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A Smart Library of Epoxide Hydrolase Variants and the Top Hits for Synthesis of (*S*)-β-Blocker Precursors**

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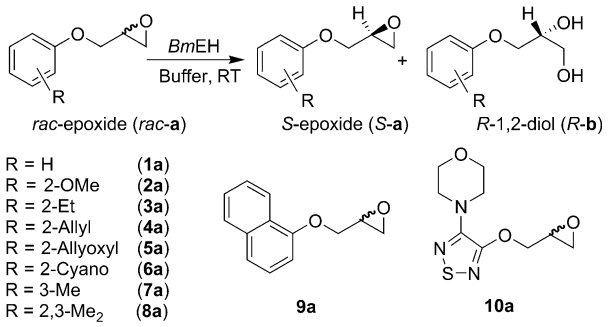
Abstract: *Microtuning of the enzyme active pocket has led to a smart library of epoxide hydrolase variants with an expanded substrate spectrum covering a series of typical β-blocker precursors. Improved activities of 6- to 430-fold were achieved by redesigning the active site at two predicted hot spots. This study represents a breakthrough in protein engineering of epoxide hydrolases and resulted in enhanced activity toward bulky substrates.*

Since the substrate specificity of enzymes limits their broad application as biocatalysts in industry, various efforts have been made toward expanding the substrate spectrum or even diversifying the reaction type of a single biocatalyst.[1] Among them, a minimalist active site redesign,[1a] based on the structural information of an enzyme, would greatly change its substrate specificity. In this study, starting from a robust epoxide hydrolase (EH) with high activity toward a simple model substrate, phenyl glycidyl ether (**1a**), we developed a small but smart library of EH variants with improved activity (by 6–430-fold) for nine typical β-blocker precursors (Scheme 1), by redesigning the active site of the enzyme for two predicted hot spots, namely Met145 and Phe128.

β-Adrenergic receptor blocking agents (β-blockers) are a group of popular drugs used widely for cardiovascular therapies.[2] β-Blockers are commercially available on the market as active pharmaceutical ingredients, primarily as racemates.[3] However, the each enantiomer of a β-blocker should be used individually because of their different pharmacokinetic and pharmacodynamic properties. The majority of β-blockers depend on their *S* enantiomer for binding with the β-adrenergic receptor, and in general the *S* enantiomers are 10–500-fold more potent than the *R* enantiomers.[4] Biocatalytic synthesis of single-enantiomer β-blockers or their precursors has been reported, and mainly involves using lipases or esterases as biocatalysts.[5] The enantioselective hydrolysis of racemic epoxides using cofactor-independent EHs is a promising approach for obtaining enantiopure epoxides or diols as key chiral precursors of β-blockers. However, EHs rarely possess sufficient activity for epoxides with bulky substituents,[6] so the industrial production of these compounds in enantiomerically pure form with EHs remains a big challenge.



Scheme 1. Typical epoxide substrates chosen to assay variants of the epoxide hydrolase *Bm*EH in hydrolysis.

In the last decades, interest in microbial EHs has arisen primarily because of their application in the synthesis of enantiopure epoxides/diols. Nevertheless, the use of EHs in the kinetic resolution of aryl glycidyl ethers for the synthesis of (*S*)-β-blockers, or other chiral drugs like Ranexa and Flivas, is still hindered by either the low activity or insufficient enantioselectivity of existing EHs. We have cloned a novel EH (*Bm*EH) from *Bacillus megaterium* ECU1001 with unusual *R* enantioselectivity and an activity of 83 U mg^{-1} protein toward the substrate phenyl glycidyl ether (**1a**).[7] However, for various substrates with a bulky substituent on the phenyl ring (Scheme 1), the activity of *Bm*EH was found to decrease significantly. For example, when *Bm*EH was employed for the resolution of Alprenolol and Propranolol precursors (**4a** and **9a**), the activity decreased to less than 5% when compared with that for **1a**. Therefore, to meet the need of chiral-β-blocker synthesis, it is imperative to alter the substrate specificity of *Bm*EH toward bulky epoxides by protein engineering.

We have recently determined the crystal structures of both *Bm*EH (PDB ID: 4NZZ) and its complex with a substrate analogue (PDB ID: 4O08), as shown in Figure 1.

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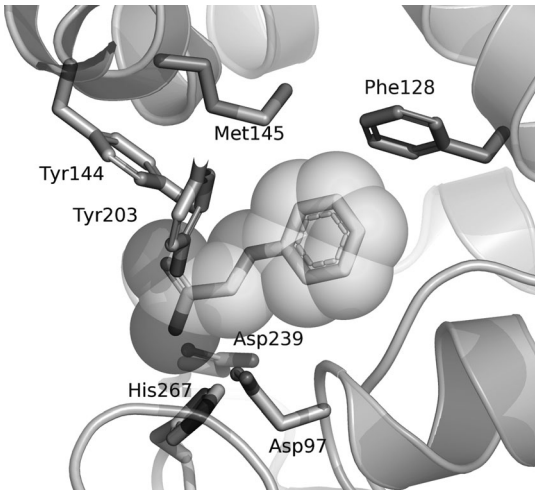

Figure 1. Locations of Met145 and Phe128 chosen for mutation around the active site of *Bm*EH. Molecule represented as space-filling model represents phenoxyacetamide (POA), an analogue of epoxide **1a**. PDB ID: 4O08.

It is worth noting that this EH, having an α/β-hydrolase fold, has a particularly deep and hydrophobic active cavity. By analyzing the active site of *Bm*EH, two residues (Met145 and Phe128) were identified as potential hot spots for enhancing the *Bm*EH activity toward the bulky substrates mentioned above. By referring to several successful examples for creating a smart library,[8] Met145 and Phe128 were mutated separately for each position using seven preselected amino acids (Ala, Cys, Ile, Leu, Ser, Thr, and Val),[9] to modify the active pocket with different levels of hindrance and hydrophobicity.

Aiming to screen for highly active *Bm*EH variants for each substrate, we first tested the activities of the lyophilized cell-free extracts (CFEs) of 15 EH variants from the library on 10 chosen epoxides using reverse-phase HPLC. As shown in Figure 2 (for more details, see also Table S1 in the Supporting Information), each of the 10 indicative substrates could find at least one variant (best hit) with much higher activity than the wild-type enzyme ($BmEH_{WT}$). The variants with mutations at Met145 showed the highest activity for the *ortho*-substituted **3a** and **4a**, whereas the Phe128 mutants were more active for the *meta*-substituted **7a** and **9a**. This outcome is consistent with the locations of these two residues relative to the phenyl ring of phenoxyacetamide (POA; Figure 1). The highest activity (47.9 $U\,mg^{-1}$ CFE) was observed for **3a** with the variant M145S, thus representing a greater than tenfold increase when compared with that of $BmEH_{WT}$ for **1a** (4.32 $U\,mg^{-1}$ CFE).

To characterize the real improvement in catalytic efficiency, $BmEH_{WT}$ and the respective best variant of each substrate were purified and assayed for their specific activities. In comparison with $BmEH_{WT}$, activities of the best hits toward **1a**–**10a** increased by 1.75- to greater than 400-fold (see Table S2), which is coincident with the results of crude enzymes (CFEs). Among all the variants, M145S for **3a** and M145A for **4a** showed the highest activities, of up to 1062 and

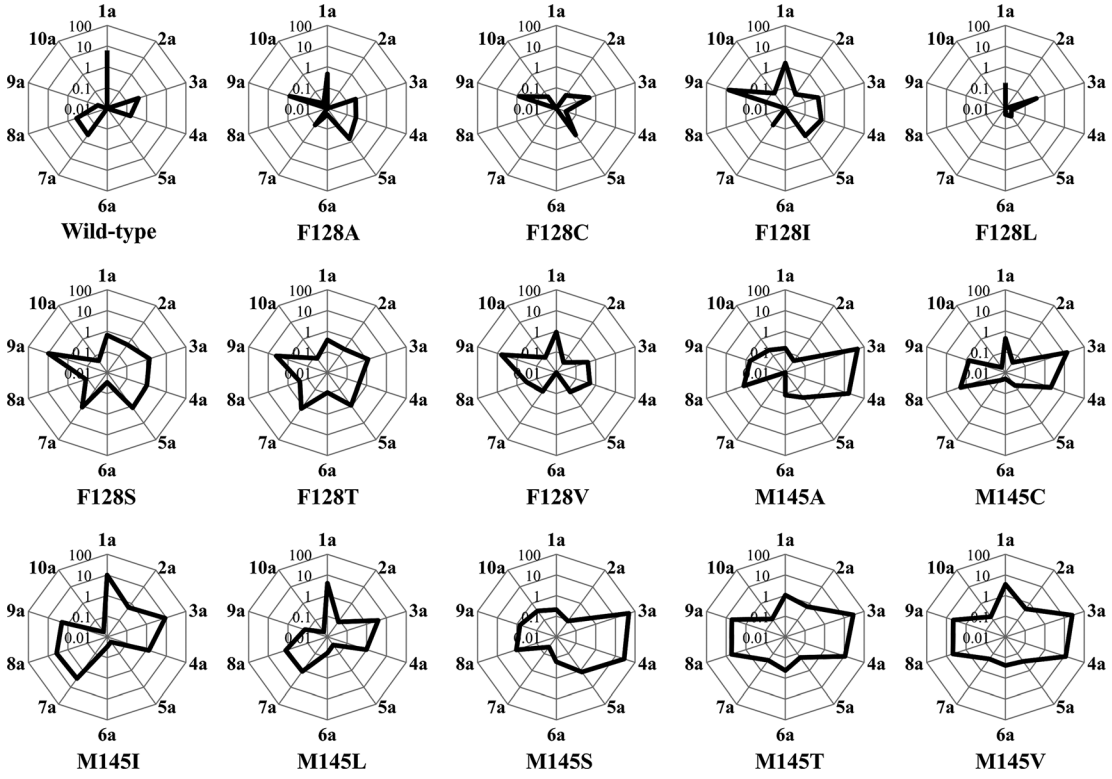

Figure 2. Overview of the *Bm*EH variants performance in hydrolytic reactions of various epoxides. Data are shown as logarithmic in the radar map. Activities equal or lower than 0.01 $U\,mg^{-1}$ cell-free extract are shown as 0.01 $U\,mg^{-1}$. For detailed accounts of the numbers, see the Supporting Information.

531 U mg^{-1} protein, respectively. Such high activity has rarely been reported in EHs, thus revealing the amazing power of biocatalyst redesign. However, the combination of F128S with M145S or M145I did not have a synergistic effect on improving the enzymatic activities, but instead caused severe protein-folding problems (see Figure S1).

Activities, which were two orders of magnitude higher, were observed with the variant M145S for the epoxides **3a**–**5a** and **10a**, and the variant F128S for the substrate **9a** (see Table S2). To elucidate the reason for the increases in activity from a biochemical perspective, the apparent steady-state kinetic parameters of these substrate–enzyme pairs was measured (Table 1). The K_M constants of the EH variants

Table 1: Apparent kinetic parameters of *Bm*EH$_{WT}$ and its variants.[a]

Substrate–Enzyme	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]	Fold[e]
3a–WT	3.96	10.8	2.73×10^3	273
3a–M145S	1.67	1245	7.46×10^5	
4a–WT	1.61	1.95	1.21×10^3	705
4a–M145S	0.82	701	8.54×10^5	
5a–WT	>20[b]	>0.22[c]	n.a.[d]	362
5a–M145S	>20[b]	>135[c]	n.a.[d]	
9a–WT	1.32	0.60	4.50×10^2	896
9a–F128S	0.49	199	4.03×10^5	
10a–WT	>20[b]	>0.10[c]	n.a.[d]	169
10a–M145S	6.53	16.7	2.55×10^3	

[a] The kinetic parameters were determined with a substrate concentration of 0.6–10 mM for **3a**, **4a**, **9a**, and 0.6–20 mM for **5a** and **10a**. See the Supporting Information for experimental conditions. [b] The K_M values were beyond the concentration range of the substrate. [c] Calculated based on the highest rate detected. [d] Not available in this case. [e] The folds of k_{cat}/K_M improved for particular variants over the wild-type. In the case of **5a** and **10a**, the folds of specific activity improvement are listed instead.

for substrates **3a**, **4a**, **9a**, and **10a** decreased significantly when compared with that of *Bm*EH$_{WT}$, thus indicating a two- to threefold stronger binding affinity. However, the variants exhibited k_{cat} values which were as much as 100–300-fold higher than that of *Bm*EH$_{WT}$. Therefore, the activity increase of the *Bm*EH variants is mainly a result of the dramatic increase in their turnover frequencies.

Subsequently, several best-variants for each substrate were examined for their enantioselectivity (see Table S3). For the majority of the substrates, the variants have a moderate to good enantioselectivity ($E = 30 \sim 200$). When compared with *Bm*EH$_{WT}$, at least for epoxides **7a** and **9a** ($E = 19$ and 25),[7b] the variants had a significantly improved enantioselectivity ($E = 70$ and 53, respectively), thus indicating greater potential for bioresolving these epoxides. As shown in Table S4, the improved enantioselectivities of *Bm*EH$_{M145I}$ for **7a** and *Bm*EH$_{F128S}$ for **9a** are mainly derived from the more dominant increases in k_{cat} toward *R* epoxides than *S* epoxides.

The activity of an enzyme for a certain substrate is determined by the structure of both protein and ligand, and might be analyzed based on hydrophobicity and steric hindrance. By ranking the substrates in the order of their hydrophobicity (log*P* values) and comparing them with the

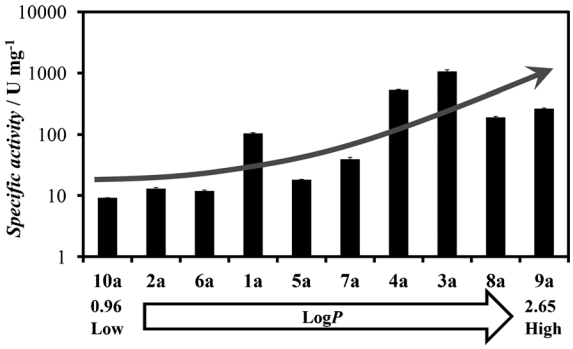

Figure 3. Relationship between the substrate polarity and the highest activity observed among *Bm*EH variants. Substrates are ranked in the order of their log*P* values (obtained from the SciFinder database; see Table S5).

highest activity observed among all the *Bm*EH variants, we found a trend: for substrates with lower hydrophobicity, usually lower enzyme activities were also observed (Figure 3). However, the epoxides **8a** and **9a** did not exhibit additional increases in activity, as expected. This observation is probably because they have substitutions on both the *ortho* and *meta* positions of the phenyl ring, thus resulting in severe steric hindrance.

Some clues could also be found from the kinetic parameters of the variants (Table 1). When *Bm*EH was successfully engineered toward a certain substrate (e.g., **3a**–**5a** and **9a**), its k_{cat} value achieved a level of 10^2–10^3 s^{-1}, but the K_M value for the substrates had more remarkable differences. For **5a** and **10a**, having hydrophilic substitutions, a K_M value which was nearly one order of magnitude higher was observed, thus the catalytic efficiency tended to be limited by its weaker substrate binding affinity. A possible explanation might be based on the deeply buried hydrophobic active site of *Bm*EH, and makes the hydrophobic interactions of the protein with the epoxides the main driving force for substrate binding.

As mentioned above, some *Bm*EH variants could effectively convert epoxides into the corresponding diols with moderate to good enantioselectivity. For epoxides **2a**–**4a** and **7a**–**9a**, the most active variants were chosen for enzymatic resolution at 10 g L^{-1} (ca. 50–60 mM). To facilitate the epoxide dissolution and to prevent spontaneous hydrolysis, a biphasic system of isopropyl ether/water (1/4, v/v) was composed for the preparative bioresolution (Table 2). Under these reaction

Table 2: Preparative bioresolution of epoxides (10 g L^{-1}) using *Bm*EH variants in a biphasic system containing 20% diisopropyl ether.[a]

Sub.	Enzyme/ Load [g L^{-1}]	Reaction Volume [L]/*t* [h]	*S* Epoxide *ee*/yield [%]	*R* Diol *ee*/yield [%]
2a	M145I/0.5	0.1/6	98.8/37.3	67.9/32.4
3a	M145S/0.3	0.1/15	97.8/38.6	71.0/51.7
4a	M145A/0.3	0.1/16	97.4/43.4	92.0/46.0
7a	M145I/0.5	1.0/11	99.5/38.7	74.9/39.8
8a	M145T/1.0	0.1/16	96.6/44.1	96.6/39.9
9a	F128S/0.2	1.0/16	99.5/44.6	99.9/37.2

[a] See the Supporting Information for experimental conditions.

conditions, even for the poorest epoxide, **3a**, for which the best variant gave a relatively low enantioselectivity, the product (*S*)-**3a** could be obtained in fairly good purity (97.8% *ee*) and an acceptable yield (38.6%). For the resolution of the epoxides **7a** and **9a** on a greater than 10 gram scale, their *S* enantiomers (>99% *ee*) were recovered in 38.7 and 44.6% yield, respectively. Furthermore, the dosage of biocatalyst could be reduced to merely 0.2 $g L^{-1}$ for **9a**, thus resulting in a substrate-to-enzyme (*S*/*E*) ratio of 50 (*w*/*w*), which is in contrast to 10 $g L^{-1}$ for *Bm*EH_{WT} resolving its best substrate, *ortho*-methyl phenyl glycidyl ether ($S/E = 3$).[7b] Notbaly, during the process of **2a** and **9a** resolution, the diols formed were rarely soluble in either aqueous or organic phase, thus making it easy to separate the epoxide from diol by simple filtration. This filtration should greatly facilitate downstream processing and improve the enantiopurity of the isolated diols. For instance, the *ee* value of **9b** recovered from filtration was as high as greater than 99%, which is in contrast to the 36% *ee* of the residual **9b** in the reaction solvent. Such an innovation is considered to be extremely suitable for practical application on the large scale.

During the course of this work, we succeeded in expanding the substrate scope of *Bm*EH by engineering two hot spots close to its active site, and it had a remarkable impact on its preference for bulky epoxides. The racemic epoxide precursors of Moprolol (**2a**), SR-59230 A (**3a**), Aprenolol (**4a**), Toliprolol (**7a**), Xibenolol (**8a**), and Propranolol (**9a**) were biochemically resolved, thus affording the *S* epoxides in 96.6–99.5% *ee* and 37–45% yield. To the best of our knowledge, no suitable EH has been reported for the majority of these practically useful epoxides.

The results of our study illustrate the significant potential of semirational design for engineering biocatalysts from the perspective of expanding the synthetic application of one single enzyme. Although the double mutation of the two hot spots might potentially provide even more active variants, simple combination of the best single-site mutants did not have a synergistic effect in our case. Therefore, the combinatorial multipoint mutation methods such as CASTing may be an alternative route to further improve our *Bm*EH.[10]

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