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An efficient single-enzymatic cascade for asymmetric synthesis of chiral amines catalyzed by ω-transaminase†

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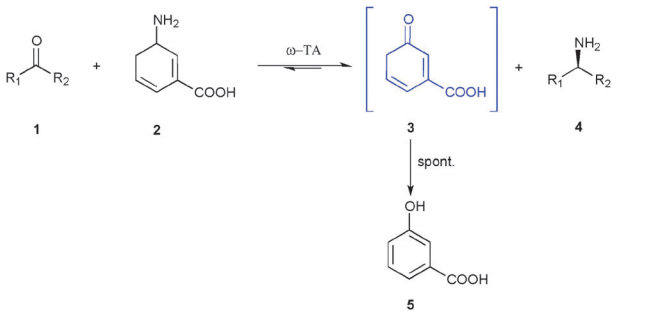
An efficient single-enzymatic cascade approach for the asymmetric synthesis of chiral amines has been developed, which applies the amino donor 3-aminocyclohexa-1,5-dienecarboxylic acid spontaneously tautomerizing to reach reaction completion with excellent ee values.

There is an increasing demand for enantiomerically pure chiral amines, which are pivotal building blocks for the development of biologically active compounds including pharmaceuticals and agrochemicals.[1–8] The use of amine transaminases, also known as ω-transaminases (ω-TAs), for the purpose of producing chiral amines has been shown to be both feasible and effective.[2,9–14] Although ω-TA catalyzed asymmetric synthesis theoretically gives a 100% yield of the desired optically pure amines, there is a disadvantage that equilibrium usually favors the substrate over the product during transamination,[2] resulting in incomplete transformation. The challenge in asymmetric synthesis with ω-TAs is thus to shift the equilibrium to the product side, which is usually achieved mainly by means of (i) overcoming problems of substrate–product inhibition, and (ii) solving issues related to poor conversion of ketones to amines.

As described by a plethora of recent papers centered on ω-TA catalyzed transaminations, much effort has been devoted to solving the issues relating to unfavored equilibrium.[15–17] This unfavored equilibrium has also been exploited to perform a complete synthesis of pyridoxamine 5′-phosphate.[18] Special interest has been shown in the area of multi-step enzyme cascade reactions where the equilibrium is usually shifted towards the product side by removing the formed ketone *via* one or several enzyme-catalyzed reactions, thus allowing a theoretical yield of 100%.[2,17,19–21] Nevertheless, multi-enzyme cascade reactions are complex systems where the reaction conditions are usually a compromise to ensure that all enzymes coexisting in the same system are active. Generally, the compromised conditions are the optimum neither for ω-TA, nor for the enzyme(s) applied in the secondary irreversible reactions, or are the optimum for one enzyme (either ω-TA or other coexisting enzymes) and suboptimal for others. Therefore, under such conditions, the enzymes display only part of their optimum performance (activity and enantioselectivity). Thus, even though multi-enzyme cascades can successfully overcome problems relating to the reaction equilibrium, the major disadvantage remains that the majority of the enzymes in the reaction system cannot show their intrinsically full activities.[2,20–27] Therefore, new and widely applicable protocols that can overcome the deficiencies mentioned above are highly desirable.

In this communication, we present a novel single-enzymatic cascade approach for the preparation of chiral amines which applies ω-TA as the biocatalyst and a commercially available 3-aminocyclohexa-1,5-dienecarboxylic acid, **2**, as the amino donor (Scheme 1). No additional enzymes, expensive cofactors or special measures are required for the purpose of pushing the equilibrium to the product side either by combining secondary irreversible reactions or removing volatile substances. In transamination, the generated ketone **3** (Scheme 1) is effectively removed by spontaneous tautomerization to 3-hydroxybenzoic acid **5**,[28,29] thus allowing a theoretical yield of 100%.



Scheme 1 Effective synthesis of chiral amines through the transamination of ketones by using an (*S*)- or (*R*)-selective ω-TA. The employment of racemic 3-aminocyclohexa-1,5-dienecarboxylic acid drives the reaction to completion *via* spontaneous and instant transformation from the product ketone to 3-hydroxybenzoic acid, pushing the equilibrium to the product side, allowing a theoretical yield of 100%.

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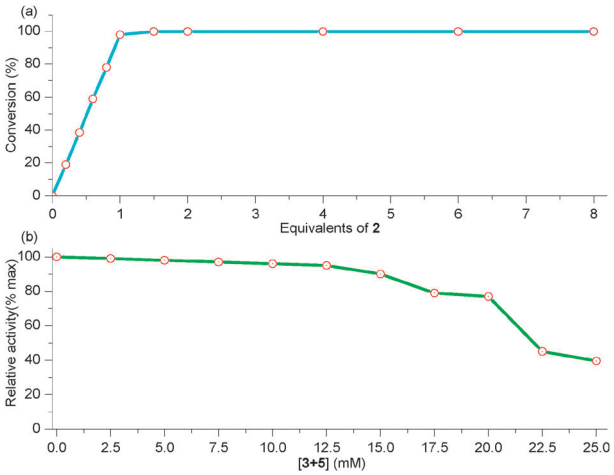


Fig. 1 (a) Profile of conversion of acetophenone *vs.* concentration of **2** (equivalents); (b) effect of the concentration of a mixture of **3** + **5** on the initial rate of Cv-ω-TA W60C obtained by following the formation of 1-phenylethylamine at 270 nm.

During transamination, prochiral ketone **1** and amino donor **2** are treated with ω-TA and pyridoxal 5′-phosphate (PLP). Successful substrates are converted to the corresponding chiral amines and ketone **3**, which is unstable and spontaneously transforms to phenol **5**. The tautomerization of **3** to **5** serves the triple purpose of (i) introducing secondary irreversible reactions, (ii) removing ketone inhibition towards ω-TA, and (iii) shifting equilibrium to the product side.

To establish the basis for the designed single-enzymatic cascade approach, initial studies were performed in HEPES buffer (50 mM, pH 7.0) using acetophenone as the model amino acceptor and the *Chromobacterium violaceum* ω-transaminase variant W60C (*Cv*-ω-TA W60C) as the biocatalyst. In our previous work we successfully obtained the *Cv*-ω-TA W60C mutant with enhanced substrate specificity, however its optimum pH value was shifted from the original 8.2 of the wild type to 7.0.[9] In transamination, the concentration of acetophenone was set at 5 mM and varied concentrations of amino donor **2** were explored to find the suitable amount of amino donor for a full conversion of acetophenone. The collected data showed that even when amino donor **2** was used at an equivalent amount, a nearly ideal conversion of up to 98% could be achieved (Fig. 1a). Reaction times were longer compared with those employing excess amino donor **2**, and when a bit more than one equivalent of amino donor **2** was introduced, for instance, 1.05 equivalent, conversions of up to >99% could be achieved (Table 1). In addition, the stoichiometric amount of amino donor will also relieve inhibition towards ω-TA, possibly caused by the excess amino donor. In the following studies, all reactions were performed with a ratio of 1 : 1.05 (prochiral ketone/amino donor) to make the prochiral ketones undergo complete conversions.

The inhibition caused by the generated co-product **3** was also evaluated, by continuously adding **5**, which tautomerizes to ketone **3**, to the reaction solution. The concentration of ketone **3** was increased and initial reaction rates *versus* varied concentrations of **3** + **5** were measured by following the formation of 1-phenylethylamine at a wavelength of 270 nm (Fig. 1b). It was observed that within the range of 0–20 mM, only a slight decline in the initial rate could be observed, and when the concentration reached 20 mM, the transaminase still maintained 80% of its highest activity. Nevertheless, when the concentration exceeded 20 mM (Fig. 1b), an obvious decline in the initial reaction rate was thus observed. As a stoichiometric amount of amino donor will be sufficient for a nearly full conversion, a maximum of 5 mM (product **5**) will be formed no matter how much amino donor **2** is used. This is far less than the upper limit (20 mM) of ketone **3** + product **5** above which inhibition occurs. The possible pH shift, which would probably result in the loss of transaminase activity, was also tested. The result showed that no detectable pH change was found, indicating that in the designed system, no deactivation caused by the pH shift exists.

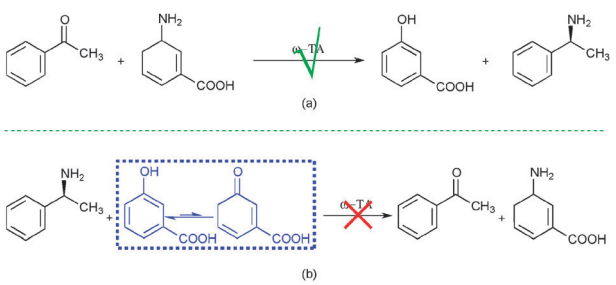
The possible reverse reaction was also investigated by employing the generated co-product **5**, which tautomerizes to ketone **3**, as the amino acceptor, (*S*)-1-phenylethylamine as the amino donor (Scheme 2a and b) and *Cv*-ω-TA W60C as the biocatalyst. Data collected showed that even after reaction for 36 h, no acetophenone was detected, indicating that no detectable reverse reaction occurred during transamination in the system. This might be the reason for why a full conversion can be achieved even when amino donor **2** was employed at only a bit more than one equivalent excess. In addition, it also indicates that there seems to be no equilibrium existing in the assay due to the tautomerization reaction.

To further explore the versatility of the approach, a group of transaminases listed in Table 1 were tested. After 12 hours, samples were taken and analyzed by GC to calculate the conversions. Similar

Table 1 Investigation of the generality of amino donor **2** when subjected to various transaminases[a]

Entry	Transaminase	Ratio (**2**/**1**)	Abs. Config[b]	Conv[c] (%)
1	*Cv*-ω-TA wild type	1 : 1	*S*	97
2	*Cv*-ω-TA wild type	1.05 : 1	*S*	>99
3	*Cv*-ω-TA W60C	1 : 1	*S*	98
4	*Cv*-ω-TA W60C	1.05 : 1	*S*	>99
5	*Cv*-ω-TA F88A/A231F	1.05 : 1	*S*	>99
6	ATA-113	1.05 : 1	*S*	>99
7	ATA-117	1.05 : 1	*R*	>99
8	ω-TA-001	1.05 : 1	*S*	>99

[a] Reactions were performed on a 1 mL scale, conditions: ketone, 5 mM; temperature, 37 °C; the pH value for all enzymes was 8.2 except for *Cv*-ω-TA W60C for which the pH value was 7.0; reaction time, 12 h. [b] Absolute configuration was determined by comparison with standard samples. [c] Conversions were determined by GC-analysis with an internal standard.



Scheme 2 (a) Asymmetric synthesis of (*S*)-1-phenylethylamine with an (*S*)-selective ω-transaminase by applying acetophenone and 3-aminocyclohexa-1,5-dienecarboxylic acid as the model amino acceptor and donor, respectively; (b) reverse reaction using 3-hydroxybenzoic acid as the amino acceptor and (*S*)-1-phenylethylamine as the amino donor.

Table 2 Asymmetric syntheses by the designed single-enzymatic cascade approach[a]

Entry	Transaminase	Product	Abs. Config[b]	pH	ee (%)	Conv[c] (%)
1	*Cv*-ω-TA W60C		*S*	7.0	>99	>99
2	*Cv*-ω-TA wild type		*S*	8.2	>99	>99
3	*Cv*-ω-TA F88A/A231F		*S*	8.2	>99	>99
4	ATA-113		*S*	8.2	>99	>99
5	ATA-117		*R*	8.2	>99	>99
6	ω-TA-001		*S*	8.2	62	>99
7[d]	*Cv*-ω-TA wild type		*S*	8.2	>99	>99
8[d]	*Cv*-ω-TA W60C		*S*	7.0	>99	>99
9[d]	*Cv*-ω-TA wild type		*S*	8.2	26	>99
10[d]	*Cv*-ω-TA W60C		*S*	7.0	41	98
11	*Cv*-ω-TA wild type		*S*	8.2	>99	>99
12	*Cv*-ω-TA W60C		*S*	7.0	>99	>99
13	*Cv*-ω-TA wild type		*S*	8.2	>99	>99
14	*Cv*-ω-TA W60C		*S*	7.0	>99	>99

[a] Unless otherwise stated, all reactions were performed on a 1 mL scale; conditions: prochiral ketone concentration, 5 mM; amino donor **2**, 5.25 mM; temperature, 37 °C; the pH value for all enzymes was 8.2 except for *Cv*-ω-TA W60C for which the pH value was 7.0; reaction time, 12 h; ee values were determined by HPLC analysis with a chiral column based on formed chiral amines. [b] Absolute configuration was determined by comparison with standard samples. [c] Conversions were determined by GC-analysis and calculated by referring to 1-phenylethanol as internal standard. [d] Prochiral ketone concentrations were set at 2 mM.

to the reaction catalyzed by *Cv*-ω-TA W60C (Table 1, entry 4), most of these reactions proceed to conversions >99% with the ratio of prochiral ketone/amino donor of 1 : 1.05. In all reactions with various ω-TAs, the substrate acetophenone was successfully converted to the corresponding (*S*)- or (*R*)-1-phenylethylamine (Table 1), though some ee values differed (Table 2, entry 1–6).

After obtaining the optimized conditions for the designed system in hand, an array of prochiral ketones were then subjected to the approach with different ω-TAs under their respective optimized conditions. The results are summarized in Table 2. As shown, all substrates were successfully converted to the corresponding chiral amines in excellent conversions. Compared with the multi-enzymatic procedure described in our previous work, which applied a large excess of isopropylamine (>310-fold),[2] the present assay requires approximately one equivalent of amino donor to successfully reach full conversions, remove equilibrium and reduce inhibition caused by both the excess amino donor and newly formed ketones.

In conclusion, we have developed a novel single-enzymatic cascade assay for the asymmetric synthesis of chiral amines employing 3-aminocyclohexa-1,5-dienecarboxylic acid, **2**, as the amino donor. Remarkably, during transamination, (i) no additional enzymes, chemicals or expensive cofactors are required aiming at shifting the equilibrium to the product side, and an ideal conversion of 100% can be achieved; (ii) reactions can proceed under the optimum conditions of the applied ω-TA; (iii) reactions can be performed with less enzyme loading as only one enzyme is required and its full activity is utilised compared with multi-enzymatic assays; (iv) the application of only one equivalent of amino donor relieves inhibition toward ω-TA caused by an excess amino donor. It is expected that the assay will be useful for many applications, especially for medical research, in pharmaceutical and green chemistry.

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