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ω-Transaminase-catalyzed kinetic resolution of chiral amines using L-threonine as an amino acceptor precursor†

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Kinetic resolution of chiral amines using L-threonine as a cosubstrate was demonstrated by a biocatalytic strategy in which (*S*)-selective ω-transaminase (ω-TA) was coupled with threonine deaminase (TD), eliminating the need to use an expensive keto acid as an amino acceptor. The coupled enzyme reaction enabled simultaneous production of enantiopure (*R*)-amine and L-homoalanine which are pharmaceutically important building blocks. To extend the versatility of this strategy to production of both enantiomers of chiral amines, (*R*)-selective ω-TA coupled with TD was employed to produce (*S*)-amine.

Production of optically pure chiral compounds has attracted growing attention in the pharmaceutical industry owing to strict regulatory requirements for preparation of single enantiomer drugs.[1–4] Among the chiral compounds, chiral amines are used as indispensable building blocks for a number of pharmaceutical drugs including (*S*)-rivastigmine for treatment of Alzheimer's disease,[5,6] dilevalol as an antihypertensive drug,[7] sitagliptin as an antidiabetic drug[8] and mexiletine as an antiarrhythmic and antimyotonic drug.[9–11] Owing to the pharmaceutical importance of the chiral amines, biocatalytic approaches to prepare the enantiopure amines such as kinetic resolution,[12–14] asymmetric synthesis[6,15–18] and deracemization[11,19] have been extensively studied for developing greener alternatives to chemical processes[4] including preferential crystallization and asymmetric catalytic hydrogenation. ω-Transaminase (ω-TA) has been proven to be a promising enzyme enabling all the three preparative approaches.[20] ω-TA displays high turnover rate, stringent enantioselectivity, high stability and no requirement of external cofactor regeneration, which renders the enzyme attractive for industrial process development.[20,21] In addition to the chiral amines, applicability of the ω-TA reactions has been extended to the preparation of unnatural amino acids of pharmaceutical interest, which is well summarized in a recent review paper.[22]

In the ω-TA reaction, an amino group of primary amines (*i.e.* amino donor) is transferred to a carbonyl group of keto acids, aldehydes or ketones (*i.e.* amino acceptor).[23,24] Asymmetric synthesis of chiral amines starting with prochiral ketones is usually preferred over kinetic resolution of racemic amines owing to a 2-fold higher maximum yield. However, several drawbacks of the asymmetric synthesis, typically very low amino acceptor reactivity of ketones and unfavorable reaction equilibrium,[16,18,25,26] render the kinetic resolution approach often more suitable for practical preparation of enantiopure amines using the ω-TA. Indeed, the kinetic resolution approach benefits by much faster reaction rate, high solubility of amine substrate at neutral pH, use of stoichiometric amounts of co-substrate and favorable reaction equilibrium.[20]

When setting up a kinetic resolution process, choice of an amino acceptor is crucial to the feasibility of industrial process scale-up. Considering amino acceptor reactivity, α-keto acids such as pyruvate and aliphatic aldehydes such as propanal would be good cosubstrate options.[25] We tested propanal, in comparison with pyruvate, for kinetic resolution of α-methylbenzylamine (α-MBA) with (*S*)-selective ω-TA from *Ochrobactrum anthropi*,[23] because propanal shows reactivity as good as pyruvate (*i.e.* 94% relative activity toward propanal compared with pyruvate)‡ but is much cheaper. Although the amino acceptor reactivities based on initial rate measurements were similar, propanal turned out to be an inefficient amino acceptor for the kinetic resolution (Fig. 1). Enantiomeric excess (ee) of (*R*)-α-MBA reached >99% with pyruvate at 3 h, whereas ee was only 57% with propanal even after the reaction for 24 h. This result is ascribed to severe enzyme inactivation by propanal.§

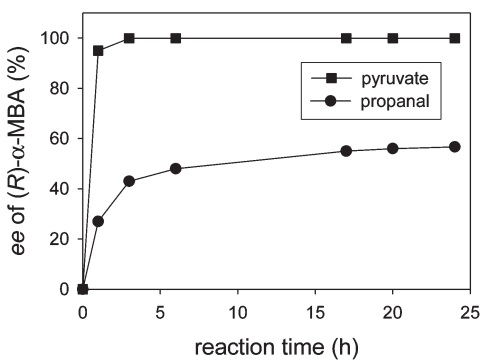

Fig. 1 Comparison of pyruvate and propanal as an amino acceptor for the kinetic resolution of α-MBA. Reaction conditions were α-MBA (100 mM), sodium pyruvate or propanal (80 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7.5) and 5 U ml^{-1} ω-TA from *O. anthropi* at 37 °C.

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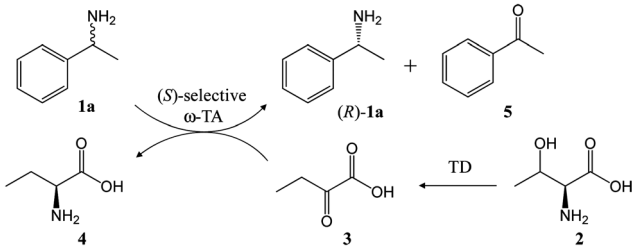
In addition to the severe enzyme inactivation, chemical toxicity of aldehydes to environments renders propanal not suited to a green process. Therefore, α-keto acids such as pyruvate should be a better option for the kinetic resolution process. Moreover, exploitation of α-keto acids as an amino acceptor provides an additional benefit to the kinetic resolution by enabling simultaneous production of two products of commercial interest rather than just one valuable product.[27,28] For example, kinetic resolution of α-MBA with pyruvate by (*S*)-selective ω-TA leads to production of enantiopure (*R*)-α-MBA as well as L-alanine. However, there is one serious problem for using α-keto acids as a cosubstrate: α-keto acids are too expensive to allow development of a cost-effective kinetic resolution process. To overcome this limitation, it is highly demanded to design a new kinetic resolution process that implements generation of the α-keto acid from a cheap precursor. The purpose of this study is to realize the one-stone-two-birds strategy, permitting production of two valuable products and exploitation of a cheap substitute for the α-keto acid.

We searched for a keto acid allowing facile generation from a cheap chemical as well as displaying high amino acceptor reactivity. We envisioned that 2-oxobutyrate was such an ideal amino acceptor based on the following reasons. First, 2-oxobutyrate can be easily generated from L-threonine by threonine deaminase (TD) as demonstrated in our previous report.[29] Second, L-threonine is one of the cheap amino acids that are produced on a million-ton scale annually.[30] Third, 2-oxobutyrate is as reactive as pyruvate which is a typical amino acceptor for all known ω-TAs. For example, the ω-TA from *O. anthropi* showed 78% relative activity toward 2-oxobutyrate compared with pyruvate.[23] Finally, L-homoalanine, the amination product of 2-oxobutyrate, is a pharmaceutically important unnatural amino acid serving as a key intermediate in the synthesis of antitubercular drug ethambutol[31] and antiepileptic drug levetiracetam.[32] Owing to the importance of L-homoalanine as a pharmaceutical intermediate, a fermentative approach to produce L-homoalanine was recently developed by redirecting a metabolic pathway of *Escherichia coli*.[33]

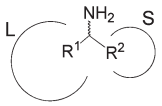
We set out to explore the feasibility of the ω-TA reaction for simultaneous production of enantiopure (*R*)-amine and L-homoalanine *via* kinetic resolution of racemic amine with concurrent stereoselective amination of 2-oxobutyrate generated from L-threonine by the action of TD. In the coupled enzyme reaction employing racemic α-MBA (**1a**) and L-threonine (**2**) as substrates (Scheme 1), (*S*)-selective ω-TA carries out oxidative deamination of (*S*)-**1a** as well as reductive amination of 2-oxobutyrate (**3**), leading to enrichment of (*R*)-**1a** and generation of L-homoalanine (**4**) and acetophenone (**5**). **2** serves as a precursor of **3** which is used as an amino acceptor for the ω-TA reaction and consequently converted to **4**.

To carry out the coupled reaction, we overexpressed and purified (*S*)-selective ω-TA from *O. anthropi* and TD from *E. coli* as described elsewhere.[23,29] Using the purified enzyme preparation of the ω-TA, we measured amino donor reactivity of five arylalkyl amines (**1a–1e**) and six alkyl amines (**1f–1k**) (Table 1). We chose pyruvate as an amino acceptor for measurements of the amino donor reactivity, because pyruvate is one of the most reactive amino acceptors and thereby has been widely used as a standard substrate for the initial rate measurements of



Scheme 1 One-pot conversion of racemic α-MBA and L-threonine to (*R*)-α-MBA and L-homoalanine using (*S*)-selective ω-TA and TD.

Table 1 Amino donor reactivity of the racemic amines[a]



Substrate	R^1	R^2	Relative reactivity[b] (%)
1a	C_6H_5	CH_3	100[c]
1b	*p*-F-C_6H_4	CH_3	91
1c	C_6H_5	CH_2CH_3	18
1d	$C_6H_5(CH_2)_2$	CH_3	70
1e			64 (153)[d]
1f	CH_3CH_2	CH_3	54
1g	Cyclopropyl	CH_3	63
1h	$CH_3(CH_2)_2$	CH_3	29
1i	$CH_3(CH_2)_5$	CH_3	146
1j	CH_3OCH_2	CH_3	60
1k	$HOCH_2$	CH_3	63

[a] Reaction conditions: racemic amine (10 mM), sodium pyruvate (10 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7.5) and 0.125 U ml^{-1} (*S*)-selective ω-TA from *O. anthropi* at 37 °C for 10 min. One unit of ω-TA activity is defined as the enzyme amount catalyzing the formation of 1 μmole of **5** in 1 min at 10 mM (*S*)-**1a** and 10 mM pyruvate. [b] Relative reactivity represents the initial reaction rate normalized by that of **1a**. L-Alanine was analyzed by HPLC to measure the initial rates. [c] Initial reaction rate for **1a** was 0.033 mM min^{-1}. [d] The value in the parenthesis represents the reactivity of (*S*)-**1e** (10 mM) relative to that of (*S*)-**1a** (10 mM), which was taken from the literature.[23]

ω-TA reactions.[25] In the previous study, we proposed that the active site of ω-TA carries two binding pockets, *i.e.* large (L) and small (S) pockets.[23,24] The S pocket was found to display a steric constraint prohibiting entry of a substituent larger than an ethyl group, whereas the L pocket can accept a bulk substituent such as a phenyl group of **1a** through a hydrophobic interaction.[23] Consistent with the two binding site model, **1c** showed a 5-fold decrease in amino donor reactivity compared with **1a** owing to the steric constraint in the S pocket. In contrast, amino donors carrying the same R^2 substituent as **1a** (*i.e.*, **1b** and **1d**) showed modest reactivity decreases. It is notable that **1e** showed a reactivity lower than **1a** although reactivity of (*S*)-**1e** was higher than (*S*)-**1a**. The discrepancy of the relative reactivities, depending on the enantiomeric purity of the amine substrate, seems to result from the different degree of enzyme inhibition by the (*R*)-enantiomer of the chiral amines. In the previous study,

Table 2 Effect of enzyme concentrations in the coupled reaction[a]

Entry	ω-TA (U mL^{-1})	TD[b] (U mL^{-1})	**4**[c] (mM)	ee of (*R*)-**1a**[d] (%)
1	1.25	9	15	28
2	2.50	9	33	65
3	3.75	9	47	90
4	1.25	18	19	24
5	1.25	27	13	26

[a] Reaction conditions: **1a** (100 mM), 2 (60 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7.5) at 37 °C for 2.5 h. [b] One unit of TD activity is defined as the enzyme amount that catalyzes the formation of 1 μmole of **3** in 1 min from **2** (50 mM). [c] Concentration determined by a standard calibration method from HPLC analysis of the GITC derivative. [d] Determined by HPLC analysis of the GITC derivatives.

we found that (*R*)-amine, of which the (*S*)-counterpart displays higher reactivity, inhibits enzyme activity more strongly.[34] This accounts for the reversal of the relative magnitude of the reactivity of **1e** and (*S*)-**1e** compared with **1a** and (*S*)-**1a**, respectively. In the case of alkyl amines except **1i**, amino donor reactivities were lower than **1a** presumably owing to weakened hydrophobic interaction in the L pocket.

To optimize coupled enzyme reactions, it is desirable to achieve reaction conditions where each enzyme reaction occurs at a similar rate, so a bottleneck step that limits the overall reaction is not present. For example, the ω-TA reaction occurring much slower than the TD reaction must result in accumulation of the reaction intermediate (**3**). In this case, increase in the ω-TA concentration allows the overall reaction to proceed faster. We examined which enzyme reaction is rate-determining by carrying out the coupled reaction at varying concentrations of either enzyme while keeping the concentration of the other one constant (Table 2). In the coupled reaction, we used a 20% higher concentration of **2** (60 mM) than the stoichiometric amount required for complete kinetic resolution of **1a** (100 mM). Increasing ω-TA concentration at a constant TD concentration (entries 1, 2 and 3) led to substantial enhancement in the production of **4** and enrichment of (*R*)-**1a**. In contrast, varying TD concentration at a constant ω-TA concentration (entries 1, 4 and 5) did not induce such a substantial change in the reaction efficiency. This result indicates that the ω-TA reaction is a rate-determining step within the enzyme concentration range used in this study. Entry 3 (3.75 U ml^{-1} ω-TA and 9 U ml^{-1} TD) among the 5 reaction conditions yielded the best reaction outcome (47 mM of **4** with >99% ee corresponding to 94% of the theoretical maximum yield).

Using the optimal enzyme concentrations (entry 3 in Table 2; 3.75 U mL^{-1} ω-TA and 9 U mL^{-1} TD), we carried out kinetic resolution of 11 chiral amines listed in Table 1. As shown in Table 3, the reaction time required to attain ee of (*R*)-amine higher than 99% was inversely correlated with the amino donor reactivity except **1i** and **1k**. All the arylalkyl chiral amines except **1c** could be completely resolved within 7 h with reaction conversion close to 50%. **1c** was not completely resolved even at 28 h (entry 3) presumably due to the drastic decrease in the amino donor reactivity compared with **1a**. Therefore, higher concentration of ω-TA (10 U mL^{-1}) without varying the TD concentration was employed to attain complete resolution of **1c**, leading to >99% ee of (*R*)-**1c** at 30 h (entry 4). Among the alkyl amines,

Table 3 Kinetic resolution of chiral amines *via* ω-TA/TD coupled reactions using **2** as an amino acceptor precursor[a]

Entry	Chiral amine	Reaction time (h)	Conversion[b] (%)	ee of (*R*)-amine[b] (%)
1	**1a**	3	49.9	>99
2	**1b**	3.5	50.5	>99
3	**1c**	28	35.1	52
4	**1c**	30[c]	50.1	>99
5	**1d**	5	50.9	>99
6	**1e**	7	50.0	>99
7	**1f**	8	50.2	>99
8	**1g**	8	50.1[d]	>99[d]
9	**1h**	9	50.5	>99
10	**1i**	24	37.4	58
11	**1i**	24[c]	50.2	>99
12	**1j**	9	50.6	>99
13	**1k**	30[c]	40.8[d]	66[d]

[a] Reaction conditions: **1a–1k** (100 mM), **2** (60 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7.5), 3.75 U ml^{-1} (*S*)-selective ω-TA and 9 U ml^{-1} TD at 37 °C. [b] Determined by a standard calibration method from HPLC analysis of GITC derivatives of corresponding amines. [c] 10 U ml^{-1} ω-TA and 9 U ml^{-1} TD were used. [d] Determined by HPLC analysis employing Marfey's reagent.

1f–h and **1j** were successfully resolved within 9 h under the optimized conditions (entry 3 in Table 2). Despite the amino donor reactivity of **1i** being higher than **1a**, **1i** led to incomplete resolution even after 24 h reaction (entry 10 in Table 3). Similar to **1c**, high ω-TA concentration was used to completely resolve **1i** (entry 11). In the case of **1k**, the kinetic resolution reaction occurred much slower than expected. **1k** showed only 40.8% conversion even after the prolonged reaction using the high ω-TA concentration (entry 13). In all the cases, the other desired product (**4**) was simultaneously formed in high enantiopurity (>99% ee).

The results in Table 3 demonstrate that kinetic resolution of chiral amines to yield (*R*)-amine can be successfully carried out using L-threonine (**2**) as a substitute for α-keto acid. To produce (*S*)-amine as well by this strategy, we investigated the coupled enzyme reaction employing (*R*)-selective ω-TA. To this end, we cloned, overexpressed and purified (*R*)-selective ω-TA from *Aspergillus terreus* identified elsewhere.[35] To render the coupled reaction executable using the (*R*)-selective ω-TA, it is crucial that the enzyme displays substantial activity toward 2-oxobutyrate (**3**). We found that the (*R*)-selective ω-TA exhibited 42% relative activity toward **3** compared with pyruvate.¶ Therefore, we carried out kinetic resolution of **1a** as a model substrate using **2** as a cosubstrate (Fig. 2). Under the reaction conditions, kinetic resolution of **1a** was completed in 5 h, resulting in >99% ee of (*S*)-**1a**. Because enantioselectivity of the ω-TA from *A. terreus* is opposite to that from *O. anthropi*, D-homoalanine of >99% ee was formed in this coupled reaction.

In summary, we have developed a facile approach to the kinetic resolution of chiral amines by coupling ω-TA with TD to enable *in situ* generation of an expensive keto acid from a readily available natural amino acid. This allowed production of two valuable products (enantiopure (*R*)-amine and L-homoalanine) from cheap substrates (racemic amine and L-threonine). Moreover, the feasibility of this strategy to obtain (*S*)-amine is also demonstrated using an ω-TA displaying opposite

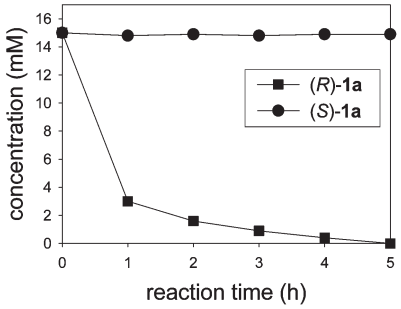


Fig. 2 Kinetic resolution of **1a** to produce (*S*)-**1a**. Reaction conditions were **1a** (30 mM), **2** (20 mM), PLP (0.1 mM), phosphate buffer (50 mM, pH 7.5), 1.5 U ml^{-1} (*R*)-selective ω-TA from *A. terreus* and 4.5 U ml^{-1} TD at 37 °C.

enantioselectivity. We expect that coproduction of the expensive amino acid would compensate for the cost increase caused by the recycling process, required for conversion of a ketone product back into a racemic amine, which has been regarded as a major shortcoming of the kinetic resolution process over the asymmetric synthesis approach. To render this strategy feasible for industrial scale-up, the production scale needs to be negotiated with commercial demand of both products.

Acknowledgements

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Notes and references

‡ Initial reaction rates for pyruvate and propanal were 92 and 86 μM min^{-1}, respectively. Reaction conditions were (*S*)-α-MBA (20 mM), amino acceptor (20 mM), phosphate buffer (50 mM, pH 7.5), 0.1 U ml^{-1} (*S*)-selective ω-TA from *O. anthropi* for 10 min at 37 °C.
§ When incubated in phosphate buffer (50 mM, pH 7.5) containing 80 mM propanal for 1 h at 37 °C, only 24% residual enzyme activity was observed. In contrast, no significant decrease in the enzyme activity was observed in the same buffer containing 80 mM pyruvate after 1 h.
¶ Initial reaction rates for pyruvate and 2-oxobutyrate were 61 and 25 μM min^{-1}, respectively. Reaction conditions were (*R*)-**1a** (20 mM), amino acceptor (20 mM), phosphate buffer (50 mM, pH 7), 60 mU ml^{-1} (*R*)-selective ω-TA from *A. terreus* for 10 min at 37 °C.

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