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Semi−Rational Engineering a Carbonyl Reductase for the Enantioselective Reduction of β-Amino Ketones

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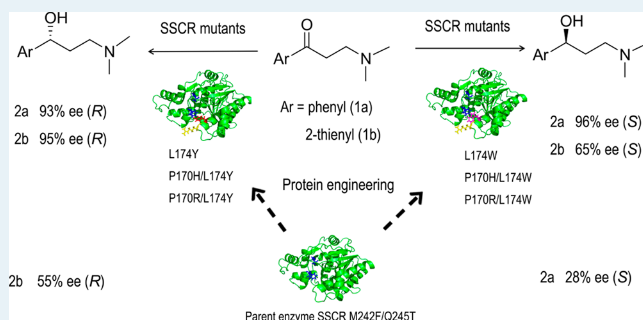
Supporting Information

ABSTRACT: Chiral 3-(dimethylamino)-1-phenylpropan-1-ol (**2a**) and 3-(dimethylamino)-1-(2-thienyl)-1-ol (**2b**) are very important intermediates for the synthesis of antidepressants. The mutant M242F/Q245T of a carbonyl reductase (SSCR) from *Sporobolomyces salmonicolor* AKU4429 catalyzed the reduction of 3-(dimethylamino)-1-phenylpropan-1-one (**1a**) to the (*S*)-**2a** (28% ee). The combinatorial active-site saturation of this enzyme resulted in two mutants P170R/L174Y and P170H/L174Y, which catalyzed the reduction of **1a** and 3-(dimethylamino)-1-(2-thienyl)-propan-1-one (**1b**) to give the (*R*)-γ-amino alcohols with up to 95% ee, respectively. The individual site saturation mutagenesis of Pro170 and Leu174 revealed that Pro170 did not significantly affect the enzyme enantioselectivity toward **1a** and **1b**, whereas residue Leu174 played a critical role in determining the enantioselectivity. Mutant L174W catalyzed the reduction of **1a** to the (*S*)-γ-amino alcohol with increased enantioselectivity from 28% ee to 96% ee, although mutant L174Y exhibited (*R*)-preference in 88% ee. For **1b**, the (*R*)-alcohol was obtained with 95% ee by using variant L174Y as the catalyst, whereas L174W exhibited (*S*)-preference in 65% ee. The kinetic studies indicated that catalytic efficiencies (k_{cat}/K_m) of these mutants were also improved. The enzyme−substrate docking provided some insights into the structural basis for the reversal of enantioselectivity by the substitution of Leu174.

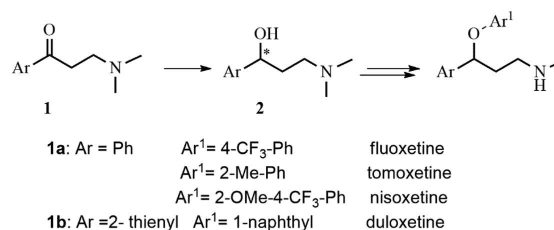


KEYWORDS: *chiral γ-amino alcohols, β-amino ketones, enzyme engineering, carbonyl reductase, enantioselective reduction*

INTRODUCTION

Chiral γ-amino alcohols are valuable building blocks for the synthesis of a class of medications referred to as selective serotonin reuptake inhibitors, which are the key antidepressant drugs in the medicinal chemistry,[1] such as fluoxetine, tomoxetine, nisoxetine, and duloxetine. This has inspired considerable efforts to search for methods of producing these γ-amino alcohols in optically pure form. Among the various approaches, the enantioselective reduction of β-amino ketones provides a straightforward method to access this class of chiral compounds. The asymmetric reduction of the prochiral β-amino ketones by chiral metal complexes or organocatalysts has been described.[2] For example, *trans*-$RuCl_2$[(*R*)-xylbinap][(*R*)-daipen] catalyzed the reduction of 3-(dimethylamino)-1-phenylpropan-1-one (**1a**) with up to 97.5% ee.[2d] However, their applications have been limited because of stringent regulatory restrictions on the level of heavy metals allowed in pharmaceutical products[3] and/or the unsatisfactory selectivity including stereoselectivity. It has been reported that the engineered ketoreductases catalyzed the conversion of β-amino ketone **1b** (Scheme 1) to the corresponding (*S*)-alcohol (**2b**) for the synthesis of duloxetine.[4] However, the patent revealed limited insight into this important enzymatic transformation, and there is no report addressing the biocatalytic reduction of other β-amino ketones, such as **1a** to the chiral alcohol **2a**, which is of high significance as a building block for production of tomoxetine, nisoxetine, and so on.

Scheme 1. Structures of β-Amino Ketones, γ-Amino Alcohols, and Related Drugs



Therefore, we initiated the search for carbonyl reductases for the biocatalytic reduction of β-amino ketones such as **1a**. The carbonyl reductases available in our laboratory were screened

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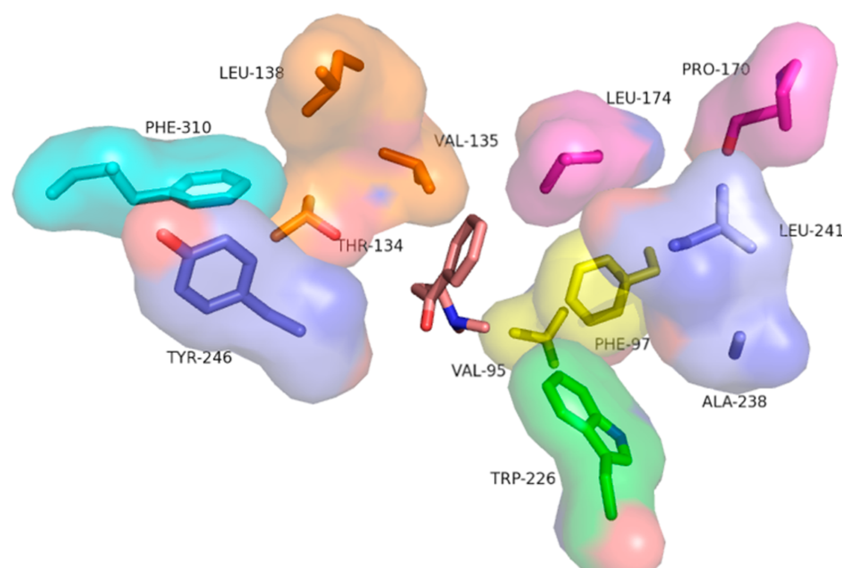

Figure 1. Docking of **1a** into the structure of the variant M242F/Q245T. The target amino acid residues of libraries A to F are shown in yellow, orange, green, magenta, blue, and cyan, respectively.

using **1a** as the substrate. Out of about 20 carbonyl reductases, only one mutant M242F/Q245T of the carbonyl reductase (SSCR) from *Sporobolomyces salmonicolor* AKU4429[5] showed detectable activity and catalyzed the reduction of **1a** to give (*S*)-**2a** in 28% ee. As such, the variant M242F/Q245T was chosen as the starting template for the enzyme engineering by using the combinatorial active-site saturation test (CAST) strategy, which has been proven to be successful in engineering enzymes,[6] in an effort to improve the catalytic activity and/or enantioselectivity of variant M242F/Q245T for the reduction of **1a**.

RESULTS AND DISCUSSION

The structure of SSCR variant M242F/Q245T was obtained in silico on the basis of the corresponding mutations of the crystal structure of wild-type SSCR containing a coenzyme NADPH (PDB code 1Y1P)[7] by using the build mutant program in the Accelrys Discovery Studio 2.5. In order to choose the amino acid residues for saturation mutagenesis, docking of **1a** into the structure of SSCR variant M242F/Q245T was performed with CDOCKER program in the Discovery Studio 2.5. Structural analysis of SSCR indicated that the two largest potential receptor sites were in the vicinity of the NADPH. Residues close to one of the (catalytic) receptor site were Val95, Phe97, Ser133, Thr134, Val135, Leu138, Tyr177, Pro206, Asn207, Tyr208, Trp226, Ala238, Tyr246, and Phe310. The other receptor sites (Phe97, Pro170, Leu174, Trp226, Ala238, and Leu241) were akin to the hydrophobic channel, which were proposed to play not only a role in allowing access to the active site but also in substrate recognition.[7] Among these residues, Tyr177, Lys181, Pro206, Asn207, and Tyr208 also participated in recognizing and binding the nicotinamide ring, and residues Tyr177, Ser133, and Lys181 formed the catalytic triad. These amino acid residues were excluded from the mutagenesis, whereas another 12 residues (Val95, Phe97, Thr134, Val135, Leu138, Pro170, Leu174, Tyr226, Ala238, Leu241, Tyr246, Phe310, Figure 1) were chosen as the target amino acid residues for mutation. Cooperative mutagenesis of the adjacent residues would more effectively affect the local configuration of secondary protein structure than individual residue substitution. For more configuration diversity of hydrophobic channel and binding pocket of SSCR, the 12 selected amino acid residues were grouped into six sets according to the structure of SSCR, namely, A (Val95/Phe97), B (Thr134/Val135/Leu138), C (Tyr226), D (Ala238/Leu241/Y246), E (Pro170/Leu174), and F (Phe310) (Figure 1).

To reduce the screening effort, we opted for NDT codon degeneracy encoding 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly) with diverse characteristic side chains. Six focused libraries at the corresponding sites were created separately and screened using **1a** as the substrate. The activities of the transformants were determined by spectrophotometrically measuring the absorbance of NADPH at 340 nm, and subsequently, the enantioselectivities of the selected mutants with higher activity than the parent enzyme were measured by chiral HPLC analysis. The absolute configurations of product alcohols were assigned by comparing the retention times with standard samples.[8] Compared to the low catalytic activity and enantioselectivity of parent enzyme, the clones with the enhanced activity and ee value were selected for further studies.

Two positive mutants P170R/L174Y and P170H/L174Y from library E (P170/L174) exhibited inverse enantiomeric preference toward substrate **1a** (Table 1). The parent enzyme catalyzed the reaction to give (*S*)-**2a** in 28% ee, whereas mutant P170R/L174Y and P170H/L74Y catalyzed the reduction of **1a**

Table 1. Enantioselectivity of Mutant SSCRs

mutants	1a	1b
parent	28 (*S*)	55 (*R*)
P170R	30 (*S*)	59 (*R*)
P170H	27 (*S*)	60 (*R*)
L174Y	88 (*R*)	95 (*R*)
P170R/L174Y	93 (*R*)	95 (*R*)
P170H/L174Y	57 (*R*)	95 (*R*)
L174W	96 (*S*)	65 (*S*)
P170R/L174W	96 (*S*)	65 (*S*)
P170H/L174W	96 (*S*)	61 (*S*)

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Table 2. Kinetic Parameters of the Template and Mutant Enzymes

	1a			1b		
enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
template	3.21 ± 0.19	8.01 ± 0.22	2.50	1.68 ± 0.09	3.14 ± 0.06	1.87
L174Y	1.48 ± 0.13	12.40 ± 0.42	8.42	1.81 ± 0.12	15.57 ± 0.42	8.62
L174W	1.02 ± 0.09	6.89 ± 0.20	6.77	1.18 ± 0.10	1.28 ± 0.03	1.09
P170R/L174Y	1.60 ± 0.08	13.30 ± 0.25	8.31	1.05 ± 0.03	14.80 ± 1.12	14.04
P170H/L174Y	0.99 ± 0.05	9.18 ± 0.17	9.28	0.84 ± 0.09	8.55 ± 0.28	10.14
P170H/L174W	2.98 ± 0.34	4.51 ± 0.46	1.51	3.39 ± 0.32	1.03 ± 0.03	0.30
P170R/L174W	0.98 ± 0.01	4.52 ± 0.14	4.61	3.54 ± 0.54	0.95 ± 0.05	0.27

to (*R*)-**2a** with ee values of 93% and 57%, respectively. For substrate **1b** (Table 1), the product alcohol (*R*)-**2b** was obtained by using the parent enzyme (55% ee) and two mutants (95% ee). The two positive mutants shared one single point mutagenesis of Leu174 to Tyr, while the proline at position 170 was mutated to Arg or His. The screening of the other libraries failed to harvest any mutant with significantly improved enantioselectivity and activity.

These results inspired us to investigate which of the two amino acid residues had such a remarkable effect or if they had synergic effect on the enzyme enantioselectivity. As such, residues Pro170 and Leu174 were individually substituted with the other 19 amino acids, and the enantioselectivity of each variant toward substrate **1a** and **1b** was measured, except P170C, which showed no catalytic activity; the other 18 substitutions of Pro170 did not exert significant effect on the enantioselectivity toward these two substrates (Figure S2A,C). In contrast, the mutation of residue Leu174 greatly affected the enantioselectivity of the enzyme and resulted in both highly *R*- and *S*-selective mutants toward substrates **1a** and **1b** (Figure S2B,D).

As shown in Table 1, the reduction of **1a** catalyzed by mutant L174Y gave (*R*)-enantiomer with 88% ee, while (*S*)-configuration product was obtained for all other mutants with up to 96% ee in the case of L174W as the biocatalyst. For the reduction of the ketone **1b**, the ee value of the corresponding *R*-alcohol was significantly increased from 55% to 95% when Leu174 was substituted with Tyr. However, mutant L174W gave the opposite (*S*)-enantiomer with moderate enantioselectivity (65% ee). Therefore, the stereochemistry of carbonyl reductase SSCR toward substrates **1a** and **1b** was effectively enhanced or inverted by change of only one residue at position 174.

It is interesting that the double mutant P170R/L174Y was highly *R*-selective (93% ee) for the reduction of **1a**, while the two corresponding single mutants P170R and L174Y were *S*-selective (30% ee) and *R*-selective (88% ee), respectively. Instead of offsetting the enantioselectivity of L174Y by P170R, an enhanced effect was observed. This was likely attributed to the cooperative effect of the two spatially adjacent amino acids. Similar phenomenon was observed for esterase by Bartsch et al.,[9] in which the double mutant E188W/M193C led to (*S*)-selective (E = 64), whereas the single mutant M193C was (*R*)-selective (E = 16) and E188W exhibited *S* selectivity (E = 26). On the other hand, the combination of P170H (27% ee, *S*) and L174Y (88% ee, *R*) led to a (*R*)-selective mutant with 57% ee, with P170H offsetting the enantioselectivity of L174Y. For the reduction of **1b**, both P170R/L174Y and P170H/L174Y exhibited same enantioselectivity with L174Y, indicating the absence of effect by P170R and P170H (Table 1).

Because mutant L174W greatly improved the enantioselectivity of the carbonyl reductase for the reduction of **1a** and inverted the enantiomeric preference for the reduction of **1b**, the double mutants P170R/L174W and P170H/L174W were created, and their enantioselectivities toward the reduction of **1a** and **1b** were evaluated. As shown in Table 1, P170R and P170H did not affect the enantioselectivity of L174W when they combined with L174W.

In order to assess the effect of the amino acid substitutions on the enzyme activities toward the *β*-amino ketones **1a** and **1b**, kinetic studies were conducted with the template reductase and mutants L174W, L174Y, P170R/L174Y, P170H/L174Y, P170R/L174W, and P170H/L174W. The K_m and k_{cat} values were calculated by nonlinear fitting, and their overall catalytic efficiencies (k_{cat}/K_m) toward **1a** and **1b** were presented in Table 2. For substrate **1a**, mutants P170R/L174W, L174W, L174Y, P170R/L174Y, and P170H/L174Y exhibited about 1.8−3.7-fold elevated k_{cat}/K_m values relative to the template enzyme, which was mainly contributed by the lower K_m. The kinetic analysis of substrate **1b** showed that L174Y, 170R/L174Y, and P170H/L174Y exhibited higher overall catalytic efficiency with up to 7.5-fold k_{cat}/K_m of the template for P170R/L174Y, while mutant L174W, P170H/L174W, and P170R/L174W had lower k_{cat}/K_m than the parent enzyme. In contrast to **1a**, the enhancement of the overall catalytic efficiency for the reduction of **1b** by these mutants was due in large part to their k_{cat}. These results suggest that residue Leu174 not only manipulate the enantioselectivity but also affect the enzyme activity toward the reduction of these two *β*-amino ketones. The mutant P170R/L174Y was applied as the biocatalyst for the reduction of **1a** and **1b**; (*R*)-**2a** and (*R*)-**2b** were obtained with 81% and 72% isolated yield, respectively.

According to the proposed catalytic mechanism of SSCR, the carbonyl oxygen atom of the substrate forms hydrogen bonds with both Tyr and Ser residues of catalytic triad Ser133-Tyr177-Lys181, resulting in its protonation by the Tyr residue, followed by the attacking of a hydrogen atom from the C^4 atom of NADPH to the carbonyl carbon atom of the substrate. In an effort to try to understand how the single mutation at residue 174 resulted in such a drastic change of enantioselectivity toward the reduction of *β*-amino ketones, the substrates **1a** and **1b** were docked into the structures of variants M242F/Q245T/L174W and M242F/Q245T/L174Y using the CDOCKER program with default settings, respectively. The structures of the mutant enzymes were obtained in silico by the mutation of wild-type in the target position with Accelrys Discovery Studio 2.5. The lowest-energy docked conformations of three enzyme−substrate complexes **1a**-NADPH-L174Y, **1a**-NADPH-L174W and **1b**-NADPH-L174Y (with relatively high enantioselectivity) selected by using the score functions of CDOCKER program are shown in Figure 2A−C, respectively.

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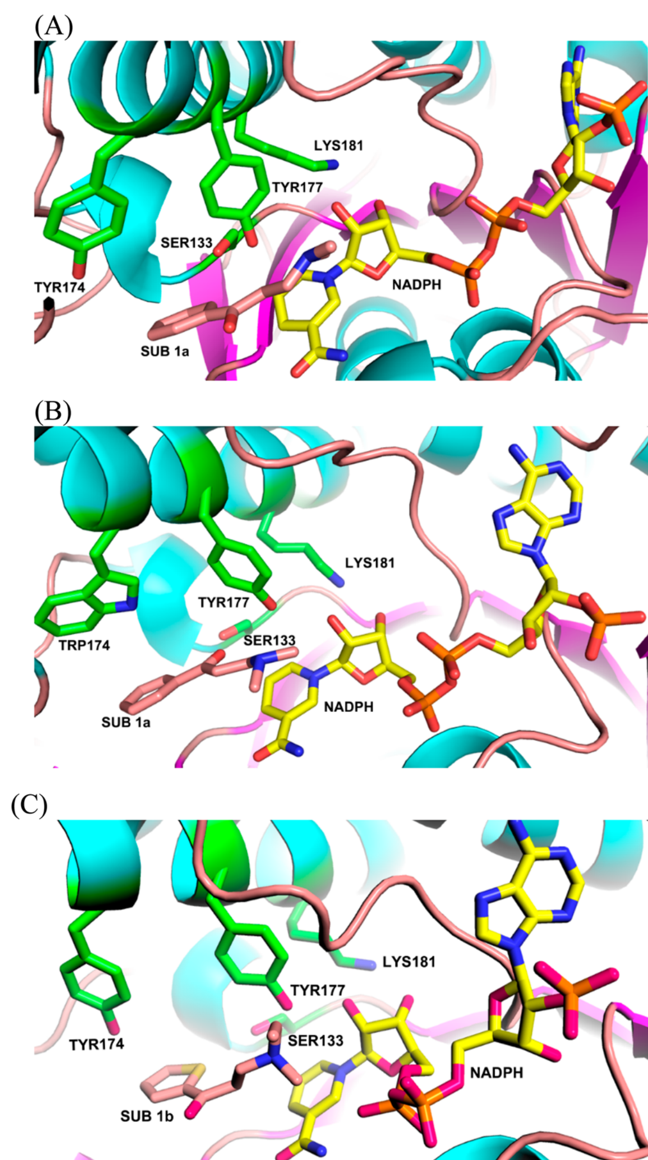

Figure 2. Docking of the substrates into the active sites of SSCR mutants. (A, B) The carbonyl oxygen atom of substrate **1a** is accommodated in a substrate-binding pocket in opposite orientations in the two mutants (toward the front and toward the back, respectively). (C) The orientation of **1b** accommodated is same to that of **1a** in L174Y.

As elucidated in the catalytic mechanism of SSCR,[5a−c,7] the configuration of reduced product is determined by attack of the nicotinamide hydride of NADPH from either the *Si*- or *Re*-face of the ketone, which leads to (*R*)- or (*S*)-alcohol, respectively.[10] In the docking studies of substrate **1a** into the SSCR enzymes, the orientation of **1a** in the template was relatively more flexible than the mutant L174W, both the *Si*- and *Re*-face attacks of hydride on the carbonyl group were possible in the least-energy conformations. This is consistent with the low enantioselectivity (28% *S*) observed for the reduction of **1a** catalyzed by the template enzyme. The nicotinamide ring of cofactor NADPH was located at the *Si*-face of **1a** in the mutant L174Y (Figure 2A), but at the *Re*-face of **1a** in the mutant L174W (Figure 2B), thus leading to the corresponding *R* and *S*-alcohol, respectively. This is in agreement with the experimental observations. The different orientations of substrate **1a** in the active site involved the opposite positioning of the oxygen of the carbonyl group. For the mutant L174W, the carbonyl oxygen was proximal to the hydrophilic portion of the catalytic site but distal in the mutant L174Y. Generally, the orientation of carbonyl oxygen away from the catalytic residues would result in the loss of activity. Although carbonyl oxygen of **1a** was distal to Tyr177, but approximate to Tyr174 in the mutant L174Y, it was thus speculated that Tyr174 might serve as the role of Tyr177 in this case.

The least-energy conformation of substrate **1b** docked into the active site of mutant L174Y (Figure 2C) showed that the NMN of the cofactor was located at the *Si*-face of **1b**, thus leading to the *R*-alcohol, which is in agreement with our experimental observation. The impact of Tyr174 on **1b** was probably the same as that toward **1a** in mutant L174Y. When the substrate **1b** was docked into the active site of the template and mutant L174W enzymes, the substrate showed relative flexible orientation in the least-energy conformations, in which the NMN of the cofactor was located at either the *Re* face or *Si* face of the ketone, and thus, lower ee values were observed for the reduction of **1b** by the template (55% ee) and mutant L174W (65% ee) enzymes. The observed alteration in stereochemistry of the reductions of **1a** and **1b** by these enzymes indicated that the substitution Leu174 with steric bulky Tyr or Trp played a significant role in determining the substrate binding. This was conjectured to be due to the hydrophobic/van der Waals forces between residue 174 and sterically hindered β-amino ketones.

In summary, two mutants P170R/L174Y and P170H/L174Y with inverted enantioselectivity and enhanced activity toward β-amino ketone **1a** and **1b** were obtained from mutant M242F/Q245T of SSCR by using the CAST strategy. They catalyzed the reduction of **1a** and **1b** to produce (*R*)-**2a** and (*R*)-**2b** with high ee, respectively. Substitution studies of P170 and L174 showed that residue 174 was critical for the alteration of stereochemistry, whereas residue 170 exerted a minimal effect on the enantioselectivity of SSCR toward **1a** and **1b**. The docking studies indicated that the close interaction between the substrate and the large steric side chain of Trp or Tyr likely contributed to the substrate orientation and thus control the reaction stereochemistry. It is remarkable that a single mutation results in such dramatic change in the enantioselectivity. Although combinational mutations of other enzymes have successfully resulted in reversal in stereoselectivity,[6d,f,11] there are few examples of reversed enzyme enantiopreference by one single-point mutation in the literature,[12,13] not to mention the carbonyl reductase.[5c,14] This study not only provides valuable information for our understanding of how to alter the enantioselectivity of this enzyme but also offers a promising starting point for further addressing the challenge in the reduction of sterically hindered β-amino ketones such as **1a** and **1b** to meet the needs of synthetic application.

EXPERIMENTAL SECTION

Enzyme−Substrate Docking. The structures of SSCR mutants were constructed based on the crystal structure of SSCR (PDB ID: 1Y1P) using the build mutants package in the Accelrys Discovery Studio 2.5 (Accelrys Inc.). The receptor proteins (SSCR mutants) were prepared by eliminating all bound water molecules, and the hydrogen atoms were added to the enzyme molecule, which was optimized by applying the force field CHARMm. The binding sphere was defined within 10.0 Å of residues Ser133, Tyr177, and Lys181 in the receptor

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protein molecule. The substrate molecules were also optimized by applying the force field CHARMm. The molecular docking procedure was performed by using CDOCKER protocol with the default settings. The molecular docking poses were ranked according to score functions which were used to predict their binding affinities and conformations of the molecules at the active site of enzymes.

Mutant Library Construction. The libraries A, B, C, D, E, and F at the corresponding sites were created by the Quick Change PCR method with the template plasmid pET22b containing the SSCR mutant M242FQ245T gene and the primers was listed in Supporting Information Table S1. PCR was carried out with KOD polymerase under the following conditions: the reaction was started at 95 °C (2 min), followed by 20 cycles — 94 °C (30 s), 55 °C (30 s), 68 °C (6 min 48 s), with a final extension at 68 °C (10 min). The reaction was carried out in a 25 μL reaction volume containing 0.2 mM of both primers, 50 ng genomic DNA, 1.0 mM MgSO4, 0.2 mM dNTP, and 1U of KOD polymerase. The PCR product was digested with *Dpn*I restriction enzyme and transformed into *E. coli* BL21(DE3) competent cell by electroporation.

Screening of Mutant Libraries. Each of the mutant colonies along with the colonies expressing the parent gene were grown in 150 μL of Luria−Bertani (LB) medium, supplemented with 100 μg/mL ampicillin at 37 °C for 12 h in a 96-well microplate. For each colony, 4 μL of the overnight culture was diluted into 300 μL of LB medium containing 100 μg/mL ampicillin and was incubated at 37 °C with shaking at 200 rpm until the optical density reached 1.2 (about 2 h). After being induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) and incubated at 37 °C for 6 h, the cultures were centrifuged at 4000 rpm and 4 °C for 30 min, and the supernatants were discarded. Each cell pellet was resuspended in 150 μL of potassium phosphate buffer (100 mM, pH 7.0) and lysed by sonication. Cell debris was precipitated by centrifugation at 4000 rpm and 4 °C for 30 min. Fifty microliters of each supernatant was used in the activity assays by spectrophotometrically monitoring the disappearance of NADPH at 340 nm with **1a** as the substrate. The colonies, which showed higher activity than the template SSCR enzyme, were subjected to further study, and their enantioselectivity toward **1a** and **1b** were determined.

Site-Directed Mutation. Site-directed mutagenesis at the sites P170 and L174 was performed with pET22b containing the SSCR M242FQ245T gene as template and pairwise primers in Supporting Information Table S2. The mutation method was the same as that described in the Mutant Library Construction section.

Purification of SSCR Mutant Enzymes. His-tagged proteins were purified on Ni-NTA column materials using an AKTA purifier 10 system with UNICORN 5 software (GE Healthcare). *E. coli* cells expressing SSCR mutant genes were incubated in LB medium containing 100 μg/mL ampicillin at 37 °C until the optical density reached 0.6 at 600 nm. After being induced by 0.1 mM IPTG and incubated at 30 °C for another 6 h, the cells were harvested by centrifugation at 4000 rpm at 4 °C for 30 min. Cell pellets were resuspended in potassium phosphate buffer (100 mM pH 8.0) containing 5% glycerin and 500 mM NaCl and were disrupted by a High Pressure Homogenizer. The clarified cell lysate was bound to the resin and then purified by column chromatography. Protein-bound resin was washed with potassium phosphate buffer (100 mM pH 8.0) containing 5% glycerin and 500 mM NaCl. The target protein was eluted with the same buffer solution but adding 500 mM imidazole. The collected fraction was desalted through dialyzing against potassium phosphate buffer (100 mM, pH 8.0). The purified proteins were collected and stored at 4 °C.

Measurement of Enantioselectivity. The enantioselectivity of the enzymatic reduction of ketones was studied by using an NADPH recycle system. The general procedure was carried out as follows: D-glucose (12 mg), D-glucose dehydrogenase (1 mg), $NADP^+$ (0.5 mg), the carbonyl reductase (500 μL), and 10 mM ketone were mixed in a potassium phosphate buffer (1 mL, 100 mM, pH 8.0), and the mixture was shaken overnight at room temperature. The mixture was adjusted to pH 10.0 with the addition of saturated sodium carbonate and extracted with ethyl acetate (1 mL). The organic solvent was removed, and the residue was dissolved in the mixture of hexane and isopropanol (90:10). The resulting solution was subjected to chiral HPLC analysis to determine ee value. Chiral HPLC analysis was performed on an Agilent 1200 series high-performance liquid chromatography (HPLC) system with AD-H column (25 cm × 4.6 mm, Daicel, Inc.). The absolute configuration of the product alcohol was identified by comparing the retention time of chiral HPLC with literature data.[2]

Kinetic Assay. The kinetic parameters were obtained by measuring the initial velocities of the enzymatic reaction and curve-fitting according to the Michaelis−Menten equation using GraphPad Prism 5 software (GraphPad Software Inc.). The enzyme activity for ketone reduction was determined by spectrophotometrically monitoring the absorbance of NADPH at 340 nm at room temperature with a molar extinction coefficient of 6220 M^{-1} cm^{-1}. The reaction was carried out in potassium phosphate buffer (100 mM, pH 8.0) with 0.5 mM NADPH. The substrate concentration range was between 0.2 and 10 mM.

Preparation of (*R*)-2a and (*R*)-2b. The *E. coli* cells expressing mutant P170R/L174Y gene were obtained as described above, and the cell free lysates were used as the biocatalyst for the reduction of substrate **1a** and **1b**. The substrate (213 mg **1a** hydrochloride or 219 mg **1b** hydrochloride), 20 mg $NADP^+$, 20 U D-glucose dehydrogenase (1 U was defined as the enzyme converting 1 μmol of $NADP^+$ to NADPH per minute with D-glucose as the substrate), and 600 mg D-glucose was added into 100 mL cell free lysate (59 U, 1 U was defined as the enzyme converting 1 μmol of NADPH to $NADP^+$ per minute with **1a** as the substrate). The resultant mixture was shaken overnight at room temperature. After the pH was adjusted to 10.0 with the addition of saturated sodium carbonate, the mixture was extracted with ethyl acetate (50 mL × 3). The organic layer was washed by saturated NaCl and dried over anhydrous Na_2SO_4. The solvent was removed to afford 146 mg (81% yield) **2a** or 134 mg (72% yield) **2b**. *R*-**2a** $[\alpha]_D$ = +28.8° (c 1.2, CH_3OH), lit[2c] $[\alpha]_D$ = +32.0° (c 1.7, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 1.79−1.85 (m, 2H), 2.30 (s, 6H), 2.44 (ddd, *J* = 4 and 8 Hz, 1H), 2.64 (ddd, *J* = 4 and 8 Hz, 1H), 4.92 (dd, *J* = 4 and 8 Hz, 1H), 7.39−7.42 (m, 5H). *R*-**2b:** $[\alpha]_D$ = +7.7° (c 2.0, CH_3OH), standard sample $[\alpha]_D$ = +8.0° (c 1.0, CH_3OH), . ^1H NMR (400 MHz, $CDCl_3$) δ 1.92−1.96 (m, 2H), 2.29 (s, 6H), 2.55 (ddd, *J* = 4 and 8 Hz, 1H), 2.62 (ddd, *J* = 4 and 8 Hz, 1H), 5.17 (dd, *J* = 4 and 8 Hz, 1H), 6.91−7.21 (m, 3H). The ^1H NMR spectra were shown in Figure S4.

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■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b00226.

Primers used in the construction of the mutant libraries and site-directed mutagenesis, enantioselectivities of the parent enzyme and its 19 variants at positions 170, 174 toward **1a** and **1b**, HPLC chromatograms of racemate standard samples of **2a**, **2b**, and the reduction products **2a**, **2b**, and the ^{1}H NMR spectrum of **2a** and **2b**.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Wolfgang, B.; Robert, S. U.S. Patent 20060009533A1, January 12, 2006. (b) Mariappan, P.; Alhasso, A.; Ballantyne, Z.; Grant, A.; N'Dow, J. *Eur. Urol.* **2007**, *51*, 67–74. (c) Robertson, D. W.; Krushinski, J. H.; Fuller, R. W.; Leander, J. D. *J. Med. Chem.* **1988**, *31*, 1412–1417. (d) Srebnik, M.; Ramachandran, P. V.; Brown, H. C. *J. Org. Chem.* **1988**, *53*, 2916–2920. (e) Wong, D. T.; Bymaster, F. P.; Horng, J. S.; Molloy, B. B. *J. Pharmacol. Exp. Ther.* **1975**, *193*, 804–811. (f) Wong, D. T.; Horng, J. S.; Bymaster, F. P.; Hauser, K. L.; Molloy, B. B. *Life Sci.* **1974**, *15*, 471–479. (g) Wong, D. T.; Robertson, D. W.; Bymaster, F. P.; Krushinski, J. H.; Reid, L. R. *Life Sci.* **1988**, *43*, 2049–2057.

(2) (a) Wang, J. H.; Liu, D. L.; Liu, Y. G.; Zhang, W. B. *Org. Biomol. Chem.* **2013**, *11*, 3855–3861. (b) Liu, D.; Gao, W.; Wang, C.; Zhang, X. *Angew. Chem., Int. Ed.* **2005**, *44*, 1687–1689. (c) Ohkuma, T.; Koizumi, M.; Muñiz, K.; Hilt, G.; Kabuto, C.; Noyori, R. *J. Am. Chem. Soc.* **2002**, *124*, 6508–6509. (d) Traff, A.; Lihammar, R.; Backvall, J. E. *J. Org. Chem.* **2011**, *76*, 3917–3921. (e) Zhao, J. F.; Dou, H. J.; Zhou, Y. H.; Qu, J. P. *Chem. J. Chinese U.* **2011**, *32*, 2331–2334. (d) Ohkuma, T.; Ishii, D.; Takeno, H.; Noyori, R. *J. Am. Chem. Soc.* **2000**, *122*, 6510–6511.

(3) Thayer, A. *Chem. Eng. News* **2005**, *83*, 55–58.

(4) Savile, C.; Gruber, J. M.; Mundorff, E.; Huisman, G. W.; Collier, S. J. Patent No. WO2010025238, July 29, 2010.

(5) (a) Zhu, D.; Hua, L. *J. Org. Chem.* **2006**, *71*, 9484–9486. (b) Zhu, D.; Yang, Y.; Buynak, J. D.; Hua, L. *Org. Biomol. Chem.* **2006**, *4*, 2690–2695. (c) Zhu, D.; Yang, Y.; Majkowicz, S.; Pan, T. H.-Y.; Kantardjieff, K.; Hua, L. *Org. Lett.* **2008**, *10*, 525–528. (d) Li, H.; Zhu, D.; Hua, L.; Biehl, E. R. *Adv. Synth. Catal.* **2009**, *351*, 583–588. (e) Li, H. M.; Yang, Y.; Zhu, D. M.; Hua, L.; Kantardjieff, K. *J. Org. Chem.* **2010**, *75*, 7559–7564.

(6) (a) Reetz, M. T.; Bocola, M.; Carballeira, J. D.; Zha, D.; Vogel, A. *Angew. Chem., Int. Ed.* **2005**, *44*, 4192–4196. (b) Reetz, M. T.; Carballeira, J. D.; Peyralans, J.; Hobenreich, H.; Maichele, A.; Vogel, A. *Chem. - Eur. J.* **2006**, *12*, 6031–6038. (c) Reetz, M. T.; Wang, L. W.; Bocola, M. *Angew. Chem., Int. Ed.* **2006**, *45*, 1236–1241. (d) Reetz, M. T.; Zheng, H. *ChemBioChem* **2011**, *12*, 1529–1535. (e) Agudo, R.; Roiban, G. D.; Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 1665–1668. (f) Wu, Q.; Soni, P.; Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 1872–1881.

(7) Kamitori, S.; Iguchi, A.; Ohtaki, A.; Yamada, M.; Kita, K. *J. Mol. Biol.* **2005**, *352*, 551–558.

(8) Zhu, Q.; Shi, D.; Xia, C.; Huang, H. *Chem. - Eur. J.* **2011**, *17*, 7760–7763.

(9) Bartsch, S.; Kourist, R.; Bornscheuer, U. T. *Angew. Chem.* **2008**, *120*, 1531–1534.

(10) Cundari, T. R.; Dinescu, A.; Zhu, D.; Hua, L. *J. Mol. Model.* **2007**, *13*, 685–90.

(11) (a) Svedendahl, M.; Branneby, C.; Lindberg, L.; Berglund, P. *ChemCatChem* **2010**, *2*, 976–980. (b) Humble, M. S.; Cassimjee, K. E.; Abedi, V.; Federsel, H.-J.; Berglund, P. *ChemCatChem* **2012**, *4*, 1167–1172.

(12) (a) Mildvan, A. S. *Biochemistry* **2004**, *43* (46), 14517–14520. (b) Reetz, M. T. *Angew. Chem., Int. Ed.* **2013**, *52*, 2658–2666. (c) Serrano, L.; Horovitz, A.; Avron, B.; Bycroft, M.; Fersht, A. R. *Biochemistry* **1990**, *29*, 9343–9352. (d) Zheng, H.; Reetz, M. T. *J. Am. Chem. Soc.* **2010**, *132*, 15744–15751.

(13) (a) Antipov, E.; Cho, A. E.; Klibanov, A. M. *J. Am. Chem. Soc.* **2009**, *131*, 11155–11160. (b) Smith, M. E. B.; Hibbert, E. G.; Jones, A. B.; Dalby, P. A.; Hailes, H. C. *Adv. Synth. Catal.* **2008**, *350*, 2631–2638.

(14) Musa, M. M.; Lott, N.; Laivenieks, M.; Watanabe, L.; Vieille, C.; Phillips, R. S. *ChemCatChem* **2009**, *1*, 89–93.

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