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One-pot one-step deracemization of amines using ω-transaminases†

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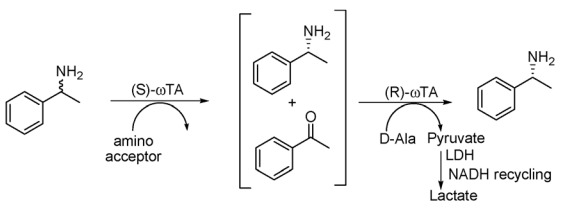
In this study, we developed a one-pot one-step deracemization method for the production of various enantiomerically pure amines using two opposite enantioselective ω-TAs. Using this method, various aromatic amines were successfully converted to their (*R*)-forms (>99%) with good conversion.

Enantiomerically pure amines are important building blocks for preparing bioactive compounds for use in pharmaceutical and chemical industries.[1] Several chemical methods have been developed for the production of chiral amines, such as hydrogenation of imines and enamines, alkylation of imines, aminohydroxylation, and reductive amination.[2,3a] Recently, deracemization processes have gained considerable attention due to their ability to produce enantiomerically pure amines from racemates with a maximum theoretical yield of 100%. Deracemization of amines *via* dynamic kinetic resolution using lipase and racemization using transition metal complexes such as Pd and Ru catalysts have been well reported.[3] Also deracemization of amines *via* enantioselective oxidation using evolved amine oxidase and non-selective reduction using an ammonia–borane complex have been employed to produce optically pure amines.[4,5f]

Among different biocatalysts, ω-transaminases (ω-TAs) have recently received a great deal of attention as promising catalysts due to their ability to produce a wide range of optically pure amines and unnatural amino acids.[5] Chiral amines are generally produced by ω-TAs through asymmetric synthesis from ketones and kinetic resolution from *rac*-amines. However, deracemization of amines using ω-TAs has, until recently, been a less-explored area. Deracemization using an ω-TA was first reported to produce an enantiomerically enriched amine using spontaneous racemization of a ketone substrate (4-oxo-3-phenylbutyric acid ethyl ester).[6] In addition, deracemization of a homoalanine using D-amino acid oxidase and an ω-TA, and racemization of amine using two opposite enantioselective ω-TAs have been reported.[7] As a more general method, deracemization using a one-pot two-step reaction was developed, in which the kinetic resolution of the racemic amine was performed using an ω-TA, followed by asymmetric synthesis of the corresponding ketone using an opposite enantioselective ω-TA.[8] One drawback of this method is the need to inactivate the ω-TA used in the kinetic resolution before starting asymmetric synthesis. Subsequently an opposite enantioselective ω-TA and its amino donor were added to the reaction mixture. To improve this process, immobilized ω-TA was employed in the first step and the immobilized ω-TA was separated easily by filtration or centrifugation after the first step.[9] Here, we report a general method, a one-pot one-step deracemization method, for production of various enantiomerically pure (*R*)-amines using two opposite enantioselective ω-TAs.

To deracemize *rac*-α-methylbenzylamine (MBA, model compound) to (*R*)-α-MBA, (*S*)-α-MBA is converted to the corresponding ketone by (*S*)-ω-TA using an amino acceptor, after which the corresponding ketone is converted back to (*R*)-α-MBA by (*R*)-ω-TA with an amino donor (Scheme 1). In the second step of the reaction ((*R*)-ω-TA reaction), (*R*)-ω-TA has negligible activity toward the amino acceptor, which is used in the (*S*)-ω-TA reaction. If not, the asymmetric reaction of (*R*)-ω-TA will not proceed efficiently because (*R*)-α-MBA will be converted to ketone by (*R*)-ω-TA using the amino acceptor.

We selected a pair of ω-TAs to carry out this reaction. Specifically, the (*R*)-selective ω-TAs from *Mycobacterium vanbaalenii* ((*R*)-ω-TAMV)[1a] and *Neosartorya fischeri* ((*R*)-ω-TANF),[1a] and



Scheme 1 Deracemization of *rac*-amine (*e.g. rac*-α-MBA) into (*R*)-amine by (*S*)- and (*R*)-selective ω-TAs.

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(*S*)-selective ω-TAs from *Polaromonas* sp. JS666 ((*S*)-ω-TAPO)[10] and *Vibrio fluvialis* JS17[7a] ((*S*)-ω-TAVF) were cloned into the pET24ma vector with an N-terminal His6-tag and effectively expressed in *Escherichia coli* BL21 (DE3).[7a] The enzymes were then purified on a Ni-NTA affinity column (Fig. S1 ESI†), after which the purified enzymes were subjected to an ω-TA activity assay performed with 10 mM α-MBA and 10 mM pyruvate in 100 mM phosphate buffer (pH 7.0) at 37 °C. Depending on the stereo-selectivity of the enzyme, either 10 mM of (*R*)- or (*S*)-α-MBA was used. One unit of activity was defined as the amount of enzyme that produced 1 μmol acetophenone per minute. The specific activities of (*R*)-ω-TAMV, (*R*)-ω-TANF, (*S*)-ω-TAPO and (*S*)-ω-TAVF were 64.7, 39.0, 2.32 and 27.3 U mg^{-1}, respectively (ESI†).

To identify a good amino acceptor of (*S*)-ω-TA, which is not a substrate for (*R*)-ω-TA, acceptor specificities of the enzymes were investigated using nine carbonyl compounds (seven α-keto acids, two esters) in the presence of 10 mM (*S*)-α-MBA (or (*R*)-α-MBA), and the produced acetophenone was analyzed to measure the initial reaction rate (Fig. 1). Interestingly, (*R*)-ω-TAs showed a narrow substrate specificity when compared to (*S*)-ω-TAs. (*R*)-ω-TAs had no activity toward **a6**, **a7**, **a8** and **a9**. Recently, Shin *et al.* examined the amino acceptor specificity of two (*R*)-ω-TAs from *Aspergillus terreus*.[11] These enzymes did not show any activity towards **a6**, **a7**, **a8** or **a9**. Since (*S*)-ω-TAs showed considerable reactivity towards **a6**, **a7** and **a8**, the active sites of (*R*)-ω-TAs might have a narrower binding pocket to accommodate the bulky side chains of keto acids than those of (*S*)-ω-TAs. The relative activities of (*S*)-ω-TAVF for **a7** and **a8** were 6.4% and 14.1% of that for **a1** (typical amino acceptor), respectively. In the case of (*S*)-ω-TAPO, **a9** was a good amino acceptor, which showed 70% reactivity of **a1**. Actually, it is the unique characteristic of (*S*)-ω-TAPO that enables it to use **a9** as an amino acceptor because TA generally shows a clear one sided preference to either **a1** or **a9**–oxaloacetate.[10] Even though (*S*)-ω-TAPO showed considerable reactivity for **a7** and **a8**, its reactivity was less than 10% of that for **a1**. Based on the reactivity (good substrate for (*S*)-ω-TA, poor substrate for (*R*)-ω-TA), **a7**, **a8**, and **a9** can be used as amino acceptors for (*S*)-ω-TA. Among them, **a9** was selected for deracemization because it is inexpensive and has high reactivity. Additionally, glutamate (aminated **a9**) can be easily removed by another coupling system (when needed). Therefore, one-step one-pot deracemization was carried out with (*S*)-ω-TAPO and two (*R*)-ω-TAs with **a9**.

Before performing the deracemization reaction, we carried out the kinetic resolution of *rac*-α-MBA (first step reaction) with (*S*)-ω-TAPO. 10 mM *rac*-α-MBA were completely resolved with 20 mM **a9** into (*R*)-α-MBA for 12 h with high enantioselectivity (E > 100) (data not shown). The asymmetric synthesis of (*R*)-ω-MBA from acetophenone (second step reaction) with (*R*)-ω-TAs was performed using D-Ala as an amino donor, in which the co-product pyruvate was removed by lactate dehydrogenase (LDH). The cofactor NADH required by LDH was recycled by employing glucose and glucose dehydrogenase (GDH).[12] When asymmetric synthesis was carried out in 1 mL of 100 mM Tris–HCl buffer (pH 8.5) containing 10 mM acetophenone, 50 mM D-Ala, 50 mM glucose, 1 mM NADH, LDH (5 U), GDH (5 U) and 0.3 mg of (*R*)-ω-TA at 37 °C, after 24 h, both (*R*)-ω-TAs quantitatively converted acetophenone to (*R*)-α-MBA (ee > 99). Since the kinetic resolution with (*S*)-ω-TAPO and the asymmetric synthesis with (*R*)-ω-TAMV (or (*R*)-ω-TANF) were carried out successfully, we carried out deracemization of *rac*-α-MBA by combining both reactions simultaneously in one pot (Fig 2). Since PLP helps to stabilize the enzyme, 0.1 mM PLP was added to the reaction mixture; and for the efficient removal of **a1**, excess of LDH (112 U) was used for the deracemization of 50 mM *rac*-α-MBA. After two days of reaction, (*S*)-ω-TAPO–(*R*)-ω-TAMV and (*S*)-ω-TAPO–(*R*)-ω-TANF gave 49.5 mM and 48.9 mM of (*R*)-α-MBA (>99%). The slightly higher conversion obtained for (*S*)-ω-TAPO–(*R*)-ω-TANF might be due to the higher specific activity of (*R*)-ω-TAMV (64.7 U mg^{-1}) compared to that of (*R*)-ω-TANF (39.0 U mg^{-1}). It is noteworthy that in this reaction scheme, deracemization can be performed without deactivating any enzyme.

After successful deracemization of *rac*-α-MBA into (*R*)-α-MBA by the one-pot, one-step reaction, we were curious to find out whether this reaction system would be applicable to other amines and unnatural amino acids. Therefore, the amino donor specificities of enzymes were examined with various compounds

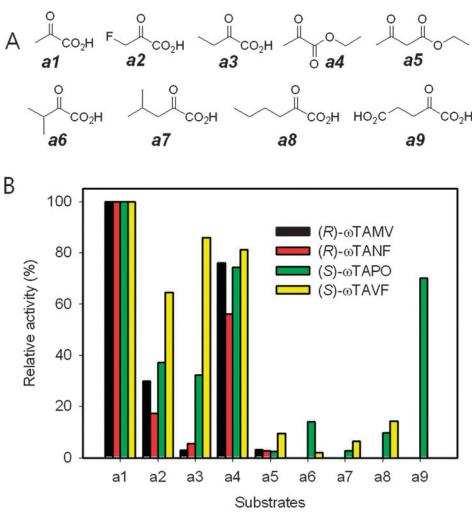

Fig. 1 Amino acceptor specificities of ω-TAs. Amino acceptors (A) and the relative reaction rate (B). Reaction conditions: 10 mM (*S*)-α-MBA (or (*R*)-α-MBA), 10 mM amino acceptor, 100 mM Tris–HCl (pH 7.5) at 37 °C. The initial reaction rate of the enzyme toward **a1** was taken as 100%. In the case of inactive substrates, the vertical bar is not visible in the graph.

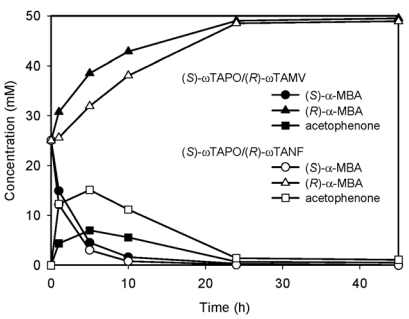

Fig. 2 Deracemization of *rac*-α-MBA. Reaction conditions: 1 mL reaction volume, 50 mM *rac*-α-MBA, 50 mM **a9**, 100 mM D-Ala, 100 mM glucose, 1 mM NADH, LDH (112 U), GDH (5 U), 0.2 mM PLP, (*S*)-ω-TAPO (0.93 mg), (*R*)-ω-TA (0.8 mg), 200 mM Tris–HCl (pH 7.5) at 37 °C.

Table 1 Deracemization of various aromatic amines[a]

	(*S*)-ω-TAPO–(*R*)-ω-TAMV		(*S*)-ω-TAPO–(*R*)-ω-TANF	
Substrate	Conv. (%)	ee (%)	Conv. (%)	ee (%)
d1	>99	>99	>99	>99
d2	82	>99	71	>99
d3	98	>99	87	>99
d4[c]	87	>99	94	>99
d5[b]	90	>99	97	>99
d6[b]	>99	>99	89	81
d7	98	>99	79	>99
d8	>99	>99	77	>99

[a] Reaction conditions: 1 mL reaction volume, 10 mM racemic substrate, 50 mM **a9**, 50 mM D-Ala, 50 mM glucose, 1 mM NADH, LDH (112 U), GDH (5 U), 0.2 mM PLP, (*S*)-ω-TAPO (1.0 mg), (*R*)-ω-TA (0.5 mg), 200 mM Tris–HCl (pH 7.5), 37 °C, 24 h reaction. Conv. and ee were determined using a Crownpak CR(+) column. Conv. is the percentage of the remaining amine concentration to the initial amine concentration. [b] (*S*)-ω-TAPO (1.86 mg), (*R*)-ω-TA (1.5 mg). [c] ee was determined using a C_{18} symmetry column after GITC derivatization.

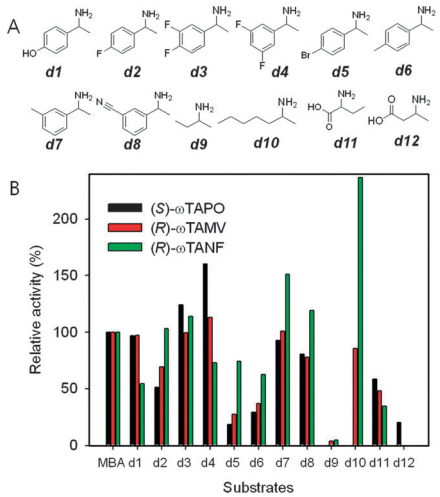

Fig. 3 Amino donor specificities of ω-TAs. Amino donors (A) and the relative reaction rate (B). Reaction conditions: 10 mM pyruvate (**a1**), 10 mM racemic amino donor, 100 mM Tris–HCl (pH 7.5) at 37 °C. The initial reaction rate of the enzyme towards *rac*-α-MBA was taken as 100%. In cases of inactive substrates, the vertical bar is not visible in the graph.

(eight aromatic amines, two aliphatic amines, two unnatural amino acids) in the presence of a 10 mM racemic amino donor and 10 mM **a1**, and the consumption rate of **a1** was analyzed using an Aminex HPX-87H HPLC column (Bio-Rad, CA) with the elution of 5 mM sulfuric acid solution. All enzymes showed considerable reactivity toward aromatic amines (**d1–d8**) and unnatural amino acids (**d11**). In the case of aliphatic amines (**d9**, **d10**), (*R*)-ω-TAMV and (*R*)-ω-TANF showed considerable activity, but (*S*)-ω-TAPO did not show any reactivity. In the case of beta-amino acid (**d11**), only (*S*)-ω-TAPO showed reactivity. These results suggested that aromatic amines can be deracemized well with ω-TAPO–(*R*)-ω-TAMV and (*S*)-ω-TAPO–(*R*)-ω-TANF, but in the case of aliphatic amine and beta-amino acid, the deracemization reaction could not be performed efficiently.

Finally, deracemization of aromatic amines (**d1–d8**) was carried out with 10 mM substrate (Table 1). In the case of **d5** and **d6**, higher amounts of ω-TAs ((*S*)-ω-TAPO; 1.86 mg, (*R*)-ω-TA; 1.5 mg) were added into the reaction mixture due to their lower reactivity ((*S*)-ω-TAPO; 1.0 mg, (*R*)-ω-TA; 0.5 mg) (Fig. 3). All racemic aromatic amines were successfully converted into (*R*)-amines (>99%) with good conversion (Table 1). All substrates except **d2** and **d4** could be deracemized into (*R*)-amines (>99%) with >97% conversion. In addition, (*S*)-ω-TAPO–(*R*)-ω-TAMV gave higher conversion than (*S*)-ω-TAPO–(*R*)-ω-TANF (except for **d5** and **d6**).

In summary, we successfully developed a one-pot one-step deracemization method using (*S*)-ω-TA and (*R*)-ω-TA through careful screening of amino acceptors for (*S*)-ω-TA. This novel deracemization method enabled various aromatic amines (α-MBA, **d1–d8**) to be converted into the (*R*)-form (>99%) with good conversion. We are currently developing a deracemization method to produce useful unnatural amino acids using ω-TAs.

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