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ω-Transaminase-catalyzed asymmetric synthesis of unnatural amino acids using isopropylamine as an amino donor†

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Eul-Soo Park, Joo-Young Dong and Jong-Shik Shin*

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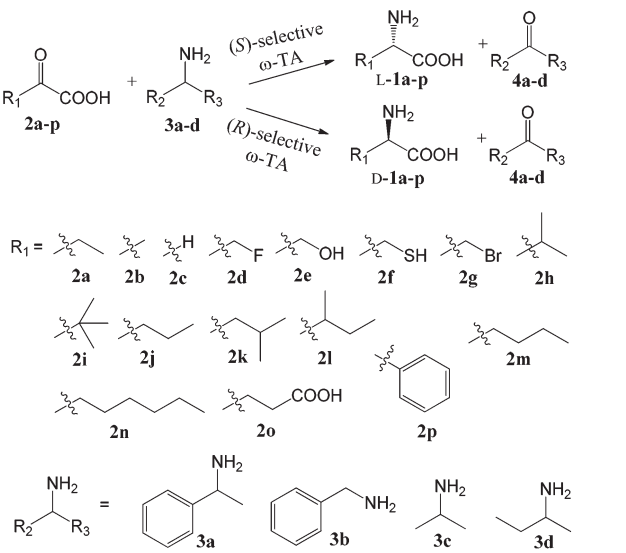
Isopropylamine is an ideal amino donor for reductive amination of carbonyl compounds by ω-transaminase (ω-TA) owing to its cheapness and high volatility of a ketone product. Here we developed asymmetric synthesis of unnatural amino acids *via* ω-TA-catalyzed amino group transfer between α-keto acids and isopropylamine.

Introduction

Nonproteinogenic amino acids, including L-α-amino acids carrying an unnatural side chain and D-α-amino acids, are gaining ever-growing importance as chiral building blocks for various pharmaceuticals, because they provide enhanced enzymatic and pharmacodynamic stability as well as structural diversity that cannot be mimicked by natural counterparts.[1–4] For example, L-homoalanine (L-**1a**) is pharmaceutically important, serving as a key intermediate for production of levetiracetam and brivaracetam (antiepileptic drugs),[5] and ethambutol (an antituberculosis drug).[6]

A number of chemocatalytic[7–10] and biocatalytic[2,3,11] methods have been developed to enable cost-effective synthesis of the unnatural amino acids. Industrial production of L-**1a** has been established by reductive amination of 2-oxobutyric acid (**2a**) using tyrosine transaminase (TTA).[2,12–14] To overcome the unfavorable reaction equilibrium, L-aspartate was employed as an amino donor because the resulting keto acid product (*i.e.* oxaloacetic acid) underwent spontaneous decarboxylation to pyruvic acid (**2b**). However, this reaction led to product contamination with L-alanine (L-**1b**) because TTA is reactive toward **2b** as well as **2a**.[2,12–14] To reduce the side product formation, the TTA reaction was coupled with acetolactate synthase capable of converting **2b** to acetolactic acid.[15] The same strategy was applied to production of D-**1a** by replacing TTA with D-amino acid transaminase (DATA).[2,12] As an alternative to addressing the unfavorable equilibrium, L-glutamic acid was used as an amino donor for a primary transaminase and the reaction equilibrium was shifted by simultaneously running an ornithine transaminase reaction which led to spontaneous cyclization of a resulting keto acid product.[14]

Contrary to the equilibrium constant (K_{eq}) close to unity for the reactions catalyzed by α-transaminase (α-TA) such as TTA and DATA,[12,16] transaminations between primary amines and α-keto acids catalyzed by ω-transaminase (ω-TA) are energetically favorable.[17] For example, K_{eq} of the transamination between **2b** and α-methylbenzylamine (**3a**) was reported to be 1130.[17] Therefore, the transaminations using primary amines as an amino donor permit thermodynamically unrestricted asymmetric amination of keto acids (Scheme 1).[18]



Scheme 1 Asymmetric reductive amination of α-keto acids using primary amines as an amino donor to produce enantiopure amino acids.

Department of Biotechnology, Yonsei University, 262 Seongsanno, Seodaemun-Gu, 120-749 Seoul, South Korea. E-mail: enzymo@yonsei.ac.kr; Fax: +82-2-362-7265; Tel: +82-2-2123-5884

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We previously demonstrated asymmetric synthesis of L-**1a** using benzylamine (**3b**) as an amino donor.[19] However, a serious drawback of this method was a high amino acceptor reactivity of the resulting deamination product, *i.e.* benzaldehyde (**4b**), which engendered a drastic reduction in the net reaction rate because of a high reverse reaction rate. For example, (*S*)-selective ω-TA from *Paracoccus denitrificans* showed 84% reactivity of **4b** relative to **2b** (*i.e.* a typical amino acceptor for most ω-TAs).[19] Therefore, a cheap amino donor, whose deamination product is either non-reactive or easily removable, is highly demanded to implement preparative asymmetric synthesis of unnatural amino acids using ω-TAs.

In this regard, isopropylamine (**3c**) is an ideal amino donor for ω-TA because its deamination product, *i.e.* acetone (**4c**), is a non-reactive amino acceptor and easy to remove owing to high volatility.[20] In addition, **3c** is much cheaper than **3b**. These beneficial features of **3c** as an amino donor have recently prompted intense research efforts to asymmetric transfer of an amino group from **3c** to prochiral ketones for preparation of chiral amines.[20–23] However, to the best of our knowledge, **3c** has not yet been exploited as an amino donor for asymmetric amination of α-keto acids although this approach is promising for cost-effective synthesis of unnatural amino acids including L-**1a** and D-**1a**. In this study, we sought to develop ω-TA-catalyzed synthesis of enantiopure unnatural amino acids from α-keto acids using **3c** as an amino donor.

Results and discussion

Although **3c** is an ideal amino donor, most ω-TAs do not exhibit substantial reactivity toward **3c**.[24,25] For example, two typical ω-TAs, *i.e.* (*S*)-selective ω-TA from *Vibrio fluvialis* JS17[24] and (*R*)-selective ω-TA from *Arthrobacter* sp. (ARTA),[25] were known to be non-reactive toward **3c**. Therefore, we set out to search for ω-TAs that could utilize **3c** as an effective amino donor. To this end, we examined the amino donor reactivities of four primary amines (two arylalkylamines and two alkylamines) toward two (*S*)-selective ω-TAs cloned in our group from *P. denitrificans* (PDTA)[19] and *Ochrobactrum anthropi* (OATA),[26,27] and (*R*)-selective ARTA[25] and its variant ($AR_{mut}TA$)[20] engineered for reductive amination of bulky ketones by **3c** (Table 1). The two (*S*)-selective ω-TAs showed reactivities of **3b** higher than (*S*)-**3a** whereas the two (*R*)-selective ω-TAs showed reactivities of **3b** much lower than (*R*)-**3a**. PDTA and ARTA showed modest reactivities toward alkylamines (*i.e.* **3c–d**). In contrast, OATA showed substantial reactivities toward the alkylamines and the reactivity of **3c** was 43% relative to (*S*)-**3a**. As expected, $AR_{mut}TA$ displayed higher reactivity toward **3c** than its parental ω-TA (*i.e.* ARTA) did. Based on the results, OATA and $AR_{mut}TA$ turned out to be suitable for asymmetric synthesis of L- and D-amino acids, respectively, using **3c** as an effective amino donor.

To ensure whether **3c** is a better amino donor than **3b**, we compared the reaction progress of the amination of **2a** catalyzed by OATA using the two amines as amino donors (Fig. 1). Despite the 6-fold higher reactivity of **3b** than **3c**, the two reactions showed similar conversions up to 30 min and then the reaction with **3b** became even slower than the one with **3c**. After a 4 h reaction, conversion reached >99% with **3c** whereas **3b** permitted 86% conversion. This result clearly corroborates that product inhibition caused by a high amino acceptor reactivity of **4b** (35% relative to **2b**)[28] is highly detrimental to efficient amination of keto acids.

It is known that most ω-TAs possess severe steric constraints in the small binding pocket which precludes entry of a substituent larger than an ethyl group.[20,24,27] To clarify the range of amino acids producible by OATA and $AR_{mut}TA$, we examined the substrate specificity of the two ω-TAs toward α-keto acids (Table 2). As expected, OATA showed substantial reactivities only toward α-keto acids carrying substituents no larger than an ethyl group, *i.e.* five α-keto acids (**2a–e**) among the sixteen tested. It was intriguing that the steric constraint of a parental enzyme toward α-keto acids was found to be conserved in $AR_{mut}TA$ in spite of the multiple mutations introduced to accommodate bulky substituents of arylalkyl ketones.[20] However, the enlarged binding pocket of $AR_{mut}TA$ permitted improved reactivities toward α-keto acids carrying a linear alkyl substituent (*i.e.* **2j**, **2m–n**).[29] It is notable that **2n** showed a reactivity even higher than **2j** and **2m**.

For asymmetric synthesis of unnatural amino acids, we decided to move forward with α-keto acids showing relative

Table 1 Amino donor reactivities of primary amines toward ω-TAs

	Relative reactivity[a] (%)			
Amines	PDTA	OATA	ARTA	$AR_{mut}TA$
3a[b]	100	100	100	100
3b	128	259	3	28
3c	7	43	2	8
3d[c]	n.r.[d]	23	2	14

[a] Relative reactivity represents the initial reaction rate (*i.e.* conversion <15%) normalized by that of (*S*)- or (*R*)-**3a**. Reaction conditions were amine (20 mM except **3d**) and **2b** (20 mM). [b] Enantiopure (*S*)- and (*R*)-**3a** (20 mM) were used for (*S*)- and (*R*)-selective ω-TAs, respectively. [c] *Rac*-**3d** (40 mM) was used for the reactivity measurements. [d] n.r.: not reactive (*i.e.* relative reactivity <1%).

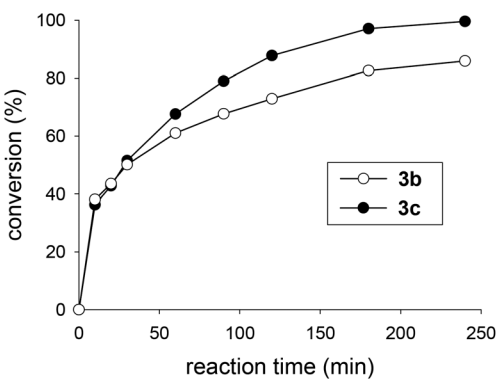


Fig. 1 Time-course monitoring of the reductive amination of **2a** using **3b** or **3c** as an amino donor. Reaction conditions were **2a** (100 mM), amine (150 mM) and OATA (5 U mL^{-1}).

Table 2 Amino acceptor specificities of OATA and $AR_{mut}TA$ toward α-keto acids

α-Keto acids	Relative reactivity[a] (%)	
	OATA	$AR_{mut}TA$
2a	13	33
2b	100	100
2c	135	36
2d	43	5
2e	14	6
2f	n.r.[b]	n.r.
2g	n.r.	n.r.
2h	n.r.	n.r.
2i	n.r.	n.r.
2j	n.r.	2
2k	n.r.	n.r.
2l	n.r.	n.r.
2m	n.r.	2
2n	n.r.	8
2o	n.r.	n.r.
2p	n.r.	n.r.

[a] Relative reactivity represents the initial reaction rate normalized by that of **2b**. Reaction conditions: α-keto acid (20 mM) and (*S*)- or (*R*)-**3a** (20 mM). [b] n.r.: not reactive.

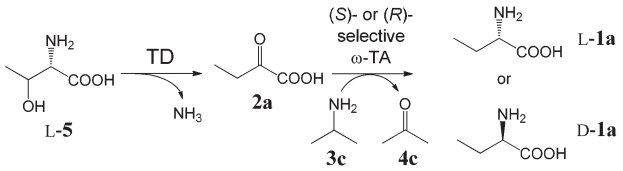
reactivities higher than 5%, *i.e.* **2a** and **2d** with OATA, and **2a–b**, **2d–e** and **2n** with $AR_{mut}TA$ (Table 3). Reductive amination of the α-keto acids (100 mM) by **3c** (1.5 molar equiv.) led to efficient synthesis of unnatural amino acids with >98% conversion and excellent enantiopurity (>99.9%) within 2 h except for **2d**. Compared with the other α-keto acids, amination of **2d** showed exceptionally slow reaction progress with both ω-TAs. We suspected that either **2d** or its amination product (*i.e.* **1d**) might induce irreversible enzyme inactivation, because we observed formation of enzyme aggregates during both amination reactions. Indeed, it turned out that **1d** induced such detrimental enzyme inactivation, because the enzyme precipitate was not observed in the incubation solution containing either **2d** or **3c**. Therefore, for efficient amination of **2d**, a method to enhance enzyme stability such as enzyme immobilization needs to be employed.

The unnatural amino acids listed in Table 3 are of industrial importance. For example, in addition to L-**1a** of pharmaceutical importance,[5] D-alanine (D-**1b**) is a building block of

Table 3 Asymmetric synthesis of unnatural amino acids from keto acids using **3c** as an amino donor[a]

Substrate	ω-TA	Reaction time (h)	Conversion[b] (%)	Product (% ee)
2a	OATA	0.5	99	L-**1a** (>99.9)
2d	OATA	10	79	L-**1d** (>99.9)
2a	$AR_{mut}TA$	0.5	99	D-**1a** (>99.9)
2b	$AR_{mut}TA$	0.2	98	D-**1b** (>99.9)
2d	$AR_{mut}TA$	10	86	D-**1d** (>99.9)
2e	$AR_{mut}TA$	2	99	D-**1e** (>99.9)
2n	$AR_{mut}TA$	0.5	99	D-**1n** (>99.9)

[a] Reaction conditions: 0.5 mL reaction mixture containing keto acid (100 mM), **3c** (150 mM) and ω-TA (50 U mL^{-1}). [b] Conversions were based on consumption of the keto acid substrate.



Scheme 2 Production of both enantiomers of **1a** from L-**5** and **3c**.

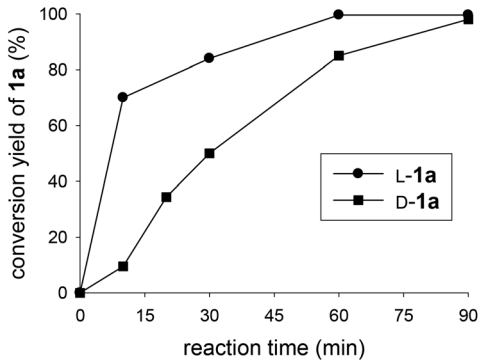


Fig. 2 Asymmetric synthesis of L- and D-**1a** from L-**5** and **3c**. Reaction conditions were L-**5** (100 mM), **3c** (200 mM), TD (5 U mL^{-1}) and ω-TA (20 U mL^{-1}) in a 1 mL reaction mixture.

Abarelix (antineoplastic)[30] and D-serine (D-**1e**) is a precursor of D-cycloserine (cognition enhancer).[31] Besides, D-fluoroalanine (D-**1d**) is recognized as an antibiotic owing to its ability to inactivate bacterial alanine racemase.[32]

Among the α-keto acids used in Table 3, **2a** is readily accessible from a cheap precursor, *i.e.* L-threonine (L-**5**), using threonine deaminase (TD) as previously demonstrated.[19] L-**5** is one of the cheap natural amino acids mass-produced by microbial fermentation.[33] Therefore, we investigated whether both enantiomers of **1a** could be prepared from L-**5** and **3c** by coupling the ω-TA reactions with TD cloned from *Escherichia coli* (Scheme 2). In this approach, the carbon skeleton and the amino group of L- and D-**1a** come from L-**5** and **3c**, respectively. The TD/OATA coupled reaction using L-**5** (100 mM) and **3c** (2 equiv.) was completed at 60 min, leading to 99% conversion yield and >99.9% ee of produced L-**1a** (Fig. 2). To produce D-**1a**, OATA was substituted by $AR_{mut}TA$. Under the same substrate conditions, the TD/$AR_{mut}TA$ reaction resulted in production of D-**1a** with 98% conversion yield and >99.9% ee at 90 min. The longer reaction time required to complete the TD/$AR_{mut}TA$ reaction presumably resulted from the lower relative reactivity of **3c** toward $AR_{mut}TA$ than that toward OATA. These results demonstrate that both enantiomers of **1a** of pharmaceutical importance can be readily prepared from cheap substrates.

We also carried out preparative-scale synthesis of L-**1a** and D-**1a** *via* the TD/ω-TA strategy in a 50 mL reaction mixture charged with L-**5** (1.79 g, 15 mmol) and **3c** (1.94 mL, 22.5 mmol). Conversions over 99% were attained within 12 and 24 h for the TD/OATA and TD/$AR_{mut}TA$ reactions, respectively. Purification and structural characterization of the desired amino acid products were performed, leading to

recovery of pure L-**1a** (1.20 g, 77.6% isolation yield, >99.9% ee) and D-**1a** (1.21 g, 78.2% yield, >99.9% ee).

Conclusions

In this work, we demonstrated efficient biocatalytic asymmetric synthesis of unnatural amino acids from corresponding keto acids using **3c** as a cosubstrate. Unlike the α-TA reactions, the ω-TA approach using the cheap achiral amino donor is thermodynamically favorable. This eliminates the need for additional enzymes to shift unfavorable equilibrium or to prevent side product accumulation, which has been compulsory in the previous α-TA-based approaches.[2,12–15] Moreover, when it comes to the synthesis of D-amino acids, a serious drawback of the α-TA approach is the requirement of an expensive D-amino acid as an amino donor (*e.g.* D-aspartic acid for DATA reactions).[2,12] However, to expand our approach to asymmetric synthesis of structurally diverse unnatural amino acids, the steric constraint in the substrate binding pocket of ω-TAs should be addressed. Although more than twenty ω-TAs have been reported, no naturally occurring enzyme capable of accepting α-keto acids carrying substituents bulkier than an ethyl group has been identified.[34,35] However, as demonstrated by AR_{mut}TA capable of accepting bulky linear aliphatic groups of α-keto acids, directed evolution combined with *in silico* modeling[20] may create an ω-TA variant of which the substrate binding pocket is redesigned in a way to accept diverse bulky substituents.

Recently, Seo *et al.* reported deracemization of *rac*-**1a** into L-**1a** using ω-TA from *V. fluvialis* JS17 coupled with D-amino acid oxidase.[36] Because they used **3b** as an amino donor for ω-TA, the severe product inhibition by **4b** had to be mitigated using a biphasic reaction system to extract the inhibitory **4b**. However, the biphasic system is often detrimental to enzyme stability. Moreover, hydrogen peroxide generated from the oxidase reaction usually leads to severe enzyme inactivation without the aid of catalase. In contrast, the TD/ω-TA approach using L-**5** and **3c** lacks such inhibitory products and affords production of both enantiomers of **1a**.

Experimental

Enzyme assay

Typical enzyme assays were carried out at 37 °C and 50 mM phosphate buffer (pH 7). One unit of ω-TA activity was defined as the enzyme amount that catalyzed formation of 1 μmol of acetophenone in 1 min at 20 mM **2b** and 20 mM (*S*)- or (*R*)-**3a**. One unit of TD was defined as the enzyme amount producing 1 μmol of **2a** in 1 min at 50 mM L-**5**.

Measurement of amino donor and acceptor reactivity

Amino donor reactivities were measured with **2b** (20 mM) and amine (20 mM except **3d**) in 50 mM phosphate buffer (pH 7). The enzyme concentrations in the reaction mixtures were 0.5, 0.7 and 0.2 U mL^{-1} for PDTA, OATA and (*R*)-selective ω-TAs, respectively. Reactions were allowed for 10 min and the initial rates (*i.e.* conversion >15%) were measured by analyzing produced L- or D-**1b**. Amino acceptor reactivities were measured with (*S*)- or (*R*)-**3a** (20 mM), α-keto acid (20 mM) and ω-TA (0.1 and 0.05 U mL^{-1} for OATA and AR_{mut}TA, respectively). The acetophenone produced was analyzed for the initial rate measurements.

Asymmetric synthesis of unnatural α-amino acids

Time-course monitoring of the reductive amination of **2a** using **3b** or **3c** as an amino donor was performed at 100 mM **2a**, 150 mM amine, 0.5 mM PLP and 5 U mL^{-1} OATA in 50 mM phosphate buffer (pH 7). The reaction volume was 500 μL and the reaction mixture was incubated at 37 °C. Aliquots of the reaction mixture (10 μL) were taken at predetermined reaction times and mixed with 60 μL acetonitrile to stop the reaction. The reaction mixtures were subjected to HPLC analysis of **2a** to measure conversions.

Asymmetric synthesis of unnatural amino acids (1 mL reaction volume) was carried out with 100 mM α-keto acid (**2a–b**, **2d–e** and **2n**), 150 mM **3c**, 0.5 mM PLP and 50 U mL^{-1} ω-TA in 50 mM phosphate buffer (pH 7) at 37 °C. Keto acids and amino acids were analyzed for measurements of conversion and enantiomeric excess, respectively.

TD/ω-TA coupled reactions

The coupled reaction for synthesis of L- and D-**1a** (1 mL reaction volume) was performed with 100 mM L-**5**, 200 mM **3c**, 0.5 mM PLP, 5 U mL^{-1} TD and 20 U mL^{-1} ω-TA in 50 mM phosphate buffer (pH 7) at 37 °C. OATA and AR_{mut}TA were employed for preparation of L- and D-**1a**, respectively. **1a** was analyzed for measurements of conversion yield and enantiomeric excess.

Preparative-scale synthesis of L- and D-**1a** was performed in a 50 mL reaction mixture containing 300 mM L-**5**, 450 mM **3c**, 0.5 mM PLP, 5 U mL^{-1} TD and 10 U mL^{-1} ω-TA (OATA or AR_{mut}TA) under magnetic stirring at 37 °C. When the conversion exceeded 99%, the reaction mixture was subjected to product isolation.

Purification and structural characterization of L- and D-1a

The pH of the reaction mixture was adjusted to 1.0 by adding 5 N HCl for protein precipitation. The reaction mixture was filtered through a glass-fritted filter funnel to remove protein precipitate. The filtrate solution was loaded on a glass column packed with Dowex 50WX8 cation-exchange resin (40 g), followed by washing with 0.1 N HCl (120 mL) and water (120 mL), and then elution with 145 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 (50 mL) and then oven-dried overnight. The purified L- and D-**1a** were structurally characterized by ^{1}H NMR, ^{13}C NMR, IR, elemental analysis and LC/MS, leading to confirmation of the recovery of the desired pure products (see ESI†).

Acknowledgements

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