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To be cited as: *ChemBioChem* 10.1002/cbic.202000491

Link to VoR: https://doi.org/10.1002/cbic.202000491

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Enantioselective Cascade Biocatalysis for Deracemization of Racemic β-Amino Alcohols to Enantiopure (*S*)-β-Amino Alcohols Employing Cyclohexylamine Oxidase and ω-Transaminase

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Dedication ((optional))

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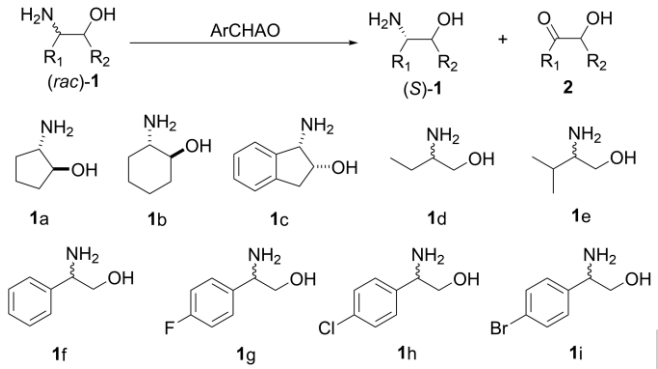
Abstract: Optically active β-amino alcohols are very useful chiral intermediates frequently used in the preparation of pharmaceutically active substances. Herein, a novel cyclohexylamine oxidase (ArCHAO) was identified from the genome sequence of *Arthrobacter* sp. TYUT010-15 with the (*R*)-stereoselective deamination activity of β-amino alcohol. ArCHAO was cloned and successfully expressed in *E. coli* BL21, purified and characterized. Substrate specific analysis revealed that ArCHAO has high activity (4.15 to 6.34 U mg^{-1} protein) and excellent enantioselectivity toward the tested β-amino alcohols. Using the purified ArCHAO, a wide range of racemic β-amino alcohols were resolved, (*S*)-β-amino alcohols were obtained in >99% ee. Deracemization of racemic β-amino alcohols was conducted by ArCHAO catalyzed enantioselective deamination and transaminase catalyzed enantioselective amination, affording (*S*)-β-amino alcohols in excellent conversion (78-94%) and enantiomeric excess value (>99%). Preparative scale deracemization was carried out with 50 mM (6.859 g L^{-1}) racemic 2-amino-2-phenylethanol, (*S*)-2-amino-2-phenylethanol was obtained in 75% isolated yield and >99% *ee*.

Optically active β-amino alcohols are very useful chiral intermediates frequently used in the preparation of pharmaceutically active substances.[1] Important examples include Xanthine analogues as phosphodiesterase type 5 (PDE5) inhibitors for the treatment of male erectile dysfunction (ED).[2] Cycloalkanediamine derivatives as activated factor X (fXa) for the therapy of thrombosis and related diseases.[3] Indinavir, an important biologically active compound as a human immunodeficiency virus (HIV) protease inhibitor.[4] In addition, Ethambutol (EMB), an important anti-tubercular drug,[5] can be prepared directly from (*S*)-(+)-2-amino-1-butanol. The importance of chiral β-amino alcohols has attracted continuous and significant attention from the synthetic groups, and a variety of chemical strategies have been developed for the synthesis of these chiral chemicals.[6] Such as Overman's aminolysis of epoxides,[7] Sharpless asymmetric aminohydroxylation of alkenes,[8] and Jacobsen's ring-opening of epoxides [9] were the most typical methods. However, in most cases, these methods often require harsh reaction conditions, laborious processes, and expensive transition metals, making the methods inconvenient and unsustainable.

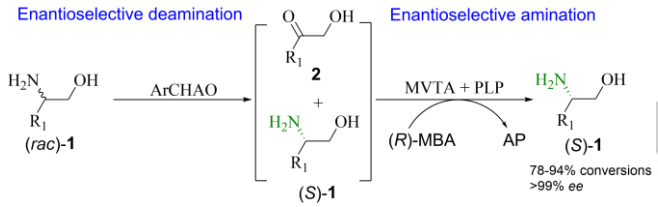
During the past two decades, biocatalysis offers a green and sustainable strategy for the synthesis of chiral β-amino alcohols. For example, chiral amino ketones asymmetric reduction,[10] racemic amino alcohols kinetic resolution,[11] and α-hydroxy ketones asymmetric reduction amination.[11h,12] Very recently, enantioselective aminohydroxylation of styrenyl olefins catalyzed by an engineered hemoprotein.[13] In addition, several cascade biocatalysis strategies combining different enzymes were also reported for chiral vicinal amino alcohol synthesis.[14] However, the limited scope of substrates, unsatisfactory product yield and enantiomeric excess, and the protection and de-protection processes made some of these methods inconvenient, alternative strategies remain of great interest.

Cyclohexylamine oxidase (CHAO) was first described in *Brevibacterium oxydans* IH-35A belongs to the flavin monooxygenase family,[15] the reaction of this enzyme only requires aerial oxygen with no need for additional co-factors. It has been utilized in the preparation of enantiopure amines via kinetic resolution or deracemization.[16] However, its synthetic application was not fully exploited, especially, the synthesis of optically active β-amino alcohols by CHAO was limited. Prompted by the wide-ranging applications of enantiopure β-amino alcohols in the pharmaceutical industry, we were interested in the exploitation of novel enzymes and new methods for the synthesis of these important chemicals. We recently isolated a new

Arthrobacter strain from the soil sample with (*R*)-selective deamination activity of β-amino alcohols.[17] Herein, a novel cyclohexylamine oxidase (ArCHAO) was successfully identified from the genome sequence of *Arthrobacter* sp. TYUT010-15. ArCHAO was heterologously expressed in the *E. coli* BL21, purified and characterized. By using the purified ArCHAO, a variety of racemic β-amino alcohols were resolved (Scheme 1) for the first time. Since the maximum theoretical yield of the kinetic resolution was only 50%, deracemization as an attractive strategy [18] was adopted for the synthesis of optically active β-amino alcohols in 100% theoretical yield. The deracemization process was conducted by ArCHAO catalyzed enantioselective deamination and transaminase catalyzed enantioselective reduction amination (Scheme 2), affording the (*S*)-β-amino alcohols in excellent conversions and *ee* values.



Scheme 1. Cyclohexylamine oxidase (ArCHAO) catalyzed kinetic resolution of racemic β-amino alcohols



Scheme 2. Deracemization of racemic β-amino alcohols by ArCHAO combined with transaminase

In order to acquire the target enzyme in the *Arthrobacter* sp. TYUT010-15. Cell-free extract of *Arthrobacter* sp. TYUT010-15 was utilized to deamination of β-amino alcohol with or without additional amine acceptor (pyruvate). However, no difference of substrate conversions was observed. Interestingly, hydrogen peroxide as the main by-product was detected from the reaction mixture. We speculated that an amine oxidase from this strain might be responsible for the deamination of β-amino alcohol. Based on the genome sequence of *Arthrobacter* sp. TYUT010-15, four amine oxidase genes were identified and cloned from *Arthrobacter* sp. TYUT010-15 (Table S1-2, Figure S1). One of the amine oxidases (Gene 4149) with significant deamination activity of 2-aminocyclohexanol was discovered (Table S3). Nucleic acid sequence alignment revealed that the discovered amine oxidase has 99% identity to a FAD-dependent oxidoreductase from *Arthrobacter* sp. TB 26 and 99% identity to a cyclohexylamine oxidase (CHAO) from *Brevibacterium oxydans*. Then, the name of the cloned enzyme was designated as ArCHAO (GenBank accession number: MT862133). While cyclohexylamine oxidase was reported for chiral amine synthesis, it is the first time that a cyclohexylamine oxidase has been identified from *Arthrobacter* sp. with the enantioselective deamination activity of β-amino alcohols.

Recombinant *E. coli* (ArCHAO) was induced with IPTG (0.5 mM) at the temperature of 20°C for 12 h. SDS-PAGE analysis revealed that the cloned recombinant ArCHAO produced in *E. coli* was in soluble form, and the enzyme was purified by using a Ni-NTA affinity column chromatography (Figure S2). The purified enzyme showed the expected molecular size of about 55 kDa. The activity of purified ArCHAO toward the 2-aminocyclohexanol **1**b was tested, the results showed that the ArCHAO has the activity of 6.0 U mg^{-1} toward (1*R*, 2*R*)-**1**b, and 2.5 U mg^{-1} toward (1*S*, 2*S*)-**1**b, demonstrated that the ArCHAO was an (*R*)-selective amine oxidase toward β-amino alcohol. Methylbenzylamine was also tested, a switch in enantiopreference to methylbenzylamine was observed, this could be explained by Cahn-Ingold-Prelog rules.[19] Then, the purified ArCHAO was characterized, the optimum pH of purified ArCHAO toward the *trans*-2-aminocyclohexanol **1**b was determined using standard assay conditions at different pHs. ArCHAO showed the maximum activity at pH 7.0 (Figure 1a). The pH stability of ArCHAO was investigated by storing the ArCHAO in 100 mM buffer with various pH values (6.0-11.0) at 20°C. The results showed that the ArCHAO was stable between pH7.0 and 10.0, >80% of residual activities were observed over a period of 24 h (Figure 1c). The optimum temperature for the deamination reaction at 30°C (Figure 1b). The temperature stability of the enzyme was investigated by storing the enzyme in sodium phosphate buffer (100 mM, pH7.0) at the temperature from 4 to 50°C for 24 h. As shown in Figure 1d, the purified ArCHAO was stable at the temperature from 4 to 30°C, and >70% residual activities of ArCHAO were observed at these temperatures over a period of 24 h. When the temperature increased to 50°C, only 10% residual activity of the enzyme was detected after incubation for 24 h.

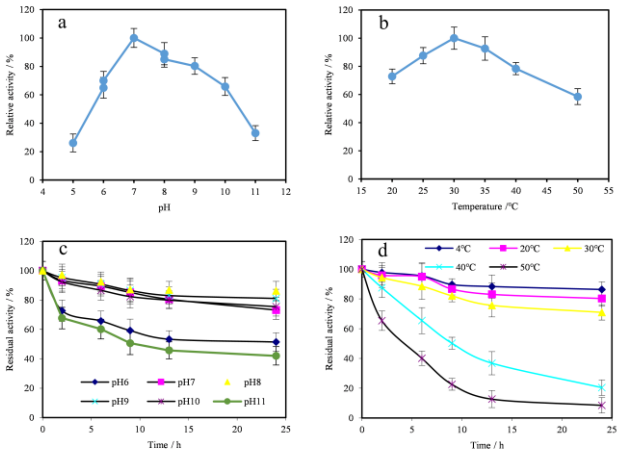


Figure 1. Effect of pH and temperature on the activity and stability of ArCHAO.

Kinetic parameters of ArCHAO were determined at various substrate concentrations with the use of Lineweaver-Burk plots and nonlinear regression analysis. As shown in Table 1, although

the affinities of ArCHAO toward (1*S*, 2*S*)-*trans*-**1**a-b (with the K_M values of 0.9 mM and 1.0 mM) were higher than (1*R*, 2*R*)- *trans*-**1**a-b (with the K_M values of 1.1 mM and 1.3 mM), the k_{cat}/K_M values of the enzyme toward (1*S*, 2*S*)-*trans*-**1**a-b (1.8 s^{-1} mM^{-1} protein and 1.5 s^{-1} mM^{-1}) were lower than (1*R*, 2*R*)-*trans*-**1**a-b (5.1 s^{-1} mM^{-1} and 4.5 s^{-1} mM^{-1}). For the substrate **1**c, the k_{cat}/K_M values were 8.2 s^{-1} mM^{-1} toward (1*R*,2*S*)-*cis*-**1**c and 1.7 s^{-1} mM^{-1} toward (1*S*,2*R*)-*cis*-**1**c with the K_M values of 0.6 mM and 0.7 mM, respectively. These results revealed that ArCHAO is more active toward (*R*)-enantiomer than (*S*)-enantiomer, further demonstrated the (*R*)-selective of the enzyme toward β-amino alcohol.

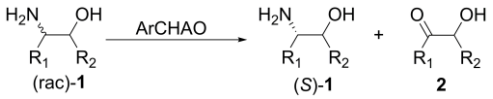
Table 1. Kinetic parameters of ArCHAO toward cyclic β-amino alcohols.[a]

Entry	Enantiomer	K_M [mM]	V_{max} [μmol mg^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM^{-1}]
1	(1*R*,2*R*)-**1**a	1.1	6.2	5.6	5.1
2	(1*S*,2*S*)-**1**a	0.9	1.8	1.6	1.8
3	(1*R*,2*R*)-**1**b	1.3	6.4	5.8	4.5
4	(1*S*,2*S*)-**1**b	1.0	1.7	1.5	1.5
5	(1*R*,2*S*)-**1**c	0.6	5.4	4.9	8.2
6	(1*S*,2*R*)-**1**c	0.7	1.3	1.2	1.7

[a] Reaction conditions: sodium phosphate buffer (100 mM, pH7.0) and six concentrations (0.5-8 mM) of substrates, shaking at 30°C for 5 min. Kinetic analysis with initial rate data obtained at the conversion lower than 5%.

Then, the purified ArCHAO was employed to kinetic resolution of racemic β-amino alcohols **1**a-i. As shown in Table 2, ArCHAO has high activity toward the cyclic β-amino alcohols (±)-*trans*-**1**a and (±)-*trans*-**1**b, with the activity of 6.1 U mg^{-1} and 6.23 U mg^{-1}, respectively. For (±)-*cis*-**1**c, the activity of the enzyme was relatively low, and 4.87 U mg^{-1} was detected. Kinetic resolution of racemic *trans*-**1**a-b and *cis*-**1**c were conducted by ArCHAO with 30-50 mM substrate concentration, (1*S*, 2*S*)-*trans*-**1**a, (1*S*, 2*S*)-*trans*-**1**b and (1*S*, 2*R*)-*cis*-**1**c were obtained in >99% *ee* and 53-58% conversion rates at 2-4 h, respectively. Four β-amino alcohols **1**f-i with different para substituent groups on an aromatic ring were tested as substrates, high activities (from 5.77 U mg^{-1} to 6.34 U mg^{-1}) of the enzyme toward these substrates were detected, kinetic resolution of (±)-**1**f-i resulted in about 50% conversion rate, leave the (*S*)-**1**f-i in >99% *ee*. Racemic **1**d and **1**e as two typical aliphatic β-amino alcohols were then tested, relative low activities (4.76 U mg^{-1} and 4.15 U mg^{-1}) were obtained, kinetic resolution of these substrates resulted in 55.4% and 53.6% conversion, leave the (*S*)-**1**d and (*S*)-**1**e in >99% *ee*. In addition, two racemic amines phenethylamine and 1,2,3,4-tetrahydro-1-naphthylamine were also resolved by ArCHAO, (*R*)-phenethylamine and (*R*)-1,2,3,4-tetrahydro-1-naphthylamine were obtained in >99% *ee* and 50% conversion (Figure S12 and Figure S13), respectively. The time courses for the kinetic resolution 50-400 mM of racemic *trans*-**1**b were shown in Figure 2. ArCHAO exhibited strong tolerance against high substrate concentration of **1**b, 55-56% conversion could be obtained when kinetic resolution 50-200 mM of **1**b at 3-12 h, even if the concentration of **1**b was increased to 400 mM, racemic *trans*-**1**b could be completely resolved by ArCHAO at 30 h. The synthetic potential of ArCHAO was then demonstrated by kinetic resolution of racemic *trans*-**1**b (400 mM) at a 50 mL-scale, (1*S*, 2*S*)-*trans*-**1**b obtained in 26.3% isolated yield (790.2 mg) and >99% *ee*. To our best knowledge, (1*S*, 2*S*)-*trans*-**1**b can be used as a building block in the synthesis of "Peptoid" cholecystokinin (CCK-B) receptor antagonists (CI-1015) for the treatment of anxiety and panic disorder.[1b]

Table 2. Kinetic resolution of racemic β-amino alcohols by ArCHAO. [a]



Sub.	Sub. [mM]	Time [h]	Specific activity [U mg^{-1}]	Conv. [%][b]	Sub. ee [%][c]
1a	50	2	6.10	56.2	>99.0 (1*S*,2*S*)
1b	50	2	6.23	58.1	>99.0 (1*S*,2*S*)
1c	30	4	4.87	53.4[d]	>99.0(1*S*,2*R*)[e]
1d	20	3	4.76	55.4	>99.0 (*S*)
1e	20	3	4.15	53.6	>99.0 (*S*)
1f	50	4	6.34	50.5	>99.0 (*S*)
1g	20	6	5.91	50.4	>99.0 (*S*)
1h	20	7	5.58	50.2	>99.0 (*S*)
1i	20	8	5.77	50.2	>99.0 (*S*)

[a] Reaction conditions: *rac*-1 (20-50 mM), ArCHAO (10 mg mL^{-1}), phosphate buffer (100 mM, pH 7.0), shaking at 30°C for 2-8 h. [b] Determined by GC. [c] Determined by chiral GC analysis. [d] Determined by HPLC. [e] Determined by chiral HPLC analysis. Error limit: <2% of the state values.

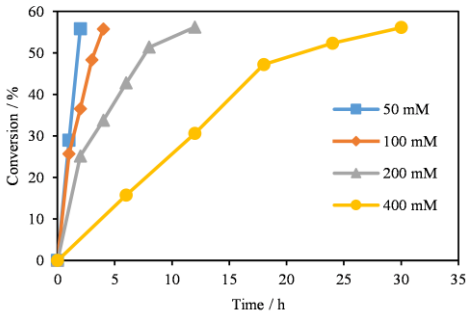
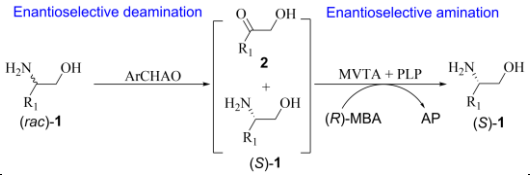


Figure 2. The time course for the kinetic resolution of racemic *trans*-**1**b by ArCHAO.

Since the highest yield of the kinetic resolution was only 50%, deracemization was chosen as an efficient process to access the chiral β-amino alcohols in a 100% theoretical yield. Herein we reported a new deracemization strategy for the preparation of chiral β-amino alcohols, the strategy was conducted by ArCHAO catalyzed enantioselective deamination and transaminase catalyzed enantioselective reduction amination (Scheme 2). While many amines have been deracemized by amine oxidases combined with various chemical reducing agents,[20] studies on the deracemization of racemic β-amino alcohols have been limited. As shown in Scheme 2, the ArCHAO mainly oxidizes the (*R*)-enantiomer to the corresponding α-hydroxy ketone **2**, which is then reduced back to the (*S*)-enantiomer **1** by an (*S*)-selective transaminase (MVTA).[11h] The cascade biocatalysis process results in eventual accumulation of the (*S*)-enantiomer in high yield and *ee*. To decrease the cost of enzyme preparation, the whole cells of *E. coli* (ArCHAO) and *E. coli* (MVTA) were used in the deracemization process. Six racemic β-amino alcohols (**1**d-i) were subjected to the new deracemization protocol using the whole cells of *E. coli* (ArCHAO) (10 g cdw L^{-1}) and *E. coli* (MVTA) (10 g cdw L^{-1}) (Table 3). (*S*)-**1**d and (*S*)-**1**f-g could be obtained in 91.3-92.9% conversions and >99% *ee* at 6-8 h with 20 mM (±)-**1**d, (±)-**1**f and (±)-**1**g, respectively. Increasing the (±)-**1**f to 50 mM, 92% conversion of **1**f could be obtained at 12 h. In the case of (±)-**1**e and (±)-**1**h-i, (*S*)-**1**e and (*S*)-**1**h-i were obtained in 78.5-85.7% conversions and >99% *ee*. To demonstrate the synthetic potential

of this new deracemization process, a 100 mL-scale preparation experiment was carried out with 50 mM racemic **1**f (686 mg). (*S*)-**1**f could be obtained in 90% conversion and >99% *ee* at 16 h. After extraction and purification, (*S*)-**1**f was obtained in 75% isolated yield (514 mg) and >99% *ee* (Figure S21).

Table 3. Deracemizatioin of racemic β-amino alcohols employing *E. coli* (ArCHAO) and *E. coli* (MVTA).[a]



Substrate	Sub. [mM]	Time [h]	Conv. [%][b]	Sub. *ee* [%][c]
1d	20	6	91.3	>99.0 (*S*)
1e	20	7	85.4	>99.0 (*S*)
1f	20	6	93.6	>99.0 (*S*)
1f	50	12	92.0	>99.0 (*S*)
1g	20	8	92.9	>99.0 (*S*)
1h	20	10	85.7	>99.0 (*S*)
1i	20	10	78.5	>99.0 (*S*)

[a] Reaction conditions: *rac*-substrates (20-50 mM), *E. coli* (ArCHAO) (10 g cdw L^{-1}), *E. coli* (MVTA) (10 g cdw L^{-1}), (*R*)-phenethylamine (10-25 mM), phosphate buffer (100 mM, pH7.0, 0.1 mM PLP), shaking at 30°C for 6-12 h. [b] Determined by GC, defined as percentage ratio of the (*S*)-β-amino alcohol product (mM) to initial racemic substrate (mM), error limit: <2% of the state values. [c] Determined by GC analysis on a chiral phase.

In summary, a novel cyclohexylamine oxidase (ArCHAO) was discovered from *Arthrobacter* sp. TYUT010-15 with the highly (*R*)-selective deamination activity toward β-amino alcohols. A variety of racemic β-amino alcohols were resolved by the purified ArCHAO, excellent conversion and *ee* value were obtained. Deracemization of racemic β-amino alcohols was conducted by ArCHAO catalyzed enantioselective deamination and transaminase catalyzed enantioselective reduction amination, various racemic β-amino alcohols were deracemized to (*S*)-β-amino alcohols in excellent conversions (78-92%) and *ee* values (>99%). Preparation experiment of deracemization was conducted with 50 mM (6.86 g L^{-1}) racemic 2-amino-2-phenylethanol, (*S*)-2-amino-2-phenylethanol was obtained in 75% isolated yield and >99% *ee*. The remarkable features of ArCHAO highlight its greater potential for the synthesis of chiral β-amino alcohols. The designed deracemization process might be generally applicable to access other important chiral amines by employing appropriate enzymes.

Acknowledgements ((optional))

This study was financially supported by the National Natural Science Foundation of China (Grant No. 21772141), the Shanxi Province Science Foundation for Youths (grant No. 201701D221042).

Keywords: cascade biocatalysis • deracemization • cyclohexylamine oxidase • transaminase • β-amino alcohols

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10.1002/cbic.202000491

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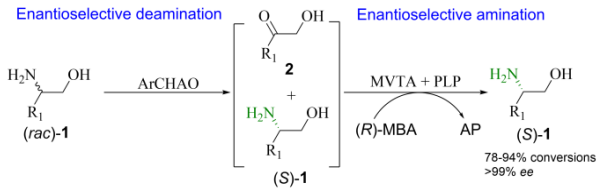

Enantioselective cascade biocatalysis for deracemization of racemic β-amino alcohols was conducted by employing cyclohexylamine oxidase and ω-transaminase. (*S*)-β-amino alcohols were obtained in excellent conversions and ee values.

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