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Biocatalytic Asymmetric Synthesis of Unnatural Amino Acids through the Cascade Transfer of Amino Groups from Primary Amines onto Keto Acids

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The widespread use of unnatural amino acids as chiral auxiliaries and ligands for asymmetric synthesis, as versatile building blocks for the assembly of combinatorial libraries, and as crucial structural motifs in a diverse range of pharmaceuticals and agrochemicals has fueled research efforts to develop efficient synthetic procedures.[1] Whereas natural amino acids, as native metabolites, are producible through microbial fermentation, metabolic redesign to enable the fermentative production of unnatural amino acids remains challenging.[2] Therefore, in addition to chemocatalytic methods,[3] various biocatalytic approaches to afford more-sustainable processes have been developed,[1b,4] including the kinetic resolution of racemic amino-acid derivatives by using hydantoinase,[5] acylase[6] and amidase[7] and the asymmetric reductive amination of keto acids by using dehydrogenase[8] and transaminase (TA).[9] Asymmetric amination is favored over kinetic resolution because it offers 100% theoretical yield and no need for the racemization of an unwanted enantiomer.[10] Dehydrogenase exploits NAD(P)H as an external cofactor, whereas TA adopts pyridoxal 5′-phosphate (PLP) as a prosthetic group to mediate amino-group transfer. Thus, TA does not require an auxiliary enzyme for cofactor recycling, which renders TAs ideal for industrial process development of asymmetric amination reactions.[11]

Recent technical advances in the biocatalytic transamination reaction have widened the choice of synthetic methods for the preparation of enantiopure amines and amino acids from achiral carbonyl precursors.[11–12] Depending on the position of the transferrable amino group relative to an intramolecular carboxy group, TAs fall into two classes: 1) α-TA, which exclusively abstracts an α-amino group, and 2) ω-TA, which can abstract an amino group from a non-α position or even from primary amines that do not contain a carboxy group.[13] It has been demonstrated that ω-TA could be used to prepare unnatural amino acids, such as L-fluoroalanine[14] and L-homoalanine,[15] from their corresponding α-keto acids. However, this strategy is seriously limited by stringent steric constraints in the active site of ω-TA, which precludes the entry of α-keto acids that contain bulkier substituents than an ethyl group.[16] For example, to date, no ω-TA is available for the asymmetric syntheses of L-*tert*-leucine and D-phenylglycine, which are valuable building blocks of pharmaceutical drugs.[17]

In contrast, α-TAs, such as branched-chain transaminase (BCTA) and D-amino-acid transaminase (DATA), have displayed broad substrate specificity toward α-keto acids,[13a,18] which is promising for the synthesis of unnatural amino acids. However, less attention has been paid to the industrial applications of α-TAs, because the reaction equilibria of most α-TA reactions are close to unity, owing to structural similarities between the substrates and the products.[9c,19] For example, the equilibrium constant (K_{eq}) for transamination between trimethylpyruvic acid (**1h**) and L-glutamic acid (L-**2j**) catalyzed by BCTA was 0.67.[20] Therefore, at least a 28-fold molar excess of compound L-**2j** should be employed to achieve a 95% yield of L-*tert*-leucine (L-**2h**), which poses a fundamental obstacle to its practical synthetic applications. To address the thermodynamic limitations of the α-TA reactions, several attempts have been made to shift the neutrally positioned equilibrium towards the products by the spontaneous or enzymatic removal of the keto-acid product.[9a–c,19,21] However, these methods remained inefficient in eliminating the thermodynamic constraints and even necessitated the adoption of an additional enzyme to suppress side-product formation, which rendered these previous approaches suboptimal for industrial frameworks.

In this study, we aimed at developing a scalable strategy for the industrial production of unnatural amino acids by bypassing the thermodynamic constraints of the α-TA reactions. There are myriad cascade biochemical reactions in which an endergonic step is driven by free-energy release from a highly exergonic reaction, such as ATP hydrolysis.[22] Inspired by this fact, we envisioned that unfavorable α-TA reactions could be driven to completion by coupling with an ω-TA reaction (Scheme 1), because ω-TA reactions between primary amines and α-keto acids are known to be energetically favorable.[23] For example, the K_{eq} value for the reaction between pyruvic acid (**1a**) and (*S*)-α-methylbenzylamine ((*S*)-α-MBA) is reported to be 1130.[23] However, a challenging problem facing the implementation of such coupled reactions is whether it is possible to find a competent amino-acid substrate that is capable of shuttling between the α-TA and ω-TA reactions. A suitable shuttling substrate should be a reactive amino donor for a given α-TA and the resulting keto acid should be a reactive amino acceptor toward an ω-TA; thus, recycling of the shuttling substrate would be efficient. Disappointingly, compound L-**2j** (i.e., a universal amino donor for most α-TAs) cannot fulfill this requirement because all known ω-TAs are not reactive at all toward α-ketoglutaric acid (**1j**).

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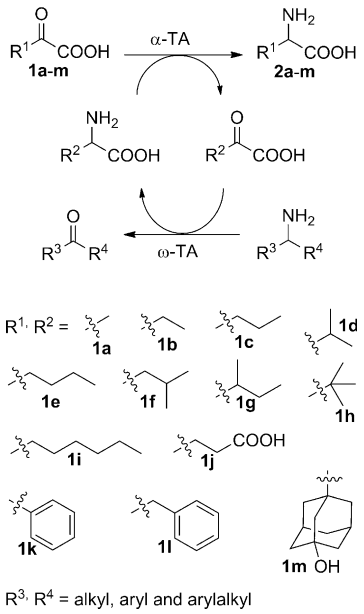

Scheme 1. The α-TA reactions were driven to completion by coupling with an ω-TA reaction for the asymmetric synthesis of unnatural α-amino acids.

We set out to examine whether the BCTA reactions, which are useful for the preparation of various unnatural amino acids that contain aliphatic side chains, could be coupled with an ω-TA reaction. To that end, we searched for a shuttling substrate by examining the amino-donor specificity of BCTA from *Escherichia coli* toward amino acids and the amino-acceptor specificity of *S*-selective ω-TA from *Ochrobactrum anthropi* (OATA)[16] towards their corresponding keto acids (Table 1). These two TAs showed opposite trends in relative reactivity with regards to the bulkiness of the side chain on the substrates, which was ascribed to the optimized substrate specificity of BCTA for the bulky side chains of branched-chain amino acids and the severe steric constraints of OATA, thereby allowing the accommodation of substituents up to an ethyl group. Despite the highest amino-acceptor reactivity of compound **1a** towards

Table 1. Dependence of the bulkiness of the R substituent on the relative reactivity of BCTA toward amino acids and OATA toward keto acids.

		R	Relative reactivity[a] [%]	
			BCTA[b]	OATA[c]
L-**2a**	**1a**		n.r.[d]	100
L-**2b**	**1b**		30	23
L-**2d**	**1d**		87	n.r.
L-**2f**	**1f**		86	n.r.
L-**2g**	**1g**		100	n.r.

[a] Relative reactivity represents the initial reaction rate (i.e., conversion $<15\%$), normalized to that of the most-reactive substrate. [b] Substrates: L-amino acid (20 mM) and compound **1j** (20 mM). [c] Substrates: (*S*)-α-MBA (20 mM) and keto acid (20 mM). [d] n.r. = not reactive (i.e., relative reactivity $<1\%$).

OATA, L-alanine (L-**2a**) could not serve as a shuttling substrate because it was a non-reactive amino donor toward BCTA. Conversely, we had to rule out the three branched-chain amino acids (L-**2d**, L-**2f**, and L-**2g**) because their corresponding keto acids (**1d**, **1f**, and **1g**) were not reactive toward OATA. However, it was encouraging to find that L-homoalanine (L-**2b**) and 2-oxobutyric acid (**1b**) showed substantial reactivities toward BCTA and OATA, respectively. This result enabled us to explore the feasibility of coupled BCTA/OATA reactions by using compound L-**2b** as the shuttling substrate, so that the thermodynamic constraint of the BCTA reaction could be eliminated by the action of OATA.

To perform the coupled BCTA/OATA reactions, three substrate components were required: 1) an α-keto acid, 2) a primary amine, and 3) a shuttling substrate (i.e., compound L-**2b**). Compared with the first two components, compound L-**2b** was added to the reaction mixture in a much lower concentration because the shuttling substrate was not consumed, but rather regenerated from the OATA reaction. As a result, compound L-**2b** mediated the cascade transfer of an amino group from the primary amine onto the target keto acid. Because the BCTA reaction was reversible, the accumulation of compound **1b** allowed a reverse reaction to occur (i.e., transamination between a desired amino-acid product and compound **1b**) and, consequently, slowed the net reaction rate. Therefore, the concentration of compound **1b** should be kept as low as possible to suppress the undesirable reverse reaction. In contrast, the reverse ω-TA reaction between compound L-**2b** and a ketone was negligible because the amino-acceptor reactivities of most ketones are very low.[16a,24] For example, OATA only showed 0.20 and 0.05% reactivities with 2-butanone and acetophenone, respectively, relative to that of compound **1a** (see the Supporting Information, Table S1).

As a proof-of-concept, we monitored the progress of the cascade reaction to produce compound L-**2h** from compound **1h** (20 mM) by using (*S*)-α-MBA (30 mM) and compound L-**2b** (2 mM, 0.1 equiv relative to compound **1h**) as co-substrates (see the Supporting Information, Figure S1). The K_{eq} value of the transamination between compounds **1h** and L-**2b** was measured to be 0.24,[25] thus predicting that the maximum conversion of compound **1h** into compound L-**2h** catalyzed by BCTA alone was only 7% under the above-specified substrate conditions. However, the unfavorable BCTA reaction could be driven to completion by simultaneously performing the ω-TA reaction (see the Supporting Information, Figure S1A), thereby resulting in the stoichiometric conversion of prochiral **1h** into enantiopure L-**2h** (*ee* > 99%) with 99% conversion. We found that compound **1b** initially accumulated and then the concentration gradually decreased (see the Supporting Information, Figure S1B). This result was presumably because the BCTA reaction was faster than the OATA reaction during the early stage of the reaction under the enzyme concentrations (i.e., both 10 U mL^{-1}). Indeed, we observed that increasing the OATA concentration at the constant BCTA level led to a decrease in the initial accumulation of compound **1b** (see the Supporting Information, Figure S2). The initially accumulated compound **1b** was completely converted back into

compound L-**2b** at the end of the cascade reaction (see the Supporting Information, Figure S1 B), thus indicating that OATA indeed served as an "equilibrium shifter", driving the efficient recycling of compound L-**2b**. Taken together with the initial dosage of compound L-**2b** relative to compound **1h**, the number of recycles of compound L-**2b** was 10.

Because the cascade amino-group transfer reaction was successful, we moved onto investigating the asymmetric syntheses of various unnatural amino acids from 100 mM of the keto-acid substrates. To this end, isopropylamine (IPA) was chosen instead of (*S*)-α-MBA as an amino donor for OATA because IPA was much cheaper than (*S*)-α-MBA and its deamination product (i.e., acetone) is highly volatile. In addition, OATA has shown substantial amino-donor reactivity towards IPA (43% reactivity relative to (*S*)-α-MBA).[26] To determine the enzyme concentrations, we performed a series of coupled reactions in which various enzyme levels, that is, 10–30 U mL^{-1} BCTA and 1–100 U mL^{-1} OATA, were used with 100 mM compound **1h**, 5 mM compound L-**2b**, and 150 mM IPA (see the Supporting Information, Table S2). The highest conversion among the 13 runs was achieved at concentrations of 20 and 50 U mL^{-1} for BCTA and OATA, respectively, which led us to use these enzyme concentrations. As shown in Table 2, unnatural L-α-amino acids that contained aliphatic side chains (Table 2, entries 1–4 and 6) were prepared with conversions higher than 94% within 18 h and excellent enantiopurities. Among these various unnatural amino acids, compound L-**2h** is an essential component of HIV-protease inhibitors[17a] and of chiral ligands for asymmetric synthesis,[27] L-norvaline (L-**2c**) is a key intermediate in perindopril (an ACE inhibitor),[28] and L-3-hydroxyadamantylglycine (L-**2m**) is a building block of saxagliptin, which is under development for the treatment of type-2 diabetes.[29] Owing to the broad substrate specificity of BCTA, the coupled reaction also enabled the efficient preparation of unnatural aromatic amino acids, such as L-phenylglycine (L-**2k**; Table 2, entry 5). Besides unnatural amino acids, the BCTA/OATA coupled reaction also enabled the efficient synthesis of natural branched-chain amino acids under the same conditions as mentioned above, that is, L-valine (L-**2d**) and L-leucine (L-**2f**) with 92 and 97% conversion, respectively, at 9 h (*ee* > 99% in both cases). Because 0.05 molar equivalents of compound L-**2b** relative to the keto-acid substrate were used, the number of recycles of the shuttling substrate was 20.

Table 2. Asymmetric synthesis of unnatural L-α-amino acids from α-keto acids by using isopropylamine as an amino donor.[a]

Entry	Substrate	*t* [h]	Conversion[b] [%]	Product (*ee* [%])
1	**1c**	9	99	L-**2c** (>99)
2	**1e**	9	97	L-**2e** (>99)
3	**1h**	18	94	L-**2h** (>99)
4	**1i**	5	98	L-**2i** (>99)
5	**1k**	12	95	L-**2k** (>99)
6	**1m**	18	94	L-**2m** (>99)

[a] Reaction conditions: α-keto acid (100 mM), compound L-**2b** (5 mM), IPA (150 mM), PLP (0.5 mM), BCTA (20 U mL^{-1}), and OATA (50 U mL^{-1}) in phosphate buffer (50 mM, pH 7). [b] Conversions were based on the consumption of the α-keto-acid substrate.

To extend this cascade-transfer concept to the production of unnatural D-amino acids, we explored the feasibility of coupling a DATA reaction with an ω-TA. Despite broad substrate specificity toward a diverse range of D-α-amino acids, the industrial implementation of DATA reactions has lagged because of the neutral equilibrium position, just like in the BCTA reactions. For example, the K_{eq} value for transamination between compound **1j** and D-homoalanine (D-**2b**) for the preparation of D-glutamic acid (D-**2j**) was 0.79.[30]

For the DATA/ω-TA coupled reactions, ω-TA should possess *R* stereoselectivity, thereby enabling the regeneration of the D-amino-acid substrate that is required for the DATA reaction. Because IPA is an ideal amino donor for ω-TA reactions, we tested three known *R*-selective ω-TAs, that is, those cloned from *Aspergillus terreus*,[31] *Aspergillus fumigatus*,[31] and *Arthrobacter* sp. (ARTA),[32] to search for a suitable ω-TA that showed substantial activity towards IPA. Disappointingly, all of these three wild-type ω-TAs showed very little reactivity of IPA (i.e., less than 1% reactivity relative to (*R*)-α-MBA). Therefore, we chose the variant of ARTA (AR_{mut}TA) that was previously engineered by Savile et al. through the laboratory for amination evolution of various bulky ketones by using IPA.[33] Indeed, the reactivity of IPA relative to (*R*)-α-MBA was 8%.[34]

To determine the optimal shuttling substrate, the amino-donor specificity of DATA from *Bacillus sphaericus* towards D-amino acids and the amino-acceptor specificity of AR_{mut}TA towards the corresponding keto acids were examined (Table 3). It has been reported that the parental enzyme of AR_{mut}TA does not show any reactivity toward α-keto acids that contain bulkier side chains than an ethyl group,[32] reminiscent of the strong steric constraints in the active-site pocket of OATA. Despite the multiple mutations to endow AR_{mut}TA to accept ketones with bulky substituents,[33] AR_{mut}TA was found to retain the steric constraints in terms of the accommodation of α-keto acids, because the amino-acceptor reactivities of compounds **1a**, **1b**, **1d**, and **1f** were similar to those observed with ARTA.[32] Taken together with the comparable reactivities of compounds D-**2a** and D-**2b** with DATA, compound D-**2a** was chosen as the shuttling amino acid for the coupled DATA/AR_{mut}TA reactions.

Table 3. Determination of a shuttling substrate for coupled DATA/AR_{mut}TA reactions.

Substrate		Relative reactivity [%]	
Amino acid	Keto acid	DATA toward amino acids[a]	AR_{mut}TA toward keto acids[b]
D-**2a**	**1a**	100	100
D-**2b**	**1b**	99	18
D-**2d**	**1d**	2	n.r.[c]
D-**2f**	**1f**	4	n.r.

[a] Substrates: D-amino acid (20 mM) and compound **1j** (20 mM). [b] Substrates: (*R*)-α-MBA (20 mM) and keto acid (20 mM). [c] n.r. = not reactive (i.e., relative reactivity < 1%).

We performed the asymmetric synthesis of seven D-amino acids from prochiral keto acids (100 mM except for compound **1l**) by using compound D-**2a** (0.05 mol equiv to the keto acid) and IPA (1.5 equiv) as co-substrates (Table 4). The coupled

Table 4. Coupled DATA/AR_{mut}TA reactions for the preparation of D-α-amino acids.[a]

Entry	Substrate	*t* [h]	Conv.[b] [%]	Product (*ee* [%])
1	**1c**	3	98	D-**2c** (>99)
2	**1d**	7	96	D-**2d** (>99)
3	**1e**	5	96	D-**2e** (>99)
4	**1f**	7	99	D-**2f** (>99)
5	**1j**	5	99	D-**2j** (>99)
6	**1k**	12	97	D-**2k** (>99)
7	**1l**[c]	3	99	D-**2l** (>99)

[a] Reaction conditions: α-keto acid (100 mM), compound D-**2a** (5 mM), IPA (150 mM), PLP (0.5 mM), DATA (5 U mL^{-1}), and AR_{mut}TA (50 U mL^{-1}) in phosphate buffer (50 mM, pH 7). [b] Conversions were based on the consumption of the keto-acid substrate. [c] Concentrations of the three substrate components were halved because the solubility of phenylpyruvic acid (**1l**) was lower than 100 mM.

DATA/AR_{mut}TA reactions enabled the efficient synthesis of D-amino acids with >96% conversions and excellent enantiopurities (>99% *ee*) within 12 h. The resulting D-amino acids include the essential building blocks of pharmaceutical compounds: D-glutamic acid (D-**2j**) for spiroglumide (treatment of bowel disorders),[35] D-phenylglycine (D-**2k**) for ampicillin (antibiotics),[17b] and D-phenylalanine (D-**2l**) for nateglinide (antidiabetes).[36] Moreover, D-valine (D-**2d**) is used for the preparation of fluvalinate (insecticide).[37] Because DATA displays a broad substrate tolerance, this DATA/AR_{mut}TA strategy provides a robust synthetic method for the preparation of a diverse range of D-amino acids.

To demonstrate the scalability of the cascade transfer concept, we performed the preparative-scale syntheses of compounds L-**2h** and D-**2k** through BCTA/OATA and DATA/AR_{mut}TA coupled reactions, respectively, in a 50 mL reaction mixture that contained the α-keto acid (300 mM), the shuttling amino acid (20 mM), IPA (450 mM), α-TA (35 U mL^{-1}), and ω-TA (30 U mL^{-1}). Conversions of over 97% were attained within 24 h. Purification and structural characterization of the amino-acid products were performed (see the Supporting Information), which confirmed the recovery of pure compounds L-**2h** (1.37 g, 70% yield, >99% *ee*) and D-**2k** (2.02 g, 89% yield, >99% *ee*).

In conclusion, we have developed an industrially scalable strategy that affords the asymmetric synthesis of a diverse range of enantiopure unnatural amino acids by using α-TAs. The thermodynamic constraint of the α-TA reactions, which has hampered their synthetic applications, could be successfully eliminated by simultaneously performing an energetically favorable ω-TA reaction as an "equilibrium shifter". This TA approach is expected to provide a competent alternative to chemocatalytic methods,[3] in particular for the preparation of enantiopure amino acids, which are required for pharmaceutical applications (i.e., >99.5% *ee*).[38] Despite the remarkable advances in asymmetric Strecker syntheses,[3b] achieving such a high enantiopurity of the products in a single catalytic step remains challenging. A typical example of the biocatalytic asymmetric synthesis of unnatural amino acids is the industrial preparation of compound L-**2h** from compound **1h** by using amino-acid dehydrogenase (AADH), coupled with formate dehydrogenase for cofactor recycling.[6a,39] The AADH approach was also applied to preparation of other unnatural amino acids, such as compounds L-**2k**[40] and L-**2m**,[41] which necessitated three substrates and a cofactor (i.e., a target keto acid, ammonia, formate, and NADH). Although a much-smaller amount of NADH was required, its compulsory use as an expensive cofactor often imposed a significant burden in terms of production costs. In contrast, the TA approach lacks such an expensive component and instead employs stoichiometric amounts of the keto acid and IPA, as well as trace amounts of a shuttling substrate. Another drawback of the AADH approach is that a D-stereoselective AADH that possesses as broad substrate specificity as DATA is not present in nature.[42] In contrast, DATA is ubiquitous in living organisms and, hence, is promising for the asymmetric synthesis of a diverse range of D-amino acids, including compound D-**2k**, as illustrated herein. We expect that our approach is generally applicable to other α-TAs, such as aspartate transaminase and aromatic amino-acid transaminase, if a suitable shuttling substrate can be exploited.

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