

Asymmetric Amination of Secondary Alcohols by using a Redox-Neutral Two-Enzyme Cascade

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Multienzyme cascade approaches for the synthesis of optically pure molecules from simple achiral compounds are desired. Herein, a cofactor self-sufficient cascade protocol for the asymmetric amination of racemic secondary alcohols to the corresponding chiral amines was successfully constructed by employing an alcohol dehydrogenase and a newly developed amine dehydrogenase. The compatibility and the identical cofactor dependence of the two enzymes led to an ingenious in situ cofactor recycling system in the one-pot synthesis. The artificial redox-neutral cascade process allowed the transformation of racemic secondary alcohols into enantiopure amines with considerable conversions (up to 94%) and >99% enantiomeric excess at the expense of only ammonia; this method thus represents a concise and efficient route for the asymmetric synthesis of chiral amines.

Enantiomerically pure chiral amines are increasingly used as important precursors for the synthesis of biologically active molecules, and the development of efficient and cost-effective methods for their asymmetric synthesis is in high demand.^[1] Compared to classic chemical methods for the manufacture of chiral amines, including asymmetric hydrogenation of enamines/imines, a variety of artificially developed enzymes could serve as a potential "green alternative" generally featuring novel activity, mild reaction conditions, high regioselectivity, high stereoselectivity, and impressive conversions.^[2] A remarkable case is the asymmetric synthesis of sitagliptin from prositagliptin ketone by using an ω -transaminase developed by protein engineering.^[3]

The preparation of chiral amines from simple and inexpensive starting materials through biocatalytic routes is attracting growing interest. A variety of exquisite enzymatic cascade pro-

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cesses encompassing enzymes such as ω -transaminase, monoamine oxidase, and lyase have been constructed.^[4] As green and renewable biobased feedstocks, alcohols are increasingly identified as suitable initial substrates for amine synthesis by metal-catalyzed amination, with some shortcomings including poor stereoselectivity, less atom efficiency, and the use of toxic and expensive metals.^[5] Initially, biocatalytic transformations of α -hydroxy acids into α -amines were studied by employing amino acid dehydrogenases and alcohol dehydrogenases.^[6] Recently, Kroutil and co-workers first reported a series of redoxneutral multienzymatic networks involving alcohol dehydrogenase (ADH), ω-transaminase, and alanine dehydrogenase for the amination of primary alcohols and secondary alcohols.^[7] Notably, in artificial multienzymatic networks, the transformation of alcohol substrates into the desired amines mainly consists of two tandem steps: one, alcohol dehydrogenation catalyzed by an ADH (for primary alcohols) or two stereoselectively complemental ADHs (for racemic secondary alcohols) to give the corresponding ketone; two, subsequent reductive amination catalyzed by an $\omega\text{-transaminase}$ by using $\mbox{\tiny L}\mbox{-alanine}$ as the amino donor. More recently, Skerra and co-workers succeeded in extending the above concept to the synthesis of a structurally more complex diamine from the biobased diol isosorbide.^[8]

The aim of this study was to construct a new redox-neutral two-enzyme cascade process for the preparation of chiral amines from racemic secondary alcohols (Scheme 1). In the



Scheme 1. A redox-neutral two-enzyme cascade for the amination of racemic secondary alcohols to chiral amines. ADH: Alcohol dehydrogenase; AmDH: Amine dehydrogenase.

cascade process, the racemic secondary alcohols were nonstereoselectively oxidized by an alcohol dehydrogenase to the corresponding ketones and then aminated by an amine dehydrogenase (AmDH) to the chiral amines by using ammonia as the amino donor. Simultaneously, the cofactor NADH formed in the first step served as the reducing agent in the reductive amination step, whereas the resulting NAD⁺ was reused for the upstream oxidation step. The newly designed two-enzyme cascade pathway consumes only ammonia and avoids the addition of alanine and an external reducing agent; it therefore



represents a more concise redox-neutral process for the synthesis of chiral amines from racemic secondary alcohols.

Amine dehydrogenases are capable of catalyzing the asymmetric reductive amination of prochiral ketones to the corresponding chiral amines, with the consumption of inorganic ammonia. AmDHs were first developed by Bommarius and coworkers through several rounds of protein engineering by using amino acid dehydrogenases as the evolution templates.^[9] More recently, Li also obtained an AmDH from Rhodococcus sp. M4 phenylalanine dehydrogenase by directed evolution.^[10] In our laboratory, an NADH-dependent leucine dehydrogenase from Exiguobactertium sibiricum (EsLeuDH) was recently identified and applied to the stereoselective synthesis of L-tert-leucine,^[11] a valuable chiral building block in the pharmaceutical industry.^[12] However, EsLeuDH has merely undetectable levels of activity towards ketone substrates in comparison with α -keto acid substrates. With the aim of further expanding its synthetic scope to give more structurally diverse products, the two-site mutation (K77S/N270L) identified by Bommarius^[9] was introduced to EsLeuDH. As a consequence, the double mutant EsLeuDH-DM (K77S/N270L) obtained by the two-site mutagenesis exhibited considerable activities towards a variety of ketones (Table 1).



To match the cofactor of *Es*LeuDH-DM, an NAD⁺-dependent ADH for the oxidation of secondary alcohols should be selected.^[13] Furthermore, an ADH with low enantioselectivity was preferable,^[14] as only 50% of the *rac*-alcohol could be oxidized by an ADH with perfect stereoselectivity.^[15] Initially, we tested approximately 20 NADH-dependent ADHs preserved in our laboratory (Table S1, Supporting Information), and an ADH (*Sc*CR) from *Streptomyces coelicolor*^[16] was identified with appreciable activity towards the desired secondary alcohols and capacity to convert both isomers of the racemic secondary alcohols into the corresponding ketones.

Further optimization of the conditions for the cascade reactions was performed by employing *rac*-2-pentanol as the model substrate. As shown in Table 2, at pH 9.5, an unusual

Table 2. Optimization of the two-enzyme cascade reaction.										
Entry	рН	NAD ⁺ [mм]	Alcohol [%] ^[b]	Ketone [%] ^[b]	Amine [%] ^[b]					
1	10.5	2.0	48.5	4.4	47.1					
2	9.5	2.0	1.6	6.1	92.3					
3	8.5	2.0	0.8	9.0	90.2					
4	9.5	1.0	2.2	4.7	93.1					
5	9.5	0.5	6.9	3.8	89.3					
6	9.5	0.2	18.9	4.0	77.1					

[a] Reaction conditions: Mixture (1 mL) of *rac*-2-pentanol (50 mM), ADH *Sc*CR (lyophilized cell-free extract, 10 mg), AmDH *Es*LeuDH-DM (lyophilized cell-free extract, 10 mg), NAD⁺ (various concentrations), and NH₄Cl/ NH₄OH buffer (2 M, various pH values) at 30 °C, 1000 rpm. [b] Compositions were analyzed by GC analysis by comparing peak areas.

condition for common ADHs,^[7,17] the cascade reaction afforded 92.3% amine (Table 2, entry 2), whereas the conversion of alcohol decreased sharply under harsher pH conditions (pH 10.5). A decrease in the NAD⁺ loading from 2.0 to 1.0 mm resulted in a slight increase in the amount of amine formed (Table 2, entry 4), which is in agreement with the fact that a higher concentration of NAD⁺ could impair the amination activity of *Es*LeuDH-DM (Figure S1). Reducing the NAD⁺ loading further, however, hindered the cascade process.

Under the optimized conditions (Table 2, entry 4), timecourse analysis revealed that the formation of 2-pentanamine reached a maximum (93%) at approximately 24 h (Figure 1), after which the remaining 2-pentanol was maintained at a relatively low level. Notably, a certain amount of 2-pentanone ($\approx 4\%$) was detected throughout the course of the cascade reaction. The high conversion of alcohol into amine indicates that the equilibrium is in favor of amine synthesis, whereas the synthesis of amine by using the ω -transaminase is commonly retarded by an adverse equilibrium position and byproduct inhibition.^[18]

To investigate the synthetic scope of the two-enzyme cascade, we tried to apply the above methodology to the conversion of various alcohols into the corresponding amines (Table 3). As both the ADH (*Sc*CR) and AmDH (*Es*LeuDH-DM) had broad substrate specificity, the secondary alcohols tested were converted into the desired corresponding amines. Consequently, over 90% conversion of the alcohols into the corre-



Figure 1. Time course of the two-enzyme cascade reaction by employing *rac-*2-pentanol as the substrate. Comp.: Composition.

Table 3. Biocatalytic synthesis of amines from alcohols through the redox-neutral two-enzyme cascade. $^{\left[a\right] }$

Entry	Substrate	Structural formula	Alcohol [%] ^[b]	Ketone [%] ^[b]	Amine [%] ^[b]	ee [%] ^[c]
1	rac- 1 a	OH	2	4	94	>99 (R)
2	rac- 2 a		23	4	73	>99 (R)
3	rac- 3 a		40	3	57	n.d. ^[d]
4	rac- 4 a	OH	3	4	93	>99 (R)
5	rac- 5 a		66	5	29	>99 (R)
6	ба	OH OH	1	2	97	n.a. ^[e]
7	rac- 7 a	OH OH	23	2	75	n.d. ^[d]
8	rac- 8 a	OH	55	2	43	n.d. ^[d]
9	9a	OH	60	5	35	n.a. ^[e]
10	rac- 10 a	OH	72	7	21	>99 (R)

[a] Reaction conditions: Mixture (1 mL) of substrate (50 mM), ADH *Sc*CR (lyophilized cell-free extract, 10 mg), AmDH *Es*LeuDH-DM (lyophilized cell-free extract, 10 mg), NAD⁺ (1 mM), NH₄Cl/NH₄OH buffer (2 M, pH 9.5) at 30 °C, 1000 rpm. [b] Compositions were analyzed by GC analysis by comparing peak areas. [c] The *ee* values of the amines were determined by GC analysis on a chiral stationary phase after derivation. [d] n.d. = not determined. [e] n.a. = not applicable.

alcohol substrates such as *rac*-2-pentanol, the cascade afforded > 99% enantiomeric excess (*ee*), and thus, this route formally represents a biocatalytic method to access optically pure chiral products from simple racemates.

The absolute configurations of the products with only one chiral center bearing an amino group were all identified to possess the (R) configuration (for details of the assignment of absolute configuration, see the Supporting Information), which is in agreement with previous reports that the developed amine dehydrogenases of this family preserve the enantiose-lectivity of the wildtypes.^[9, 10]

In summary, by coupling an amine dehydrogenase (EsLeuDH-DM) with a nonstereoselective alcohol dehydrogenase, a redox self-sufficient and concise two-enzyme cascade process was constructed that was capable of aminating racemic secondary alcohols into the desired optically pure chiral amines, with considerable conversions (up to 94%) and >99%enantiomeric excess. To the best of our knowledge, this is the first report of the enzymatic synthesis of chiral amines from alcohols by employing a redox-neutral two-enzyme cascade consisting of an alcohol dehydrogenase and an amine dehydrogenase. Further attempts at protein engineering and process development may be necessary to improve the performance of the one-pot transformation and to minimize the waste of salts. We envision that this elegant enzymatic route might represent an easy and efficient biocatalytic route for the production of highly valued chiral amines.

Experimental Section

General information

Both the wildtype *Es*LeuDH and the ADH (*Sc*CR) were cloned into *E. coli* BL21 (DE3) cells by using plasmid pET-28a (+) as previously described^[11,16] and were stocked at -80 °C.

GC analysis for determining the conversion was performed with a Shimadzu-2014 gas chromatograph equipped with a flame ionization detector (FID) and an Agilent J&W DB-1701 capillary column $(30 \text{ m} \times 0.25 \text{ mm}, 0.25 \text{ }\mu\text{m})$ by using nitrogen as the carrier gas; for determination of the enantiomeric excess values, an Agilent J&W CP-Chiralsil-DEX CB capillary column (25 m×0.25 mm, 0.25 µm) was equipped. To measure the conversion, the relative amount (reported in %) of each component (alcohol, ketone, and amine) was determined according to the proportion of each peak area in the total peak area of the three compositions. The enantiomeric excess values of the amine products were determined through GC analysis on a chiral stationary phase after derivatization by comparison with commercially available reference materials. For derivatization, samples in CH₂Cl₂ (1 mL) were supplemented with acetic anhydride (100 $\mu L)$ and pyridine, then shaken for 4 h (16 $^\circ C$, 180 rpm), after which water (500 µL) was added to hydrolyze the excess amount of acetic anhydride. The organic phase was extracted and finally dried with anhydrous MgSO₄ before GC analysis on a chiral stationary phase.

One-pot procedure

sponding amines was achieved for three alcohol substrates (i.e., *rac*-1 a, *rac*-4 a, and 6 a), and for some racemic secondary

An analytical-scale one-pot reaction mixture (1 mL) containing NH_4CI/NH_4OH buffer (2 m, pH 9.5), alcohol substrate (50 mm), NAD⁺



(1 mm), and lyophilized cell-free extracts of *Es*LeuDH-DM (10 mg) and *Sc*CR (10 mg). For optimization of the two-enzyme cascade process, the pH of the NH₄Cl/NH₄OH buffer (2 m) was varied from 8.5 to 10.5, and the concentration of NAD⁺ was varied from 0.2 to 2.0 mm. The mixture was shaken at 30 °C, 1000 rpm for 24 h, after which it was quenched by adding NaOH (10 m, 200 μ L) and extracted with CH₂Cl₂ (1 mL, for once). The organic extract was dried with anhydrous MgSO₄ and analyzed by GC.

A preparative-scale mixture (10 mL) containing NH₄Cl/NH₄OH buffer (2 m, pH 9.5), *rac*-**1a** substrate (44.1 mg, 0.5 mmol), NAD⁺ (10 µmol), lyophilized *Es*LeuDH-DM cell-free extract (100 mg), and lyophilized *Sc*CR cell-free extract (100 mg). The mixture was shaken at 30 °C, 250 rpm for 24 h, after which NaOH solution (10 m, 2 mL) was added to stop the reaction. The resulting mixture was extracted with CH₂Cl₂ (3×10 mL), and the extract was combined, dried with anhydrous MgSO₄, and concentrated under reduced pressure. Then, the remaining was supplemented with HCl ethereal solution (2 m, 2 mL) to precipitate the amine hydrochloride. The final precipitate was evaporated again under reduced pressure and dried under vacuum to give **1c**-HCl. Yield: 13.2 mg (21%). ¹H NMR (400 Hz, CDCl₃): δ = 0.96 (t, 3 H, *J* = 7.3 Hz), 1.41 (d, 3 H, *J* = 6.6 Hz), 1.46 (q, 2 H, *J* = 7.5 Hz), 1.56–1.65 (m, 1 H), 1.75–1.84 (m, 1 H), 3.26–3.42 (m, 1 H), 8.06–8.60 ppm (br.m, 3 H).^[19]

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