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Molecular Basis for the High Activity and Enantioselectivity of the Carbonyl Reductase from *Sporobolomyces salmonicolor* toward α-Haloacetophenones

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ABSTRACT: In an effort to develop a practical method for the synthesis of optically pure 2,2,2-trifluoro-1-phenylethanol, we found that the carbonyl reductase (SSCR) from *Sporobolomyces salmonicolor* showed excellent activity and enantioselectivity toward the halogenated acetophenones. Especially, SSCR exhibited more than 1000 times higher activity toward α,α,α-trifluoroacetophenone than unsubstituted acetophenone, a strikingly different observation from the previously well-studied alcohol dehydrogenase (LBADH) from *Lactobacillus brevis*. Enzyme-substrate docking and site-directed mutagenesis studies revealed the molecular basis for the high enzyme activity and enantioselectivity of SSCR toward the α-halogenated

acetophenones. The hydrogen bond of the Asn207 side chain with the substrate halogen atom and the XH/π interaction of the substrate phenyl group with the side chains of Ser222/Thr223 resulted in the formation of the highly reactive conformation of α-halogenated acetophenones in the active site of the enzyme. (*S*)-2,2,2-Trifluoro-1-phenylethanol was prepared in excellent isolated yield and enantiomeric excess from the reduction of α,α,α-trifluoroacetophenone with mutant T209A. These results suggest that tuning the interactions between the halogen atoms/phenyl group of the substrate and the amino acid residues of enzyme would lead to valuable mutants for the practical synthesis of β-haloalcohols.

KEYWORDS: α-haloacetophenones, carbonyl reductase, enzymatic reduction, site-directed mutagenesis, substrate-binding mechanism

Introduction

Stereoselective reduction of aromatic ketones with α-halo groups is of particular interest, since the corresponding chiral halohydrins (β-haloalcohols) are key-intermediates in the synthesis of a number of biologically active compounds.[1-4] Chemical asymmetric reduction of mono- or multi- halomethyl ketones has been widely studied. For example, chiral oxazaborolidine,[5] Ru-complex,[6-7] Ir-complex,[8] Fe-complex,[9] Corey–Bakshi–Shibata (CBS) catalyst[10] and cinchonine-modified Pt/Al_2O_3[11] have been used as the catalysts for the asymmetric reduction of α,α,α-trifluoroacetophenone, and the enantiomeric excess (ee) value of chiral alcohol

ranges from 8% to 99%. Recently, carbonyl reductases (alcohol dehydrogenases) have also been extensively used in the synthesis of chiral alcohols, and proved to be a powerful tool.[12-15] Carbonyl reductases from *Pseudomonas* sp.[16] *Kluyveromyces thermotolerans*,[17] *Rhodococcus ruber*,[18] *Lactobacillus brevis*[18] and *Scheffersomyces stipitis* CBS 6045[19] have been used as the biocatalyst for the reduction of α,α,α-trifluoroacetophenone, and the range of the product ee value is 50 to 99%. The highest specific activity reported for the reduction of α,α,α-trifluoroacetophenone was 124 U/mg, but the ee value was only 50%.[19]

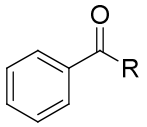
In an effort to develop a practical method for the synthesis of optically pure 2,2,2-trifluoro-1-phenylethanol, we screened the reductases available in our laboratory. Fortunately, a carbonyl reductase (SSCR) from *Sporobolomyces salmonicolor*,[20] which belongs to short-chain alcohol dehydrogenase, showed an enzyme activity of 31.24 U/mg and enantioselectivity of >98% ee. However, its specific activity toward acetophenone was low (0.03 U/mg) with the ee of the product alcohol being 42%.[21] It has been reported that the specific activity of the alcohol dehydrogenase (LBADH) from *Lactobacillus brevis* toward α,α,α-trifluoroacetophenone was only 20% of that for acetophenone.[22] The opposite observations stimulated our curiosity about how the halogen atoms at the α-position of acetophenone affect the enzyme activity and enantioselectivity. Therefore, acetophenones with different kinds and number of halogen atoms were examined with the enzyme SSCR. The enzyme-substrate docking and site-directed mutagenesis

studies were performed to understand the structural basis for the observed enzyme activity and enantioselectivity.

Results and Discussion

The specific activities of purified SSCR enzyme toward six α-halogenated acetophenones together with α-hydroxyacetophenone (Figure 1) were determined by measuring the absorbance decrease of NADPH in the reaction system. The ee values of the product alcohols were measured by chiral HPLC analysis of the product mixtures. The results are summarized in Table 1. As observed in our previous studies,[21] SSCR showed relatively low specific activity (0.03 U/mg) and enantioselectivity toward acetophenone (**1a**). It was surprising that the enzyme specific activity (31.24 U/mg) toward α,α,α-trifluoroacetophenone (**1g**) was enhanced more than 1000 times compared to that with **1a** as substrate, and the ee of the product alcohol was also enhanced from 42% (*R*, 1-phenylethanol) to 98% (*S*, 2,2,2-trifluoro-1-phenylethanol). When the α-position was substituted with halogen atoms, the enzyme specific activity was enhanced at least 223 times. Especially, for substrates **1e**, **1f** and **1g** with more than two halogen atoms, the enzyme specific activity was increased up to 1041 folds. Furthermore, the ee values of the product alcohols were also significantly improved from 42% (*R*, 1-phenylethanol) to 87 – >98%. Rosen *et al* studied the influence of the CF_3 group on the activity and enantioselectivity of various alcohol dehydrogenases, and found that the CF_3 group exerted negative or no influence on the enzyme activity.[23] In contrast, the carbonyl reductases from *Pseudomonas* sp. strain PED, *Leifsonia* sp. S749, *Kluyveromyces*

thermotolerans and *Scheffersomyces stipitis* CBS 6045 showed moderate activity enhancement (7 to 60 times higher) toward **1g** compared to **1a**.[16, 17, 19, 24] The steric and electronic effects of different α-substituted ketones on the specific activity of LBADH were studied in details.[22] LBADH showed higher enzyme activity toward the acetophenones with one or two hydrogen atoms being substituted with electron-withdrawing groups such as F, Cl and Br, although the activity enhancement is not significant as SSCR (Table 1). SSCR showed nearly 10 fold activity enhancement when the α-position of substrate was substituted by hydroxyl group (**1h**),[25] while LBADH exhibited the opposite trend. The acetophenones with mono-halogenation were better substrates of LBADH than those with two halogen atoms at α-position because of the steric effect. Following this tendency, the activity of LBADH toward **1g** is only 20% of that for **1a**.[22] This is strikingly different from the observation for SSCR, which are more than 1000 times more active toward α,α,α-trifluoroacetophenone than acetophenone.



1a R=CH_3; **1b** R=CH_2Cl; **1c** R=CH_2Br; **1d** R=CHF_2;
1e R=$CHCl_2$; **1f** R= CF_2Cl; **1g** R=CF_3; **1h** R=CH_2OH

Figure 1. Structures of acetophenone derivatives used in this study.

Table 1. Asymmetric reduction of α-haloacetophenones with SSCR

substrate (R group)	ee%/conformation by SSCR	Relative activity of SSCR [a]	Relative activity of LBADH [b] [22]

1a (CH_3)	42 *R*	1.0	1.0
1b (CH_2Cl)	98 *S*	284.3	23.6
1c (CH_2Br)	87 *S*	247.0	5.5
1d (CHF_2)	>98 *S*	223.7	7.8
1e ($CHCl_2$)	>98 *S*	606.7	4.7
1f (CF_2Cl)	>98 *S*	510.7	n.d. [c]
1g (CF_3)	>98 *S*	1041.3	0.2
1h (CH_2OH)	92 *S* [25]	9.3	0.1

[a] The relative activity of wt SSCR toward acetophenone (0.03 U/mg) is defined as 1.0.

[b] The relative activity of LBADH toward acetophenone (k_{cat}/K_m 16.1 $mM^{-1}\cdot s^{-1}$) is defined as 1.0.

[c] n.d. means "not determined".

In order to understand the structural basis of the striking increase in the enzyme activity and enantioselectivity of SSCR toward α,α,α-trifluoroacetophenone, the ketones **1a** and **1g** were docked into the substrate-binding site of SSCR and LBADH, respectively. Based on the CDOCKER_ENERGY and the CDOCKER_INTERACTION_ENERGY, two most possible conformations of **1a** and one most possible conformation of **1g** in the active site of SSCR were obtained (Figures 2A and 2B). The conformation of **1a** in the active center of LBADH was obtained directly from the crystal data,[26] and docking of **1g** into the active site of LBADH gave one most possible conformation (Figures 2C and 2D).

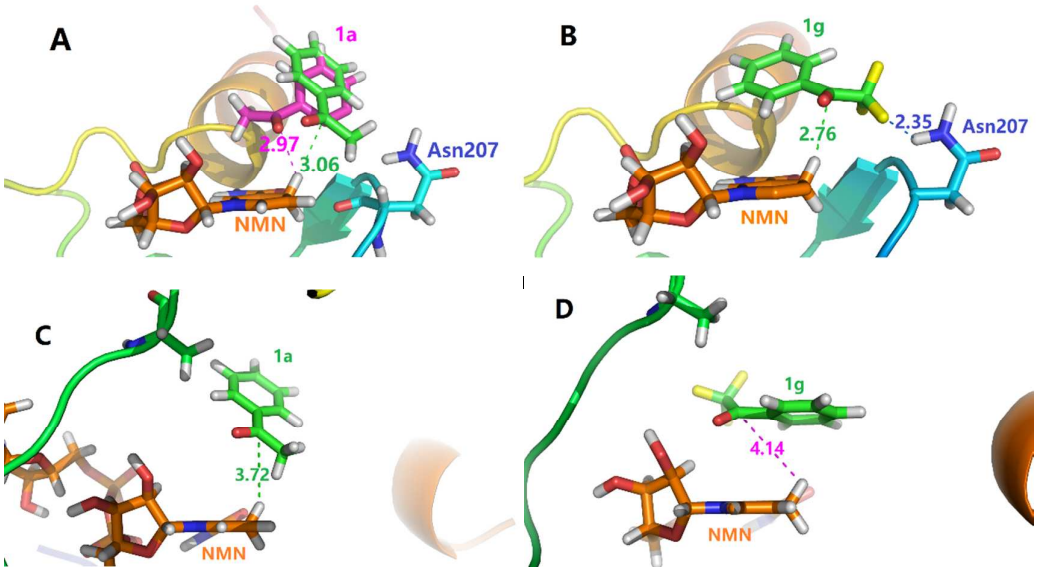


Figure 2. Docking of substrate **1a** and **1g** into the active site of the wt SSCR and LBADH. A) The two most possible conformations of **1a** in SSCR shown in green or purple, and oriented with the *Re* or *Si* face toward the cofactor, respectively. B) The one most possible conformation of **1g** in SSCR shown in green, and oriented with the *Si* face toward the cofactor (fluorine atom is shown in yellow). C) The conformation of **1a** LBADH shown in green color and oriented with *Re* face toward the cofactor. D) The conformation of **1g** in LBADH shown in green color and oriented with *Re* face toward the cofactor.

As shown in Figure 2A, the distances between the carbon atom of the carbonyl group of **1a** and C4-H of cofactor NMN in the two conformations were 2.97 and 3.06 Å, respectively. The methyl group of **1a** could swing in the opposite direction when the phenyl group was located at the same position. The little difference of the distances in the two different conformations of **1a** is consistent with the experimental observation of low stereoselectivity (42% ee) for acetophenone. It is quite different

that only one most possible conformation of **1g** in the active site was obtained, and the distance of carbon atom of carbonyl group of **1g** with C4-H of NMN was 2.76 Å. The shorter distance suggests higher enzyme activity of SSCR toward **1g**, and the higher frequency of the conformation of **1g** leading to the *S*-enantiomer of the product alcohol is in line with the experimental stereoselectivity (>98% ee) of SSCR toward **1g**. In this reactive conformation, the residue Asn207 of SSCR and the fluorine of **1g** forms hydrogen bond with the distance of fluorine atom of **1g** and hydrogen atom of Asn207 being only 2.35 Å. These docking studies suggest that Asn207 may be responsible for the high activity and enantioselectivity of SSCR toward **1g**. And in general, the α-halogen atom possibly forms hydrogen bond with hydrogen atom of Asn207 of SSCR, resulting in higher activity and enantioselectivity toward the α-halogenated acetophenones. In contrast, the distances of carbon atom of carbonyl group of **1a** and **1g** with C4-H of NMN in LBADH were 3.72 and 4.14 Å, respectively. This is consistent with the observed enzyme activity of LBADH toward these two substrates. [22]

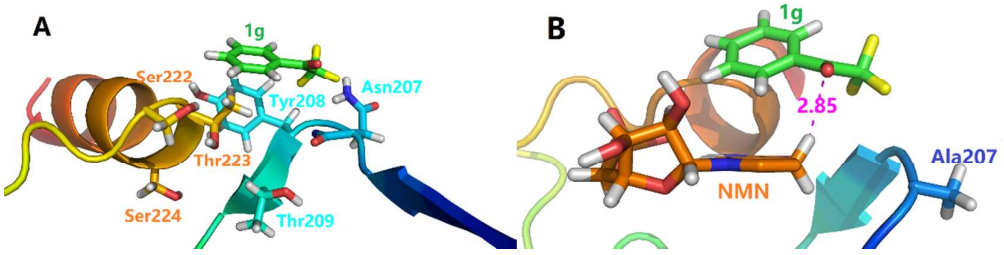


Figure 3 A) Docking of substrate **1g** into the active site of wt SSCR, showing the amino acid residues of SSCR around **1g**. B) Docking of substrate **1g** into the active site of mutant SSCR N207A. C) View of the docking results at the backward direction of A), only Ser222 and Thr223 were shown in sticks form. D) Docking of substrate **1g** into the active site of mutant SSCR S222A.

From the docking results, it can be seen that residues N207, Y208 and T209 are on the side of α,α,α-trifluoromethyl group, and N207 forms hydrogen bond with fluorine atom to anchor the trifluoromethyl group. In addition, S222, T223 and S224 locate on the side of phenyl group, and at least one of them should have some interaction with phenyl group. These interactions make the C4-H only at the *Si* face of carbonyl group (Figure 3A). In order to clarify the role of the amino acid residues around **1g** in determining the enzyme activity and enantioselectivity toward the α-halogenated acetophenones, mutations of residues N207, Y208, T209, S222, T223 and S224 to alanine were conducted. The enzyme activity of six mutants toward α-halogenated acetophenones were measured, and the results are summarized in Table 2 and 3. As shown in Table 2, although N207A mutation slightly increased the enzyme activity toward acetophenone, the enzyme activities of mutant N207A toward the

α-halogenated acetophenones and α-hydroxyacetophenone were significantly reduced. Furthermore, the mutation of Asn207 only slightly reduced the ee of the product alcohols in most cases (from >98% to 93% for **1g**). On the other hands, mutation of Y208 and T209 to Ala showed quite different effects on the enzyme activity from N207A. For mutant Y208A, the specific activity for acetophenone was greatly reduced, while mutant T209A exerted minimal effect on the activity toward the unsubstituted acetophenone. Mutation of Y208 and T209 to Ala enhanced the enzyme activity toward the α-halogenated acetophenones in most cases. Especially, T209A showed the highest enzyme activity (120.0 U/mg) and >98% ee for substrate **1g**.

Table 2. Asymmetric reduction of α-haloacetophenones with SSCR mutants

substrate (R group)	WT		N207A		Y208A		T209A	
	relative activity [a]	product ee %	relative activity	product ee %	relative activity	product ee %	relative activity	product ee %
1a (CH_3)	1.0	42 *R*	1.3	34 *R*	0.1	16 *S*	0.9	50 *R*
1b (CH_2Cl)	284.3	98 *S*	1.5	87 *S*	2.7	90 *S*	6.1	96 *S*
1c (CH_2Br)	247.0	87 S	42.3	68 *S*	396.7	67 S	513.3	93 S
1d (CHF_2)	223.7	>98 *S*	57.0	94 *S*	1536.0	98 *S*	1717.3	>98 *S*
1e ($CHCl_2$)	606.7	>98 *S*	57.5	98 *S*	352.5	>98 *S*	648.8	>98 *S*
1f (CF_2Cl)	510.7	>98 *S*	73.6	97 *S*	2603.5	>98 *S*	1988.6	>98 *S*
1g (CF_3)	1041.3	>98 *S*	195.9	93 *S*	1184.7	97 *S*	4001.1	>98 *S*
1h (CH_2OH)	9.3	92 *S*	1.7	84 *S*	0.2	97 *S*	4.5	88 *S*

[a] The relative activity of wt SSCR toward acetophenone (0.03 U/mg) is defined as 1.0.

For the mutants S222A, T223A and S224A located at the side of phenyl group, the enzyme activities toward the α-halogenated substrates were also increased in most cases, similar to the results observed for mutants Y208A and T209A. These amino acid residues also exert some effect on the enantioselectivity of SSCR. For mutants S222A and T223A, the enantioselectivity of the biotransformation was quite different from the wt SSCR even though the enzyme activity was kept high. When Ser222 was mutated to Ala, the enantioselectivity was reduced for almost all tested ketones and the enantiopreference reversal was observed for substrates **1a** and **1c**.

Table 3. Asymmetric reduction of α-haloacetophenones with SSCR mutants

substrate (R group)	WT		S222A		T223A		S224A	
	Relative activity [a]	ee %	Relative activity	ee %	Relative activity	ee %	Relative activity	ee %
1a (CH_3)	1.0	42 *R*	0.3	53 *S*	1.0	42 *R*	2.1	18 *R*
1b (CH_2Cl)	283.3	98 *S*	3.4	47 *S*	14.7	91 *S*	15.3	95 *S*
1c (CH_2Br)	247.0	87 S	420.6	23 *R*	945.1	85 *S*	526.5	82 *S*
1d (CHF_2)	223.7	>98 *S*	530.8	87 *S*	1535.7	99 *S*	605.8	98 *S*
1e ($CHCl_2$)	606.7	>98 *S*	953.7	98 *S*	783.1	98 *S*	2197.3	>99 *S*
1f (CF_2Cl)	510.7	>98 *S*	448.5	82 *S*	448.3	82 *S*	976.6	98 *S*
1g (CF_3)	1041.3	>98 *S*	1381.0	81 *S*	1381.5	81 *S*	2926.7	98 *S*
1h (CH_2OH)	9.3	92 *S*	3.7	69 *S*	3.7	69 *S*	5.9	86 *S*

[a] The relative activity of wt SSCR toward acetophenone (0.03 U/mg) is defined as 1.0.

Among the tested mutants, mutant N207A showed obviously reduced enzyme activity. The kinetic parameters of mutant N207A together with wild-type enzyme and mutant T209A toward **1g** were determined (Table 4). The wt SSCR had the lowest K_m (0.32 mM). The mutant T209A showed higher K_m, but the highest k_{cat} (51.3 s^{-1}). Mutant N207A had the smallest k_{cat} and increased K_m, resulting in a decrease of the catalytic efficiency to almost only one-tenth of that of the wild-type enzyme. These results suggest that residue N207 plays an important role for the high activity of SSCR toward the α-halogenated acetophenones.

Table 4. Kinetic Parameters of wt SSCR and mutant enzymes

enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
wt SSCR	0.32	20.4	63.6
N207A	0.89	5.9	6.6
T209A	1.63	51.3	31.5

In order to understand how the mutant N207A demolished the enzyme activity of SSCR toward the α-halogenated acetophenones, substrate **1g** was docked into the active site of mutant N207A. By using the standard docking protocol, **1g** in the active center of mutant N207A is shown in Figure 3B. Compared to Figure 3A, in which the amino acid residue Asn207 wiggles toward **1g** and forms a hydrogen bond with the fluorine atom, the amino acid residue Ala207 in Figure 3B is away from **1g**. The distance of carbon atom of carbonyl group of **1g** with C4-H of NMN is increased

from 2.76 Å to 2.85 Å when Asn207 is mutated to alanine. The demolishment of hydrogen bond between Asn207 and **1g** makes the distance between the carbonyl group of **1g** and C4-H of NMN longer, and should be the reason of the reduced enzyme activity.

According to the docking results of **1g** into wt SSCR (Figure 3C), we reasoned that the hydrogen atom at the side chain of Ser222 and Thr223 should form the XH/π interactions [27] with the phenyl group, thus reducing the mobility of the phenyl group and locating the *Si* face of the carbonyl group toward NMN. When **1g** was docked into mutant S222A, we found that the direction of the carbonyl group of **1g** has two opposite orientations, one was the *Si* face (same as wt SSCR in Figure 3C), and the other was the *Re* face (Figure 3D). In both conformations, the trifluoromethyl group interacted with Asn207. Due to the smaller methyl side chain in the mutant S222A, the XH/π interaction of the side chain with phenyl group was interrupted or weaken, and the rest part of substrate tended to rotate, resulting in the reduced enantioselectivity.

Therefore, we can deduce that the side chain of Asn207 forms hydrogen bond with the halogen atom when an α-haloacetophenone as the substrate enters the active center, anchoring the halomethyl group and making the substrate close to the cofactor. At the meantime, the side chains of Ser222 and Thr223 form XH/π interaction with the phenyl group of the substrate, fixing the phenyl group at right position. These multiple interactions make the substrate oriented in a highly reactive conformation in the enzyme active center, thus leading to high activity and enantioselectivity.

Since 2,2,2-trifluoro-1-phenylethanol and its derivatives are important intermediates for the synthesis of some pharmaceuticals, [1, 3-4] the reduction of α,α,α-trifluoroacetophenone was conducted with mutant T209A and the GDH cofactor regeneration system at preparative scale. The substrate was transformed completely at 100 g/L concentration in 5 h, and the optically pure product alcohol was isolated in 95% yield, demonstrating the application potential of the mutant SSCR enzymes in the synthesis of pharmaceutically important 2,2,2-trifluoro-1-phenylethanol.

Conclusion

The enzymatic reduction of α-halogenated acetophenones including α,α,α-trifluoroacetophenone is of synthetic importance. In this study, a different pattern of the effects of α-halogenation on the enzyme activity from the previously well-studied alcohol dehydrogenase LBADH was observed for SSCR. The site-directed mutagenesis studies revealed that the enzyme activities of mutant N207A toward α-halogenated acetophenones were significantly reduced, suggesting the important role of this residue in determining the enzyme activity toward α-haloacetophenones. The enzyme-substrate docking studies imply that the hydrogen bond (between the side chain of Asn207 of SSCR and the halogen atom of substrate) and the XH/π interactions (between the substrate phenyl group and the side chains of Ser222 and Thr223) may be responsible for the formation of the highly reactive conformation of α-halogenated acetophenones in the active site of the enzyme. These results provide the molecular basis for the high activity and enantioselectivity of

SSCR toward α-haloacetophenones with **1g** as an example. One of the mutants, T209A with the highest enzyme activity and excellent enantioselectivity toward α,α,α-trifluoroacetophenone, was applied in the preparation of (*S*)-2,2,2-trifluoro-1-phenylethanol with excellent isolated yield and ee. These results suggest that the hydrogen-bonding of the halogen atom and the XH/π interactions of the substrate phenyl group with amino acid residues are critical in determining the activity and stereoselectivity for the enzymatic reduction of halogenated acetophenones, thus providing valuable information to guide the future engineering of carbonyl reductases for efficient synthesis of β-halogenated alcohols.

Experimental Section

General

Carbonyl reductases SSCR, and glucose dehydrogenase (GDH) were prepared as previously described. [20, 28] The α-halogenated acetophenones and the other ketones were purchased from commercial sources and the cofactors were obtained from Roche. The racemic alcohol standard samples were prepared by the reduction of the corresponding ketones with sodium borohydride. The substrate conversions and product ee values were determined by HPLC analysis using a Chiralcel OD-H column (Daicel Co., Japan; 4.6 mm x 250 mm). The enzyme activities toward the reduction of ketones were measured using a SpectraMax M2 microplate reader (Molecular Devices). The ^{1}H NMR spectra were measured on Brucker Avance 400 using $CDCl_3$ as the solvent. When the reaction was conducted, $NADP^+$ not NADPH was added, because NADPH could be regenerated by GDH and D-glucose.

The KOD-Plus-DNA Polymerase was obtained from TOYOBO CO., LTD. Restriction enzyme Dpn I was bought from Thermo Scientific. The oligonucleotides were synthesized and DNA sequencing was conducted by BGI. Plasmid mini kit used for extracting plasmid and PCR extraction kit were bought from Omega Bio-tek.

Flexible Docking

The enzyme structure used as the receptor target in this study was the SSCR protein structure (PDB ID: 1Y1P) bound to NADPH, [29] and LBADH structure (PDB ID: 1zk4) [26] bound to NADPH and acetopehone. Flexible dockings of acetophenone and α,α,α-trifluoroacetophenone into SSCR and LBADH were performed using flexible docking protocol and the structures of N207A, S222A mutants were obtained by using the standard mutation protocol of Discovery Studio V4.1 (Biovia – former Accelrys, USA). The protein active site was determined from PDB site records, and the active site was defined by a sphere of 11 Å radius. Other parameters were kept on default settings in Flexible docking.

Construction of SSCR Mutants

The mutant genes were constructed by using PCR amplification methods with KOD-Plus-DNA Polymerase as decribed in the manual. Based on the SSCR sequence, appropriate primers were designed. The PCR reaction conditions were as follows: 94°C 2 min, (95°C 15 sec, 58°C 30 sec, 68°C 8 min) × 20 cycles, 68°C 10 min. The PCR products were analyzed on agarose gel by electrophoresis and purified using a Omega Bio-tek Cycle-Pure kit. The purified PCR products were digested with *Dpn*I restriction enzyme at 37 ℃ for more than 1 h to remove template DNA. After

digestion, the genes were purified again using Cycle-Pure kit. The purified products were transformed to commercial *E. cloi* BL21(DE3) by heat shock. Transformation mixtures were spread on agar plates containing 100 μg/mL ampicillin and grown overnight at 37°C. The cultures containing the corresponding mutant genes were confirmed by DNA sequencing (BGI).

Purification of wt and Mutant SSCR Proteins

E. coli BL21 (DE3) cells expressing wt SSCR and mutant genes were cultivated in 5 mL LB medium containing 100 μg/mL ampicillin overnight at 37°C. The overnight culture was inoculated into 600 mL L-B medium containing 100 μg/mL ampicillin and grown at 37°C. The culture was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 0.1 mM when OD600 reached 0.6-0.8, and then allowed to grow for additional 16 h at 30°C. After centrifugation at 6000 × *g* for 15 min at 4°C, the cells were washed with phosphate buffer (50 mM, pH 7.5), and resuspended in a phosphate buffer (50 mM pH 7.5) containing 0.5 M of NaCl. The cells were lysed by high pressure homogenizer and the supernatant was collected by centrifugation at 8000 × *g* for 20 min at 4°C. The supernatant was filtered with 0.22 μm filter. Protein purification was performed using an AKTA purifier 10 system with UNICORN 5 software (GE Healthcare). The wt and mutant proteins were purified using affinity chromatography with Ni Sepharose^{TM} 6 Fast Flow column (GE, USA). The column was preequilibrated with buffer (50 mM phosphate buffer, 0.5 M NaCl, pH 7.5). The sample (70 mL) was loaded with a flow rate of 3.0 mL/min. After washing with 50 ml of the equilibrium buffer, the bounded

target protein was washed with 50 – 75 mM imidazole solution containing 0.5 M NaCl and 50 mM sodium phosphate (pH 7.5). The target protein fractions were collected and desalted through ultrafiltration. The pure enzyme solutions were stored at -80°C after adding 10% glycerin and 0.1 mM DTT.

Measurement of the Activity of wt and Mutant Enzymes and the Product ee Values

The specific activity of the purified carbonyl reductase SSCR and its mutants toward the reduction of α-substituted acetophenones was determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of an excess amount of ketones. The activity was measured at room temperature in a 96-well plate, in which each well contained ketone (6.25 mM), NADPH (0.40 mM) and 10% v/v DMSO in sodium phosphate buffer (100 mM, pH 7.0, 190 μL). The reaction was initiated by the addition of the carbonyl reductase (10 μL solution containing 0.5-20 μg of enzyme) in 5 min. The specific activity (U/mg) was defined as the number of micromoles of NADPH converted in 1 min by 1 mg of enzyme.

Sodium phosphate buffer (1 mL, 100 mM, pH 7.0) containing 10% DMSO (v/v), 10 mM substrate, 30 mM Glucose, 0.5 mM $NADP^+$, 0.5-2 mg SSCR or its mutant and 4 U GDH was shaken at room temperature. The reaction was stopped after overnight by addition of equal amount of ethyl acetate. The organic phase was separated and the solvent was removed. The resulting sample was analyzed by chiral HPLC to determine the ee value of the product alcohol.

Measurement of Kinetic Parameters for Substrate 1g

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. The reaction was carried out in potassium phosphate buffer (100 mM, pH 7.0) with 0.5 mM NADPH. The substrate concentration range was between 0.1 and 10 mM.

Enzymatic Reduction of α,α,α-Trifluoroacetophenone

A solution of 5 g α,α,α-trifluroacetophenone in DMSO (5 mL) was added into a sodium phosphate buffer (100 mM, pH 7.0, 45 mL) containing carbonyl reductase SSCR T209A 50 U (1 U was defined as the number of micromoles of NADPH converted in 1 min with **1g** as substrate), lyophilized powders of GDH 50 U, $NADP^+$ (5 mg), and 12 g D-glucose. The reaction mixture was stirred at room temperature with TLC monitoring from time to time. The pH was controlled at 7.0 by adding 2 M NaOH. After complete consumption of the substrate, the reaction mixture was extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford the optically pure product, (*S*)-2,2,2-trifluoro-1-phenylethanol (4.8 g, purity >95%, 95% yield), without further purification. ^1H NMR (600 MHz, $CDCl_3$) δ 5.01 (q, J = 6.9, 12 Hz, 1H), 7.26–7.57 (m, 5H).

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Notes

The authors declare no competing financial interest.

Supporting Information Available

^{1}H NMR spectrum of (*S*)-2,2,2-trifluoro-1-phenylethanol. This information is available free of charge on the ACS Publications website.

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REFERENCES

1. Kawase, M.; Sakagami, H.; Kusama, K. α-Trifluoromethylated Acyloins Induce Apoptosis in Human Oral Tumor Cell Lines. *Bioorg. Med. Chem. Lett.* **1999,** 9, 3113-3118.

2. Kirk, K. L. Fluorine in Medicinal Chemistry: Recent Therapeutic

Applications of Fluorinated Small Molecules. *J. Fluorine Chem.* **2006,** 127, 1013-1029.

3. Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarais, S.; Duong, L. T.; Falgueyret, J. P.; Kimmel, D. B.; Lamontagne, S.; Leger, S.; LeRiche, T.; Li, C. S.; Masse, F.; McKay, D. J.; Nicoll-Griffith, D. A.; Oballa, R. M.; Palmer, J. T.; Percival, M. D.; Riendeau, D.; Robichaud, J.; Rodan, G. A.; Rodan, S. B.; Seto, C.; Therien, M.; Truong, V. L.; Venuti, M. C.; Wesolowski, G.; Young, R. N.; Zamboni, R.; Black, W. C. The Discovery of Odanacatib (MK-0822), A Selective Inhibitor of Cathepsin K. *Bioorg. Med.Chem. Lett.* **2008,** 18, 923-928.

4. Swallow, S. Fluorine in Medicinal Chemistry. *Prog. Med. Chem.* **2015,** 54, 65-133.

5. Corey, E. J.; Link, J. O.; Bakshi, R. K. A Mechanistic and Structural Analysis of the Basis for High Enantioselectivity in the Oxazabotolidine-catalyzed Reduction of Trihalomethyl Ketones by Catecholborane. *Tetrahedron Lett.* **1992,** 33, 7107-7110.

6. Ohkuma, T.; Koizumi, M.; Doucet, H.; Pham, T.; Kozawa, M.; Murata, K.; Katayama, E.; Yokozawa, T.; Ikariya, T.; Noyori, R. Asymmetric Hydrogenation of Alkenyl, Cyclopropyl, and Aryl Ketones. $RuCl_2$(xylbinap)(1,2-diamine) as a Precatalyst Exhibiting a Wide Scope. *J. Am. Chem. Soc.* **1998,** 120, 13529-13530.

7. Jolley, K. E.; Zanotti-Gerosa, A.; Hancock, F.; Dyke, A.; Grainger, D. M.; Medlock, J. A.; Nedden, H. G.; Le Paih, J. J. M.; Roseblade, S. J.; Seger, A.; Sivakumar, V.; Prokes, I.; Morris, D. J.; Wills, M. Application of Tethered

Ruthenium Catalysts to Asymmetric Hydrogenation of Ketones, and the Selective Hydrogenation of Aldehydes. *Adv. Synth. Catal.* **2012,** 354, 2545-2555.

8. Cano, I.; Tschan, M. J. L.; Martínez-Prieto, L. M.; Philippot, K.; Chaudret, B.; van Leeuwen, P. W. N. M. Enantioselective Hydrogenation of Ketones by Iridium Nanoparticles Ligated with Chiral Secondary Phosphine Oxides. *Catal. Sci. Technol.* **2016,** 6, 3758-3766.

9. Li, Y.; Yu, S.; Wu, X.; Xiao, J.; Shen, W.; Dong, Z.; Gao, J. Iron Catalyzed Asymmetric Hydrogenation of Ketones. *J. Am. Chem. Soc.* **2014,** 136, 4031-4039.

10. Korenaga, T.; Nomura, K.; Onoue, K.; Sakai, T. Rational Electronic Tuning of CBS Catalyst for Highly Enantioselective Borane Reduction of Trifluoroacetophenone. *Chem. Commun.* **2010,** 46, 8624-8626.

11. Pereñiguez, R.; Santarossa, G.; Mallat, T.; Baiker, A. Hydrogenation of 2,2,2-Trifluoroacetophenone: Molecular Insight into the Role of Solvent in Enantioselection. *J. Mol. Catal. A: Chem.* **2012,** 365, 39-49.

12. Nakamura, K.; Yamanaka, R.; Matsuda, T.; Harada, T. Recent Developments in Asymmetric Reduction of Ketones with Biocatalysts. *Tetrahedron: Asymmetry* **2003,** 14, 2659-2681.

13. Moore, J. C.; Pollard, D. J.; Kosiek B.; Devine, P. N. Advances in the Enzymatic Reduction of Ketones. *Acc. Chem. Res.* **2007,** 40, 1412-1419.

14. Strohmeier, G. A.; Pichler, H.; May, O.; Gruber-Khadjawi, M. Application of Designed Enzymes in Organic Synthesis. *Chem. Rev.* **2011,** 111, 4141-4164.

15. Ni, Y.; Xu, J. H. Biocatalytic Ketone Reduction: A Green and Efficient Access to Enantiopure Alcohols. *Biotechnol. Adv.* **2012,** 30, 1279-1288.

16. Bradshaw, C. W.; Fu, H.; Shen, G.-J.; Wong, C.-H. A *Pseudomonas* sp. Alcohol Dehydrogenase with Broad Substrate Specificity and Unusual Stereospecificity for Organic Synthesis. *J. Org. Chem.* **1992,** 57, 1526-1532.

17. Xu, G.-C.; Yu, H.-L.; Zhang, X.-Y.; Xu, J.-H. Access to Optically Active Aryl Halohydrins Using a Substrate-Tolerant Carbonyl Reductase Discovered from *Kluyveromyces thermotolerans*. *ACS Catal.* **2012,** 2, 2566-2571.

18. Borzecka, W.; Lavandera, I.; Gotor, V. Synthesis of Enantiopure Fluorohydrins Using Alcohol Dehydrogenases at High Substrate Concentrations. *J. Org. Chem.* **2013,** 78, 7312-7317.

19. Shang, Y.-P.; Chen, Q.; Kong, X.-D.; Zhang, Y.-J.; Xu, J.-H.; Yu, H.-L. Efficient Synthesis of (*R*)-2-Chloro-1-(2,4-dichlorophenyl)ethanol with a Ketoreductase from *Scheffersomyces stipitis* CBS 6045. *Adv. Synth. Catal.* **2017,** 359, 426-431.

20. Kataoka, M.; Sakai, H.; Morikawa, T.; Katoh, M.; Miyoshi, T.; Shimizu, S.; Yamada, H. Characterization of Aldehyde Reductase of *Sporobolomyces salmonicolor*. *Biochem. Biophys. Acta* **1992,** 1122, 57-62.

21. Zhu, D.; Hua, L. Enantioselective Enzymatic Reductions of Sterically Bulky Aryl Alkyl Ketones Catalyzed by a NADPH-Dependent Carbonyl Reductase. *J. Org. Chem.* **2006,** 71, 9484-9486.

22. Rodríguez, C.; BorzUęcka, W.; Sattler, J. H.; Kroutil, W.; Lavandera, I.; Gotor, V. Steric *vs*. Electronic Effects in the *Lactobacillus brevis* ADH-catalyzed Bioreduction of Ketones. *Org. Biomol. Chem.* **2014,** 12, 673 - 681.

23. Rosen, T. C.; Feldmann, R.; Dünkelmann, P.; Daußmann, T. Bioreductive Synthesis of Perfluorinated Chiral Alcohols. *Tetrahedron Lett.* **2006,** *47,* 4803-4806.

24. Inoue, K.; Makino, Y.; Itoh, N. Production of (*R*)-chiral Alcohols by a Hydrogen-transfer Bioreduction with NADH-dependent *Leifsonia* Alcohol Dehydrogenase (LSADH). *Tetrahedron: Asymmetry* **2005,** 16, 2539-2549.

25. Chen, X.; Mei, T.; Cui, Y.; Chen, Q.; Liu, X.; Feng, J.; Wu, Q.; Zhu, D. Highly Efficient Synthesis of Optically Pure (*S*)-1-phenyl-1,2-ethanediol by a Self-Sufficient Whole Cell Biocatalyst. *ChemistryOpen* **2015,** 4, 483-488.

26. Niefind, K.; Müller, J.; Rieble, B.; Hummel, W.; Schomburg, D. The Crystal Structure of *R*-specific Alcohol Dehydrogenase from *Lactobacillus brevis* Suggests the Structural Basis of its Metal Dependency. *J. Mol. Biol.* **2003,** 327, 317-328.

27. Nishio, M.; Hirota, M.; Umezawa, Y. The CH/π Interaction: Evidence, Nature, and Consequences, 1st ed.; Wiley-VCH: New York, **1998**; 80-87.

28. Makino, Y.; Negoro, S.; Urabe, I.; Okada, H. Stability-increasing Mutants of Glucose Dehydrogenase from *Bacillus megatarium* IWG3. *J. Biol. Chem.* **1989,** 264, 6381-6385.

29. Kamitori, S.; Iguchi, A.; Ohtaki, A.; Yamada, M.; Kita, K. X-ray Structures of NADPH-dependent Carbonyl Reductase from *Sporobolomyces salmonicolor*

Provide Insights into Stereoselective Reductions of Carbonyl Compounds. *J. Mol. Biol.* **2005,** 352, 551-558.

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