 2. Analysis of effects of mutations in evolved FDHs on halocyclase activity.

				Mutations[a]	
Entry	Enzyme	yield (%)[b]	e.r.[c]	Active Site	Other
1	RebH	2	n/a	-	-
2	1-PVM	7	59:41	-	S2P/M71V/K145M
3	2T	7	57:43	-	N467T
4	3-S	12	65:35	N470S	-
5	4V	12	72:28	-	A442V
6	4V+S	88	96:4	F111S	P2S (reversion)
7	4V+S K79A	2	n/a	K79A	-
8	4V+S K79A[d]	20	50:50	K79A	-

[a]Mutations relative to the parent in the previous row. [b]Determined by LC/MS using p-bromoanisole internal standard. [c]Determined by chiral LC/MS. Reported yields and e.r. values are the average of triplicate measurements. [d]Reaction conducted without added glutathione.

Docking simulations were conducted to examine how F111S/L and N470S might affect FDH halocyclase activity. Several studies have established that substrates typically bind to FDHs such that the site of halogenation projects toward a conserved active site lysine residue (K79 in RebH).[22] Geometry optimized structures of **1** and the corresponding cationic brominated intermediate (Fig. 1b) were docked into the structure of previously-evolved RebH variant 3-LSR (PDB ID 4LU6), which possesses many of the mutations present in 4V+S, 4PL E461G, and 7L, but notably not F111S/L or N470S (PDB ID 4LU6). Low energy poses in each case show the substrate alkene (Fig. S5a) or the intermediate bromine substituent (Fig. 4a) projecting toward K79. In the docked intermediate structure, the bromine substituent is only 3.4 Å from K79, but the propanoic acid

substituent adopts an extended conformation to avoid a steric clash with F111 that places the carboxylate 4.3 Å from the benzylic cation that it must attack for halocyclization to occur. Similar binding is observed using the N470S variant of 3-LSR (not shown), so it is not clear how this mutation affects halocyclase activity.

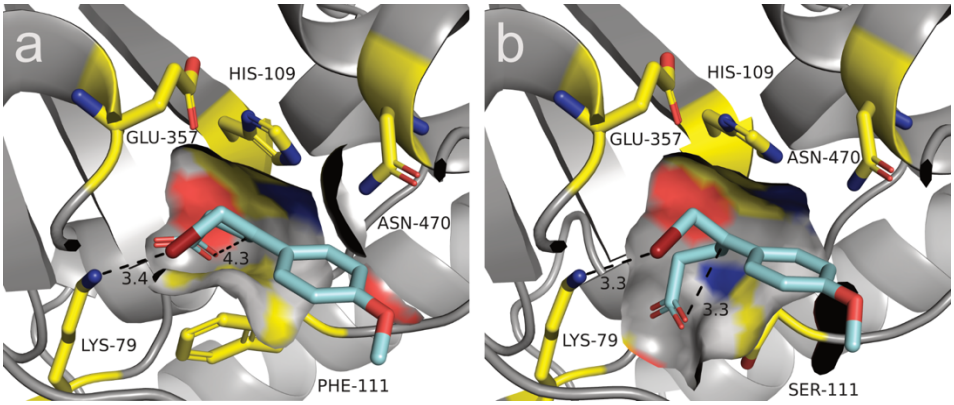


Figure 4. Docking poses for the cationic intermediate generated upon bromination of **1** in the structure of RebH variant a) 3-LSR and b) 3-LSR F111S. Key active site residues, including K79 and F/S111 are shown in yellow and a surface rendering of several residues is provided to illustrate the space created by F111S. The $K79_{\varepsilon\text{-amino}}$-Br and C_{benzyl}-$O_{carboxylate}$ distances are shown.

The F111S mutation, on the other hand, allows the propanoic acid substituent to fold under both the intermediate (Fig. 4b) and the substrate structures (Fig. S5b) in a conformation consistent with *syn* addition of carboxylate and Br^+ to the olefin in a *pro-S* configuration (similar docking was observed for the F111V variant, not shown). While low enantioselectivity for the *p*-fluorine-substituted substrate **3** was observed (Fig. 3), the major product had the *S*-configuration based on comparison to authentic enantio-enriched product, providing experimental support for the simulated binding poses (Figure S6). The bromine substituent in the intermediate structure remains 3.3 Å from K79, but the carboxylate is also only 3.3 Å from the benzylic site. Notably, this distance is 2.5 Å in the calculated transition state for chlorolactonization of the analogous substrate lacking the *p*-methoxy substituent (Fig. S5c).[13] This similarity suggests that the F111S/V mutations allow **1** to bind in orientation required for halocyclization that is otherwise blocked, which explains the large improvement in halocyclase activity and selectivity that results from F111S in the 4V+S lineage.

Finally, steady state kinetics were used to compare FDH halocylase activity to the native aromatic halogenation activity of these enzymes. Variant 4V+S catalyzes bromolactonization of **1** with a k_{cat} of 0.36 min^{-1} and a K_M of 0.47 mM (Fig. S7). The similarity of these values to those for native FDH catalysis (k_{cat} ~ 0.5-3 min^{-1})[22] highlight the facility with which the FDH active site can be reconfigured to enable topologically distinct chemical transformations. Previous studies of FDH-catalyzed aromatic chlorination established that mutating a conserved active site lysine (K79 in RebH) abolishes activity, and this was also observed for bromolactonization of **1** catalyzed by 4V+S K79A (Table 2, Entry 7; Fig. S8). Importantly, however, in the absence of glutathione, bromolactonization activity for the K79A mutant was restored, but the reaction produced racemic product and was significantly slower than that of 4V+S in the presence of glutathione (Table 2, Entry 8). No reaction was observed in the absence of FDH, indicting that this reaction did not result from side reactions involving reduced flavin cofactor, O_2, and halide ions in solution.

Combined with our finding that the selectivity of less active enzymes like 3-LR can be increased by increasing substrate concentration (Fig. S2), these results suggest that FDHs can release HOBr into solution even after it migrates into the active site (Figure 1a)[22]. This process is exaggerated in the absence of K79 and can be mitigated by tuning the active site so that olefin substrates react before HOBr is released as in 4V+S or by reducing the reactivity of HOBr in solution using increased pH or added glutathione.

In summary, we have characterized the first examples of olefin halocyclization catalyzed by an FDH and the first examples of enantioselective halocyclization on simple, achiral substrates catalyzed by any enzyme. High yields and enantioselectivity could be achieved using enzymes previously engineered for arene halogenation, though notably not using a number of wild-type FDHs. While halocyclization is a key step in the biosynthesis of several natural products, all examples reported to date are catalyzed by VHPOs.[16] Our results indicate that FDHs should also be considered as viable catalysts for halocyclization in natural product biosynthesis. Given that many natural products contain aliphatic carbocycles and heterocycles bearing halogen substituents in orientations consistent with installation via halocyclization,[4] it will be interesting to see if this possibility has been exploited in nature. Uncovering this new manifold of FDH reactivity also led to the finding that FDHs can release freely diffusible HOX from their active sites, indicating that caution is warranted when evaluating the activity of FDHs toward non-native substrates that may be reactive toward this species. This side reaction can be mitigated by controlling reaction pH or including reducing agents like glutathione, however, paving the way for exploration of other non-native transformations. Given the range of different halocyclization reactions and other organic transformations that proceed via oxidative halogenation,[11] this expansion of FDH catalytic activity bodes well for the development of a wide range of biocatalytic halogenation reactions.

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Author Contributions
D.M. completed all biocatalysis screening, optimization, kinetics, and characterization. B.F.F. optimized the mass spectrometry assay for initial biocatalyst screening. Y.J. assisted with substrate synthesis and product characterization. J.C.L conceived and directed the project and completed the docking simulations.

Competing Interests
The authors declare no competing interests.

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