One-Pot Conversion of L-Threonine into L-Homoalanine: Biocatalytic Production of an Unnatural Amino Acid from a Natural One

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Abstract: A novel biocatalytic process for production of L-homoalanine from L-threonine has been developed using coupled enzyme reactions consisting of a threonine deaminase (TD) and an ω -transaminase (ω -TA). TD catalyzes the dehydration/deamination of L-threonine, leading to the generation of 2-oxobutyrate which is asymmetrically converted to L-homoalanine via transamination with benzylamine executed by ω -TA. To make up the coupled reaction system, we cloned and overexpressed a TD from Escherichia coli and an (S)-specific w-TA from Paracoccus denitrificans. In the coupled reactions, Lthreonine serves as a precursor of 2-oxobutyrate for the ω -TA reaction, eliminating the need for employing the expensive oxo acid as a starting reactant. In contrast to a-transaminase reactions in which use of

Introduction

There has been a growing demand for optically pure compounds as chiral intermediates, chiral auxiliaries and resolving agents in the pharmaceutical and chemical industry.^[1] A recent survey made with 128 drug candidates under R&D development showed that as many as half of the drugs are chiral and contain two chiral centers on an average.^[2] Regulatory requirements for enantiomeric purities of the pharmaceutical compounds are usually higher than 99.5%,^[1a] which often requires complicated multistep procedures *via* organic synthesis strategies.^[1b,2] Therefore, most chemical and pharmaceutical companies have been expanding their R&D capacities in the biocatalysis sector.^[1a,2-3]

Unnatural amino acids attract ever-growing attention in pharmaceutical development owing to their use as building blocks for the synthesis of a number of chiral drugs.^[4] Because of difficulties in the fermenamino acids as an exclusive amino donor limits complete conversion, amines are exploited in the ω -TA reaction and thus maximum conversion could reach 100%. The ω -TA-only reaction with 10 mM 2-oxobutyrate and 20 mM benzylamine resulted in 94% yield of optically pure L-homoalanine (*ee* > 99%). However, the ω -TA-only reaction did not produce any detectable amount of L-homoalanine from 10 mM Lthreonine and 20 mM benzylamine, whereas the ω -TA reaction coupled with TD led to 91% conversion of L-threonine to L-homoalanine.

Keywords: asymmetric synthesis; coupled enzyme reactions; L-homoalanine; threonine deaminase; ω transaminase

tative production of unnatural amino acids, several biocatalytic approaches have been explored.^[5] These fall into two classes depending on the substrate chirality: (i) kinetic resolution of chemically modified forms of racemic amino acids^[5a] using amidases,^[6] hydantoinases^[7] and acylases,^[8] and (ii) asymmetric synthesis of enantiopure amino acids from achiral oxo acids^[9] using dehydrogenases (DHs)^[10] and transaminases (TAs).^[11] The asymmetric conversion is usually preferred because the theoretical maximum yield is 100% which is two times higher than that for the kinetic resolution.^[12] Besides, the asymmetric synthesis can do without racemization of an unwanted enantiomer which should be included in the kinetic resolution approach.^[13]

However, there are technical problems that remain to be resolved for the development of a cost-effective asymmetric synthesis of unnatural amino acids. First, prochiral oxo acid substrates are not readily available compared to racemic amino acids that can be easily

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prepared by Strecker synthesis.^[14] Second, driving the reaction to completion often requires an additional enzymatic reaction. For example, cofactor dependence of the DH reaction mandates coupling with a second DH for cofactor recycling.^[10] In contrast, TAs do not pose such a cofactor problem, which adds up to compelling features over the DH process. However, equilibrium positions for the TA reactions are usually neutral between substrates and products, which has made the industrial applications of TA reactions lag behind in spite of their high turnover rate, high enantioselectivity, broad substrate specificity and no cofactor requirement.^[11c,15]

TAs catalyze the transfer of an amino group from an amino acid to an oxo acid and thereby play an important role in amino acid metabolism.^[15,16] The enzyme can be classified into α -TA and ω -TA according to the relative position of the amino group to be transferred with respect to the carboxyl group of the substrate.^[15,16] Compared to an α -TA executing transamination exclusively between amino acids and oxo acids, an ω -TA exhibits distinctive substrate specificity enabling the oxidative deamination of primary amines.^[15] Therefore, in contrast to the neutral equilibrium position for most α -TA reactions (i.e., K_{eq} ≈ 1), the thermodynamic equilibrium for ω -TA reactions can be directed to completion using amines as an amino donor.^[17] This led us to develop several ω -TA processes for the production of enantiomerically pure chiral amines via kinetic resolution.[18]

In this report, we expand the product spectrum of ω -TA by demonstrating a novel cost-effective process for the production of L-homoalanine (L-1) which is one of the pharmaceutically important unnatural amino acids. L-1 serves as a key intermediate for the production of the antituberculosis drug ethambutol^[19] and the antiepileptic drug levetiracetam.^[20] In this study, the production process consists of two enzymatic reactions as shown in Scheme 1: conversion of Lthreonine (L-2) to 2-oxobutyrate (3a) by a threonine deaminase (TD) and asymmetric transfer of an amino group from benzylamine (4a) to 3a by an (S)-enantioselective ω -TA. The net reaction employs L-2 and 4a as substrates, yielding ammonia, benzaldehyde (3b) and L-1. The coupled reactions overcome the aforementioned obstacles to the asymmetric synthesis of amino acids. First, the TD reaction coupled with the ω -TA reaction permits substitution of the expensive oxo acid substrate (3a) by L-2 which is a cheap natural amino acid produced on a million-ton scale annually.^[5a] Second, use of the amine compound (4a) as an amino donor unleashes the thermodynamic constraint of the TA reactions, allowing high conversion yield without incorporation of another enzyme to remove one of the products as reported elsewhere.^[11] As a result, the coupled reaction system established a onepot conversion of a cheap natural amino acid into a



Scheme 1. Enzymatic coupled reactions to convert L-2 to L-**1.** The oxo acid substrate **3a** is supplied from L-**2** by the action of TD and then undergoes asymmetric amination by (S)-specific ω -TA with a concomitant deamination of **4a**.

pharmaceutically valuable unnatural amino acid without thermodynamic limitation to the final conversion.

Results and Discussion

Cloning and Functional Expression of TD and ω -TA

To carry out the coupled reactions shown in Scheme 1, we set out to clone two genes encoding TD and (S)-specific ω -TA into expression vectors. We chose a TD from Escherichia coli (ilvA) because the enzyme has been well characterized^[21] and homologous expression in E. coli cells was expected to guarantee highly functional expression. The *ilvA* gene was amplified by colony PCR using E. coli DH5a cells as a template and then cloned into a pET21a(+) expression vector using NdeI and XhoI restriction sites, leading to pET21-TD. Seven transformants were subjected to DNA sequencing and sequence alignment with the *ilvA* gene from E. coli K-12 showed that all transformants shared a single nucleotide mutation (G754A) which led to a D252N amino acid substitution. This seems to be a background mutation carried by the E. coli DH5a strain in our laboratory. Cellfree extracts prepared with E. coli BL21(DE3) cells transformed with pET21-TD displayed high TD activity, typically 300-750 U/mL, which was comparable to the expression result reported elsewhere.^[21a]

In the case of ω -TA, a BLASTP search (http:// blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out to identify a candidate gene using the protein sequence of (*S*)-specific ω -TA from *Vibrio fluvialis* JS17 (VF ω -TA) which has been extensively studied.^[22] Note that *E. coli* lacks an ω -TA activity. Among the homologous genes, we chose a putative class III aminotransferase from *Paracoccus denitrificans* PD1222 (94% identity to VF ω -TA) for gene cloning. Sequence

alignment with the ω -TAs from V. fluvialis JS17^[22] and Pseudomonas putida^[23] (see Figure S1 in the Supporting Information) shows that four invariant residues identified from sequence alignment of 32 TAs^[16] are conserved in the TA from P. denitrificans. These include D256 (H-bond with PLP), K285 (active site lysine) and R415 (salt bridge with the carboxylate of oxo acid substrates).^[24] The TA gene was cloned into pET21a(+) vector using the same procedures as those for the *ilvA* cloning, resulting in pET21-pdTA. However, enzyme activity from E. coli BL21(DE3) cells harboring the pET21-pdTA was too low to carry out further experiments. We presumed that the heterologous protein expression is toxic to cells and thus the transformants are prone to a spontaneous plasmid loss. To provide higher plasmid stability, the selection maker was changed from the ampicillin marker used in the pET21a(+) vector to a kanamycin marker.^[25] To this end, XbaI/XhoI-cut of the pET21-pdTA was inserted into a pET28a(+) vector digested with the same enzymes. To verify functional expression of the TA gene from the resulting vector (pET28-pdTA), cell-free extracts prepared with E. coli BL21(DE3) transformants were subjected to ω -TA activity assay performed with 20 mM (S)- α -ethylbenzylamine [(S)-**4b**] and 20 mM pyruvate (**3c**). Indeed, the cell-free extracts displayed high activity (typically 2-5 U/mL), showing that the TA gene cloned from P. denitrificans falls into a ω -TA class.

Substrate Specificity of PD ω-TA

The ω-TA cloned from P. denitrificans (PD ω-TA) was assigned to class III aminotransferase but its enzymatic properties have not been reported to date. To explore the suitability of the new ω-TA for the production of L-1, we first examined substrate specificity. Among six amino acceptors tested (three oxo acids and three aldehydes), 3c (a typical amino acceptor for ω -TAs) showed the highest reactivity (Table 1). The amino acceptor reactivity of 3a was as good as that of **3c**, indicating that PD ω -TA is promising for the production of L-1 from 3a. Trimethylpyruvate (3d) showed little reactivity, suggesting that the active site of PD ω-TA has a steric constraint in accommodating bulky side chains of oxo acids. Compared to α-TA reactions in which oxo acids are exclusively used as an amino acceptor, w-TAs show distinctive reactivity toward aldehydes.^[26] Indeed, PD ω-TA showed high reactivities toward an aromatic (3b) and short-chain aliphatic aldehydes (3e and 3f).

Given that **3a** is a good substrate for PD ω -TA, its amino donor specificity was investigated with six amine compounds (two chiral and four achiral) to select the best amino donor. As listed in Table 2, amines bearing an aromatic side chain [**4**a, (*S*)-**4b** and Table 1. Amino acceptor specificity of PD ω -TA.

Substrate			Relative reactivity [%] ^[a]
	\mathbf{R}^1	\mathbf{R}^2	
3a	CH ₃ CH ₂	СООН	100 ^[b]
3b	C_6H_5	Η	91.2 ± 13.6
3c	CH ₃	COOH	109.2 ± 12.2
3d	(CH ₃) ₃ C	COOH	<1
3e	CH_3CH_2	Н	48.1 ± 3.1
3f	$CH_3(CH_2)_2$	Η	95.4 ± 13.5

^[a] Relative reactivity represents the initial rate normalized by that for **3a** using (*S*)-**4b** as an amino donor.

^[b] Initial reaction rate for **3a** was $0.027 \pm 0.005 \text{ mM min}^{-1}$.

Table 2. Amino donor specificity of PD ω-TA.

Substrate	R^1 R^2		Relative reactivity [%] ^[a]
	\mathbf{R}^1	\mathbf{R}^2	
4a	C ₆ H ₅	Н	100 ^[b]
(S)- 4b	C_6H_5	CH_2CH_3	11.8 ± 5.4
(R)- 4b	C_6H_5	CH_2CH_3	< 0.1
(S)- 4 c	C_6H_5	CH_3	79.2 ± 12.6
(R)- 4c	C_6H_5	CH_3	0.5 ± 0.1
(<i>R</i> , <i>S</i>)-4d	CH_3CH_2	CH_3	0.7 ± 0.4
4e	CH_3CH_2	Н	0.7 ± 0.3
4f	$CH_3(CH_2)_2$	Η	0.5 ± 0.2

^[a] Relative reactivity was normalized by the initial reaction rate for **4a** using **3c** as an amino acceptor.

^[b] Initial reaction rate for **4a** was $0.12 \pm 0.1 \text{ mM min}^{-1}$.

(S)-4c] exhibit high reactivities, whereas short-chain aliphatic amines [(R,S)-4d, 4e and 4f] are inert. Compound 4a was found to be the best amino donor, leading us to select 4a as an amino donor for production of L-1.

PD ω -TA showed stringent stereoselectivity toward (S)-amines over the (R)-counterparts as shown in Table 2. Initial reaction rates for (R)-4b and (R)-4c were less than 1% of those for (S)-4b and (S)-4c, respectively. The excellent (S)-stereoselectivity of PD ω -TA was a prerequisite for asymmetric transfer of an amino group from 4a to 3a, leading to production of enantiopure L-1 as shown in Scheme 1.

Kinetic Investigations to Optimize Reaction Conditions

Enzymes often display complicated kinetic properties. A thorough understanding of such kinetic behavior including enzyme inhibition is crucial in determining optimal reaction conditions. TD is a typical enzyme

exhibiting feedback inhibition and activation by endproducts of relevant metabolic pathways, i.e., isoleu-cine and valine, respectively.^[21,27] Although these amino acids are not present in the reaction medium for the coupled reactions shown in Scheme 1, the allosteric regulation raises a possibility of enzyme inhibition by L-1 owing to the structural similarity to isoleucine and valine. Indeed, L-1 is known to act as an inhibitor with a dissociation constant (K_D) of 12.7 mM^[21b] which is comparable to the K_D value for its own substrate L-2 (8.0 mM).^[21a] The low K_D value for L-1 suggests that conversion of L-2 to 3a would better be completed before L-1 is accumulated by transamination to a level high enough to repress the TD activity. This led us to use a TD concentration much higher than that of ω -TA, so the L-1 generation to a level detrimental to the TD activity is delayed until most L-2 is converted to 3a.

 ω -TA is known to exhibit enzyme inhibition at high concentrations of substrates and products,^[17,28] whereas TD from *E. coli* does not display such a behavior.^[21] We examined if the ω -TA activity is adversely affected by high concentrations of substrates, that is, **3a** and **4a**. As shown in Figure 1a, **3a** was found to exert substrate inhibition. The enzyme reaction rate declined at concentrations higher than 40 mM. In contrast, **4a** did not show enzyme inhibition up to 150 mM (Figure 1b). In the case of product inhibition, **3b** provoked severe enzyme inhibition, whereas L-1 did not affect enzyme activity up to 300 mM. Residual enzyme activity was 19% at 5 mM **3b**. Taken together, enzyme inhibition by the keto product was identified as a major hurdle to carrying out efficient ω -TA reactions, which was also observed in the previous studies for the kinetic resolution of chiral amines.^[18]

Optimal pH for the Coupled Reactions

The coupled reactions shown in Scheme 1 consist of two enzymes which show different optimal pHs. The optimal pH for the TD from *E. coli* was reported to be 7.^[21] However, the PD ω -TA showed the highest activity at pH 9.0 as shown in Figure 2a. The alkaline pH for the optimal enzyme activity was also observed with VF ω -TA,^[22] which is likely to result from the increase in effective concentration of the deprotonated amino donor that benefits the formation of an external aldimine.^[26] Because enzyme stability is another important factor in choosing an operational pH for long-term enzyme reactions, we examined the pH dependence of the enzyme stability (Figure 2b). Although the pH optimum for ω -TA activity is 9, enzyme stability is greatly reduced at alkaline pHs.



Figure 1. Dependence of ω -TA activity on the concentrations of substrates (3a and 4a) and products (3b and L-1). a) Substrate inhibition by 3a. b) Effect of the concentrations of 4a. c) Product inhibition by 3b. d) Effect of the concentrations of L-1.

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Figure 2. Effect of pH on a) the enzyme activity and b) the enzyme stability of PD ω -TA. Relative activity represents normalized initial rates measured at 10 mM 3a, 10 mM 4a and 50 mM phosphate buffer (pH 7).



Figure 3. Enhancement of PD ω -TA stability by PLP. Enzyme incubation was carried out in 50 mM phosphate buffer (pH 7) at 37 °C.

Residual enzyme activities were 84 and 8% after 2-h incubation at pH 7 and 9, respectively. Taken together with the optimal pH for the TD, this result led us to employ pH 7 as the medium pH for the coupled reactions in Scheme 1.

Because TA is a typical pyridoxal 5'-phosphate (PLP)-dependent enzyme, its stability is greatly affected by the presence of PLP in the reaction medium.^[22] As shown in Figure 2b, enzyme activity decreased by 16% after 2 h-incubation at pH 7. However, the activity loss was completely protected by $1 \mu M$ PLP (Figure 3). At the PLP concentrations higher than $10 \,\mu\text{M}$, the enzyme activities were even substantially improved. The activity enhancement might be ascribed to PLP binding and subsequent reactivation of the apoenzyme that is dissociated from pyridoxamine 5'-phosphate (PMP). Note that TA is present in two forms; E-PLP in which PLP is covalently attached to the active site lysine and E-PMP where PMP is noncovalently bound to the enzyme. The stability result indicates that at least 1 µM PLP should be supplemented for long-term operational stability.

Production of L-1 from L-2 by the Coupled Reactions

Before carrying out the coupled reactions, we examined individual enzymatic conversions. As shown in Figure 4, stoichiometric conversion of L-2 to 3a by TD was observed. For example, starting with 10 mM L-2, the residual concentration of L-2 was 3.2 mM and that of 3a produced was 6.5 mM after 10 min reaction. The stoichiometric conversion was also observed with transamination between 3a and 4a (Figure 5). To ensure complete amination of 3a, the concentration of 4a was 2-fold higher than that of 3a. After 5-h reaction, conversion of 3a to L-1 reached 94% and the enantiometric excess of L-1 was higher than 99%.

In the coupled reactions shown in Figure 6, to minimize the aforementioned inhibition of TD activity by L-1, the TD concentration was 5-fold higher than that used in the single conversion reaction shown in Figure 4. Owing to the high TD concentration, L-2 is depleted much faster compared to the reaction in Figure 4 and the residual amount was not detectable after 20 min. The concentration of **3a** increased at the



Figure 4. Conversion of L-2 to **3a** by TD. *Reaction conditions:* 10 mM L-2, 1 U/mL TD and 50 mM phosphate buffer (pH 7).

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Figure 5. Production of L-1 from 3a and 4a using the ω -TA reaction. *Reaction conditions*: 10 mM 3a, 20 mM 4a, 2 U/mL ω -TA, 1 μ M PLP and 50 mM phosphate buffer (pH 7).



Figure 6. Production of L-1 from L-2 and 4a using the coupled enzyme reactions. *Reaction conditions:* 10 mM L-2, 20 mM 4a, 5 U/mL TD, 2 U/mL ω -TA, 1 μ M PLP and 50 mM phosphate buffer (pH 7).

early stage of the coupled reactions and began to decrease at 20 min, indicating that generation of **3a** from L-**2** by TD was much faster than conversion of **3a** to L-**1** by ω -TA as we intended. Conversion yield of **3a** was 91% after 5-h reaction and the overall reaction progress based on L-**1** concentration was very similar to that shown in Figure 5. This result is indicative that the conversion of L-**2** to **3a** did not limit the ω -TA reaction and L-**2** effectively served as the precursor of **3a**. When either TD or ω -TA was employed alone in the reaction mixture containing L-**2** and **4a**, no detectable amount of L-**1** was produced.

Conclusions

Recently, ω -TA is gaining increasing interest owing to its great utility for the production of chiral amines *via* kinetic resolution and asymmetric synthesis.^[29] This study clearly shows that product pipeline obtainable from the ω -TA reactions can be extended to one of the pharmaceutically important unnatural amino acids (L-1) with the expensive oxo acid problem resolved by coupling with the TD reaction for generation of the oxo acid substrate from a cheap amino acid. Our approach exploiting w-TA for asymmetric conversion achieves two technical advances over the previous $DH^{[10]}$ and α -TA^[11] reactions for amino acid production. First, compared to DH reactions, use of an expensive cofactor and an additional DH for cofactor recycling is not necessary.^[10] Second, in contrast to the α -TA reactions in which complete conversion is frustrated by unfavorable reaction equilibrium, no additional enzyme is required to remove one of the products for driving the reaction to completion.^[11] Moreover, in contrast to the α -TA reactions in which the residual amino acid used as an amino donor is often troublesome in the product separation, benzylamine used as a cosubstrate in our approach can be simply removed from the aqueous reaction mixture by base treatment followed by solvent extraction.

As the purpose of this study is to explore the feasibility of one-pot conversion from L-2 to L-1, we did not implement extractive reaction processes to mitigate the ω -TA inhibition by **3b**. However, the development of large-scale processes involving high substrate concentrations for future work should address strategies to overcome the enzyme inhibition problem, that is, biphasic reaction systems or enzyme membrane reactors with a separate extraction module as previously studied for kinetic resolutions of chiral amines.^[18a,b]

Experimental Section

Gene Cloning

Cloning of the *ilvA* gene from *E. coli* and the ω -ta gene from P. denitrificans PD1222 into pET expression vectors (Novagen, WI) was carried out by standard molecular biology techniques.^[30] Oligonucleotide primers for the PCR amplification were purchased from Bioneer, Inc. (Daejon, Korea). Enzymes for the cloning experiments including Taq DNA polymerase and restriction enzymes were from New England Biolabs (Edison, NJ). E. coli DH5a cells were used for all cloning experiments. The coding regions of the *ilvA* and ω -ta genes were amplified by colony PCR using E. coli DH5a and P. denitrificans PD1222 cells as templates, respectively. Primers for the PCR amplification of ilvA were forward (5'-GATATACATATGGCTGACTCGCAACCCCTG-3') and reverse (5'-GTGGTG<u>CTCGAG</u>ACCCGCCAAAAAGAA-3'). Primers for the ω -ta gene amplification were forward (5'-GATATACA (5'-TATGAACCAACCGCAAAGC-3') and reverse GTGGTG<u>CTCGAG</u>GGCCACCTCGGCAAA-3'). The forward and reverse primers carry NdeI and XhoI restriction sites as underlined, respectively. The amplified genes were cloned into NdeI/XhoI-linearized vector pET21a(+), resulting in pET21-TD and pET21-pdTA. Cloning products were confirmed by restriction analysis and DNA sequencing. In the case of ω -TA, enzyme activity from the pET21-pdTA was too low to carry out more experiments. Therefore, to exchange the selection marker, XbaI/XhoI-cut of the pET21-pdTA was inserted into a pET28a(+) vector digested with the same enzymes. The resulting plasmid (pET28-pdTA) was used for further experiments.

Functional Expression and Preparation of Cell-free Extracts

E. coli BL21(DE3) cells transformed with pET21-TD or pET28-pdTA were cultivated in 300 mL LB medium supplemented with appropriate antibiotics. When the cell OD₆₀₀ reached approximately 0.5, enzyme expression was induced by IPTG (final concentration = 1 mM) and the cells were allowed for overnight cultivation at 37 °C. Cells were harvested by centrifugation (10,000×g, 20 min, 4 °C) and resuspended in 15 mL resuspension buffer (Tris-HCl 50 mM, NaCl 50 mM, β-mercaptoethanol 1 mM, PMSF 0.1 mM, PLP 20 μ M, pH 7.0). The cell suspension was subjected to ultrasonic cell disruption and then the supernatant solution obtained after centrifugation (17,000×g, 30 min, 4 °C) was employed as cell-free extracts for enzyme reactions.

Enzyme Assay

Unless otherwise specified, enzyme assays were carried out at 37 °C and pH 7 (50 mM phosphate buffer). Typical reaction volume was 200 μ L and the reaction was stopped by adding 75 μ L of 16% (v/v) perchloric acid after 10 min reaction. One unit of TD activity was defined as the enzyme amount catalyzing the formation of 1 μ mol of **3a** in 1 min at 50 mM L-**2**. One unit of ω -TA activity was defined as the enzyme amount catalyzing the formation of 1 μ mol of propiophenone in 1 min at 20 mM (*S*)-**4b** and 20 mM **3c**. For TD and ω -TA activity assays, **3a** and propiophenone, respectively, were analyzed by HPLC.

Substrate Specificity

Initial reaction rates were compared to examine substrate specificity. Six amino acceptors and eight amino donors were tested. To examine amino acceptor specificity, 10 mM (S)-4b and 10 mM amino acceptor were used. Initial reaction rates were measured by analyzing propiophenone, which were normalized by the initial rate for 3a. In the case of amino donor specificity, 10 mM amino donor and 10 mM 3c were used. Initial reaction rates were measured by analyzing L-alanine, which was normalized by the initial rate for 4a. Conversions were less than 10% in the initial rate measurements which were independently triplicated.

Substrate and Product Inhibition

Initial reaction rates were measured to examine substrate and product inhibition in the ω -TA reaction between **3a** and **4a**. In all cases, 0.09 U/mL PD ω -TA and 50 mM phosphate buffer (pH 7.0) were used. Reaction conditions to examine substrate inhibition by **3a** and **4a** were 2–150 mM **3a** and 20 mM **4a**, and 10 mM **3a** and 10–150 mM **4a**, respectively. Product inhibition by **3b** was measured at 10 mM **3a**, 10 mM **4a** and 0–5 mM **3b**. Reaction conditions to examine product inhibition by L-1 were 10 mM **3a**, 10 mM **4a** and 0–300 mM L-1. Initial reaction rates were measured by analyzing **3b**. To examine product inhibition by **3b**, initial rate calculations were carried out by subtracting initial concentrations of **3b** from final ones in the reaction mixtures. Reaction rates represent mean values of at least three independently measured initial rates.

Determination of Optimal pH

Initial reaction rates using 10 mM **3a** and 10 mM **4a** were measured to determine the optimal pH for PD ω -TA. Buffer species (final concentration = 50 mM) used were citrate (pH 5–6), potassium phosphate (pH 7, 11) and borate (pH 8–10). To measure enzyme stability at different pH values, ω -TA was incubated in the buffer solutions at 37 °C. After the predetermined incubation time, aliquots of the enzyme solution (10 μ L) were added to 190 μ L reaction mixture containing 10 mM **3a**, 10 mM **4a** and 50 mM phosphate buffer (pH 7) to measure initial rates.

Enzymatic Conversions

For time-course monitoring of single-step conversions and coupled enzyme reactions, aliquots of reaction mixture (100 μ L) were taken at predetermined reaction times and subjected to HPLC analysis. Typical initial volume of the reaction mixtures was 2 mL and the reaction mixture was incubated under magnetic stirring at 37 °C. For reaction mixture preparation to carry out the coupled enzyme reactions, 25 μ L of 400 U/mL TD solution and 800 μ L of 5 U/mL ω -TA solution were mixed with 200 μ L of 100 mM L-2, 200 μ L of 200 mM 4a (pH adjusted to 7), 2 μ L of 1 mM PLP and 200 μ L of 500 mM phosphate buffer (pH 7). Distilled water was additionally added to set the reaction volume to 2 mL. Aliquots of the reaction mixture were taken 7 times for 5 hours, which were subjected to HPLC analysis for time-course monitoring of L-1, L-2 and 3a.

Analytical Methods

All HPLC analyses were performed with a Waters HPLC system (Milford, MA). Analysis of propiophenone and 3b was performed using a Symmetry HPLC column (Waters, USA) with isocratic elution of 60% methanol/40% water (0.1% trifluoroacetic acid in both) at 1 mLmin⁻¹. Detection was done with a UV detector tuned at 254 nm. Retention times were 5.5 min for **3b** and 8.7 min for propiophenone. Quantitative analysis of amino acids was carried out with a Ultrasphere ODS column (Beckman, USA) after derivatization with *o*-phthaldialdehyde as described elsewhere.^[31] Linear gradient elution was done from 9 to 19% acetonitrile/Na₂HPO₄ buffer (12.5 mM, pH 7.2) for 25 min at a flow rate of 1.5 mL min⁻¹. UV detection was done at 330 nm. Retention times were 24.4 min for alanine and 19.3 min for 2 and 26.8 min for 1. Chiral analysis of 1 was carried out using the Symmetry HPLC column after derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate as described elsewhere.^[32] Isocratic elution of 50% methanol/ 50% NaH₂PO₄ buffer (10 mM, pH 3.0) was used at 1 mLmin⁻¹. UV detector was set to 250 nm. Retention times were 6.2 min for L-1 and 8.4 min for D-1. Compound 3a was analyzed with an Aminex HPX-87H column (Bio-Rad,

USA) using isocratic elution of 5 mM of H_2SO_4 solution at a flow rate of 1.0 mLmin⁻¹. Column oven temperature was set to 40 °C and UV detection was carried out at 210 nm. Retention time for **3a** was 5.9 min.

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