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Overcoming Thermodynamic and Kinetic Limitations of Aldolase-Catalyzed Reactions by Applying Multienzymatic Dynamic Kinetic Asymmetric Transformations**

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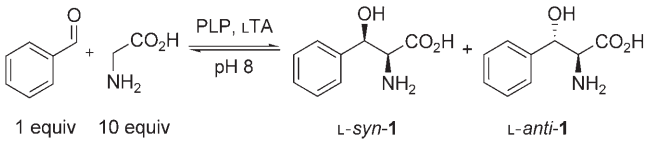
Nature uses enzymes for both the formation and cleavage of C–C bonds within aldols.[1] More than 40 aldolases have been characterized to date, and their application offers biocatalytic access to α-hydroxycarbonyl compounds.[2] However, the usefulness of this transformation for synthetic purposes is often limited by the position of the equilibrium (thermodynamic limitation).[3] Furthermore, kinetic limitations also exist, which means that the reaction rates for the formation of the stereoisomers are sometimes not sufficiently different to ensure high stereoselectivities.

Herein we show how these limitations can be overcome for the studied transformation—the synthesis of β-hydroxy-α-amino acids—by the application of threonine aldolases (TAs) to catalyze the reaction of glycine with appropriate aldehydes. All glycine-dependent aldolases require pyridoxal-5′-phosphate (PLP) to catalyze the reversible aldol reaction of glycine and the acceptor aldehyde. We describe herein the transformation of benzaldehyde to phenylserine (**1**, Scheme 1) as a model system. Several natural L- and D-threonine aldolases (LTA/DTA, EC 4.1.2.5)[4] and a recently engineered DTA (starting from alanine racemase)[5] have been discovered so far, and it has been shown that all TAs can transform a variety of aromatic and aliphatic aldehydes.[6] If a tenfold excess of glycine over benzaldehyde is used (LTA, optimized conditions) an 85% yield is achieved, since the equilibrium is shifted to the side of the aldol.[7] The process can be optimized by continual removal of the product to ensure high yields.[8]



Scheme 1. Synthesis of L-phenylserine (**1**) using L-threonine aldolase.

A still better approach to overcome the thermodynamic limitations is to pull the transformation to the aldol side of the equilibrium by an irreversible reaction of the initially formed β-hydroxyamino acid **1**. LTA, DTA, and most enzymes involved in amino acid metabolism/catabolism are highly C_α selective (L/D), while the C_β selectivity is disappointingly low for both LTA and DTA (kinetic limitation, *syn*/*anti*).[6] In recent studies we showed that the kinetic limitation was overcome by using DTA from *Alcaligenes xylosoxidans*,[9] which yielded diastereomerically pure D-*syn*-**1**.[7] For LTA, the enantioselectivity at the carbon atom bearing the amino group is high ($ee > 99\%$), whereas the selectivity for the transformation of the aldehyde to a hydroxy group is low; this leads to a diastereomeric ratio L-*syn*-**1**/L-*anti*-**1** of 60:40.[7] A consecutive and C_β-selective step would also help to overcome this limitation.

L-Amino acid decarboxylases are known to be highly substrate specific. Only L-tyrosine decarboxylase (L-TyrDC, PLP-dependent) was shown to tolerate an additional hydroxy group in the β position and to be tolerant towards aromatic substituents.[10] L-TyrDC is highly selective with respect to the NH_2-bound carbon atom, and only the L enantiomer is transformed. Thus, L-TyrDC can be utilized to resolve chemically synthesized DL-*syn*-**1** into 2-amino-1-phenylethanol ((*R*)-**2**) in a maximum yield of 50%, with D-*syn*-**1** left behind.[10] The addition of DTA enabled the untransformed diastereomer (D-*syn*-**1**) to be recycled by degrading it to benzaldehyde and glycine in a so-called parallel kinetic resolution process.[11,12]

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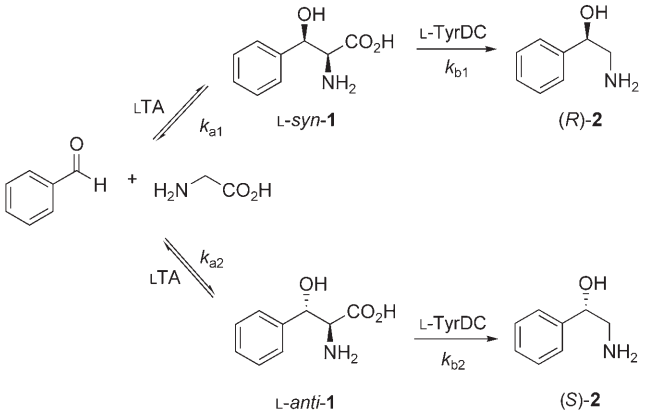
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

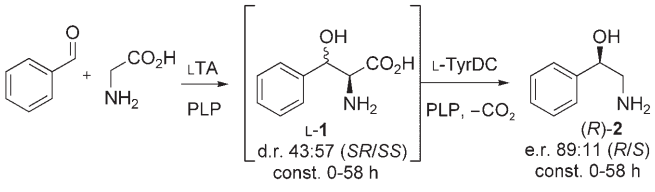
However, besides this simple resolution of DL-*syn*-**1**, the thermodynamic limitations can be overcome by a combination of an LTA-catalyzed aldol reaction with an in situ decarboxylation using L-TyrDC. As a consequence, the aldol equilibrium is shifted to the L-aldol products, thus resulting in high conversions. L-TyrDC (from *Enterococcus faecalis* V583)[13] and LTA (from *Pseudomonas putida*)[14] were cloned and overexpressed in *Escherichia coli*. In our model reaction we investigated the unprecedented bienzymatic synthesis of **2** from benzaldehyde and glycine by utilizing a reversible LTA-catalyzed aldol reaction (forming the intermediate product L-*syn*-**1**/L-*anti*-**1**) with subsequent irreversible decarboxylation (Scheme 2). The product of this



Scheme 2. Synthesis of 2-amino-1-phenylethanol (**2**) using L-threonine aldolase and L-tyrosine decarboxylase.

formal one-pot aminomethylation of benzaldehyde retains the hydroxy-substituted stereogenic center, while "losing" the second center—as the amino function is transformed into a terminal group. By this means, the moderate C_β selectivity of the aldolase is drastically improved because of the high *syn*/*anti* selectivity of L-TyrDC (10:1, see the Supporting Information). The reaction sequence described is to the best of our knowledge the first example of a bienzymatic resolution of diastereomers. A similar process involving an organocatalytic protocol was referred to as a dynamic kinetic asymmetric transformation (DYKAT).[15,16] For dynamic processes it is of utmost importance that the equilibration (k_{a1} and k_{a2}) is much faster than the irreversible step (k_{b1} and k_{b2}) so as to obtain yield high selectivities and to reach the theoretical yield of 100%.[17] Equilibration studies using LTA showed the *syn*–*anti* interconversion of **1** to be fast (see the Supporting Information).

In a first approach to overcome the thermodynamic limitation by this means we tested the compatibility of LTA and L-TyrDC. Indeed, the equilibrium reaction of benzaldehyde and glycine to form L-*syn*-**1**/L-*anti*-**1** was successfully shifted towards the product side by the irreversible decarboxylation. The conversion of benzaldehyde into the intermediate L-*syn*-**1**/L-*anti*-**1** (8%) and the final amino alcohol (*R*)-**2** (91%) was complete after 58 h. (Scheme 3). Hence, the combination of LTA and L-TyrDC promises to be suitable for the quantitative conversion of aldehydes into 1,2-amino alcohols. Interestingly, the moder-

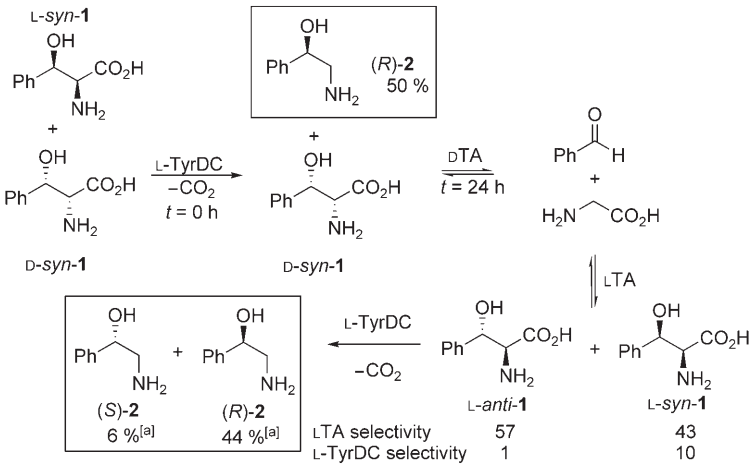


Scheme 3. Conversion of benzaldehyde into the intermediates L-*syn*-**1**/L-*anti*-**1** and the final product (*R*)-**2**. Reaction conditions: benzaldehyde 100 mM, glycine 1 M, LTA 38 U, L-TyrDC 0.4 U, PLP 50 μM, pH 5.5, 25°C; yield, diastereomeric ratio (d.r.), and enantiomeric ratio (e.r.) were determined by HPLC.

ate C_β selectivity of LTA (*R*/*S* 60:40) in the aldol reaction was slightly altered (*R*/*S* 43:57) in the bienzymatic process. This finding can be explained by the fact that L-*syn*-**1** (2*S*,3*R*) is converted into (*R*)-**2** at a faster rate than is L-*anti*-**1** (2*S*,3*S*) to (*S*)-**2**, thus shifting the *syn*/*anti* ratio of the intermediate products L-*syn*-**1**/L-*anti*-**1**.

This approach resulted in the low selectivity at the β position of **2** being improved to 89:11 (*R*/*S*) by stereoselective decarboxylation. The stereomeric ratios of L-**1** (d.r.) and **2** (e.r.) were constant throughout the reaction, which implies that the dynamic process is efficient.

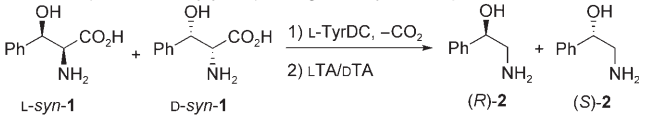
The resolution of DL-*syn*-**1** by using the described one-pot, two-enzyme DYKAT is not possible because of the strict L selectivity of LTA and L-TyrDC. Only L-*syn*-**1** is transformed in a so called kinetic asymmetric transformation (KAT), which leaves the D enantiomer behind. High *ee* values (>99%) of the amino alcohol (*R*)-**2** can consequently be obtained, but only in a maximum yield of 50%. A novel one-pot, three-enzyme protocol (Scheme 4) was investigated to overcome this limitation, but with the high selectivity retained. The initial KAT (L-TyrDC) of L-*syn*-**1** yielded



Scheme 4. Theoretical outcome for the synthesis of (*R*)-**2** by using a KAT/DYKAT protocol (88% *ee*: 94% (*R*)-**2**, 6% (*S*)-**2**). [a] Estimated C_β selectivity of the coupled LTA/L-TyrDC reaction: 89:11 (*R*/*S*; see Scheme 3).

enantiopure (*R*)-**2** (50%). Addition of the aldolases LTA and DTA[9] after 24 h resulted in a three-enzyme DYKAT (L-TyrDC, LTA/DTA) which converted the remaining D-*syn*-**1** (50%) into benzaldehyde and glycine, and thereafter into L-*syn*-**1**/L-*anti*-**1** and finally (*R*)-**2**/(*S*)-**2**. Theoretically, the quantitative resolution of DL-*syn*-**1** by this protocol with delayed addition of LTA and DTA should give an *ee* value of 88% for amino alcohol **2** (*R*, Scheme 3), when the results of the one-pot, two-enzyme approach are considered (*R*/*S* 89:11, Scheme 3). The following application of this protocol showed that enantioenriched **2** (82% *ee R*) was obtained in only 44% yield at pH 5.5 if all three enzymes were added at the beginning (Table 1, entry 1). The low yield might be

Table 1: Synthesis of (*R*)-**2** by using a KAT/DYKAT protocol.[a]



Entry	pH	Yield **2** [%] (5 h)	*ee* (*R*) [%] (5 h)	Yield **2** [%] (80 h)	*ee* (*R*) [%] (80 h)
1[b]	5.5	23	80	44	82
2[c]	5.5	46	>99	67	>99
3[b]	6.0	15	72	47	82
4[c]	6.0	39	>99	57	>99
5[b]	6.5	13	80	28	80
6[c]	6.5	35	>99	58	>99
7[b]	7.0	3	>99	11	82
8[c]	7.0	25	>99	50	>99
9[b]	7.5	<1	>99	4	>99
10[c]	7.5	10	>99	24	>99
11[c,d]	5.5	n.d.[f]	n.d.[f]	58[e]	>99

[a] Conditions: 1-mL reaction volume, DL-*syn*-**1** 100 mM, glycine 900 mM, PLP 50 μM, $MnCl_2$ 50 μM, 25 °C, LTA 19 U, DTA 6 U, L-TyrDC 0.4 U; yield and *ee* values were determined by HPLC. [b] Addition of LTA/DTA at $t = 0$ h. [c] Addition of LTA/DTA after $t = 24$ h. [d] 5-mL reaction scale. [e] Yield of isolated product after $t = 100$ h. [f] n.d. = not determined.

explained by inhibitory effects of $MnCl_2$ or DTA on L-TyrDC; these effects will be investigated in more detail in the near future. However, delayed addition of LTA/DTA (after 24 h) yielded enantiopure **2** (Table 1, entries 2 and 11, *ee* > 99% *R*) as well as improved conversion (HPLC 67%, isolated product 58%). Similar results were obtained at pH 6.0 and 6.5 (Table 1, entries 3–6). Higher pH values (7.0 and 7.5, entries 7–10) resulted in a decreased yield because of the low activity of L-TyrDC at pH > 6.5 (optimum pH: 5.5). The excellent *ee* values of **2** together with the good yields (pH 5.5–6.5) show that all three enzymes collaborate well. Furthermore, the kinetic limitation—low C_β selectivity in the LTA-catalyzed reaction—is overcome and enantiopure (*R*)-**2** is obtained in yields of over 50%.

In summary, the protocols described herein overcome the thermodynamic (DYKAT) and the kinetic (KAT/DYKAT) limitations of reactions catalyzed by L-threonine aldolase. The first bienzymatic DYKAT involves an aldolase with low selective and a decarboxylase with high diastereoselectivity. By applying this approach we were able to shift the aldol reaction of glycine and benzaldehyde to quantitative conversion and to improve the low C_β selectivity. A KAT/DYKAT protocol starting from DL-*syn*-**1** gave enantiopure amino alcohol (*R*)-**2** in good yield. Currently, we are testing other acceptor compounds in this novel one-pot aminomethylation to obtain valuable aromatic 1,2-amino alcohols.

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