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Efficient Asymmetric Synthesis of Chiral Amines by Combining Transaminase and Pyruvate Decarboxylase

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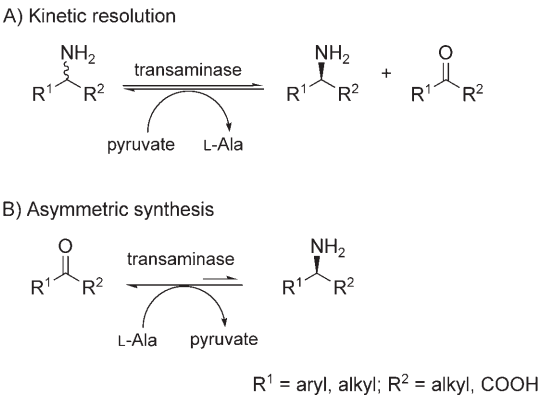
Dedicated to Prof. Karl Schügerl on the occasion of his 80th birthday.

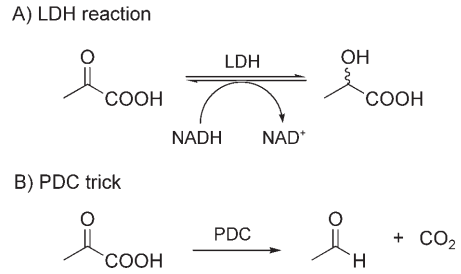
Chiral amines and amino acids play an important role in the pharmaceutical, agrochemical and chemical industry. They are frequently used as synthons for the preparation of various pharmaceutically active substances and agrochemicals, or as resolving agents for chiral acids. Consequently, there is a need for efficient methods to obtain the desired *R* or *S* enantiomer in an optically pure form.[1]

The most frequently used enzymatic method for the production of optically active amines is the kinetic resolution of racemic starting material by enantioselective hydrolysis of, for example, *N*-acyl amides by peptidases, amidases or lipases.[2] Alternatively, transaminases can be used in kinetic resolution (Scheme 1 A).[3] The maximum yield in all of these processes is limited to 50% unless a racemization step is included to enable a dynamic kinetic resolution. One example is the deracemization of an amine racemate with a monoamine oxidase.[4]



Scheme 1. Transaminases (TA) can be used in kinetic resolution or asymmetric synthesis to obtain optically pure amines or amino acids.

In principle, ω-transaminases (TA) can also be used in an asymmetric synthesis of amines by starting from readily available prostereogenic ketones in combination with suitable amino donor molecules, for example, L-alanine (Scheme 1 B).[5] Although ω-transaminases exhibit excellent enantioselectivity in general, they are not widely used in asymmetric synthesis, although in this case a 100% yield is theoretically possible. The problem with the asymmetric synthesis is that the equilibrium of the reaction lies strongly on the side of substrate ketone, which means that only small amounts of chiral product amine are formed. A typical example is the amination of acetophenone with alanine to (*S*)-α-methylbenzylamine, where the equilibrium constant was reported to be 8.8×10^{-4}. Shifting the equilibrium to the desired direction is the key requirement for utilizing transaminases efficiently in asymmetric synthesis. The asymmetric synthesis of α-amino acids with α-transaminases is less dramatic because the equilibrium constant is ≈1. Still, the equilibrium needs to be shifted in order to reach a conversion >50%. If alanine is used as the amine donor then the equilibrium shift can be achieved by removing the co-product pyruvate with the enzyme lactate dehydrogenase (LDH).[5] A disadvantage of the reduction of pyruvate to lactate by LDH is that the enzyme requires NADH, and cofactor recycling is necessary (Scheme 2 A). The direct use of whole cells is hampered by the fact that an undesired side reaction, (e.g., the reduction of ketone to alcohol) can occur. Another option is the condensation of pyruvate by acetolactate synthase to acetolactate, which subsequently decarboxylates spontaneously to acetoin.[6]



Scheme 2. Removal of pyruvate by the use of A) lactate dehydrogenase (LDH) or B) pyruvate-decarboxylase (PDC) to shift the equilibrium in transaminase-catalysed asymmetric synthesis.

In this report we show that the equilibrium can be efficiently shifted by the use of pyruvate decarboxylase (PDC) as illustrated in Scheme 2 B. Several PDCs that originate from yeast and bacteria are commercially available, as they can be used in the synthesis of ephedrine precursors and a range of α-hydroxy ketones.[7]

The major advantage of PDC in contrast to LDH is that it requires no cofactor recycling, and the reaction products are highly volatile, which allows for the desired shift of equilibri-

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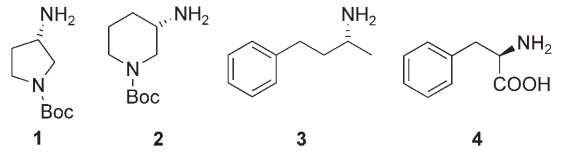
um. We have chosen three PDCs of different origin and several chiral amines as products to verify this novel concept in transaminase-catalyzed asymmetric syntheses (Scheme 3). The ω-TA



Scheme 3. Amines **1**–**3** and amino acid **4** were produced in the TA and PDC-catalysed asymmetric synthesis from the corresponding ketones.

and PDC have different pH optima (ω-TA: pH 9, PDC: pH 6) so the coupled reactions were carried out at pH 7 where both enzymes are still active. Initially, the effect of the pyruvate removal by PDC was investigated in the asymmetric synthesis of 1-*N*-Boc-3-aminopyrrolidine (**1**). This compound was chosen as a model substrate because substituted 3-aminopyrrolidines show interesting pharmacological activity,[8] and are used as synthons for semisynthetic cephalosporines.[9]

The transamination reactions of ketone 1-*N*-Boc-3-oxopyrrolidine by ω-TA from *Vibrio fluvialis* (Vfl-TA) were compared in the presence of one of the three PDCs: *Zymomonas mobilis* (Zmo-PDC), *Zymobacter palmae* (Zpa-PDC)[10] and a recombinant PDC of undisclosed origin that was obtained from Biocatalytics (BC-PDC). The same amount of PDC (1.7 U, ADH assay) and TA (0.11 U) was used in all reactions. By using an equimolar amount of the aminodonor (10 mM DL-alanine) and ketone, the conversion with TA alone reached a plateau after 1 h, and did not exceed 5.5–6.0%. In contrast, the conversion increased already to 17–18% when Vfl-TA was used in combination with one of the PDCs; Zpa-PDC was the most active pyruvate decarboxylase. Figure 1 shows that an increase in the alanine concentrations leads to a substantial increase in product formation, and yields of up to 96% of amine **1** were obtained.

The combination of Vfl-TA with Zpa-PDC was further investigated for the efficient asymmetric synthesis of amines **2** and **3**. In each case, substantially enhanced conversions were found that were in the same range or higher compared to the known use of LDH (Table 1).

Finally, a 'broad-range' D-amino acid transaminase was used in the asymmetric synthesis of D-phenylalanine **4**. The conversion did not exceed 50% when only the transaminase was used, but 86% conversion was achieved when BC-PDC[11] was added to shift the equilibrium (Table 1).

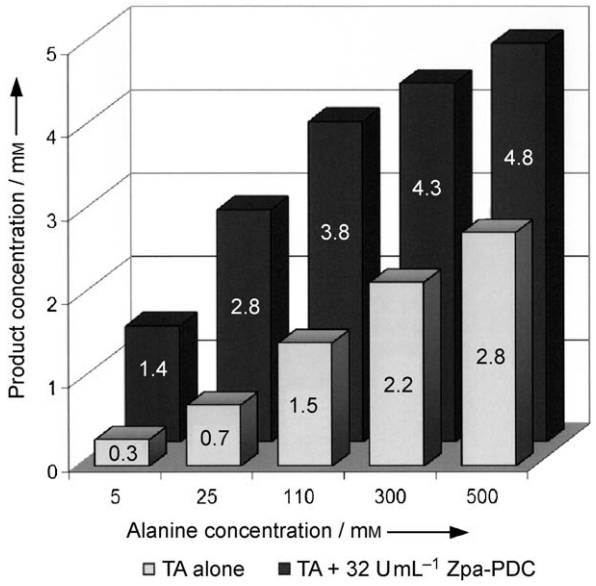


Figure 1. Comparison of asymmetric synthesis of **1** by using transaminase alone or in the presence of PDC to shift the equilibrium. A product concentration of 5 mM corresponds to 100% conversion.

In summary, a highly efficient methodology for the asymmetric synthesis of optically pure amines and one amino acid was developed. Compared to the known procedure that uses lactate dehydrogenase for the shift of equilibrium, this PDC-trick has the major advantage that no cofactor recycling is required, yields are in the same range or even higher, and easily removable, highly volatile byproducts are formed.

Experimental Section

Chemicals and enzymes: All chemicals were from Sigma–Aldrich, Germany. *S. cerevisae* PDC and *S. cerevisae* alcohol dehydrogenase were from Fluka. The transaminase from *V. fluvialis* was obtained from Julich Chemical Solutions (now Codexis Inc. Redwood City, CA, USA) as a liquid. The amount of the enzyme that was used was according to the manufacturer recommendation. The broad range D-amino acid transaminase and one pyruvate decarboxylase, PDC 101, were from BioCatalytics (now Codexis Inc.). PDCs from

Table 1. Asymmetric synthesis of different amines/amino acids.

Product	Conversion [%]			Enantiomeric purity
	TA[a]	TA and PDC[a,b]	TA and LDH[a,b]	*ee* [%]
1	27 (±3)	80 (±5)	80 (±5)	99 (±0.2)
2	22 (±4)	40 (±4)	34 (±4)	98 (±0.5)
3	9 (±2)	45 (±5)	40 (±20)	88.5 (±0.2)
4	47 (±3)	86 (±2)	58 (±3)	96.6 (±0.2)

[a] Reaction conditions to produce **1**–**3**: 5 mM ketone, 110 mM L-alanine, Vfl-transaminase. For the synthesis of **4**, 5 mM phenylpyruvate, 11 mM DL-alanine and "broad range" D-amino acid transaminase were used. The amounts of BC-PDC and LDH were 3 U mL^{-1}. [b] For comparison, the same amounts (32 U mL^{-1}) of PDC and LDH were used in the synthesis of **1**–**3**.

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Zymomonas mobilis and *Zymobacter palmae* were recombinantly expressed in *E. coli*.[10]

Activity measurement of PDC: One unit of PDC was defined as the amount of enzyme that catalyzes the formation of 1 μmol acetaldehyde from pyruvate per minute. The assay buffer for the activity measurement of PDC contained Na_2HPO_4 (50 mM), citrate (50 mM), $MgCl_2$ (5 mM), thiamine pyrophosphate (0.1 mM), and was adjusted to pH 7. The reaction was carried out in a 1-mL cuvette, The reaction buffer (800 μL) was mixed with NADH (50 μL, 3 mM), alcohol dehydrogenase (50 μL with an activity of 200 U mL^{-1} according to the manufacturer) and cell extract (50 μL) was diluted as necessary. The reaction was started by the addition of pyruvate (50 μL, 100 mM), and the decrease of the absorption at 340 nm was followed.

Biocatalysis: All biocatalytic reactions were done at 37 °C in sodium phosphate buffer (50 mM, pH 7) that contained pyridoxal-5′-phosphate (0.1 mM), thiamine pyrophosphate (0.1 mM) and $MgCl_2$ (5 mM). The end-concentration of 1-*N*-Boc-3-oxopyrrolidine and the other ketones was 5 mM.

Analysis of reaction products: The concentration of 1-*N*-Boc-3-aminopyrrolidine **1** was measured by capillary electrophoresis. The reaction was stopped by adding an aliquot of the reaction mixture (100 μL) to a NaOH solution (100 μL, 0.1 M) that contained α-methylbenzylamine (0.2 mM) as an internal standard, followed by extraction with CH_2Cl_2 (200 μL). Subsequently the organic layer (100 μL) was extracted with triethylammonium phosphate buffer (200 μL, 10 mM, pH 3.0) that now contained both analyte and standard. The separation was done on a Beckman PACE-MDQ system equipped with a fused silica capillary (60 cm length, 10 cm to the detector, 50 μm inner diameter) and a PDA detector. A pressure of 0.5 psi was applied for 5 s for the injection. The background electrolyte contained triethylammoniumphosphate (50 mM, pH 3.0). A voltage of 30 kV was applied for 4 min for separation, and the compounds were detected at 190 nm. The capillary that was used for the analysis of the other amines **2**–**3** and phenylalanine **4** was dynamically coated with CElixir (MicroSolTech, Eatontown, NY, USA) according to the manufacturers instructions. The reaction was stopped by mixing the sample (40 μL) with HCl (160 μL, 12.5 mM) that included α-methylbenzylamine (1 mM) as an internal standard. The separation protocol was the same as described above.

Analysis of optical purity of products: The enantiomeric excess values of **1** and **2** were analysed by gas chromatography. After extraction of the amine with CH_2Cl_2, derivatisation to the trifluoroacetamide was performed by adding a 20-fold excess of trifluoroacetic anhydride. After purging with nitrogen to remove excess anhydride and residual trifluoroacetic acid, the derivatised compound was dissolved in CH_2Cl_2 (50 μL) and analysed by using a Shimadzu GC14 A that was equipped with a Heptakis-(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin column (25 m × 0.25 mm). The retention times were 13.5 min for the *S* enantiomer and 19 min for the *R* enantiomer at an oven temperature of 180 °C.

The enantiomeric excess values of **3** and **4** were determined by capillary electrophoresis on a PACE-MDQ system that was equipped with a fused silica capillary (30 cm length, 10 cm to the detector, 50 μm inner diameter). Amine **3** was extracted from the reaction solution as describe above for the activity measurement. Before analysis of amino acid **4**, proteins were removed with a phenol-chloroform extraction prior to injection. Enantiomer separation was achieved by the addition of 5% highly-sulphated-γ-cyclodextrin (Beckman–Coulter, Fullerton, CA, USA) to the running buffer as a chiral selector. A voltage of 15 kV (reverse polarity) was applied and the compounds were detected at 200 nm. The migration times were 5.2 min for (*R*)-**4**, 8.0 min for (*S*)-**4**, and 2.0 min and 2.3 min for the enantiomers of **3**, respectively.

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Keywords: amines · asymmetric synthesis · enzyme catalysis · pyruvate decarboxylase · transaminase

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