Advanced Synthesis & Catalysis

Accepted Article

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To be cited as: *Adv. Synth. Catal.* 10.1002/adsc.201900178

Link to VoR: http://dx.doi.org/10.1002/adsc.201900178

10.1002/adsc.201900178

FULL PAPER

DOI: 10.1002/adsc.201((will be filled in by the editorial staff))

Chemoenzymatic Approach to (*S*)-1,2,3,4-Tetrahydroisoquinoline Carboxylic Acids Employing D-Amino Acid Oxidase

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Received: ((will be filled in by the editorial staff))

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201######.((Please delete if not appropriate))

Abstract. Optically pure 1,2,3,4-tetrahydroisoquinoline carboxylic acids constitute an important class of building blocks for the synthesis of natural products and synthetic pharmaceuticals. However, redox deracemization of racemic 1,2,3,4-tetrahydroisoquinoline carboxylic acids as an attractive method is still challenging for the lack of suitable oxidoreductases. Herein, a D-amino acid oxidase from *Fusarium solani* M-0718 (*Fs*DAAO) with broad substrate scope and excellent enantioselectivity was exploited through genome mining, and applied for the kinetic resolution of a number of racemic 1- and 3-carboxyl substituted tetrahydroisoquinolines to yield the corresponding (*S*)-enantiomers with excellent enantiomeric excess (*ee*) values (up to > 99%). By using *Fs*DAAO in combination with ammonia-borane in one pot, deracemization of these racemic carboxyl-substituted tetrahydroisoquinolines was achieved with conversions up to > 98% and > 99% *ee*. Preparative-scale deracemization of racemic 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid was also demonstrated with good isolated yields (82% and 73%, respectively) and *ee* > 99%. Our study provides an effective method for the synthesis of enantiomeric pure 1,2,3,4-tetrahydroisoquinoline carboxylic acids. This method is expected to provide access to chiral carboxyl-substituted 1,2,3,4-tetrahydroquinolines and 1,2,3,4-tetrahydro-ß-carbolines.

Keywords: biotransformations; chiral tetrahydroisoquinoline; D-amino acid oxidase; deracemization; kinetic resolution

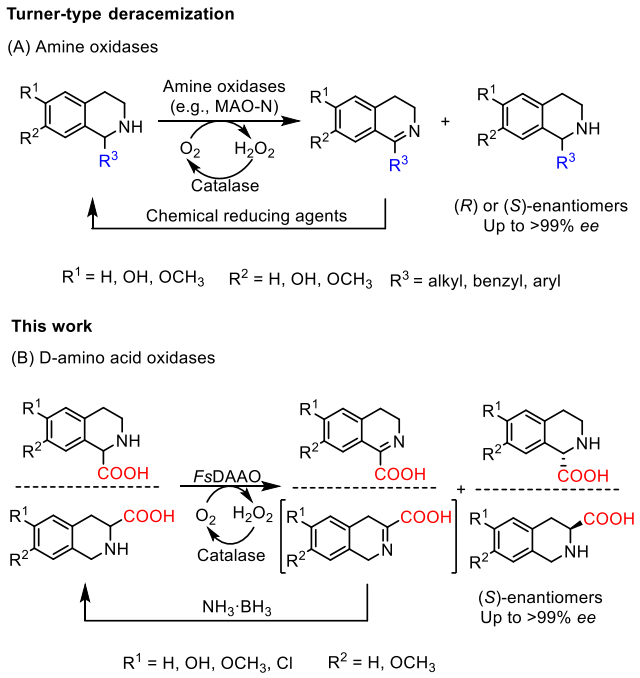
Introduction

Chiral 1,2,3,4-tetrahydroisoquinolines (THIQs) are widely distributed in various natural bioactive alkaloids and also serve as important intermediates in pharmaceutical chemistry[1]. Compounds containing THIQ skeleton exhibit a variety of biological activities, such as antitumor[2], antimalarial[3], anthelmintic[4], antihypertensive[5], targeting of the central nervous system[6], and inhibiting Keap1-Nrf2 protein-protein interactions[7]. Their biological activities are occasionally stereoselective in terms of their potency, metabolism and bitter taste [4] [8].

Considerable effort has been devoted to the enantioselective preparation of chiral THIQs. Chemical asymmetric synthesis method are important and frequent sources of these compounds[1a, 5b, 9]. However, the use of hazardous materials, such as toxic organic reagents or transition metal catalysts, remains a challenge for industrial application. As an alternative to chemical approaches, attention has been gradually directed to elegant and environmentally-friendly enzymatic methods. In recent years, the preparation of chiral non-carboxyl substituted THIQs have been intensively investigated using redox biocatalysis[10]. Most notably are the applications of recombinant amine oxidase variants developed by Turner and coworkers, which have been used in the chemo-enzymatic synthesis of chiral 1-alkyl-, benzyl- or aryl-substituted THIQs (Scheme 1A) [10g-j]. These enzymatic routes are cost-effective and generally produce excellent enantioselectivity and yield. However, to the best of our knowledge, these biocatalytic approaches mainly focused on non-carboxyl substituted THIQs. The preparation of chiral carboxyl-substituted THIQs, especially 1-substituted THIQs, is still challenging since the actions of suitable oxidoreductases on these compounds had not been described. Accordingly, it is highly desirable to develop novel methods for the preparation of chiral carboxyl-substituted THIQs in both academia and industry.

Encouraged by the Turner-type deracemization[11], we attempted to design a biocatalytic process to transform the racemic carboxyl-substituted THIQs into optically-pure products using an oxidoreductase in combination with a nonselective chemical reducing

agent. The process contained two steps: 1) one of the enantiomers should be oxidized to the corresponding imino acid with high enantioselectivity; 2) the imino acid should be transformed into racemic mixture via nonselective in situ reduction. The enantioselective oxidation and nonselective reduction should run simultaneously and efficiently. The challenge is the exploitation of robust enzymes for the first step. There has been no previous report regarding the bio-oxidation of the model substrate 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid. However, its analogue, 1-methyl-tetrahydroisoquinoline, is the substrate of monoamine oxidases MAO-N-D5 and LG-I-D11, which can be used in a more generally manner for the oxidation of C-N bond to C=N double bonds in different amines lacking carboxyl group[10h, 12]. For those amines bearing carboxyl group, L-amino acid oxidases (LAAO, EC 1.4.3.2), L-amino acid deaminases (LAAD), D-amino acid oxidases (DAAO, EC 1.4.3.2) and NADH/NADPH-dependent amino acid dehydrogenases (AADH, EC 1.4.1.X) are known to catalyze the equivalent type of bio-oxidation transformation[13].



Scheme 1. Chemo-enzymatic deracemization of racemic THIQs using (A) amine oxidases (e.g., MAO-N) or (B) D-amino acid oxidases (*Fs*DAAO).

Herein, we describe the first example of the DAAO catalyzed bio-oxidation of racemic carboxyl-substituted THIQs with a preference for the (*R*)-enantiomer (Scheme 1B). An enzyme library containing LAAOs, LAADs, DAAOs, and AADHs was constructed and screened through genome mining. *Fs*DAAO, which was derived from *Fusarium solani* M-0718 and initially expressed for the production of 7-aminocephalosporanic acid (7-ACA)[15], was achieved. The substrate specificity toward a number of racemic 1- and 3-carboxyl substituted THIQs with different substitutions on the phenyl ring was investigated. Using *Fs*DAAO, we successfully constructed a kinetic resolution system for the highly enantioselective synthesis of (*S*)-1- and 3-carboxyl substituted THIQs. Furthermore, we combined the *Fs*DAAO with a nonselective chemical reducing agent, ammonia-borane ($NH_3 \cdot BH_3$), thus providing a novel and general method for the efficient synthesis of optically pure carboxyl-substituted THIQs.

Results and Discussion

Exploitation and Purification of *Fs*DAAO with Excellent Enantioselectivity toward *rac*-1a

To identify an enzyme with good activity and excellent enantioselectivity toward these sterically hindered substrates, a genome mining-based library construction strategy was applied. Considering the structural characteristics of these substrates, which bear a carboxyl group substituent on the chiral carbon atom, an enzyme library containing ten DAAOs, six LAAOs, four LAADs, and fourteen AADHs from different microorganisms was constructed. These oxidoreductases were overexpressed in *Escherichia coli* BL21 (DE3). Compared with the AADHs, the other three types of enzymes were rather difficult to overexpress, and most were expressed as a partial or total inclusion body. Nevertheless, the activity of the crude extract toward the natural substrates was further measured and the results revealed that four enzymes were expressed without activity among the 34 tested enzymes (Supporting Information, Table S1). After confirming the functional expression of 30 candidate enzymes, we tested their activity on racemic 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (*rac*-**1a**) using crude extract. As a result, four DAAOs of the tested enzymes showed different activities toward *rac*-**1a** with strict R-configuration enantioselectivity (*ee* > 99% with 50% conversion) (Table 1). These four recombinant DAAOs with a His-tag at the N-terminal were then purified using nickel-affinity chromatography, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Supporting Information, Figure S2), indicating a subunit size of approximately 43 kDa. Their specific activities toward *rac*-**1a** were further compared. As shown in Table 1, *Fs*DAAO displayed the highest activity of 0.84 ± 0.05 U/ml and 19.02 ± 0.09 U/mg, and was selected for further research.

Characterization of *Fs*DAAO

The enzymatic properties of *Fs*DAAO were characterized to investigate the biotransformation conditions suitable for this enzyme. The activity and stability of purified *Fs*DAAO were measured at various pH levels and temperatures.

10.1002/adsc.201900178

Table 1. Enzyme screening for the oxidation of *rac*-**1a**[a].

Entry	Source	Activity (U/ml)[b]	Specific activity (U/mg)[b]	Conversion (*c*)(%)[c]	ee_s(%)[d]
1	*Fusarium solani* M-0718	0.84 ± 0.05	19.02 ± 0.09	50	> 99 (*S*)
2	*Fusarium graminearum* CS3005	0.24 ± 0.01	4.34 ± 0.15	50	> 99 (*S*)
3	*Fusarium poae* 2516	0.21 ± 0.02	4.63 ± 0.32	50	> 99 (*S*)
4	*Trigonopsis variabilis* CBS 4095	0.04 ± 0.01	0.85 ± 0.06	50	> 99 (*S*)

[a] Reaction conditions (1ml): *rac*-**1a** (10 mM), crude extract (10 mg/mL) and catalase (0.75 mg/mL) in Na_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 8.0). Reactions were incubated at 30 ℃ with 600 rpm shaking for 24h.

[b] Enzyme activity or specific activity was determined using HPLC method.

[c] Conversion(*c*) value was determined by chiral HPLC analysis. *c* = [(the initial *rac*-**1a** - the residual *rac*-**1a**)/the initial *rac*-**1a**] × 100%.

[d] The substrate *ee* (ee_s) was determined by chiral HPLC analysis, and configuration was determined by comparison with literature date or by analogue. ee_s=[((*S*)-**1a** - (*R*)-**1a**)/((*S*)-**1a** + (*R*)-**1a**)]×100%.

The enzyme maintained relatively high activity (more than 80%) between pH 8.0 and 9.0 (Supporting Information, Figure S3A) and was stable from pH 6.0 to 11.0 (retained more than 90% activity) for 24 h at 4 °C (Supporting Information, Figure S3B). The specific activities from 20 to 60 °C were then measured. The enzyme exhibited a maximum activity at 45 °C and 62% relative activity at 30 °C (Supporting Information, Figure S3C). The thermostability of *Fs*DAAO was assessed at 30, 40 and 50 °C (Supporting Information, Figure S3D). The half-lives of the enzyme were calculated to be 165 h at 30 °C, 114 h at 40 °C and 36 h at 50 °C, indicating good thermal stability, especially under mild reaction conditions. Taken together, pH 8.0 and 30 °C were considered as the biotransformation conditions. The kinetic parameters of *Fs*DAAO toward *rac*-**1a** were determined under the same conditions, indicating a catalytic efficiency constant (*k*cat/*K*m) of 47.14 s^{-1} mM^{-1}.

Investigation of Substrate Specificity

Having preliminarily established the *Fs*DAAO-mediated enantioselective biotransformation system, we were curious to explore the potential application of this system in biocatalysis. Initially, we examined the oxidative dehydrogenation activity of *Fs*DAAO toward a range of substrates comprising THIQs, D-amino acids and their derivatives (Figure 1). The relative specific activity was measured by employing the coupled *o*-dianisidine/peroxidase method with purified enzyme at pH 8.0 and 30 °C. The activity of *Fs*DAAO for *rac*-**1a** (19.66 ± 0.4 U/mg) was defined as 100%, and the activities relative to *rac*-**1a** were calculated.

In general, *Fs*DAAO exhibited strict stereospecificity toward D-amino acids, as found in other DAAOs[15]. Among these D-amino acids, *Fs*DAAO appears to prefer substrates with large hydrophobic side chains, such as D-phenylalanine ((*R*)-**12**) and D-methionine ((*R*)-**14**). The presence of hydrophilic group in the side chains, such as hydroxyl group on the phenyl ring, significantly reduced the activity. For instance, the relative specific activity toward D-tyrosine ((*R*)-**13**) (198%) was only about one third of that toward D-phenylalanine ((*R*)-**12**) (564%).

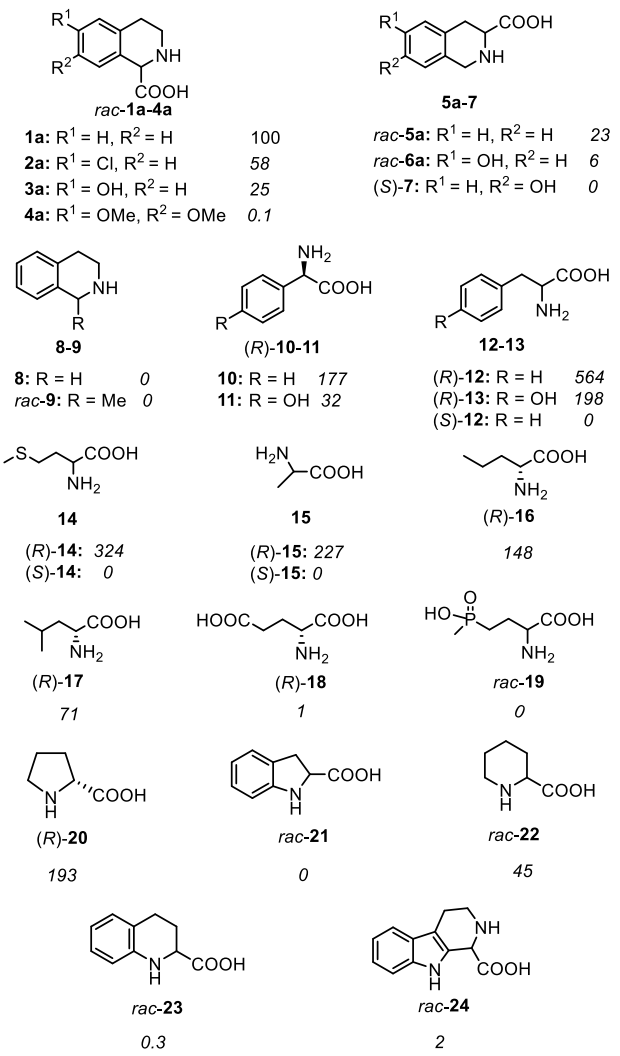


Figure 1. Relative specific activity of *Fs*DAAO on different substrates. The specific activities were measured in triplicate using the coupled *o*-dianisidine/peroxidase method, and each value (italicized number) represents mean ± standard deviation.

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This trend was also observed in the oxidation of 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acids **2a**-**4a**. The substituent groups on the phenyl ring affect the activities**,** which are of the following order: 6-chlorine > 6-hydroxyl > 6,7-methoxyl substituents. Compared with *rac*-**1a**, *Fs*DAAO exhibited much lower activity toward 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*rac*-**5a**). No activity was measured with non-carboxyl substituted THIQs, such as 1,2,3,4-tetrahydroisoquinoline (**8**) or 1-methyl-1,2,3,4-tetrahydroisoquinoline (**9**). The enzyme also displayed some activity toward racemic 1,2,3,4-tetrahydroquinoline-2-carboxylic acid (*rac*-**23**) and 1,2,3,4-tetrahydro-ß-carboline-1-carboxylic acid (*rac*-**24**), in which the tetrahydroquinoline and tetrahydro-ß-carboline cores are privileged motifs found in numerous bioactive molecules[16]. Unfortunately, racemic indoline-2-carboxylic acid (*rac*-**21**) was not a suitable substrate.

Kinetic Resolution of 1- and 3-Carboxyl Substituted THIQs

After investigation of the substrate specificity, we applied *Fs*DAAO for the kinetic resolution of a group of racemic 1- and 3-carboxyl substituted THIQs with different substitutions on the phenyl ring, which was the interest of this study. To evaluate the stability of substrate, a series of control experiments featuring all reaction conditions but lacking *Fs*DAAO were carried out. After incubation at pH 8.0 and 30 °C for 24 h, chiral HPLC analysis revealed that the concentrations of *rac*-**1a**-**6a** remained unchanged and no side product was observed, confirming that these substrates were stable under the mild conditions. To study the effect of pH on substrate racemization, (*S*)-**1a** was incubated at 30 °C in Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0) or Na_2CO_3-$NaHCO_3$ buffer (pH 10.0). After 24 h, chiral HPLC analysis revealed that no (*R*)-enantiomer was observed at pH 8.0. However, significant racemization occurred at pH 10.0. Hence, kinetic resolution reactions were performed at pH 8.0 and 30 °C. As shown in Table 2, racemic substrates **1a**-**5a** were resolved completely into *S*-enantiomers with excellent enantioselectivity (*ee* > 99%) and conversions (50%). Exceptionally, even with prolonging the reaction time, reducing the amount of substrate, or adding more enzyme, a relatively low *ee* (88%) was obtained with 46% conversion when using *rac*-**6a** as the substrate, which was attributed to the product inhibition of *Fs*DAAO.

One-pot Synthesis of (*S*)-carboxyl Substituted THIQs via Chemo-enzymatic Deracemization

Compared with chiral 3-carboxyl substituted THIQs, which could be synthesized from the corresponding chiral amino acids (e.g., L-phenylalanine)[5b], asymmetric synthesis of chiral 1-carboxyl substituted THIQs are more difficult. Having obtained the *Fs*DAAO and applied it for the kinetic resolution of carboxyl-substituted THIQs successfully, we attempted to investigate a novel method for the synthesis of (*S*)-carboxyl substituted THIQs, especially for the 1-carboxyl substituted THIQs, by combination of *Fs*DAAO with a chemical reducing agent in one pot.

To assess the feasibility, we first analyzed the oxidative dehydrogenation product of (*R*)-**1a.** Purified *Fs*DAAO-catalyzed oxidation of (*R*)-**1a** was performed and the product was analyzed using NMR and HR-MS. All of these results confirmed that the product was 3,4-dihydroisoquinoline-1-carboxylic acid (**1b**), which is essential for the deracemization reaction.

Then three commonly used chemical reductive agents, including sodium borohydride ($NaBH_4$), sodium cyanoborohydride ($NaCNBH_3$), and $NH_3{\cdot}BH_3$, were incubated with **1b** at pH 8.0 and 30 °C, respectively. As a result, **1b** was successfully racemized to *rac*-**1a**. However, compared with $NaBH_4$, $NaCNBH_3$ and $NH_3{\cdot}BH_3$ were milder and more water-stable in the preliminary experiments, which was consistent with previous reports[18], and were selected for further research.

On the basis of the mechanistic studies, deracemization of *rac*-**1a** employing *Fs*DAAO in

Table 2. Results of *Fs*DAAO-catalyzed kinetic resolution reactions[a].

Entry	Substrate (R^1 =, R^2 =)	Concentration (mM)	Time (h)	Conversion (*c*)(%)[b]	ee_s(%)[c]/ Configuration	*E*[d]
1	**1a** (H, H)	10	1	50	> 99 (*S*)	> 200
2	**2a** (Cl, H)	10	1	50	> 99 (*S*)	> 200
3	**3a** (OH, H)	10	1	50	> 99 (*S*)	> 200
4	**4a** (OMe, OMe)	5	5	50	> 99 (*S*)	> 200
5	**5a** (H, H)	10	9	50	> 99 (*S*)	> 200
6	**6a** (OH, H)	5	6	47	88 (*S*)	> 200

[a] Reaction conditions (1ml): racemic substrates (10 or 5 mM), purified *Fs*DAAO (0.1 mg/mL), flavine adenine dinucleotide (FAD) (100 μM) and catalase (0.75 mg/mL) in Na_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 8.0). Reactions were shaken at 30 ℃, 600 rpm.

[b] Conversion (*c*) was determined by chiral HPLC analysis. c = [(the initial *rac*-**a** - the residual *rac*-**a**)/the initial *rac*-**a**] × 100%.

[c] The ee_s was determined by chiral HPLC analysis, and configuration was determined by comparison with literature data or by analogue. ee_s = [((*S*)-**a** - (*R*)-**a**)/((*S*)-**a** + (*R*)-**a**)] × 100%.

[d] The *E* values were determined from *c* and ee_s according to the equation: $E = \ln[(1 - c)\cdot(1 - ee_s)]/\ln[(1 - c)\cdot(1 + ee_s)]$[17].

Table 3. Chemo-enzymatic deracemization of *rac*-**1a**-**6a** employing *Fs*DAAO [a].

Entry	Substrate	Concentration (mM)	*Fs*DAAO (mg/mL)	Reductive agent [equivalents]	Time (h)	Conversion (*C*) (%)[b]	ee_s (%)[c]/ Configuration
1	**1a**	10	0.1	$NaCNBH_3$ [5-10]	5	50-60	> 99 (*S*)
2	**1a**	10	0.1	$NH_3{\cdot}BH_3$ [4]	5	> 96	> 99 (*S*)
3	**2a**	10	0.1	$NH_3{\cdot}BH_3$ [4]	5	> 94	> 99 (*S*)
4	**3a**	10	0.1	$NH_3{\cdot}BH_3$ [4]	10	> 94	> 99 (*S*)
5	**4a**	5	0.1	$NH_3{\cdot}BH_3$ [4]	10	> 96	> 99 (*S*)
6	**5a**	10	0.1	$NH_3{\cdot}BH_3$ [8]	24	40	> 99 (*S*)
7	**5a**	10	0.1	$NH_3{\cdot}BH_3$ [20]	24	75	> 99 (*S*)
8	**6a**	5	0.2	$NH_3{\cdot}BH_3$ [40]	24	28	87 (*S*)

[a] Reaction conditions (1ml): racemic substrates (10 or 5 mM), purified *Fs*DAAO (0.1 or 0.2 mg/mL), FAD (100 μM), catalase (0.75 mg/mL), $NH_3{\cdot}BH_3$ (4-40 equivalents) or $NaCNBH_3$ (5-10 equivalents) in Na_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 8.0). Reactions were shaken at 30 ℃, 600 rpm.

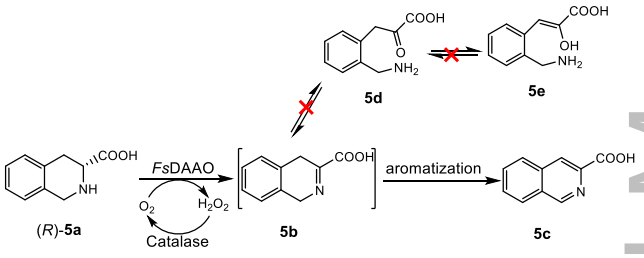
[b] Conversion (*C*) of (*R*)-enantiomer to (*S*)-enantiomer was determined by chiral HPLC analysis. *C* = [(the residual (*S*)-**a** - the initial (*S*)-**a**)/the initial (*R*)-**a**] × 100%.

[c] The ee_s was determined by chiral HPLC analysis, and configuration was determined by comparison with literature data or by analogue. ee_s = [((*S*)-**a** - (*R*)-**a**)/((*S*)-**a** + (*R*)-**a**)] × 100%.

combination with $NaCNBH_3$ or $NH_3{\cdot}BH_3$ was performed in one pot. An initial set of experiments using purified *Fs*DAAO in combination with 5-10 equivalents of $NaCNBH_3$ was carried out for the deracemization of *rac*-**1a** (Table 3, entry 1). The chiral HPLC analysis revealed that excellent optically pure (*S*)-**1a** (*ee* > 99%) was obtained in 5 h, however, the conversion of (*R*)-**1a** to (*S*)-**1a** only reached 50-60%. In contrast to the results obtained using $NaCNBH_3$, 4 equivalents of $NH_3{\cdot}BH_3$ afforded conversion up to > 96% thus made it ideal for our chemo-enzymatic deracemization process (Table 3, entry 2).

With these results in hand, we proceeded to investigate the deracemization of 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid derivatives employing the *Fs*DAAO-$NH_3{\cdot}BH_3$ cascade. As shown in Table 3, racemic substrates **2a**-**4a** were transformed into (*S*)-enantiomers at high conversions (up to > 96%) and *ee* > 99%. Compared with the alcalase-catalyzed dynamic kinetic resolution of the racemic ester[19], the preparation of (*S*)-**4a** using this cascade was more attractive in terms of the enantioselectivity and reaction efficiency.

We also investigated the chemo-enzymatic deracemization of racemic 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*rac*-**5a**) and its derivative (*rac*-**6a**). Substrate **5a** was first tested with 8 equivalents of $NH_3{\cdot}BH_3$. After 24 h of incubation, chiral HPLC analysis revealed that (*R*)-**5a** was completely oxidized, however, only 40% of (*R*)-**5a** was converted into (*S*)-**5a** (Table 3, entry 6). We speculated that the proposed oxidation product of (*R*)-**5a**, 1,4-dihydroisoquinoline-3-carboxylic acid (**5b)**, may have been unstable and transformed into keto acid (**5d**) or enolic acid (**5e**), which subsequently led to low efficiency in the reduction process. To shed light on this process, we further analyzed the oxidative dehydrogenation product of (*R*)-**5a** using NMR and HR-MS. Unexpectedly, all of the data indicated that the final product was isoquinoline-3-carboxylic acid (**5c**). On the basis of these results and the previous reports[20], we proposed the *Fs*DAAO-catalyzed oxidation and aromatization reaction (Scheme 2): (*R*)-**5a** was oxidized to 1,4-dihydroisoquinoline-3-carboxylic acid (**5b**), followed by aromatization to produce isoquinoline-3-carboxylic acid (**5c**).



Scheme 2. Biocatalytic aromatization of (*R*)-**5a**.

To further improve the conversion, more reducing agent may be needed to ensure the immediate reduction of the intermediate **5b**. Hence, 20 equivalents of $NH_3{\cdot}BH_3$ were used to produce 75% conversion of (*R*)-**5a** to (*S*)-**5a** with excellent enantioselectivity (*ee* > 99%). Unfortunately, only 28% of (*R*)-**6a** was converted to (*S*)-**6a** with relatively low *ee* (88%) using 40 equivalents of $NH_3{\cdot}BH_3$.

Effect of Substrate Concentration on the Deracemization Reaction

The effect of substrate concentration on the deracemization reaction was studied to evaluate the potential of this chemo-enzymatic cascade (Table 4). The ratio of purrified *Fs*DAAO to substrate was kept unchanged. 50 mM of **1a** could be transformed into (*S*)-**1a** with high conversion (> 98%) and excellent enantioselectivity (*ee* > 99%) in 5 h. When the concentration of **1a** was further increased to 60 or 100 mM, the conversion only reached 92% or 60% at 5 h, which was attributed to the slight insolubility of **1a** at high concentration. Prolonging the reaction time could facilitate the improvement of conversion and *ee*

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(data not shown). A similar phenomenon was also observed for the deracemization of **5a**. 40 mM of **5a** could be transformed with 78% conversion. Further increasing the concentration of **5a** to 50 mM resulted in 69% conversion at 24 h.

Table 4. Effect of **1a** and **5a** concentration on the deracemization reaction[a].

Entry	Substrate (mM)	*Fs*DAAO (mg/ml)	Conversion (*C*)(%)[b]	ee_s (%)[c]/ Configuration
1	**1a** (20)	0.2	> 98%	> 99 (*S*)
2	**1a** (40)	0.4	> 98%	> 99 (*S*)
3	**1a** (50)	0.5	> 98%	> 99 (*S*)
4	**1a** (60)	0.6	92	97 (*S*)
5	**1a** (80)	0.8	88	95 (*S*)
6	**1a** (90)	0.9	76	84 (*S*)
7	**1a** (100)	1.0	60	66 (*S*)
8	**5a** (20)	0.2	76	> 99 (*S*)
9	**5a** (40)	0.4	78	> 99 (*S*)
10	**5a** (50)	0.5	69	95 (*S*)

[a] Reaction conditions (1ml): *rac*-**1a** (20-100 mM) or **5a** (20-50 mM), purified *Fs*DAAO (0.2-1.0 mg/mL), FAD (100 μM), catalase (0.75 mg/mL), $NH_3{\cdot}BH_3$ (4 or 20 equivalents) in Na_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 8.0). Reactions were incubated at 30 ℃ with 600 rpm shaking for 5 or 24 h, respectively.

[b] Conversion (*C*) of (*R*)-enantiomer to (*S*)-enantiomer was determined by chiral HPLC analysis.

[c] The ee_s was determined by chiral HPLC analysis.

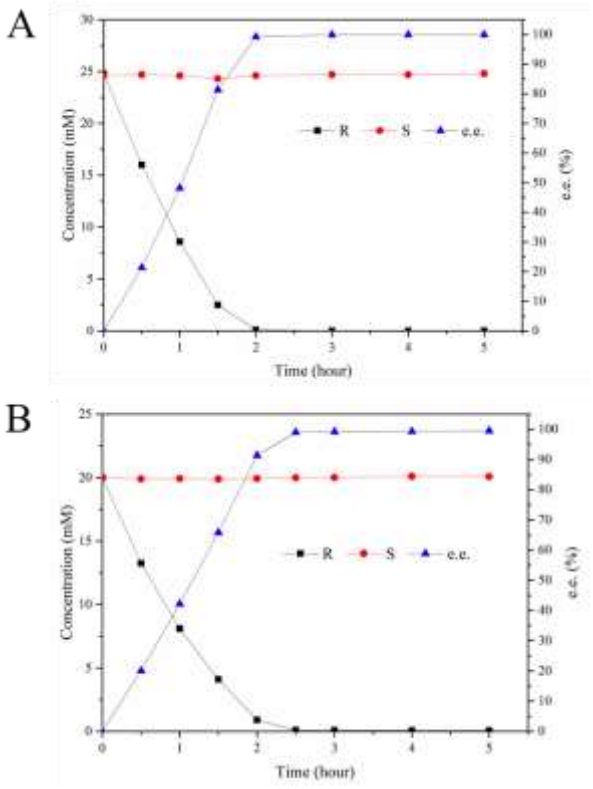

Figure 2. The time-course studies of the kinetic resolution of *rac*-**1a** (A) and *rac*-**5a** (B).

Time-course Studies of Kinetic Resolution and Deracemization Reaction

To understand the process of kinetic resolution and deracemization of carboxyl-substituted THIQs, time-course studies were carried out with 50 mM of *rac*-**1a** or 40 mM of *rac*-**5a** using *Fs*DAAO crude extract as a biocatalyst in an amplified reaction volume (20 or 30 mL). As shown in Figure 2, the chiral HPLC analysis showed that (*R*)-**1a** or **5a** were quickly transformed in the first 2 or 2.5 h, achieving complete conversion in the next 2 h, while the concentration of (*S*)-**1a** or **5a** remained nearly unchanged. Excellent enantiomeric purity of (*S*)-**1a** or **5a** (*ee* > 99%) and high conversion (50%) were obtained in the kinetic resolution process.

In the process of chemo-enzymatic deracemization of *rac*-**1a** (Figure. 3A), after 2.5 h of incubation, 95% of (*R*)-**1a** was converted to (*S*)-**1a** in the presence of 4 equivalents of $NH_3{\cdot}BH_3$ as the reducing agent, and the conversion reached up to > 98% with *ee* > 99%. For deracemization of *rac*-**5a** (Figure. 3B), 20 equivalents of $NH_3{\cdot}BH_3$ produced 75% conversion of (*R*)-**5a** to (*S*)-**5a** within 4 h. After 6 h, 78% conversion was achieved with excellent enantioselectivity (*ee* > 99%).

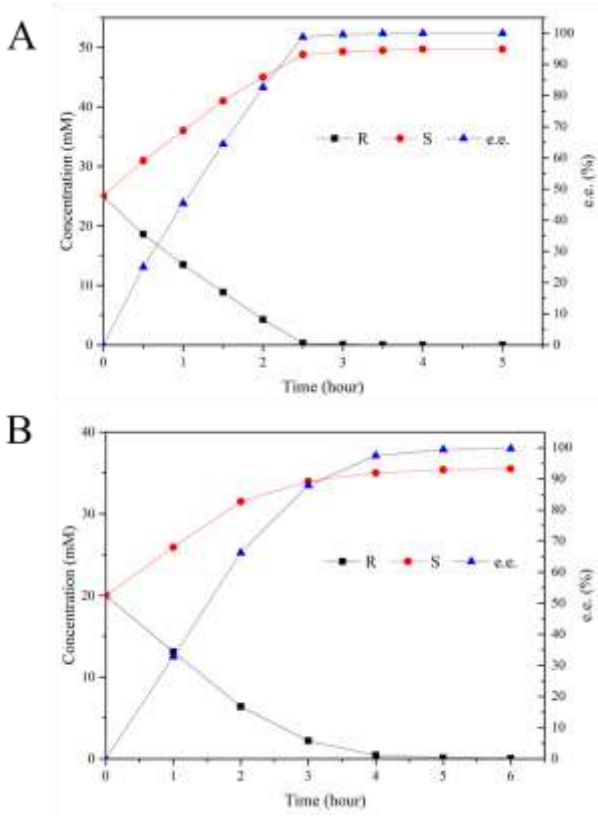

Figure 3. The time-course studies of chemo-enzymatic deracemization of *rac*-**1a** (A) and *rac*-**5a** (B).

Preparative-scale Deracemization of *rac*-1a and *rac*-5a

To demonstrate the synthetic capability of the investigated transformation, preparative-scale chemo-enzymatic deracemization of *rac*-**1a** (500 mg) or *rac*-**5a** (400 mg) was performed in a 50 or 60 ml reaction system to produce enantiopure (*S*)-**1a** or (*S*)-**5a** at pH 8.0 and 30 °C. As expected, chiral HPLC showed that (*R*)-**1a** was almost completely converted into (*S*)-**1a** within 6 and 79% of (*R*)-**5a** was transformed to (*S*)-**5a.** (*S*)-**1a** and (*S*)-**5a** were finally obtained with good isolated yields (82% and 73%, respectively) and excellent enantioselectivity (*ee* > 99%).

Conclusion

In summary, we exploited a D-amino acid oxidase *Fs*DAAO with excellent enantioselectivity toward the 1- and 3-carboxyl substituted THIQs while keeping a broad substrate scope. Using the recombinant *Fs*DAAO as the biocatalyst, a general method for efficient deracemization of 1,2,3,4-tetrahydroisoquinoline carboxylic acids was developed that provides a complementary synthetic method to the preparation of chiral THIQs. Optically pure (*S*)-**1a** and **5a** were obtained in good yields (82% and 71%, respectively) and with excellent enantioselectivity (up to >99% *ee*). The *Fs*DAAO-catalyzed deracemization is expected to provide access to the chiral carboxyl-substituted 1,2,3,4-tetrahydroquinolines and 1,2,3,4-tetrahydro-ß-carbolines. Further protein engineering of the *Fs*DAAO to modulate the enantioselectivity and expand the substrate scope is ongoing in our laboratory.

Experimental Section

Chemicals

Cofactor FAD, formaic acid (FA), $NaBH_4$ and $NaCNBH_3$ were purchased from Aladdin (Shanghai, China). Cofactor nicotinamide adenine dinucleotide phosphate ($NADP^+$), NADPH, nicotinamide adenine dinucleotide (NAD^+) and NADH were purchased from Bontac Bio-engineering Co., Ltd. (Shenzhen, China). Tryptone and yeast extract were obtained from Oxoid (Basingstoke, England). Substrates racemic 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid hydrochloride (*rac*-**1a)** and (*R*)-**1a** were provided by Tongli Biomedical Co., Ltd. (Suzhou, China). Substrates racemic 6-chloro-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid hydrochloride (*rac*-**2a)** and racemic 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid hydrochloride (*rac*-**4a**) were purchased from BOC sciences(Shirley, NY). Substrates racemic 6-hydroxy-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (*rac*-**3a**), racemic 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*rac*-**5a**), (*R*)-**5a**, racemic 6-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid hydrate (*rac*-**6a)**, (*S*)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid hydrate (**7**), racemic 1,2,3,4-tetrahydroquinoline-2-carboxylic acid hydrochloride (*rac*-**23**) and racemic 1,2,3,4-tetrahydro-ß-carboline-1-carboxylic acid (*rac*-**24**) were purchased from J&K scientific Ltd (Beijing, China). Substrates 1,2,3,4-tetrahydroisoquinoline (**8**), racemic 1-methyl-1,2,3,4-tetrahydroisoquinoline (**9**), D-phenylglycine (**10**), D-4-hydroxy-phenylglycine (**11**), D or L-phenylalanine (**12**), D-tyrosine (**13**), D or L-methionine (**14**), D or L-alanine (**15**), D-norvaline (**16**), D-leucine (**17**), D-glutamic acid (**18**), phosphinothricin (**19**), D-proline (**20**), racemic indoline-2-carboxylic acid (*rac*-**21**), racemic pipecolinic acid (*rac*-**22**), methanol (MeOH) and methyl cyanide (MeCN), diethyl amine(DEA) for HPLC, *o*-dianisidine dihydrochloride, horseradish peroxidase(HRP), catalase from bovine liver, $NH_3{\cdot}BH_3$, isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin and ampicillin were purchased from Sigma-Aldrich (Munich, Germany). All other reagents compounds used were of analytical grade and commercially available.

Cloning, Expression, Purification and Characterization of Target Enzymes

Candidate DAAO, LAAO, LAAD and AADH genes were cloned and overexpressed in *E. coli* BL21 (DE3). Their activity toward natural substrates and model substrate *rac*-**1a** were detected using chromogenic assay, HPLC method or monitoring the changes in the absorbance of NADPH or NADH at 340 nm. The DAAOs were purified by His-tag affinity chromatography. The enzymatic properties of *Fs*DAAO was characterized by using HPLC method or a coupled *o*-dianisidine/peroxidase method. All these methods were described in the Supporting Information in detail.

Substrate Specificity

The standard substrate concentration was 10 mM, except for 2 mM of (*R*)-**13** and *rac*-**24**. The specific activities were measured in triplicate using the coupled *o*-dianisidine/peroxidase method.

Analytical-scale Kinetic Resolution and Deracemization of *rac*-1a-6a

The kinetic resolution of racemic substrates were performed at pH 8.0 and 30 ℃. Unless stated otherwise, a typical 1 mL reaction mixture contained purified *Fs*DAAO (0.1-0.2 mg/mL), FAD (100 μM), catalase (0.75 mg/mL), and substrate *rac*-**1a**-**6a** (10 mM), except for **4a** (5 mM) or **6a** (5 mM).

For deracemization of racemic substrates **1a**-**6a,** 5-10 equivalents of $NaBH_4$, 5-10 equivalents of $NaCNBH_3$, or 4-40 equivalents of $NH_3{\cdot}BH_3$ were used and the other components in the reaction were the same with the kinetic resolution system. Reactions were shaken at 30℃, 600 rpm.

Effect of Substrate Concentration on Deracemization Reaction

The Effect of substrate concentration on deracemization reaction were determined with different concentrations of *rac*-**1a** (20, 40, 50, 60, 80, 90, 100 mM) or **5a** (20, 40, 50 mM). The ratio of purified *Fs*DAAO to substrate was kept unchanged. 4 or 20 equivalents of $NH_3{\cdot}BH_3$ were used, respectively. The reaction were incubated at 30℃ with 600 rpm shaking for 5 or 24 h.

Time-course Experiments

The kinetic resolution reaction was carried out in a 50 mL three-necked flask equipped with a magnetic stirrer. The *rac*-**1a** hydrochloride salt (214 mg, 1 mmol) or *rac*-**5a** (213 mg, 1.2 mmol) were dissolved in 10 or 24 mL Na_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 8.0). The pH was carefully adjusted to a value of pH 8.0 with ammonium hydroxide. The lyophilized *E.coli* cells containing *Fs*DAAO from 200 mL ferments was resuspended in 25 or 10 mL Na_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 8.0), and the crude lysate was prepared by sonication and centrifugation. 10 or 6 ml

crude lysate and 50 mg catalase were added to the substrate solution. The pH was kept at 8.0. The reactor was kept at 30℃ through a thermostatic water bath. The reaction was monitored by chiral HPLC.

For the deracemization reaction, 124 mg (4 mmol) or 741 mg (24 mmol) $NH_3{\cdot}BH_3$ was used. The other components and reaction conditions were the same with the kinetic resolution reaction.

Preparative-scale Deracemization of *rac*-1a and 5a

The preparative-scale deracemization reaction was carried out in a 100 mL three-necked flask equipped with a magnetic stirrer. The *rac*-**1a** hydrochloride salt (500 mg, 2.34 mmol) or *rac*-**5a** (400 mg, 2.26 mmol) were dissolved in 25 or 48 ml water. The pH was carefully adjusted to a value of pH 8.0 with ammonium hydroxide. 289 mg (9.36 mmol) or 1395 mg (45.2 mmol) $NH_3{\cdot}BH_3$ was added, respectively. The lyophilized *E.coli* cells containing *Fs*DAAO from 200 or 400 mL ferments was resuspended in 25 or 20 mL water, and the crude lysate was prepared by sonication and centrifugation. 25 or 12 mL crude lysate and 100 mg catalase were added to the substrate solution. The reactor was kept at 30℃ through a thermostatic water bath. The pH was kept at 8.0. The reaction was monitored by chiral HPLC. Upon the completion of reaction, the proteins were removed through ultrafiltration. The solution was freeze dried, taken up in hot ethanol and hot filtered to remove unreacted $NH_3{\cdot}BH_3$. The major parts of ethanol were evaporated off under vacuum, and the residue was washed with warm water. The mixture was concentrated so that compound (*S*)-**1a** or (*S*)-**5a** was recrystallized as white solid.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 21676240).

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FULL PAPER

Chemoenzymatic Approach to (*S*)-1,2,3,4-Tetrahydroisoquinoline Carboxylic Acids Employing D-Amino Acid Oxidase

Adv. Synth. Catal. **Year**, *Volume*, Page – Page

Shuyun Ju, Mingxin Qian, Gang Xu, Lirong Yang, and Jianping Wu*