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Investigation of one-enzyme systems in the ω-transaminase-catalyzed synthesis of chiral amines

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ABSTRACT

ω-Transaminase (TA) catalyzed asymmetric syntheses of amines were carried out in the one enzyme systems with wild-type enzymes (*S*)-TA from *Pseudomonas aeruginosa*, (*S*)-TA from *Paracoccus denitrificans* and (*R*)-TA from *Aspergillus terreus*. The scope of amine donors and aromatic carbonyl substrates was thoroughly explored. Among the range of potential amino donors, 2-propylamine, 2-butylamine and 1-phenylethylamine were found as promising candidates, which gave superior conversions in the amination reactions compared to other donors. Various prochiral aromatic ketones were accepted as substrates by the investigated enzymes. In most cases, good to excellent conversions (up to 98%) to the amine products with excellent e.e.-values (>99.9% for (*S*) or (*R*)) were obtained by the action of a single enzyme and an appropriate amino donor. (*S*)-TA from *Paracoccus denitrificans* was found to accept bulky ketones, e.g. 1-indanone, α- and β-tetralone or 2-acetonaphthone, in the asymmetric amination. In some cases the enantiomeric excesses in the amination reactions were dependent on the amino donor. Moreover, the influence of the pH, temperature and cosolvents on the outcome of reactions was additionally investigated.

1. Introduction

Amine transaminases or ω-transaminases (ω-TA) are biocatalysts of great importance for the production of chiral primary amines, which represent a major class of compounds used in pharmaceutical industry and (bio)chemistry [1,2]. ω-TAs catalyze the transfer of an amino group from an amino donor onto a carbonyl moiety, utilizing pyridoxal-5′-phosphate (PLP) as cofactor. Thereby the reaction mixture consists of two amines (an amino donor and a product) and two carbonyl compounds (a ketone substrate and a by-product) (Scheme 1). Both, (*S*)- and (*R*)-transaminases have been found and well described so far [3–5]. The enzymes are highly stereoselective and, thus, have great potential for the direct asymmetric amination, where chiral amines are generated with high enantiomeric excesses (e.e.) directly from an achiral ketone using inexpensive amino donors [4,6,7].

Limitations arising during the asymmetric synthesis of amines, such as unfavourable equilibrium, substrate and product inhibition, poor thermostability, insufficient substrate specificity and sometimes low enantioselectivity of the catalyst, still have to be overcome for an efficient production of a wide range of amines on industrial scale [8]. Recently, medium [9–14] as well as protein engineering [15–17] was applied to broaden the scope of applications of transaminases towards bulky substrates, e.g. aminotetralin [15], sitagliptin [16,18] and to decrease the substrate and product inhibition [19]. In order to shift the equilibrium to the product side, several methods are used [4,6,10]: (a) addition of an excess of amino donor; (b) in situ removal of a product or a by-product [4]; (c) auto-degradation of the by-product [20,21]; (d) use of enzymatic cascades [22] and application of whole cell catalysis [23].

It was shown that the major hurdles in the asymmetric synthesis of amines from ketones lie mainly in the selection of the right amino donor [24]. Most approaches use alanine as amino donor. As the underlying reaction equilibrium favours ketone formation, additional efforts are required to shift the equilibrium towards the synthesis of the desired chiral amines. The coupling of a transamination reaction to a second reaction where the by-product

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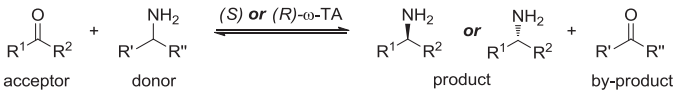


Scheme 1. Asymmetric synthesis of (*S*)- and (*R*)-amines using ω-transaminases.

is consumed is the preferred method to drive the reaction towards the production of an amine. Among the most often used methods to accomplish this endeavour is the removal of formed pyruvate from the reaction mixture using either lactate dehydrogenase [25] or alanine dehydrogenase [9]. Pyruvate decarboxylase [26] and acetolactate synthase [12] were also employed for the degradation of pyruvate to shift the equilibrium towards the amination reaction. On the other hand, the use of several enzymes in one pot is not always desirable for an industrial application, as it increases the cost of the overall process and often requires an additional step for cofactor regeneration. Other amino donors investigated so far led to a more favourable product equilibrium compared to alanine. Inexpensive 2-propylamine can be used as alternative donor [19,27–29] and the formed by-product acetone might be removed by yeast alcohol dehydrogenase [10] or by evaporation [19]. In principle, chiral amines such as 1-aminoindane, 1-aminotetralin, 1-phenylethylamine (1-PEA) or achiral 1-phenylmethanamine are thermodynamically favourable for the transamination and good donors for ω-TA, however due to high costs they cannot be applied on a large scale [30]. Thus, the search for alternative amino donors, which would be efficient in reductive amination, inexpensive and lead to a by-product that can be easily removed from the reaction mixture, is of great importance.

Herein, we present our research on three wild-type ω-transaminases (*S*)-TA *Pseudomonas aeruginosa* (Pae) [31], (*S*)-TA *Paracoccus denitrificans* (Pde) [22,32,33] and (*R*)-TA *Aspergillus terreus* (Ate) [5,34]. The enzymes were applied for the synthesis of aromatic chiral amines using different amine donors. Moreover, the substrate spectrum, enzyme inhibition and influence of the reaction conditions on the conversion and enantioselectivity were investigated. Proper selection of the amino donor/amino acceptor pair is the essential factor in the successful transamination.

2. Experimental

2.1. General

All chemicals were purchased from Sigma Aldrich, Fluka, Acros Organics, TCI Europe or Alfa Aesar, unless stated otherwise. All enzymes for genetic work were purchased from Fermentas (now part of Thermo Scientific Molecular Biology). HPLC analyses were conducted on an Agilent 1100 or Agilent 1200 instrument. More detailed information is provided in the supporting information.

2.2. Production of ω-transaminases

The gene (gene ID: 115385557) encoding the ω-(*R*)-transaminase (XP_001209325) from *A. terreus* was ordered codon-optimized for *E. coli* from GeneArt (Life Technologies). The gene was recloned into the expression vector pMS470Δ8-vector via the restriction sites *Nde*I and *Hin*dIII. The genes (gene ID: 9951072 and 119386691) encoding the ω-(*S*)-transaminases (AE004893) from *P. aeruginosa* and (YP_917746) from *P. denitrificans* were PCR amplified from plasmids provided by DSM and ligated via the restriction sites *Nde*I and *Hin*dIII into pMS470Δ8-vector. The three constructs were transformed into *E. coli* TOP10F' cells (Invitrogen).

E. coli TOP10F' cells harbouring pMS470-Ate, pMS470-Pae or pMS470-Pde were grown in LB medium supplemented with ampicillin (80 μg/ml) at 37 °C. Expression of recombinant protein was initiated by addition of 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) to OD600 ~0.8 cultures, and cultivation was continued at 25 °C for 20 h. Protein expression and localization in the supernatant was confirmed by SDS-PAGE. The cells were harvested, resuspended in cold buffer (50 mM potassium phosphate buffer pH 7.5 containing 0.1 M PLP) and disrupted by sonication (Branson Sonifier S-250; 6 min, 80% duty cycle, 7 output control). The cell lysate was centrifuged for an hour at 50,000 g to remove unbroken cells and insoluble material. The cell free lysate was filtered through a 0.45 μm syringe filter and concentrated to about 40 mg/ml using Vivaspin 20 Centrifugal Filter Units (10,000 Da molecular-weight cut-off; Sartorius). The total protein concentration was analyzed by Bradford protein assay (Biorad).

2.3. General procedure for ω-transaminase catalyzed reactions

Sodium phosphate reaction buffer (50 mM, pH 7.5) containing 0.1 mM PLP was mixed with the ketone substrate (10 or 50 mM) and the appropriate amino donor (0.1–0.5 M). The pH was adjusted to 7.5 with phosphoric acid before adding the enzymes. The reactions were started by adding 50 μl of cell-free extract (CFE, contains approximately 2 mg of protein) and then shaken at 30 °C for 24 h.

2.4. Analysis of reactions

2.4.1. Determination of conversions

50 μl from the reaction mixture were added to 950 μl of an acetonitrile/water mixture (50:50) containing 0.01% formic acid. Then the solution was centrifuged at 13000 rpm for 5 min and analyzed by rp-HPLC. HPLC-analysis was carried out on a Purospher Star RP C18 (250 × 4.0 mm, 5 μm, Merck, Darmstadt) column at 30 °C and a flow rate of 0.80 ml/min using varying mixtures of eluent A (water + 0.01% HCOOH) and eluent B (acetonitrile). UV detection at 210 nm was applied. Where necessary, appropriate response factors obtained with reference compounds were used to calculate the conversions.

2.4.2. Determination of enantiomeric excesses

The enantiomeric excess of amines was determined after derivatization with Marfey's reagent as following: 20 μl of the reaction mixture were mixed with 50 μl of an *N*-α-[2,4-dinitrophenyl-5-fluorophenyl]-L-alanine amide solution (1% (w/v) in acetone) and 10 μl saturated $NaHCO_3$. After incubation of the mixture at 40 °C for 1 h, 10 μl 2 M HCl and 920 μl acetonitrile were added and the sample was centrifuged for 5 min prior to analysis. HPLC-analysis was carried out on a Purospher Star RP C18 (250 × 4.0 mm, 5 μm, Merck, Darmstadt) column at 30 °C and a flow rate of 1.00 ml/min using a 50:50 mixture of eluent A (water + 0.01% HCOOH) and eluent B (acetonitrile). UV detection was performed at 338 nm.

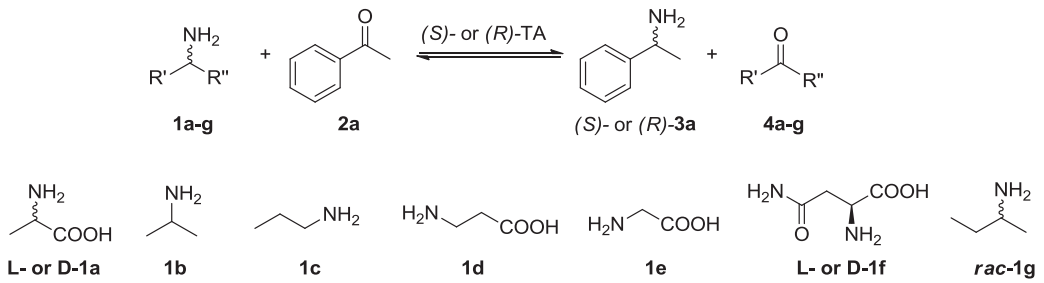
2.4.3. Determination of enzyme inhibition by pyruvate

The reactions were performed in a 1.5 ml UV cuvette and the mixture consisted of 5–50 mM pyruvate, 50 mM 1-phenylethylamine, 0.1 mM PLP and 0.03 mg purified (*S*)-TA Pde in 50 mM phosphate buffer pH 7.5. The initial reaction rates were measured spectro-photometrically by monitoring the formation of 1-phenylethanone at 300 nm ($\varepsilon = 0.28\ cm^2\ \mu mol^{-1}$) [35].

3. Results and discussion

3.1. Investigation of the donor range

The choice of a proper amino donor for the asymmetric synthesis of chiral amines with ω-TAs is of great importance. The amino donor must be efficient in the desired transformation, inexpensive, well soluble and the corresponding carbonyl product should



Scheme 2. Amination of 1-phenylethanone (**2a**) with a range of amino donors **1a–g** catalyzed by ω-transaminases.

not decrease the enzyme activity. Furthermore, an easy removal of the carbonyl by-product from the reaction mixture to shift the equilibrium is highly desirable. Fig. 1 summarizes the relative conversions of 1-phenylethanone (**2a**) to 1-phenylethylamine (**3a**) in the presence of various amino donors and three ω-transaminases (Scheme 2). Short chain aliphatic amines such as 2-propylamine (**1b**), 1-propylamine (**1c**) and *rac*-2-butylamine (**1g**) gave up to three times higher conversions compared to reactions with alanine (**1a**). Amino acids β-alanine **1d** and asparagine **1f** were poorly accepted as donors.

Based on the results presented in Fig. 1, 2-propylamine (**1b**) and *rac*-2-butylamine (**1g**) were selected as promising amino donors for the following experiments. Fortunately, their low price and the low boiling points of the corresponding keto compounds acetone and 2-butanone, which can be removed from the reaction mixture by evaporation and distillation [11,16,36] or further converted by other enzymes (e.g. alcohol dehydrogenase [10]) makes an application feasible. Alanine (**1a**) and 1-phenylethylamine (**3a**) were additionally tested as amino donors.

3.2. Investigation of the substrate spectrum

Various prochiral aromatic ketones **2a–m** were tested in the asymmetric amination to investigate the substrate spectrum of (*S*)-TA Pae, (*S*)-TA Pde and (*R*)-TA Ate (Scheme 3). Five times excess of amino donor **1** over ketone substrates was used in order to shift the equilibrium towards the desired chiral amino product. Other methods for shifting the reaction to the product side were not applied at this stage.

In most cases, good to excellent conversions to amines with excellent e.e. values were obtained when (*S*)-TA Pae and (*R*)-TA Ate were used (Table 1). In general, substrates are transformed better

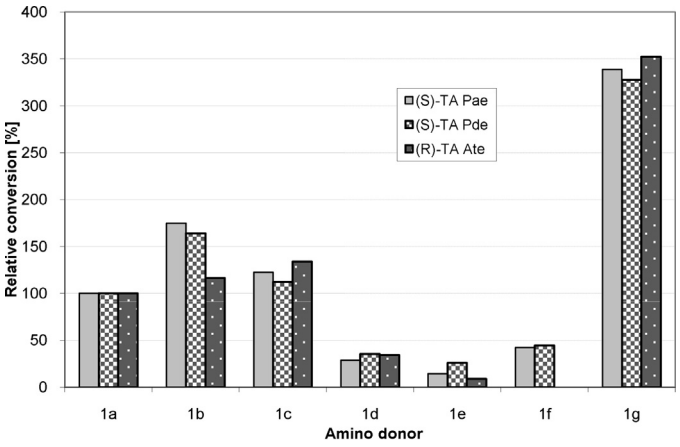


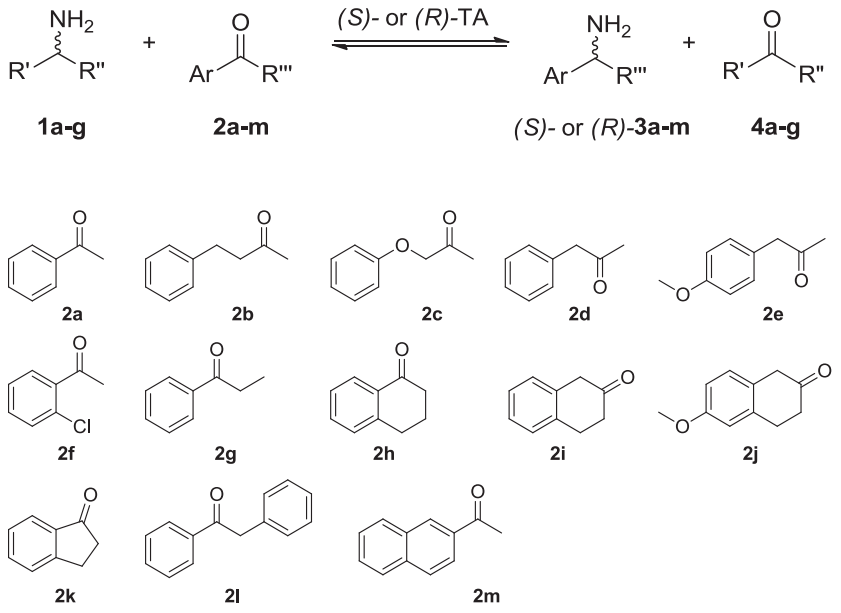
Fig. 1. Amino donor specificity of ω-TAs in the amination reactions of 1-phenylethanone (**2a**). Conditions: 10 mM **2a**, 50 mM **1a–f** or 100 mM donor ***rac*-1 g**, 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM sodium phosphate buffer, pH 7.5, 30 °C, 24 h.

when they possess a methyl next to the carbonyl group (Table 1, **2d** vs **2 g**). As proposed before by a two-binding-site model, such substrates properly fit into the small and large binding pockets of the active site of ω-transaminases [37,38]. Electron-donating groups (e.g. methoxy) at the aromatic ring do not significantly affect the conversion (Table 1, **2d** vs **2e**). It was recently shown, that (*R*)-TA Ate prefers aliphatic substrates with a chain length of at least six carbon atoms and aromatic ketones with hydroxy substituent [34,39]. Conversions and in some cases the enantioselectivity depends on the amino donor used. This might be explained by putative conformational changes in the active site of enzymes after deamination of the amino donor. Usually higher conversions (up to 90%) were obtained with donors **1g** or **3a**. For some amino donor/amino acceptor combinations, conversions of more than 90% were reached (e.g. **2c**–**1g**, **2c**–**3a**, **2d**–**3a**). Thereby, amine (*R*)-**3c**, a precursor for the anti-arrhythmic drug mexiletine, and other valuable enantiopure amines were obtained in a cost-effective procedure using one enzyme and inexpensive amino donors. However, for some substrates, the yields need to be improved by introducing additional steps for the removal of the (by)product.

Compared with the literature data where an additional LDH/GDH system was applied to shift the reaction equilibrium towards the amine products, the results obtained with (*R*)-TA Ate were in case of **2a–c,e** only slightly worse than reported by Mutti et al. [34] but significantly better than those by Schätzle et al. [39] for **2a** and **2b**. The reported conversions of ketones **2a–c** in reactions with (*S*)-TA Pde [32] are higher but in case of ketones **2b** and **2c** we also found decent values up to >80%. In the case of bulky substrates such as **2h–m**, no conversions were obtained with (*S*)-TA Pae and (*R*)-TA Ate. On the other hand, (*S*)-TA Pde exhibited broad substrate specificity and accepted most of the ketones tested. Only bulky-bulky 2-phenylacetophenone (**2l**) was not converted (Table 2). The stereoselectivities of (*S*)-TA Pde catalyzed reactions were in general not as good as those of the other ω-TAs tested in this study, which showed perfect enantioselectivities (Table 1). This effect is probably due to the larger active site of the enzyme, which allows more binding modes of the bulky substrate, thereby leading to reduced stereoselectivity [32,40]. (*S*)-TA Pde, in spite of the high sequence similarity (94%) to the well investigated (*S*)-TA from *Vibrio fluvialis*, was shown before to have a broader substrate specificity compared to the other known ω-TAs [40].

3.3. Donor influence on e.e.'s in (S)-TA Pde-catalyzed reactions

In our study, for some (*S*)-TA Pde catalyzed amination reactions of aromatic ketones, the e.e.'s were dependent on the donor used. This might be caused by the high flexibility of the active site to undergo conformational changes upon forming a donor amine-PLP complex. Low e.e.'s were usually obtained when alanine was the donor. In the course of amination reactions of 4-phenyl-2-butanone (**2b**) and 1-phenoxy-2-propanone (**2c**) in the presence of L-alanine (**1a**), the e.e.'s continuously decreased (Fig. 2). Remarkably, an equal ratio of the (*S*)- and (*R*)-amine product was obtained after 24 h in



Scheme 3. Asymmetric amination of aromatic ketones **2** with different amino donors **1** catalyzed by ω-transaminases.

Table 1
Conversions and enantiomeric excess of the amines obtained by the asymmetric synthesis catalyzed by (*S*)-TA Pae, (*S*)-TA Pde and (*S*)-TA Ate.

Ketone substrate	Amino donor	(*S*)-TA Pae		(*S*)-TA Pde		(*R*)-TA Ate	
		c [%]	e.e. [%] (*S*)	c [%]	e.e. [%] (*S*)	c [%]	e.e. [%] (*R*)
2a	**1a**	10	>99	19	>99	10	>99
	1b	18	>99	29	>99	11	>99
	1g	35	>99	32	>99	36	>99
2b	**1a**	18	>99	6	4	18	>99
	1b	35	>99	62	62	46	>99
	1g	67	>99	67	70	65	>99
	3a	92	>99	82	91	85	>99
2c	**1a**	22	>99	15	68	30	>99
	1b	55	>99	63	94	65	>99
	1g	67	>99	67	98	92	>99
	3a	68	>99	65	>99	96	>99
2d	**1a**	12	>99	5	14	17	>99
	1b	57	>99	53	>99	55	>99
	1g	80	>99	72	>99	94	>99
	3a	90	>99	83	>99	92	>99
2e	**1a**	14	>99	1	20	13	>99
	1b	26	>99	41	94	43	>99
	1g	32	>99	30	>99	74	>99
	3a	65	>99	38	>99	75	>99
2f	**1a**	3	>99	6	>99	12	>99
	1b	20	>99	15	>99	66	>99
	1g	12	>99	30	>99	78	>99
	3a	14	>99	51	>99	80	>99
2g	**1a**	<0.1	–	1	>99	<0.1	–
	1b	<0.1	–	4	>99	14	>99
	1g	<0.1	–	3	>99	13	>99
	3a	2	–	2	>99	12	>99

Reaction conditions: 10 mM ketone (**2**), 50 mM (for **1a**, **1b**) or 100 mM (for *rac*-**1g**, *rac*-**3a**) amino donor, 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM sodium phosphate buffer, pH 7.5, 30 °C, 24 h.

Table 2
Conversions and enantiomeric excess of bulky amines obtained by asymmetric synthesis catalyzed by (*S*)-TA Pde.

Donor Substrate	1a		1b		1g		3a	
	c [%]	e.e. [%]	c [%]	e.e. [%]	c [%]	e.e. [%]	c [%]	e.e. [%]
2h	<0.1	–	<0.1	–	<0.1	–	11	>99
2i	6	–	36	>99	47	>99	57	>99
2j	<0.1	–	16	>99	19	>99	16	>99
2k	<0.1	–	<0.1	–	4	–	21	83
2l	<0.1	–	<0.1	–	<0.1	–	<0.1	–
2m	<0.1	–	4	>99	4	>99	3	>99

Reaction conditions: 10 mM ketone (**2**), 50 mM (for **1a**, **1b**) or 100 mM (for *rac*-**1g**, *rac*-**3a**) amino donor, 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM phosphate buffer, pH 7.5, 30 °C, 24 h.

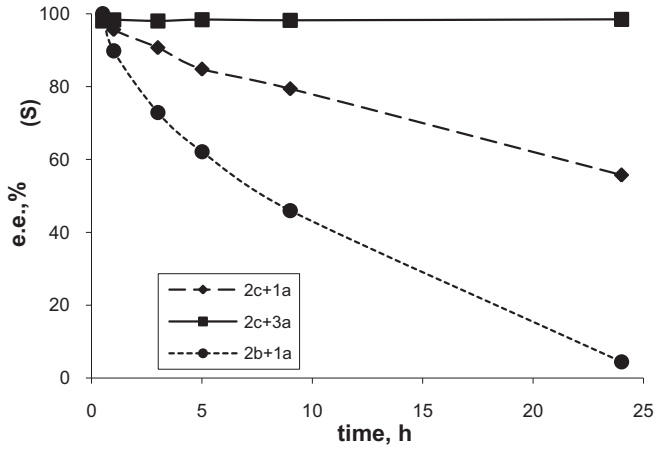


Fig. 2. Development of e.e.'s during amination reactions catalyzed by (*S*)-TA Pde. Conditions: 25 mM 1-phenoxy-2-propanone (**2c**) or 4-phenyl-2-butanone (**2b**), 125 mM L-alanine (**1a**) or 250 mM *rac*-1-PEA **3a**, 2 U/ml CFE, 0.1 mM PLP, 50 mM sodium phosphate buffer, pH 7.5, 30 °C.

the reaction of **2b** with **1a** catalyzed by (*S*)-TA Pde. With **3a** as donor, no decrease of the e.e. was observed (Fig. 2).

On the other hand, a strong inhibition of (*S*)-TA Pde was found by increasing the pyruvate concentration (Fig. 3). Most likely, pyruvate generates an abortive complex with the improper enzyme form, which does not further react, but forms non-productive bindings (e.g. pyruvate with E-PLP) [1,37,41]. This complex might stay in the active site, thus changing its conformation and affecting the proper binding of the amino acceptor substrate. This seems to be relevant for the aromatic substrates which have at least one methylene group between the aromatic ring and the carbonyl-group (e.g. **2b**, **2d**, **2e**). Thus, low enantioselectivity is presumably a result of unfavoured coordination of one enantiomer in a catalytically productive conformation, due to lack of space in the active site [42]. Enzyme inhibition by the carbonyl product was earlier identified as a major hurdle for carrying out efficient transamination reactions with the (*S*)-TA from *V. fluvialis*. The effect was described to be significantly depending upon the sources of the ω-TAs [22,43]. Thus, continuous removal of pyruvate from the reaction mixture is important not only for shifting the equilibrium, but also for avoiding enzyme inhibition.

3.4. Bulky amines as donors in the transamination reaction

The asymmetric synthesis of bulky amines from ketones is a rather difficult task due to thermodynamic limitations. In the presence of bulky ketones **2h–m**, no conversions were detected when using (*S*)-TA Pae and (*R*)-TA Ate (Section 3.2). Steric limitations and/or relatively low electrophilicity of the carbonyl carbon seem to be the reason for this outcome. To find out whether these enzymes can act on bulky amino substrates, we applied them in reactions with pyruvate and 1-phenylethanone (Scheme 4). Remarkably, with the single exception of 1-(1-naphthyl)ethylamine (**3m**) all tested bulky amines were accepted by (*S*)-TA Pae and (*R*)-TA Ate as donors (Table 3). 1-Aminoindane (**2k**) was identified as the most efficient donor among those tested. Previous observations showed that the activity of a ketone as amino acceptor is inversely related to amino donor activity of the corresponding amine. It was postulated that structural changes upon binding of a substrate and amination of the PLP cofactor in the active site of enzyme occur, which prevents entering of the bulky ketones as substrates [24,44,45].

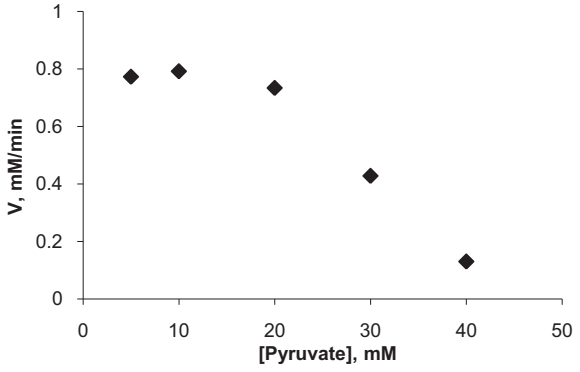


Fig. 3. Dependence of the initial reaction rate on the pyruvate concentration. Conditions: 50 mM 1-PEA (**3a**), 5–50 mM pyruvate, 0.03 mg (*S*)-TA Pde, 0.1 mM PLP, 50 mM sodium phosphate buffer pH 7.5, observation at λ = 300 nm.

Table 3
Amino donor reactivity of bulky aromatic amines in the ω-TA catalyzed amination of 1-phenylethanone and pyruvate.

Amine donor	Ketone acceptor	Conversion [%]	
		(*S*)-TA Pae	(*R*)-TA Ate
3h	pyruvate	8	28
	1-phenylethanone	4	9
3k	pyruvate	19	89
	1-phenylethanone	16	65
3l	pyruvate	9	2
	1-phenylethanone	3	1
3m	pyruvate	<0.1	<0.1
	1-phenylethanone	<0.1	<0.1

Reaction conditions: 10 mM pyruvate or 1-phenylethanone, 50 mM *rac*-amine (**3h–m**), 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM sodium phosphate buffer, pH 7.5, 30 °C, 24 h.

3.5. Effect of the pH and the reaction temperature

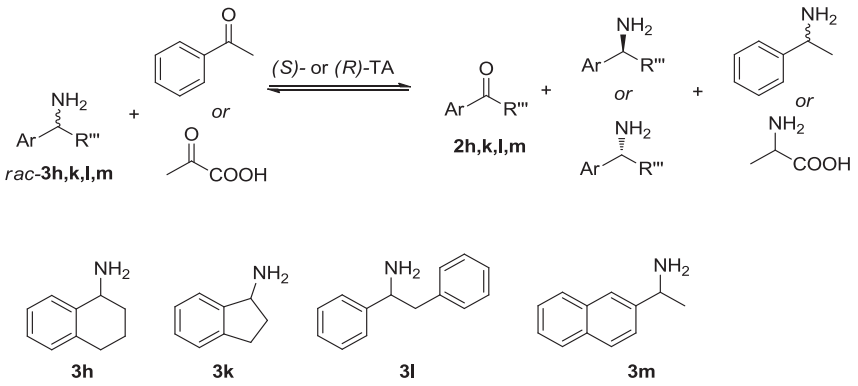
The influence of the pH on the asymmetric amination of 1-phenoxy-2-propanone (**2c**) using 2-butylamine (**1g**) as a donor amine in the presence of (*S*)-TA Pae, (*S*)-TA Pde or (*R*)-TA Ate is shown in Table 4. The optimal pH for the (*S*)-specific enzymes was found as 7.5. Increasing the pH to 9 leads to lower conversions and e.e.'s. (*R*)-TA Ate was stable within the pH range tested and the conversion was even improved by increasing the pH.

Thermostability of ω-TAs is required to perform the reactions at higher temperatures in order to remove ketone by-products by distillation, when using 2-propylamine (**1b**) or 2-butylamine (**1g**) as donors. The wild type (*S*)-TA Pde turned out to lose its activity upon increasing the reaction temperature from 30 °C to 40 °C. The enantioselectivity also dropped significantly (Table 5). (*S*)-TA Pae and (*R*)-TA Ate were not affected by the increase of the reaction temperature.

Table 4
Effect of the pH on the asymmetric amination of 1-phenoxy-2-propanone (**2c**) catalyzed by (*S*)-TA Pae, (*S*)-TA Pde and (*R*)-TA Ate.

pH	(*S*)-TA Pae		(*S*)-TA Pde		(*R*)-TA Ate	
	Conv. [%]	e.e. [%] (*S*)	Conv. [%]	e.e. [%] (*S*)	Conv. [%]	e.e. [%] (*R*)
7.0	61	>99	61	98	90	>99
7.5	67	>99	67	98	92	>99
8.0	65	>99	63	94	95	>99
9.0	15	>99	33	86	96	>99

Reaction conditions: 10 mM 1-phenoxy-2-propanone (**2c**), 100 mM 2-butylamine (**1g**), 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM sodium phosphate buffer, pH 7.0–8.0, 50 mM sodium carbonate buffer, pH 9.0, 30 °C, 24 h.



Scheme 4. Kinetic resolution of bulky aromatic amines **3** catalyzed by (*S*)-TA Pae and (*R*)-TA Ate.

Table 5
Effect of the reaction temperature on the asymmetric amination of 1-phenoxy-2-propanone (**2c**) catalyzed by (*S*)-TA Pae, (*S*)-TA Pde and (*R*)-TA Ate.

Enzyme	Amino donor	30 °C		40 °C	
		Conv. [%]	e.e. [%]	Conv. [%]	e.e. [%]
(*S*)-TA Pae	**1b**	55	>99	49	>99
	1 g	67	>99	70	>99
	3a	68	>99	70	>99
(*S*)-TA Pde	**1b**	63	94	25	93
	1 g	67	98	17	88
	3a	65	>99	37	97
(*R*)-TA Ate	**1b**	65	>99	75	>99
	1 g	92	>99	87	>99
	3a	96	>99	99	>99

Reaction conditions: 10 mM 1-phenoxy-2-propanone (**2c**), 50 mM (for **1b**) or 100 mM (for *rac*-**1g**, *rac*-**3a**) amino donor, 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM sodium phosphate buffer, pH 7.5, 24 h.

3.6. Effect of the substrate concentration

Accurate understanding of the substrate inhibition is beneficial when selecting optimal substrate concentrations in reactions. As shown before, pyruvate led to substantial inhibition in ω-TA catalyzed reactions, especially when (*S*)-TA from Pde was used (Section 3.3). Inhibition by substrates is common in ω-TA catalyzed reactions [37]. The influence of substrate concentration on the amination of 4-phenyl-2-butanone (**2b**) was investigated by subjecting various amounts of ketone to the reactions. Increasing the ketone as well as the amino donor concentration has negative effects on the conversion. This factor is significantly stronger in the presence of 1-phenylethylamine (**3a**) compared to 2-butylamine (**1g**) (Fig. 4). Most likely, multiple inactivation/inhibition of the enzymes by a carbonyl substrate, a donor amine, and a keto by-product occur. At low concentrations of substrates ([**2b**] < 50 mM), the conversion in the presence of donor **3a** is higher than in the case of **1g**. However, with increasing concentrations of **2b** and the amino donor, inhibition is much stronger in the case of **3a**. The additional inhibition caused by **3a** is perhaps enhanced by the corresponding by-product 1-phenylethanone (Fig. 4). Thus, when higher concentrations must be applied, **1g** is the preferred donor amine compared to **3a**.

3.7. Effect of cosolvents

For the successful biocatalytic amination, it is important to apply substrates at high concentration. However, bulky ketones are only poorly soluble in water, thus feeding strategies or addition of cosolvents are required [46]. We tested a range of water-miscible solvents in the amination reaction of almost insoluble 4-phenyl-2-butanone **2b** (50 mM) with **3a** as amino donor in the presence of (*S*)-TA from Pae, (*S*)-TA from Pde and (*R*)-TA from Ate. For all ω-TAs tested, the presence of cosolvents in the reactions led to reduced enzyme activities, which resulted in lower conversions, whereas the e.e.'s remained unchanged (Table 6, entries 2–6). In the presence of 15% v/v of water-miscible solvents like DMSO or methanol, the enzymes retained some activity, however, the conversion was significantly decreased. Obviously, all three wild-type ω-TAs have only little tolerance towards cosolvents and therefore improved enzyme variants have to be developed. The influence of water-immiscible organic solvents was also investigated (Table 6, entries 7–13). Such cosolvents can be applied for two-phase reaction systems which would allow a) to remove the ketone co-product (e.g. 1-phenylethanone (**2a**) if **3a** is used as a donor) from the reaction mixture to shift the equilibrium and b) to reduce the inhibition effects of ketones at high concentration [47,48]. Low concentrations of SDS (sodium dodecyl sulphate), which is supposed to minimize the inhibition effect [49], gave similar conversions to the reactions without an additive (Table 6, entry 14).

Among all additives tested, only cyclohexane and SDS might be useful for the amination reactions catalyzed by ω-TAs. They were applied for the synthesis of (*R*)-4-phenyl-2-butylamine (**3b**), a precursor for the antihypertensive drug dilevalol, starting from achiral ketone **2b** and **3a** as amino donor catalyzed by (*R*)-TA Ate (Fig. 5).

(*R*)-**3b** was obtained after 4 days with 50% conversion and >99% e.e. Cyclohexane slows down the reaction which is most likely due to limited substrate transfer from the organic to the aqueous phase.

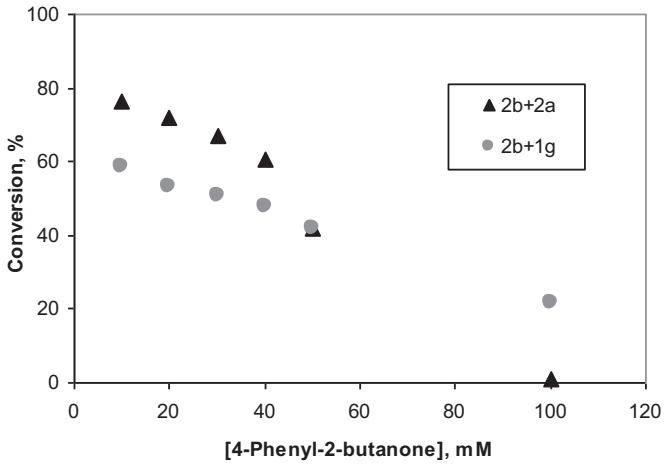


Fig. 4. Influence of the 4-phenyl-2-butanone (**2b**) concentration on the conversion in amination reaction with (*R*)-TA Ate. Conditions: 5–100 mM 4-phenyl-2-butanone (**2b**), 10 eq. amino donor **1g** or **3a**, 2 U/ml ω-TA (CFE), 0.1 mM PLP, 50 mM phosphate buffer, pH 7.5, 30 °C, 24 h.

Table 6
Effect of cosolvents and SDS on the asymmetric amination of 4-phenyl-2-butanone (**2b**) catalyzed by ω-transaminases.

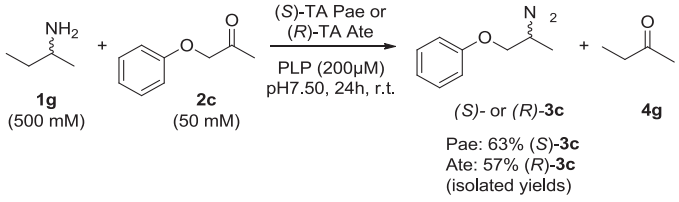
	Cosolvent/Additive	(*S*)-TA Pae		(*S*)-TA Pde		(*R*)-TA Ate	
		Conv. [%]	e.e. [%] (*S*)	Conv. [%]	e.e. [%] (*S*)	Conv. [%]	e.e. [%] (*R*)
1	no	11	>99	11	>99	42	>99
2	DMSO	3	>99	2	>99	6	>99
3	MeOH	1	>99	0	–	2	>99
4	Dioxane	0	–	0	–	1	n.d.
5	DMF	0	–	0	–	2.0	n.d.
6	Acetonitrile	0	–	0	–	0	–
7	Cyclohexane	5	>99	2	n.d.	22	>99
8	MTBE	3	>99	0	–	0	–
9	THF	0	–	0	–	0	–
10	2-Me-THF	0	–	0	–	0	–
11	Toluene	0	–	3	n.d.	2	n.d.
12	Octanol	1	>99	1	n.d.	6	>99
13	Benzene	1	n.d.	0	–	2	n.d.
14	SDS	8	>99	8	>99	38	>99

Reaction conditions: 50 mM 4-phenyl-2-butanone (**2b**), 250 mM 1-PEA (**3a**), 2 U/ml ω-TA (CFE), 15% (v/v) solvent or 3% SDS (sodium dodecyl sulphate), 0.5 mM PLP, 50 mM phosphate buffer, pH 7.5, 30 °C, 24 h.

3.8. Preparative scale synthesis of (S)- and (R)-1-phenoxypropan-2-amine

To show the applicability of the one-enzyme method, we have conducted preparative scale syntheses of (*S*)- and (*R*)-1-phenoxypropan-2-amine (**3c**), a mexiletine analogue, using (*S*)-TA Pae and (*R*)-TA Ate (Scheme 5; see supporting data).

After 24 h reaction time using a 10-fold excess of racemic 2-butylamine (**1g**), 63% of (*S*)-**3c** respectively 57% of (*R*)-**3c** with e.e.'s > 99.5% could be isolated. These yields match the reported value obtained with a three-enzyme system at similar scale [25] which exemplifies the applicability of the method described in this work.



Scheme 5. Preparative scale synthesis of (*S*)- and (*R*)-1-phenoxypropan-2-amine **3c** using (*S*)-TA Pae and (*R*)-TA Ate respectively.

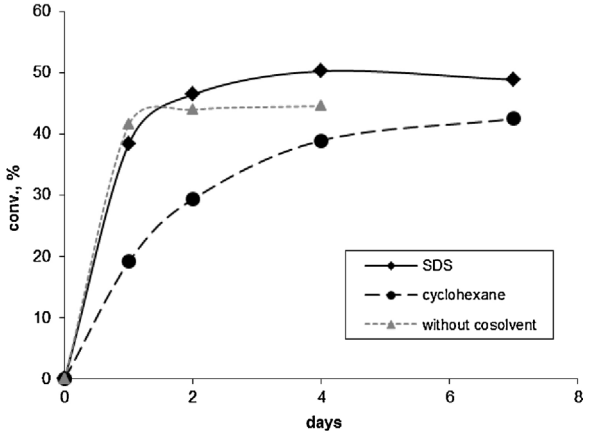


Fig. 5. Influence of additives on amination reactions of 4-phenyl-2-butanone (**2b**) catalyzed by (*R*)-TA Ate in the presence of cyclohexane or SDS. Conditions: 50 mM 4-phenyl-2-butanone (**2b**), 250 mM 1-PEA (**3a**), 2 U/ml ω-TA (CFE), 30% (v/v) cyclohexane or 3% SDS, 0.5 mM PLP, 50 mM sodium phosphate buffer, pH 7.5, 25 °C.

4. Conclusion

We have investigated the amino donor specificity of three ω-transaminases in a one-enzyme system and show that chiral amines can be obtained with varying conversions depending on the nature of the keto-substrate and the amino donor. For some methyl ketones containing an aromatic residue, the optically pure amines were obtained with high yields when an excess of 2-butylamine or 1-phenylethylamine was used as amino donor. No further steps for shifting the equilibrium were required, which makes the overall process more cost efficient compared to published multi-enzyme-based methods. When using alanine as amino donor in (*S*)-TA Pde-catalyzed reactions, the enantioselectivity of products was significantly different to those obtained in the presence of other donors. This observation indicates that many structural and mechanistic factors play a role when carrying out a transaminase reaction. Fortunately, the broad substrate acceptance of all enzymes tested makes the biocatalytic synthesis of chiral amino compounds feasible. Further improvement of the enzymes by means of protein engineering may in addition be applied if the desired properties are not available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.06.015.

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