Upgrading Nature's Tools: Expression Enhancement and Preparative Utility of the Halogenase RebH

James T. Payne, Jared C. Lewis*

Department of Chemistry, University of Chicago, Chicago, IL 60637, USA
Fax +1(773)7020805; E-mail: jaredlewis@uchicago.edu

Received: 07.12.2013; Accepted after revision: 25.02.2014

Abstract: Enzymes often excel at performing green, selective chemical reactions, and thus offer key advantages over many chemocatalytic processes. We recently reported the enhanced expression of a regioselective halogenase, RebH, in *E. coli*, as well as its use in the preparative halogenation of a range of substrates. These improvements have made it possible to begin directed evolution to improve further the activity and substrate scope of RebH. Chemistry is in need of a regioselective halogenation catalyst, and this work illustrates the potential and recent gains made towards using enzymes for this goal.

Key words: halogenation, catalysis, enzymes, directed evolution, protein engineering

James Payne (left) received his BS in chemistry in 2010 from the University of Illinois at Urbana-Champaign, from which he also received a BA in astronomy in the same year and worked under the mentorship of Prof. You-Hua Chu. He is currently pursuing a PhD at the University of Chicago under the guidance of Prof. Lewis.
Jared Lewis (right) received his BS in chemistry in 2002 from the University of Illinois at Urbana-Champaign, where he worked under the mentorship of Prof. Eric Oldfield. He then earned his PhD in 2007 from the University of California, Berkeley under the joint guidance of Prof. Robert Bergman and Prof. John Ellman. From 2007 to 2010 he was a postdoctoral fellow at the California Institute of Technology in the lab of Prof. Frances Arnold. In 2010, Prof. Lewis joined the faculty at the University of Chicago. His research focuses on the catalytic functionalization of C–H bonds that are difficult to functionalize with current methods, and his lab takes three main approaches toward this goal: organometallic chemistry, the directed evolution of natural enzymes, and the development of artificial metalloenzymes.

While only 200 halogenated natural products were known in 1973, today over 4000 have been discovered.[1] Along with this increased awareness of halogenation in the natural world, there has been an increased application of halogenation in research and industry. Today, halogenation is a ubiquitous and vital process in the development of biologically active compounds; an estimated one quarter of all pharmaceuticals and agrochemicals are halogenated.[2] In addition, halogenated intermediates are common in pharmaceutical synthesis, especially as substrates for cross-coupling reactions,[3] of which examples abound in both drug discovery[4,5] and industrial-scale drug production.[6] Aside from synthetic utility, halogenation can also lead to drastic changes in the biological activity of compounds. This point is illustrated by the effects of chlorination or bromination in drugs as diverse as antibiotics,[7] apoptosis inhibitors,[8] and psychoactive compounds[9] (Figure 1). Halogenation has also been demonstrated to have a profound impact on the metabolic properties of drug compounds[10] and, as such, is widely employed during lead optimization and drug design to improve pharmacokinetic profiles. In a particularly notable example, the microsomal clearance of over 220,000 compounds was examined, and it was observed that chlorination at one position could increase the metabolic half-life of a drug, while chlorination at different positions would *decrease* the metabolic half-life.[10]

Despite the utility of halogenated compounds, current methods to install halogen atoms leave much to be desired, since they often require prefunctionalized or activated starting materials, or lengthy, wasteful, multistep functional group conversion sequences.[11] For example, aromatic halogenation is commonly accomplished by electrophilic aromatic substitution (EAS), which, in addition to requiring harsh, environmentally unfriendly reagents and reaction conditions, cannot selectively halogenate electronically disfavored positions, and can lead to product mixtures.[12] This often renders EAS unsuitable for the derivitization of drug leads. Whereas a number of oxidative methods have been developed to mitigate the environmental impact of EAS, none of these approaches offer a solution to the lack of site-selectivity.[13] To this day, exploration of halogenated derivatives of drug leads often relies on time-consuming and expensive syntheses from available halogenated starting materials, in many cases necessitating a unique synthetic path to explore each differently halogenated analogue.[9]

Not surprisingly, given the importance and paucity of existing options for selective halogenation, several groups have explored potential methods for this transformation. In 2010, the Hartwig group published a method for site-

SYNLETT 2014, 25, 1345–1349
Advanced online publication: 03.04.2014
DOI: 10.1055/s-0033-1341024; Art ID: ST-2013-P1118-SP

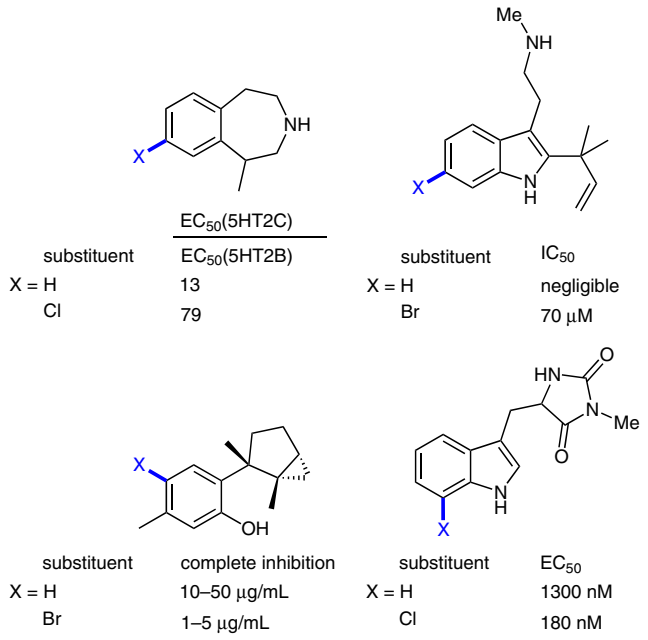
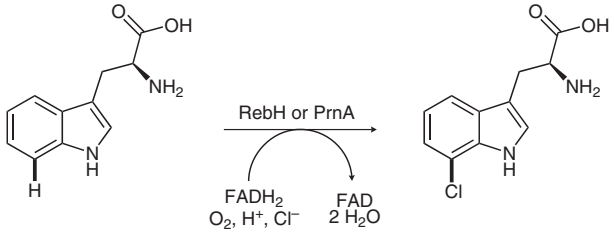


Figure 1 Examples of aromatic halogenation improving biological activity. From top-left, clockwise: lorcaserin (antiobesity drug), chlorination increases specificity for $5HT_{2C}$ receptor;[9] desformylflustrabromine, bromination effects inhibition of bacterial biofilm growth;[7a] necrostatin, chlorination increases inhibition of cellular necrosis;[8] laurinterol, bromination increases inhibition of bacterial growth.[7b]

selective borylation of several nitrogen-containing heterocycles, primarily indoles.[14] This borylation process provides a functional handle for allylation, cross-coupling, and also chlorination and bromination. Halogenation, however, requires a three-step sequence of ruthenium-catalyzed silyation of the indole nitrogen, iridium-catalyzed borylation of the indole 7-position, and copper-catalyzed halogenation. Although the 7-chlorination of indole was accomplished in this manner, this method illustrates the difficulty of traditional chemocatalytic, site-selective halogenation. Five steps and three metals are required, and the indole nitrogen is used as a directing group – indoles functionalized at the 1-position would not be compatible with this method. Furthermore, only the 7-position is functionalized by this method, and modifying this procedure to allow 6-, 5-, or 4-borylation would prove difficult or impossible. From this, we see that an optimal halogenation catalyst is not only site-selective, but also easily modifiable in its site-selectivity. In addition, an optimal catalyst is also efficient, scalable, environmentally benign, and tolerant of a wide range of substrates (and furthermore, modifiable in its substrate scope). These characteristics are hard to find together in a catalyst for any reaction, let alone halogenation.

Thankfully, there already exist catalysts that fulfill many of these criteria. Natural enzymes capable of oxidative halogenation have been discovered;[15] among these, a subclass of enzymes called FAD-dependent halogenases (FDHs) are able to perform *regioselective* arene halogenations.[16] Their regioselectivity arises from the fact that the active halogenating intermediate, a haloamine adduct formed with an active site lysine residue, is proximal only to a single C–H bond on the bound substrate.[17] These FDHs utilize sodium chloride instead of chlorine gas as a chlorine source, operate in water at room temperature rather than in chlorinated solvents at high temperatures and pressures, and use no heavy metals, which is especially attractive in the synthesis of pharmaceuticals, for which the removal of residual metals can be expensive and reduce yield.[18] Because these FDHs are genetically encoded catalysts, they can be altered through the process of directed evolution.[19] In this process, mutations are introduced into the genetic code, the resultant mutant enzymes are screened for improved properties, and those that are improved are used as the starting point for another round of mutation and selection. This process may be repeated as many times as necessary to obtain a mutant with sufficiently improved properties. The enzymes themselves are inherently green and regioselective, while the process of directed evolution offers the facile modifiability of the substrate scope, regioselectivity, and other properties that are necessary components of an ideal catalyst.

Particularly notable are several tryptophan halogenases, which are capable of selectively halogenating the 5-, 6-, or 7-position on the indole ring; of these, the tryptophan 7-halogenases PrnA[20] and RebH[21] are the most studied (Scheme 1). This natural tryptophan 7-halogenation has been exploited for the derivatization of drug molecules by the Goss group, who integrated PrnA into the biosynthetic pathway for pacidamycin, a uridyl peptide antibiotic.[22] The resultant chloropacidamycin was further derivatized by using cross-coupling chemistry to produce a range of arylated pacidamycin analogues. However, the utility of this method is limited to derivatizing products that integrate L-tryptophan into their synthetic pathways, and the ability to use halogenases on other substrates would substantially increase the number of accessible compounds. The substrate scope of wild-type PrnA has been explored, and it was found to halogenate a range of substituted indoles, including N-Ω-methyltryptamine, 5-methyltryptamine, and 3- and 5-methylindole.[23] With the exception of the native substrate L-tryptophan, however, the other indole substrates were all halogenated at the most electronically activated 2-position. In addition, several other substrates tested were not halogenated, including gramine, 1-methyltryptamine, and indole itself. Whereas tryptophan itself is not the most interesting target for ha-



Scheme 1 General reaction scheme for 7-chlorination of L-tryptophan catalyzed by PrnA or RebH

logenation, the indole moiety is highly prevalent in pharmaceuticals, and regioselective halogenation of one of these tryptamines at an electronically deactivated position would have been a promising start for evolution towards drug scaffolds. The first piece of evidence that RebH might be a useful alternative for regioselectivity came from the O'Connor group. This group had previously integrated RebH into the alkaloid-producing plant Madagascar periwinkle in order to produce halogenated alkaloids, but they found a buildup of 7-chlorotryptophan, because tryptophan decarboxylase is a bottleneck for alkaloid production.[24] To avoid this bottleneck, the authors sought to halogenate tryptamine directly, rather than exploit tryptophan halogenation followed by subsequent decarboxylation.[25] Several active site residues were mutated to a range of different amino acids until a mutant was found that preferentially halogenated tryptamine over tryptophan. Characterization of the halogenated product showed that tryptamine was selectively halogenated at the 7-position, offering the first evidence of selective halogenation of an unnatural substrate at an electronically disfavored position.

We then sought to explore the substrate scope of RebH even further, both to reexamine the range of accepted substrates that were halogenated only at the 2-position by PrnA as well as those not accepted by PrnA, and to explore new scaffolds in which we are interested. Because of the wide range of substrates we wished to test, and the preparative scale on which we wished to perform the reactions, it was first necessary to improve the expression of RebH in *E. coli*. We focused on *E. coli* as a host because it is easily manipulated with high transformation efficiencies, which greatly facilitates directed evolution. Through coexpression of RebH with the GroEL and GroES chaperone proteins,[26] more than 100 mg of soluble enzyme were isolated per liter of culture, which is more than a sevenfold improvement over the reported conditions.[27] In addition, RebF, the flavin reductase partner to RebH, was conjugated to the maltose binding protein (MBP) solubility tag.[28] This MBP-RebF conjugate afforded more than eleven-fold greater yields than those reported for RebF, and fortuitously retained full reductase activity without cleavage of the MBP moiety. Using large quantities of both enzymes obtained in this manner, along with a commercially available glucose dehydrogenase to complete the cofactor regeneration system,[29] we began to explore the substrate scope of wild-type RebH. In addition to the halogenation of L-tryptophan and tryptamine, both reported by O'Connor, we found that RebH halogenated 5-methyltryptamine and *N*-Ω-methyltryptamine as reported for PrnA. Furthermore, RebH also halogenated gramine, 1-methyltryptamine, and indole, all of which PrnA was reported to not accept, as well as D-tryptophan, tryptophol, 2-methyltryptamine, the tricyclic alkaloid tryptoline, and several naphthalenes, none of which had been tested as halogenation substrates for either RebH or PrnA. Most significantly, we performed 10 mg scale bioconversions of eleven of these substrates, isolated and characterized the products

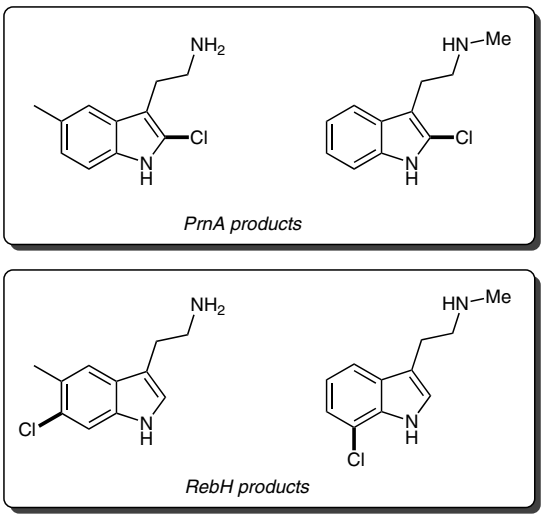


Figure 2 Products of aromatic chlorination catalyzed by PrnA or RebH. PrnA-catalyzed chlorination occurs at the electronically most activated 2-position[23] for both substrates, whereas RebH-catalyzed halogenation occurs selectively at the 6-position for 5-methyltryptamine[27] and the 7-position for *N*-Ω-methyltryptamine (unpublished results); both sites are electronically disfavored relative to the 2-position.

using conventional chemical techniques (Figure 2), and found that, unlike for PrnA, many of these substrates were selectively halogenated at electronically disfavored positions (Figure 3). In many of these cases, such as tryptamine, tryptophol, and 2-methyltryptamine, only the product of 7-halogenation was observed, as seen for the native substrate, L-tryptophan. For 5-methyltryptamine, however, only 6-halogenation was observed, whereas a mix of 5- and 6-chlorinated products of tryptoline were isolated. We think that these alternative selectivities will offer useful synthetic starting points and provide insights into ways to alter the selectivity of RebH through directed evolution.

Prior to our work on the expression enhancement of RebH in *E. coli*, studies on protein engineering, and especially directed evolution, of halogenases were greatly hindered by low yields of soluble enzyme. To alter the behavior of RebH with unnatural substrates, the O'Connor group introduced a mutation to cause RebH to preferentially halogenate tryptamine, as described above. To change the regioselectivity of PrnA, the van Pée group changed a residue to one present in PyrH, a tryptophan 5-halogenase, and observed a mix of 7- and 5-halogenation in an approximately 2:1 ratio.[30] Despite the fact that FDHs had been known for over a decade, these two studies represented the limit of protein engineering to alter substrate scope or regioselectivity of FDHs, and no directed evolution had been reported. Following our expression enhancement, we were able to obtain sufficient quantities of soluble RebH to perform the preparative-scale bioconversions described above, as well as a 100 mg bioconversion of L-tryptophan by using the crude lysate from *E. coli* expressing RebH to demonstrate the preparative utility of the en-

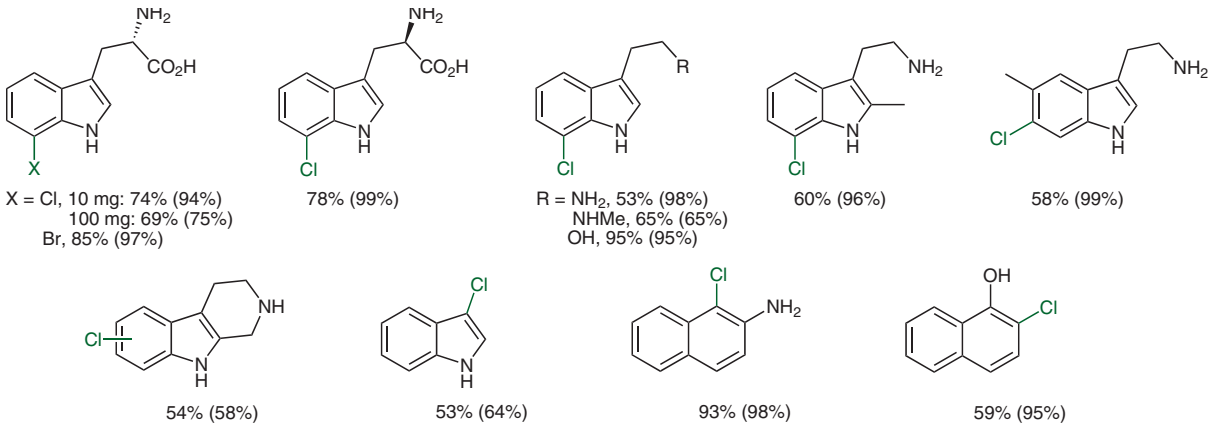


Figure 3 Products isolated from preparative-scale RebH-catalyzed aromatic halogenation reactions, with isolated yields shown (HPLC conversions shown in parentheses)[27]

zyme without purification. Another effect of this expression enhancement was that reasonable levels of conversion of L-tryptophan could be observed when the lysate from only 1 mL of culture was used. During directed evolution, 96-well plates, in which each well contained 1 mL of culture of a different mutant, were used for high-throughput protein expression and screening. Our expression enhancement thus allowed us to begin the directed evolution of RebH.

It has been shown that the thermostability of an enzyme tends to promote its evolvability;[31] random mutations introduced into an enzyme tend to be destabilizing, so the more stable the enzyme is initially, the higher the mutational load it can tolerate. As only single mutations had been introduced to RebH in the past, we were unsure of the mutational load that RebH would tolerate and therefore the extent to which mutations could be introduced to improve various catalytic parameters of this enzyme. Our group thus set out to improve the stability of RebH. By using error-prone PCR, libraries of RebH mutants were generated, which were expressed in 96-well culture plates and screened (by high-throughput HPLC) for improved activity after heat pre-treatment. By using this method, a mutant was found after three rounds of evolution with a melting temperature (measured by circular dichroism) nearly 18 °C higher than wild-type RebH[32] (Figure 4). It was further found that several thermostabilized mutants gave higher conversions on several unnatural substrates at elevated temperatures, in several cases giving higher conversions than wild-type RebH does at its optimal temperature, apparently owing to the longer life of these mutants. The thermostabilized mutants developed through this process offer a potentially improved starting point for evolving other properties of RebH.

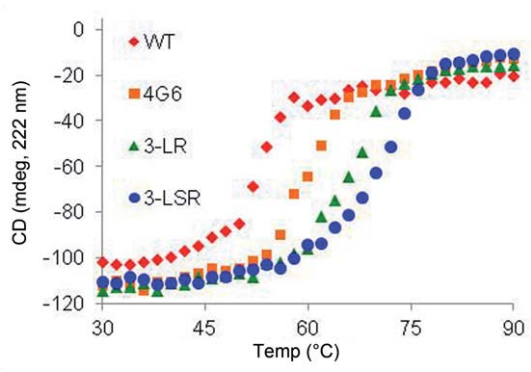


Figure 4 Thermal denaturation curves (measured by CD) of wild-type RebH and three thermostabilized mutants, showing an 18 °C increase in T_m. Mutant 4G6 has the following amino acid mutations: S2P, M71V, K145M, E423D, and E461G. 3-LR has the 4G6 mutations, as well as S130L and Q494R mutations. 3-LSR has all the aforementioned mutations, plus N166S (from ref. 32).

Halogenases offer an environmentally benign, site-selective alternative to conventional chemocatalytic techniques, which suffer from a lack of site-selectivity and require harsh reaction conditions. Natural halogenases are hindered in their application in the laboratory by their low activity and narrow substrate scope, and attempts to make halogenases more useful through directed evolution have, in turn, been hindered by the low expression of soluble enzyme in *E. coli*. By using chaperone proteins, our group has successfully overcome the low expression of soluble RebH, a tryptophan 7-halogenase, and demonstrated that RebH is capable of site-selective halogenation of a remarkably broad range of substrates. Our group has also accomplished the first reported directed evolution of RebH, improving its thermostability as a starting point for subsequent evolution. Future efforts will focus on the generation of mutants to accomplish site-selective halogenations of complex drug targets to improve the biological activities of these compounds, such as those described in the introduction. Evolved halogenases will hopefully soon enable a biological exploration of many potentially interesting drug targets that would otherwise have been overlooked due to the difficulty of their syntheses.

Acknowledgment

We acknowledge financial support from the University of Chicago, the NIH through a Pathways to Independence Award (5R00GM087551), and the Searle Scholars Program (11-SSP-202).

J.T.P. acknowledges fellowship support from an NIH Chemistry and Biology Interface training grant (T32 GM008720).

References and Notes

(1) (a) Gribble, G. W. *J. Chem. Educ.* **2004**, *81*, 1441. (b) Gribble, G. W. *Chemosphere* **2003**, *52*, 289.
(2) Herrera-Rodriguez, L. N.; Khan, F.; Robins, K. T.; Meyer, H.-P. *Chem. Today* **2011**, *29*, 31.
(3) Suzuki, A. *J. Organomet. Chem.* **1999**, *576*, 147.
(4) Wipf, P.; Lim, S. *J. Am. Chem. Soc.* **1995**, *117*, 558.
(5) Garg, N. K.; Caspi, D. D.; Stoltz, B. M. *J. Am. Chem. Soc.* **2004**, *126*, 9552.
(6) Anderson, B. A.; Becke, L. M.; Booher, R. N.; Flaugh, M. E.; Harn, N. K.; Kress, T. J.; Varie, D. L.; Wepsiec, J. P. *J. Org. Chem.* **1997**, *62*, 8634.
(7) (a) Bunders, C. A.; Minvielle, M. J.; Worthington, R. J.; Ortiz, M.; Cavanagh, J.; Melander, C. *J. Am. Chem. Soc.* **2011**, *133*, 20160. (b) Sims, J. J.; Donnell, M. S.; Leary, J. V.; Lacy, G. H. *Antimicrob. Agents Chemother.* **1975**, *7*, 320.
(8) Degterev, A.; Hitomi, J.; Germscheid, M.; Ch'en, I. L.; Korkina, O.; Teng, X.; Abbott, D.; Cuny, G. D.; Yuan, C.; Wagner, G.; Hedrick, S. M.; Gerber, S. A.; Lugovskoy, A.; Yuan, J. *Nat. Chem. Biol.* **2008**, *4*, 313.
(9) Smith, B. M.; Smith, J. M.; Tsai, J. H.; Schultz, J. A.; Gilson, C. A.; Estrada, S. A.; Chen, R. R.; Park, D. M.; Prieto, E. B.; Gallardo, C. S.; Sengupta, D.; Dosa, P. I.; Covel, J. A.; Ren, A.; Webb, R. R.; Beeley, N. R. A.; Martin, M.; Morgan, M.; Espitia, S.; Saldana, H. R.; Bjenning, C.; Whelan, K. T.; Grottick, A. J.; Menzaghi, F.; Thomsen, W. J. *J. Med. Chem.* **2008**, *51*, 305.
(10) Sun, H.; Keefer, C. E.; Scott, D. O. *Drug Metab. Lett.* **2011**, *5*, 232.
(11) Sasson, Y. *Formation of Carbon-Halogen Bonds (Cl, Br, I)*, In *Patai's Chemistry of Functional Groups*; Wiley–VCH: Weinheim, **2009**.
(12) Smith, K.; El-Hiti, G. A. *Curr. Org. Synth.* **2004**, *1*, 253–274.
(13) Podgoršek, A.; Zupan, M.; Iskra, J. *Angew. Chem. Int. Ed.* **2009**, *48*, 8424.
(14) Robbins, D. W.; Boebel, T. A.; Hartwig, J. F. *J. Am. Chem. Soc.* **2010**, *132*, 4068.
(15) (a) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; Garneau-Tsodikova, S.; Walsh, C. T. *Chem. Rev.* **2006**, *106*, 3364. (b) Butler, A.; Sandy, M. *Nature* **2009**, *460*, 848.
(16) van Pée, K.-H.; Patallo, E. P. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 631.
(17) Yeh, E.; Blasiak, L. C.; Koglin, A.; Drennan, C. L.; Walsh, C. T. *Biochemistry* **2007**, *46*, 1284.
(18) Woodley, J. *Trends Biotechnol.* **2008**, *26*, 321.
(19) For recent reviews, see: (a) Shivange, A.; Marienhagen, J.; Mundhada, H.; Schenk, A.; Schwaneberg, U. *Curr. Opin. Chem. Biol.* **2009**, *13*, 19. (b) Bommarius, A.; Blum, J.; Abrahamson, M. *Curr. Opin. Chem. Biol.* **2011**, *15*, 194. (c) Romero, P.; Arnold, F. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 866.
(20) Keller, S.; Wage, T.; Hohaus, K.; H?lzer, M.; Eichhorn, E.; van Pée, K.-H. *Angew. Chem. Int. Ed.* **2000**, *39*, 2300.
(21) Yeh, E.; Garneau, S.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3960.
(22) Roy, A. B.; Grüschow, S.; Cairns, N.; Goss, R. J. M. *J. Am. Chem. Soc.* **2010**, *132*, 12243.
(23) H?lzer, M.; Burd, W.; Reissig, H.-U.; van Pée, K.-H. *Adv. Synth. Catal.* **2001**, *343*, 591.
(24) Runguphan, W.; Qu, X.; O'Connor, S. E. *Nature* **2010**, *468*, 461.
(25) Glenn, W. S.; Nims, E.; O'Connor, S. E. *J. Am. Chem. Soc.* **2011**, *133*, 19346.
(26) Mayhew, M.; da Silva, A. C. R.; Martin, J.; Erdjument-Bromage, H.; Tempst, P.; Hartl, F. U. *Nature* **1996**, *379*, 420.
(27) Payne, J. T.; Andorfer, M. C.; Lewis, J. C. *Angew. Chem. Int. Ed.* **2013**, *52*, 5271.
(28) Cabrita, L.; Dai, W.; Bottomley, S. P. *BMC Biotechnol.* **2006**, *6*, 12.
(29) Wichmann, R.; Vasic-Racki, D. *Adv. Biochem. Engin./Biotechnol.* **2005**, *92*, 225.
(30) Lang, A.; Polnick, S.; Nicke, T.; William, P.; Patallo, E. P.; Naismith, J. H.; van Pée, K.-H. *Angew. Chem. Int. Ed.* **2011**, *50*, 2951.
(31) Bloom, J. D.; Labthavikul, S. T.; Otey, C. R.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 5869.
(32) Poor, C. B.; Andorfer, M. C.; Lewis, J. C. *Improving the Halogenase Activity and Stability of RebH via Directed Evolution*; manuscript submitted for publication.

