Biocatalysis

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Directed Evolution of RebH for Site-Selective Halogenation of Large Biologically Active Molecules**

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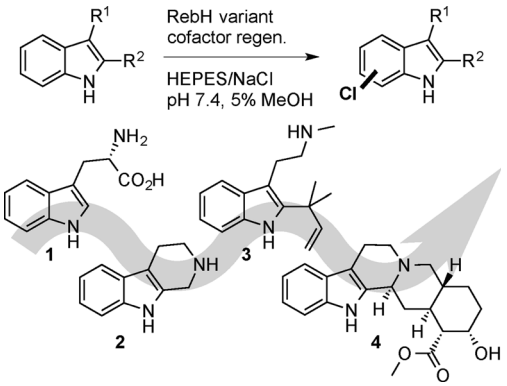
Abstract: *We recently characterized the substrate scope of wild-type RebH and proceeded to evolve variants of this enzyme with improved stability for biocatalysis. The substrate scopes of both RebH and the stabilized variants, however, are limited primarily to compounds similar in size to tryptophan. A substrate walking approach was used to further evolve RebH variants with expanded substrate scope. Two particularly notable variants were identified: 3-SS, which provides high conversion of tricyclic tryptoline derivatives; and 4-V, which accepts a broad range of large indoles and carbazoles. This constitutes the first reported use of directed evolution to enable the functionalization of substrates not accepted by wild-type RebH and demonstrates the utility of RebH variants for the site-selective halogenation of biologically active compounds.*

Halogenated aromatic compounds are essential building blocks in chemical synthesis[1] and are commonly found in pharmaceuticals[2] and agrochemicals;[3] an estimated one quarter of all such compounds are halogenated.[4] This is in part because halogenation can significantly affect molecular pharmacology, which is highlighted by the effects of chlorination or bromination on a diverse range of drugs spanning antibiotics,[5] anticancer agents,[6] and psychoactive compounds.[7] It has also been demonstrated that the presence of a halogen substituent can greatly alter drug metabolism. In one study of the microsomal clearance of over 220000 compounds, chlorination was found to predictably increase or decrease the metabolic half-life of a drug, depending on the location of the substitution.[8]

Despite the importance of halogen substituents, conventional arene halogenation methods, such as those proceeding through electrophilic aromatic substitution (EAS), often suffer from poor regioselectivity and require harsh reaction conditions.[9] It is therefore notable that flavin-dependent halogenase (FDH)-catalyzed arene halogenation, proposed to proceed through EAS,[10] provides high regioselectivity and uses air and halide salts as the terminal oxidant and halogen source, respectively. We recently reported that the tryptophan 7-halogenase RebH, an FDH from the rebeccamycin biosynthetic pathway,[11] can halogenate a range of indoles and naphthalenes on a preparative scale.[12] In contrast to previous reports for another FDH, PrnA,[13] RebH was found to halogenate many of these substrates at sites other than those most electronically activated, thus providing regioselectivity that could not be obtained from conventional EAS. The unnatural substrates halogenated by RebH in both our[12] and others'[14] work were, however, comparable in size to the native substrate, tryptophan (**1**, Scheme 1), and increasing



Scheme 1. General scheme for RebH-catalyzed halogenation and the substrates used for directed evolution through substrate walking. HEPES = 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid.

structural differences led to lower turnover numbers and less favorable kinetic parameters.[12] Given a recent report in which cross-linked RebH aggregates were used for gram-scale halogenation,[15] the narrow substrate scope and low activity of RebH with unnatural substrates stand as key limitations to its general utility for preparative halogenation.

Several groups have used RebH and other FDHs in metabolic engineering efforts involving tryptophan halogenation and the conversion of halogenated tryptophan into halogenated natural product derivatives.[16] A point mutant of RebH has been reported to preferentially halogenate tryptamine over tryptophan, and direct halogenation of this smaller alkaloid precursor has aided the aforementioned metabolic engineering efforts.[17] On the other hand, no examples in which FDHs are used for late-stage halogenation of complex biologically active compounds on a preparative scale have been reported, presumably owing to substrate scope limitations. We recently used directed evolution to engineer thermostable RebH variants that provide increased conversion of several unnatural substrates.[18] By using

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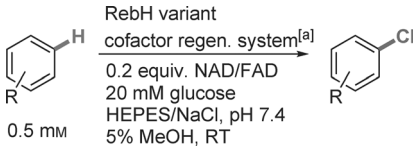
techniques developed for this effort, and starting from one of the stable variants identified, we sought to evolve the substrate scope of RebH through substrate walking (Scheme 1). This approach involves improving enzyme activity with a known substrate with structural homology to a target substrate, identifying variants that fortuitously possess increased activity with the target substrate, and repeating this process to gradually evolve the substrate scope of the enzyme.[19]

We found that RebH variant 1-PVM from our thermostability lineage provided increased conversion of numerous substrates, including tryptamine and tryptoline (**2**, Scheme 1, Table 1). Initially, we intended to further improve 1-PVM activity with **2**, since we hoped that variants with improved activity with this tricyclic molecule would be active with larger tryptophan-based natural products. Both RebH and 1-PVM, however, provided insufficient conversion of **2** for reliable UPLC analysis under the conditions used for high-throughput screening (crude lysate in 96-well plates). Because 1-PVM is both more stable and has nearly twofold higher activity with **2** than RebH (Table 1), it was used as the parent for our first round of evolution. We hoped that improving 1-PVM activity with **1**, which was similar to that of RebH and could be detected by UPLC, would provide variants with sufficient activity with **2** to enable further evolution using this substrate.

A library of 1-PVM variants, each containing an average of 1–2 amino acid mutations, was generated by error-prone PCR,[18] and 1080 colonies were picked and expressed in 96-well plates. The halogenation of **1** was assayed by UPLC, and any variants that provided increased conversion relative to 1-PVM, which was included in each plate, were submitted to a secondary screen for increased conversion of **2**. All hits were verified by gene sequencing and large-scale reactions conducted with purified enzyme. From this library, one variant possessing a single N467T mutation (and therefore called 2-T) gave a modest 1.2-fold increase in the conversion of **1** and, when submitted to the secondary screen, showed a 1.3-fold increase in the conversion of **2**. Although also modest, this improvement elevated the conversion of **2** to nearly 10% under the conditions used for screening. We found that at this level of conversion, we could reliably detect 1.5-fold increases in the conversion of **2** and we thus decided to screen directly against **2** in our next round of mutagenesis.

The second-round library was constructed as described above but with 2-T as the parent, and 1080 variants were again picked and expressed. Reactions were conducted with **2** as the substrate, and any variants that showed 1.5-fold or higher activity in lysate were grown and purified to confirm their activity. Several mutations giving activity above this threshold were found, and one of these, N470S, led to nearly 7-fold higher conversion of **2**. This mutation and a second mutation identified in this round of screening, G112S, were combined to give variant 3-SS, which provided 9-fold higher conversion of **2** relative to 2-T.

Preparative chlorination of **2** by using 1 mol% 3-SS afforded 93% conversion into 6-chlorotryptoline (Scheme 2,



Scheme 2. General Scheme for RebH-variant-catalyzed arene halogenation. [a] The cofactor regeneration system consisted of 0.5 mol% MBP-RebF and 50 U ml^{-1} glucose dehydrogenase. NAD = nicotinamide adenine dinucleotide, FAD = flavin adenine dinucleotide, MBP = maltose-binding protein.

Table 2). This represents a significant improvement over our previously reported chlorination of **2** with 10 mol% RebH, which gave a nearly 1:1 mixture of 6- and 7-chlorotryptoline at only 58% conversion;[12] only trace 7-chlorination was observed when using 3-SS. Many of the mutations found in 3-SS are in the enzyme active site (N467T, N470S, and G112S; see Figure S2 in the Supporting Information) and could affect the binding of **2** and thus the halogenation selectivity with this substrate.[21] Steady-state kinetic analysis of these variants was conducted to probe the effects of these mutations on the kinetic parameters (Table 3). We found that the catalytic efficiency scaled with the increased conversion observed for successive variants. While this effect appears to have been solely due to increased k_{cat} values for variants 1-PVM and 2-T, the final variant, 3-SS, gave greatly increased k_{cat} and greatly decreased K_m values, thus resulting in a catalytic efficiency nearly 70-fold greater than that of the wild-type enzyme.

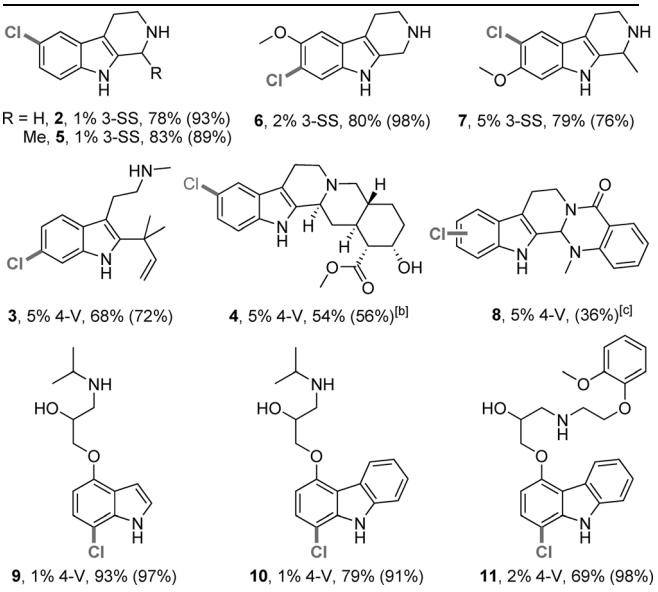
We further explored the substrate scope of 3-SS by examining its activity with a range of additional substrates. We found that 3-SS is particularly effective for halogenating

Table 1: Representative conversion (and enzyme loading) values for the substrates used in the substrate-walking-based mutagenesis of RebH.

Generation	Enzyme	Mutation[a]	Method (substrate)[b]	%Conv. 1 (%Loading)[c]	%Conv. 2 (%Loading)	%Conv. 3 (%Loading)	%Conv. 4 (%Loading)
0	RebH	–	–	53 (0.2)	3 (0.5)	<1 (5)	1 (5)
1	1-PVM	S2P/M71V/K145M	epPCR[d] (**1**)	43 (0.2)	5 (0.5)	<1 (5)	5 (5)
2	2-T	N467T	epPCR (**1**)	53 (0.2)	7 (0.5)	<1 (5)	9 (5)
3	3-SS	G112S/N470S	epPCR (**2**)	22 (0.2)	64 (0.5)	6 (5)	9 (5)
3	3-S	S112G	point mut.(**3**)[e]	43/5[f] (0.2)	50 (0.5)	29 (5)	39 (5)
4	4-V	A442V	epPCR (**3**)	39/3[f] (0.2)	43 (0.5)	48 (5)	38 (5)

[a] Mutations relative to the parent in the previous row. [b] Method used to introduce mutations (and substrate used in the screening effort. [c] Conversion of the substrates as determined by UPLC (and mol% loading values for the enzymes used). [d] Error-prone PCR. [e] Point mutation introduced through SOE PCR.[20] [f] The second number refers to conversion to the dihalogenated product.

Table 2: Yields for 10 mg RebH-variant-catalyzed halogenation reactions[a]



[a] Substrate numbers, mol% enzyme loading, yields of isolated product, (and conversion values determined by UPLC) are provided. [b] In addition to the major product of 10-chlorination shown, 11-chlorination was also observed to afford a minor product. [c] Multiple closely eluting products were observed and were not individually isolated, so only total conversion is shown.

Table 3: Kinetic data for the chlorination of tryptoline (**2**) by RebH variants.

Variant	Mol%	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [$min^{-1}\,\mu M^{-1}$]
RebH	5	0.013	144	9.05×10^{-5}
1-PVM	5	0.040	215	1.86×10^{-4}
2-T	3	0.056	236	2.37×10^{-4}
3-SS	0.5	0.537	8.6	6.24×10^{-3}

tricyclic indoles similar to **2** (Table 2). Eleagnine (**5**), an alkaloid from *Chrysophyllum albidum* with potent analgesic effects,[22] has the same structure as **2** but with an additional methyl group at the 1-position. We found this added steric bulk distal from the indole moiety interesting because we aimed to further expand the substrate scope of RebH to encompass compounds significantly larger in the region of this methyl group. Although RebH shows very low conversion of **5**, 3-SS showed high activity with this compound (over 60-fold higher than that observed with wild-type RebH; Table 4). With only 1 mol% enzyme loading of 3-SS, 89% conversion of **5** was seen on the 10 mg scale. We found that 3-SS also gave good conversion of two tryptoline derivatives with substituents on the benzene ring of the indole moiety: pinoline (6-methoxytryptoline; **6**), a metabolite with monoamine oxidase A inhibition activity,[23] which was chlorinated solely at the 7-position; and tetrahydroharmine (7-methoxyeleagnine; **7**), an alkaloid from *Banisteriopsis caapi* with antiviral and antifungal activity,[24] which was chlorinated solely at the 6-position. These two substrates show that these RebH variants are able to tolerate substituents at multiple positions on the indole moiety, which occur frequently in biologically active natural products.

Table 4: Ratio of activity improvement for the best variant versus wild-type RebH for each substrate in Table 2.

Substrate	Variant	Mol%	Activity Ratio[a]
Tryptoline (**2**)	3-SS	0.5	65.5
Eleagnine (**5**)	3-SS	0.5	67.1
Pinoline (**6**)	3-SS	0.5	2.0
Tetrahydroharmine (**7**)	3-SS	5	17.6
Desbromo-dFBr (**3**)	4-V	5	N/A[b]
Yohimbine (**4**)	4-V	5	38.0
Evodiamine (**8**)	4-V	5	16.5
Pindolol (**9**)	4-V	0.2	1.3
Carazolol (**10**)	4-V	0.2	4.9
Carvedilol (**11**)	4-V	0.5	8.2

[a] Activity ratio is the ratio of the conversion seen with the variant tested versus wild-type RebH. Reaction conditions were those shown in Scheme 2. [b] The wild-type enzyme showed no quantifiable activity and thus a ratio cannot be determined.

The goal of our substrate-walking effort was to expand the substrate scope of RebH to include biologically active compounds that are significantly larger than L-tryptophan or the tryptoline derivatives described above. One substrate in particular caught our interest: deformylflustrabromine (dFBr), a tryptamine metabolite first isolated from *Flustra foliacea*. dFBr is a potent inhibitor of bacterial biofilm formation, but only when it possesses the 6-bromo substituent on its indole moiety; no detectable biofilm inhibition is observed when this substituent is not present.[5] To the best of our knowledge, however, the biological activities of the 4-, 5-, and 7-bromo isomers and the chlorinated analogues have not been reported. The preparation of these compounds for subsequent biological evaluation seemed like a prime target for RebH variants given the substrate scope outlined above. We synthesized **3**,[25] the non-halogenated indole analogue of dFBr, and while RebH shows no quantifiable activity with this compound, 3-SS did provide low conversion. Notably, we found that variant 3-S, which lacks the G112S mutation but still possesses N470S, gave 5-fold higher conversion of **3** than did 3-SS.

Given that the conversion of **3** afforded by 3-S was sufficient to observe low activity in crude lysate in 96-well plates, we screened for improved activity with **3** in our third round of evolution, just as was done with **2** in our second round. After verifying the hits with purified enzyme, one variant was found with nearly 1.7-fold increased activity with **3**. This variant, 4-V, possesses a single A442V mutation that is quite far removed from the active site of the enzyme. It is well established that mutations distant from enzyme active sites can have profound effects on their activity,[26] and such mutations are especially difficult to find with structure-guided mutagenesis. We used 4-V for the preparative chlorination of **3** and found that this enzyme provides exclusively 6-chlorination, thereby providing a facile synthesis of the 6-chloro analogue of dFBr. The high activity of 4-V with **3**, despite the bulk of the inverse prenyl group, encouraged us to explore the substrate scope of this variant. We first looked at two pentacyclic compounds with well-studied biological activities:

yohimbine (**4**), an alkaloid from *Catharansus roseus* that is also an α_2 adrenergic receptor antagonist;[27] and evodiamine (**8**), an alkaloid from the *Tetradium* family of plants.[28] Both of these compounds are significantly larger than any others we had assayed to this point—**4** has over twice the molecular weight of **2**—but to our surprise, both showed significant conversion with 4-V (Table 2). While 4-V produces two distinct monochlorinated derivatives of **8**, as well as one dichlorinated derivative, only monochlorinated products were observed with **4**, and at a sufficient level of conversion for a preparative-scale halogenation. After performing the preparative-scale reaction, we observed that **4** is chlorinated to 56% conversion in a roughly 3:1 ratio at the 10- and 11-positions, respectively.

Given that we had demonstrated that RebH variants could accept greatly expanded bulk distal to the indole moiety of these unnatural substrates, as well as substitution at the 5- and 6-positions on the indole moiety, we decided to explore the impact of large substitutions at the 4-position of the indole ring. Pindolol (**9**), a nonselective beta blocker,[29] possesses a sizeable substituent with alcohol and amine functionalities linked via an ether linkage at the indole 4-position. We found that 4-V was able to fully convert this compound into the 7-chlorinated product, even at lower (<1%) enzyme loading. A similar compound, carazolol (**10**),[30] possesses a carbazole rather than an indole moiety, and we found that this compound was selectively halogenated at the analogous position. This was the first time we had observed the selective conversion of a carbazole in high yield by any RebH variant, and encouraged by this result, we decided to test carvedilol (**11**), a carbazole that is a nonselective beta blocker/alpha-1 blocker and has an even bulkier substituent at the 5-position (analogous to the indole 4-position).[31] Carvedilol has a molecular weight of over 400 g mol^{-1}, twice the size of the native tryptophan and even larger than **4**. Despite this added steric bulk, we found that 4-V was able to again give full conversion of **11** at 2% enzyme loading and with exquisite selectivity to give only a single chlorinated product. The confined nature of the RebH active site in crystal structures with bound tryptophan substrate[32] makes rationalizing the binding of the larger substrates explored in this work difficult, but it may be that some of these, particularly **11**, are not fully contained within the active site.

In summary, beginning from a thermostablized variant of RebH, 1-PVM, RebH variants with significantly expanded substrate scope were identified by using only three rounds of random mutagenesis and screening against progressively larger substrates. These variants catalyzed the selective halogenation of a number of unnatural substrates displaying a range of sizes, substitutions, and biological activities. Given the established impact that halogenation has on the biological activities of compounds and the dearth of methods to perform selective halogenations such as those we have described, we believe that these variants could prove useful for producing high-value derivatives of biologically active compounds. While the mutations discovered offer great insight for future targeted libraries,[33] the discovery of crucial mutations distant from the active site, such as A442V in variant 4-V, highlight the value of random mutagenesis in discovering important mutations that are difficult to predict.[26] We hope to gain insight into the role of this mutation and the others identified during our evolution efforts by using protein crystallography with bound substrates.

Keywords: biocatalysis · directed evolution · halogenase · RebH · substrate walking

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[1] *Metal-Catalyzed Cross-Coupling Reactions* (Eds.: F. Diederich, P. J. Stang), Wiley-VCH, New York, **1998**.
[2] a) M. Z. Hernandes, S. M. T. Cavalcanti, D. R. M. Moreira, W. F. de Azevedo Junior, A. C. L. Leite, *Curr. Drug Targets* **2010**, *11*, 303–314; b) K. Naumann, *Pest Manage. Sci.* **2000**, *56*, 3–21.
[3] P. Jeschke, *Pest Manage. Sci.* **2010**, *66*, 10–27.
[4] L. N. Herrera-Rodriguez, F. Khan, K. T. Robins, H.-P. Meyer, *Chem. Today* **2011**, *29*, 31.
[5] C. A. Bunders, M. J. Minvielle, R. J. Worthington, M. Ortiz, J. Cavanagh, C. Melander, *J. Am. Chem. Soc.* **2011**, *133*, 20160.
[6] P. G. Williams, G. O. Buchanan, R. H. Feling, C. A. Kauffman, P. R. Jensen, W. Fenical, *J. Org. Chem.* **2005**, *70*, 6196–6203.
[7] B. M. Smith, J. M. Smith, J. H. Tsai, J. A. Schultz, C. A. Gilson, S. A. Estrada, R. R. Chen, D. M. Park, E. B. Prieto, C. S. Gallardo, D. Sengupta, P. I. Dosa, J. A. Covel, A. Ren, R. R. Webb, N. R. A. Beeley, M. Martin, M. Morgan, S. Espitia, H. R. Saldana, C. Bjenning, K. T. Whelan, A. J. Grottick, F. Menzaghi, W. J. Thomsen, *J. Med. Chem.* **2008**, *51*, 305.
[8] H. Sun, C. E. Keefer, D. O. Scott, *Drug Metab. Lett.* **2011**, *5*, 232.
[9] K. Smith, G. A. El-Hiti, *Curr. Org. Synth.* **2004**, *1*, 253–274.
[10] C. Dong, S. Flecks, S. Unversucht, C. Haupt, K.-H. van Pée, J. H. Naismith, *Science* **2005**, *309*, 2216–2219.
[11] E. Yeh, S. Garneau, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3960–3965.
[12] J. T. Payne, M. C. Andorfer, J. C. Lewis, *Angew. Chem. Int. Ed.* **2013**, *52*, 5271–5274; *Angew. Chem.* **2013**, *125*, 5379–5382. Note: we previously referred to the 6- and 7-halogenation of tryptoline as 5- and 6-halogenation, by analogy to the numbering used for L-tryptophan, but here we use the established numbering for carbazole derivatives.
[13] M. Hölzer, W. Burd, H.-U. Reißig, K.-H. van Pée, *Adv. Synth. Catal.* **2001**, *343*, 591–595.
[14] M. Frese, P. H. Guzowska, H. Voß, N. Sewald, *ChemCatChem* **2014**, *6*, 1270–1276.
[15] M. Frese, N. Sewald, *Angew. Chem. Int. Ed.* **2014**, *54*, 298–301.
[16] a) A. B. Roy, S. Grüschow, N. Cairns, R. J. M. Goss, *J. Am. Chem. Soc.* **2010**, *132*, 12243; b) W. Runguphan, X. Qu, S. E. O'Connor, *Nature* **2010**, *468*, 461; c) C. Sánchez, L. Zhu, A. F. Braña, A. P. Salas, J. Rohr, C. Méndez, J. A. Salas, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 461–466.
[17] W. S. Glenn, E. Nims, S. E. O'Connor, *J. Am. Chem. Soc.* **2011**, *133*, 19346–19349.
[18] C. B. Poor, M. C. Andorfer, J. C. Lewis, *ChemBioChem* **2014**, *15*, 1286–1289.
[19] a) R. Fasan, Y. T. Meharenna, C. D. Snow, T. L. Poulos, F. H. Arnold, *J. Mol. Biol.* **2008**, *383*, 1069–1080; b) C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman, G. J. Hughes, *Science* **2010**, *329*, 305–309.
[20] K. L. Heckman, L. R. Pease, *Nat. Protoc.* **2007**, *2*, 924–932.
[21] A. Lang, S. Polnick, T. Nicke, P. William, E. P. Patallo, J. H. Naismith, K.-H. van Pée, *Angew. Chem. Int. Ed.* **2011**, *50*, 2951–2953; *Angew. Chem.* **2011**, *123*, 3007–3010.
[22] T. O. Idowu, E. O. Iwalewa, M. A. Aderogba, B. A. Akinpelu, A. O. Ogundaini, *J. Biol. Sci.* **2006**, *6*, 1029–1034.

[23] R. Pähkla, J. Harro, L. Rägo, *Pharmacol. Res.* **1996**, *34*, 73 – 78.
[24] H. Song, Y. Liu, Y. Liu, L. Wang, Q. Wang, *J. Agric. Food Chem.* **2014**, *62*, 1010 – 1018.
[25] S. K. Adla, G. Golz, P. G. Jones, T. Lindel, *Synthesis* **2010**, 2161 – 2170.
[26] a) A. Shimotohno, S. Oue, T. Yano, S. Kuramitsu, H. Kagamiyama, *J. Biochem.* **2001**, *129*, 943 – 948; b) B. Spiller, A. Gershenson, F. H. Arnold, R. C. Stevens, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12305 – 12310; c) P. A. Romero, F. H. Arnold, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 866 – 876; d) R. Fasan, M. M. Chen, N. C. Crook, F. H. Arnold, *Angew. Chem. Int. Ed.* **2007**, *46*, 8414 – 8418; *Angew. Chem.* **2007**, *119*, 8566 – 8570.
[27] S. W. Tam, M. Worcel, M. Wyllie, *Pharmacol. Ther.* **2001**, *91*, 215 – 243.
[28] Y. Kobayashi, Y. Nakano, M. Kizaki, K. Hoshikuma, Y. Yokoo, T. Kamiya, *Planta Med.* **2001**, *67*, 628 – 633.
[29] W. H. Aellig, *Br. J. Clin. Pharmacol.* **1982**, *13*, 187S – 192S.
[30] R. B. Innis, F. M. A. Correa, S. H. Snyder, *Life Sci.* **1979**, *24*, 2255 – 2264.
[31] P. C. Stafylas, P. A. Sarafidis, *Vasc. Health Risk Manage.* **2008**, *4*, 23 – 30.
[32] E. Bitto, C. A. Bingman, S. Singh, G. N. Phillips, Jr., *Proteins* **2008**, *70*, 289 – 293.
[33] a) M. T. Reetz, M. Bocoa, J. D. Carballeira, D. Zha, A. Vogel, *Angew. Chem. Int. Ed.* **2005**, *44*, 4192 – 4196; *Angew. Chem.* **2005**, *117*, 4264 – 4268; b) J. C. Lewis, S. M. Mantovani, Y. Fu, C. D. Snow, R. S. Komor, C.-H. Wong, F. H. Arnold, *ChemBioChem* **2010**, *11*, 2502 – 2505.

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