SUPPORTING INFORMATION for

# Engineering carbonyl reductase for one-pot chemobiocatalytic enantioselective synthesis of value-added N-containing chiral alcohol from N-acetyl-D-glucosamine

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**Biological and chemical materials**

NAG (97%), β-nicotinamide adenine dinucleotide (NADH, 98%), 2-acetylfuran (99%), 2-acetylpyrazine (98%), 3-acetylpyridine (98%), acetophenone (98%), (*R*)-1-phenylethanol (98%), (*R*)-1-(4-methoxyphenyl)ethanol (95%), racemic 1-(4-chlorophenyl)ethanol (97%), racemic 1-(4-bromophenyl)ethanol (98%), racemic 1-(4-methoxyphenyl)ethanol (95%) and ethyl (*R*)-2-hydroxy-4-phenylbutyrate (98%) were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). 4′-Chloroacetophenone (97%), 4′-bromoacetophenone (98%), racemic 1-phenylethanol (98%), 4′-methoxyacetophenone (99%) and dimethyl sulfoxide (99%) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Ethyl 2-oxo-4-phenylbutyrate (95%) was obtained from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). (*R*)-1-(2-Furyl)ethanol (95%) was obtained from Jizhi Biochemical Technology Co., Ltd. (Shanghai, China). Racemic 1-(3-pyridyl)ethanol (98%) was obtained from Titan Technology Co., Ltd. (Shanghai, China). Racemic 1-(2-pyrazinyl)ethanol (95%) was obtained from Bide Pharmatech Technology Co., Ltd. (Shanghai, China). Ethyl (*S*)-2-hydroxy-4-phenylbutyrate (98%) was obtained from Haohong Biomedicine Technology Co., Ltd. (Shanghai, China). Racemic 1-(2-furyl)ethanol was synthesized from the reduction of 2-acetylfuran by $NaBH_4$.[1] To a solution of 2-acetylfuran (0.2 g) in ethanol (10 mL), $NaBH_4$ (0.04 g) was slowly added at 0 °C. The reaction mixture was stirred at room temperature for 4 h and the solid was removed via filtration. Upon solvent evaporation, racemic product was afforded. DMA was provided by Tianjin Fuyu Fine Chemical Co., Ltd. (Tianjin, China). D-Glucose was bought from Tianjin Damao Chemical Reagent Co., Ltd. (Tianjin, China). Glycine was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Calcium chloride and sodium bicarbonate were obtained from Guangzhou Chemical Reagent Factory (Shanghai, China). Other

chemicals were from commercial sources and were of the highest purity available. The plasmids harboring *Sc*CR (GenBank accession number NC003888.1) was kindly donated by Prof. Gao-Wei Zheng of East China University of Science and Technology.

**Site-Directed Mutagenesis**

Site-directed mutagenesis was carried out by polymerase chain reaction (PCR) using mutagenic primers (Table S4) and plasmid pET28a-*Sc*CR as the template according to the manufacturer's instructions of Q5 High-Fidelity DNA polymerase. The PCR conditions were as following: 98 °C and 2 min, (98 °C and 10 s, 72 °C and 15 s, 72 °C and 3 min 20 s) × 25 cycles, 72 °C and 5 min, 16 °C and 30 min. Two μL NEB CutSmart Buffer and 2 μL Dpn I were added in 3 μL PCR reaction mixture and the digestion was carried out at 37 °C for more than 4 h. After Dpn I digestion, the PCR products (1 μL) was transformed into 100 μL of *E. coli* BL21 (DE3)-competent cells, and the transformants verified by DNA sequencing were incubated for protein expression.

**Enzyme Purification**

The cells overexpressing the target enzyme were lysed by ultrasonication in an ice bath (350 w; 15 min, 3 second ON, 5 second OFF cycles), and the supernatant was collected by centrifugation (12 000 rpm, 20 min) at 4 °C. Upon centrifugation, the sample was loaded onto a HisTrap HP column (GE Healthcare, USA) and subsequently eluted with elution buffer (0.4 M imidazole, 0.1 M sodium phosphate, 0.5 M NaCl, pH 7.0) at a flow rate of 4 mL/min. The target protein fraction was desalted on a HiTrap Desalting column (GE Healthcare, USA) with desalting buffer (pH 7.0, 0.1 M sodium phosphate buffer). Protein concentration was measured by the Bradford assay.[2]

**Enzyme Assay**

The enzyme activity was determined spectrophotometrically by measuring the changes in NADH at 340 nm under pH 7 and 30 °C using a Shimadzu UV2550 spectrophotometer (Japan). The reaction was carried out in 0.4 mL phosphate buffer (0.1 M, pH 7) containing an appropriate amount of enzyme, 1 mM 3A5AF, and 0.2 mM NADH. One unit (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per minute under the above reaction conditions. The protein concentration was measured with Bradford method, using bovine serum albumin as the standard.

**Molecular docking and molecular dynamics (MD) simulation**

Substrate 3A5AF was docked into the crystal structure of the WT ScCR (PDB ID: 5H5X) using Autodock. Homology modelling was performed to generate the initial structure of variant M3 in complex with 3A5AF using crystal structure of the WT enzyme as the template.

To get the mechanistic insights into the increased catalytic activity upon mutation, molecular dynamics simulations were performed. The modelling structures of the WT enzyme and variant M3 in complex with 3A5AF were used as the starting structures for the MD simulation, respectively.

The protonation states of titratable residues were assigned on the basis of pKa values using H++.The force field parameter for each system was built using Tleap module in Amber20. The Amber ff14SB force field was employed for the protein residues and the force field parameters for 3A5AF were generated via antechamber package and Gaussian using B3LYP/6-31G* level of theory (Table S1). Each simulation system was solvated in a TIP3P water box and extended 10 Å from the protein surface. $Na^+$ ions were added to neutralize the total charge of each system.

NAMD was used for running all simulations. The particle-mesh Ewald method was used to compute the long-range electrostatic interactions, and the van der Waals interactions were treated with a switching function for smoothly turned off. Each system was minimized for 20,000 steps and then heated gradually from 0 K to 300 K in the NVT ensemble. The NPT ensemble was applied for the followed equilibration and production simulations. Finally, trajectories from 0-100 ns were gathered for data analysis. The Molecular Mechanics-Poisson Boltzmann Surface Area (MM/PBSA) approach was used to estimate the substrate binding affinity.

**Preparative-scale synthesis and characterization of (*R*)-3A5HEF**

0.6 g NAG (452 mM), 0.6 g $CaCl_2$ and 0.6 g [Tyr]Cl were added into 2 mL DMA, and the dehydration reaction was performed at 160 °C for 10 min with stirring. Then, the reaction mixture was cooled, followed by 12-fold dilution using phosphate buffer (0.1 M, pH 7) and tuning pH to 7 by $NaHCO_3$. The final volume of reaction mixture was around 72 mL. Then, purified *Sc*CR M4 (0.8 mg/mL), NADH (0.2 mM), GDH CFE (0.05 mg/mL), D-glucose (40 mM) were supplemented and the enzymatic reaction was conducted at 30 °C and 150 r/min. After 48 h, the reaction mixture was boiled to inactivate enzymes, followed by filtering. The filtrate was subjected to extraction by ethyl acetate (72 mL) for 3 times. The organic phases were combined and evaporated, followed by silica column chromatography by petroleum ether/ethyl acetate (1:2, v/v; Rf = 0.2). 3A5HEF of 193 mg was obtained, which was characterized by NMR (Figure S8), and MS (Figure S9). HPLC (Figure S7) and NMR (Figure S8) analyses show the high purity of the obtained product. The specific rotation value of the obtained (*R*)-3A5HEF was determined via an automatic polarimeter P810 (Hanon Instruments, Inc., China). (*R*)-3A5HEF was dissolved in ethanol at the final concentration of around 1.9 mg/mL. The assay was performed at 589 nm and 25 °C in 1 dm polarimeter tube. The specific rotation of +6.9 was obtained, which is consistent with our previous results.[3]

**Analytical methods**

The reaction mixtures were analyzed on a Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA) by using a reversed phase HPLC (Waters, USA) equipped with a Waters 515 pump and a Waters 996 photodiode array detector. The detailed analysis conditions and the retention times were summarized in Table S3.

Product e.e. values were determined by HPLC/GC (Agilent, USA) using chiral columns. The detailed conditions were listed as follows. The products were extracted from the reaction mixtures with 400 μL of ethyl acetate for determining e.e. values. The product absolute configurations were determined by comparing the retention times with those of the authentic standards and literature.

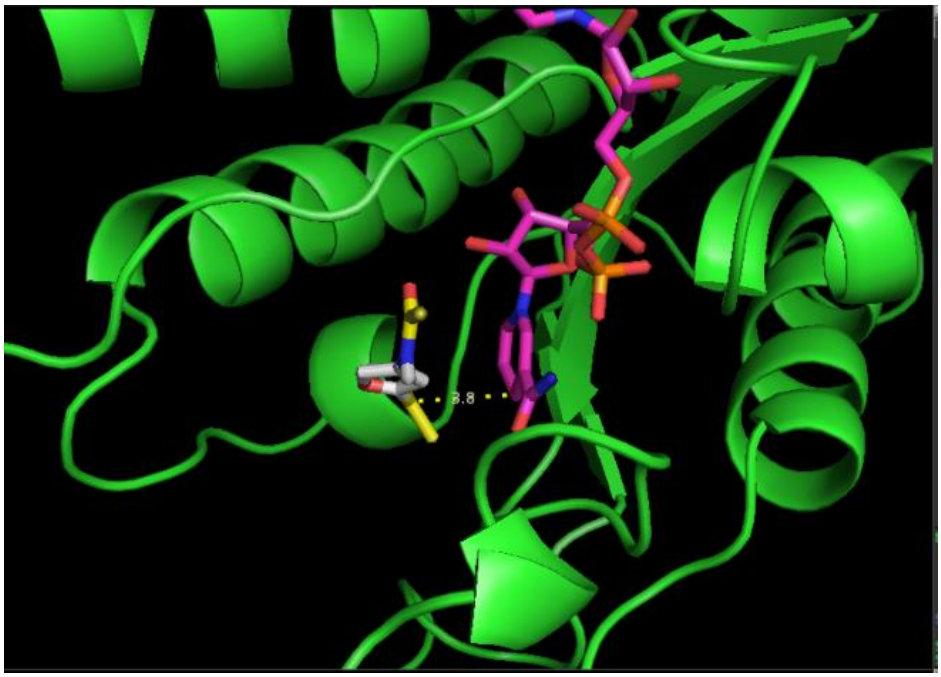


**Figure S1.** The results of *Sc*CR docking with 3A5AF and NADH

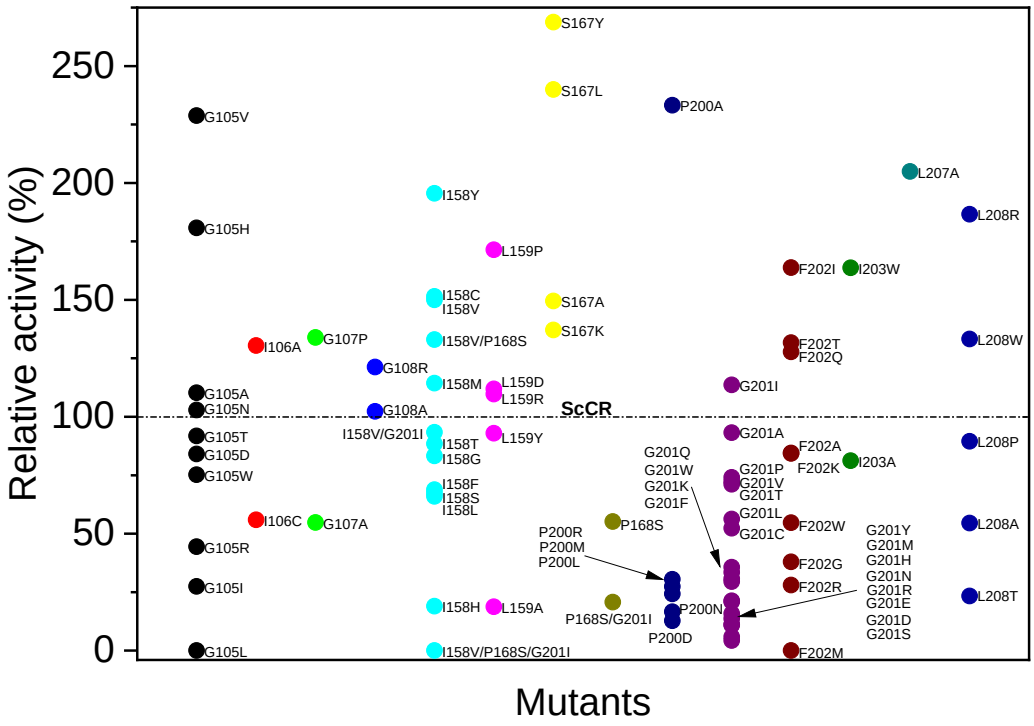



**Figure S2.** The relative activities of the variants in the form of CFE.

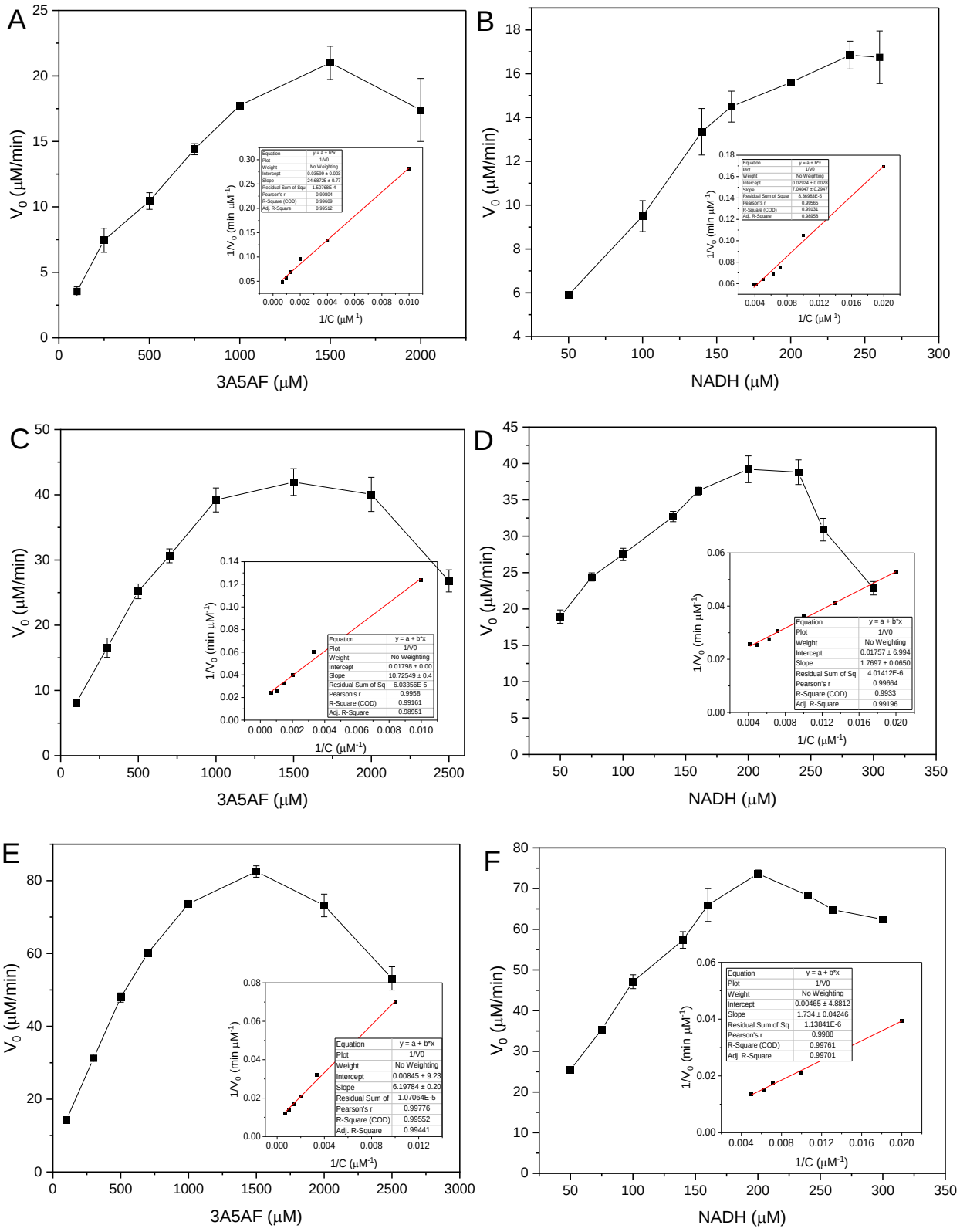



**Figure S3.** The kinetic studies of ScCR and its variants: (A) effect of 3A5AF concentrations on the ScCR activity; (B) effect of NADH concentrations on the ScCR activity; (C) effect of 3A5AF concentrations on the M3 activity; (D) effect of NADH concentrations on the M3 activity; (E) effect of 3A5AF concentrations on the M4 activity; (F) effect of NADH concentrations on the M4 activity. Reaction conditions: the designated concentration of 3A5AF, 0.2 mM NADH (or the designated concentration of NADH and 1mM 3A5AF), 0.4 mL phosphate buffer (0.1 M, pH

7), appropriate amount of purified enzyme, 30 °C.

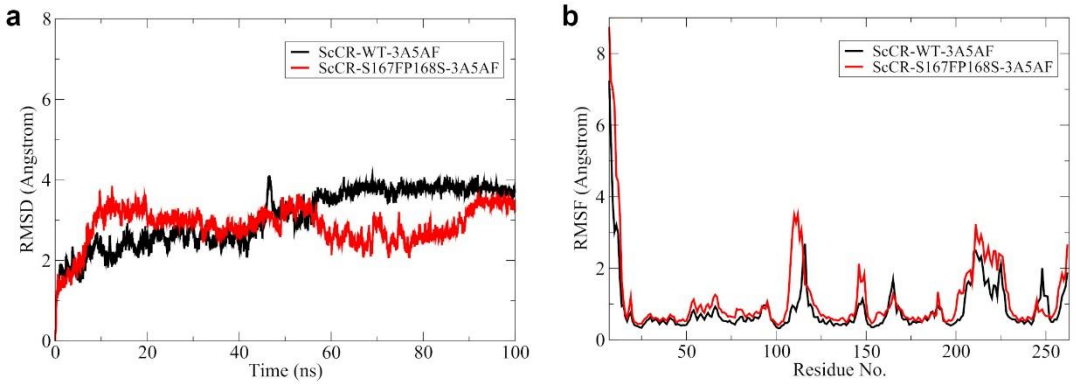



**Figure S4.** The root mean square deviation (RMSD) (a) and the root mean square fluctuation (RMSF) (b) of the WT enzyme and variant M3 in complex with 3A5AF.

**Table S1**. Force field parameters for 3A5AF: (a) Partial charges (b) Bond, angle and torsion parameters.

**(a) Partial charges**

| Index | Name | Coordinate x | Coordinate y | Coordinate z | Type | | | Partial charges |
|---|---|---|---|---|---|---|---|---|
| 1 | C1 | -0.662 | -0.719 | 0.014 | cc | 1 | MOL | 0.354479 |
| 2 | C2 | 0.279 | 0.364 | 0.014 | cc | 1 | MOL | -0.33328 |
| 3 | C3 | 2.963 | 1.865 | 0.003 | c3 | 1 | MOL | -0.39645 |
| 4 | C4 | -4.309 | 0.225 | -0.027 | c3 | 1 | MOL | -0.60954 |
| 5 | C5 | 1.495 | -0.217 | 0.001 | cd | 1 | MOL | 0.023855 |
| 6 | H1 | -2.536 | -1.534 | 0.031 | hn | 1 | MOL | 0.377585 |
| 7 | C6 | 0.078 | -1.843 | 0.001 | cd | 1 | MOL | -0.18052 |
| 8 | C7 | 2.86 | 0.357 | -0.006 | c | 1 | MOL | 0.69274 |
| 9 | C8 | -2.817 | 0.472 | 0.011 | c | 1 | MOL | 0.946671 |
| 10 | O1 | -2.35 | 1.574 | 0.002 | o | 1 | MOL | -0.63118 |
| 11 | O2 | 3.822 | -0.349 | -0.017 | o | 1 | MOL | -0.5471 |
| 12 | O3 | 1.383 | -1.552 | -0.007 | os | 1 | MOL | -0.18062 |
| 13 | N1 | -2.06 | -0.661 | 0.027 | n | 1 | MOL | -0.77904 |
| 14 | H2 | 0.047 | 1.404 | 0.022 | ha | 1 | MOL | 0.23434 |
| 15 | H3 | -0.181 | -2.88 | -0.004 | h4 | 1 | MOL | 0.206524 |
| 16 | H4 | -4.817 | 1.123 | 0.294 | hc | 1 | MOL | 0.164419 |
| 17 | H5 | -4.604 | -0.605 | 0.606 | hc | 1 | MOL | 0.164419 |
| 18 | H6 | -4.611 | 0.001 | -1.045 | hc | 1 | MOL | 0.164419 |
| 19 | H7 | 4.007 | 2.142 | -0.003 | hc | 1 | MOL | 0.10942 |
| 20 | H8 | 2.485 | 2.276 | 0.886 | hc | 1 | MOL | 0.10942 |
| 21 | H9 | 2.471 | 2.288 | -0.867 | hc | 1 | MOL | 0.10942 |

**(b) Bond, angle and torsion parameters**

**DIHE**

| Type | $N_{path}$ | $V_n/2$ (kcal/mol) | $\gamma$ (degrees) | n | |
|---|---|---|---|---|---|
| cc-cd-os-cd | 2 | 2.100 | 180.000 | 2.000 | same as X -c2-os-X , penalty score=232.0 |
| c -cd-os-cd | 2 | 2.100 | 180.000 | 2.000 | same as X -c2-os-X , penalty score=232.0 |
| h4-cd-os-cd | 2 | 2.100 | 180.000 | 2.000 | same as X -c2-os-X , penalty score=232.0 |

**MPROPER**

| Type | $V_n/2$ (kcal/mol) | $\gamma$ (degree) | n | |
|---|---|---|---|---|
| cc-cd-cc-n | 1.1 | 180.0 | 2.0 | Using the default value |
| cc-cd-cc-ha | 1.1 | 180.0 | 2.0 | Same as X -X -ca-ha, penalty score= 38.9 (use general term)) |
| c -cc-cd-os | 1.1 | 180.0 | 2.0 | Using the default value |
| cc-h4-cd-os | 1.1 | 180.0 | 2.0 | Same as X -X -ca-ha, penalty score= 67.2 (use general term)) |
| c3-cd-c -o | 10.5 | 180.0 | 2.0 | Using general improper torsional angle X- X- c- o, penalty score= 6.0) |
| c3-n -c -o | 10.5 | 180.0 | 2.0 | Using general improper torsional angle X- X- c- o, penalty score= 6.0) |
| c -cc-n -hn | 1.1 | 180.0 | 2.0 | Using general improper torsional angle X- X- n-hn, penalty score= 6.0) |

**Table S2.** Substrate binding energies of 3A5AF in the WT enzyme and variant M3 computed via MM-PBSA method.

| **Energy (kcal/mol)** | WT | M3 |
|---|---|---|
| $\Delta E_{vdW}$ | -22.6 ± 2.2 | -26.4 ± 3.3 |
| $\Delta E_{elec}$ | -1.1 ± 3.7 | -10.1 ± 4.4 |
| $\Delta E_{PB,elec}$ | 12.5 ± 3.4 | 24.7 ± 5.4 |
| $\Delta E_{npolar}$ | -2.3 ± 0.1 | -2.5 ± 0.1 |
| $\Delta E_{disper}$ | 0 ± 0 | 0 ± 0 |
| $\Delta G_{bind}$ | -13.4 ± 2.5 | -14.4 ± 4.2 |

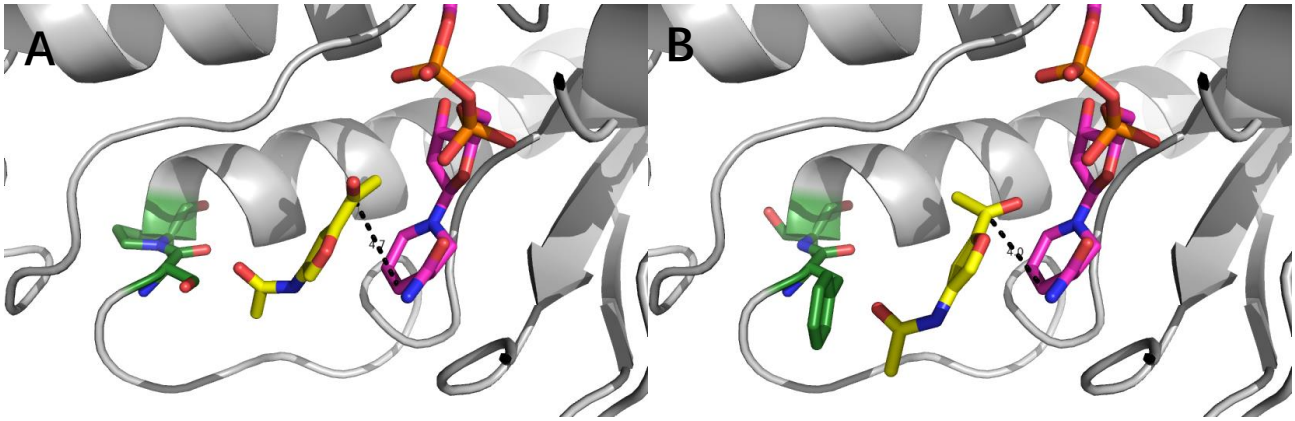



**Figure S5.** Molecular docking of *Sc*CR (A) and M3 variant (B) with 3A5AF and NADH

As shown in Figure S5, the substrate binding free energy of the M3 variant is equal to that of the parent (-4.8 kcal/mol), suggesting the slight effect of the mutation of the two sites S167/P168 on the $K_m$ value. It is in good agreement with the kinetic studies (Table 1). Based on the analysis of the substrate binding mode and hydride transfer from NADH, both the parent and engineered enzymes could produce the (*R*)-product. Nevertheless, the distance between $C_{NADH}$ and the C atom present in the carbonyl group of the substrate was decreased from 4.7 Å to 4.0 Å upon mutation (Figure S5), allowing for easier hydride transfer between substrate and NADH and thus improving the reaction rate. It is also consistent with the kinetic results.

**Table S3.** Effect of $Ca^{2+}$ on the stability of the variants

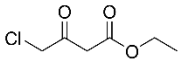
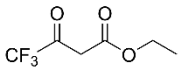
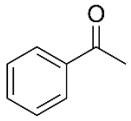
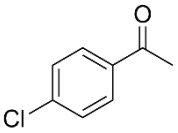
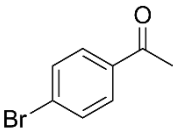
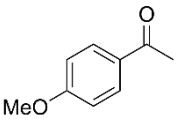
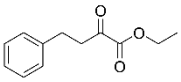
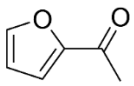
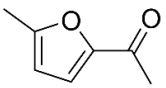
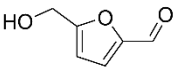
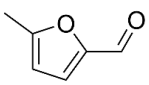
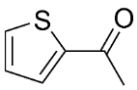
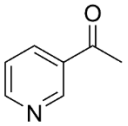
| Chemical | Residual activity (%) | |
|---|---|---|
| | M3 variant | M4 variant |
| $CaCl_2$ | 88 | 95 |

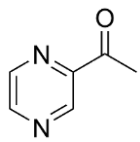
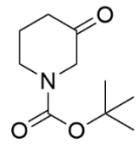
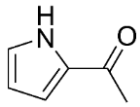
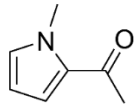
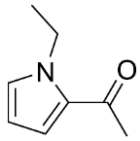
Conditions: The enzyme was incubated in 0.1 M, pH 7 phosphate buffer containing 40 mM $CaCl_2$ at 30 °C for 4 h, the residual activity of the enzyme was determined by the standard assay.

**Table S4.** Estimation of the waste generated in the preparative-scale synthesis

| | Component | *E* factor contribution (kg waste per kg product) |
|---|---|---|
| Chemical dehydration | $CaCl_2$ | 3.1 |
| | IL | 3.1 |
| | DMA | 27.4 |
| | Humin, et al. | 1.8 |
| Enzymatic reduction | buffer salt | 4.8 |
| | NADH | 0.05 |
| | enzymes | 0.3 |
| | glucose | 2.7 |
| Purification | petroleum ether | 1243.5 |
| | ethyl acetate | 4737.8 |
| Sum | | 6024.7 |

**Table S5.** The activities of *Sc*CR and its mutants toward various carbonyl compounds

| Entry | Substrate | WT enzyme | M3 variant | | M4 variant | |
|---|---|---|---|---|---|---|
| | | Specific activity (mU/mg) | Specific activity (mU/mg) | Fold change | Specific activity (mU/mg) | Fold change |
| 1 |  | 54826 | 46225 | 0.8 | 48351 | 0.9 |
| 2 |  | 747 | 1945 | 2.6 | 3267 | 4.4 |
| 3 |  | 207 | 1075 | 5.2 | 2003 | 9.7 |
| 4 |  | 872 | 20450 | 23.5 | 35453 | 40.7 |
| 5 |  | 1269 | 19312 | 15.2 | 20658 | 16.3 |
| 6 |  | 1298 | 939 | 0.7 | 1846 | 1.4 |
| 7 |  | 12601 | 29368 | 2.3 | 31432 | 2.5 |
| 8 |  | 17 | 64 | 3.8 | 58 | 3.4 |
| 9 |  | 43 | 242 | 5.6 | 413 | 9.6 |
| 10 |  | 49 | 94 | 1.9 | 51 | 1.0 |
| 11 |  | 23 | 87 | 3.8 | 191 | 8.3 |
| 12 |  | 32 | 156 | 4.8 | 162 | 5.0 |
| 13 |  | 1172 | 3911 | 3.3 | 3659 | 3.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 14 |  | 248 | 1018 | 4.1 | 1295 | 5.2 |
| 15 |  | 390 | 81 | 0.2 | 237 | 0.6 |
| 16 |  | 3 | 8 | 3.0 | 0 | 0.0 |
| 17 |  | n.a. | 1 | - | 2 | - |
| 18 |  | n.a. | 1 | - | 2 | - |

Reaction conditions: 1 mM substrate, 0.2 mM NADH, appropriate amount of purified enzyme, 0.4 mL phosphate buffer (0.1 M, pH 7), 30 °C。n.a., no activity.

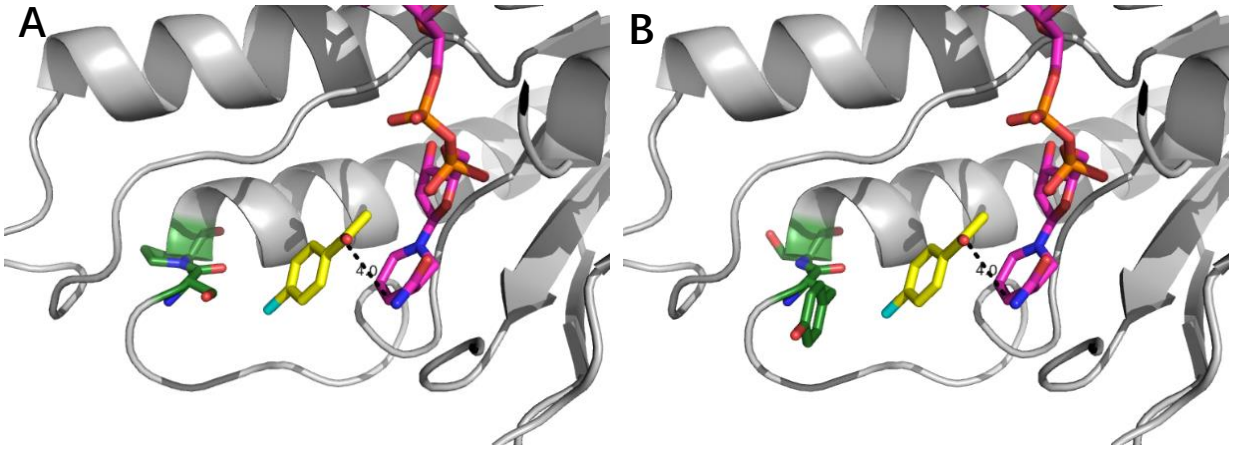



**Figure S6.** Molecular docking of *Sc*CR (A) and variant M4 (B) with 4'-chloroacetophenone and NADH

**Table S6.** Detection wavelength and retention times of substrates and products

| Substrates | HPLC methods | Wavelength and retention time | |
|---|---|---|---|
| | | Substrates | Products |
| **1a** | a | 228 nm | 210 nm |
| | | 11.1 min | 7.7 min |
| **1b** | c | 270 nm | 214 nm |
| | | 8.7 min | 7.9 min |
| **1c** | d | 230 nm | 210 nm |
| | | 13.1 min | 9.4 min |
| **1d** | e | 270 nm | 265 nm |
| | | 16.2 min | 9 min |
| **1e** | b | 241 nm | 210 nm |
| | | 6.1 min | 4.7 min |
| **1f** | b | 245 nm | 222 nm |
| | | 9.7 min | 7 min |
| **1g** | b | 247 nm | 222 nm |
| | | 10.5 min | 7.5 min |
| **1h** | b | 270 nm | 224 nm |
| | | 6.1 min | 4.7 min |
| **1i** | b | 210 nm | 210 nm |
| | | 13.3 min | 8.3 min |

a：acetonitrile/0.41% $(NH_4)_2SO_4$ aqueous solution with pH 3.5 (2/8, v/v), 0.6 mL/mim

b：acetonitrile/0.02% phosphoric acid aqueous solution (6/4, v/v), 0.6 mL/min

c：acetonitrile /0.4% $(NH_4)_2SO_4$ aqueous solution with pH 3.5 (4/6, v/v), 0.5 mL/min

d：acetonitrile/20 mM, pH 7.5 phosphate buffer (containing 0.1% (v/v) triethylamine）(2/8, v/v), 0.6 mL/min

e：methanol/25 mM, pH 7 phosphate buffer（containing 0.1% (v/v) triethylamine）(1/9, v/v), 1 mL/min

**Table S7.** Primers for mutants

| Mutation site | | Primer sequence (5'-3') |
|---|---|---|
| L207A | F | gctCTCAAGACCATGGACGAGGCCGCCTACAA |
| | R | TCCATGGTCTTGAGagcGGGGGTGTCGATGAAGCC |
| G105V | F | AACAACGCCgtaATCGGCGGCCCCAGCGCCCC |
| | R | CCGATtacGGCGTTGTTGACGGCGAGGTCCAG |
| I158Y | F | AACGTCGCCTCCtatCTCGGCTCGGTCGGCTTC |
| | R | AGataGGAGGCGACGTTCACGATCGAGCCGCC |
| P200A | F | TgctGGCTTCATCGACACCCCCCTGCTCAAGA |
| | R | TGTCGATGAAGCCagcACCGACCGCGTTGATCCG |
| G105H | F | AACAACGCCcatATCGGCGGCCCCAGCGCCCC |

| | | |
|---|---|---|
| | R | CCGATatgGGCGTTGTTGACGGCGAGGTCCAG |
| F202I | F | TCCGGGCattATCGACACCCCCCTGCTCAAGA |
| | R | TGTCGATaatGCCCGGACCGACCGCGTTGATC |
| L208R | F | CCCCTGagaAAGACCATGGACGAGGCCGCCTA |
| | R | ATGGTCTTtctCAGGGGGGTGTCGATGAAGCC |
| I203W | F | TCCGGGCTTCtggGACACCCCCCTGCTCAAGA |
| | R | TGTCccaGAAGCCCGGACCGACCGCGTTGATC |
| L159P | F | TCCATCccaGGCTCGGTCGGCTTCGCCGGCTC |
| | R | ACCGAGCCtggGATGGAGGCGACGTTCACGAT |
| S167W | F | GGCtggCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGccaGCCGGCGAAGCCGACCGA |
| S167H | F | GGCcatCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGatgGCCGGCGAAGCCGACCGA |
| S167E | F | GGCgaaCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGttcGCCGGCGAAGCCGACCGA |
| S167T | F | GGCaccCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGggtGCCGGCGAAGCCGACCGA |
| S167V | F | GGCgtaCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGtacGCCGGCGAAGCCGACCGA |
| S167G | F | GGCggtCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGaccGCCGGCGAAGCCGACCGA |
| S167N | F | GGCaatCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGattGCCGGCGAAGCCGACCGA |
| S167I | F | GGCattCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGaatGCCGGCGAAGCCGACCGA |
| S167P | F | GGCccgCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGcggGCCGGCGAAGCCGACCGA |
| S167D | F | GGCgatCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGatcGCCGGCGAAGCCGACCGA |
| S167C | F | GGCtgtCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGacaGCCGGCGAAGCCGACCGA |
| S167F | F | GGCtttCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGaaaGCCGGCGAAGCCGACCGA |
| S167M | F | GGCatgCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGcatGCCGGCGAAGCCGACCGA |
| S167R | F | GGCagaCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGtctGCCGGCGAAGCCGACCGA |
| S167Q | F | GGCcagCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGctgGCCGGCGAAGCCGACCGA |
| S167L | F | GGCctgCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGcagGCCGGCGAAGCCGACCGA |
| S167K | F | GGCaaaCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGtttGCCGGCGAAGCCGACCGA |
| S167Y | F | GGCtatCCCGCCTACGTCGCCGCCAAGCACGG |

| | | |
|---|---|---|
| | R | ACGTAGGCGGGataGCCGGCGAAGCCGACCGA |
| S167A | F | GGCgctCCCGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCGGGagcGCCGGCGAAGCCGACCGA |
| I158V | F | ACGTCGCCTCCgtaCTCGGCTCGGTCGGCTTC |
| | R | AGtacGGAGGCGACGTTCACGATCGAGCCGCC |
| S167F/P168S | F | TCGGTCGGCTTCGCCGGCTTCTCTGCCTACGTCGCC |
| | R | GGCGACGTAGGCAGAGAAGCCGGCGAAGCCGACCGA |
| S167Y/P168S | F | TCGGTCGGCTTCGCCGGCTACTCTGCCTACGTCGCC |
| | R | GGCGACGTAGGCAGAGTAGCCGGCGAAGCCGACCGA |
| I158V/S167F/P168S | F | CGGCttttcaGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCtgaaaaGCCGGCGAAGCCGACCGAG |
| I158V/S167Y/P168S | F | CGGCtattcaGCCTACGTCGCCGCCAAGCACGG |
| | R | ACGTAGGCtgaataGCCGGCGAAGCCGACCGAG |

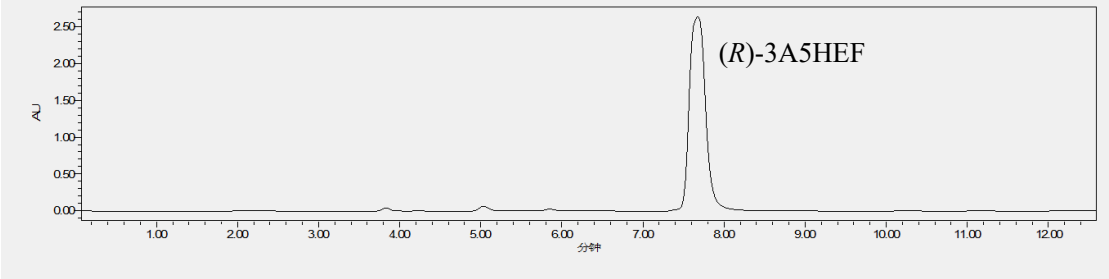
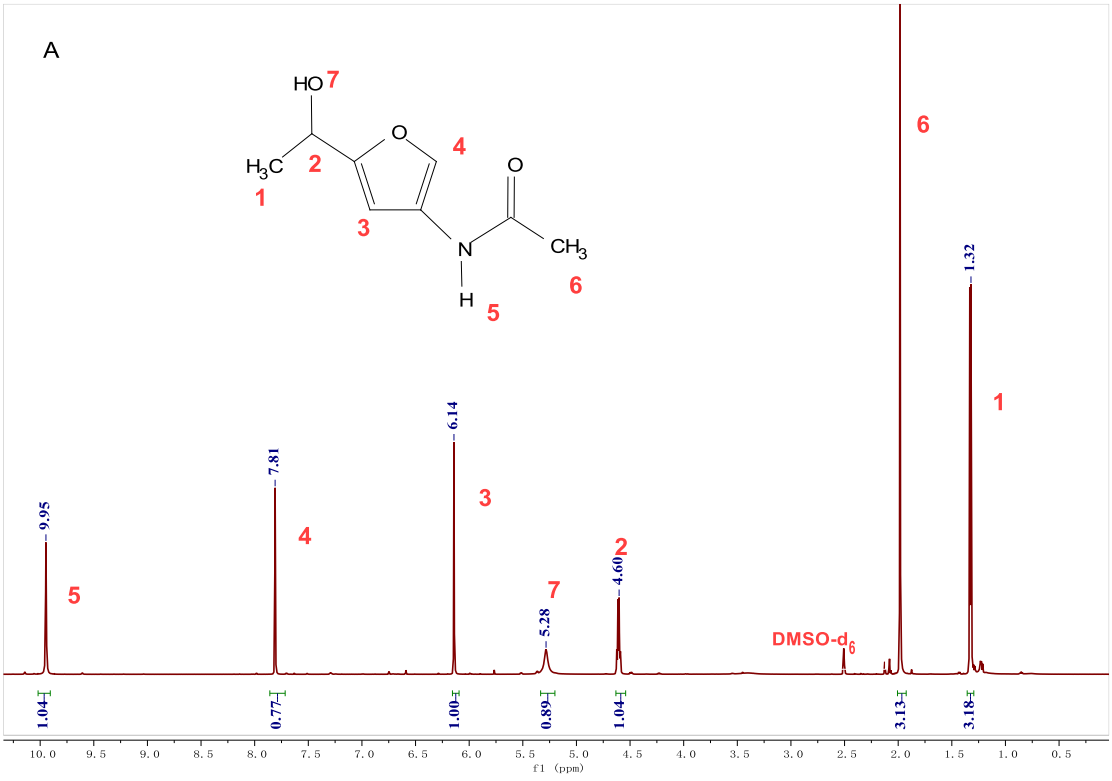



Figure S7. HPLC chromatogram of (*R*)-3A5HEF obtained in the preparative-scale synthesis



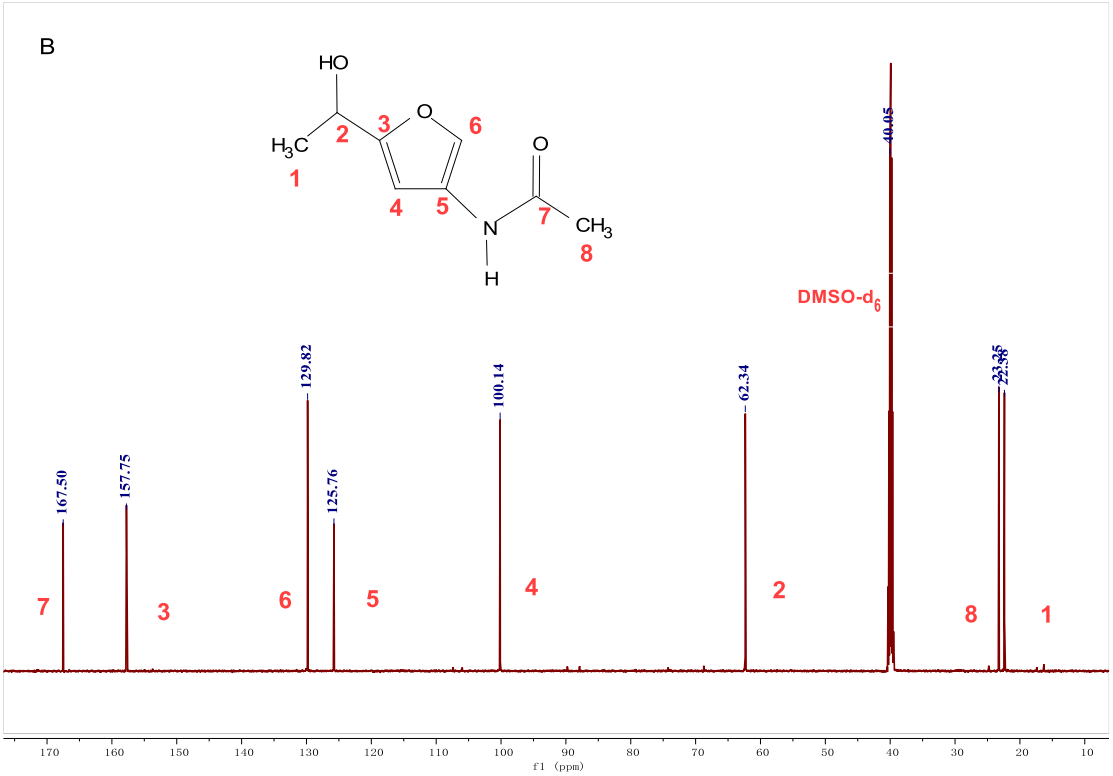



Figure S8. $^{1}H$ (A) and $^{13}C$ NMR (B) spectra of (*R*)-3A5HEF obtained in the preparative-scale synthesis.

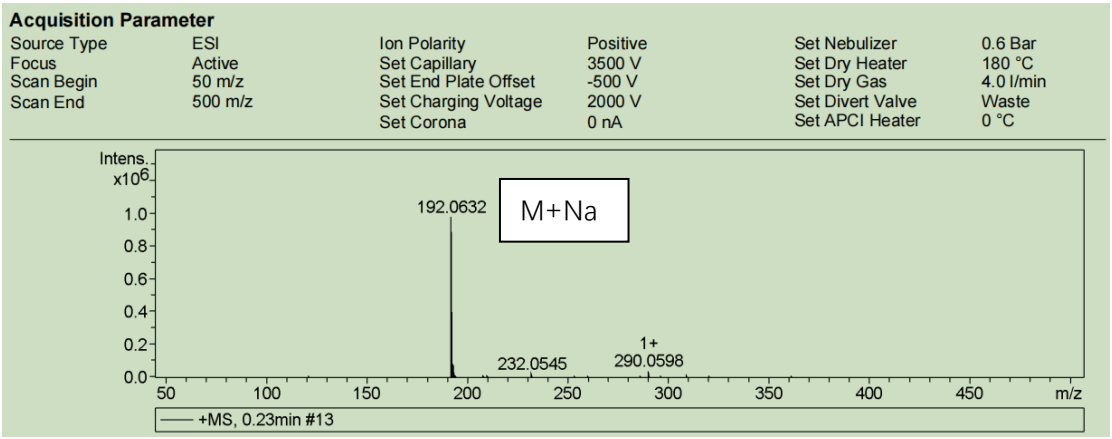
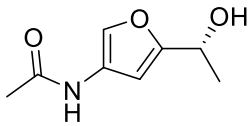


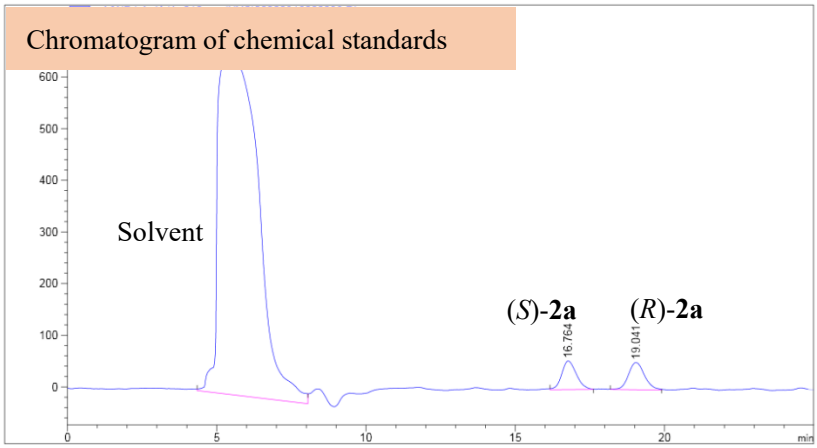

**Figure S9.** UPLC-MS spectra of (*R*)-3A5HEF (M, 169.07) obtained in the preparative-scale synthesis.

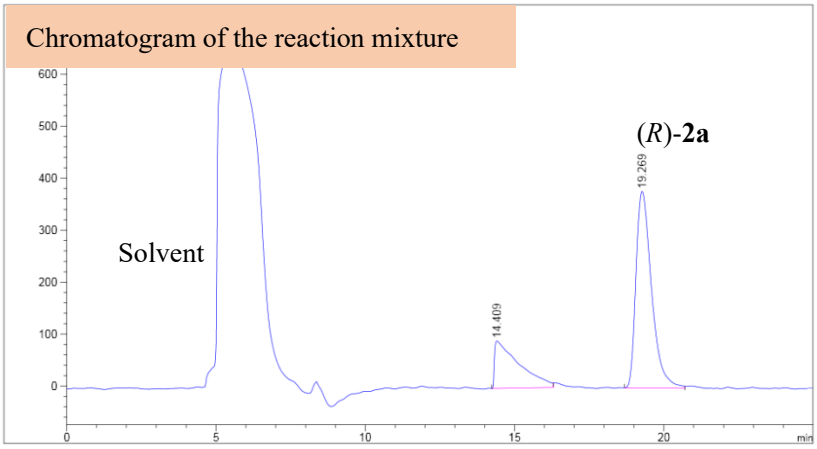
**Determining product ee values**

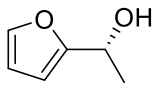



(*R*)-**2a**

HPLC Conditions. Column: Chiralcel OJ-H, Daicel Chemical Industries, Ltd., Eluent: Hexanes/isopropanol (86/14); Flow rate: 0.7 mL/min; Detection: UV 210 nm.

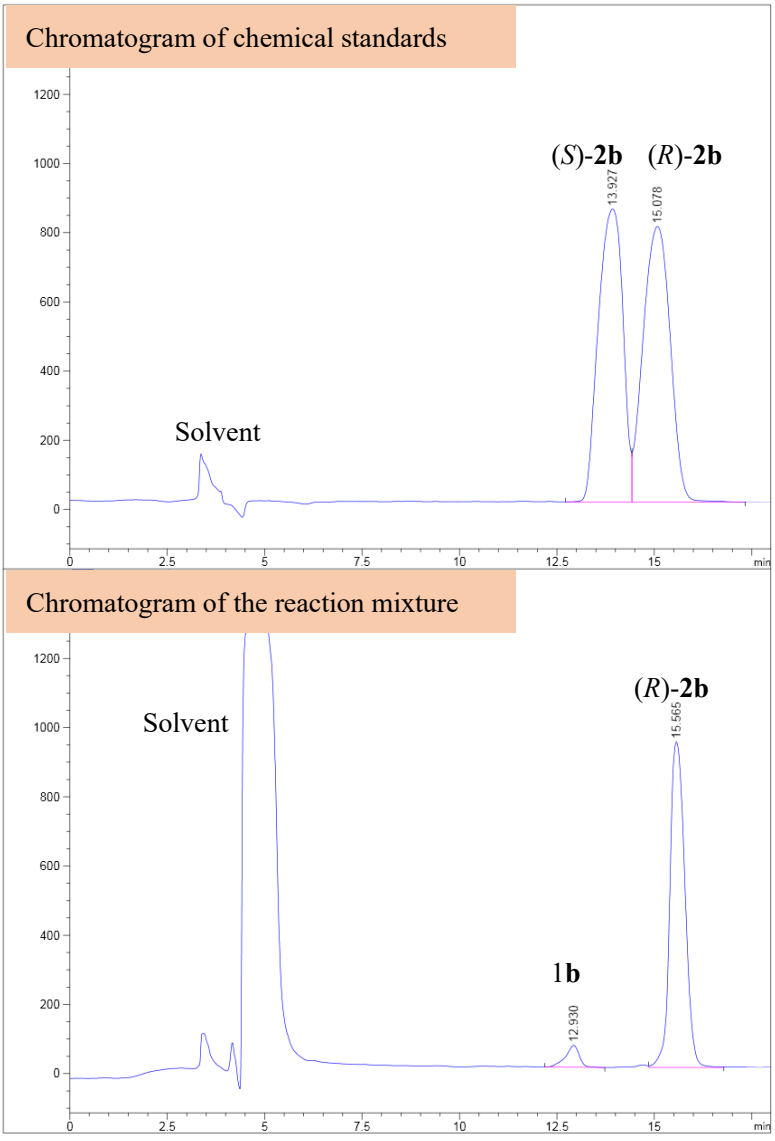


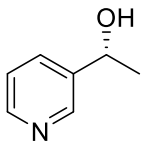





(*R*)-**2b**

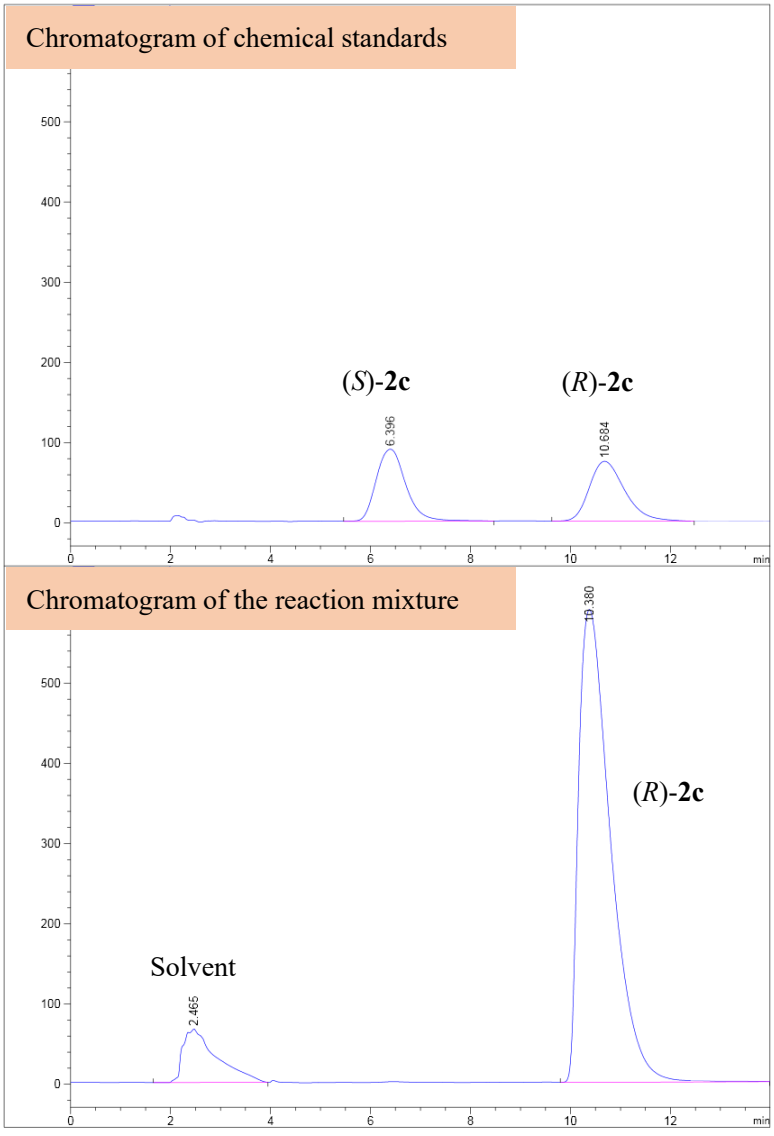
HPLC Conditions: Column: Chiralcel OJ-H, Daicel Chemical Industries, Ltd., Eluent: Hexanes/isopropanol (98/2); Flow rate: 1.0 mL/min; Detection: UV 210 nm. (*R*)-**2b** = 15.0 min; (*S*)-**2b** = 13.9 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards.

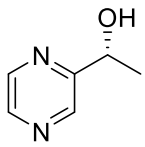





(*R*)-**2c**

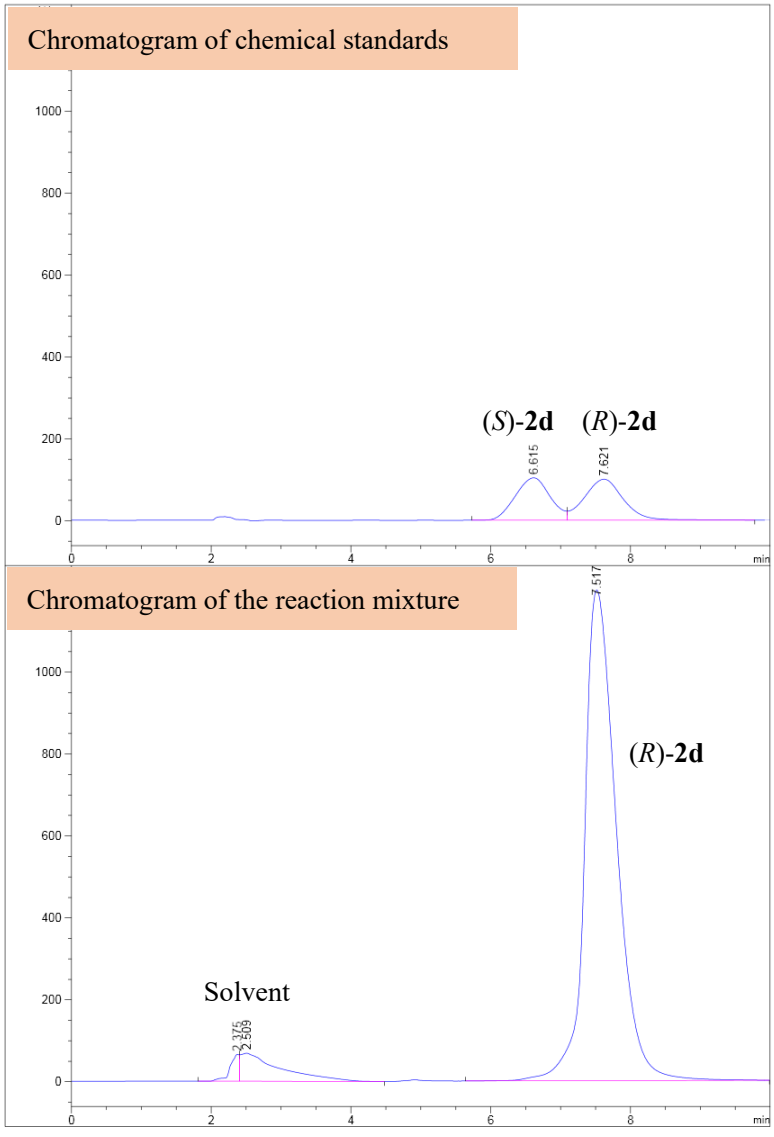
HPLC Conditions: Column: Chiralcel OB-H, Daicel Chemical Industries, Ltd., Eluent: Hexanes/isopropanol (90/10); Flow rate: 1.0 mL/min; Detection: UV 254 nm. (*R*)-**2c** = 10.6 min; (*S*)-**2c** = 6.3 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards and literature.[4]

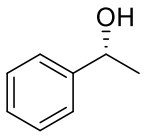




(*R*)-**2d**

HPLC Conditions: Column: Chiralcel OB-H, Daicel Chemical Industries, Ltd., Eluent: Hexanes/isopropanol (92/8); Flow rate: 1.0 mL/min; Detection: UV 254 nm. (*R*)-**2d** = 7.6 min; (*S*)-**2d** = 6.6 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards and literature.[4]

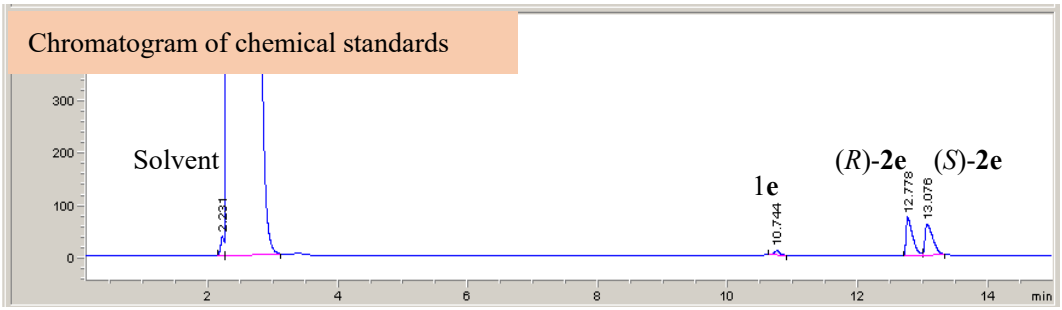




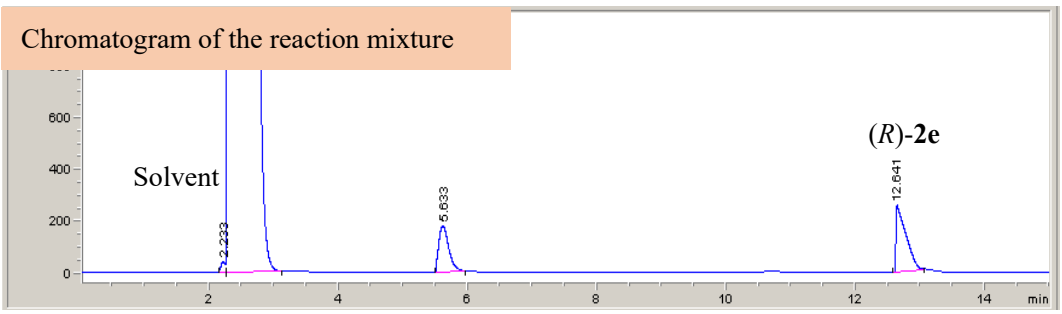

(*R*)-**2e**

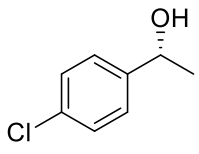
GC conditions: Column: HP Chiral 10B, Agilent Technoligies, Co., Ltd. Temperature conditions: initial temperature 100 °C, 2 °C/min to 126 °C, holding 2 min; injector 250 °C, detector 250 °C; and 0.8 mL/min $N_2$ flow. The injection volume was 1.0 uL with a split ratio of 2:1. (*R*)-**2e** = 12.7 min; (*S*)-**2e** =13.0 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards.

Chromatogram of chemical standards




Chromatogram of the reaction mixture

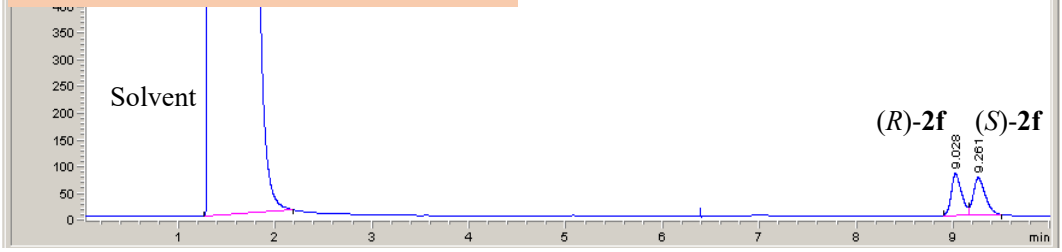




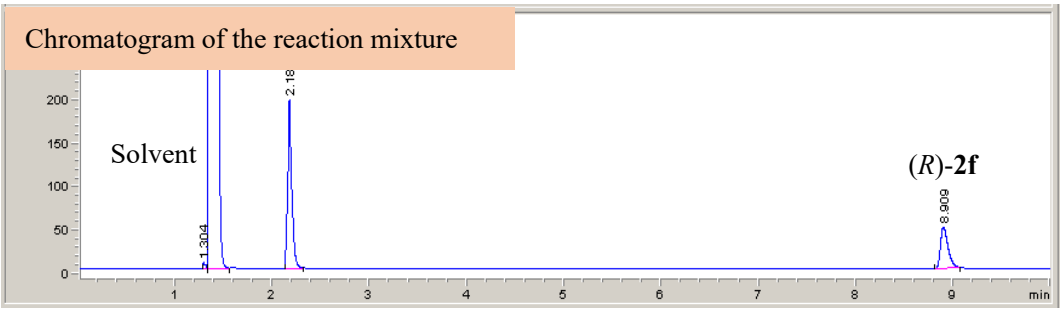

(*R*)-**2f**

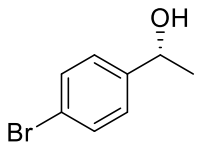
GC conditions: Column: HP Chiral 10B, Agilent Technoligies, Co., Ltd. Temperature conditions: initial temperature 130 °C, 2 °C/min to 150 °C; injector 250 °C, detector 250 °C; and 1.5 mL/min $N_2$ flow. The injection volume was 1.0 uL with a split ratio of 30:1. (*R*)-**2f** = 9.0 min; (*S*)-**2f** =9.2 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards and literature.[5]

Chromatogram of chemical standards




Chromatogram of the reaction mixture

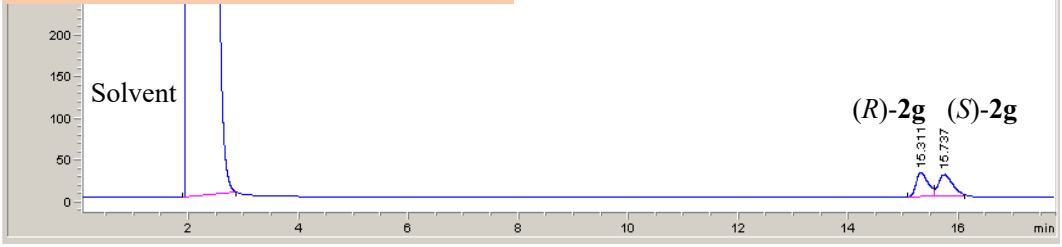




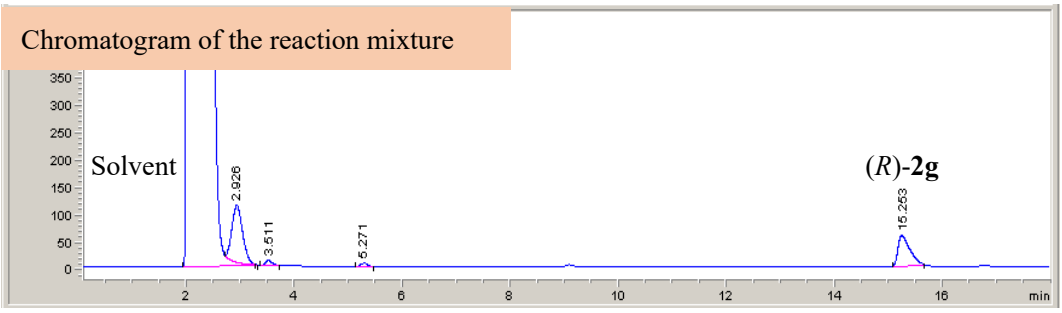

(*R*)-**2g**

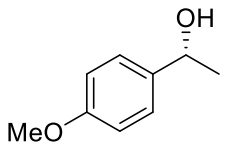
GC conditions: Column: HP Chiral 10B, Agilent Technoligies, Co., Ltd. Temperature conditions: initial temperature 150 °C, holding 17 min; injector 250 °C, detector 250 °C; and 0.8 mL/min $N_2$ flow. The injection volume was 1.0 uL with a split ratio of 2:1. (*R*)-**2g** = 15.3 min; (*S*)-**2g** =15.7 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards and literature.[5]

Chromatogram of chemical standards




Chromatogram of the reaction mixture

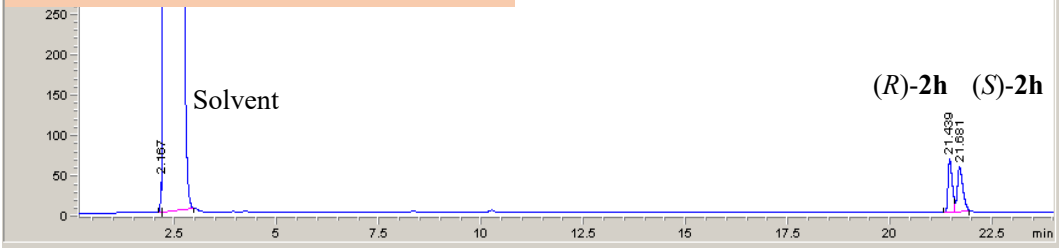




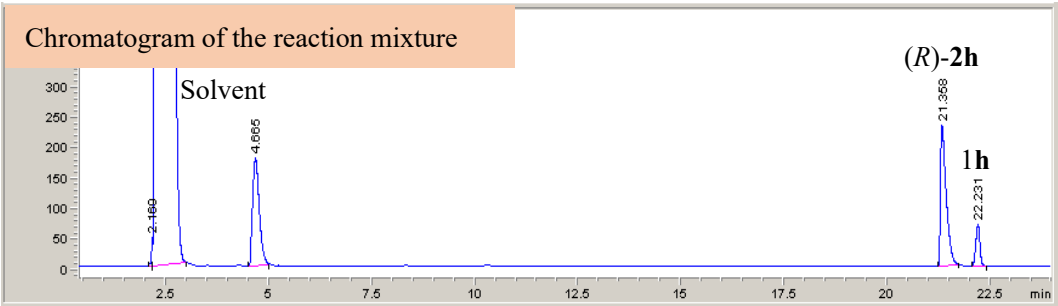

(*R*)-**2h**

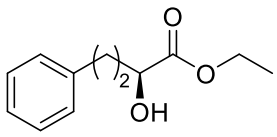
GC conditions: Column: HP Chiral 10B, Agilent Technoligies, Co., Ltd. Temperature conditions: initial temperature 110 °C, 2 °C/min to 150 °C, holding 4 min; injector 250 °C, detector 250 °C; and 0.8 mL/min $N_2$ flow. The injection volume was 1.0 uL with a split ratio of 2:1. (*R*)-**2h** = 21.4 min; (*S*)-**2h** =21.6 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards.

Chromatogram of chemical standards




Chromatogram of the reaction mixture

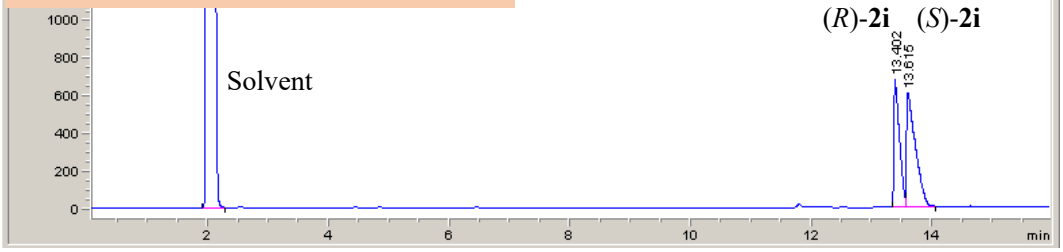




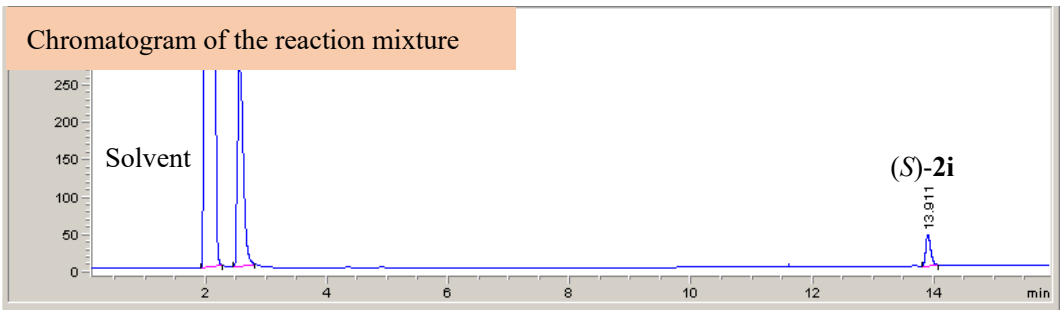
(*S*)-**2i**

GC conditions: Column: CP-Chiralsil-DEX-CB, Agilent Technoligies, Co., Ltd. Temperature conditions: initial temperature 140 °C, 2 °C/min to 170 °C, holding 1 min; injector 250 °C, detector 250 °C; and 2.5 mL/min $N_2$ flow. The injection volume was 1.0 uL with a split ratio of 2:1. (*R*)-**2i** = 13.4 min; (*S*)-**2i** =13.6 min. The product absolute configurations were determined by comparing the retention times with those of the authentic standards.

Chromatogram of chemical standards




Chromatogram of the reaction mixture

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2. M. M. Bradford, *Anal. Biochem.* , 1976, **72**, 248-254.
3. Y.-C. Hao, M.-H. Zong, Z.-L. Wang and N. Li, *Bioresour. Bioprocess.*, 2021, **8**, 80.
4. M. Pal, G. Srivastava, L. S. Moon and R. S. Jolly, *Bioresource technology*, 2012, **118**, 306-314.
5. P. Wei, Z.-W. Guo, X.-L. Wu, S. Liang, X.-Y. Ou, P. Xu, M.-H. Zong, J.-G. Yang and W.-Y. Lou, *Molecular Catalysis*, 2019, **479**, 110613.