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One Pot Asymmetric Synthesis of (*R*)-Phenylglycinol from Racemic Styrene Oxide via Cascade Biocatalysis

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(*R*)-Phenylglycinol is an important chiral building block for pharmaceutical and fine chemical industry, and its efficient synthesis from cheap and commercially available starting materials is challenging and highly desirable. Herein, a new three-step one-pot cascade system employing epoxide hydrolase, glycerol dehydrogenase, and ω-transaminase was designed for the asymmetric synthesis of (*R*)-phenylglycinol from racemic styrene oxide. A cofactor self-sufficient system employing AlaDH/L–Ala was utilized for the regeneration of expensive cofactor NAD^+ and removal of by-product pyruvate. Furthermore, *in situ* product removal by cation resin adsorption was used to drive the thermodynamic equilibrium of the cascade reaction to the direction of product generation. Finally, optically pure (*R*)-phenylglycinol was successfully produced from racemic styrene oxide with high yield (81.9%) and excellent enantioselectivity (99% *ee*).

Chiral β-amino alcohols are widely used as important building blocks and structural motifs in pharmaceutical and fine chemical industry and also serve as chiral auxiliaries for organic synthesis.[1] Although numerous chemical methods have been developed for the efficient synthesis of chiral amino alcohols, these processes suffered from the requirement of complicated reaction steps,[2] expensive metal catalysts,[3] and unsatisfactory enantioselectivity and product yield.[4] Due to the inherent non-toxicity, mild reaction conditions, and high selectivity (chemo-, regio-, and enantioselectivity), biocatalysis has become a useful tool for the green synthesis of chiral β-amino alcohols and great progress has been achieved, including lipase-catalysed kinetic resolution of racemic amino alcohols,[5] transaminase-mediated kinetic resolution of racemic amino alcohols[6] or asymmetric amination of hydroxyl ketones,[7] and carbonyl reductase-catalysed asymmetric reduction of amino ketones.[8] However, the high cost and limited availability of the substrates and the low yield (50% theoretical yield) of the product in kinetic resolution hinder the practical application of these biocatalytic methods. Therefore, it is interesting to develop novel and economic chiral β-amino alcohols synthesizing routes from cheap and commercially available starting materials.

Recently, multi-enzyme cascade reactions in one pot have found widespread applications in organic synthesis, since they can avoid the costly, time-consuming, and yield-reducing purification of intermediates and the generation of large amounts of wastes.[9] Several biocatalytic cascade processes have been developed for the biosynthesis of chiral amino alcohols, including the biotransformation of benzaldehyde to (1*R*,2*S*)-norephedrine (NE) and (1*R*,2*R*)-norpseudoephedrine (NPE),[10] the conversion of glycolaldehyde to (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT),[11] as well as the transformation of terminal alkenes to (*S*)-2-amino-1-phenylethanol.[12]

Since epoxides are cheap and readily available in large quantity from commercial sources, the direct asymmetric ring opening of racemic epoxides to chiral β-amino alcohols represents a promising alternative.[13] However, the utilization of expensive (*R*)-(+)-1-phenylethylamine as the amino donor for transaminase make the economy of this bioprocess challenging. In this work, we envisaged a new three-step one-pot biocatalytic process for the asymmetric synthesis of chiral (*R*)-phenylglycinol from racemic styrene oxide (Scheme 1). In this biocatalytic cascade process, racemic styrene oxide was hydrolysed by an enantioconvergent epoxide hydrolase to (*R*)-phenylglycol, which was then stereoselectively oxidized to 2-hydroxyacetophenone by glycerol dehydrogenase, and asymmetric amination of 2-hydroxyacetophenone by transaminase using the cheap and accessible L-alanine as the amino donor gave (*R*)-phenylglycinol. For *in situ* removal of the by-product pyruvate, and recycle the expensive cofactor NAD^+, an internal self-sufficient regeneration system employing alanine dehydrogenase (AlaDH) was introduced. Furthermore, *in situ* product removal by cation exchange resin was adopted to drive the thermodynamic equilibrium of the reaction.

The prerequisite to construct an efficient multi-enzyme cascade system is the acquisition of highly efficient biocatalyst for each reaction step. Since the epoxide hydrolase mutant *Vr*EH2$_{M263V}$ from *Vigna radiate* could catalyse the enantioconvergent hydrolysis of racemic styrene oxide (in which Cα of the epoxide ring is preferentially attacked in one enantiomer, giving the product with an inverted configuration, while Cβ is

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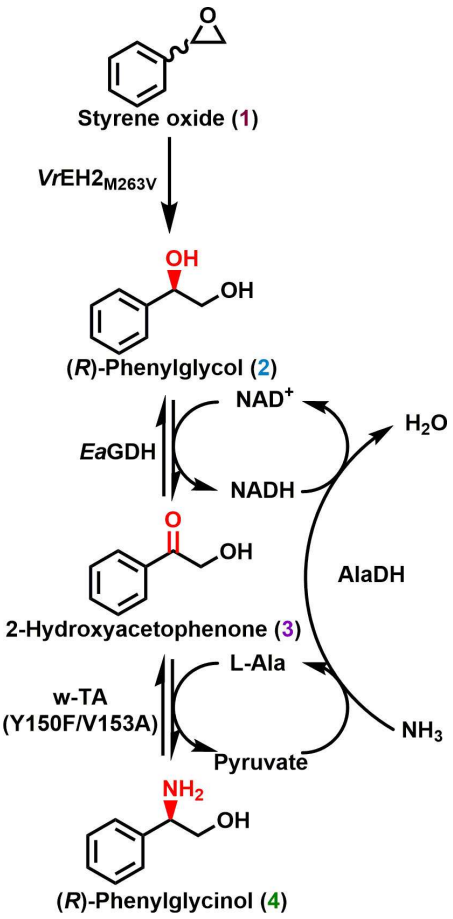

Scheme 1. A new multi-enzyme cascade for the asymmetric synthesis of enantiopure (*R*)-phenylglycinol from racemic styrene oxide. *Vr*EH2$_{M263V}$: Epoxide hydrolase; *Ea*GDH: Glycerol dehydrogenase; ω-TA$_{Y150F/V153A}$: ω-Transaminase; AlaDH: Alanine dehydrogenase.

preferentially attacked in the other enantiomer affording the diol in a retained configuration, thereby affording the enantioenriched vicinal diols in a theoretical yield of 100%[14]) to enantiopure (*R*)-phenylglycol with >99% conversion and 97.1% *ee* (Figure S6, supporting information), and could be easily obtained in soluble form through heterologous expression in *E. coli*,[15] it was selected as the catalyst for the first step. Although glycerol dehydrogenase from *Enterobacter aerogenes* (*Ea*GDH)[16] and carbonyl reductase from *Candida parapsilosis* (*Cp*CR)[17] were both reported to be able to stereoselectively oxidize (*R*)-phenylglycol to 2-hydroxyacetophenone, *Ea*GDH showed not only much higher specific activity (160 mU/mg for *Ea*GDH and 7.8 mU/mg for *Cp*CR), but also better stability under the operational conditions (Table S1, supporting information). Therefore, *Ea*GDH was considered to be a promising candidate catalyst for the second step. As for the third step, although three ω-transaminase mutants (ω-TA$_{Y150F/V153A}$, ω-TA$_{Y150M/V153A}$, and ω-TA$_{F85L/Y150F/V153A}$) from *Vibrio fluvialis* JS17 were reported to be capable of catalysing the asymmetric amination of 2-hydroxyacetophenone to (*R*)-phenylglycinol, ω-TA$_{Y150F/V153A}$ was selected due to its better performance in the synthesis of (*R*)-phenylglycinol.[7c]

Table 1. The activities of component enzymes for the cascade reaction.[a]

Enzymes	Substrate	Coenzyme	Specific activity [U/mg]
*Vr*EH2$_{M263V}$	Styrene oxide		2.43
*Ea*GDH	(*R*)-Phenylglycol	NAD^+	0.16
ω-TA$_{Y150F/V153A}$	2-Hydroxyacetophenone	PLP	0.09
AlaDH	Pyruvate	NADH	1200

[a] Reaction conditions: 1 mL of reaction mixture containing glycine–NaOH (100 mM, pH 8.0), 20 mM substrate, 0.2 mM NADH for AlaDH, 1 mM NAD^+ for *Ea*GDH, 0.1 mM PLP for ω-TA$_{Y150F/V153A}$, and appropriate amount of purified enzyme.

After choosing the appropriate biocatalysts (*Vr*EH2$_{M263V}$, *Ea*GDH, and ω-TA$_{Y150F/V153A}$) for each step of the cascade system (Table 1), the reaction conditions of each single step including buffer constituents and pH were then optimized. Excellent conversion (99%) was observed with styrene oxide hydrolysis (20 mM) in the pH range of 7.0–8.5 within 2 h, and the buffer constituent seems to have neglect effect on the conversion (Table S2, supporting information). As for (*R*)-phenylglycol oxidation, in order to recycle the expensive cofactor NAD^+, an NAD^+ regeneration system employing AlaDH with NH_4Cl as ammonia donor was introduced.[18] The conversion could reach as high as 99% in glycine/NaOH buffer with pH ranging from 7.5–8.5 within 4 h, replacement of buffer constituent, higher pH or lower NH_4Cl concentration significantly reduce the conversion (Table S3, supporting information). For transamination, L-Ala was utilized as the amino donor, since the by-product pyruvate can severely inhibit transaminase activity,[19] therefore, *in situ* removal of pyruvate is very critical not only for keeping the transaminase activity but also for pushing the reaction equilibrium.[20] As a result, AlaDH was used for the reductive amination of pyruvate with NADH as cofactor. With this design, in one hand, the by-product pyruvate was removed immediately, furthermore, the amino donor L-alanine could be regenerated. High conversion (83–92%) could be observed in the pH range of 7.5–8.5 regardless of the buffer constituent. Specifically, after 20 h transformation, (*R*)-phenylglycinol was produced with 92% conversion and 99% *ee* from 10 mM 2-hydroxyacetophenone in glycine–NaOH (100 mM, pH 8.0) buffer (Table S4, supporting information). However, the conversion significantly reduced to 41% at pH 9.0. Based on the above results, glycine–NaOH (100 mM, pH 8.0) buffer was chosen for the cascade reaction.

Previous to the establishment of the cascade process, the stability of each component enzyme was investigated under the operational conditions. *Ea*GDH, ω-TA$_{Y150F/V153A}$, and AlaDH all showed excellent stability retaining more than 70% of original activity after incubation in 100 mM glycine–NaOH buffer (pH 8.0) at 30 °C for 40 h. Epoxide hydrolase *Vr*EH2$_{M263V}$ showed moderate stability, and retained more than 50% of original activity after 8 h. The moderate to excellent stability of the component enzyme ensures the operation of cascade reaction.

The biocatalytic cascade system was then performed using lyophilized crude powders of *Vr*EH2$_{M263V}$, *Ea*GDH, ω-TA$_{Y150F/V153A}$, and AlaDH in one pot, and (*R*)-phenylglycinol was successfully

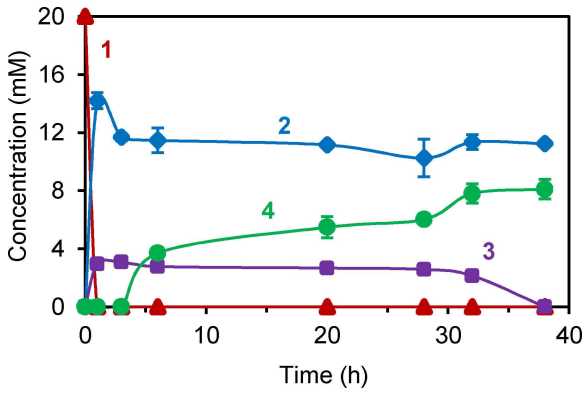

Figure 1. Time course of the cascade reaction for the synthesis of (*R*)-phenylglycinol from styrene oxide. Styrene oxide (1, ▲), (*R*)-phenylglycol (2, ♦), 2-hydroxyacetophenone (3, ■), (*R*)-phenylglycinol (4, ●). Reaction conditions (20 mL): styrene oxide 20 mM, L-Ala 200 mM, NH_4Cl 250 mM, *Vr*EH2$_{M263V}$ 4 U/mL, *Ea*GDH 2 U/mL, *Ala*DH 2 U/mL, ω-TA$_{Y150F/V153A}$ 2 U/mL, NAD^+ 2 mM, PLP 0.5 mM, 100 mM glycine–NaOH buffer, pH 8.0, 30 °C. All biotransformations were performed in triplicate and error bars refer to ± s.d.

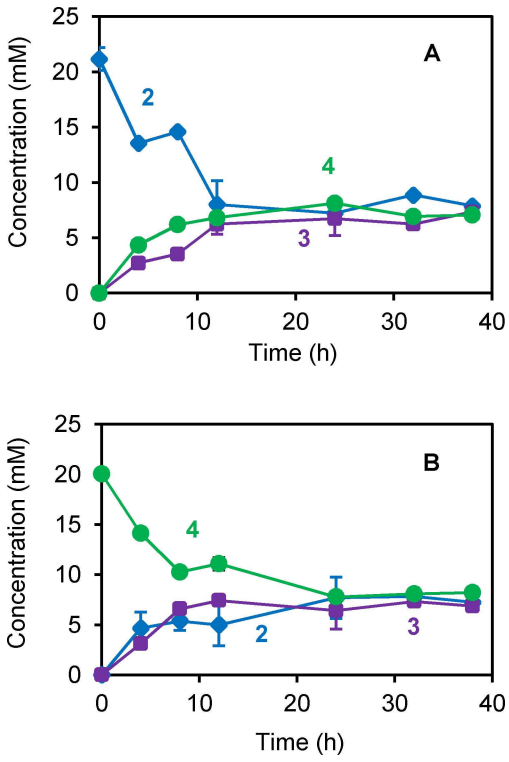

Figure 2. Investigation of the thermodynamic equilibrium of the cascade reaction. (*R*)-Phenylglycol (2, ♦), 2-hydroxyacetophenone (3, ■), (*R*)-phenylglycinol (4, ●). (A) Time course of the cascade reaction from (*R*)-phenylglycol to (*R*)-phenylglycinol. Reaction conditions (20 mL): (*R*)-phenylglycol 22.3 mM, L-Ala 200 mM, NH_4Cl 250 mM, *Ea*GDH 2 U/mL, *Ala*DH 2 U/mL, ω-TA$_{Y150F/V153A}$ 2 U/mL, NAD^+ 20 mM, NADH 20 mM, PLP 0.5 mM, 100 mM glycine–NaOH buffer, pH 8.0, 30 °C. (B) Time course of the cascade reaction from (*R*)-phenylglycinol to (*R*)-phenylglycol. Reaction conditions (20 mL): (*R*)-phenylglycinol 22.3 mM, L-Ala 200 mM, NH_4Cl 250 mM, *Ea*GDH 2 U/mL, *Ala*DH 2 U/mL, ω-TAY$_{150F/V153A}$ 2 U/mL, NAD^+ 20 mM, PLP 0.5 mM, NADH 20 mM, 100 mM glycine–NaOH buffer, pH 8.0, 30 °C. All biotransformations were performed in triplicate and error bars refer to ± s.d.

produced from racemic styrene oxide (20 mM) with 40.5 % yield and 99 % *ee* (Figure 1 and Figure S5, supporting information). The low yield of target product was mainly due to the accumulation of (*R*)-phenylglycol (12 mM). Therefore, the dosage of *Ea*GDH and ω-TA$_{Y150F/V153A}$ were increased from 2 U/mL to 4 U/mL and the catalyst ratio between *Ea*GDH and ω-TA$_{Y150F/V153A}$ was also increased from 1:1 to 1:4 aiming to further improve the conversion of (*R*)-phenylglycol to (*R*)-phenylglycinol (Table S5, supporting information). However, there was still about 8–12 mM (*R*)-phenylglycol remained in the reaction mixture, indicating that catalyst loading of *Ea*GDH and ω-TA$_{Y150F/V153A}$ was not the predominant reason for the accumulation of such reaction intermediate.

Since *Ea*GDH and ω-TA$_{Y150F/V153A}$ catalysed reactions are both reversible, the thermodynamic equilibrium might be the main reason for the accumulation of the intermediate (*R*)-phenylglycol. To verify our hypothesis, forward and reverse reactions of the cascade system employing (*R*)-phenylglycol or (*R*)-phenylglycinol as substrate, respectively, were investigated (Figure 2). Starting from 22.3 mM (*R*)-phenylglycol, 7.06 mM (*R*)-phenylglycinol was produced with 7.35 mM 2-hydroxyacetophenone, while 7.21 mM (*R*)-phenylglycol was produced with 6.87 mM 2-hydroxyacetophenone starting from 22.3 mM (*R*)-phenylglycinol. The concentrations of three reactants in the forward and reverse reactions were almost identical, suggesting that thermodynamic equilibrium of the cascade system caused the accumulation of intermediate (*R*)-phenylglycol and therefore resulting in low yield of the target product.

In situ product removal by resin adsorption has been proven to be a useful strategy in driving the thermodynamic equilibrium to the product, since the resin is nontoxic, inexpensive, easy to handle, and recyclable.[21] Three types of cation exchange resin including HD-8, C160, and C104plus were subjected to adsorption experiment. Interestingly, macroporous strong acid cation exchange resin HD-8 was observed to be a good adsorbent for the target product (*R*)-phenylglycinol, and could only selectively adsorb (*R*)-phenylglycinol, while the other reaction components were not absorbed by HD-8 (Figure S1 & S2, supporting information). Therefore, resin HD-8 was selected for the *in situ* removal of (*R*)-phenylglycinol to drive the thermodynamic equilibrium. To this end, a new bio-reactor system coupling the three-step cascade reaction with the *in situ* product removal for the synthesis of (*R*)-phenylglycinol from racemic styrene oxide was constructed (Figure S7, supporting information). As can be seen from Figure 3, the accumulation of intermediate (*R*)-phenylglycol was significantly decreased from 60 % to 16.5 % due to the efficient adsorption of product to the resin, and no product could be detected in the reaction mixture. The product was then eluted from the cation exchange resin HD-8 with 2 M NaOH, and after follow up purification steps, (*R*)-phenylglycinol was isolated with 81.9 % yield, which is obviously superior to that without *in situ* product removal (40.5 %), suggesting that the thermodynamic equilibrium of the reaction was efficiently driven to the product with *in situ* product removal strategy.

In summary, a new three-step one-pot cascade system involving enantioconvergent hydrolysis of racemic styrene

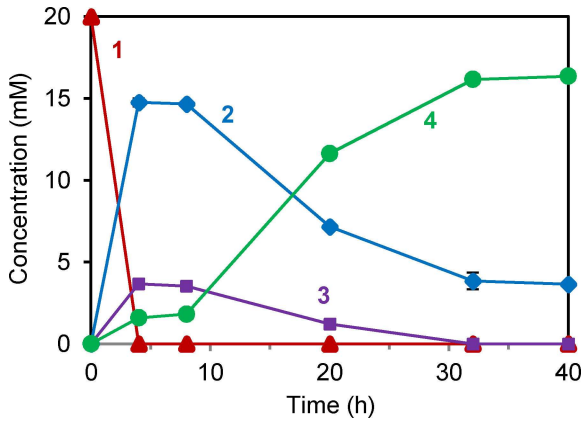

Figure 3. Time course of the cascade reaction from racemic styrene oxide to (*R*)-phenylglycinol coupled with *in situ* product removal using cation resin HD-8. Styrene oxide (1, ▲), (*R*)-phenylglycol (2, ♦), 2-hydroxyacetophenone (3, ■), (*R*)-phenylglycinol (4, ●) recovered from resin. Reaction conditions (100 mL): Racemic styrene oxide 20 mM, L-Ala 200 mM, NH_4Cl 250 mM, *Vr*EH2$_{M263V}$ 4 U/mL, *Ea*GDH 2 U/mL, *Ala*DH 2 U/mL, ω-TA$_{Y150F/V153A}$ 2 U/mL, NAD^+ 2 mM, PLP 0.5 mM, 100 mM glycine–NaOH buffer, pH 8.0, 30 °C. All biotransformations were performed in triplicate and error bars refer to ± s.d.

oxide by epoxide hydrolase, followed by stereoselective oxidation of (*R*)-phenylglycol by glycerol dehydrogenase, and asymmetric amination of 2-hydroxyacetophenone by ω-transaminase was designed for the preparation of (*R*)-phenylglycinol with excellent *ee* from the cheap and commercially available racemic styrene oxide. AlaDH and L-alanine was introduced to the biocatalytic cascade system to regenerate the expensive cofactor NAD^+ and remove the by-product pyruvate. In addition, *in situ* product removal by resin adsorption was coupled with the cascade reaction to drive the thermodynamic equilibrium to the product and improve the product yield. Protein engineering of ω-transaminase to improve its catalytic activity, thereby increasing the overall efficiency of this cascade and the extension of this cascade for the other chiral β-amino alcohols are currently under progress. The present study affords a new practical method for the synthesis of optically pure (*R*)-phenylglycinol, and might be generally applicable for the preparation of other pharmaceutically important and value-added chemicals by combining appropriate enzymes in a similar way.

Experimental Section

Construction of Recombinant *E. coli* Cells

The genes of *Vr*EH2$_{M263V}$ and *Ea*GDH were cloned in plasmid pET-28a (+), respectively, and the competent cells of *E. coli* BL21 (DE3) were then transformed with the resultant recombinant plasmids as described previously.[15,16] The gene of AlaDH was amplified by PCR from the genome DNA of *Bacillus megaterium* ECU1001 with the primers reported for alanine dehydrogenase from *Bacillus megaterium* WSH-002 and cloned in the plasmid pET-28a (+), and competent cells of *E. coli* BL21 (DE3) was then transformed with the recombinant plasmid.[22] The gene of ω-TA$_{Y150F/V153A}$ was cloned in plasmid pET-24b (+) and transformed into *E. coli* BL21 (DE3) competent cells as described in literature.[7e]

Analytic Methods

Quantification of Reaction Components

The concentrations of styrene oxide, phenylglycol, hydroxyacetophenone, and phenylglycinol were determined by GC analysis with a Shimadzu-2014 gas chromatograph equipped with a flame ionization detector (FID) and an Agilent Rxi®-5Sil MS capillary column (25 m × 0.25 mm, 0.25 μm) by using nitrogen as the carrier gas. The temperature for injector and detector was 280 °C, and the column temperature was first set at 80 °C, rise to 140 °C at 10 °C/min and hold for 2 min, and finally rise to 180 °C at 20 °C/min and hold for 5 min. The concentration of L-alanine, pyruvate, NAD^+, and NADH was measured by HPLC analysis with a Shimadzu-2010A liquid chromatogram equipped with UV-detector and C18 column (4.6 mm × 250 mm), eluted with acetonitrile:20 mM KH_2PO_4 (10:90, v/v) at a flow rate of 0.4 mL/min and detected at 254 nm. The column temperature was 30 °C.

Determination of Enantiomeric Excess

The enantiomeric excess (*ee*) value of phenylglycinol was determined by GC analysis with a Shimadzu-2014 gas chromatograph equipped with an Agilent J&W CP-Chiralsil-DEX CB capillary column (25 m × 0.25 mm, 0.25 μm). The temperature for injector and detector was 280 °C, and the column temperature was first set at 80 °C, rise to 140 °C at 10 °C/min and hold for 2 min, and finally rise to 180 °C at 20 °C/min and hold for 5 min. While the *ee* value of phenylglycol was determined by HPLC analysis with a Shimadzu-2010A liquid chromatogram equipped with an OD column (4.6 mm × 250 mm), eluted with hexane: 2-propanol (85:15, v/v) at a flow rate of 0.6 mL/min and detected at 254 nm. The column temperature was 30 °C. The absolute configuration of (*R*)-phenylglycol and (*R*)-phenylglycinol was established by comparison with commercial standards.

Enzyme Assay

Epoxide Hydrolase

The hydrolysis reaction (1 mL in a 2-mL Eppendorf tube) was carried out in 100 mM glycine–NaOH buffer (pH 8.0) containing 20 mM racemic styrene oxide and a certain amount of enzyme. After incubation at 30 °C for 15 min, the reaction was stopped by adding 50 μL of H_2SO_4. The sample was assayed by HPLC. One unit (U) of enzyme activity was defined as the amount of *Vr*EH2$_{M263V}$ generating 1 μmol product per minute under the standard assay conditions.

Glycerol Dehydrogenase and Alanine Dehydrogenase

The oxidation activity of *Ea*GDH and reduction activity of AlaDH were measured by monitoring the change of absorbance of NADH at 340 nm. Reactions were performed in a 1-mL cuvette supplemented with 1 mL of 100 mM glycine–NaOH buffer (pH 8.0) containing 20 mM substrate, 0.2 mM NADH or 2 mM NAD^+ and a certain amount of enzyme at 30 °C. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation or reduction of 1 μmol substrate in one minute.

ω-Transaminase

The transamination activity of ω-transaminase was measured as described previously.[23] Reaction was performed in a 1-mL cuvette supplemented with 1 mL of 100 mM glycine–NaOH buffer (pH 8.0) containing 10 mM substrate, 0.1 mM PLP, 10 mM (*R*)-phenylglycinol and a certain amount of enzyme at 30 °C.

Biocatalytic Cascade Reaction in One Pot

The reaction mixture (20 mL in a 100-mL shake flask or 100 mL in a 500-mL shake flask) containing styrene oxide (20 mM, 1% v/v DMSO as co-solvent), NAD^+ 2 mM, PLP 1 mM, NH_4Cl 250 mM, L-ALa 200 mM, *Vr*EH2$_{M263V}$ (4 U/mL), *Ea*GDH (2 U/mL), ω-TA$_{Y150F/V153A}$ (2 U/mL), *Ala*DH (2 U/mL) and 100 mM glycine–NaOH buffer was magnetically stirred at 30 °C for 40 h. The reaction sample (200 μL) was mixed with NaOH (10 M, 20 μL), and extracted with ethyl acetate (0.6 mL). The organic extract was dried with anhydrous $MgSO_4$ and analysed by GC.

Biocatalytic Cascade Reaction Coupled with *In Situ* Product Adsorption

A column (134.8 mm length × 26.4 mm diameter) filled with 25 g resin HD-8 was integrated with the reactor to selectively adsorb the product (*R*)-phenylglycinol (Figure S7). Multi-enzyme cascade reaction was carried out under identical conditions as described in previous section and the 100 mL reaction mixture flowed through the column at 2.5 mL/min and the effluent reaction mixture flowed back to the reactor at 2.4 mL/min. After 40 h transformation, the resin was eluted by NaOH solution (2 M, 100 mL). The eluent was saturated with NaCl, and extracted with ethyl acetate for three times (3 × 100 mL). The organic phases were combined and dried over anhydrous Na_2SO_4, and evaporation of the organic solvent under reduced pressure afforded the crude product (*R*)-phenylglycinol. The crude product was then purified by flash chromatography on a silica gel column to give (*R*)-phenylglycinol as a white solid in 81.9% yield and 99% *ee*. ^{1}H NMR (600 MHz, $CDCl_3$):[24] δ 7.38–7.30 (m, 4H), 7.30–7.26 (m, 1H), 4.04 (dd, J = 8.3, 4.3 Hz, 1H), 3.74 (dd, J = 10.9, 4.3 Hz, 1H), 3.56 (dd, J = 10.8, 8.3 Hz, 1H), 2.19 (br, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 142.77, 128.77, 127.65, 126.59, 68.11, 57.48.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · asymmetric synthesis · cascade reaction · *in situ* product removal · (*R*)-phenylglycinol

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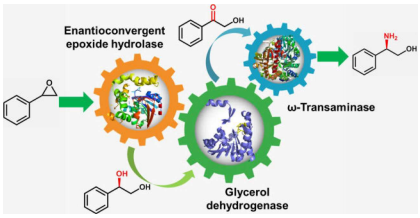
3 steps in 1 pot: A new three-step one-pot cascade for the asymmetric synthesis of (*R*)-phenylglycinol from racemic styrene oxide was developed. An internal self-sufficient regeneration system employing AlaDH was introduced to recycle the cofactor NAD^+ and remove the by-product pyruvate. Furthermore, in situ product removal by cation exchange resin was adopted to drive the reaction equilibrium.



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One Pot Asymmetric Synthesis of (*R*)-Phenylglycinol from Racemic Styrene Oxide via Cascade Biocatalysis