Protein Engineering for Enhanced Enantioselectivity of Carboxylesterase Est924 Toward Ethyl 2-Arylpropionates

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Research

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Abstract

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) are the world's most used drugs with anti-inflammatory, analgesic and antipyretic effects. 2-arylpropionate drugs are a very important class of chiral drugs in NSAIDs. In the pharmaceutical industry, there is an increasing demand for esterase-mediated chiral resolution of racemic 2-arylpropionate esters due to the high activity and low side effects of the (S)-enantiomers. Esterases are important biocatalysts for chemical synthesis owing to their enantioselectivity, regioselectivity, broad substrate specificity and no need for cofactors. Several bHSL family esterases have been used to resolve racemic 2-arylpropionates. However, each reported enzyme was able to synthesize only one NSAIDs drug, which does not meet the industry requirement for catalysts and limits their application.

Results

Through careful analysis of the structural information and molecular docking, we present the discovery of key residues that controlled the enantioselectivity of bHSL carboxylesterases to ethyl 2-arylpropionates. Est924 was identified as a bHSL family esterase and a promising catalyst for kinetic resolution of racemic ethyl 2-arylpropionates with sight (R)-stereopreference. Using Est924 as the starting enzyme, protein engineering at these key residues was conducted, and the substitution of A203 was shown to affect the enantioselectivity. The stereopreference of the mutant M1(A203W) was inverted to ethyl 2-(S)-arylpropionates, and this stereopreference was further improved in variant M3(I202F/A203W/G208F). In addition, the optimal variant, M3, was also suitable for the resolution of ibuprofen ethyl ester and ketoprofen ethyl ester, and their efficient (S)-isomers were synthesized.

Conclusions

Our results suggested that Est924 variants could kinetically resolve economically important racemates for industrial production and further offer the opportunity for the rational design of enzyme enantioselectivity.

Background

Chirality is one of the essential properties of natural substances, and enantiomers are ubiquitous in nature. Different enantiomers of a biologically active molecule generally have different physiological properties, such as different degrees or different types of biological activities, or even opposite bioactivities or toxic side effects [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the world's most used drugs with anti-inflammatory, analgesic and antipyretic effects. 2-arylpropionate drugs (known as profen drugs) are a very important class of chiral drugs in NSAIDs, including ibuprofen, naproxen, ketoprofen, flurbiprofen, fenoprofen, and loxoprofen, among others. Profen drugs contain a stereogenic center at the carbon alpha of the carboxyl function and therefore racemic (R)- and (S)-enantiomers which
are equivalent in mass. A large number of pharmacological studies on the relative activities of the two enantiomers showed that (S)-enantiomers have significantly higher clinical effects, and dexketoprofen, (S)-ibuprofen and (S)-naproxen are most widely used commercially [2].

Due to differences in physiological functions of isomers, the Food and Drug Administration (FDA) requested manufacturers to separate each enantiomer for specific usage [3]. Biocatalysts have been under development for several decades to replace chemical-based synthesis because they have advantages in economic feasibility and environmental friendliness [4]. Carboxylesterase (E.C. 3.1.1.1), as the main group of natural biocatalysts for the cleavage and formation of ester bond, has good stability, wide substrate specificity and high chemoselectivity, stereoselectivity and regioselectivity. It is widely used as an environmentally-friendly biocatalyst for the synthesis of active pharmaceutical intermediates and fine chemicals [5]. Several research groups have already described enzymatic routes for the production of (S)-2-arylpropionates. Choi et al. cloned an esterase from Pseudomonas fluorescens KCTC1767 with high activity and strict selectivity to hydrolyze rac-ketoprofen ethyl ester (27,000 U) to produce the (S)-enantiomer (>99% ee) [6]. The esterase from Pseudomonas sp. KCTC 10122BP exhibited a strict selectivity (>99%) and high activity (2360 U/mg) towards (S)-ketoprofen ethyl ester [7]. Thermostable esterase (Est3) from Sulfolobus solfataricus could resolve racemic ketoprofen methyl ester and (R)-configured acid with an ee value of 80% at a conversion rate of 20% in 10.5 h [8]. Carboxylesterase SsoEST1 from S. solfataricus P1 was confirmed to resolve naproxen methyl ester, yielding highly optically pure (S)-naproxen (90% ee) [9]. Directed evolution technology is commonly used to improve the activity and selectivity of enzymes, and is also suitable for enhancing the enantioselectivity of esterases in kinetic resolution of 2-arylpropionate esters. Kim et al. used error-prone PCR (epPCR) and site-directed saturation mutagenesis to improve the selectivity of Est-AF toward (S)-ketoprofen ethyl ester. E value of the mutant V138G/L200R was increased from 0.7 (wild type) to 19.5 [10]. Kim et al. modified the esterase Est25, and the enantioselectivity of the mutant Est25F72G/L255W was improved to 60.1 (E value) compare with the parent Est25 (E=1.1) [11]. Ngo et al. conducted site-directed mutations on Est-Y29 originating from the metagenomic library. The ee value of (S)-ketoprofen of the mutant (F125W) was increased to 85%, and the E value was correspondingly increased from 4.48 to 20.39 [12]. Esterases are thus potential biocatalysts for the synthesis of NSAIDs with high optical purity. However, each reported enzyme was able to synthesize only one NSAIDs drug, which does not meet the industry requirement for catalysts and limits their application.

Inspired by the broad-spectrum substrate of the bHSL carboxylesterases [13] and high pharmaceutical activity of (S)-2-arylpionic acids, we screened key residues that controlled the enantioselectivity of bHSL carboxylesterases to ethyl 2-arylpropionates, via careful analysis of the structural information and molecular docking. Subsequently, Est924 (GenBank accession number MW460906), a bHSL family esterase, was set as starting enzyme. It was cloned from the soil metagenomic library and displayed a promiscuous substrate spectrum, including phthalates and ethyl 2-arylpropionates with slight R-stereopreference [14]. The hotspots were mutated through rational design and site-directed mutagenesis. The enantioselectivity of mutant M1(A203W) was inverted to the (S)-naproxen ethyl ester. Variant M3
(I202F/A203W/G208F) resolved racemic naproxen ethyl ester with higher enantioselectivity and produced (S)-naproxen with 91% ee. Furthermore, M3 was confirmed to resolve other ethyl (S)-2-arylpropionates with high selectivity. The products dexketoprofen and (S)-ibuprofen were obtained with 85%ee and 87%ee respectively. This is the first report that esterases can be used in the synthesis of several NSAIDs drugs, including (S)-ibuprofen, (S)-naproxen and dexketoprofen. Our results provide the molecular basis of the enantioselectivity of bHSL family esterases against (S)-2-arylpropionic acids, indicating a broad effect on the development of esterases for the synthesis of NSAIDs with diverse structures.

**Results And Discussion**

**Substrate specificity and enantioselectivity of Est924**

It is widely known that bHSLs are promiscuous by nature[15]. For instance, esterase EH1 (PDB: 5JD4) belongs to the bHSL family and demonstrates with a promiscuous substrate specificity [13]. The substrate specificity of Est924 was determined at 30 °C in pH 8.0 buffer by using racemic ethyl (R, S)-2-arylpropionates as substrates. As shown in Figure 1 and Table 1, Est924 could hydrolyze all tested substrates, indicating that it had a wide range of substrates. In the pharmaceutical industry, there is an increasing demand for esterase-mediated chiral resolution of racemic 2-arylpropionate esters due to the high activity and low side effects of the (S)-enantiomers. The enantioselectivity of Est924 was subsequently determined using (R) and (S)-naproxen ethyl ester as substrates. Similar to Est25 [16] and SsoEST1 [9], wild-type Est924 had activity toward (R)-naproxen ethyl ester and (S)-naproxen ethyl ester, with a slight preference for (R) -enantiomer (10%eeR).

**Structural analysis of bHSL family esterases**

Enzymes with the broad-spectrum substrate applicability generally owe this to their larger active pocket, similar to bHSL family esterases [13]. According to the classification of the Esther database [17], 111 esterase structures of the bHSL family were resolved. We superimposed the crystal structures of Est25 (PDB: 4J7A) and 41 wild-type enzymes of bHSL family (Additional file 1: Figure S2). Three-quarters of bHSL family esterases had highly similar crystal structures, although the lowest sequence similarity observed between 5IQ3 and 3D7R was only 11.7% (Additional file 1: Table S3). The residue L255 of Est25 attracted our attention, because the variant Est25L255W reversed the enantioselectivity of the racemic ketoprofen ethyl ester, resulting in (S)-ketoprofen with 80% ee [18]. Residue L255 was located at the loop between helix 10 and 11 with an isobutyl side chain extension to catalytic serine (Figure 2A). The enantiomer discrimination by enzymes is a very accurate mechanism, which often involves a few amino acids located at the active site. Based on structural information, about three-quarters of the 41 analyzed structures contain the same loop, indicating that it is conserved in the bHSL family (Additional file 1: Table S3). Alternatively, carboxylesterase SsoEST1 has the same residue (L198) as Est25 (L255), but selectively produces (S)-naproxen when racemic naproxen methyl was used as a substrate [9]. This indicates that other residues in the active pocket probably control the stereoselectivity of the α carbon. By docking (R)-naproxen ethyl ester into Est25 (Figure 2B), several residues around the naphthalene ring
were selected to test whether they played a role in the stereoselectivity of the enzymes, due to the opposite stereoselectivity of Est25 and SsoEST1. As shown in Table 2, the residues at position A2 in all crystal structures were hydrophobic amino acids, and the residues at position B1 exhibited lower conservation (Figure 2C). Since residues at position A2 and position B1 directly extend their side chains into the α carbon of substrates, their low conservation suggested that these residues might be responsible for the different stereoselectivity of enzymes.

**Site analysis and rational design of Est924 variants**

Est924 can hydrolyze a variety of ethyl 2-arylpropionates, demonstrating the same enantioselectivity as Est25. We simulated the structure of Est924 using the crystal structure of Est25 as a template (Figure 3A). The key residues of Est924 that might control its stereopreferences were analyzed. The selectivity of Est25 mutants with L255W to (S)-ketoprofen ethyl ester was significantly improved, because tryptophan has the largest side chain, which increases the binding steric hindrance of the (R)-enantiomer [11]. Superposition of the Est924 and Est25\textsubscript{F72G/L255W} crystal structure (5IQ3) revealed that the A203 with a small side chain in Est924 might be responsible for the low enantioselectivity. The Est924 residue was transferred to Est25\textsubscript{F72G/L255W} by A203W substitution to investigate whether the variant Est924\textsubscript{A203W} could make (S)-naproxen as the main product (Figure 3B, yellow residues). Similarly, the structure of SsoEst1 was also simulated and superimposed with the Est25\textsubscript{F72G/L255W}. The imidazole group of H202 in SsoEst1 occupies the indole ring of W255, which might be the second key residue responsible for the enantioselectivity of the enzymes (Figure 3C, yellow residues). Therefore, G208 at position B1 was also selected as a mutation site to prove our hypothesis. In addition, because the steric hindrance and hydrophobicity of A203 was lower than the phenylalanine in Est25 (F254), SsoEst1 (F197) and Est3 (F203), A203 was also selected for mutation.

**The enzyme activity and enantioselectivity of esterases**

The enzyme activity and enantioselectivity of esterases were measured using (R, S)-naproxen ethyl ester as substrate by HPLC. Firstly, A203 was mutated to tryptophan according to the structural analysis and results obtained by Kim et al [11]. As predicted, variation of residue A203 significantly impacts enantioselectivity, and the variant M1 (A203W) turned to (S)-selective, producing (S)-naproxen with 60% ee\textsubscript{p}. The variant Est924\textsubscript{G208F} also reversed the stereoselectivity and hydrolyzed (S)-naproxen ethyl ester with moderate selectivity, yielding (S)-naproxen with 52% ee\textsubscript{p}. These results indicated that both positions (204 and 208) were effective in improving the selectivity of Est924. Variant M2 with A203W and G208F was highly (S)-selective, producing (S)-naproxen with 90% ee\textsubscript{s}. Finally, the variant with mutation at A203 still retained the (S)-enantioselectivity as high as M2, and variant M3 (I202F/A203W/G208F) with 91% ee\textsubscript{s} was obtained.

To clarify why the enantioselectivity of the variants were reversed and increased, we investigated the specific activity (mM/mg/min) for biocatalysis reactions using wild-type Est924, M1, G208F, M2 and M3 with (R)-naproxen ethyl ester and (S)-naproxen ethyl ester as substrates. Est924 showed the lowest
enantioselectivity among esterases (Table 3, list 1), which mainly caused similar hydrolysis activity as compared to the enantiomers. M1 retained 90% activity of Est924 on (S)-naproxen ethyl ester, and 15% activity against (R)-enantiomer (Table 3, list 2). Therefore, the enantioselectivity of M1 was reversed and (S)-naproxen was formed as the main product. M3 showed the highest enantioselectivity among the esterases examined. It was almost inactivated in its ability to process (R)-naproxen ethyl ester, but still retained 80% of the activity of Est924 when (S)-naproxen ethyl ester was used as a substrate (Table 3, list 5). Subsequently, the structure of the optimal variant M3 was simulated and docked with (R) and (S)-naproxen ethyl ester, respectively. As shown in Figure 4A, (S)-naproxen ethyl ester fitted well with the active pocket but failed to obtain an effective docking of (R)-enantiomer with M3. Next, we docked (R) and (S)-naproxen ethyl ester into the structure of Est924, as shown in Figure 4B. The naphthalene ring of the (R)-enantiomer pointed to the cavity formed by A203 and G208. The distance between the protonated Nε atom of the active histidine residue and the ester oxygen atom (dNE-O) was usually taken as a geometrical probe to indicate enantioselectivity [20]. The dNE-O of Est924 with (R) and (S)-naproxen ethyl ester were measured in the most favorable binding mode. The ΔdNE-O of (R)-naproxen ethyl ester (3.6Å) was 0.5Å shorter than (S)-naproxen ethyl ester (4.1Å), which corresponded to the slight (R)-stereopreference of Est924. Both M1 (A203W) and M3 had a steric hindrance effect on the (R)-isomer, preventing it from being effectively bound. In addition, the molecular docking results also suggested that (S)-naproxen ethyl ester was not affected by the newly generated steric hindrance from large side chain amino acids in M3. However, mutations reduced the activity pockets of enzymes which were not conducive to the entry of substrates, so their activity on (S)-naproxen ethyl ester were reduced. This suggested that the enantioselectivity reversal of the enzyme was due to steric hindrance effect that made it difficult for the enzyme to bind (R) -enantiomer, which was consistent with the results of specific activity tests.

Resolution of racemic ibuprofen ethyl ester and ketoprofen ethyl ester with M3

In addition to (S)-naproxen, (S)-ibuprofen and (S)-ketoprofen are also widely used. A variety of esterases have been used to produce them through the resolution of corresponding racemic esters [18, 21-25]. Est924 could effectively hydrolyze both racemic ibuprofen ethyl ester and ketoprofen ethyl ester (Table 1). The optimal variant (M3) was tested for enantioselective resolution of these racemic esters. The results showed that M3 preferred to hydrolyze their (S)-conformation esters, generating (S)-ibuprofen (85%ee) and (S)-ketoprofen (87%ee), respectively. To our knowledge, Est924 was the first reported esterase that resolved more than two 2-arylpropionate esters with high enantioselectivity. These results showed that we successfully generated Est924 variants with inverted and highly increased enantioselectivity toward ethyl (S)-2-arylpropionates and provided Est924 mutants with potential application in industrial biocatalysts.

Conclusion

In this study, we carefully analyzed the structure of 41 wild-type esterases of the bHSL family. Their crystal structures are highly similar, although with only low to moderate sequence similarity. Several
residues that may reverse and enhance the enantioselectivity of the enzyme toward (S)-2-aryl propionate esters were found through docking (R) and (S)-naproxen ethyl ester into the crystal structure of Est25. Est924, an esterase of the bHSL family, was set as the starting enzyme, because it could hydrolyze a variety of ethyl 2-arylpropionates with light (R)-enantioselectivity. By protein engineering, the key position A2 was substituted by tryptophan, and the substrate binding pockets were reshaped due to its large and hydrophobic side chain; this switched its stereoselectivity to (S)-2-aryl propionate esters. In addition, another key residue at position B1 was mutated to phenylalanine, which further improved the stereoselectivity of the enzyme. Mutant M3 has A2 and B1 di-substitution, could resolve racemic naproxen ethyl ester, ketoprofen ethyl ester and ibuprofen ethyl ester with high (S)-enantioselectivity. It was the first reported esterase that could be used to synthesize (S)-naproxen, dexketoprofen and (S)-ibuprofen, which were the three highly effective and low side effects non-steroidal anti-inflammatory drugs.

**Methods**

**Mutation library construction of Est924 and screening assay**

Selected residues of Est924 were mutated by site mutagenesis using primers in Additional file 1: Table S1 with wild-type Est924 plasmid as a template. The introduction of single site mutations into Est924 was constructed using the QuickChange kit (Stratagene, CA, USA) by following the manufacturer’s instructions. The PCR product was incubated with DpnI (New England Biolabs, MA, USA) at 37 °C for 1 h to separate the parental template. Variants were transformed into *Escherichia Coli* BL21 (DE3) competent cells. The recombinants were picked up and inoculated on lysogeny broth (LB) solid medium containing 10 mM racemic naproxen ethyl ester. We picked recombinants with translucent zones and those with transparent zones smaller than Est924. The standard expression and purification conditions were followed to obtain mutant enzymes. The racemic naproxen ethyl ester was subsequently used as substrate and the enantiomeric excess (ee) of products was analyzed by HPLC. The extracted product was purified by flash column chromatography with petroleum ether and ethyl acetate to separate acid and ester. The acid was esterified by the previously described method [26] and the enantiomeric excess of the product was analyzed using a chiral compound analytical column (Additional file 1: Table S2) mounted on HPLC (Agilent, CA, USA) and UV detector at 214 nm.[27] The enantioselectivity of the esterase was expressed as the enantiomeric ratio (E) and calculation of E-value was performed using the method of Kim et al [11]. Finally, the genes of the enzymes with increased enantioselectivity were verified by DNA sequencing (Sangon Biotech, Shanghai, China).

**Expression and purification of Est924 and variants**

A single colony was picked up and grown in LB supplemented with kanamycin (50 μg/mL). The overnight culture was used to inoculate main cultures (800 mL LB medium containing 50 μg/mL kanamycin) to an initial OD600 of 0.05. Cells were grown at 37 °C to an OD600 of 0.6, and IPTG was added to a final concentration of 0.1 mM. The cultures were incubated at 30 °C for an additional 10 h, and cells were
harvested by centrifugation at 10,800 g at 4 °C for 20 min. Cell pellets were washed with a NaCl solution (0.9%, w/v) and frozen at -20 °C for later purification.

The cell pellets were thawed on ice, resuspended in 10 ml lysis buffer (50 mM phosphate buffer containing 300 mM NaCl and 10 mM imidazole, pH 8.0) per gram of cell pellet and disrupted by sonication (Sonicator QSonica Q500 Ultra Sonicator) at 0 °C. Cell debris was removed by centrifugation at 18,000 g for 30 min at 4 °C. The supernatant was filtered through a 0.25 μm PVDF filter and was loaded onto a Ni-NTA column (Thermo scientific) equilibrated with lysis buffer. The column was then washed with six column volumes of wash buffer (50 mM phosphate buffer containing 300 mM NaCl and 20 mM imidazole, pH 8.0) after loading of the filtered lysate. Finally, the protein was eluted with elution buffer (50 mM phosphate buffer containing 300 mM NaCl and 250 mM imidazole), and fractions containing target enzyme (determined by SDS-PAGE) were pooled and dialyzed at 4 °C against Tris-HCl buffer (20 mM, pH 7.5). Protein expression and purity were assessed by SDS-PAGE (Additional file 1: Figure S1).

**Enzymatic activity assay**

Esterase activity was measured using the standard assay: 50 μg purified esterase was reacted with 10 mM racemic ethyl 2-arylpropionates in 200 mM Tris-HCl buffer (pH 8.0) with 0.1% Triton X-100 for 30 min at 30 °C. The reaction was terminated by adjusting the pH to 2 using 6 M HCl and extract with ethyl acetate. The organic layers were dried over anhydrous Na₂SO₄ and evaporated under a vacuum. Conversion ratio was measured using NMR. The enantiomeric excess of products was analyzed by HPLC as described above.

**Structure modeling and molecular docking**

Homology modeling of Est924, and M3 (I202F/A203W/G208F) were performed using the SWISS-MODEL (https://swissmodel.expasy.org) online system using esterase Est25 (PDB id: 4J7A) as a template (sequence similarity: 49%) [28, 29]. The structure of SsoEst1 was simulated in the same manner using 5LK6 as template (sequence similarity 91.78%). The final models were validated by PROCHECK (http://services.mbi.ucla.edu/PROCHECK) and Verify 3D [30]. Pymol was employed to visualize and analyze the 3D structure of Est924 and its mutants.

Est924 and M3 were docked with (R) and (S)-naproxen using the Surflex-Dock module of Sybyl-X 2.0 program, respectively. All docking calculations were set to equal parameters (20 poses each), only using the pose that was highest ranked by Surflex-Dock.

**Abbreviations**

ee, enantiomeric excess

IPTG, isopropyl-β-d-thiogalactopyranoside
NSAIDs, Non-steroidal anti-inflammatory drugs

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files. The gene sequences of Est924, Est25, SsoEST1 and EST3 were available in the GenBank with accession number of MW460906, Q4TZQ3, A0A3G8EMI7 and Q97VU2, respectively.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

LXL: have analyzed the structures, screened the key residues and done HPLC analysis. ZM: have constructed site mutations, screened clones, and tested activity and selectivity. FXJ: have written and revised the manuscript. FY: have conceived the study, supervised the experiments. All authors have read and approved the manuscript.

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References

1. Lough WJ, Drayer D, Brittain H, Jozwiak K, Wainer I, Cox G, et al. Drug stereochemistry, analytical methods and drug pharmacology. 3rd ed. London: CRC Press; 2012.
2. Toledo MV, Briand LE. Relevance and bio-catalytic strategies for the kinetic resolution of ketoprofen towards dexketoprofen. Crit Rev Biotechnol. 2018; 38:778-800.
3. Tomaszewski J, Rumore MM. Stereoisomeric drugs: FDA’S policy statement and the impact on drug development. Drug Dev Ind Pharm. 1994; 20:119-139.
4. Sheldon RA, Woodley JM. Role of biocatalysis in sustainable chemistry. Chem Rev. 2018; 118:801-838.
5. Romano D, Bonomi F, de Mattos MC, de Sousa Fonseca T, de Oliveira MdCF, Molinari F. Esterases as stereoselective biocatalysts. Biotechnol Adv. 2015; 33:547-565.
6. Sunna A, Hunter L, Hutton CA, Bergquist PL. Biochemical characterization of a recombinant thermoalkalophilic lipase and assessment of its substrate enantioselectivity. Enzyme Microb Technol. 2002; 31:472-476.
7. Kim GJ, Lee EG, Gokul B, Haeh MS, Prenda D, Choi GS, et al. Identification, molecular cloning and expression of a new esterase from Pseudomonas sp. KCTC 10122BP with enantioselectivity towards racemic ketoprofen ethyl ester. J Mol Catal B: Enzym. 2003; 22:29-35.
8. Kim S, Lee SB. Thermostable esterase from a thermoacidophilic archaeon: purification and characterization for enzymatic resolution of a chiral compound. Biosci Biotechnol Biochem. 2004; 68:2289-2298.
9. Sehgal AC, Kelly RM. Strategic selection of hyperthermophilic esterases for resolution of 2-arylpropionic esters. Biotechnol Progr. 2003; 19:1410-1416.
10. Kim J, Kim S, Yoon S, Hong E, Ryu Y. Improved enantioselectivity of thermostable esterase from Archaeoglobus fulgidus toward (S)-ketoprofen ethyl ester by directed evolution and characterization of mutant esterases. Appl Microbiol Biotechnol. 2015; 99:6293-6301.
11. Kim J, Seok Sh, Hong E, Yoo TH, Seo M-d, Ryu Y. Crystal structure and characterization of esterase Est25 mutants reveal improved enantioselectivity toward (S)-ketoprofen ethyl ester. Appl Microbiol Biotechnol. 2017; 101:2333-2342.
12. Ngo TD, Oh C, Mizar P, Baek M, Park K-s, Nguyen L, et al. Structural basis for the enantioselectivity of esterase Est-Y29 toward (S)-ketoprofen. ACS Catal. 2019; 9:755-767.
13. Martínez-Martínez M, Coscolín C, Santiago G, Chow J, Stogios PJ, Bargiela R, et al. Determinants and prediction of esterase substrate promiscuity patterns. ACS Chem Biol. 2018; 13:225-234.
14. Liu YY, Liu XL, Zhao M, Fan XJ. The enzymatic properties of esterase from metagenomic sources and the degradation of phthalates. Acta Sci Natur Univ Sunyatsen. 2020; 59:41-50.
15. Pascale M, Arnaud C. Enzymatic activity and protein interactions in Alpha/Beta hydrolase fold proteins: moonlighting versus promiscuity. Protein Peptide Lett. 2012; 19:132-143.
16. Kim YJ, Choi GS, Kim SB, Yoon GS, Kim YS, Ryu YW. Screening and characterization of a novel esterase from a metagenomic library. Protein Expres Purif. 2006; 45:315-323.
17. Lenfant N, Hotelier T, Velluet E, Bourne Y, Marchot P, Chatonnet A. ESTHER, the database of the α/β-hydrolase fold superfamily of proteins: tools to explore diversity of functions. Nucleic Acids Res. 2013; 41:D423-D429.
18. Yoon S, Kim S, Park S, Hong E, Kim J, Kim S, et al. Improving the enantioselectivity of an esterase toward (S)-ketoprofen ethyl ester through protein engineering. J Mol Catal B: Enzym. 2014; 100:25-31.

19. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res. 2004; 14:1188-1190.

20. Henke E, Bornscheuer UT, Schmid RD, Pleiss J. A molecular mechanism of enantiorecognition of tertiary alcohols by carboxylesterases. ChemBioChem. 2003; 4:485-493.

21. Kim GJ, Choi GS, Kim JY, Lee JB, Jo DH, Ryu YW. Screening, production and properties of a stereospecific esterase from Pseudomonas sp. S34 with high selectivity to (S)-ketoprofen ethyl ester. J Mol Catal B: Enzym. 2002; 17:29-38.

22. Choi GS, Kim JY, Kim JH, Ryu YW, Kim GJ. Construction and characterization of a recombinant esterase with high activity and enantioselectivity to (S)-ketoprofen ethyl ester. Protein Expres Purif. 2003; 29:85-93.

23. Sathishkumar M, Jayabalan R, Mun SP, Yun SE. Role of bicontinuous microemulsion in the rapid enzymatic hydrolysis of (R,S)-ketoprofen ethyl ester in a micro-reactor. Bioresour Technol. 2010; 101:7834-7840.

24. Memarpoor-Yazdi M, Karbalaei-Heidari HR, Doroodmand MM. Enantioselective hydrolysis of ibuprofen ethyl ester by a thermophilic immobilized lipase, ELT, from Rhodothermus marinus. Biochem Eng J. 2018; 130:55-65.

25. López G, Chow J, Bongen P, Lauinger B, Pietruszka J, Streit WR, et al. A novel thermoalkalostable esterase from Acidicaldus sp. strain USBA-GBX-499 with enantioselectivity isolated from an acidic hot springs of Colombian Andes. Appl Microbiol Biotechnol. 2014; 98:8603-8616.

26. Jin JN, Lee SH, Lee SB. Enzymatic production of enantiopure ketoprofen in a solvent-free two-phase system. J Mol Catal B: Enzym. 2003; 26:209-216.

27. Godinho LF, Reis CR, Rozeboom HJ, Dekker FJ, Dijkstra BW, Poelarends GJ, et al. Enhancement of the enantioselectivity of carboxylesterase A by structure-based mutagenesis. J Biotechnol. 2012; 158:36-43.

28. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 2018; 46:W296-W303.

29. Li PY, Zhang Y, Xie BB, Zhang YQ, Hao J, Wang Y, et al. Structural and mechanistic insights into the improvement of the halotolerance of a marine microbial esterase by increasing intra- and interdomain hydrophobic interactions. Appl Environ Microbiol. 2017; 83:e01286-01217.

30. Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, Thornton JM. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR. 1996; 8:477-486.

Tables
### Table 1. Hydrolytic activity of carboxylesterase Est924 for ethyl 2-arylpropionates

| Entry | Substrate                        | Conversion (%) | Specific activity (mM/mg/min) | ee%  |
|-------|----------------------------------|----------------|-----------------------------|------|
| 1     | Ibuprofen ethyl ester            | 95             | 1.5                         | 20%(R)|
| 2     | (S)-Naproxen ethyl ester         | 95             | 3.3                         | N.D. |
| 3     | (R)-Naproxen ethyl ester         | 90             | 3.5                         | N.D. |
| 4     | Ketoprofen ethyl ester           | 97             | 3.5                         | 5%(R)|
| 5     | Flurbiprofen ethyl ester         | 85             | 2.7                         | N.D. |
| 6     | Fenoprofen ethyl ester           | 95             | 3.1                         | N.D. |
| 7     | Zaltoprofen ethyl ester          | 82             | 2.3                         | N.D. |
| 8     | Carprofen ethyl ester            | 82             | 2.4                         | N.D. |
| 9     | Ethyl 2-phenylpropionic acid     | 90             | 2.7                         | 5%(R)|

### Table 2. Key residues in crystal structures of bHSL carboxylesterases
| PDB entry | A1  | A2  | B1  | B2  |
|-----------|-----|-----|-----|-----|
| Est924    | I203| A203| G208| I212|
| 1EVQ      | L205| L206| M210| F214|
| 1JJI      | I209| L210| I214| F218|
| 1JKM      | F259| L260| G264| L268|
| 2C7B      | S205| L206| L210| F214|
| 2YH2      | I208| L209| L213| F217|
| 3AIK      | F197| L198| H202| F206|
| 3D7R      | V202| L203| G207| I211|
| 3DNM      | L206| A207| T211| M215|
| 3GA7      | G215| L216| D220| Y224|
| 3H17      | M193| V194| G198| M202|
| 3QH4      | A209| F210| A214| M218|
| 3WJ1      | F197| L198| H202| F206|
| 4J7A      | F254| L255| T259| M263|
| 4KRY      | G215| L216| D220| Y224|
| 4OB6      | F207| L208| M212| F216|
| 4V2I      | W206| L207| A211| F215|
| 4XVC      | S194| V195| R199| I203|
| 4YPV      | F207| L208| G212| F216|
| 5GMR      | S196| V197| R201| F205|
| 5HC0      | L239| L240| N244| A248|
| 5IQ3      | F254| W255| T259| M263|
| 5JD4      | F209| L210| L214| F218|
| 5JD5      | I219| L220| D224| F228|
| 5L2P      | F203| L204| L208| Y212?|
| 5MII      | G238| L239| E243| A247|
| 6AAE      | Y202| L203| G207| F221|
| 6I8D      | F209| L210| H214| F218|
Table 3. Comparison of enzyme activity and enantioselectivity of Est924 and its mutants

| Entry | Esterase               | Specific activity (mM/mg/min) | E value |
|-------|------------------------|-----------------------------|---------|
|       |                        | (R)-Naproxen | (S)-Naproxen |         |
| 1     | Est924                 | 3.5          | 3.3          | 1.2 (R) |
| 2     | A203W                  | 0.5          | 3.1          | 12 (S)  |
| 3     | G208F                  | 0.8          | 3.2          | 5.6 (S) |
| 4     | A203W/G208F            | 0.04         | 2.6          | 60 (S)  |
| 5     | I202F/A203W/G208F      | 0.02         | 2.5          | 67 (S)  |