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Expanding the Reactivity of Flavin Dependent Halogenases Toward Olefins via Enantioselective Intramolecular Haloetherification and Chemoenzymatic Oxidative Rearrangements

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Abstract

Of the different classes of halogenases characterized to date, flavin dependent halogenases (FDHs) are most associated with site-selective halogenation of electron rich arenes and enol(ate) moieties in the biosynthesis of halogenated natural products. This capability has made them attractive biocatalysts, and extensive efforts have been devoted to both discovering and engineering these enzymes for different applications. We have established that engineered FDHs can catalyze different enantioselective halogenation processes, including halolactonization of simple alkenes with a tethered carboxylate nucleophile. In this study, we expand the scope of this reaction to include alcohol nucleophiles and a greater diversity of alkene substitution patterns to access a variety of chiral tetrahydrofurans. We also demonstrate that FDHs can be interfaced with ketoreductases to enable halocyclization using ketone substrates in one-pot cascade reactions and that the halocyclization products can undergo subsequent rearrangements to form hydroxylated and halogenated products. Together, these advances expand the utility of FDHs for enantio- and diastereoselective olefin functionalization.

Graphical Abstract

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Supporting Information

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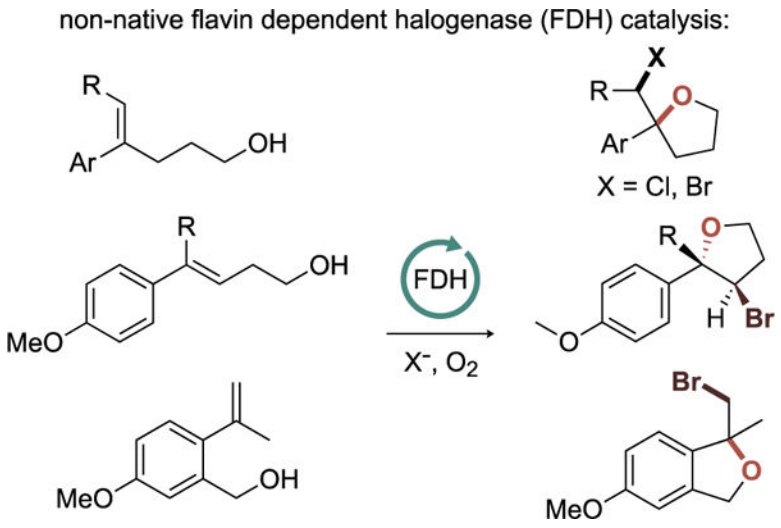
supporting_information (PDF containing supplementary figures, complete experimental procedures, and relevant characterization)



Keywords

halogenase; flavin; halocyclization; chemoenzymatic synthesis; enzyme cascade

Body

In nature, flavin-dependent halogenases (FDHs) catalyze halogenation of electron rich arenes and enol(ate) moieties using halide anions (typically $X^- = Cl^-$ and Br^-) as a halogen source and dioxygen as a terminal oxidant.[1] These mild reaction conditions and the high site selectivity of FDHs toward their native substrates and even many non-native substrates have made them attractive biocatalysts.[2–4] Our group and others have established that variants of these enzymes can be engineered to halogenate different non-native substrates or different sites on a given substrate with high (i.e. >90%) selectivity.[5] We also established that FDHs can catalyze enantioselective desymmetrization of methylenedianilines[6] and atroposelective[7] halogenation of 3-aryl-4(3*H*)-quinazolinones; however, these transformations share a similar mechanism involving electrophilic attack by an active site halogenating agent, likely HOX bound within the FDH active site, and *ipso* deprotonation (Figure 1A).[8–10]

Inspired by the wide range of transformations known to proceed via electrophilic attack of different nucleophiles by electrophilic halogen species,[11] we recently discovered that engineered FDHs can also catalyze enantioselective halolactonization of olefins (Figure 1B)[12]. This finding significantly broadened the catalytic repertoire of FDHs and revealed that their active sites can tolerate intermediate and transition state structures that are topologically distinct from those associated with electrophilic aromatic substitution. This activity also provided a starting point for assessing whether different olefin geometries, nucleophiles, and linkers between the olefins and pendant nucleophiles,[13] could be tolerated by FDHs to further broaden the scope of enzymatic halocyclization.

Because our initial report only explored carboxylate nucleophiles, which are deprotonated and therefore highly nucleophilic under the conditions required for biocatalysis,[14] we were particularly interested in evaluating the reactivity of less activated nucleophiles.

Chemoenzymatic halocycloetherification has been achieved using vanadium-dependent haloperoxidases, but racemic products are obtained from this approach.[15] With some notable exceptions,[16,17] however, enantioselective catalysis of halocycloetherification remains challenging using small molecule catalysts,[18–23] so we hoped that FDHs might be able to address this outstanding methodology challenge. Herein, we report that FDHs can indeed catalyze halocyclooetherification of electron rich styrenes bearing pendant alcohols to generate chiral tetrahydrofurans in high yield and with high enantioselectivity (Figure 1C). We also show that 2-(4-methoxyphenyl)-2-(bromomethyl)tetrahydrofurans can rearrange via nucleophilic attack on a phenonium intermediate leading to net chemoenzymatic oxidative rearrangements of the starting olefins. Finally, we demonstrate that alcohol nucleophiles can be generated from the parent ketones and cyclized in a ketoreductase/FDH cascade. These results further broaden the substrate scope of FDH-catalyzed halocyclization and establish that FDHs can be used to access chiral tetrahydrofurans and dihydrobenzofurans that are commonly found in a range of natural products and other biologically active compounds.[24–26]

We previously found that variants of the FDH RebH provided good conversion and enantioselectivity for halolactonization of 4-arylpent-4-enoic acids,[12] so we analyzed the activity of a panel of 50 RebH variants and other WT FDHs[27] toward the corresponding alcohol **1** (Table 1, Figure S1). Despite the lower nucleophilicity of its pendant alcohol, **1** undergoes 5-*exo*-trig cyclization to give **1a** in good yields using several previously reported FDHs. While variant 4V+S[28] was optimal for halolactonization,[12] improved selectivity was observed for variant 4PL[29] (Table 1, entries 1 and 3). Low selectivity for the opposite product enantiomer was observed for variant 6TL T52V,[29] providing a starting point for directed evolution to improve this sense of asymmetric induction (Table 1, entry 2). Notably, improved yields were obtained using 4PL when the reaction time was decreased to 2 h. While glutathione and catalase, HOX and H_2O_2 scavengers, respectively, had only a minor effects on enantioselectivity, they were included in further reactions due to their beneficial effects on halolactonization reactions[12] and to avoid any potential substrate-dependent background reactions involving these reactive oxygen species, which can form during FDH catalysis.[27,30]

The observed decrease in the yield of **1a** at longer reaction times suggested that degradation of this compound could be occurring. Analysis of the reaction mixtures revealed that 1-(*p*-methoxyphenyl)-5-hydroxypentan-2-one (**1b**) was indeed forming (Figure 2A, Figure S2), which was surprising since it lacks a halogen substituent and requires structural rearrangement of the starting olefin. While this reaction was avoided by simply quenching the reactions following complete halogenation of the olefin starting material, it was facilitated by heating the completed haloetherification reaction mixtures to 50 °C. Related reactivity was reported for cyclic acetals of α-haloacetophenones, which undergo rearrangement to the corresponding 1-aryl-5-bromopentan-2-ones via a proposed phenonium intermediate (Figure 2B).[32] In this reaction, the $ZnBr_2$ is believed to serve as a Lewis acid to assist with abstraction of the Br substituent and could also help to mediate the nucleophilic attack on the phenonium intermediate. We found that **1a** produced via FDH catalysis could be efficiently converted to brominated compound **1c** under these

conditions (Figure 2C). While we hoped that reaction of trisubstituted alkene **8** might lead to chiral α-substituted ketones, racemic **8c** was obtained from this reaction despite the fact that moderate enantioselectivity is observed for the bromoetherification step (*vide infra*), suggesting that the rearrangement process is not stereospecific.

With conditions to avoid this non-selective rearrangement process in hand, we next investigated the substrate scope of FDH-catalyzed halocycloetherification (Chart 1). Reactions involving 1,1-disubstituted alkenes generally proceed with good yield and selectivity as long as the styrene possesses an electron donating group *para* to the olefin. For example, while *p*-methoxyphenyl substituted substrate **1** gives a 91% yield of **1a** and 91:9 e.r., phenyl substituted substrate **2** provides no detectable products under the reaction conditions; only unreacted starting material remains. This trend was previously observed for the analogous halolactonization reactions, but low yields of cyclized products were still obtained for phenyl- and *p*-fluorophenyl-substituted substrates.[12] We attributed the lower yields for these relatively electron deficient substrates to the difficulty of accessing the transition state required for the concerted nucleophile-assisted alkene activation pathway that such substrates have been shown to follow.[14] The lack of reaction for the corresponding alcohols suggests that while the concerted pathway could still occur to some extent with a more potent carboxylate nucleophile, it was no longer possible with alcohols, and only substrates with electron donating groups that would allow for a stepwise pathway were suitable for FDH catalysis. Alternatively, if these reactions proceed via bromiranium intermediates,[13] substrates like **2** that lack electron donating groups would also be expected to react less readily. Substrate **3**, which has a methoxy group in the *ortho* position, was also problematic and gave **3a** in low yield and e.r. Substrates **4**-**7** all cyclize in greater than 90% yield and >92:8 e.r., and *p*-acetamide substituted substrate **7** provided particularly high selectivity for **7a**. This reaction was scale up to 28 mL scale with 2 mM substrate loading to provide 86% yield of **7a** (14 mg) with 99:1 e.r. Trisubstituted substrate **8** (64:36 E/Z) also cyclizes in good yield to give **8a** as a 72:28 mixture of diastereomers (largely reflecting the E/Z ratio of the starting material) and moderate enantioselectivity. Substrate **9** gives 2,2',5,5'-tetrasubstituted tetrahydrofuran **9a** in moderate yield and selectivity. Finally, chloroetherification of substrate **1** was also achieved to give **1d** in 70% yield and 99:1 e.r. using NaCl in place of NaBr under otherwise standard reaction conditions.

We also found that 1,2-disubstituted alkenes **10** and **11** undergo haloetherification to give **10a** and **11a** as single diastereomers (Figure 3A). The analogous halolactonization reactions provided only trace conversion, so this finding demonstrates that FDHs can accommodate 5-*endo*-trig cyclization and provides access to 2,3-disubstituted and 2,2',3-trisubstituted tetrahydrofurans. While 4PL provided these products in 72% and 84% yield, respectively, modest enantioselectivity was observed in both cases. Variants 2RFQ F111S[29] and 3LR[33] provided increased selectivity at the expense of yield (Figure 3A). Finally, α-methyl styrene **12**,[17] which possesses a pendant benzyl alcohol nucleophile, undergoes cyclization catalyzed by 4PL to give chiral 1,3-dihydroisobenzofuran **12a** in 74% yield and 78:22 e.r. (Figure 3B).

While substrates **1**-**12** involve achiral alcohol nucleophiles, secondary alcohols introduce the possibility of using FDHs to control the diastereoselectivity of bromoetherification involving chiral nucleophiles.[19] We envisioned that such alcohols could be accessed via

reduction of the corresponding ketones using a ketoreductase (KRED).[34] These enzymes are NAD-dependent and require reduced cofactor supplied by a cofactor regeneration system like the glucose/glucose dehydrogenase (GDH) system that we use to drive FAD reduction or by oxidation of a sacrificial alcohol like isopropanol (IPA). We therefore anticipated that KREDs could be used in a one-pot cascade reaction with FDHs to enable sequential ketone reduction/bromoetherification with the respective enzymes controlling the enantioselectivity of the former and the diastereoselectivity of the latter. Indeed, we found that simply adding different KREDs to our standard reaction conditions with a 10-fold excess of NAD relative to our usual loading and with IPA in place of DMSO as a co-solvent led to high yields of bromoetherification catalyzed by 4PL starting from ketone **13** (Figure 3C). Depending on the KRED used, modest selectivity for either diastereomer could be obtained, and high enantioselectivity for the major diastereomer was observed.

The FDH variants with haloetherification activity described in this study were identified from an initial screen of 50 WT and engineered FDHs on substrate **1** (Figure S2) followed by secondary screens of a set of these enzymes on individual substrates (Figures S4-S11). Four engineered FDHs (4PL-F111S, 2RFQ-F111S, 4PL, and 3LR) were sufficient to provide the haloetherification yields and selectivities outlined above (Figure S12). The first three of these enzymes contain the F111S/L and N470S mutations that imparted halolactonization activity to RebH variants and were predicted by docking simulations to favor the formation of the *pro*-R form of the halolactonization product in previously reported docking simulations.[12] Observation of the (R)-**7a** by X-ray crystallography (Figure S13) is consistent with these simulations and suggests that alcohol substrates that undergo 5-exo-trig cyclization could bind analogously to the corresponding carboxylic acid substrates. Variant 3-LR doesn't possess any of these mutations, but it was only used in the 5-endo-trig cyclization of **11**, which provided a low yield of **11a** relative to formation of **10a** by 2RFQ F111S (Figure 3A). This finding, and the fact that FDHs exhibited very different selectivity toward substrates **10** and **11** (Figures S10/S11), suggests that the methyl substituent of **11** hinders 5-endo-trig cyclization but that mutations in 3-LR somehow facilitate this reaction with modest enantioselectivity. Further analysis of FDH activity and selectivity toward different halocyclization reactions will be required to understand the subtleties of this non-native reactivity.

Conclusion

This study establishes that FDH-catalyzed halocyclization can be extended to substrates bearing alcohol nucleophiles in addition to the previously reported carboxylate nucleophiles. It also shows that these enzymes can tolerate different styrene substitution patterns to enable the synthesis of di-, tri-, and tetra-substituted tetrahydrofurans. Good yields and enantioselectivity are obtained as long as styrenes with electron donating aromatic groups are used, and promising diastereoselectivity was obtained for a trisubstituted olefin. Related efforts to catalyze bromoetherification of 1,1-disubstituted alkenes via 5-*exo*-trig cyclization using chiral Lewis bases revealed that geminal dimethyl substituents on the pendant alcohol were required to achieve even modest enantioselectivity.[19] Olefin-to-olefin bromiranium ion exchange was proposed to degrade the selectivity of these reactions, but such exchange would not be possible within the FDH active site, perhaps explaining

the high selectivities for simple styrenes in the current study. This feature should aid efforts to engineer FDHs with improved haloetherification selectivity on tri-substituted olefins, substrates that undergo 5-endo-trig cyclization, and additional chloroetherification reactions, all which give modest selectivity using existing FDHs. Extending the scope of FDH-catalyzed halocyclization to substrates lacking electron donating groups will likely require more significant sculpting of the active site to accommodate the nucleophile-assisted alkene activation pathway proposed for such substrates.[14] We also characterized novel rearrangements of the initial bromoetherification products that give rise to hydroxylated and brominated ketones, providing initial hints at how enzymatic bromination could be used to further elaborate compounds in a chemoenzymatic sense. Likewise, we showed that KREDs could be used to simplify access to the alcohol nucleophiles required for bromoetherification involving secondary alcohols, showing how these enzymes can be readily integrated with FDHs in cascade processes. Together, these results highlight the utility of FDHs and (chemo)enzymatic sequences and cascades for olefin functionalization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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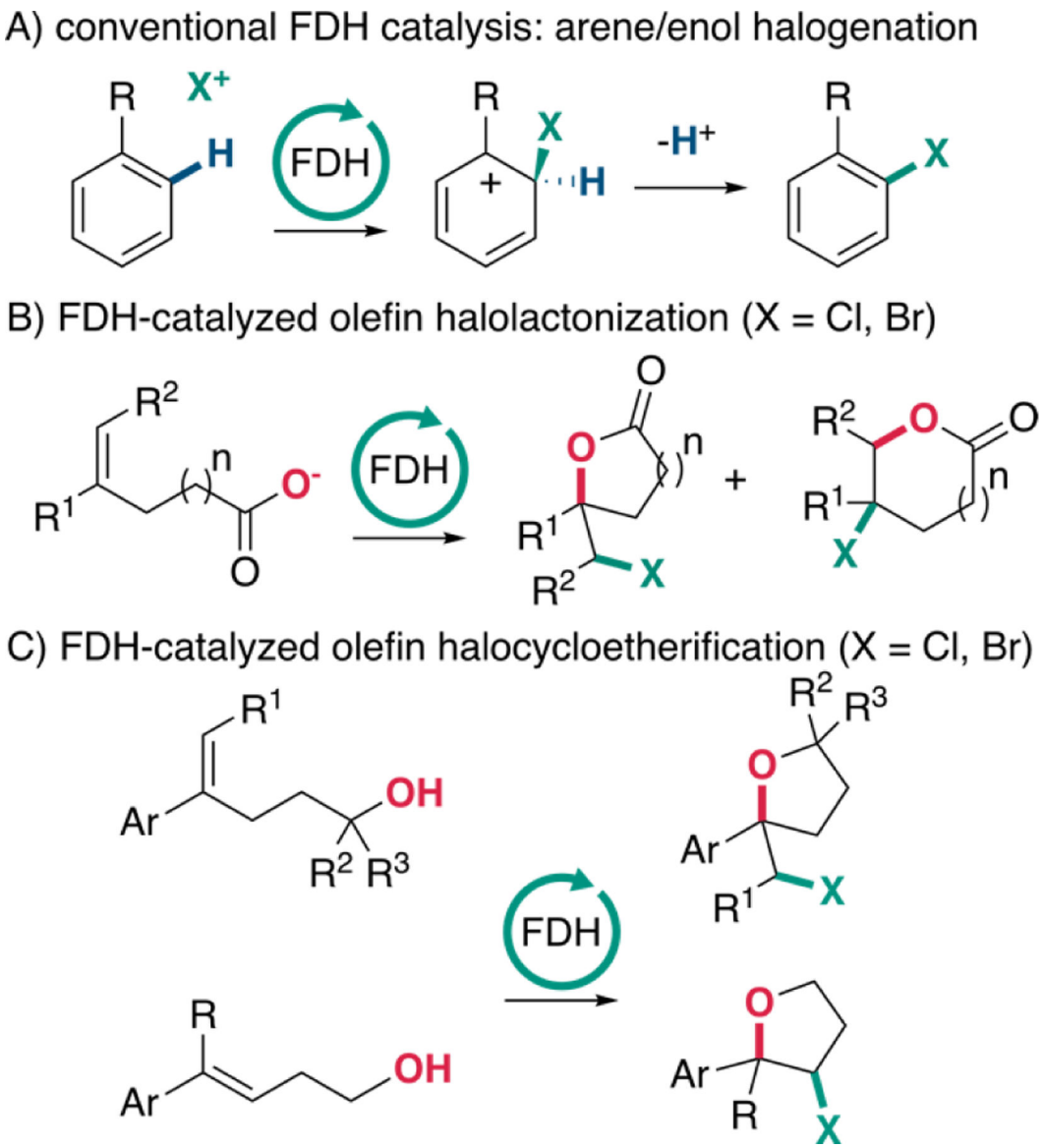


Figure 1.

A) Conventional FDH catalysis: arene/enol halogenation. B) Previously reported FDH-catalyzed halolactonization with X = Cl, Br.[12] C) FDH-catalyzed halocycloetherification with X = Cl, Br.

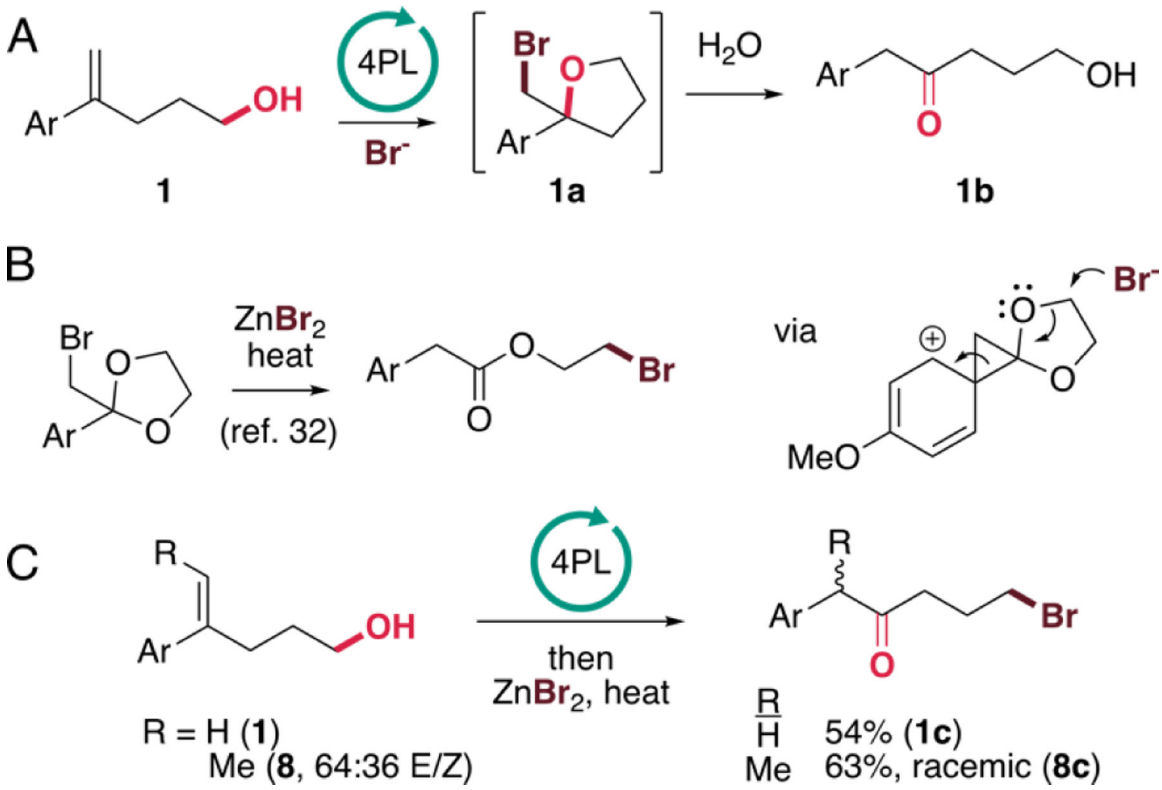


Figure 2.

A) Chemo-enzymatic bromoetherification/rearrangement/hydroxylation of **1a** using biocatalysis conditions shown in Table 1. B) Reported Zn(II)-catalyzed rearrangement of cyclic acetals.[32] C) Chemoenzymatic bromoetherification/rearrangement/bromination of **1** and **8** (64:36 E/Z).

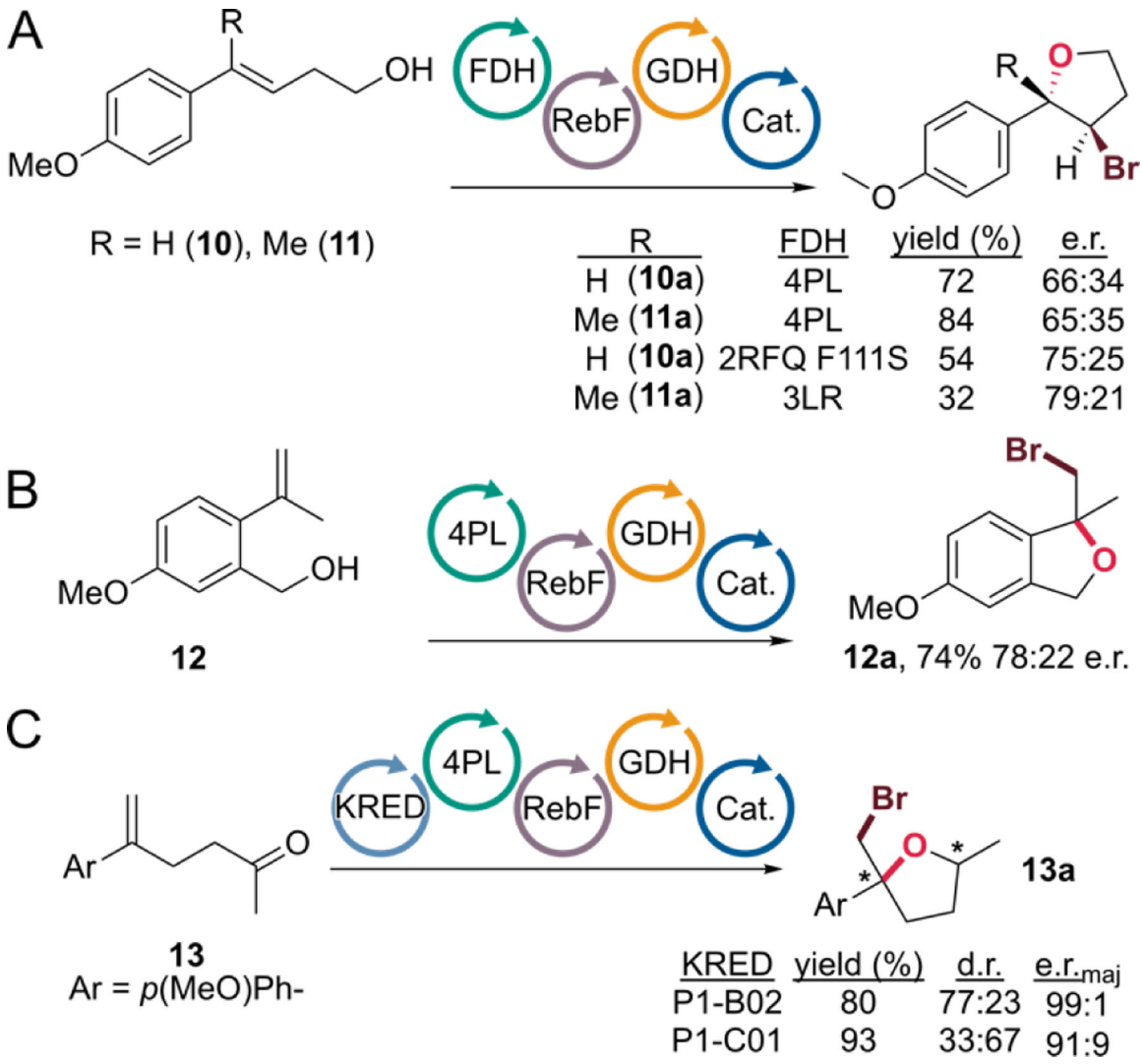


Figure 3.

FDH-catalyzed bromoetherification A) via 5-*endo*-trig cyclization, B) involving a pendant benzyl alcohol nucleophile, and C) starting from ketones using a ketoreductase/FDH cascade. $e.r._{maj}$ indicates the e.r. of the major diastereomer of **13a**. See supporting information for reaction details.

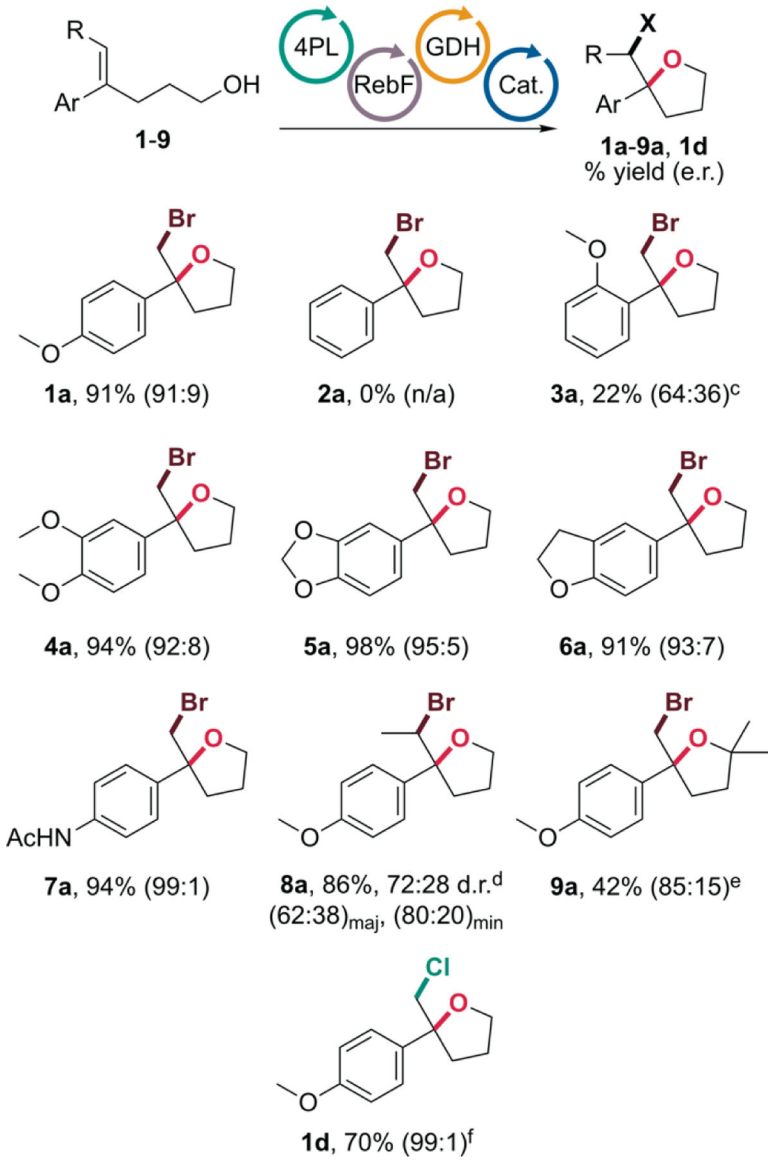


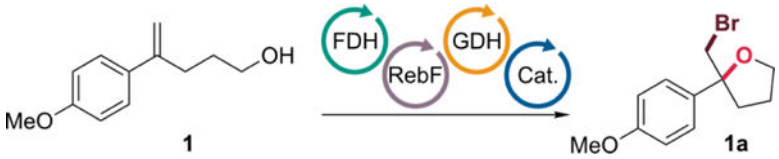
Chart 1.

Representative scope of FDH-catalyzed haloetherification of 1,1-disubstituted alkenes[a,b]

[a]Reactions conducted at room temperature for times specified in the supporting information using 1 mM substrate, 5 mM NaBr, 5 mol% FDH, 100 μM NAD and FAD, 20 mM glucose, 1 mM glutathione, 2.5 μM RebF, 9 U/mL GDH, and 35 U/mL catalase in 200 mM tricine, pH 7.5. [b]Yields determined by LC/MS relative to internal standard; selectivities determined by chiral HPLC. [c]Variant 4PL-F111S was used in place of 4PL. [d]A 64:36 mixture of E/Z-**8** was used.[e] Variant 2RFQ-F111S was used in place of 4PL. [f]5 mM NaCl used in place of NaBr.

Table 1.

Yields and enantioselectivities for bromoetherification of **1** using representative FDHs[a]



Entry	FDH	Time (h)	Yield (%)[a]	e.r.[b]
1	4V+S	18	66	79:21
2	6TL T52V	18	48	36:64
3	4PL	18	68	91:9
4	4PL	2	88	91:9

[a]Reactions conducted using 1 mM **1**, 5 mM NaBr, 5 mol% FDH, 100 μM NAD and FAD, 20 mM glucose, 1 mM glutathione, 2.5 μM flavin reductase (RebF),[31] 9 U/mL glucose dehydrogenase (GDH), and 35 U/mL catalase (Cat.) in 200 mM tricine, pH 7.5.

[b]Yields determined by LC/MS relative to internal standard; selectivities determined by chiral HPLC.