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Arene Halogenation

Regioselective Arene Halogenation using the FAD-Dependent Halogenase RebH**

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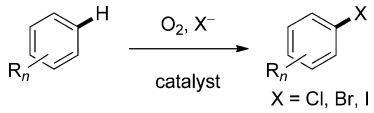
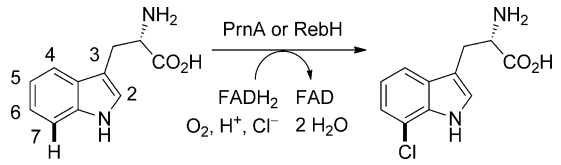
Halogenated organic compounds are essential building blocks for chemical synthesis[1] and play important roles as pharmaceuticals[2] and agrochemicals.[3] Despite the utility of these compounds, installing halogen atoms frequently necessitates the use of activated or prefunctionalized starting materials and wasteful multistep functional group conversion sequences.[4] Arene halogenation through electrophilic aromatic substitution (EAS) requires harsh reaction conditions using stoichiometric reagents and suffers from poor regioselectivity in many cases.[5] Oxidative methods to catalytically generate halogenating agents from halide salts have been developed, but none of these have solved the aforementioned selectivity problem, and few utilize oxygen as a terminal oxidant (Scheme 1).[6]



Scheme 1. General scheme for oxidative halogenation.

New, more efficient methods for oxidative halogenation would significantly improve the syntheses of a wide range of chemicals and are therefore highly desirable.[6] Given the high efficiency and selectivity often associated with enzyme-catalyzed reactions, we were drawn to several classes of enzymes that catalyze oxidative halogenation reactions.[7] Particularly notable in this regard are the FAD-dependent halogenases, which were first characterized by van Pée and co-workers in the late 1990s.[8] Extensive mechanistic investigation of these enzymes by both van Pée and Walsh, focusing predominately on tryptophan halogenases, has clarified their mechanism.[9] In short, they utilize $FADH_2$ supplied by a NAD-dependent flavin reductase to reduce oxygen to water with concomitant oxidation of halide to the corresponding hypohalous acid (HOX). This species migrates through a tunnel within the enzyme to form a stable chloramine adduct with an active-site lysine. Tryptophan binding proximal to this species enables regioselective EAS of this substrate.[10] Importantly, these enzymes are able to override the electronic preference of tryptophan for halogenation at the 2-position, and homologs have been identified that catalyze selective 5-, 6-, or 7-halogenation of the indole ring.[8]

While FAD-dependent halogenases thus have the potential to improve a number of problems with current oxidative halogenation methods, they remain largely unexplored for preparative synthesis. Characterization of their activity in vitro has typically involved analytical scale reactions of tryptophan,[9] but van Pée and co-workers also explored the substrate scope of the tryptophan-7-halogenase PrnA expressed in *Pseudomonas fluorescens* (Scheme 2).[11] A



Scheme 2. RebH- or PrnA-catalyzed 7-chlorination of tryptophan.

variety of substituted indoles were accepted by the enzyme, but halogenation invariably occurred at the electronically most activated indole 2-position for all substrates except tryptophan. These same researchers were able to alter the selectivity of PrnA to produce 5-chloro- and 5-bromotryptophan by mutating a single active-site residue, but 7-halogenation remained the dominant reaction.[12] More recently, several groups have utilized a second tryptophan-7-halogenase, RebH,[9a,13] in metabolic engineering efforts aimed at converting halogenated tryptophan into more complex structures.[14] As part of one such effort, O'Connor and co-workers demonstrated that tryptamine is accepted by RebH and is halogenated at the 7-position,[15] which contrasts with the preference for halogenation of the 2-position of this substrate by PrnA.[11]

Inspired by these examples, we set out to explore the synthetic utility of RebH in preparative halogenation reactions. We focused on this enzyme and its cognate flavin reductase, RebF, due to reports of their expression in *E. coli*,[13] which facilitates both enzyme production and genetic manipulation. However, while both RebH and RebF were indeed expressed in *E. coli* using published procedures,[13,16] low enzyme yields hindered their synthetic utility. SDS-PAGE analysis of the soluble and insoluble fractions obtained

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following cell lysis and centrifugation revealed that essentially all of the expressed protein was insoluble (Figure 1, lanes 1/2 and 6/7).

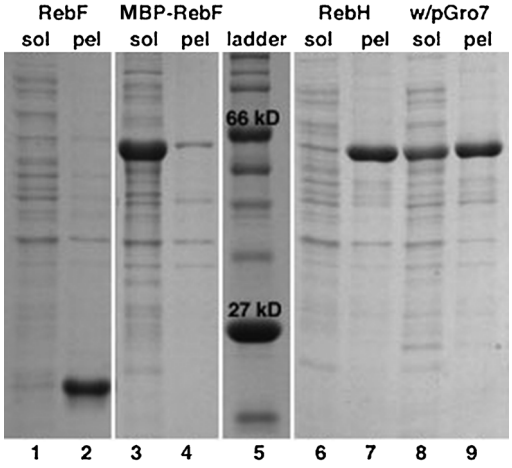


Figure 1. SDS-PAGE illustrating difference in expression of various RebH and RebF preparations. Lanes show soluble (sol) and insoluble (pel) fractions obtained from expression of RebH/F using different conditions. Lanes 1 and 2, RebF (MW = 21 kDa), literature protocol.[13] Lanes 3 and 4, MBP-RebF (MW = 63 kDa). Lane 5, ladder. Lanes 6 and 7, RebH (62 kDa), literature protocol.[13] Lanes 8 and 9, RebH co-expressed with pGro7.

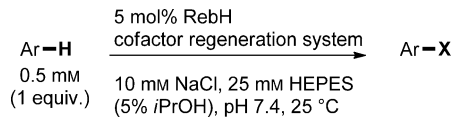
On the other hand, fusing RebH and RebF to maltose-binding protein (MBP),[17] a protein known to produce soluble fusion constructs with insoluble proteins,[18] provided high levels of soluble RebH/F-MBP (Figure 1, lanes 2/3, only MBP-RebF shown). The RebF-MBP fusion protein retained high activity for the NAD-dependent reduction of FAD to $FADH_2$ ($k_{cat,RebF} = 32\ min^{-1}$; $k_{cat,MBP\text{-}RebF} = 80\ min^{-1}$),[19] but, as is commonly observed,[20] the RebH–MBP fusion protein displayed strongly reduced activity compared to wild-type RebH. While protease-mediated cleavage of MBP from MBP-RebH was achieved, co-expression of RebH with a variety of chaperone proteins was explored to simplify the production of this enzyme.[21] The combination of chaperones GroEL and GroES (pGro7) enabled significant improvement of protein solubility, and additional optimization of cell culture conditions led to the expression of high levels of RebH in *E. coli* (Figure 1, lanes 8/9). The yields for isolated RebH and MBP-RebF according to the various procedures employed are shown in Table 1.

Ready access to large quantities of RebH and MBP-RebF enabled optimization of conditions for preparative halogenation reactions (Scheme 3, Table 2). To mitigate cofactor expense, a number of cofactor regeneration strategies were explored.[22] The best results were obtained using a system comprised of a glucose dehydrogenase, which uses glucose to reduce NAD to NADH, and MBP-RebF, which uses NADH to reduce FAD to $FADH_2$ for RebH catalyzed halogenation. Using this system under optimized reaction conditions, 74% isolated yield of L-7-chlorotryptophan (**1**, 94% HPLC conversion observed) was achieved using L-tryptophan as a substrate on a 10 mg scale, clearly demonstrating that these enzyme reactions can be conducted on a preparative scale.

Table 1: Yields of RebH/F under different conditions.[a]

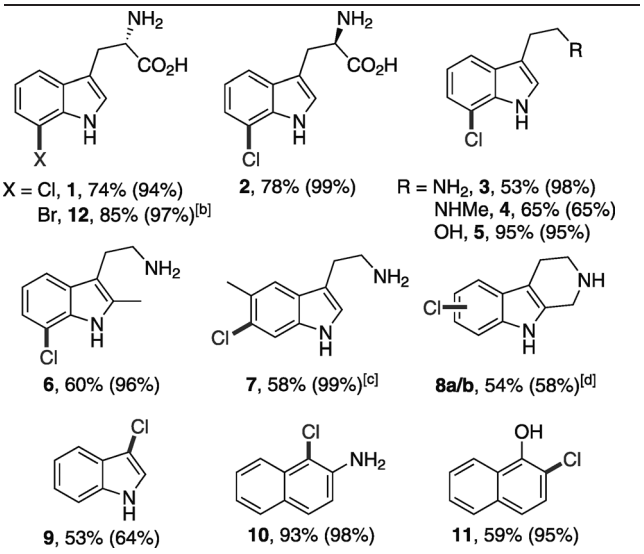
Conditions	RebH [mg L^{-1}]	RebF [mg L^{-1}]
Literature conditions[13]	15	3
With pGro7 (RebH) or as MBP fusion (RebF)	111	33

[a] Yields determined by bicinchoninic acid (BCA) assay.



Scheme 3. General scheme for RebH-catalyzed arene halogenation.

Table 2: Representative yields for preparative RebH-catalyzed aromatic halogenation reactions.[a]



[a] Yields of isolated products and HPLC conversions (in brackets) are provided. The cofactor regeneration system consisted of 0.2 equiv FAD, 0.2 equiv NAD, 0.5 mol% RebF, 50 U mL^{-1} glucose dehydrogenase, and 40 equiv glucose. [b] 100 mM NaBr was used in place of 10 mM NaCl. [c] 6,7-dichloro-5-methyltryptamine was also isolated in 23% yield. [d] 10 mol% RebH loading was used. A nearly 1:1 mixture of 5- and 6-halogenation was observed.

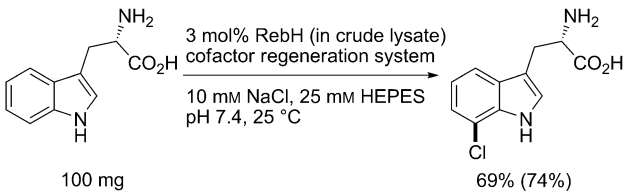
Preparative halogenation of several additional substrates was explored to demonstrate the scope of this method. Different co-solvents (5% v/v), including DMSO, *i*PrOH, and THF, did not impact the rate or conversion of tryptophan halogenation, and their use allowed for facile introduction of hydrophobic substrates into the aqueous reaction medium.[23] In contrast to previously published results using PrnA,[11] RebH halogenates a range of 3-substituted indoles at electronically disfavored sites (i.e. not C2) in high yields. For example, D-tryptophan was converted to D-7-chlorotryptophan (**2**) in 78% yield (Table 2). Halogenation of the 7-positions of tryptamine, previously demonstrate by O'Connor and co-workers on analytical scale,[15] *N*-Ω-methyltryptamine,

tryptophol, and 2-methyltryptamine was also observed in good yields (**3, 4, 5**, and **6**). Interestingly, 6-halogenation of 5-methyltryptamine was observed in 58% yield (**7**), indicating that additional sites of the indole benzene ring can be halogenated despite the presence of the more electron-rich pyrrole ring.[12] At longer reaction times, dihalogenation of these substrates occurred,[24] so reaction monitoring was required to ensure isolation of high yields of mono-halogenated products. Low conversion (9–12%) of 1-methyl-L-tryptophan and gramine to mono-halogenated products was also observed, although the site of halogenation for these substrates was not determined.

Halogenation of a number of bicyclic and tricyclic carbocycles and heterocycles lacking pendent functionality was also examined. The tricyclic 2,3-disubstituted indole tryptoline was converted to a nearly 1:1 mixture of 5- and 6-chlorotryptoline (**8a/b**; Table 2), providing a promising starting point for evolving halogenases with activity on more complex heterocyclic compounds.[25] Comparing this result with those for 2-methyltryptamine (7-halogenation) and 5-methyltryptamine (6-halogenation) also demonstrates that different sites of the benzene ring of nonnatural indole substrates can be halogenated by RebH.[12] Again contrasting with reports for PrnA,[11] RebH provided high conversion of indole to halogenated products. This substrate was particularly susceptible to dihalogenation, and the reported 53% yield of 3-chloroindole (**9**) is the maximum obtained before this process became significant. Substituted naphthalenes were also viable substrates, and monochlorinated compounds **10** and **11** were isolated in high yields. While these latter substrates were halogenated at their most activated sites, they nonetheless illustrate the ability of RebH to accept substrates significantly different from tryptophan.

To further demonstrate the synthetic utility of RebH, we confirmed that the bromination capabilities of this enzyme[13] could be translated to the preparative scale, and addition of NaBr to the reaction medium provided 7-bromotryptophan (**12**) in 85% isolated yield. The reaction scale was also increased to enable chlorination of 100 mg of tryptophan using crude cell lysate, rather than purified enzyme, as a catalyst (Scheme 4). While a significant decrease in conversion was observed relative to the 10 mg reaction conducted using purified enzyme, a 69% product yield was still obtained. A strong dependence of yield on the reaction surface area/volume ratio was also observed and suggests that improvements may be achieved by controlling dissolved oxygen concentration.[26]

Finally, the differing catalytic efficiency of RebH on representative substrates was examined by conducting reactions with enzyme loadings below those required for maximal substrate conversion (see Supporting Information for representative kinetic data). As expected, decreased total turnover numbers (TTN) were observed for nonnatural substrates (Table 3). The increasing K_m values for tryptamine and tryptoline are consistent with their increasing structural variation from tryptophan. The large structural differences between these substrates and 2-aminonaphthalene make direct comparisons difficult, but the relative efficacy of this substrate presumably results from its strong electronic activation. These data clearly show that significant improvements to catalyst efficiency or stability are desirable and provide a benchmark for further RebH optimization.



Scheme 4. Preparative halogenation of tryptophan on 100 mg scale (cofactor regeneration system described in Table 2).

Table 3: Catalytic parameters for halogenation of select substrates.[a]

Substrate	RebH [mol%]	Conv. [%]	TTN	k_{cat}[b] [min^{-1}]	K_m[b] [μM]
L-tryptophan[c]	0.5	75	165	1.1	7.3
tryptamine	1	15	19	0.023	9.0
tryptoline	5	30	3.6	0.027	216
2-aminonaphthalene	1	32	26	0.59	14

[a] 75 μL reactions were conducted as described in Table 2 using the indicated RebH loading and 0.5 mM phenol as an internal standard, quenched with an equal volume of methanol, and analyzed by HPLC. [b] Initial rate data were used to construct Hanes–Woolf plots to determine k_{cat} and K_m. [c] Reported values for the k_{cat} and K_m of RebH on L-tryptophan are 1.4 min^{-1} and 2.0 μM,[13] respectively.

Several notable differences between the reactivity of RebH and that reported for PrnA deserve comment given the significant homology of both the complete sequences (55%) and the active sites (see below) of these two enzymes. First, PrnA was reported to have no activity on indole, gramine, or 1-methyl-L-tryptophan,[11] whereas RebH halogenated each of these substrates. The simplest explanation for this difference is that low conversion using PrnA precluded identification of the halogenated products. RebH has higher catalytic efficiency than PrnA (reported k_{cat} values on tryptophan are 1.4 min^{-1} versus 0.1 min^{-1}),[27] and our improved reaction conditions would have further improved this advantage.

Second, PrnA was reported to catalyze 2-halogenation of both 5- and *N*-Ω-methyltryptamine,[11] while RebH catalyzes 6- and 7-halogenation of these substrates, respectively. Crystal structures for both of these enzymes bound to FAD, chloride, and tryptophan are available.[16,9d] Analysis of residues (24 total) within 5 Å of the substrate tryptophan shows only one pair of residues (N467 and L456) that differ in identity and only one additional pair of residues (N464 and N453) displaying a notable conformational difference (Figure 2). In RebH, the side chain of N467 forms a water-mediated hydrogen bond to the substrate tryptophan. Similar hydrogen bonding could help position other substrates for halogenation at electronically disfavored sites during catalysis. The structurally analogous residue in PrnA is L456, which cannot form a hydrogen bond, and this difference may explain the apparent differences in selectivity between PrnA and RebH.

In summary, co-expression of the halogenase RebH with GroEL/ES and fusion of the flavin reductase RebF to MBP

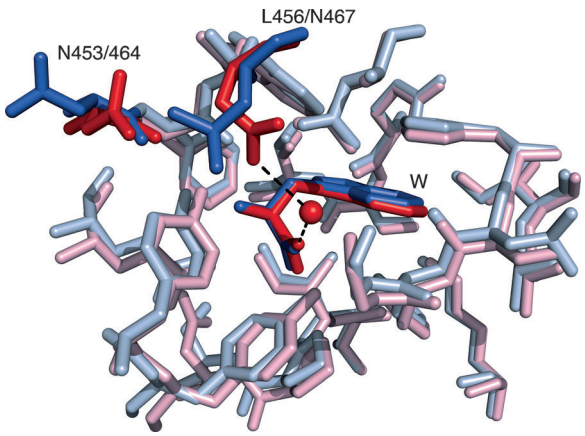


Figure 2. Selected residues and a water molecule located within 5 Å of tryptophan in PrnA (purple/lavender) and RebH (red/pink). PDB files 2A1 and 2AQJ used for RebH[16] and PrnA,[9d] respectively. Bold colors denote the tryptophan substrates (W), residues with different identities (L456/N467) or conformations (N453/464) in PrnA or RebH, respectively, and a water molecule.

enabled production of both enzymes on scales sufficient for preparative biocatalysis without the need for specialized fermentation equipment or bioreactors. Optimized reaction conditions, including *i*PrOH co-solvent and a cofactor regeneration system, were used to enable efficient halogenation using air, sodium chloride, and glucose as the only stoichiometric reagents. Halogenation, including both chlorination and bromination, of a range of medicinally relevant indoles and naphthalenes was possible on up to 100 mg scale with high yields. These results contrasted somewhat with those reported for PrnA, a structurally homologous halogenase, which provided a narrower substrate scope and only enabled halogenation of nonnatural substrates at their most electronically activated positions. Overall, this work demonstrates the unique potential of RebH as a catalyst for regioselective oxidative halogenation, and work is currently underway to further expand the substrate scope and improve the activity of this enzyme.

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