Efficient production of \((R)-3\)-TBDMSO glutaric acid methyl monoester by manipulating the substrate pocket of \textit{Pseudozyma antarctica} lipase B†

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Optically pure \((R)-3\)-substituted glutaric acid methyl monoesters are multifunctional chiral building blocks used in the pharmaceutical industry. In the current study, a combined \textit{in silico}/mutagenesis approach was used to improve the performance of \textit{Pseudozyma antarctica} lipase B (CALB) as a biocatalyst in the asymmetric synthesis of \((R)-3\)-t-butyl-dimethyl-silyloxy (TBDMSO) glutaric acid methyl monoester \((R)\)-J6. Candidate amino acids that likely affected the enantioselectivity of CALB were identified by substrate structure analysis. Mutant variants were screened \textit{in silico}; CALB enantioselectivity was reversed and enhanced based on molecular docking analyses, followed by reshaping of the substrate pocket. EFS CALB variant, generated by semi-rational design and selected by high-throughput screening, exhibited high \(R\)-selectivity with an ee \((\text{enantiomeric excess})\) value of 85%, while the wild-type (WT) CALB showed \(S\)-selectivity; the \(k_{\text{cat}}/K_{M}\) of EFS towards \((R)\)-J6 increased 14-fold, from 0.59 to 8.29 mM\(^{-1}\) s\(^{-1}\). Compared with WT CALB, the affinity of EFS for 3-TBDMSO glutaric anhydride increased 2.31-fold. By optimizing the fermentation conditions for the yeast host for protein production and enzyme immobilization conditions, the hydrolytic activity of EFS was increased to 2401.5 ± 5.3 U mL\(^{-1}\) and 2706.7 ± 11.4 U g\(^{-1}\), respectively. The yield of \((R)\)-J6 generated by the EFS variant in non-aqueous media increased to 55 ± 1.6 g L\(^{-1}\), with an ee\(_{R}\) value of 98.5%. Semi-rational design was hence successfully employed to generate gram quantities of \((R)-3\)-substituted glutaric acid monoesters with enormous potential and high ee.

**Introduction**

Optically pure \((R)-3\)-substituted glutaric acid monoesters \((R)\)-J6 are attractive building blocks for the synthesis of a number of pharmaceutically important compounds, including pitavastatin, fluvastatin, atorvastatin, and rosuvastatin. Among these, rosuvastatin inhibits hydroxymethylglutaryl-CoA reductase and has few side-effects.\(^1\) \((R)\)-J6 can be obtained by four approaches: chemical synthesis,\(^2\) hydrolysis of dialkyl-3-substituted glutaric acids using hydrolases,\(^3\) kinetic resolution of racemates,\(^4\)\(^5\) and desymmetrization of prochiral compounds.\(^6\) To date, \((R)\)-J6 is mainly prepared by chemical synthesis;\(^7\) \((S)-1\)-phenethylamin is used to catalyze the asymmetric reduction of 3-substituted glutaric acid monoesters, with a space-time yield of 13.6 g L\(^{-1}\) h\(^{-1}\), low yield (54.9%), and a low enantiomeric excess \((\text{ee} ; 80\%)\) at −78 °C.

Fortunately, biocatalysis provides an attractive alternative to chemical synthesis and is environmentally friendly. \((R)\)-J6 may be prepared using pig liver esterase and Novozym 435.\(^8\) The space-time yield of such a reaction is high (≥4.5 g L\(^{-1}\) h\(^{-1}\)) but the selectivity for the \((R)\)-isomer is low (Table 1). \(\alpha\)-Chymotrypsin is also employed in \((R)\)-J6 preparation, hydrolyzing the dialkyl-3-substituted glutaric acid with a high ee\(_{R}\) of 97%.\(^9\) However, this approach comprises six steps, starting with diethyl-3-t-butyl-dimethyl-silyloxy (TBDMSO) glutaric acid (conversion, 65.4%; isolated yield, 53.2%), and the 3-substituent group of the substrate appreciably affects the catalytic efficiency and enantioselectivity of the enzyme.\(^9\) Thus, \(\alpha\)-chymotrypsin performs poorly with TBDMSO as the 3-substituent group of the substrate. Furthermore, the process entails high production costs, and two high-selectivity biocatalysts \((\alpha\)-chymotrypsin and cephalosporin C acetyl esterase\) are required.\(^9\) Moreover, \(\alpha\)-chymotrypsin exhibits low activity and selectivity during \((R)\)-J6 preparation in an organic solvent, as described by us in a previous study.\(^9\)

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With a theoretical yield close to 100%, enzymatic desymmetrization of prochiral compounds is currently recognized as an efficient route for the preparation of optically pure compounds. Therein, the hydrophobic substrates are dissolved in organic solvents and the desired products are subsequently easily isolated, purified, and dried.\textsuperscript{11-13} Therefore, the research focus has shifted from chemical synthesis to the desymmetrization of 3-substituted glutaric acid anhydrides by biocatalysts.\textsuperscript{14} Literature search, however, revealed a lack of one type of enzyme that would meet the industry requirements; the reactions catalyzed by the available biocatalysts are characterized by low titers, low yields, and low ee values.\textsuperscript{14} 

### Materials and methods

**Chemicals**

3-TBDMSO glutaric anhydride was purchased from Henan Yuchen Fine Co., Ltd. (Henan, China). Hexane and iso-propanol (high-performance liquid chromatography grade) were purchased from Fisher Chemical (Fairlawn, NJ, USA); other chemicals and solvents (analytical grade) were obtained from local suppliers. An R\textsubscript{J\textsubscript{6}} standard and racemic 3-TBDMSO glutaric acid methyl monoester was gifts from Chanyoo PharmaTech Co., Ltd. (Nantong, China). All other chemicals were of analytical grade and obtained from companies in China. A Daicel Chiralpak AD-H column (4.6 × 250 mm) was purchased from Daicel Chiral Technologies Co., Ltd. (Shanghai, China).

**Bacterial strains, plasmids, and enzymes**

The calB gene (GeneBank accession no. Z30645.1) was amplified by polymerase chain reaction (PCR) from the chromosome of *P. antarctica* JCM3941 purchased from the Japan Collection of Microorganisms (JCM; RIKEN, Saitama, Japan). A fragment of the gene (ca. 1029 bp) was amplified using the following primers: 5'-CGCTCGAGAATGAGACTACCTTCCGGTTCGGACCG-3' (forward) and 5'-TGCTCTAGATTAGGGGTTGACGATCCG-3' (reverse; the underlined regions are XhoI and XbaI restriction sites, respectively). A recombinant plasmid vector pMD19-T-CALB was constructed and used as a parental vector for the generation of enzyme variants.\textsuperscript{15} *Escherichia coli* JM109 [recA supE hsr Δ(lac-pro)] was used as the host for plasmid amplification. *Pichia pastoris* GS115 strain (Invitrogen, Carlsbad, CA, USA) served as the host for CALB production. The expression vector pGPAPZzaA was purchased from Novagen (Madison, WI, USA). LTAq DNA polymerase, PrimeSTAR DNA polymerase, and restriction enzymes were from TakaRa (Dalian, China). AvrII (Thermo Fisher Scientific, Waltham, MA, USA) was used to linearize the recombinant plasmids. *E. coli* was incubated in lysogeny broth (LB) medium at 37°C, 200 rpm, for 24 h. *P. pastoris* GS115 and *P. antarctica* JCM3941 were inoculated into yeast extract peptone dextrose (YPD) medium (10 g L\textsuperscript{-1} of yeast extract, 20 g L\textsuperscript{-1} of peptone, and 20 g L\textsuperscript{-1} of glucose), and grown at 30°C on a rotary shaker (200 rpm) for 2 d.

### Table 1: Comparison of the asymmetric synthesis of 3-substituted glutaric acid monoesters

| Catalyst          | Substrate load (g L\textsuperscript{-1}) | Time (h) | Catalyst load (g L\textsuperscript{-1}) | Yield\textsuperscript{a} (%) | Temp. (°C)/strategy | ee (%)/config. | STY\textsuperscript{b} (g L\textsuperscript{-1} h\textsuperscript{-1}) | Ref. |
|-------------------|------------------------------------------|----------|------------------------------------------|------------------------------|---------------------|----------------|----------------------------------|------|
| (S)-1-Phenylalanin| 45                                       | 2        | 44                                       | 54.9                         | –78 (CS)\textsuperscript{d} | 80 (R)         | 13.60                             | 2, 7 and 28 |
| Novozym 435       | 45                                       | 64       | 20                                       | 92.6                         | 30 (H)\textsuperscript{f} | 95.6 (R)       | 0.654                             | 29   |
| PLE               | 50                                       | 6        | 7.5                                      | 76                           | NG\textsuperscript{g} \textsuperscript{f} | 22 (S)         | 6.330                             | 3 and 30 |
| z-Chymotrypsin    | 50                                       | 48       | 20                                       | 56                           | NG\textsuperscript{g} \textsuperscript{f} | 97 (R)         | 0.677                             | 3 and 10 |
| Novozym 435       | 52                                       | 24       | 50                                       | 35 (AS)\textsuperscript{b}   | 30 (AS)\textsuperscript{b} | <22            | 4.800                             | 9 (PS)\textsuperscript{c} |
| Novozym 435       | 200                                      | 50       | 52.6                                     | 35 (AS)\textsuperscript{b}   | 98 (S)               | 2.030          | 1.152                             | 29   |
| EF5 mutant        | 60                                       | 48       | 80                                       | 92.1                         | 5 (AS)\textsuperscript{b} | 98.5 (R)       | 1.152                             | This work |

\textsuperscript{a} Note: This value refers to the isolation yield. \textsuperscript{b} STY, space-time yield (g L\textsuperscript{-1} h\textsuperscript{-1}). \textsuperscript{c} NG, not given. \textsuperscript{d} CS, chemical synthesis. \textsuperscript{e} PS, previous study. \textsuperscript{f} H, hydrolysis. \textsuperscript{g} AS, asymmetric synthesis.

\textsuperscript{16} The three-dimensional structure of the redesigned mutants was calculated by the Molegro Virtual Docker software.\textsuperscript{17} Only ligand molecules were considered to be flexible during the docking simulation, and only the free energy of their best orientation was used to compute the docking free energy. The cavity volume of the redesigned mutants was calculated by the Molegro Virtual Docker software.\textsuperscript{18}
For site-directed mutagenesis, candidate amino acids, excluding the catalytic triad (Ser105, His224, and Asp187), were changed to ones that occurred with high frequency in the homologous sequence (ESI Table S1†). Introduction of specific amino acid substitutions into CALB was performed using primer pairs listed in ESI Table S2.24,25 PCR products were digested by DpnI (Thermo Fisher Scientific) and used to transform E. coli JM109; the transformants were selected on LB agar plates containing 100 μg mL⁻¹ of ampicillin. The introduced mutations were confirmed by DNA sequencing (Sangon Biotech, Shanghai, China), and the mutated sequences were inserted into the pGAPZzA vector. The pGAPZzA-CALB-variant plasmids were linearized by AvrII and introduced into the host, P. pastoris GS115, by electroporation.21

CALB variant library expression in P. pastoris and biocatalyst preparation

Degenerate NNN, NDK, and NDT codons were used to create CALB variant libraries based on the results of docking analysis; the primers used for the semi-saturation/saturation mutagenesis of A141S-A283V are shown in ESI Table S3.† The prepared plasmid libraries were introduced into P. pastoris GS115 by electroporation,22 the cells were then incubated at 30 °C in YPD medium (1 mL) for 2 h, which was followed by plating on YPD agar plates containing zeocin (100 μg mL⁻¹). The plates were then incubated at 30 °C for 3 d. Single colonies were picked, inoculated into conical, deep 96-well plates, and incubated for 3 d, with shaking at 200 rpm, at 29 °C. Each well contained YPD (800 μL) supplemented with zeocin (100 μg mL⁻¹). Clones were verified by PCR using a pair of validation primers (pGAP-forward, 5'-TCCCTATTTCAATCATTGA3'; and 3'-AOX1-Rev, 5'-GCAATGGCATTCTGACATCC3'). After gene expression, the yeast cells were pelleted by centrifugation (2500 × g, 10 min). The supernatant was harvested by aspiration and used directly for optical screening. Master plates were stored at −70 °C prior to further analysis or cultivation. The best-performing CALB variants were purified on a nickel-affinity column (Ni Sepharose 6 Fast Flow, Amersham Biosciences, Freiburg, Germany), and prepared for kinetic analysis.29 The enzyme solution was immobilized on D101 macroporous resin (Shanghai Hualing Resin Factory, Shanghai, China) according to the manufacturer’s guidelines and the pellets were freeze-dried.

Hydrolytic activity determinations and enantioselectivity screening

The hydrolytic activity assay was performed by monitoring the amount of released p-nitrophenol during the hydrolysis of 10 mM p-nitrophenyl butyrate (p-NPB) in 50 mM Tris–HCl buffer (pH 8.0) at 410 nm and 30 °C.22 One unit of hydrolytic activity (1 U) was defined as the amount of enzyme that generated 1 μmol of p-nitrophenol per min. The protein concentration was determined using Bradford’s method, with bovine serum albumin as the standard.23

Preliminary screening. The main purpose of the preliminary screening was to improve the hydrolytic activity of variant proteins with the R-isomer; the screening was only performed with (R)-methyl mandelate to reduce the subsequent workload.

The screening buffer [120 μL; 100 mM potassium phosphate, 10% (v/v) methanol, and 4% (v/v) Triton X-100, pH 7.4] and (R or S)-methyl mandelate (10 μL; 100 mM in methanol) were premixed as screening buffer I. The yeast library supernatant (20 μL) and screening buffer I (150 μL) were dispensed into a microtiter plate, and incubated at 30 °C for 15 min, following which the absorbance was measured at 655 nm. Individual enantiomers of the substrate were hydrolyzed in parallel reactions.

Detailed screening. The potential R-selectivity of candidate proteins was further investigated by hydrolysis of the racemic mandelic acid methyl ester. The hydrolytic activities with (R)-methyl mandelate (A_R) and (S)-methyl mandelate (A_S) were calculated at 655 nm, and the total activity (A_total) was determined at 410 nm by the p-NPB assay (ESI Fig. S1†). The R/S value (A_R/A_S) was then determined; the variants with high R/S values were deemed to be superior to other variants. The variants with high R/S values (see below) were then screened for hydrolysis of R-J_6 to investigate their specificity toward R-J_6; the activity of the most promising variants was finally verified during asymmetric alcoholysis of 3-TBDMSO glutaric anhydride.

Kinetics of CALB variants. The kinetic parameters were obtained by measuring the initial velocities of the enzymatic hydrolysis reaction. Enzymes assays with 96 μL of CALB variants were carried out in 0.1 M Tris–HCl buffer (pH 8.0), 4 μL of ethanol with increasing concentration of substrate. For substrate of p-nitrophenyl butyrate (p-NPB), the concentration range was between 1 mM and 25 mM; the increasing concentration of R-J_6 (substrate) was from 0.2 mM to 10 mM. Lineweaver-Burk plots were plotted to determine K_m and V_max of CALB variants. All measurements were taken in triplicate to evaluate the standard deviation.

Characterization of variant function and analytical methods

Imnobilized CALB variants were characterized using a model esterification reaction for (R)-J_6 synthesis; the reaction was conducted in 10 mL capped flasks containing methyl tert-butyl ether and equal concentrations of substrates. The mixtures were incubated in batches for 36 h on an orbital shaker (200 rpm) at 5 °C, and the system water activity was adjusted by using appropriate mesh sieves.24,25 The products were analyzed using a high-performance liquid chromatograph (Waters E2695, Waters Corporation, Milford, MA, USA) equipped with an AD-H column and an ultraviolet detector (Waters Corporation). The mobile phase consisted of 94% hexane and 6% iso-propanol with 0.01% (v/v) acetic acid, filtered through a 0.22 μm membrane before use. Samples (10 μL) were injected into the chromatograph (25 °C detection temperature and 1 mL min⁻¹ flow rate); the detection took 12 min. The retention times of R-J_6 and S-J_6 were 6.1 and 6.6 min, respectively; racemic 3-TBDMSO glutaric acid methyl monooester (racemic J_6) served as an internal standard. The ee_R and ee_S values were defined as follows: ee_R = [(R - S)/(R + S)] × 100% and ee_S = [(S - R)/(R + S)] × 100%, wherein R and S represent the concentrations of (R)-J_6 and (S)-J_6, respectively.
Results and discussion

Potential CALB derivatives with improved enantioselectivity

To select the key amino acid residues that pronouncedly affect CALB enantioselectivity, the docking energies of different 3-hydroxy protecting groups on wild-type (WT) CALB were first compared in Table 2. The highest and lowest docking energy values were −124.33 kcal mol⁻¹ (TBDPSO⁻) and −72.92 kcal mol⁻¹ (OH⁻), respectively. Specific amino acid residues were identified by analyzing the interaction (H-bonds and salt bridges) of these structures [(R)-TBDPSO⁻, (R)-OH⁻, and (R)-J₆] with WT CALB. Thr42, Ile189, Asp134, Thr138, Gln157, and Gln106 were selected as candidate residues that appeared twice and interacted with WT CALB via an H-bond or a salt bridge.

Similarly, 3-TBDMsO glutaric acid monoalkyl esters with different alcohol moieties as ligands were docked into WT CALB (Table 3); amino acid residues Ala141, Leu278, Trp104, Val190, Ser47, Ala282, and Ile285 were selected. Collectively, 13 amino acid residues were selected as candidate residues to be substituted.

Enhancing the enantioselectivity of CALB by manipulating the substrate pocket

The enantioselectivity of CALB is affected by the affinity, and the substrate pocket.²⁵,²⁶ Hence, to increase the affinity of CALB for the R-isomer, the enantiomer products R_J₆ and S_J₆ were docked in silico into thirteen potential enzyme variants (the acceptors); the respective docking energies of (R)_J₆ and (S)_J₆ are listed in ESI Table S4. The ratios of the docking energies of (R)_J₆ to (S)_J₆ (E_R/E_S) were then calculated and used as an index to compare the potential variants in silico: the higher of E_R/E_S value, the higher the R-selectivity. Consequently, mutants S47N (E_R/E_S = 1.138) and A141S (E_R/E_S = 1.119), with the highest E_R/E_S values, were identified. Of the two, the (R)_J₆ docking energy was lower for A141S (−92.01 kcal mol⁻¹), i.e., the mutant exhibited a higher affinity for (R)_J₆, and was chosen for further analysis. The titer and ee values of R_J₆ were 4.4 ± 0.3 g L⁻¹ and −63%, respectively, for WT CALB; and 10.1 ± 0.6 g L⁻¹ and 17.6%, respectively, for the A141S mutant.

To manipulate the substrate pocket by altering the its cavity volume, the amino acid residue A282 and the adjacent amino acids, A281 and A283, which limit the size of the substrate pocket,²⁷ were substituted. The cavity volumes for the variants were calculated using the Molegro Virtual Docker software²⁸ (Table 4); when A281 was changed to A281E, A281V, or A281G, the E_R/E_S decreased to 1.051, 0.976, and 0.953, respectively; the cavity volume decreased to 57.344, 55.088, and 68.608 Å³, respectively; and the M value [M = (E_R/E_S) × cavity per volume] was 0.0183, 0.0177, and 0.0139 Å⁻³, respectively. Similarly, when A282 was changed to A282G, A282L, or A282N, the M value

Table 2 Effect of 3-OH protecting group on the enantioselectivity of CALB

| Protecting group | R-Isomer structure | Docking energy (kcal mol⁻¹) | Related amino acids⁸ |
|------------------|--------------------|-----------------------------|----------------------|
| OH⁻              | (R)-OH⁻_J₆         | −72.92                      | Gln106 Gln157 Thr138 Asp134 Thr42 |
| Acetyl⁻          | (R)-J₆             | −76.34                      | —                    |
| TBDMSO⁻          | (R)-J₆             | −96.99                      | Asp134 Gln106 Gln157 Val154 Ile189 |
| Benzoyl⁻         | —                  | −86.48                      | —                    |
| TIPSO⁻           | —                  | −104.20                     | —                    |
| TBDPSO⁻          | (R)-TBDPSO⁻_J₆     | −124.33                     | Ile285 Thr42 Trp104 Ile189 Thr138 Leu140 |
| TMSO⁻            | —                  | −86.98                      | —                    |

⁸ Note: Related amino acids are the ones interacting with the R-isomer structure via H bond or salt bridge, excluding the catalytic triad (Ser105, His224, and Asp187).
was 0.0185, 0.0080, and 0.0140 \(^{\circ}\)A/C0, respectively. When A283 was changed to A283V, A283L, or A283S, the \(M\) value was 0.0205, 0.0171, and 0.0155 \(^{\circ}\)A/C0, respectively.

Among these, the mutant A141S-A283V had the highest \(M\) value (0.0205 \(^{\circ}\)A/C0), indicating the highest \(R\)-selectivity. This protein was then overproduced in \(P.\ \text{pastoris}\); its hydrolytic activity and enantioselectivity were determined as 16.06 U mL/C0 and 60% (ee\(R\) value), respectively, and were greater by 47.8% and 56.8%, respectively, than the corresponding parameters of the A141S mutant (ESI Fig. S2†).

Furthermore, the protein structure of the A141S-A283V mutant has changed from an \(\alpha\)-helix to \(\beta\)-sheet, decreasing the distance between \(R\)-J6 and Ser105 (from 4.7 \(^{\circ}\)A to 3.3 \(^{\circ}\)A, compared with the WT CALB) (Fig. 1B and C).

### Table 3  Effect of alcohol moiety on the enantioselectivity of CALB

| Alcohol moiety | Compound structure | Docking energy (kcal mol\(^{-1}\)) | Related amino acids\(^a\) |
|---------------|-------------------|-----------------------------------|--------------------------|
| EtOH          | ![EtOH structure](image) | −89.34                           | —                        |
| Methanol      | ![Methanol structure](image) | −96.19 Gln106 Gln157 Val154 Ile189 Val190 Lue278 Thr40 |
| n-Propanol    | ![n-Propanol structure](image) | −101.67                           | —                        |
| i-Propanol    | ![i-Propanol structure](image) | −90.25                           | —                        |
| \(n\)-Butanol | ![\(n\)-Butanol structure](image) | −104.40 Gln106 Thr40 Gln157 Ala282 Ile189 Val190 Leu278 Ser47 |
| \(t\)-Butanol | ![\(t\)-Butanol structure](image) | −90.44                           | —                        |
| i-Butanol     | ![i-Butanol structure](image) | −86.97                           | —                        |
| Phenylmethanol| ![Phenylmethanol structure](image) | −115.07 Ala141 lle285 lle189 Trp104 Leu140 Gln157 Ala282 Asp134 Ser47 |
| 2-Phenylethanol| ![2-Phenylethanol structure](image) | −86.71                           | —                        |
| 1-Phenylethanol| ![1-Phenylethanol structure](image) | −83.25 Ala141 lle285 lle189 Thr40 Val154 |

\(^a\) Note: Related amino acids are the ones interacting with the \(R\)-isomer structure via H bond or salt bridge, excluding the catalytic triad (Ser105, His224, and Asp187).

### Table 4  Docking free energy and product enantiomers of the variants

| Variant       | \(E_R/E_S\)^a | Cavity volume (\(^{\circ}\)A\(^3\)) | \(M\) (\(^{\circ}\)A/C0) |
|---------------|--------------|-----------------------------------|--------------------------|
| WT\(^b\)      | 0.975        | 99.840                            | 0.0098                   |
| A141S         | 1.119        | 66.560                            | 0.0168                   |
| A141S-A281E   | 1.051        | 57.344                            | 0.0183                   |
| A141S-A281V   | 0.976        | 55.088                            | 0.0177                   |
| A141S-A281G   | 0.953        | 68.608                            | 0.0139                   |
| A141S-A282G   | 1.006        | 54.272                            | 0.0185                   |
| A141S-A282L   | 0.958        | 119.296                           | 0.0080                   |
| A141S-A282N   | 0.839        | 59.940                            | 0.0140                   |
| A141S-A283V   | 1.083        | 52.736                            | 0.0205                   |
| A141S-A283L   | 0.952        | 55.808                            | 0.0171                   |
| A141S-A283S   | 0.867        | 55.808                            | 0.0155                   |

\(^a\) Note: \(E_R/E_S\) represents the ratio of the docking energy of the \(R\)-isomer to the \(S\)-isomer. \(^b\) WT, wild-type CALB. \(^* M = \frac{E_R}{E_S} \times (\text{cavity volume} - 1)\).

Among these, the mutant A141S-A283V had the highest \(M\) value (0.0205 \(^{\circ}\)A/C0), indicating the highest \(R\)-selectivity. This protein was then overproduced in \(P.\ \text{pastoris}\); its hydrolytic activity and enantioselectivity were determined as 16.06 U mL\(^{-1}\) and 60% (ee\(R\) value), respectively, and were greater by 47.8% and 56.8%, respectively, than the corresponding parameters of the A141S mutant (ESI Fig. S2†).

Furthermore, the protein structure of the A141S-A283V mutant has changed from an \(\alpha\)-helix to \(\beta\)-sheet, decreasing the distance between \(R\)-J6 and Ser105 (from 4.7 \(^{\circ}\)A to 3.3 \(^{\circ}\)A, compared with the WT CALB) (Fig. 1B and C).

### Construction and characterization of the EF5 mutant

Compared with WT CALB, the volume of the stereoselectivity pocket of the A141S-A283V variant obviously decreased (domains 1 and 2 in Fig. 1). Hence, to further increase the enantioselectivity of CALB, potential amino acid candidates close to domains 2 or 3 (Fig. 1C) were selected to further reshape the substrate pocket. Val190, Ile189, Asp134, and Thr138, all...
located in domain 3, which accommodates the free carboxyl group of \( R \)-J₆; and Leu278, Ala282, Ser47, and Thr42, located in the hydrophobic tunnel (domain 2), were all selected for modification (Fig. 2). A combinational mutagenesis approach was employed to further increase the ee value of the A141S-A283V mutant. A141S-A283V variants were obtained and

Fig. 1 Comparison of the A141S-A283V mutant and WT CALB. (A) Structural comparison between the A141S-A283V mutant and WT CALB. The structures of the A141S-A283V mutant and WT CALB are shown in red and blue, respectively, and the catalytic triad (S105, D187, and H224) is in yellow. Models of the active site for WT CALB (C) and the A141S-A283V mutant (B). In both cases, \((R)-3\)-TBDSO glutaric acid methyl monoester \((R\)-J₆\) is covalently bound to the enzyme. Hydrogen bonds are indicated with black lines. Ser105 is represented in stick form, where the C, H, N, S, and O atoms are in magenta, white, blue, yellow, and red, respectively.

Fig. 2 Generation of highly \( R \)-selective variants. (A) Amino acids selected for the combinational mutagenesis. (B) Domain 3 (Val190, Ile189, Asp134, and Thr138) and domain 2 (Leu278, Ala282, Ser47, and Thr42). (C) \((R)-J_6\) production by CALB variants.
WT 2.05 ± 0.05 26.57 ± 1.74 12.94 6.28 ± 0.61 3.69 ± 0.05 0.59
A141S 4.59 ± 0.16 25.29 ± 1.48 5.51 1.29 ± 0.10 4.26 ± 0.08 3.30
A141S-A283V 15.98 ± 1.52 21.72 ± 1.16 1.36 1.22 ± 0.08 4.71 ± 0.09 3.86
EF5 18.15 ± 2.09 17.62 ± 0.76 0.97 0.60 ± 0.02 4.96 ± 0.11 8.29

Table 5 Kinetic parameters of the different CALB variants

**Production of (R)-J6 by EF5**

The effect of glucose concentration, glucose feeding, pH, and temperature on the specific activity of EF5 variant were then investigated (ESI Fig. S3 and S4†). Under optimum fermentation conditions (pH 6.0, 29 °C, glucose as the inducer, and glucose feeding concentration of 25 g L⁻¹), the activity of EF5 variant was 1114 ± 8.7 U ml⁻¹. The activity of the variant was further increased to 2401.5 ± 5.3 U ml⁻¹ by increasing the gene copy number upon the addition of 800 ng ml⁻¹ zeocin. When the enzyme variant was immobilized under optimal conditions [macroporous resin D101 as the carrier, 25 : 1 (v/w) ratio of the enzyme solution to the carrier, pH 8.2, 37 °C, 5 h adsorption time], the activity of immobilized EF5 increased to 2706.7 ± 11.4 U g⁻¹ (ESI Fig. S7).

The conditions for the enzymatic transformation of 3-TBDMSO glutaric anhydride to (R)-J6 by the EF5 variant were then investigated. The titer of (R)-J6 increased but the enantioselectivity of the immobilized EF5 catalyst decreased with increasing temperature; at temperature below 5 °C, the obtained (R)-J6 titer was 9.56 ± 1.7 g L⁻¹, with the ee₉ value of 95.6% (Fig. 4A). As shown in Fig. 4B, the titer of (R)-J6 increased but the enantioselectivity of EF5 decreased with the molar ratio of 3:0; the obtained (R)-J6 titer was 20.2 ± 1.7 g L⁻¹, with the ee₉ value of 69.7%. With increasing EF5 variant concentrations, the titer and ee value of (R)-J6 increased. When 80 g L⁻¹ of the immobilized EF5 variant was used in a 10 L reaction system, the titer and ee values of (R)-J6 reached 55.28 ± 1.6 g L⁻¹ and 98.5%,
respectively (Fig. 5); in comparison, the ee value was 60% during S-J6 preparation with WT CALB (Fig. 2C). These results indicated that an excess of EF5 biocatalyst is required to obtain a high-optical purity product.

Conclusions
In summary, in the current study, the ee value of R-J6 was increased by manipulating the substrate pocket of CALB using a semi-rational design strategy. The key amino acid candidates Ala141, Ala283, Thr138, and Ser47 were identified by analyzing the relationship between WT CALB and the target product structure (the 3-substituent group and alcohol moiety). The A141S-A283V mutant was generated in silico screening, and displayed R-selectivity (with ee of 60%) during asymmetric alcoholysis of 3-TBDMSO glutaric anhydride. The ee value was further increased to 85% in a reaction catalyzed by the EF5 mutant, which was generated by combining mutagenesis with high-throughput screening. The $k_{cat}/K_M$ value of the EF5 mutant toward R-J6 increased 14-fold compared with WT CALB. Upon optimization of the fermentation conditions and increase the gene copy number, the activity of EF5 increased to 2401.5 U g$^{-1}$; after immobilization, EF5 activity increased to 2706.7 U g$^{-1}$. By optimizing the enzymatic reaction system, the R-J6 titer increased to 55.28 g L$^{-1}$, with the ee value of 98.5%. These results indicate that the employed semi-rational design strategy is a promising approach for redesigning other biocatalysts and it has a great potential for the production of R-J6 on an industrial scale.

Conflict of interest
There are no conflicts to declare.

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References
1 J. W. Blasetto, E. A. Stein, W. V. Brown, R. Chitra and A. Raza, Am. J. Cardiol., 2003, 91, 3–10.
2 T. Konoike and Y. Araki, J. Org. Chem., 1994, 59, 7849–7854.
3 E. E. Jacobsen, B. H. Hoff, A. R. Moen and T. Anthonsen, J. Mol. Catal. B: Enzym., 2003, 21, 55–58.
4 F. Wang, S. Hou, Q. Wang, P. Wang, J. Liu, B. Yang and Y. Wang, Adv. Appl. Microbiol., 2015, 5, 493.
5 E. Rogalska, C. Cudrey, F. Ferrato and R. Verger, Chirality, 1993, 5, 24–30.
6 W. M. Liu, Y. Hu, Y. Zhang, Y. Ma and H. Huang, Biotechnol. Bioprocess Eng., 2014, 19, 449–455.
7 M. Wolberg, W. Hummel, C. Wandrey and M. Muller, Angew. Chem., Int. Ed., 2000, 39, 4306–4308.
8 M. López-Garcia, I. Alfonso and V. Gotor, Tetrahedron: Asymmetry, 2003, 14, 603–609.
9 H. J. Wang, Z. B. Li, X. X. Yu, R. D. Chen, X. L. Chenabc and L. M. Liu, RSC Adv., 2015, 5, 75160–75166.
10 R. Metzner, W. Hummel, F. Wetterich, B. König and H. Groger, Org. Process Res. Dev., 2015, 19, 635–638.
11 E. Garcia-Urdiales, I. Alfonso and V. Gotor, Chem. Rev., 2011, 111, Pr110–Pr180.
12 J. M. Palomo and Z. Cabrera, Curr. Org. Synth., 2012, 9, 791–805.
13 A. Fryszkowska, M. Komar, D. Koszelewski and R. Ostaszewski, Tetrahedron: Asymmetry, 2005, 16, 2475–2485.
14 A. Fryszkowska, M. Komar, D. Koszelewski and R. Ostaszewski, Tetrahedron: Asymmetry, 2006, 17, 961–966.
15 J. K. Yang, L. Y. Liu, J. H. Dai and Q. Li, PLoS One, 2013, 8, e53939.
16 M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. Gallo Cassarino, M. Berton, L. Bordoli and T. Schwede, Nucleic Acids Res., 2014, 42, W252–W258.
17 K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes and I. Vorobyov, J. Comput. Chem., 2010, 31, 671–690.
18 D. H. Priscilla, D. Roy, A. Suresh, V. Kumar and K. Thirumurugan, Chem.-Biol. Interact., 2014, 210, 77–85.
19 X. D. Kong, S. Yuan, L. Li, S. Chen, J. H. Xu and J. Zhou, Proc. Natl. Acad. Sci. U. S. A., 2014, 111, 15717–15722.
20 X. J. Li, R. C. Zheng, H. Y. Ma and Y. G. Zheng, Appl. Microbiol. Biotechnol., 2014, 98, 2473–2483.
21 A. K. Vadhana, P. Samuel, R. M. Berin, J. Krishna, K. Kamatchi and S. Meenakshisundaram, Enzyme Microb. Technol., 2013, 52, 177–183.
22 R. Margesin, G. Feller, M. Hammerle, U. Schinner and F. Schinner, Biotechnol. Lett., 2002, 24, 27–33.
23 M. M. Bradford, Anal. Biochem., 1976, 72, 248–254.
24 B. Wang, J. Liu, X. L. Tang, C. Cheng, J. L. Gu, L. Y. Dai and H. W. Yu, Tetrahedron Lett., 2010, 51, 309–312.
25 E. Corey and M. C. Noe, J. Am. Chem. Soc., 1996, 118, 319–329.
26 F. Hou, T. Miyakawa, M. Kataoka, D. Takeshita, S. Kumashiro, A. Uzura, N. Urano, K. Nagata, S. Shimizu and M. Tanokura, Biochem. Biophys. Res. Commun., 2014, 446, 911–915.
27 R. T. Otto, H. Scheib, U. T. Bornscheuer, J. Pleiss, C. Sylldatk and R. D. Schmid, J. Mol. Catal. B: Enzym., 2000, 8, 201–211.
28 T. Rosen, M. Watanabe and C. H. Heathcock, J. Org. Chem., 1984, 49, 3657–3659.
29 I. Høegh, S. Patkar, T. Halkier and M. T. Hansen, Can. J. Bot., 1995, 73, 869–875.
30 L. Novak, J. Rohály, L. Poppe, G. Hornyánszky, P. Kolonits, I. Zelei, I. Fehér, J. Fekete, É. Szabó and U. Záhorszky, Liebigs Ann. Chem., 1992, 1992, 145–157.