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Manipulating the Expression Rate and Enantioselectivity of an Epoxide Hydrolase by Using Directed Evolution

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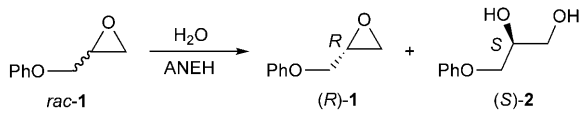
Dedicated to Carmen Najera on the occassion of her 60th birthday

We describe here a strategy to improve the expression efficiency and enantioselectivity of *Aspergillus niger* epoxide hydrolase (ANEH) by directed evolution. Based on a blue-colony screening system using the LacZα (β-galactosidase α peptide) complementation solubility reporter, several ANEH variants out of 15000 transformants from a random-mutagenesis library were identified that show improved recombinant expression in *E. coli*. Among them, Pro221Ser was subsequently used as a template for iterative saturation mutagenesis (ISM) at sites around the ANEH binding pocket. Following four rounds of ISM, a highly enantioselective mutant was identified that catalyzes the hydrolytic kinetic resolution of racemic glycidyl phenyl ether with a selectivity factor of $E=160$ in favor of the (*S*)-diol compared to WT ANEH characterized by $E=4.6$. Expression of this mutant is 50 times higher than that of WT ANEH. It also serves as an excellent stereoselective catalyst in the hydrolytic kinetic resolution and desymmetrization of several other structurally diverse epoxides.

Introduction

Directed evolution, or laboratory evolution as it is sometimes called, permits the manipulation of essentially any property of enzymes, including substrate acceptance, stereoselectivity and stability.[1] The most popular gene-mutagensis methods are error-prone polymerase chain reaction (epPCR), saturation mutagenesis, and DNA shuffling. The application of these molecular biological techniques can always be expected to lead to some degree of catalyst improvement, depending upon the amount of time that is invested in exploring protein sequence space and in evaluating the respective mutants. Because the bottleneck of laboratory evolution is the screening (selection) step,[2] interest in devising better methods and strategies for generating higher-quality libraries has increased in recent years.[1,3,4] We have defined "library quality" in terms of the frequency of improved mutants in a given library and the actual degree of catalyst improvement that these hits induce.[1a,4] High quality thus means less screening effort. Our contribution regarding methodology development in directed evolution is iterative saturation mutagenesis (ISM).[1a,4,5] It is a knowledge-driven approach according to which sites comprising one or more amino acid positions are randomized by using saturation mutagenesis followed by a screening step in which the respective libraries are assayed for a given enzyme property. A hit in one library is then used as a template to perform saturation mutagenesis at another site, and the process is continued until the desired degree of catalyst improvement has been achieved. In a given ISM Scheme many pathways can be chosen, but it is not necessary to explore all of them.[1a] However, the choice of appropriate sites is crucial. For this purpose structure-based guidelines have been proposed that depend upon the protein property to be manipulated, namely stereoselectivity,[5] substrate acceptance (rate),[5] or thermostability.[6] In the case of substrate scope and/or stereoselectivity, we have proposed the combinatorial active-site saturation test (CAST), according to which sites around the binding pocket are chosen for ISM.[1a] The first example of ISM based on CASTing was the enhancement of enantioselectivity of the epoxide hydrolase from *Aspergillus niger* (ANEH) as a catalyst in the hydrolytic kinetic resolution of glycidyl phenyl ether (*rac*-**1**), the selectivity factor favoring (*S*)-**2** increasing from $E=4.6$ (wild-type, WT) to $E=115$ in five ISM steps (Scheme 1).[5a] Only small libraries



Scheme 1.

were necessary; the total number of transformants screened was less than 20000. No tradeoff regarding thermostability was observed. Subsequently, the underlying reason for the observed efficacy was traced to the occurrence of pronounced cooperative (synergistic) effects operating between sets of mutations at each evolutionary step, as demonstrated by the ex-

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perimentally generated fitness landscape and the quantitative analysis of epistatic effects along a given pathway.[4b] Comparison with traditional approaches based on epPCR as a "shotgun" method in this particular case[7] underscored the superiority of ISM, as in several other studies.[1a, 5b–d]

In spite of the success of ISM in improving the stereoselectivity of ANEH, the system proved to have limited practical application due to the relatively low expression rate of the enzyme in *E. coli*.[5a, 7] The efficiency of protein expression in heterologous hosts depends upon many factors, which are indeed complex.[8] It is therefore not surprising that numerous approaches have been described in the quest to boost the expression efficiency of proteins, including directed evolution.[1, 9] In many cases of observed expression improvement, protein engineering actually focused on a different parameter, for example, rate or stability, the induced mutations leading (serendipitously?) to higher expression.[9] Indeed, when focusing on rate using some kind of analytical technique during the screening of supernatants in the wells of microtiter plates, the apparently higher rate may in fact be due partially or solely to increased amounts of protein, brought about by the particular mutations.[1] Careful analysis of the results is necessary to pinpoint all effects, as shown by several studies.[9] Therefore, if directed evolution is to be targeted toward enhancing expression rate, then a screening process is necessary which is specific for identifying this particular parameter ("you get what you screen for").[1, 2, 9] In the present study we take this approach in an effort to improve the expression efficiency of ANEH in *E. coli*. In a second step, one of the improved mutants is employed as a starting point for manipulating stereoselectivity in the test reaction of *rac*-**1** using CASTing-based ISM.

Results and Discussion

Enhancing expression efficiency of ANEH

Because it is not obvious how to apply ISM when wanting to enhance expression efficiency, we turned to epPCR,[1] a prerequisite being the establishment of an appropriate screening system.[2] Several high-throughput screening methods based on fusion reporter systems are known, such as the green fluorescent protein (GFP) folding report method,[10] the N-terminal fusion system with chloramphenicol acetyltransferase[11] and the LacZα (β-galactosidase α peptide) complementation solubility reporter assay.[12] We opted for a screening protocol based on structural complementation between the α- and ω-fragments of β-galactosidase (β-gal; Figure 1). It should be pointed out that the deleted lacZ is provided by the *E. coli* host. In this study *E. coli* DH5a was used.

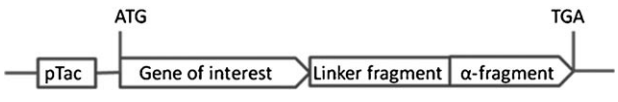


Figure 1. The complementary construct of fused ANEH-linker-α fragment in the pMALC4X plasmid.

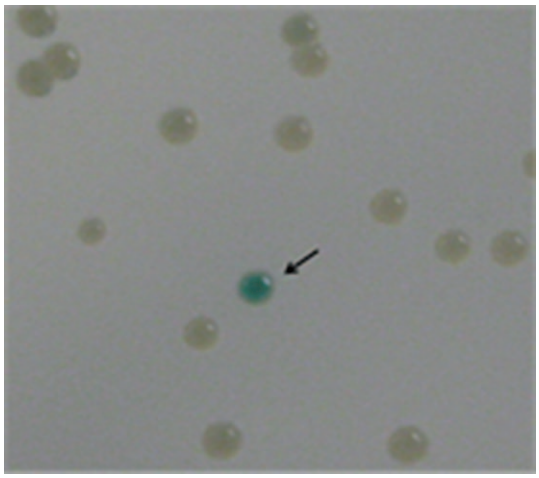


Figure 2. A blue-colony screening system was used to identify ANEH mutants leading to higher expression. Arrow shows a hit in LB agar plate containing X-gal.

By using this screening system (Supporting Information), variants with improved expression can be identified on LB agar plates containing X-gal. Blue colonies indicate mutants leading to high expression (Figure 2). This efficient pre-screen was subsequently checked by performing expression on a large scale and by quantifying efficiency.

epPCR at a low mutation rate was chosen as the mutagenesis method,[1] which addresses the whole enzyme ANEH randomly (although some bias can be expected). A total of 15 000 transformants were screened by using the blue-colony screening system. Fourteen variants having one or more mutations were identified by picking blue colonies, and these were evaluated quantitatively for β-gal activity (Table 1). This data was obtained with pMALC4X; the fused tags were removed when ANEH and its mutants were expressed in the pET system. During the expression screening process, the β-galactosidase activities were measured when the ANEH gene was fused with the lacZ fragment. As can be seen, several hits were identified

Table 1. ANEH mutants detected in the initial epPCR library showing enhanced expression rate.

	Mutants[a]	Mutation	β-Gal activity [U/OD_{600}]
1	EH87	Arg219Trp/Ala233Thr	29.1
2	EH94	Arg219Gly	31
3	EH101	Pro43His/Arg219Met	33
4	EH103	Ala220Val/Phe340/Leu/Val415Ala	8.0
5	EH107	Ala220Thr/Ser226Pro	18.5
6	EH116	Ala220Val	17.6
7	EH117	Arg219Trp	25.2
8	EH126	Pro222Thr/Ile262Val	10.2
9	EH135	Asp161Gly/Arg219Gly	22.3
10	EH145	Thr33Asn/Val197Ile/Pro222Ser	16.7
11	EH146	Val211Ile/Arg219Trp	33.6
12	EH147	Pro221Ser	47.1
13	EH150	Arg219Lys	45.3
14	EH153	Pro222Leu	30.6
15	WT		5.8

by this process, the best one was mutant EH147, which is characterized by a single amino acid exchange Pro221Ser.

The results are encouraging, but the expression of the ANEH mutants in the original plasmid pMALC4XEH is still not ideal, as indicated by SDS-PAGE analyses. To boost the expression further, pET22b was used under the action of promoter T7. All variants were cloned into pET22b and induced with 0.1 mM IPTG. Pronounced differences in SDS-PAGE were observed. Unfortunately, many of these variants expressed in pET22b proved to be insoluble. Therefore, to increase the solubility of the target enzyme, chaperone plasmids were applied, in these cases by using mutants EH117 (Arg219Trp) and EH147 (Pro221Ser). Of the five kinds of chaperone plasmids tested, the plasmid of pKJE7 expressing chaperone dnaK-dnaJ-grpE significantly improved the solubility of variant EH147 (Supporting Information), which was then chosen for further studies. Mutation Pro221Ser is in a loop far removed from the catalytic triad Asp192/Tyr251/Tyr314. In the hydrolytic kinetic resolution of *rac*-**1**, mutant EH147 was found to be only slightly *S*-selective ($E=5$), which is essentially identical to the performance of WT ANEH ($E=4.6$). This set the stage for improving the stereoselectivity of mutant EH147.

Enhancing enantioselectivity of ANEH

To choose optimal CAST sites for saturation mutagenesis, we first performed induced fit docking of the model substrate *rac*-**1** by using the "mutated" structure of variant EH147 (Pro221Ser) based on the crystal structure of WT ANEH.[13] Nine residues were identified for potential randomization, namely Leu215, Ala217, Arg219, Phe244, Leu249, Thr317, Thr318, Leu349, and Cys350 (Figure 3A). The next step required a decision concerning how to group these amino acid positions into appropriate sites, keeping in mind the screening effort. One could opt for nine single-residue sites, a strategy that we had successfully applied in the directed evolution of the enoate-reductase YqjM.[5b] Using NNK codon degeneracy (N: adenine/cytosine/guanine/thymine; K: guanine/thymine) encoding all 20 canonical amino acids, this would entail nine randomization libraries, each requiring about 100 transformants to be screened for 95% library coverage assuming the absence of amino acid bias.[1a,4] In more recent studies, however, we discovered that randomization at sites composed of more than one amino acid position constitutes the better strategy,[5c–e] which was traced to the occurrence of cooperative effects operating between the newly introduced amino acids within a given site and between sets of mutations in the ISM process.[4b] To keep the screening effort as low as possible, we decided to delete one of the residues (Ala217) from further consideration and focused on the remaining eight, which were grouped into four sites, each composed of two amino acid positions: A (Leu215/Arg219), B (Leu349/Cys350), C (Thr317/Thr318), and D (Phe244/Leu249; Figure 3B). These sites are very similar, but not quite identical to the previously utilized CAST sites (note that the numbering system of the sites is different from the previous study).[5a]

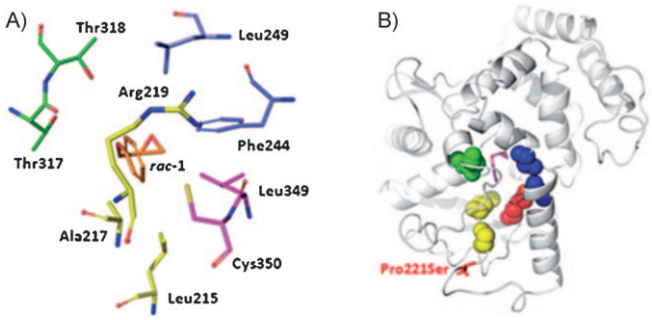


Figure 3. A) CAST analysis of ANEH mutant Pro221Ser based on the crystal structure of WT ANEH,[13] into which mutation Pro221Ser was "added"; induced fit docking of *rac*-**1** (purple) then led to the identification of nine residues for possible saturation mutagenesis. B) Grouping eight of the nine identified residues into four potential CAST sites, A (Leu215/Arg219), B (Leu349/Cys350), C (Thr317/Thr318), and D (Phe244/Leu249).

When choosing NNK codon degeneracy, requiring in each library the screening of about 3000 transformants for 95% coverage,[4b,6] the total screening effort would amount to about 12000 measurements. Because this does not include any ISM steps and already exceeds the screening limit arbitrarily set by us, we opted for NDT codon degeneracy (D: adenine/guanine/thymine; T: thymine), encoding 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly). They constitute a balanced mixture of building blocks having polar, nonpolar, charged, uncharged, aromatic, and nonaromatic side chains. NDT requires for 95% library coverage the screening of only 430 transformants.[4b,6] We settled for 480, which means that the total screening effort involves only 1920 transformants. As a first step, we focused on site D and performed randomization by using the QuikChange protocol[14] but using NDT codon degeneracy.[4a,6] After screening 480 transformants for enantioselectivity of the kinetic resolution of *rac*-**1**, an improved mutant EH181 ($E=7$) was identified that was characterized by two new mutations Phe244Cys/Leu249Phe. At this stage a decision had to be made regarding further mutagenesis experiments, that is, to continue with EH181 in an ISM process or to first generate the other three possible randomization libraries at sites A, B, and C. Because the libraries are quite small, it really did not matter. We opted for ISM exploration by starting from EH181. When using the gene of this variant as a template, either site A, B, or C can be chosen in the next randomization experiment. We arbitrarily chose pathway D→A→C→B. As Figure 4 reveals, this ISM pathway provides a highly enantioselective mutant (EH222) displaying an *E* value of 160 in favor of (*S*)-**2**. To our surprise, saturation mutagenesis at site C led to the insertion of extra residues (*Pro-Thr-Ala-Ser-Ala-Pro-His-Thr-Tyr-Arg-Glu-Phe-Ile*). Unintended insertions may well occur during the PCR process as part of the QuikChange protocol, but are hardly ever reported.[1,14] Indeed, in most cases the likelihood of observing this phenomenon is low because it usually has an adverse effect and will therefore not be detected in the screening process. Rather than discarding the result and turning to other saturation mutagenesis libraries, we prefer to report this observation. In the same library the

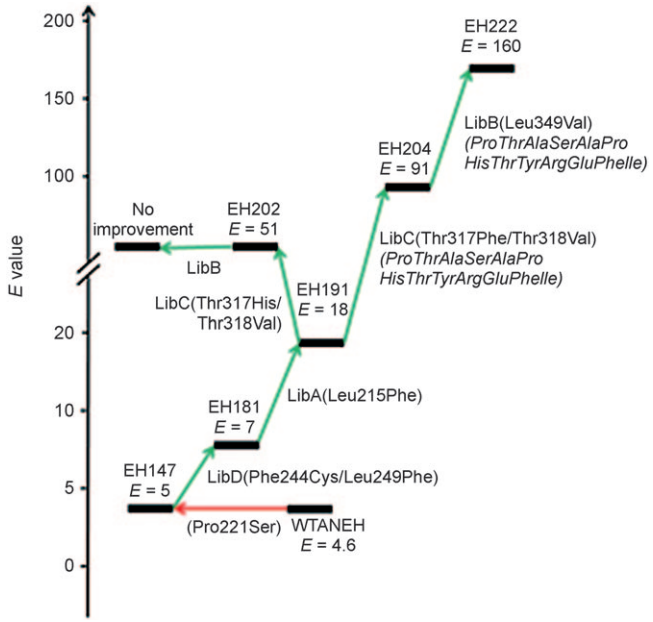


Figure 4. Multiple pathways explored in the quest to boost the expression rate of WT ANEH by using epPCR (red pathway) and enantioselectivity of the respective ANEH mutant as a catalyst in the hydrolytic kinetic resolution of *rac*-**1** by using ISM (green pathways). Extra residues (*ProThrAlaSerAlaPro-HisThrTyrArgGluPhelle*) appear in EH204 and EH222, indicating an inserting sequence produced by QuikChange.[14]

second best variant in terms of stereoselectivity was identified, namely EH202 characterized by $E = 51$, which is also a notable degree of enantioselectivity. However, upon continuing ISM by subsequently visiting site B, no significant improvement was detected. Table 2 and Figure 4 summarize the results, which required the screening of a total of only 2880 transformants. Further exploration of ISM pathways is possible, but because the goal of acceptable stereoselectivity had been reached, we decided at this point to leave this to future work.

As delineated earlier, induced-fit docking was performed to identify appropriate CAST residues. However, to interpret the stereochemical results on a molecular level with any degree of confidence, extensive molecular dynamics (MD) simulations need to be carried out, an important topic for future work. Moreover, the unexpected loop insertion in library C and its influence on stereoselectivity also have to be considered.

To check whether the best mutant EH222 ($E = 160$) maintains the improved expression found in the initial mutant EH147, the respective control experiments were performed. We obtained 9.1 mg enzyme per gram of wet pellet by following affinity purification, which is 50-times higher than in the case of WT ANEH.

Investigation of substrate scope

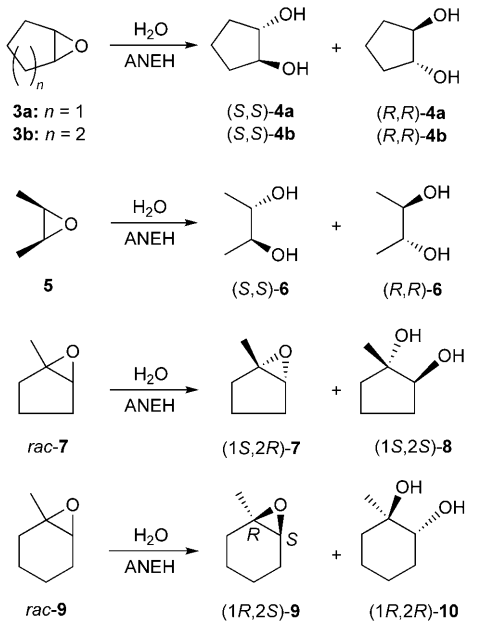
In an effort to see how the best mutant EH222 performs as a catalyst in the hydrolytic desymmetrization of *meso*-configurated epoxides,[5d] compounds **3a**, **3b**, and **5** were tested as substrates. Table 3 reveals that the substrate scope of mutant EH222 is quite broad, and the activity and improved enantioselectivity are generally distinctly higher than WT ANEH. Moreover, in hydrolytic kinetic resolution of *rac*-**7** and *rac*-**9** (Scheme 2),[5d] mutant EH222 again shows much better performance than WT ANEH, which behaves stereorandomly in these cases.

Table 3. Catalytic performance of mutant EH222 compared to WT ANEH under standard conditions (4 h).

	WT ANEH		EH222	
Subtrates	Conv. [%]	*ee* [%]	Conv. [%]	*ee* [%]
3a	0		70	94 (*S*,*S*)
3b	82	79 (*S*,*S*)	94	97 (*S*,*S*)
5	0		72	95 (*S*,*S*)
rac-**7**	39	0	46	56 (1*S*,2*S*)
rac-**9**	8	0	15	46 (1*R*,2*R*)

Table 2. Typical ANEH mutants arising from an arbitrarily chosen ISM process D→A→C→B starting from mutant EH147 (Pro221Ser) produced by epPCR for higher expression, their performance as catalysts was tested in the hydrolytic kinetic resolution of *rac*-**1**. In all cases NDT codon degeneracy was employed as part of the QuikChange protocol[14] encoding an amino acid alphabet of 12 amino acids (see text).

Site	Mutant	Mutations	*ee* [%] (*S*)	Conv. [%]	*E* value
	WT				
D	EH147	Pro221Ser	54	40	5
	EH181	Pro221Ser/Phe244Cys/Leu249Phe	69	23	7
	EH182	Pro221Ser/Leu249Ser	55	53	6
	EH183	Pro221Ser/Phe244Tyr/Leu249Arg	70	23	7
A	EH185	Pro221Ser/Phe244His/Leu249His	66	24	6
C	EH191	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe	86	27	18
	EH202	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe/Thr317His/Thr318Thr	93	40	51
	EH203	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe/Thr317Ile/Thr318Ser	92	35	41
	EH204	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe/Thr317/Thr318(*PheValProThrAlaSerAlaProHisThrTyrArgGluPhelle*)	96	41	91
	EH205	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe/Thr317Val/Thr318Ile	85	43	24
B	EH207	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe/Thr317Val/Thr318Ser	92	18	29
	EH222	Pro221Ser/Phe244Cys/Leu249Phe/Leu215Phe/Thr317Phe/Thr318Val(*ProThrAlaSerAlaProHisThrTyrArgGluPhelle*)-Leu349Val	97	45	160



Scheme 2.

Conclusions

We have demonstrated that it is possible to exploit the techniques of directed evolution[1] to achieve two very different goals, namely increasing the expression rate and enhancing the stereoselectivity of an enzyme. Both parameters are crucial in biotechnology. In principle, it is conceivable that either one of the two properties can be optimized first followed by the second optimization. In the present study we focused on expression first, and then turned to the improvement of enantioselectivity. As a test case the epoxide hydrolase from *Aspergillus niger* (ANEH) was used as a catalyst in the hydrolytic kinetic resolution of glycidyl phenyl ether (*rac*-**1**), WT leading to $E=$ 4.6 (*S*).[5a,7] Expression in *E. coli* was notably improved by screening a 15000-membered epPCR-based library, providing mutant Pro221Ser. The latter was subsequently used in a limited number of iterative saturation mutagenesis experiments (less than 3000 transformants screened), leading to several enantioselective mutants characterized by selectivity factors ranging between 51 and 160 in favor of the (*S*)-diol. The expression efficiency of the best mutant was found to be 50 times higher than that of WT ANEH. Thus, our strategy proved to be successful, but it remains to be seen if the opposite order of events delivers similar results, or whether it is less or perhaps even more efficient. In future studies the reasons for catalyst improvement also need to be illuminated on a molecular level.

Experimental Section

General: The restriction enzymes were purchased from New England Biolabs. KOD Hot Start DNA Polymerase was obtained from Novagen. The oligonucleotides were synthesized by Invitrogen. Plasmid preparation kit and PCR purification kit were bought from Qiagen. Chaperone plasmid set was bought from TaKaRa. All commercial chemicals were purchased from Sigma–Aldrich. Commercially unavailable chemicals were synthesized and characterized by GC-MS and NMR spectroscopy. Terrific Broth (TB; per liter) containing: tryptone (12 g), yeast extract (24 g), glycerol (4 mL), KH_2PO_4 (2.31 g), K_2HPO_4 (12.54 g). Lysis buffer was prepared by using potassium phosphate buffer (50 mM, pH 7.4) containing lysozyme (1 mg mL^{-1}, AppliChem) and DNaseI (0.1 mg mL^{-1}, 4066 U mg^{-1}, AppliChem).

Recombinant plasmid construction: The primers of UpEH_linker_pMALC4X and DownEH_linker_pMALC4X were used to amplify the gene of epoxide hydrolase of *Aspergillus niger* (ANEH). The "GSAGSAAGSGAS" peptide, which is encoded by 5′-ggatccgctggctccgctgctggttctggcgcaagc-3′ was used as a linker between ANEH and the α-fragment of β-galactosidase, the PCR was performed for 26 cycles at 95 °C, 30 s; 52 °C, 30 s and 72 °C for 1.5 min. The primers of upVector_pMALC4X and DownVector_pMALC4X were used to amplify the vector fragment of pMALC4X. The PCR was performed for 26 cycles at 95 °C, 30 s; 52 °C, 30 s and 72 °C for 6 min. The original templates were digested by DpnI twice within 6 h. The purified PCR products of the ANEH gene and vector fragment of pMALC4X were ligated according to enzymatic assembly procedures.[15] The assembly products were then transformed into chemically competent DH5α cells. The recombinant plasmid was confirmed by sequencing.

Construction and screening of epPCR-based random mutagenesis library for expression efficiency: Random mutagenesis library was constructed by GeneMorph II Random Mutagenesis Kit. The primers of upEPgenepMALC4X and downEPgenepMALC4X were used to amplify the ANEH gene. A low range of mutation frequency (0 ~4.5 mutations per kb) was utilized. Twenty-five cycles were run by PCR at 95 °C, 1 min, 60 °C, 1 min, and 72 °C for 2 min. The primers of upEPpMALC4X and downEPpMALC4X were used to amplify the vector fragment of pMALC4X by thirty cycles at 95 °C, 1 min, 60 °C, 1 min, and 72 °C for 6 min. The original templates were digested by DpnI twice within 6 h. The purified PCR products of the ANEH gene and vector pMALC4X were ligated based on enzymatic assembly procedures. The assembly product was transformed to the electroporation-competent DH5α cells. The transformed product was plated on LB agar plates containing 50 μg mL^{-1} carbenicillin, 20 μg mL^{-1} X-gal, and 0.1 mM IPTG. The plates were kept at 37 °C in an incubator overnight and moved into a 30 °C incubator for about 15 h to allow the blue colonies to appear.

Measurement of β-galactosidase activity: WT and mutants obtained from the blue-colony screening system were cultured in LB medium containing 50 mg mL^{-1} carbenicillin at 37 °C. The expression of target protein was induced with 0.1 mM IPTG for 4 h at 30 °C when the OD of the culture reached 1.0. The activity of β-galactosidase was measured by an adapted method by using ONPG (*O*-nitrophenyl-β-D-galactopyranoside) as a substrate.[12] An aliquot of expression culture (1 mL) was centrifuged and washed twice by using potassium phosphate buffer (50 mM, pH 7.0) and resuspended in potassium phosphate buffer (1 mL). An aliquot of resuspended cells (0.5 mL) was taken for reaction. Reactions were initiated by adding *O*-nitrophenyl-β-D-galactopyranoside (ONPG) solution (0.25 mL; 4.0 mg mL^{-1} dissolved in potassium phosphate buffer) and incubated at 37 °C for 10 min. Reactions were quenched by adding 1 M Na_2CO_3 (0.25 mL). After centrifugation, the supernatant's absorption at 420 nm was measured. *O*-Nitrophenol (ONP) was used to make standard curve. One unit of enzyme was equiva-

lent to 1 μmol ONP liberated from ONPG per minute per OD_{600} of culture.

Construction and screening of iterative saturation mutagenesis libraries for enantioselectivity: The mutant gene of ANEH from random mutagenesis library was cloned into pET22b to construct and screen ISM libraries. The primers of up_22bEH_ANEH and down_22bEH_ANEH were used to amplify the mutated ANEH gene. The primers of up_22bEH_Vector and down_22bEH_Vector were used to amplify vector pET22b. The cloning steps were carried out based on enzymatic assembly procedures described as above. The constructed plasmids were confirmed by sequencing.

Electroporation-competent cells containing five different chaperone plasmids (chap1, pG-KJE8; chap2, pGro7; chap3, pKJE7; chap4, pG-Tf2; chap5, pTf16) were prepared. Mutant ANEH cloned into pET22b were applied to transform different competent cells of BL21Gold (DE3) containing each different chaperone plasmid. The expression of mutant ANEH together with chaperones was performed according to TaKaRa protocols.

Iterative saturation mutagenesis libraries were constructed by QuikChange. Twenty-two cycles were run by PCR at 95 °C, 30 s, 60 °C, 30 s, and 72 °C for 3 min. The parent template was digested by *Dpn*I twice within 6 h. The purified products were transformed electroporation-competent cells of BL21Gold (DE3).

Transformants grown on LB agar plates were picked and cultured overnight in 96-deep-well plates containing LB (800 μL) and 50 μg mL^{-1} carbenicillin, 20 μg mL^{-1} chloramphenicol, and 2 mg mL^{-1} L-arabinose at 37 °C with shaking (800 rpm). An aliquot of culture (100 μL) was transferred into 96-deep-well plates containing TB (700 μL), 0.1 mM IPTG, 50 μg mL^{-1} carbenicillin, 20 μg mL^{-1} chloramphenicol, and 2 mg mL^{-1} L-arabinose. The expression of transformants was performed for 6 h and at 30 °C.

An aliquot of expression culture (150 μL) was transferred into a 96-deep-well plate, then phosphate buffer mixed with 13.6 mM *rac*-**1** (containing 8.1% *v/v* MeCN; 600 μL) was added. The reaction was performed at 30 °C with shaking at 500 rpm for 1 h. The plates were centrifuged for 20 min and supernatant (150 μL) was transferred into a new 96-deep-well plate filled with EtOH (150 μL). The plates were again centrifuged at 4 °C for 1 h. An aliquot of supernatant (150 μL) was transferred into a microtiter plate. These plates were sealed with aluminum stick seal and subjected to a medium-throughput chiral HPLC assay.

Investigation of substrate scope: The typical reaction procedure for enzymatic hydrolysis of epoxide is as follows: WT and ANEH mutants were expressed in TB at 30 °C for 4 h with 0.1 M IPTG and 2 mg mL^{-1} L-arabinose as inducers. The pellets of each 1.0 mL of culture were harvested by centrifuging. The cells were broken by the addition of 400 μL lysis buffer. Then 5.0 mM of epoxides (200 mM stock solution in acetonitrile) were applied to each reaction for 10 h at 30 °C with shaking (400 rpm). The products and residual substrates were extracted with decane (1 mM) in EtOAc (400 μL). For conversion measurement, an aliquot of solvent layer (1 μL) was analyzed by GC. Then, the extraction was carried out by using EtOAc (3×400 μL). The EtOAc layer (ca. 1200 μL) was collected and concentrated by speed vacuum to a final volume of 150 μL. An aliquot of EtOAc (1 μL) was analyzed by chiral GC column.

Expression and purification: The expression of mutant and WT was induced by using 0.1 mM IPTG in 300 mL TB containing 50 μg mL^{-1} carbenicillin at 30 °C, 20 μg mL^{-1} chloramphenicol and 2 mg mL^{-1} L-arabinose for 6 h. The cells were harvested and washed twice by using potassium phosphate buffer (50 mM, pH 7.4). The pellets were resuspended with the same buffer at the ratio of 8 mL potassium phosphate buffer per gram of cells. The cells were disrupted by sonication (Bandelin, 120 seconds, 40% pulse, on ice broth), and a clear supernatant was obtained by centrifugation at 10 000 rpm and 4 °C for 1 h. Recombinant epoxide hydrolases were purified by affinity chromatography (HisTrap HP, 5 mL, GE Healthcare). The impurities were removed by ten column volumes of washing buffer (50 mM potassium phosphate buffer, pH 7.4, 500 mM NaCl and 40 mM imidazole) at 2 mL min^{-1} flow rate. His-tagged epoxide hydrolases were obtained by using three column volumes of elution buffer (50 mM potassium phosphate buffer, pH 7.4, 500 mM NaCl and 500 mM imidazole). The elution fractions were concentrated by Amicon Ultra-15 (10 K Nominal Molecular Weight Limit, Minipore). PD-10 desalting column (5 mL, GE Healthcare) was then utilized to remove imidazole from concentrated enzyme solution.

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