Biocatalysis

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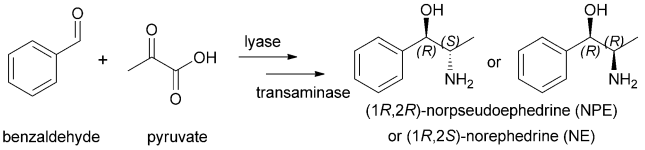
# Two Steps in One Pot: Enzyme Cascade for the Synthesis of Nor(pseudo)ephedrine from Inexpensive Starting Materials**

*Torsten Sehl, Helen C. Hailes, John M. Ward, Rainer Wardenga, Eric von Lieres, Heike Offermann, Robert Westphal, Martina Pohl, and Dörte Rother**

A challenging task for chemical researchers in the next decade is the development of cleaner and more environmentally friendly reactions.[1] The traditional chemical syntheses of enantiomerically pure compounds often require multistep protocols with protection–deprotection steps as well as the isolation of potentially unstable intermediates, lowering the yields and sustainability of the overall process.[2]

Phenylpropanolamines, members of the amphetamine family of ephedra alkaloids, are compounds with multifunctional applications but challenging syntheses routes. The stereoisomers norpseudoephedrine (NPE) and norephedrine (NE) are used as building blocks for the preparation of ligands and chiral auxiliaries in organic syntheses[3] and also have direct applications as pharmaceutically active molecules.[4] Reported synthetic approaches to these compounds have disadvantages such as relatively expensive reagents, multistep preparative routes, and only moderate enantio- and diastereoselectivity.[5] Recently, a novel highly stereoselective method was described for the synthesis of all phenylpropanolamine isomers with *ee* and *de* values exceeding 99%.[6] Norephedrine isomers were accessible in four steps (40% yield) and norpseudoephedrine in seven steps (35% yield) starting from 2-phenyl-2-trimethylsilyloxyacetonitrile.

Synthetic enzyme cascades are valuable alternative routes for the stereoselective production of fine chemicals. Since the chemo- and stereoselectivities are typically high, the isolation of by-products and reaction intermediates can be circumvented[7] and thus the eco-efficiency increased.[8] Here we present an enzymatic one-pot two-step reaction for the synthesis of stereomerically pure (1*R*,2*S*)-NE and (1*R*,2*R*)-NPE from benzaldehyde and pyruvate (Scheme 1). A number of different ways to perform enzyme cascade reactions have already been described (for more details see Chapter 1 in the Supporting Information).[1b,2a,8a–c,9] Our one-pot two-step reaction combines many advantages of known synthesis strategies like high stereoselectivities, inexpensive starting materials, high step economy (only two steps), and an equilibrium shift without addition of further enzymes or cosubstrates.




***Scheme 1.*** One-pot two-step reaction for the synthesis of norpseudoephedrine (NPE) and norephedrine (NE).

In the first step pyruvate is decarboxylated and subsequently ligated to benzaldehyde yielding (*R*)-phenylacetylcarbinol ((*R*)-PAC). The reaction is catalyzed by the thiamine diphosphate (ThDP)-dependent acetohydroxyacid synthase I (AHAS-I) from *E. coli* which performs the decarboxylation of pyruvate and the subsequent carboligation without releasing the hydroxyethyl-ThDP (see Scheme 2).[10] (*R*)-PAC is obtained with high stereoselectivity ($ee > 98\%$) and can be converted directly to the desired (1*R*,2*S*)-NE and to (1*R*,2*R*)-NPE in the second step of the cascade (reductive amination) by selectively using (*S*)- and (*R*)-selective ω-transaminases (TAs), respectively. In our previous work a set of 18 different (*S*)-selective wild-type (*S*)TAs had been screened for the conversion of 2-hydroxy ketones.[11] For the reductive amination of (*R*)-PAC, the *Cv*-(*S*)TA from *Chromobacterium violaceum* gave the most promising results. To gain access to (1*R*,2*R*)-NPE, seven different (*R*)-selective (*R*)TAs from Enzymicals AG (see Chapter 2 in the Supporting Information) were tested.

The enzymatic reductive amination requires an amine donor as a cosubstrate. Through the clever combination of cosubstrates (here: alanine) and enzymes, the resulting by-product (here: pyruvate) of the second reaction step can serve as the substrate for the first step. This novel type of cascade design is referred to as a "recycling cascade" (Scheme 2 and Chapter 1 in the Supporting Information). We determined the

[*] M. Sc. T. Sehl, Dr. E. von Lieres, H. Offermann, Dipl.-Biotechnol. R. Westphal, Prof. Dr. M. Pohl, Dr. D. Rother
Institute of Bio- and Geosciences
IBG-1: Biotechnology, Forschungszentrum Jülich GmbH
Leo-Brandt-Strasse 1, 52425 Jülich, (Germany)
E-mail: do.rother@fz-juelich.de
Prof. H. C. Hailes
Department of Chemistry, University College London
20 Gordon Street, London WC1H OAJ (UK)
Prof. J. M. Ward
The Advanced Centre for Biochemical Engineering
Department of Biochemical Engineering, University College London
Torrington Place, London, WC1E 7JE (UK)
Dr. R. Wardenga
Enzymicals AG
Walther-Rathenau-Strasse 49a, 17489 Greifswald (Germany)

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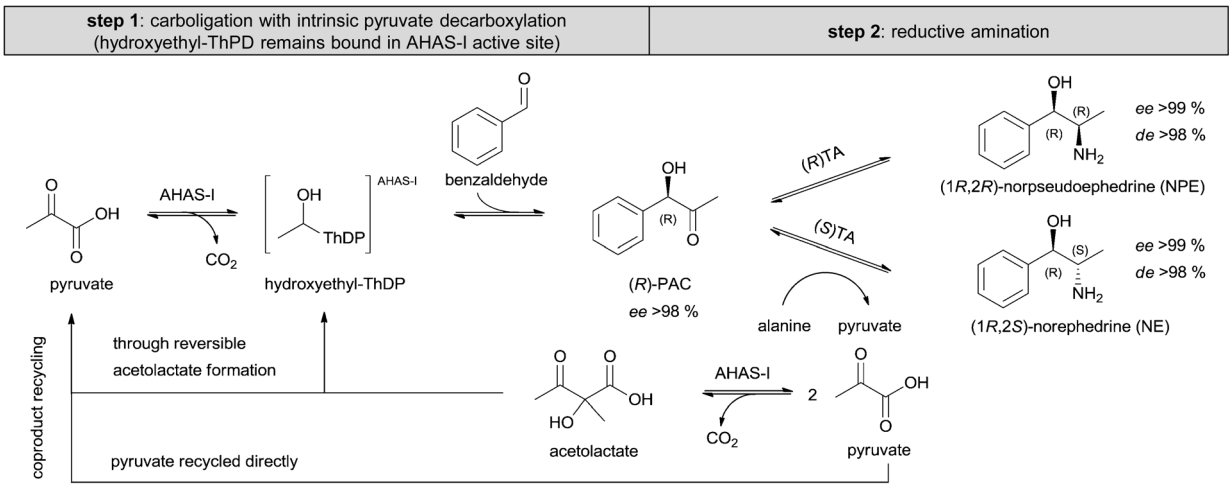


***Scheme 2.*** One-pot two-step reaction for the synthesis of nor(pseudo)ephedrine performed as a recycling cascade combining acetohydroxyacid synthase I (AHAS-I) and a (*S*)- or (*R*)-selective ω-transaminases ((*R*)TA, (*S*)TA).

thermodynamic equilibrium constant for the reductive amination of PAC with alanine as the amine donor to be $2.31 \times 10^{-3}$ (Chapter 4 in the Supporting Information). Consequently, when equimolar concentrations of alanine and PAC are used and the by-product pyruvate is not removed, a theoretical conversion of less than 5% is obtained (see Chapter 4.4 in the Supporting Information). In our reaction setup, pyruvate can be removed by two different carboligation reactions mediated by AHAS-I: 1) the carboligation with benzaldehyde yielding PAC or 2) a carboligation with another pyruvate molecule yielding acetolactate. The reversible reaction giving acetolactate is kinetically favored, whereas the reaction equilibrium lies on the side of PAC formation.[10] Thus, acetolacetate is a suitable substrate for the carboligation of (*R*)-PAC by the cleavage reaction to pyruvate and hydroxyethyl-ThDP.

A challenge in this one-pot two-step cascade is the fact that the starting material benzaldehyde might serve as a substrate for AHAS-I as well as for the ω-transaminases. As a consequence of the higher chemical reactivity of aldehydes relative to ketones and steric constraints in the active site of ω-TAs, it was not possible to find an enzyme among the 25 screened ω-TAs for which the reductive amination of PAC was kinetically favored over the reductive amination of benzaldehyde. The most promising (*S*)-selective transaminase *Cv*-(*S*)TA has a roughly 17-fold higher initial rate in the reaction with benzaldehyde than with PAC (Figure 1 A). As a consequence, in a one-pot two-step cascade reaction where AHAS-I and *Cv*-(*S*)TA were added simultaneously, 98% of the benzaldehyde was converted to benzylamine (Figure 2 A). However, in the case of the (*R*)-selective ω-TAs we could surprisingly identify enzymes for which the simultaneous one-pot two-step reaction provided (1*R*,2*R*)-NPE with conversions of up to 85% (Figure 2 A). The initial rate activities of the ω-TA from *Aspergillus terreus* (*At*-(*R*)TA) for reactions with PAC and benzaldehyde were on the same order of magnitude (Figure 1 B), but roughly ten times lower than the initial rates for the (*R*)-PAC formation

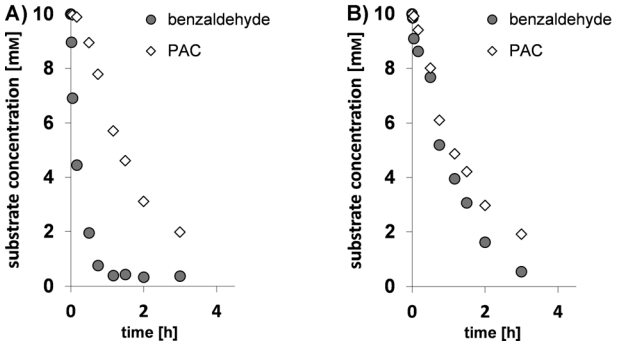


***Figure 1.*** Conversion curves for the reductive amination of benzaldehyde (10 mM) and PAC (10 mM) by A) ω-TA *Cv*-(*S*)TA (1 mg mL$^{-1}$) and by B) *At*-(*R*)TA (1 mg mL$^{-1}$), respectively. The reaction was carried out in 100 mM HEPES (pH 7.5 with 200 μM pyridoxal-5′-phosphate (PLP), 50 μM flavine adenine dinucleotide (FAD), 100 μM ThDP, 5 mM $MgCl_2$) containing (*S*)- or (*R*)-α-methylbenzylamine (10 mM) as amine donor.

catalyzed by AHAS-I. These differences suffice to reduce the amount of formed by-product (benzylamine) to merely 10%.

In line with these experimental data, the NE/benzylamine ratio is low when the one-pot two-step reaction is performed as a simultaneous cascade including a recycling step. Here, both enzymes were added simultaneously to a mixture of 20 mM benzaldehyde, 10 mM pyruvate, and 50 mM alanine. Since no further pyruvate was added, product concentrations higher than 10 mM (NE or NPE) are only possible by the successful recycling of pyruvate that is generated by deamination of alanine. Remarkably, with *At*-(*R*)TA about 14 mM (1*R*,2*R*)-NPE and only 5.5 mM benzylamine were formed in this simultaneous recycling mode, while in case of the *Cv*-(*S*)TA the major product is benzylamine.

Although benzylamine can be separated from NPE and NE by column chromatography (mobile phase EtOAc/MeOH/$NH_3$ = 85:10:5), it is more advantageous to reduce

  

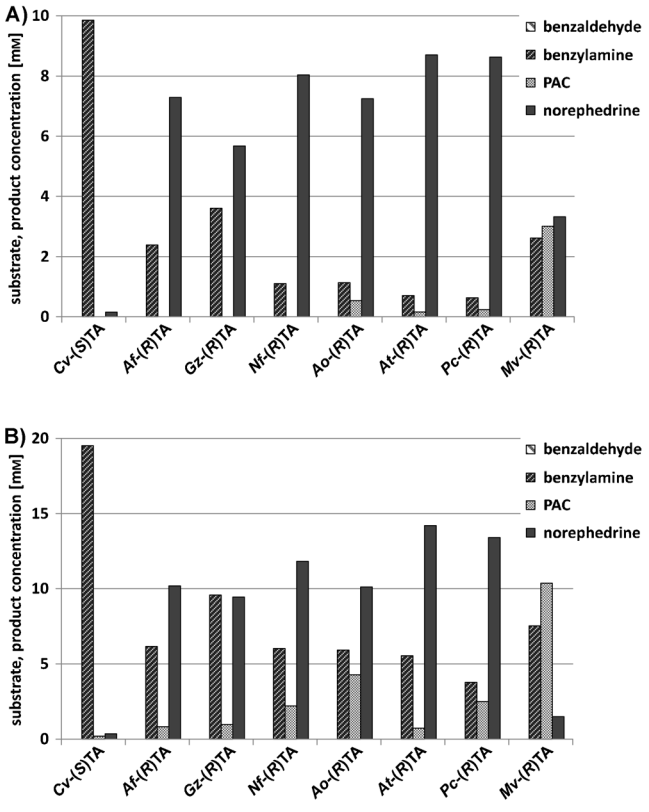


***Figure 2.*** A) One-pot two-step simultaneous cascade and B) one-pot two-step simultaneously recycling cascade with *Cv*-(*S*)TA and seven (*R*)-selective TAs. For the one-pot two-step reaction 10 mM benzaldehyde, 10 mM pyruvate, and 50 mM D- or L-alanine were dissolved in 100 mM HEPES (pH 7.5 with 200 μM PLP, 50 μM FAD, 100 μM ThDP, 5 mM $MgCl_2$) and the two enzymes (AHAS-I and ω-TA) were added simultaneously. The recycling cascade reactions (B) were performed analogously with 20 mM benzaldehyde instead of 10 mM.

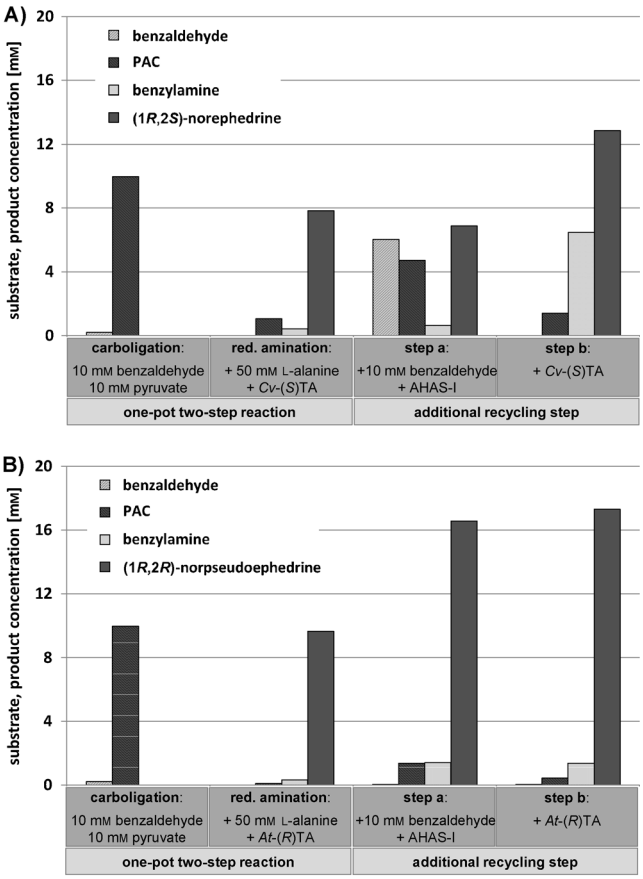


***Figure 3.*** Synthesis of A) (1*R*,2*S*)-NE and B) (1*R*,2*R*)-NPE performed as a sequential one-pot two-step reaction with an additional recycling step (time-dependent reaction curve can be found in Chapter 6 in the Supporting Information). Reaction conditions: 100 mM HEPES (pH 7.5 with 200 μM PLP, 50 μM FAD, 100 μM ThDP, 5 mM $MgCl_2$), 25 °C, 100 rpm. One-pot two-step reaction: Carboligation (1 h): 10 mM benzaldehyde, 10 mM pyruvate, 0.5 mg mL$^{-1}$ AHAS; reductive amination (12 h): +50 mM alanine, +1 mg mL$^{-1}$ TA. Recycling step:step a: +10 mM benzaldehyde, +0.5 mg mL$^{-1}$ AHAS-I (A: 1.5 h, B: 5 h); step b: +1 mg mL$^{-1}$ TA (A: *Cv*-(*S*)TA, 12 h, B: *At*-(*R*)TA, 5 h).

the formation of byproducts by appropriate process engineering in order to increase process efficiency. There are two general ways to perform a cascade reaction: one is the already described simultaneous mode, the other one the so-called sequential mode, where the catalysts are added consecutively.[9] In our sequential synthetic enzyme cascade, the limiting step is the reductive amination. In order to circumvent this bottleneck, we optimized the reaction parameters of the reductive amination step regarding pH, temperature, the concentrations of transaminase and AHAS-I, and the amine donor/PAC ratio (see Chapter 5 in the Supporting Information). For the enzyme combination *Cv*-(*S*)TA/AHAS-I conversions exceeding 80% could be achieved under optimized cascade conditions (pH 7.5, 25 °C, 1 mg mL$^{-1}$ *Cv*-(*S*)TA, 0.5 mg mL$^{-1}$ AHAS-I, alanine/PAC = 5:1).

These optimized conditions were applied in the one-pot two-step sequential mode. Here, the transaminase was added after the benzaldehyde had been completely consumed in the AHAS-I-catalyzed carboligation step (after 1 h 100% conversion was achieved, Figure 3A). This increased the conversion of (1*R*,2*S*)-NE from 2% (Figure 2A: one-pot two-step simultaneous cascade) to 78% (7.8 mM, Figure 3A) with the combination AHAS-I/*Cv*-(*S*)TA. Under these conditions the undesired by-product benzylamine amounted to less than 0.5 mM (Figure 3A). Upon subsequent addition of further 10 mM of benzaldehyde and fresh AHAS-I, PAC was formed in a second carboligation step. Since no further pyruvate was added, this result demonstrates that the recycling of pyruvate, generated by deamination of alanine, was successful in this sequential enzyme recycling cascade mode. However, the reaction resulted in the formation of only 4.7 mM PAC (47% conversion), which is most likely due to the instability of acetolactate. If the latter is chemically decarboxylated to acetoin, it is no longer available for PAC formation. Moreover, acetoin (and probably also acetolactate) can act as substrates for *Cv*-(*S*)TA as described previously.[11] Further, neither the NE nor the benzylamine concentration increased significantly, which suggests almost complete inactivation of *Cv*-(*S*)TA (Figure 3A). Addition of fresh *Cv*-(*S*)TA started the reaction again yielding 12.9 mM (1*R*,2*S*)-NE ($de > 98\%$, $ee > 99\%$). This corresponds to roughly 65% of the possible

  

product concentration (related to a total benzaldehyde concentration of 20 mM, Figure 3A).

In the case of *At*-(*R*)TA the cascade reaction is even more efficient (Figure 3B): (1*R*,2*R*)-NPE is accessible in the one-pot two-step sequential cascade with conversions greater than 96% and very high stereomeric purity ($de > 98\%$, $ee > 99\%$). After addition of another 10 mM benzaldehyde and fresh AHAS-I, the *At*-(*R*)TA was still active. Thus, without addition of further transaminase, 16.6 mM (1*R*,2*R*)-NPE (83% conversion) was obtained in 5 h by the complete one-pot two-step recycling cascade. Further addition of *At*-(*R*)TA did not considerably increase the final product concentration.

In summary, we have developed a strategy for the synthesis of (1*R*,2*S*)-NE and (1*R*,2*R*)-NPE. Both compounds are accessible in a biocatalytic one-pot two-step reaction in high stereoisomeric purity ($de > 98\%$, $ee > 99\%$) from inexpensive starting materials without isolation of the intermediate product. Additionally, these cascade reactions can be performed in a novel "recycling mode" in which the coproduct of the second step is removed without addition of further catalysts or cosubstrates and recycled as a substrate for the first cascade step.

By combining reaction and process optimization, the sequential cascade consisting of AHAS-I and *Cv*-(*S*)TA provided (1*R*,2*S*)-NE with a conversion of 80% (8.0 mM). For the production of (1*R*,2*R*)-NPE we could identify (*R*)-selective ω-TAs catalyzing the one-pot two-step cascade in a simultaneous mode with conversions up to 85% (8.5 mM). Moreover, in the sequential mode, formation of the side product (benzylamine) is reduced and (1*R*,2*R*)-NPE was obtained with a conversion exceeding 96% within 13 h. In the recycling step (addition of another 10 mM benzaldehyde, but no pyruvate) a total concentration of 16.6 mM (1*R*,2*R*)-NPE (83% conversion) was observed after further 5 h reaction time.

The recycling mode can be applied to any set of reactions for which a clever combination of cosubstrates and coproducts is possible, such that the coproducts of one reaction can be reused as substrates for the other. This approach optimizes the atom economy of the reaction by reducing the waste production.

## Experimental Section

L-alanine (Merck), D-alanine (Sigma Aldrich), and pyruvate (Sigma–Aldrich) were of > 99% purity. Benzaldehyde (Sigma–Aldrich) was freshly distilled before use. The preparation of the catalysts *Cv*-(*S*)TA and AHAS-I is described in the supporting information. (*R*)-selective TAs are commercially available from Enzymicals AG (Germany) as lyophilized crude cell extracts. Descriptions of the reaction details, reaction analysis, cascade optimizations, and equilibrium determination can be found in the Supporting Information.

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