Article

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# Understanding Flavin-Dependent Halogenase Reactivity via Substrate Activity Profiling

Mary C. Andorfer,[1] Jonathan E. Grob,[2] Christine E. Hajdin,[2] Julia R. Chael,[1] Piro Siuti,[2] Jeremiah Lilly,[2] Kian L. Tan,[2,*] Jared C. Lewis[1,*]

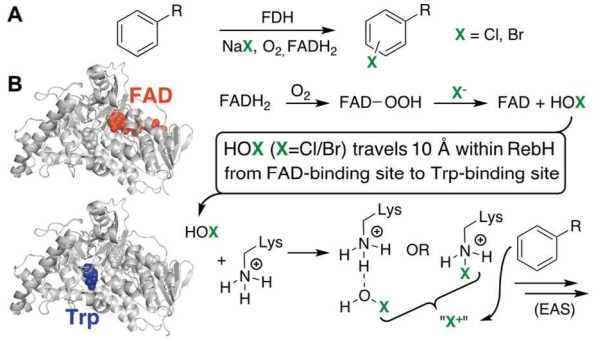
[1]Department of Chemistry, University of Chicago, Chicago, IL 60637. [2]Global Discovery Chemistry, Novartis Institutes for Biomedical Research, 250 Massachusetts Ave, Cambridge, MA 02139.

**ABSTRACT:** The activity of four native FDHs and four engineered FDH variants on 93 low molecular weight arenes was used to generate FDH substrate activity profiles. These profiles provided insights into how substrate class, functional group substitution, electronic activation, and binding impact FDH activity and selectivity. The enzymes studied could halogenate a far greater range of substrates than previously recognized, but significant differences in their substrate specificity and selectivity were observed. Trends between the electronic activation of each site on a substrate and halogenation conversion at that site were established, and these data, combined with docking simulations, suggest that substrate binding can override electronic activation even on compounds differing appreciably from native substrates. These findings provide a useful framework for understanding and exploiting FDH reactivity for organic synthesis.

*KEYWORDS: halogenase, flavin, C-H functionalization, biocatalysis, site selective*

## Introduction

The importance of halogenation for the synthesis and function of organic compounds has driven extensive efforts to identify[1,2] and engineer[3] halogenases to selectively install halogen substituents. Among the several classes of halogenases identified to date, flavin-dependent halogenases (FDHs) have proven particularly promising in this regard.[4] FDHs for which *in vitro* activity has been established halogenate electron rich arenes (Scheme 1A). While some FDHs require substrates linked to carrier proteins, many have activity on free substrates, which greatly simplifies biocatalysis and engineering efforts.[1]




**Scheme 1.** (A) General scheme for FDH-catalyzed halogenation. (B) Mechanism for generation of proposed halenium for electrophilic aromatic substitution (EAS) within FDHs.

Tryptophan halogenases (Trp-FDHs), including RebH,[5] PrnA,[6] and PyrH,[7] are perhaps the best studied FDHs with activity on free substrates. Mechanistic studies on RebH and PrnA suggest that these enzymes promote electrophilic aromatic substitution of enzyme-bound tryptophan by a formal halenium ion ($X^+$) donor, which is proposed to be either a lysine-derived haloamine (K79 in RebH)[8] or bound HOX[9] (Scheme 1B). This mechanism is consistent with the fact that relatively electron rich substrates are halogenated. Other factors are clearly important, however, as less electronically activated sites can be halogenated in the presence of more electronically activated sites.[10]

A number of fungal halogenases with activity on phenols and anisoles have also been identified.[1] For example, Rdc2, an FDH from *C. chiversii*, was found to halogenate a variety of resorcylic acid lactone substrates, the acyclic natural product curcumin, and two hydroxyisoquinolines.[11,12] GsfI, a homologue of Rdc2 from *P. aethiopicum*, catalyzes a selective chlorination in the biosynthesis of griseofulvin.[13] No information on how the substrate scope of these enzymes compare with Trp-FDHs has been reported.

Despite the sequence and structural homology of Trp-FDHs, we[14,15] and others[16,17] have observed significant differences in their activity toward non-native substrates. Likewise, the conservation of key sequence motifs in FDHs in general belies the apparent specificity of Trp, fungal, and likely other FDHs for distinct substrate classes based on the limited exploration of substrates reported to date.[1] We therefore became interested in establishing detailed profiles[18,19] of FDH activity toward a diverse panel of substrates (substrate activity profiles). These profiles provided insights into how substrate class, functional group

substitution, electronic activation, and binding impact FDH activity and selectivity. These data, in turn, provide a useful framework for understanding and exploiting FDH reactivity in organic synthesis.

## Results

### *FDHs and Substrates Selected for Activity Profiles*

FDHs were selected to evaluate differences in the activity and selectivity between wild-type Trp-FDHs (RebH, Thal),[20,21] engineered RebH variants (0K, 4V, 6TL, and 10S),[10,15] and fungal halogenases (Rdc2 and GsfI)[11,13]. The scope of RebH has been examined to a limited extent,[15,20,22] but far less is known about the activity of Thal, a Trp-6-FDH, other than its ability to halogenate D- and L-tryptophan[21]. Similarly, while Rdc2 activity has been confirmed on a handful of non-native phenol-containing substrates,[11,12] there are no previous reports of GsfI activity on non-native substrates. RebH variant 0K was created by replacing active site residue Glu461 with Lys based on the success of the analogous mutation in PrnA to increase activity on benzoic acids.[16] Variant 4V was developed to accept larger, biologically active compounds,[15] while variants 6TL and 10S were engineered during an effort[10] to alter the selectivity of tryptamine halogenation.

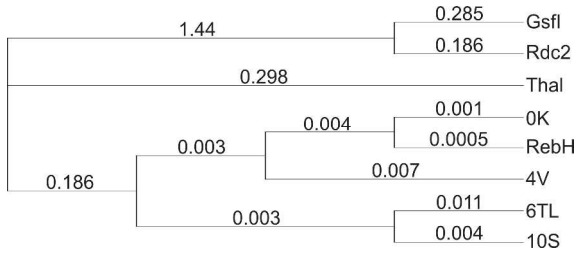


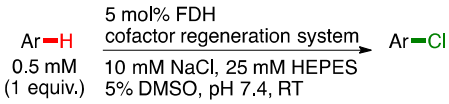

**Figure 1.** Phylogenetic tree of FDHs examined. Branch labels display amino acid substitutions per site.

When the sequence homology of these 8 FDHs is assessed, they fall into two categories according to organism of origin (Figure 1). RebH and Thal, both of which are bacterial enzymes, have much higher sequence identities to one another (61% identity, 99% query coverage) than RebH and the fungal FDH, Rdc2 (43% identity, 48% query coverage). We hypothesized this dramatic difference in primary sequence could lead to significant differences in scope, even beyond that previously observed between wild-type and engineered Trp-FDHs.

A substrate panel[18,23] comprised of 86 *N*-containing compounds (panel 1, Table S1) was used to evaluate FDH activity. Because halogenation with FDHs is believed to proceed via electrophilic aromatic substitution,[6] substrates in this panel contained at least one electronically-activated site. In addition, substrates were selected to probe significant steric and electronic variation, as well as functional group substitution, across a range of substrate classes, including anilines, indoles, azoles or pyrroles. A second panel (panel 2) of seven phenols and anisoles was also constructed, taking into consideration the native substrates of the fungal FDHs.[1] The 93 compounds contained within these two panels constitute the largest survey of FDH substrate scope to date. Moreover, these compounds are representative of those commonly used for fragment based drug design,[24] so understanding their reactivity toward FDHs could provide information on FDH activity toward motifs found in pharmaceuticals.

### *FDH Substrate Activity Profiles*

The eight FDH genes were co-expressed with the GroEL/ES genes, and the resulting FDHs were purified as previously described for RebH.[20] This protocol produced high yields of all eights FDHs, including the fungal enzymes GsfI (~35 mg/L) and Rdc2 (~60 mg/L). Purified enzyme was then use to conduct two analytical bioconversions on each substrate (Scheme 2). One set of reactions was allowed to proceed overnight while the other was quenched after 1 hour to ensure that monohalogenation could be observed on more reactive substrates for which dihalogenation is possible.[15,25] Despite differences in optimal reaction conditions for the FDHs, conditions were standardized to directly compare conversions. Reactions were analyzed by LCMS, and conversion data were used to generate substrate activity profiles (Fig. 2, Table S1).




**Scheme 2. General scheme for FDH bioconversions.**

### *Trp-FDH Activity on Anilines, Indoles, Pyrroles, and Azoles*

The Trp-FDHs exhibited broad scope toward panel 1 substrates; 67% of these electron-rich arenes were halogenated to some extent (Fig. 2). Notably, activity on a wide range of substituted anilines was observed, indicating that the narrow range of anilines evaluated to date[16] could be significantly expanded. The Trp-FDHs also demonstrated remarkable functional group tolerance by accepting substrates containing amines, alcohols, esters, amides, sulfonamides, nitriles, thioethers, pyridines, quinolones, azoles, and pyrroles. Qualitatively similar scope was observed for Thal, 6TL, and RebH, which each halogenated 40-47% of substrates examined with >1% conversion. In contrast, the scope and activity for variants 10S and 0K are more limited (>1% conversion for 23-24% of substrates). In the case of 0K, this could result from the fact that this enzyme was designed for higher activity on benzoic acid substrates,[16] but few panel 1 substrates contain acidic functionality. Variant 10S was evolved to halogenate the 5-position of tryptamine with high selectivity, while 6TL, an intermediate in the 10S lineage, halogenated the 5-, 6-, and 7-positions of tryptamine with similar efficiency.[10] The improved scope of 6TL relative to 10S is thus consistent with other examples in which evolutionary intermediates serve as more general catalysts than variants with high activity/selectivity on target substrates.[26,27] Interestingly, the FDH with the broadest scope toward panel 1 substrates was 4V. This variant was engineered to accept large indole-containing compounds,[15] and this appears to have enabled activity on a wider range of substrates than was used in the original engineering effort.

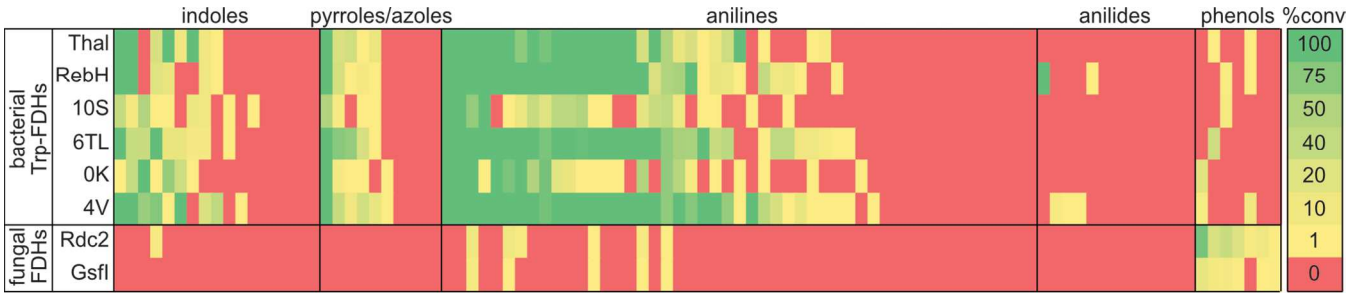



**Figure 2.** Substrate activity profiles in heat map form for eight FDHs on substrates in panels 1 (indoles, pyrroles, azoles, anilines, anilides) and 2 (phenols). Maximum conversion is shown for each enzyme-substrate pair (for complete data sets at both time points, see Table S1.).

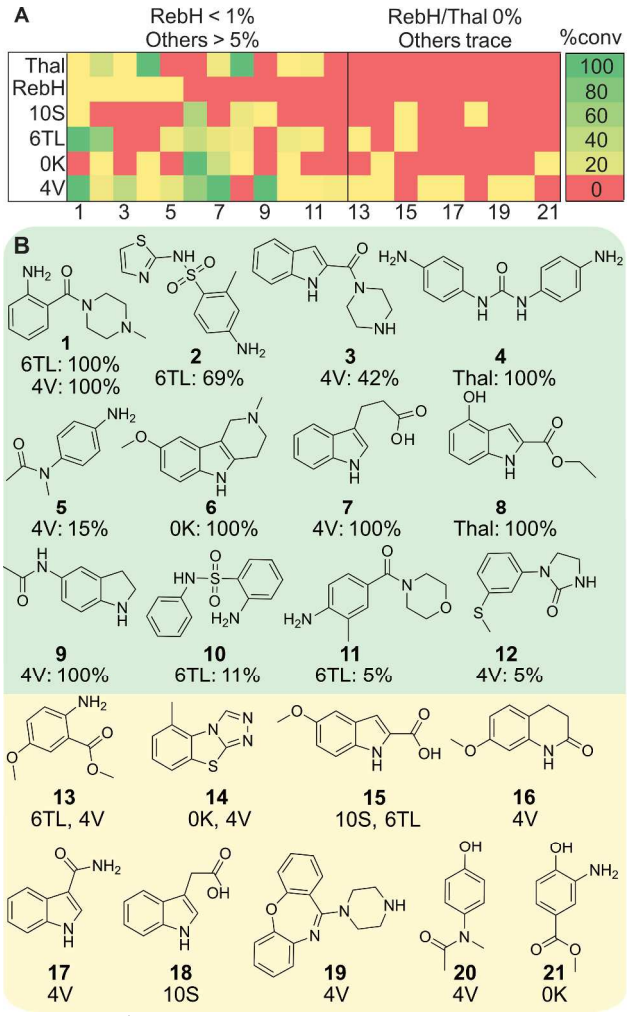



**Figure 3.** A) Conversion data from initial activity profile for selected substrates and B) Structures depicted in heat map. Compounds for which >5% conversion was observed are highlighted in green; those halogenated to a trace extent are highlighted in yellow.

Closer examination of the activity profiles revealed significant differences in the specific substrates halogenated by each Trp-FDH (Fig. 3A). Many instances arose in which significant conversion (>5%) was observed for Thal or the engineered variants on substrates where trace or no activity was observed for RebH (Fig. 3B, **1-12**). A number of cases were also observed in which neither RebH nor Thal displayed any conversion on a substrate, but trace activity was seen for the engineered halogenases (Fig. 3B, **13-21**). This trace activity, confirmed by LCMS using chlorine isotope patterns, provides a starting point for evolving enzymes with good activity on these different compound classes.[15] We have previously demonstrated the feasibility of this with FDHs.[10,15] Moreover, this finding clearly shows that even minor changes in halogenase sequence can significantly impact specificity and scope, which highlights the importance of evaluating multiple enzymes on a given substrate(s) to find active catalysts, be it for preparative reactions or for starting points for evolution.[26,28,29]

*Comparison of Trp-FDH and Fungal FDH Activity*

While many panel 1 substrates were accepted as substrates by the Trp-FDHs, very few (7%) were halogenated by the fungal FDHs (Fig. 2). For this reason, the activity of these enzymes toward panel 2 substrates was evaluated. Many of these less nucleophilic substrates[30] were not halogenated to a significant extent by the Trp-FDHs; however, all were halogenated by at least one of the fungal FDHs (Fig. 4). Rdc2 and GsfI, in contrast to Trp-FDHs, have a preference for the less electron rich substrates[30], which indicates selectivity is more complex than a simple electronic activation. The practical consequence is that expanded substrate scope can be achieved by investigating diverse enzyme sets.

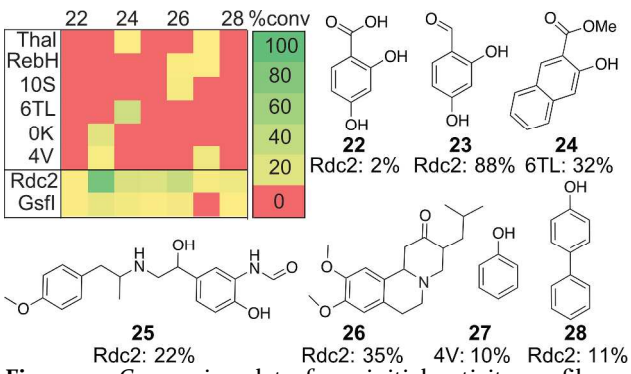



**Figure 4.** Conversion data from initial activity profile and structures for substrates **22-28**.

*Selectivity of FDH Halogenation*

The conversion data for substrates from panels 1 and 2 provide valuable insights into the scope and specificity of the eight FDHs in this study. To determine whether site selective halogenation occurred in reactions of non-native substrates, however, RebH- and Rdc2-catalyzed reactions

that provided >5% conversion under high throughput screening conditions (36 total) were conducted on a 1-10 mg scale. Products for 29 of these reactions were obtained from preparative LCMS. Comparable conversion to the initial substrate panel evaluation was generally observed (Fig. 2). Characterization of these products by NMR spectroscopy and HRMS established that most of these substrates (22) were halogenated at a single position with >95% selectivity (Fig.5, representative examples; Table S2, all products).

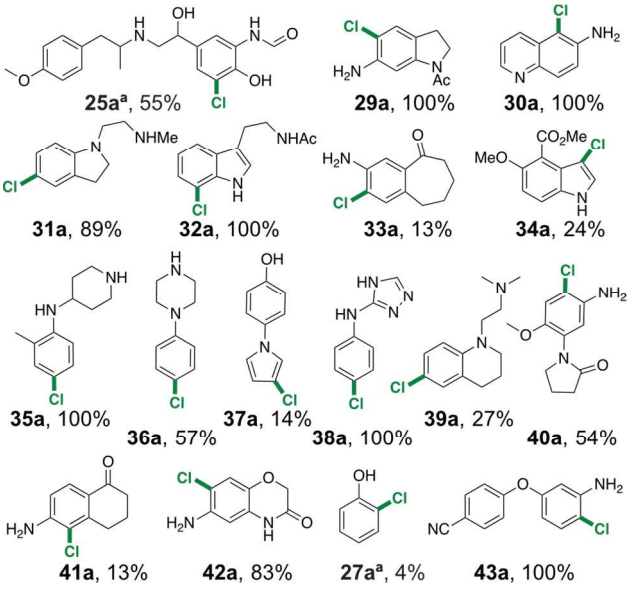



**Figure 5.** Conversions for substrates with multiple electronically activated sites that provided a single halogenated product. Reactions were conducted on 1-10 mg scale using either RebH (**29a-43a**) or Rdc2[a] (**25a**, **27a**). See Table S2 for full product list. Conversion and selectivity for **27a** determined by comparison with authentic material.

*Quantitative Evaluation of Substrate Activation*

As previously noted, FDH catalyzed halogenation is believed to proceed via electrophilic aromatic substitution.[6] Substrates bound within the FDH active site are presumably bound such that a single site is located proximal to an electrophilic halogen species (a Lys79-chloramine and hypohalous acid have been proposed)[8,9] that formally transfers a halenium ion ($X^+$) to the substrate. This enables Trp-FDHs to halogenate the benzo ring of tryptophan over the more reactive pyrrolo ring. The selectivity of the reactions whose products are shown in Figure 5 are similarly remarkable given that more than one site on each of substrates might reasonably be considered sufficiently activated for electrophilic aromatic substitution.[30] Importantly, however, this level of catalyst control was achieved on diverse substrates without the need for multiple halogenase engineering efforts.[10]

We previously used calculated halenium affinity (HalA)[31] values to quantitate the extent to which the observed selectivity of RebH variants overrides the electronic preference of tryptamine toward (hypothetical) halogenation by $Cl^+$ at different positions.[10] This approach is analogous to several previous examples in which calculated electronic parameters were used to rationalize the regioselectivity of electrophilic aromatic substitution reactions involving small molecule reagents.[30,32-34] HalA values for each $sp^2$ hybridized carbon on each substrate for which halogenation selectivity was unambiguously assigned were calculated (29 substrates, Table S2), and a plot of conversion versus HalA at each site was constructed (Fig. 6).

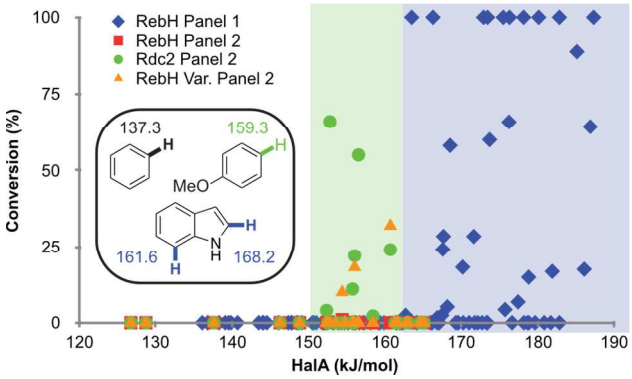



**Figure 6.** Conversion versus halenium affinity (HalA) for each $sp^2$ carbon on each substrate in Table S2. HalA ranges for panel 1 substrates halogenated by RebH and panel 2 substrates halogenated by Rdc2 are highlighted in blue and green, respectively. The inset shows representative HalA values for common arenes (not substrates).

Several useful trends can be obtained from this plot. Good conversion of panel 1 substrates by RebH (Fig. 6, blue diamonds), occurs only at sites with HalA values >160 kcal/mol. Poor conversion of panel 2 substrates, which all possess HalA values <164 kcal/mol, was observed for RebH (Fig. 6, red squares), but Rdc2 provides significant conversion of panel 2 substrates at sites with HalA values as low as 152 kcal/mol (Fig. 6, green circles). Similarly, engineered RebH variants were able to halogenate sites on panel 2 substrates with HalA values as low as 154 kcal/mol (Fig. 6, orange triangles). Sites on substrates with HalA values above the minimum for each enzyme, however, were often not halogenated, so other factors clearly impact FDH selectivity. In short, our initial data suggest that there is a minimum electronic activation (HalA~154 kJ/mol) necessary for halogenation by the FDHs examined, and this can serve as a coarse filter for identifying potential new substrates for these enzymes. To better understand the selectivity of the reactions substrate binding must be considered (*vida infra*).

*Substrate Docking*

Deviation of FDH selectivity from outcomes expected based on electronic effects alone likely results in part from the unique binding of different substrates within FDH active sites.[7,10,16] Sixteen substrates for which the selectivity of RebH- was established (Fig. 5) were therefore docked into a multi-conformer model of RebH to determine if computationally inexpensive methods[35,36] could improve on a purely electronic view of RebH selectivity. Docking was performed using ICM Molsoft docking[37] and ROCS pharmacophore overlay[38]. The former

employs a Monte Carlo minimization algorithm combined with empirical energy terms to model van der Waals forces, hydrophobic properties, and electrostatics.[39] This procedure returns multiple energetically favorable poses of a ligand within a target, but it is blind to known binding interactions. Given the existence of several crystal structures of RebH-tryptophan complexes, the ROCS pharmacophore overlay approach, which aligns query molecules to a given template based on molecular shape and charge,[38] was also used. As in our previous work, catalytically relevant poses were taken to be those in which an $sp^2$ hybridized carbon was positioned proximal to Lys79 in the RebH active site.[10]

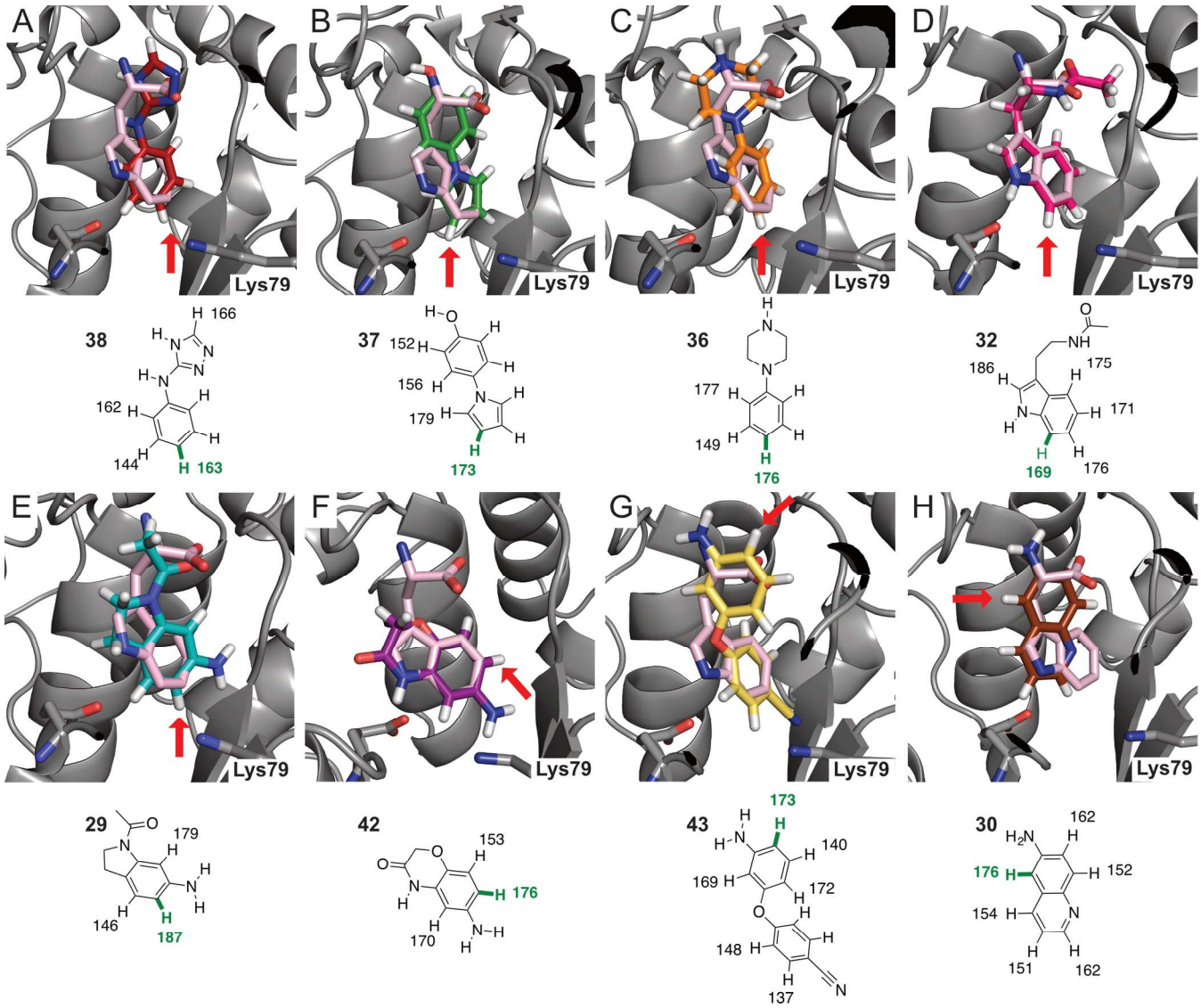



**Figure 7.** Poses observed for ROCS pharmacophore overlay. Tryptophan in the RebH-tryptophan complex (PDB 2OA1) is shown in pink. Lys79 is labeled in each model, and the site of halogenation for each substrate is indicated with a red arrow. Structures for each substrate are shown with HalA values for each site and the site halogenated in green.

In general, neither method provided significant predictive ability regarding substrate specificity (i.e. whether or not a given substrate would be halogenated). Many unreactive substrates were nonetheless predicted to bind in the RebH active site. In addition, while ICM Molsoft docking returned several poses for each substrate within the RebH active site, very few of these appeared catalytically relevant. This was likely due to both the relatively large size of the binding pocket and the relatively small size of the compounds, allowing for multiple high scoring (energetically feasible) docking poses.[36] ROCS pharmacophore overlay, on the other hand, provided a number of interesting results regarding the site selectivity of substrates that *were* halogenated (Fig. 7, representative examples). Perhaps most notably, panels A-D show cases where the site halogenated does not have the highest HalA, but is predicted to bind proximal to Lys79. This selectivity is inconsistent with purely electronic effects (substrate control) but is consistent with predicted binding within the active site (catalyst control), suggesting that

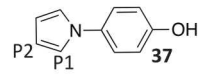
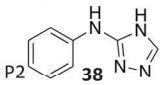
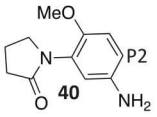
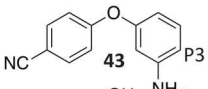
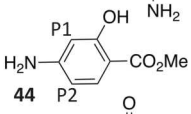
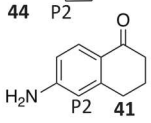
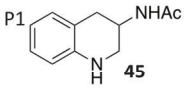
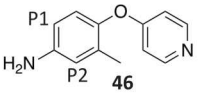
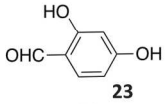
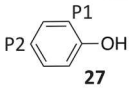
FDHs could be useful for obtaining novel selectivity on diverse structures. Moreover, the pharmacophore overlay predicts that this selectivity results from different polar functional groups distal to the site of halogenation binding to the pocket occupied by the substrate amino acid moiety in the crystallized RebH-tryptophan complex. Similar anchoring effects have been exploited for other enzymes,[40] but these results suggest that a range of different functional groups and even heterocycles can fulfill this role in RebH.

Several substrates, including the one shown in panel E, were predicted to bind such that the most electronically activated site was proximal to Lys79, but cases in which electronic effects appear to outweigh any predicted binding effects were also found. In panels F-H for example, substrates were halogenated at the most electronically activated site even when this site was predicted to bind distal to Lys79. Presumably, in these cases, alternate substrate orientations within the RebH active site can be sampled, leading to halogenation at the most activated site(s). While ICM Molsoft docking tended to underrepresent binding modes similar to native Trp binding, pharmacophore overlay emphasized such binding modes, both of which reduce predictive utility. When combined with HalA values, however, computationally inexpensive docking simulations appear to provide insight into the observed selectivity of FDH-catalyzed halogenation on non-native substrates in many cases.

*Controlling Site Selectivity Using Different FDHs*

The catalyst control exhibited by RebH toward various substrates also suggests that different FDHs could be used to produce different halogenated products. To explore this possibility, halogenation of several substrates with multiple electronically activated sites that were selectively halogenated by RebH or Rdc2 was also examined using 4V, 6TL, and 10S (Table 1, entries 1-6). Two reactions in which RebH provided mixtures of compounds were also examined to determine if other enzymes could improve selectivity (Table 1, entries 7 and 8). Finally, reactions of two phenols were also examined (Table 1, entries 9 and 10). To compare the product profiles of different halogenases with those produced by a common stoichiometric chlorinating reagent, these reactions were also conducted with *N*-chlorosuccinimide (NCS)[41].

**Table 1.** Relative product distributions for halogenation of representative substrates using FDHs and NCS. Sites of halogenation (P1-3) are indicated for select substrates.

| Entry | Substrate | Species | %1 | %2 | %3 | % di | % conv. |
|---|---|---|---|---|---|---|---|
| 1 |  **37** | NCS | 100 | 0 | - | - | 97 |
| | | RebH | 0 | 100 | - | - | 55 |
| 2 |  **38** | NCS | 18 | 41 | 29 | 13 | 87 |
| | | RebH | 0 | 100 | 0 | 0 | 32 |
| | | 6TL | 17 | 83 | 0 | 0 | 51 |
| 3 |  **40** | NCS | 7 | 65 | - | 28 | 86 |
| | | RebH | 0 | 100 | - | 0 | 35 |
| 4 |  **43** | NCS | 41 | 20 | 30 | 9 | 80 |
| | | RebH | 2 | 0 | 98 | 0 | 94 |
| 5 |  **44** | NCS | 63 | 37 | - | - | 94 |
| | | 6TL | 3 | 97 | - | - | 100 |
| 6 |  **41** | NCS | 10 | 90 | - | - | 98 |
| | | RebH | 0 | 100 | - | - | 15 |
| | | 6TL | 93 | 7 | - | - | 81 |
| | | 4V | 70 | 30 | - | - | 22 |
| 7 |  **45** | NCS | 2 | 25 | - | 73 | 65 |
| | | RebH | 50 | 0 | - | 50 | 50 |
| | | 6TL | 58 | 42 | - | 0 | 44 |
| 8 |  **46** | NCS | 52 | 43 | - | 5 | 94 |
| | | RebH | 30 | 70 | - | 0 | 74 |
| | | 4V | 77 | 23 | - | 0 | 81 |
| 9 |  **23** | NCS | 37 | 52 | - | 11 | 62 |
| | | Rdc2 | 75 | 18 | - | 7 | 44 |
| | | GsfI | 91 | 9 | - | 0 | 2 |
| 10 |  **27** | NCS | 23 | 75 | - | 2 | 94 |
| | | Rdc2 | 100 | 0 | - | - | 4 |
| | | 4V | 0 | 100 | - | - | 10 |

Substrate selectivity profiles for the different halogenating species are presented in Table 1, with results for enzymes that provide similar profiles removed for clarity. Altered site-selectivity was observed not only between FDHs, but also between the FDHs and NCS. A complete switch in site-selectivity is observed using different FDHs for substrates such as **27**, **41** and **46**. A remarkable example of this is phenol (**27**), for which Rdc2 halogenates exclusively ortho to the alcohol, while 4V only halogenates para. This particular example highlights how effective enzymatic catalyst control can be even on simple non-native substrates. For many substrates within this subset, an FDH gave a single isomer when mixtures were seen with NCS (Table 1; entries 2-6, 10). In addition, NCS often begins to di-halogenate substrates to significant amounts before complete consumption of starting material. This problem is circumvented by using FDHs, which typically have decreased activity on halogenated products (Table 1; entries 2-4, 7). FDHs are also catalytic and use chloride as a halide sources and air as a terminal oxidant.[2]

For certain substrates, such as **46** (Table 1; entry 8), different ratios of two mono-chlorinated products can be observed for NCS, RebH and 4V. The use of FDHs presents the unique opportunity in such cases to tune existing selectivity through directed evolution.[10] For example, RebH halogenates primarily *para* to the methyl group, but is not highly selective for this site (70%). RebH could be tuned to give better selectivity at this position. On the

other hand, 4V halogenates primarily *ortho* to the methyl group (77%) and could be used as a starting point to selectivity halogenate this position is desired.

**Discussion**

In this work, the substrate scope of several FDHs was probed by measuring their activity toward diverse panels of low molecular weight aromatic compounds. The resulting substrate activity profiles contain information that cannot be obtained by studying enzyme activity on native substrates or simple derivatives of these substrates.[18,19,23] While substitution of compounds distal to sites of reaction is commonly used to probe electronic effects in organic reactions (e.g. LFERs),[42] this can perturb substrate binding as well as electronic effects within an enzyme active site,[43] leading to spurious correlations between substitution and activity. The points in Fig. 6 corresponding to substrate sites with sufficient electronic activation (as indicated by calculated HalA values) but for which halogenation was not observed likely exemplify this phenomenon. On the other hand, sampling a sufficient range of substrate classes, substituents, and substitution patterns provides general information regarding the halogenating ability of different enzymes. Analogous efforts on other enzymes could prove similarly enlightening.

The small set of FDHs examined provided remarkably broad substrate scope considering the substantial variation of the substrates evaluated relative to the native substrates of these enzymes. A wide range of functional groups were tolerated, but it is worth noting that this would not necessarily be apparent from evaluating individual substrates. For example, the simple ethylsulfonamide-substituted aniline **51** (Table S1) was not halogenated, but sulfonamides **2** and **10** were both halogenated, showing that minor changes in substituents distal to reaction sites can significantly impact substrate reactivity. Similarly, compounds **21** (Fig. 3) and **44** (Table 1, entry 5) are regioisomeric methyl (aminohydroxybenzoates), but while **44** is halogenated in high conversion by multiple FDHs (100% with 4V, 56% with 6TL, 55% with RebH), **21** was halogenated only by 0K to a trace amount. Examining activity on diverse substrates[23] can account for these differences and provide an accurate picture of the types of functional groups and fragments that can potentially be halogenated. The molecular context in which these moieties appear, however, will clearly influence their impact on FDH catalysis.

Subsequent analysis of substrates that undergo halogenation can then be used to rationalize how substrate properties impact FDH selectivity. Each $sp^2$ carbon on each substrate constitutes a potential reaction site, so conversion values for each site can be plotted against steric or electronic descriptors. The use of calculated HalA values[31] in this work indicated clear cutoffs in electronic activation that are required for substrate halogenation by FDHs, but electronics alone could not explain the observed selectivity in most cases. Docking simulations provided further insight into selectivity when combined with HalA values, and more rigorous computational methods (e.g. MD simulations)[44] could significantly improve the predictive models for FDH activity and selectivity.

These results constitute the largest examination of FDH substrate scope and selectivity to date and significantly improve our understanding of the types of substrates that can be halogenated using these enzymes. The range of substrates that could be halogenated clearly attests to the potential for halogenases evolved for one application to find utility toward another. Moreover, this broad substrate scope should facilitate the identification of novel FDHs, since native substrates are not necessarily required to confirm FDH activity.[18] Given the unique scope and selectivity of the enzymes examined in this study relative to one another and to NCS, the use of probe substrates for functional characterization of putative FDHs holds great promise for expanding the utility of enzymatic halogenation.[1]

## AUTHOR INFORMATION

**Corresponding Author**

*Department of Chemistry, University of Chicago, Chicago, IL 60637
E-mail: *jaredlewis@uchicago.edu*
*Global Discovery Chemistry, Novartis Institutes for Biomedical Research, 250 Massachusetts Ave, Cambridge, MA 02139
E-mail: kian.tan@novartis.com

**Author Contributions**

All authors have given approval to the final version of the manuscript.

## ASSOCIATED CONTENT

**Supporting Information**

A full description of materials, a complete substrate list, experimental details, and characterization of all compounds and enzymes are provided in the Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

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## ABBREVIATIONS

FDH, flavin-dependent halogenase; Trp-FDH, tryptophan-flavin-dependent halogenase; EAS, electrophilic aromatic substitution; HalA, halenium affinity; NCS, *N*-chlorosuccinamide; LFER, linear free energy relationship

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