Advanced Synthesis & Catalysis

DOI: 10.1002/adsc.201400185

Deracemization of Amino Acids by Coupling Transaminases of Opposite Stereoselectivity

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Received: February 19, 2014; Revised: April 4, 2014; Published online: June 20, 2014

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201400185.

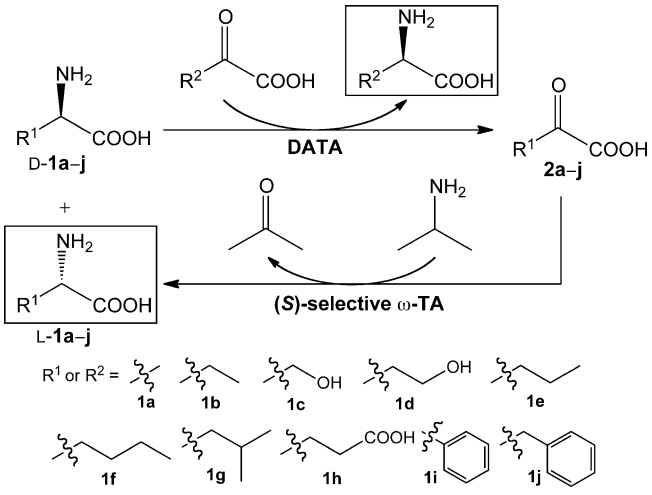
Abstract: Biocatalytic deracemization of amino acids without relying on oxidase-based deamination of an unwanted enantiomer was demonstrated by coupling α- and ω-transaminases displaying opposite stereoselectivity. This strategy employs isopropylamine and a keto acid as cosubstrates and is free of generation of hydrogen peroxide which is troublesome in the conventional oxidase-based methods.

Keywords: amino acids; biocatalysis; coupled enzyme reaction; deracemization; transaminases

There has been an increasing demand for optically pure amino acids as essential chiral building blocks of diverse pharmaceutical drugs, agrochemicals and chiral ligands.[1] Despite remarkable technical advances in chemocatalytic asymmetric synthesis, it remains challenging to achieve the one-step synthesis of amino acids that are enantiopure enough to fulfill pharmaceutical purposes (i.e., *ee* >99.5%).[2] Therefore, aside from natural L-amino acids producible by microbial fermentation,[3] most industrial processes to produce enantiopure amino acids rely on biocatalytic strategies such as asymmetric reductive amination of keto acids,[4] kinetic resolution by exploiting different reaction rates of the two amino acid enantiomers[5] and deracemization by carrying out chiral inversion of an unwanted enantiomer.[6] Because of the high production costs of keto acids, the asymmetric amination approach is usually disfavored over the other two methods using racemic amino acid substrates that are cheaply prepared by a Strecker synthesis. Contrary to the kinetic resolution approach, deracemization does not mandate chemical modification of the racemic substrate as also demonstrated for the production of chiral amines.[7] Moreover, it affords 100% theoretical yield without addition of racemase that is essential in the kinetic resolution to improve reaction yield over 50%.[6a,c]

Deracemization of amino acids consists of two reactions: (i) stereoselective deamination of an unwanted enantiomer by amino acid oxidase to generate a prochiral keto acid intermediate[8] and (ii) subsequent amination of the keto acid back to an amino acid of desired chirality by asymmetric biocatalysis or to racemic mixture by non-stereoselective chemical reduction.[6a–c] A crucial problem in the current deracemization methods is rapid enzyme inactivation engendered by a reactive oxygen species resulting from the oxidase reaction (i.e., H_2O_2), which necessitates addition of a high dosage of catalase.[9]

To cope with this drawback, we envisaged that a deracemization process free of H_2O_2 generation could be implemented using two transaminases (TAs) of opposite stereoselectivity (Scheme 1). As a proof-of-con-



Scheme 1. Deracemization of racemic amino acids into L-enantiomers using D-amino acid transaminase (DATA) and (*S*)-selective ω-transaminase (ω-TA). This strategy affords one-pot production of two enantiopure amino acids of opposite chirality, enclosed by boxes.

cept, we set out to design a coupled enzyme reaction enabling chiral inversion of a D-amino acid substrate into an L-enantiomer accompanied by asymmetric conversion of a keto acid cosubstrate into another D-amino acid, resulting in simultaneous production of two enantiopure amino acids of opposite chirality. To this end, we chose D-amino acid transaminase (DATA), cloned from *Bacillus sphaericus*,[10] for the deamination of the unwanted D-enantiomer. DATA is known to exhibit broad substrate specificity toward various D-amino acids and keto acids but not to amine compounds.[11] The keto acid intermediate, resulting from the DATA reaction, is asymmetrically aminated to an L-amino acid by an (*S*)-selective ω-transaminase (ω-TA) using a primary amine as an amino donor.

Although the DATA reaction is not thermodynamically favorable [i.e., equilibrium constant (K_{eq}) close to unity], the whole reaction can be driven to completion by the highly exergonic ω-TA reaction between a keto acid and a primary amine.[10] For example, K_{eq} for the amination of pyruvic acid (**2a**) by α-methylbenzylamine (α-MBA) or isopropylamine was reported to be 1130 or 67, respectively.[12] Isopropylamine was chosen as an amino donor for the reductive amination of the keto acid intermediate by ω-TA, because the resulting ketone (i.e., acetone) is easily removable owing to its high volatility as previously demonstrated.[13] Accordingly, we used ω-TA cloned from *Ochrobactrum anthropi* (OATA) because of its high activity toward isopropylamine [i.e., 43% reactivity relative to (*S*)-α-MBA].[4d]

The deracemization procedure requires three substrate components, that is, racemic amino acid and at least 0.5 molar equiv. of keto acid and isopropylamine. To ensure production of enantiopure D-amino acid from the keto acid cosubstrate, it is essential to use a keto acid that completely lacks reactivity toward OATA. It is known that α-ketoglutaric acid (**2h**), a universal amino acceptor for α-transaminases (α-TAs) including DATA and branched-chain transaminase (BCTA), is inert to all native ω-TAs known to date.[14] Moreover, **2h** is cheap and its amination product by DATA [i.e., D-glutamate (D-**1h**)] is an important pharmaceutical intermediate of spiroglumide for treatment of bowel disease.[15]

To examine whether the deracemization strategy was technically feasible, we monitored the reaction progress of the deracemization of *rac*-homoalanine (*rac*-**1b**; 100 mM) using **2h** (70 mM) and isopropylamine (70 mM) as cosubstrates (Figure 1). L-**1b** is a key intermediate for the production of levetiracetam and brivaracetam (antiepileptic drugs),[16] and ethambutol (an antituberculosis drug).[17] Concentration and enantiopurity of L-**1b** reached 98 mM and >99% *ee*, respectively, within 3 h. Stoichiometric conversion of D-**1b** into **2b** and then L-**1b** was observed

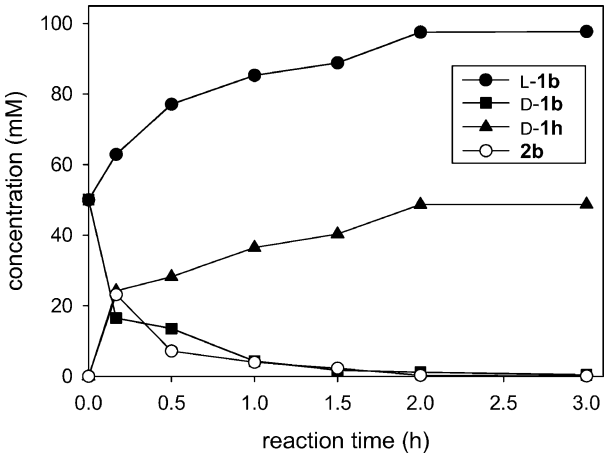


Figure 1. Time-course monitoring of deracemization of *rac*-**1b** into L-**1b** using **2h** and isopropylamine as cosubstrates. The reaction was catalyzed by D-amino acid transaminase (DATA) (4 U/mL) and ω-transaminase cloned from *Ochrobactrum anthropi* (OATA) (20 U/mL).

because the concentration sum of L-**1b**, D-**1b** and **2b** was kept constant around 100 mM throughout the reaction. Initial build-up of **2b** indicates that the DATA reaction proceeded faster than the OATA reaction at the given enzyme concentrations. This was in agreement with faster consumption of **2h** than that of isopropylamine (Supporting Information, Figure S1). However, **2b** disappeared at the end of the reaction, indicating that the DATA reaction could be completed by the exergonic ω-TA reaction. In addition to the deracemization product (i.e., L-**1b**), the coupled enzyme reaction afforded the simultaneous production of D-**1h** (>99% *ee*) to the level (49 mM) close to a theoretical limit (i.e., 0.5 molar equiv. to the deracemization product). Note that D-**1h** is easily separable against L-**1b** by using anion-exchange chromatography.

As the deracemization concept worked, we applied the strategy to various racemic amino acids using the same cosubstrates (entries 1–7 in Table 1). In addition to OATA, we also used (*S*)-selective ω-TA cloned from *Paracoccus denitrificans* (PDTA) because of much higher activities toward keto acids carrying bulky side chains (compare entries 5 and 6).[18] All the coupled enzyme reactions led to complete deracemization of natural (entries 1, 2 and 7) as well as unnatural amino acids (entries 3–6) into L-enantiomers (*ee* > 99%) with concomitant production of enantiopure D-**1h**. We also examined the feasibility of using a keto acid cosubstrate other than **2h**, so the deracemization reaction led to coproduction of other valuable D-amino acids (entries 8 and 9). To this end, we tested phenylglyoxylic acid (**2i**) and phenylpyruvic acid (**2j**) that are inert to the ω-TAs. Indeed, our strategy afforded the one-pot production of unnatural L-amino acids as well as D-phenylglycine (D-**1i**; entry 8) and D-

Table 1. Deracemization of amino acids into L-enantiomers accompanied by asymmetric synthesis of chemically different D-amino acid.[a]

Entry	Substrates	ω-TA	Reaction time [h]	Products [mM, % *ee*][d]
1	*rac*-**1a**+**2h**	OATA	10	L-**1a** (99, >99)+D-**1h** (49, >99)
2	*rac*-**1c**+**2h**	OATA	25	L-**1c** (100, >99)+D-**1h** (48, >99)
3	*rac*-**1d**+**2h**	OATA	25	L-**1d** (95, >99)+D-**1h** (49, >99)
4	*rac*-**1e**+**2h**	OATA	25	L-**1e** (98, >99)+D-**1h** (50, >99)
5[b]	*rac*-**1f**+**2h**	OATA	20	L-**1f** (49, >99)+D-**1h** (25, >99)
6[b]	*rac*-**1f**+**2h**	PDTA	12	L-**1f** (50, >99)+D-**1h** (25, >99)
7[b]	*rac*-**1g**+**2h**	PDTA	10	L-**1g** (50, >99)+D-**1h** (24, >99)
8[c]	*rac*-**1b**+**2i**	OATA	25	L-**1b** (99, >99)+D-**1i** (24, 99)[e]
9[c]	*rac*-**1e**+**2j**	PDTA	18	L-**1e** (100, >99)+D-**1j** (48, >99)

[a] *Reaction conditions*: racemic amino acids (100 mM), keto acid (70 mM), isopropylamine (70 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7), 4 U/mL DATA and 20 U/mL (*S*)-selective ω-TA at 37 °C.
[b] Concentrations of the three substrate components were halved because of the solubility of **1f** and **1g** lower than 100 mM.
[c] Concentration of DATA was 40 U/mL.
[d] Quantitative analyses of amino acid enantiomers were carried out by HPLC after chiral derivatization.
[e] Due to the solubility of D-**1i** lower than 25 mM, a white precipitate of D-**1i** was observed during the reaction.

phenylalanine (D-**1j**; entry 9) which are valuable building blocks of antibiotics[19] and pharmaceutical drugs,[20] respectively.

We moved forward to investigate whether our approach could afford deracemization of racemic amino acid into a D-enantiomer by inversing stereoselectivity of both TAs used in the coupled enzyme reactions. To this end, we used an L-stereoselective BCTA cloned from *Escherichia coli*[10] and an engineered variant of (*R*)-selective ω-TA from *Arthrobacter* sp. ($AR_{mut}TA$).[13a] We chose **2h** again as a keto acid cosubstrate, because **2h** is one of the most reactive amino acceptors toward BCTA. Owing to the substantial activity of $AR_{mut}TA$ toward isopropylamine [i.e., 8% reactivity relative to (*R*)-α-MBA],[4d] we kept using isopropylamine as an amine cosubstrate. Note that BCTA cannot utilize isopropylamine as an amino donor, because BCTA belongs to the α-TA family that can transfer an amino group only from α-amino acids.[14] The BCTA/$AR_{mut}TA$ coupled reactions led to deracemization of various amino acids into D-enantiomers in over 99% *ee* within 20 h, except for **1d** (Table 2). Contrary to our expectation, enantiopurities of the resulting L-**1h** were not high (i.e., between 68 and 88% *ee*). We suspected that D-**1h** was generated from an unwanted side reaction between **2h** and isopropylamine by $AR_{mut}TA$. Indeed, $AR_{mut}TA$ showed a non-negligible activity to **2h** (i.e., 0.6% reactivity relative to **2a**)[21] although its parental enzyme is completely inert to **2h**.[22]

To demonstrate the scalability of the deracemization strategy, we carried out a preparative-scale reaction to convert *rac*-**1b** into L-**1b** with concurrent asymmetric synthesis of D-**1h** from **2h** in 50 mL reaction mixture charged with *rac*-**1b** (1.55 g, 15 mmol), **2h** (1.24 g, 8.5 mmol), isopropylamine (1.29 mL, 15 mmol), DATA (500 U, 91 mg) and OATA (1000 U, 121 mg). Enantiopurity of L-**1b** reached over 99% *ee* at 9 h. Product isolation was performed by ion-exchange column chromatography.

Structural characterizations of the resulting amino acids were performed (see the Supplementary Infor-

Table 2. Deracemization of amino acids into D-enantiomers using branched-chain transaminase (BCTA)/ω-transaminase from *Arthrobacter* sp. ($AR_{mut}TA$) coupled reactions.[a]

Entry	Substrate	Reaction time [h]	Products (mM, % *ee*)[b]
1	*rac*-**1a** +**2h**	20	D-**1a** (100, >99)+L-**1h** (54,[c] 83)
2	*rac*-**1b** + **2h**	15	D-**1b** (99, >99)+L-**1h** (53,[c] 85)
3	*rac*-**1c**+**2h**	15	D-**1c** (99, >99)+L-**1h** (52,[c] 88)
4	*rac*-**1d**+**2h**	25	D-**1d** (99, 96)+L-**1h** (58,[c] 68)
5	*rac*-**1e**+**2h**	20	D-**1e** (98, >99)+L-**1h** (54,[c] 87)
6[d]	*rac*-**1f**+**2h**	20	D-**1f** (49, >99)+L-**1h** (28,[c] 85)

[a] *Reaction conditions*: racemic amino acids (100 mM), **2h** (70 mM), isopropylamine (70 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7), 4 U/mL BCTA and 20 U/mL $AR_{mut}TA$ at 37 °C.
[b] Quantitative analyses of amino acid enantiomers were carried out by HPLC after chiral derivatization.
[c] Concentrations represent sum of both enantiomers of **1h**.
[d] Concentrations of the three substrate components were halved because of the low solubility of **1f**.

mation), leading to recovery of L-**1b** (1.28 g, 79.5% isolation yield, >99% *ee*) and D-**1h** (0.89 g, 82.6% isolation yield, > 99% *ee*).

In summary, we report the first example of an H_2O_2-free deracemization of amino acids using α-TA/ω-TA coupled reactions. Despite low K_{eq} values of the α-TA reactions close to unity, complete deracemization could be achieved due to the equilibrium shift driven by a highly exergonic ω-TA reaction as we had proved in the previous study.[10] We demonstrated that this strategy was applicable to the deracemization of amino acids into L- as well as D-enantiomers, depending on the stereoselectivity of the TAs. Conventional oxidase-based deracemization methods require multi-enzyme systems (e.g., oxidase coupled with two dehydrogenases) and are susceptible to severe enzyme inactivation by H_2O_2 in the absence of a high catalase activity.[6a,c] In contrast, our approach employs only two enzymes and is free of the H_2O_2-induced enzyme activity loss, which is expected to benefit enzyme cost reduction for industrial process development. Moreover, generation of another enantiopure amino acid as a valuable side product (e.g., D-**1h**, D-**1i** and D-**1j** from the DATA reactions) might render the TA-based deracemization process more cost-effective.

Experimental Section

Chemicals

Pyruvic acid was obtained from Kanto Chemical Co. (Tokyo, Japan). Isopropylamine was purchased from Junsei Chemical Co. (Tokyo, Japan). Materials used for preparation of culture media including yeast extract, tryptone and agar were purchased from Difco. (Spark, MD, USA). All other chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

Overexpression and Purification of Transaminases

Overexpression and purification of His_6-tagged transaminases (DATA, OATA, BCTA and $AR_{mut}TA$) were carried out as described elsewhere with minor modifications.[10] Detailed procedures are described in the Supporting Information.

Enzyme Assay

Unless otherwise specified, enzyme assays were carried out at 37 °C and pH 7 (50 mM phosphate buffer). One unit of (*S*)-selective ω-TA activity is defined as the enzyme amount that catalyzes the formation of 1 μmole of acetophenone in 1 min at 20 mM **2a** and 20 mM (*S*)-α-MBA. In the case of $AR_{mut}TA$, 20 mM (*R*)-α-MBA was used as an amino donor. One unit of BCTA activity is defined as the enzyme amount catalyzing the formation of 1 μmole of **2b** in 1 min at 20 mM trimethylpyruvate and 20 mM L-**1b**. One unit of DATA activity is defined as the enzyme amount that catalyzes the formation of 1 μmole of **2b** in 1 min at 20 mM **2a** and 20 mM D-**1b**. Typical reaction volume was 100 μL and the enzyme reaction was stopped after 10 min by adding 600 μL acetonitrile. For initial rate measurements, acetophenone and **2b** for ω-TA and α-TA activity assays, respectively, were analyzed by HPLC.

Small-Scale Coupled Enzyme Reactions

Unless otherwise specified, the DATA/(*S*)-selective ω-TA coupled reactions (reaction volume = 1 mL) were carried out at 100 mM *rac*-amino acid, 70 mM **2h**, 70 mM isopropylamine, 0.1 mM PLP, 4 U/mL DATA and 20 U/mL (*S*)-selective ω-TA in 50 mM phosphate buffer (pH 7). The reaction mixture was incubated at 37 °C. In the case of **1f** and **1g**, substrate concentrations were 50 mM *rac*-amino acid, 35 mM **2h** and 35 mM isopropylamine. When a keto acid co-substrate other than **2h** was used, the DATA concentration was increased to 40 U/mL.

The BCTA/$AR_{mut}TA$ coupled reactions were carried out at 100 mM *rac*-**1a–e**, 70 mM **2h**, 70 mM isopropylamine, 0.1 mM PLP, 4 U/mL BCTA and 20 U/mL $AR_{mut}TA$ in 50 mM phosphate buffer (pH 7). In the case of **1f**, the deracemization reaction was carried out at 50 mM *rac*-**1f**, 35 mM **2h** and 35 mM isopropylamine. The reaction volume was 1 mL and the reaction mixture was incubated at 37 °C.

Aliquots of reaction mixture (30 μL) were taken at predetermined reaction times and mixed with 180 μL acetonitrile to stop the reaction. The reaction mixtures were subjected to HPLC analysis for measurement of conversion and enantiomeric excess.

Preparative-Scale Deracemization and Product Isolation

Preparative-scale deracemization of *rac*-**1b** was carried out at 37 °C under magnetic stirring in oven-dried beakers charged with 50 mL reaction mixture containing *rac*-**1b** (1.55 g, 300 mM), **2h** (1.24 g, 170 mM), isopropylamine (1.29 mL, 300 mM), PLP (0.1 mM), DATA (10 U/mL), OATA (20 U/mL) and potassium phosphate (50 mM, pH 7.0). When the enantiomeric excess of L-**1b** exceeded 99%, the reaction mixture was subjected to product isolation.

The pH of the reaction mixture was adjusted to 13.0 by adding 30% ammonia solution for protein precipitation. The reaction mixture was filtered through a glass-fritted filter funnel to remove the protein precipitate. The pH of the filtrate solution was adjusted to 7.0 by adding 5 N HCl. The resulting solution was loaded on a glass column packed with Dowex 1X8 anion-exchange resin (40 g), followed by washing with water (100 mL) and then eluted with 150 mL of 0.1 N HCl. To recover D-glutamate (D-**1h**), the elution fraction was loaded on a glass column packed with Dowex 50WX8 cation-exchange resin (40 g), followed by washing with 0.1 N HCl (100 mL) and water (100 mL), and then elution was done with 150 mL of 10% ammonia solution. The elution fractions were pooled and evaporated at 30 °C and 0.1 bar. The resulting solids were washed with EtOH (30 mL) and then oven-dried overnight.

For the isolation of L-**1b**, the pass-through solutions from the anion-exchange column prior to elution by 0.1 N HCl were collected and the pH was adjusted to 1.0 by adding 5 N HCl. The resulting solution was loaded on the Dowex 50WX8 cation-exchange column, followed by washing with

0.1 N HCl (100 mL) and water (100 mL), and then was eluted with 10% ammonia solution (150 mL). The elution fractions were pooled and evaporated at 30 °C and 0.1 bar. The resulting solids were washed with EtOH (30 mL) and then oven-dried overnight.

Analytical Methods

All the HPLC analyses were performed on a Waters HPLC system (Milford, MA, USA). Keto acids were analyzed using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) with isocratic elution of 5 mM H_2SO_4 solution at 0.5 mL min^{-1}. Column oven temperature was set to 40 °C and UV detection was done at 210 nm. Retention times of keto acids were 11.3 (**2a**), 12.0 (**2b**), 9.8 (**2c**), 14.2 (**2e**), 17.7 (**2f**), 15.3 (**2g**), 10.0 (**2h**), 14.7 (**2i**) and 21.0 min (**2j**).

Analysis of acetophenone was performed using a Symmetry column C18 (Waters Co.) with isocratic elution of 60% methanol/40% water/0.1% trifluoroacetic acid at 1 mL min^{-1}. UV detection was done at 254 nm. Retention time of acetophenone was 3.8 min.

For quantitative chiral analysis of amino acids, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) was used for amino acid derivatization.[23] Details of the chiral HPLC analysis are provided in the Supporting Information.

Acknowledgements

This work was supported by the Advanced Biomass R&D Center (ABC-2011-0031358) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

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