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Deracemization of Racemic Amines to Enantiopure (*R*)- and (*S*)-amines by Biocatalytic Cascade Employing ω-Transaminase and Amine Dehydrogenase

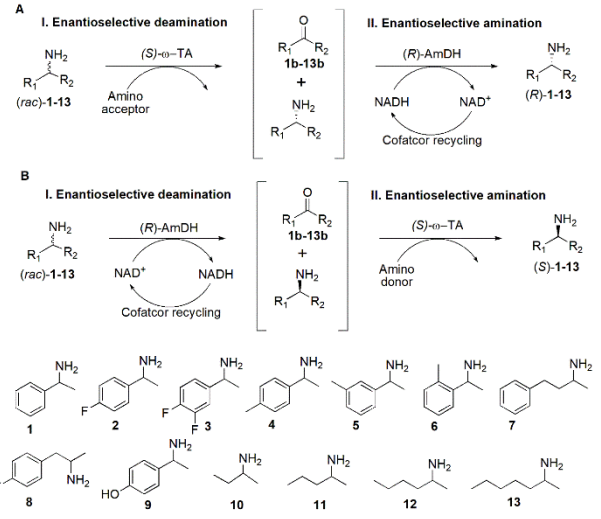
Sanghan Yoon,† Mahesh D. Patil,† Sharad Sarak, Hyunwoo Jeon, Geon-Hee Kim, Taresh P. Khobragade, Sihyong Sung, and Hyungdon Yun*

Abstract: A one-pot deracemization strategy for α-chiral amines is reported involving an enantioselective deamination to the corresponding ketone followed by a stereoselective amination by enantiocomplementary biocatalysts. Notably, this cascade employing a ω-transaminase and amine dehydrogenase enabled the access to both (*R*)-and (*S*)-amine products, just by controlling the directions of the reactions catalyzed by them. A wide range of (*R*)-and (*S*)-amines was obtained with excellent conversions (>80%) and enantiomeric excess (>99% *ee*). Finally, preparative scale syntheses led to obtain enantiopure (*R*)- and (*S*)-**13** with the isolated yields of 53 and 75%, respectively.

Enantiopure chiral amines have been described as indispensable constituents of nearly 50% small molecule pharmaceuticals.[1] The chemical synthesis of enantiopure chiral amines is challenging owing to the use of toxic intermediates, formation of large number of by-products, thereby complicating the system and ultimately increasing the overall costs.[2] In this context, biocatalytic methods have been recognized as major 'green technologies' that provide sustainable methods for the synthesis of enantiopure chiral amines. Moreover, the judicial integration of chemical syntheses and 'greener' enzymatic approaches is highly desirable for the increasing demands of the enantiopure amines in pharmaceutical, fine chemicals, and agricultural industries.[2,3]

The practical application of one of the widely used strategies for the synthesis of chiral amines, i.e. kinetic resolution, is limited by the maximum theoretical yield of 50%.[4] On the other hand, deracemization is an attractive strategy, especially when racemic amines can easily be accessed compared to their corresponding prochiral ketones.[5] Deracemization strategies enable to achieve theoretical complete conversion by employing two stereocomplementary enzymes.[6] Since the successful demonstration of lipase-catalyzed dynamic kinetic resolution of amines,[7] various other biocatalysts have been explored for their application in deracemization for the synthesis of chiral amines. For instance, the group of Turner used the variants of monoamine oxidase from *Aspergillus niger* in tandem with nonselective chemical reductants for the deracemization of a wide range of primary, secondary and tertiary amines.[8] Recently, coupling of a nonselective chemical reducing agent with reductive aminases has been shown to efficiently deracemize racemic amines.[9] Yasukawa et al. generated *R*-stereoselective amine oxidase from porcine kidney D-amino acid oxidase and used it for the deracemization of α-methylbenzylamine.[10] Recent years have evidenced effective deracemization protocols for the synthesis of important pharmaceuticals such as mexiletine,[11] rasagiline and salsolidine,[9] to name a few.

Various enzymes such as ω-transaminases (ω-TAs) and amine dehydrogenases (AmDHs)[12] have emerged as viable options for the biocatalytic syntheses of chiral amines. Although ω-TAs have been well documented in deracemization;[11,13] the utility of AmDHs in deracemization protocols to produce enantiopure amines is still unexplored. Although, the synthesis of (*R*)-amines have been sucessfullyl carried out by easier strategies such as AmDH-catalyzed reductive amination of ketones.[1a,1d] Also, recent years have evidenced the improved array of new (*R*)-seletive ω-TAs for the scalable synthetic applications.[13d] Since AmDHs exhibit exclusive selectivity towards (*R*)-amines,[1d, 12a] they open a new avenue of a class of enzymes representing enantiocomplementarity to well reported (*S*)-selective ω-TAs. We envisaged to utilize (*R*)-AmDHs in tandem with enantiomerically complementary (*S*)-ω-TA to constitute a deracemization cascade enabling the access to both the enantiomers [(*R*)- and (*S*)] of a range of chiral amines. We herein report a one-pot deracemization strategy which proceeds *via* two steps: 1) Enantioselective deamination of racemic amine substrates to corresponding ketones and 2) Stereoselective amination of the ketones generated in step 1 yielding enantiopure amine products with theoretical yield of 100% [**Scheme 1**].



Scheme 1. Deracemization of racemic amines to enantiopure A) (*R*)-amines and B) (*S*)-amines using a cascade employing (*S*)-ω-transaminase and (*R*)-amine dehydrogenase

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It is worth emphasizing that both the enantiomers [(*R*)- and (*S*)-] of the amine products could be easily accessed, notably using the same pair of enantiocomplementary biocatalysts, just by controlling the directions of the constituent reactions catalyzed by applied biocatalysts.

We commenced this study with the establishment of deracemization protocol for the synthesis of (*R*)-amines [**Scheme 1A**]. To examine the AmDH-catalyzed enantioselective amination, two (*R*)-AmDHs, *Rs*-AmDH (developed from L-phenylalanine dehydrogenase of *Rhodococcus* sp.)[14a] and chimeric AmDH (Chi-AmDH)[14b] were cloned, expressed and purified [**ESI**]. *Rs*-AmDH and Chi-AmDH have been reported to exhibit good activity toward aromatic and aliphatic ketone substrates, respectively.[14] Thus, 4-phenyl-2-butanone **7b** and 2-heptanone **13b** were used as model substrates for *Rs*-AmDH and Chi-AmDH, respectively. To select the operational pH, we examined the reactivity of purified AmDHs at varying pH from 7.0 to 10.5. *Rs*-AmDH and Chi-AmDH exhibited the maximum specific activity of 353 and 305 mU/mg toward **7b** and **13b**, respectively at pH 9.5 [**Fig. S1 and Fig. S2**]. To ensure the efficient regeneration of NADH cofactor, formate dehydrogenase (FDH) from *Pseudomonas* sp. 101 (Uniprot ID. No. P33160.3) and glucose dehydrogenase (GDH) from *Bacillus subtilis* (Uniprot ID. No. P12310-1)[14c] systems were tested, wherein FDH consistently performed better than GDH system and >99% conversion of both the model substrates could be achieved [**Fig. S3 and Fig. S4**].

Since whole-cell systems are advantageous in enabling simpler handling and cost-effective biotransformations,[15,16] the applicability of the AmDH-FDH reaction system was tested in whole-cell transformations. A reaction was carried out with varying amounts of cells (0.5 to 10 mg_{DCW}/mL), wherein 5 mg_{DCW}/mL of *Rs*-AmDH cells (One-cell two-vector system co-expressing *Rs*-AmDH and FDH) achieved comparatively better conversion (96%) than that by Chi-AmDH cells (One-cell two-vector system co-expressing Chi-AmDH and FDH) (80%). Nevertheless, 10 mg_{DCW}/mL of both *Rs*-AmDH and Chi-AmDH cells could successfully transform >99% of their corresponding substrates in 24 h [**Fig. S5 and Fig. S6**].

Once the AmDH system was instituted, we turned our attention on establishing the ω-TA system. For the selection of efficient ω-TA catalyzing enantioselective deamination of racemic amines, whole cells separately expressing four ω-TAs (3.33 mg_{DCW}/mL each) were reacted with 20 mM *rac*-2-aminoheptane (*rac*-**13**) and *rac*-4-phenyl-2-aminobutane (*rac*-**7**) in the presence of 20 mM pyruvate and 0.1 mM PLP. While all the four ω-TAs efficiently catalyzed the enantioselective deamination of 13 and yielded 10 mM (50% conversion) (*R*)-**13**, only ω-TAVF and ω-TAPO exhibited better activity towards **7** [**Fig. S7 and Fig. S8**]. Moreover, the concentration of desired (*R*)-enantiomer in the case of ω-TAVF decreased over the time. Thus, ω-TAPO was used in the subsequent experiments.

Having the individual AmDH and ω-TA whole-cell systems in hand, we further checked their application in tandem for the deracemization of two model substrates i.e. *rac*-**7** using *Rs*-AmDH - ω-TAPO and *rac*-**13** using Chi-AmDH - ω-TAPO system. Both, *Rs*-AmDH - ω-TAPO and Chi-AmDH - ω-TAPO systems could achieve good conversions and yielded 84.4% (*R*)-**7** and 86% (*R*)-**13**, respectively from their corresponding racemic substrates in 24 h. Organic co-solvents such as DMSO have been reported to improve the solubility of the hydrophobic substrates and thereby yields of the enzymatic syntheses.[17] The use of DMSO in the present study improved the generation of (*R*)-**7** and (*R*)-**13**; and complete conversions were achieved when the concentration of DMSO was increased up to 15 % (v/v) [**Fig. S9 and Fig. S10**].

The successful deracemization of racemic model substrates to enantiopure amine products encouraged us to check the applicability of this system for the synthesis of wide range of (*R*)-amines. A panel of 13 racemic amine substrates was tested, among which complete conversion was achieved only for four substrates [System 1 in **Table 1**]. Low conversions albeit of excellent *ee* values obtained for the racemic substrates implied that the activity of AmDH was inadequate. Furthermore, low expression level of AmDH was identified as a limiting factor for the modest conversions achieved for majority of the substrates [**Fig. S11**].

In order to increase the expression levels of AmDHs, the gene encoding AmDH in pET-duet vector was transferred to pET-24ma vector, and that of FDH in pET-24ma was transferred to PQE80L vector [**Fig. S12**]. The vector system used for the expression of (*S*)-ω-TAPO was unaltered. This newly designed vector strategy improved the expression levels of AmDHs [**Fig. S13**]. Although expression level of FDH marginally reduced, the limiting factor of poor AmDH expression was circumvented, which ultimately resulted in excellent conversions (~80-100%) of all the racemic substrates, except one, to their corresponding (*R*)-amines with excellent enantioselectivities (>99 *ee*) [System 2 in **Table 1**].

Table 1. Deracemization of racemic amines to (*R*)-amines using the whole-cell biocatalytic cascade employing (*S*)-ω-TAPO and (*R*)-AmDH

Sub.	(*S*)-ω-TAPO – (*R*)-Rs-AmDH				(*S*)-ω-TAPO – (*R*)-Chi-AmDH			
	System 1[a]		System 2[b]		System 1[a]		System 2[b]	
	Conv (%)[c]	ee^R (%)	Conv (%)[c]	ee^R (%)	Conv (%)[c]	ee^R (%)	Conv (%)[c]	ee^R (%)
rac-**1**	66.9	>99	96.5	>99	72.9	>99	87.7	>99
rac-**2**	66	>99	100	>99	73	>99	94.8	>99
rac-**3**	66.7	>99	100	>99	71.6	>99	81.8	>99
rac-**4**	63.4	>99	100	>99	70.2	>99	95.7	>99
rac-**5**	63.6	>99	100	>99	73	>99	96.4	>99
rac-**6**	72.5	>99	100	>99	72.5	>99	96.9	>99
rac-**7**	100	>99	100	>99	100	>99	100	>99
rac-**8**	100	>99	100	>99	100	>99	100	>99
rac-**9**	72.6	>99	100	>99	68	>99	89.5	>99
rac-**10**	52.3	>99	61.1	>99	69	>99	75.7	>99
rac-**11**	77.1	>99	79.4	>99	87.8	>99	96.9	>99
rac-**12**	100	>99	100	>99	100	>99	100	>99

rac-**13**	100	>99	100	>99	100	>99	100	>99

Reaction conditions: 10 mM racemic substrate, 10 mM pyruvate, 0.1 mM PLP, 3.3 mg_{DCW}/mL ω-TAPO cells, 30 mg_{DCW}/mL AmDH-FDH cells, 37 °C, 100 mM Glycine buffer (pH 9.5) and 200 mM ammonium formate, 15% (v/v) DMSO, 24 h

[a] System 1: AmDH-pET-duet; FDH-pET-24ma; (*S*)-ω-TAPO- pET-24ma

[b] System 2: AmDH-pET-24ma; FDH-PQE80L; (*S*)-ω-TAPO- pET-24ma

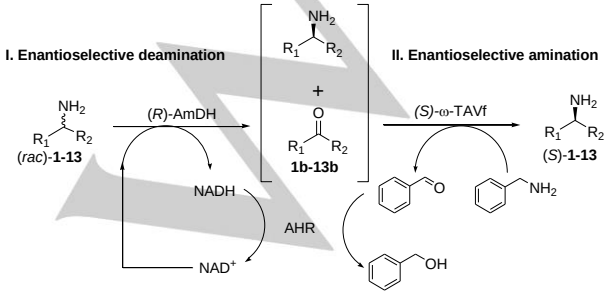
[c] Conversion was defined as percentage ratio of the (*R*)-amine product to initial racemic amine substrate;[13a]

$$\frac{[(R)\text{-amine product (mM)}]}{[\text{Initial racemic amine substrate (mM)}]} \times 100$$

…

Having successfully demonstrated the deracemization of racemic amines to their corresponding (*R*)-amines, we further decided to check the usability of this system for the deracemization of racemic amine substrates to their corresponding (*S*)-enantiomers. For the synthesis of (*S*)-amines, the first step of enantioselective deamination was catalyzed by (*R*)-AmDH, followed by the enantioselective amination by (*S*)-ω-TA [**Scheme 1B**]. However, compared to the deracemization of racemic amines to (*R*)-amines, the equilibrium of both the constituent reactions in the synthesis of (*S*)-amines is highly unfavourable. For instance, the first step of AmDH-catalyzed enantioselective deamination for the synthesis of (*S*)-amines is unfavorable since the pertinent function of AmDHs is the reductive amination of ketones to amines and not the deamination of racemic amines to their corresponding ketones.[12] Moreover, ω-TA-catalyzed asymmetric synthesis of enantiopure amines from prochiral ketones also suffers from a unfavorable reaction equilibrium compared to that of kinetic resolution of racemic amines.[18]

To establish the ω-TA system catalyzing the enantioselective amination of prochiral ketone substrates to their corresponding chiral amines, four purified (*S*)-ω-TAs were tested for their reactivity towards model substrates **7b** and **13b** using alanine as an amino donor. Alanine dehydrogenase (AlaDH) was used to recycle pyruvate (deaminated alanine) back to alanine and to regenerate nicotinamide cofactor used in the first step of AmDH-catalyzed enantioselective deamination. However, AlaDH system performed poorly when AmDH and (*S*)-ω-TA were combined for the deracemization of racemic amines [Data not shown]. We thus decided to use benzylamine as an amino donor, wherein the deaminated benzylamine i.e. benzaldehyde is utilized by aldehyde reductase (AHR) from *Synechocystis* sp.[19] to give benzyl alcohol and regenerate NAD^+ cofactor for AmDH [**Scheme 2**].



Scheme 2. Deracemization of racemic amines to enantiopure (*S*)-amines using an AHR recycling system

To check the applicability of the AHR system in *Rs*-AmDH-catalyzed enantioselective deamination, a reaction was carried out using 10 mM *rac*-**7** in the presence of 10 mM benzaldehyde. This reaction yielded 5 mM **7b** (50% Conv.), implying that AHR could efficiently regenerate NAD^+ for the AmDH-catalyzed enantioselective deamination [**Fig. S14**]. Following this, the efficiency of AHR system was tested for another constituent reaction of this deracemization protocol, i.e. (*S*)-ω-TA-catalyzed enantioselective amination of prochiral ketones to their corresponding (*S*)-amines. Three purified (*S*)-ω-TAs were examined for their reactivity towards model ketone substrate **7b** using the AHR system and varying pH from 8.5-9.5. Among the tested enzymes, (*S*)-ω-TAVF exhibited marginally better activity irrespective of the reaction pH [**Fig. S15**]. (*S*)-ω-TAVF could successfully transform 94.5% of **7b** to (*S*)-**7** at pH 8.5.

Having successfully demonstrated the individual constituent reactions i.e. AmDH-catalyzed enantioselective deamination of racemic amine substrate and ω-TA-catalyzed enantioselective amination of prochiral ketone, we further checked their application in combination for the deracemization of model substrate *rac*-**7** to (*S*)-**7**. Although excellent conversion of 90% could be achieved, mediocre enantiomeric excess only of 91% was attainable [**Fig. S16**]. Unreacted amounts of (*R*)-**7** in the reaction mixture suggested that the activity of AmDH was inadequate and the poor *ee* values obtained could be consequent to the poor drive of the reaction. Ideally, generating a driving force for the forward reaction is a key episode in the cascade reactions.[16] We anticipated that the addition of trivial quantity of benzaldehyde could help to drive the reaction in the forward direction, as more substrate would be easily available for AHR and thereby efficient regeneration of nicotinamide cofactor would be enabled. Thus, 0.2 mM benzaldehyde was added in the subsequent experiments. As benzylamine serves as an amino donor, increasing concentration of benzylamine was anticipated to drive the ω-TA-catalyzed reaction towards the formation of (*S*)-amines. Thus, further experiments were performed with the increasing amounts of benzylamine ranging from 20 to 60 mM and fixed amount of benzaldehyde (0.2 mM) [**Fig. S17**]. However, the increasing concentration of benzylamine negatively affected the activity of AHR [**Fig. S18**]. Thus, further experiment was performed by varying the amounts of benzaldehyde and fixing the benzylamine concentration at 20 mM. It was observed that increasing the concentration of benzaldehyde beyond 0.2 mM decreased the ee of the (*S*)-amine product. The best combination (20 mM benzylamine and 0.2 mM benzaldehyde) resulted in the improved conversion to 100% and *ee* to 95.4% [**Fig. S19**].

Following the successful improvements in conversion and *ee* values using the purified enzyme system, we extended this deracemization protocol to a whole-cell system expressing AmDH-AHR and (*S*)-ω-TA. The addition of 1 mM of benzaldehyde resulted in the excellent conversion of 100 and *ee* of >99% [**Fig. S20**].

The robustness of the established deracemization protocol was evaluated for the synthesis of a wide range of (*S*)-amines. A panel of 13 racemic amine substrates was tested, wherein this deracemization protocol could successfully catalyze the synthesis of (*S*)-amine products with excellent conversions (~80-100%) and enantioselectivities (>99% *ee*) [**Table 2**].

Table 2. Deracemization of racemic amines to (*S*)-amines using the whole-cell biocatalytic cascade employing (*R*)-AmDH and (*S*)-ω-TAVF

Substrate	(S)-ω-TAVF– (*R*)-Rs-AmDH		(S)-ω-TAVF– (*R*)-Chi-AmDH	
	Conv (%)[a]	ee^S (%)	Conv (%)[a]	ee^S (%)
rac-**1**	100	>99	98	97
rac-**2**	100	>99	100	>99
rac-**3**	100	>99	100	>99
rac-**4**	100	>99	100	>99
rac-**5**	100	>99	100	>99
rac-**6**	100	>99	100	>99
rac-**7**	100	>99	100	>99
rac-**8**	100	>99	100	>99
rac-**9**	100	>99	100	>99
rac-**10**	61.4	23	80.4	61
rac-**11**	76.0	52	85.0	71
rac-**12**	98.0	97	100	>99
rac-**13**	100	>99	100	>99

Reaction conditions: 10 mM racemic substrate, 20 mM Benzylamine, 1 mM Benzaldehyde, 0.1 mM PLP, 10 mg_{DCW}/mL ω-TAVF Cells, 27 mg_{DCW}/mL AmDH-AHR Cells, 37 °C, 100 mM Tris buffer (pH 8.5), 15% (v/v) DMSO, 24 h

[a] Conversion was defined as percentage ratio of the (*S*)- amine product to initial racemic amine substrate;[13a] $\frac{[(S)-\text{amine product (mM)}]}{[\text{Initial racemic amine substrate (mM)}]} \times 100$

Finally, to demonstrate the synthetic applicability of the biocatalytic cascade, deracemization of a model substrate *rac*-**13** was carried out on a preparative-scale [20 mM in 25 mL reaction volume; 57.5 mg] [ESI, Section 4] using the reaction conditions optimized for analytical-scale reactions. The results demonstrated that complete deracemization of *rac*-**13** substrate could be achieved to obtain enantiopure (*R*)-**13** and (*S*)-**13** (*ee* >99%) [**Fig. S21 and Fig S22**]. *R*)-**13** and (*S*)-**13** from these reactions were isolated with the yields of 53 and 75% respectively [**Fig. S23**]. These results confirm that this biocatalytic cascade is promising for the preparative scale deracemization of racemic substrates to obtain valuable enantiopure amine compounds.

In summary, we have successfully developed an efficient biocatalytic cascade catalyzing the deracemization of racemic amines by employing AmDH and (*S*)-ω-TA. This reaction enabled the access to a range of chiral amine products, notably to both the enantiomers, with excellent conversions ranging from 80-100% and enantioselectivities (>99%). It is worth emphasizing that the absolute configuration of the amine products could be easily switched, just by controlling the directions of the constituent reactions of the biocatalytic cascade catalyzed by the same pair of enantiocomplementary enzymes. The broad substrate specificity of both the enzymes i.e. ω-TA and AmDH imply that the biocatalytic systems employing these enzymes, such as demonstrated herein, would enjoy large applicability in biocatalytic syntheses for chiral amines.

Experimental Section

Details of the experimental procedures are provided in the Supporting Information.

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Keywords: Biocatalysis • chiral amines • deracemization • transaminases • amine dehydrogenases

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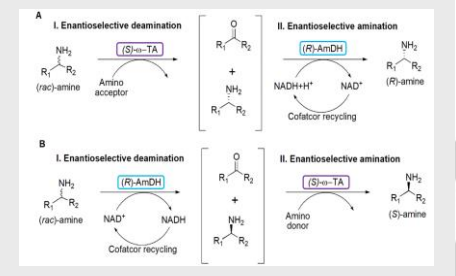
COMMUNICATION

Novel one-pot deracemization cascade, employing amine dehydrogenase and ω-transaminase, is reported for the synthesis of enantiopure amines with excellent conversions



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Deracemization of Racemic Amines to Enantiopure (*R*)- and (*S*)-amines by Biocatalytic Cascade Employing ω-Transaminase and Amine Dehydrogenase