Efficient 2-step biocatalytic strategies for the synthesis of all nor(pseudo)ephedrine isomers

--- Supplementary Information ---

Table of contents:

1.) Catalyst preparation - crude cell extract, purified enzyme and lyophilized whole cells

All catalysts were cloned and heterologously expressed in *Escherichia coli* as described below. If not otherwise indicated, the same cell stocks were either lyophilized directly (lyophilized whole cells) or disrupted and the target protein was lyophilized after a previous chromatographical purification

1.1) AHAS-I - purified enzyme and lyophilized whole cells

AHAS-I was cloned, expressed and purified as described before.[3] The purity of AHAS-I lyophilized powder was >90 % with a protein content of 15 % (w/w).

1.2) *Ap*PDC-E469G - lyophilized whole cells

*Ap*PDC variant E469G (pyruvate decarboxylase from *Acetobacter pasteurianus*) was cloned and expressed as described before.[4]

1.3) *Cv*-(*S*)TA - purified enzyme and lyophilized whole cells

For the preparation of purified lyophilized *Cv*-(*S*)TA the cloning[5], expression[6], preparation of the cell-free extract[7] and the purification were performed as previously described.[3] The enzyme was obtained in purity >90 % and the lyophilized powder had a protein content of 70 % (w/w). Lyophilized whole cells were prepared by an optimized protocol for expression. *E. coli* BL21(DE3) was transformed with the HIS-tagged *Cv*-(*S*)TA coding plasmids.[5] A pre-culture was grown overnight in lysogeny broth (LB) medium containing 50 mg L^{-1} kanamycin at 37 °C. The main culture was inoculated in Terrific Broth medium (containing 4 % (v/v) glycerin and 50 mg L^{-1} kanamycin) to an OD_{600nm} of 0.1 in a 10 L bioreactor (Labfors, Infors AG, Switzerland). The pH (7.0) and temperature (30 °C) of the cultivation were constant while the gas exposure rate (0-600 L h^{-1}, 21 % oxygen) and the stirring rate (400-1.200 rpm) were varied to keep the oxygen partial pressure in a range of 20-30 %. Enzyme expression was induced by addition of 50 µM isopropyl-β-D-thiogalactopyranoside at an OD_{600nm} of 3. Cells were harvested after 20 h and stored at -20 °C. These cells were either lyophilized directly or used for protein purification according to the protocol described before. The purity of purified *Cv*-(*S*)TA was >90 % and the lyophilized powder hat a protein content of ~70 % (w/w).

1.4) *At*-(*R*)TA – lyophilized crude cell extract

The (*R*)-selective transaminase *At*-(*R*)TA is commercially available by Enzymicals AG as lyophilized crude cell extract.[8]

1.5) *R*ADH and *Lb*ADH – purified enzyme and lyophilized whole cells

The preparation of *R*ADH cells and optional protein purification steps were performed as indicated elsewhere.[9] *Lb*ADH was expressed and purified as described in the work of Kulishova *et al.*.[10]

1.6) Expression level of enzymes in lyophilized whole cells, crude cell extracts and purity of purified enzymes

The expression levels of the target proteins *Ap*PDC-E469G, AHAS-I, *Cv*-(*S*)TA, *R*ADH and *Lb*ADH in lyophilized whole cells (LWC) of *E. coli* (see ESI chapter 1.1-1.3 and 1.5) were analyzed with SDS-PAGE. In addition, the purity of *At*-(*R*)TA from *E. coli* as crude cell extract (ESI chapter 1.4) and the purity of purified lyophilized enzymes were analyzed with the same method. For the sample preparation of LWC, a suspension of 1.66 mg_{LWC} $mL_{SDS\text{-}buffer}^{-1}$ (w/v, SDS-buffer: 1-fold NuPAGE® LDS Sample Buffer) was heated for 25 min at 95 °C in an Eppendorf Thermomixer. For samples of crude cell extract and purified proteins, a solution of 1.5 $mg_{protein}$ $mL_{SDS\text{-}buffer}^{-1}$ was heated for 2 min at 95 °C in Eppendorf Thermomixer. 10 µL of these solutions were added to a NuPAGE® Novex® 4-12% Bis-Tris Protein Gel (1.0 mm, 12 well; Thermo Fisher Scientific Inc.). SDS-PAGE and staining of the gel was performed in accordance with the NuPAGE® user manual.

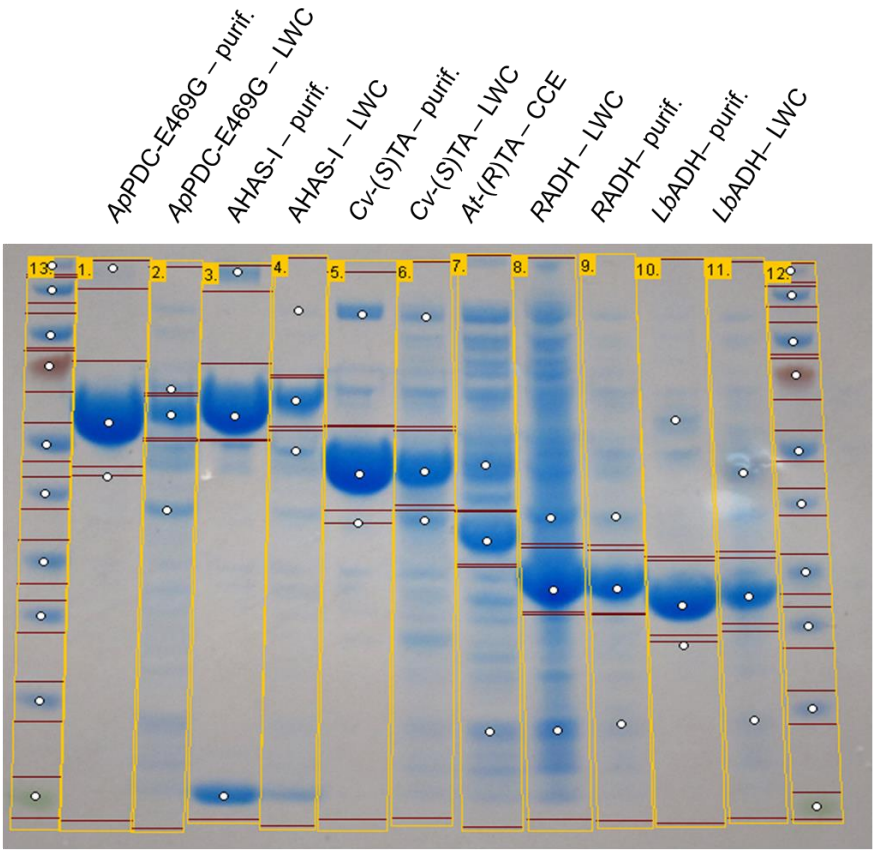


Figure 1 Expression level of enzymes in lyophilized whole cells (LWC) or crude cell extracts (CCE) and purity of purified enzymes (purif.) determined by SDS-PAGE.

The bands' intensities of the target protein relative to the sum of all proteins in the lane were quantified by using the freeware tool "GelAnalyzer 2010a" to give a more precise estimation of the protein expression level and protein purities:

Table 1 Estimation of expression levels and protein purities

	purified protein	**lyophilised whole cells**
*Ap*PDC-E469G	>95 %,	~40 %,
AHAS-I	>90 %,	~52 %,
Cv-(*S*)TA	>90 %,	~52 %,
*R*ADH	~54 %	~45 %
*Lb*ADH	~80 %,	~60 %.
	crude cell extract	
At-(*R*)TA	~28 %,	

2.) Reaction analytics

2.1) Quantification of benzaldehyde, phenylacetylcarbinol (PAC), acetophenone and 1-phenylpropane-1,2-dione

PAC (R_t = 8.7 min) and benzaldehyde (R_t = 17.1 min) were quantified by achiral HPLC-analysis as described before.[3, 7] For the quantification of acetophenone and 1-phenylpropane-1,2-dione, 20 µL reaction solution were added to 180 µL acetonitrile and analysed using a LiChrospher RP-8 reverse phase column, 250x4 mm, 5 µm pore-size column with a linear gradient of 45 % (v/v) acetonitrile in H_2O (containing 0.3 % (v/v) H_3PO_4). Typical retention times (200 nm) for acetophenone were 7.2 min and for 1-phenylpropane-1,2-dione 4.4 min.

2.2) Quantification of benzylamine, nor(pseudo)ephedrine isomers and 1-amino-1-phenyl-propan-2-one (APPO)

For the quantification of benzylamine, nor(pseudo)ephedrine and 1-amino-1-phenyl-propan-2-one (APPO) the reaction was extracted by the addition of 50 µL NaOH (1 M) to a 100 µL reaction solution. The solution was centrifuged (1000 rpm, Eppendorf centrifuge 5424, 3 min) and the supernatant (125 µL) extracted with 125 µL ethyl acetate containing 0.1 µL mL^{-1} decane as internal standard. The organic phase was analyzed by chiral gas chromatography with a Chirasil-DEX CB column (25 m x 320 µm x 25 µm) at a constant column temperature of 150 °C. Typical retention times were: decane = 1.8 min, benzylamine = 2.1-2.4 min, APPO = 4.4 min and nor(pseudo)ephedrines = 6.3-6.9 min.

2.3) Absolute configuration of all nor(pseudo)ephedrine isomers

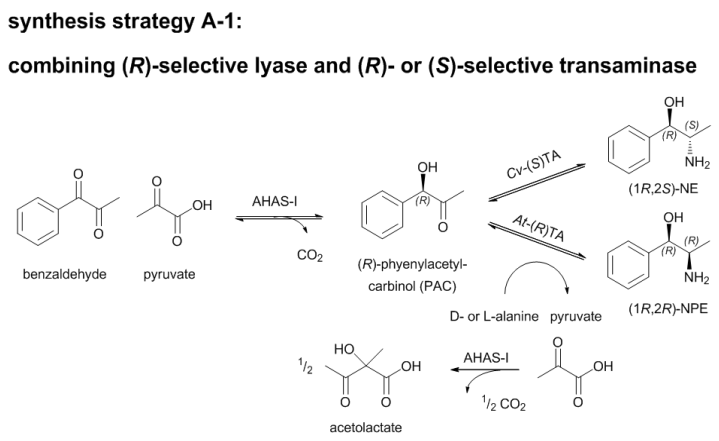
The absolute configuration of all nor(pseudo)ephedrine isomers was determined as described previously.[3] Typical retention times of the four possible isomers on the Chirasil-DEX CB column (25 m x 0.25 mm x 0.25 µm) with a constant column temperature of 130 °C were: (1*S*,2*S*)-norpseudoephedrine = 24.4 min, (1*R*,2*R*)-norpseudoephedrine = 25.7 min, (1*S*,2*R*)-norephedrine = 28.0 min, (1*R*,2*S*)-norpseudoephedrine = 29.5 min.

2.4) Determination of the enantiomeric excess of (*R*)- and (*S*)-PAC

The configuration of (*R*)- and (*S*)-PAC was determined according to literature.[4] Typical retention times for the enantiomers were: (*R*)-PAC = 11.40 min and (*S*)-PAC = 11.95 min.

3.) Reaction procedure

3.1) Synthetic strategy A-1



3.1.1) Reaction optimization for the 1-pot 2-step sequential cascade combining AHAS-I and *At*-(*R*)TA

In order to determine the minimum amount of AHAS-I (purified lyophilized enzyme - see 1.1) and *At*-(*R*)TA (lyophilized crude cell extract – sell 1.3) required for the 1-pot 2-step sequential cascade, these enzymes were applied in different concentrations. In the first step, 20 mM benzaldehyde and 20 mM pyruvate were used (in 100 mM HEPES pH 7.5 with 200 µM PLP, 50 µM FAD, 100 µM ThDP, 5 mM $MgCl_2$) were used, and the concentration of AHAS-I was varied between 0.5 mg mL^{-1} and 5 mg mL^{-1}. Each reaction was performed in a volume of 1.5 mL in 2 mL closed glass vials (CS-Chromatographie Service GmbH, Germany) at 25 °C and 300 rpm shaking in a Eppendorf Thermomixer with a glass vial adapter (Eppendorf AG, Germany). After 90 min reaction time, 100 mM D-alanine and *At*-(*R*)TA were added in different concentrations (0-1 mg mL^{-1}) without isolation or quantification of the reaction intermediate (*R*)-PAC. The complete reaction was analyzed concerning the (1*R*,2*R*)-NPE product concentration after another 12 h. For the statistical data analysis and the data interpolation (see main article - Figure 1), the Kriging method was used as previously described.[3]

3.1.2) 1-Pot 2-step sequential cascade combining AHAS-I and *At*-(*R*)TA with high substrate concentrations

The 1-pot 2-step sequential synthesis of (1*R*,2*R*)-NPE with benzaldehyde and pyruvate substrate concentrations of up to 100 mM was performed with optimized enzyme concentrations for AHAS-I (purified lyophilizate) and *At*-(*R*)TA (lyophilized crude cell extract). For the carboligation step, 0.025 $mg_{protein}\,mL^{-1}\,mM^{-1}{}_{BA}$ of AHAS-I was added to 25, 50, 75 or 100 mM benzaldehyde with equimolar concentrations of pyruvate (Pyr). All reactions were performed in a volume of 1.5 mL in 2 mL closed glass vials (CS-Chromatographie Service GmbH, Germany) at 25 °C and 300 rpm in a Eppendorf Thermomixer with a glass vial adapter (Eppendorf AG, Germany). The reaction buffer 100 mM HEPES (pH 7.5) contained 200 µM PLP, 50 µM FAD, 100 µM ThDP, and 5 mM $MgCl_2$. After 90 min reaction time 0.02 $mg_{protein}\,mL^{-1}\,mM^{-1}{}_{BA}$ of *At*-(*R*)TA and 2.5 mM mM_{BA}^{-1} D-alanine were added. The solution was analyzed after another 12 h reaction time and concentrations of benzaldehyde, benzylamine, PAC and (1*R*,2*R*)-NPE were quantified as described (see ESI chapter 2.1 and 2.2).

3.1.4) Synthesis of (1*R*,2*S*)-NE combining AHAS-I and *Cv*-(*S*)TA with lyophilized whole cells and purified enzymes

The AHAS-I catalyzed carboligation of 10 mM benzaldehyde with 10 mM pyruvate was performed in 100 mM HEPES (pH 7.5) containing 50 µM FAD, 100 µM ThDP, and 5 mM $MgCl_2$ with either 0.5 $mg_{protein}$ mL^{-1} purified enzyme or 5 mg_{cells}/mL lyophilized whole cells. Reactions were carried out in 1.5 mL reaction volume in 2 mL glass vials at 25 °C (see ESI chapter 3.1.1). Reactions with LWC were performed at an increased shaking rate of 800 rpm. The decrease of benzaldehyde and the formation of PAC were analyzed after different time points between 5 min and 1 h (see ESI chapter 2.1). Separately, the reductive amination of 10 mM (*R*)-PAC (provided by BASF with an *ee* = 84 %)[7] with 10 mM (*S*)-α-methylbenzylamine (MBA) catalyzed by the *Cv*-(*S*)TA was investigated with either 1 $mg_{protein}$ mL^{-1} purified enzyme or 5 mg_{cells} mL^{-1} lyophilized whole cells. The reactions were performed in 100 mM HEPES (pH 7.5) containing 200 µM PLP in a reaction volume of 1.5 mL (2 mL glass vials, 25 °C, and 300 rpm or 800 rpm in the case of LWC, respectively (see ESI chapter 3.1.1). At different time points (between 10 min and 12 h) the reaction was analyzed regarding conversion of PAC (see ESI chapter 2.1).

3.1.4) Synthesis of (1*R*,2*S*)-NE: Reduction of the lag-phase by per-incubation of lyophilized whole cells in buffer

In order to investigate if an observed lag phase in the synthesis of (1*R*,2*S*)-NE from (*R*)-PAC by *Cv*-(*S*)TA containing lyophilized whole cells (see main text, Figure 4) can be reduced and to determine, if necessary rehydration of the cells is causing this lag-phase, 10 mg_{cells} mL^{-1} lyophilized whole cells were pre-incubated for 2 h in 100 mM HEPES (pH 7.5) containing 200 µM PLP at 25 °C and 800 rmp shaking rate of 800 rpm in 2 mL glass vials. After 2 h the cell suspension was diluted with the same volume of 20 mM (*S*)-α-MBA and 20 mM (*R*)-PAC to a final concentration of 5 mg_{cells} mL^{-1} lyophilized whole cells, 10 mM (*S*)-α-MBA and 10 mM (*R*)-PAC. Analogously to the reactions described before (see ESI chapter 3.1.1, and main article Figure 4) lyophilized whole cells were given directly to a solution of 10 mM (*S*)-α-MBA and 10 mM (*R*)-PAC without pre-incubation. Both reactions were performed in a reaction volume of 1.5 mL in 2 mL glass vials at 25 °C with a shaking speed of 800 rpm. The conversion of PAC was analyzed at different time points (see ESI chapter 2.1).

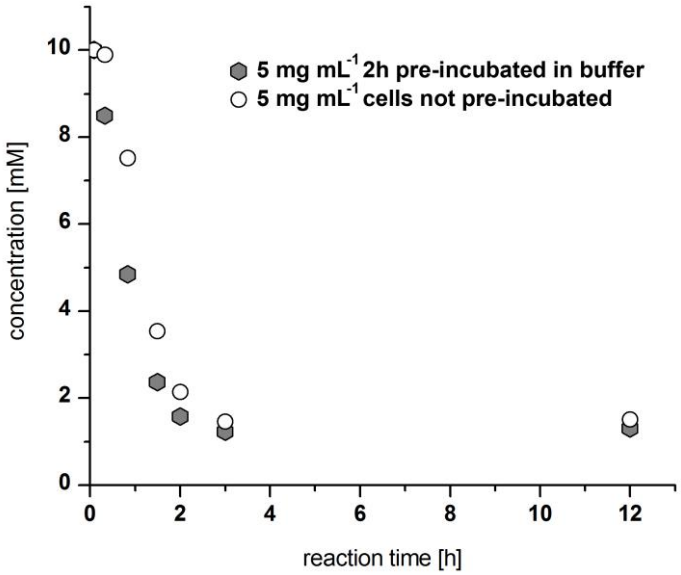
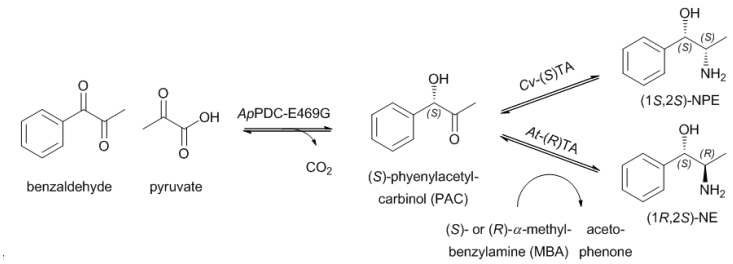


Figure 2 *Cv*-(*S*)TA catalyzed reductive amination of 10 mM (*R*)-PAC in the presence of 10 mM (*S*)-α-MBA catalyzed with lyophilized cells (5 mg mL^{-1}) wich were either per-incubated for 2 h in reaction buffer (dark combs) or added directly to a solution of the reactants (white dots).

3.2) Synthetic strategy A-2

synthesis strategy A-2:

combining (*S*)-selective lyase and (*R*)- or (*S*)-selective transaminase



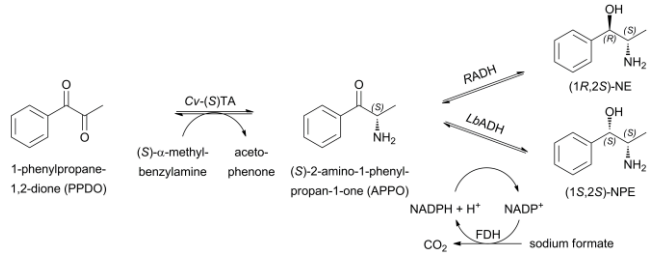
3.2.1) 2-Step cascade for the synthesis of (1*S*,2*S*)-NPE or (1*S*,2*R*)-NE

For the synthesis of (1S,2S)-NPE or (1S,2R)-NE the first reaction step, the carboligation of 40 mM benzaldehyde with 400 mM pyruvate, was conducted with the *Ap*PDC variant E469G. The reaction was performed in 100 mM potassium phosphate buffer (pH 7) containing 2.5 mM $MgSO_4$, 100 µM ThDP and 0.5 mg mL^{-1} purified enzyme. The reactions were carried out in glass vials at 25 °C. The reaction mixture was extracted with ethyl acetate after 48 h and dried over magnesium sulfate. After evaporation of the solvents, flash chromatography (eluent: petrol ether : ethyl acetate = 85 : 15) was performed. The compound (*S*)-PAC was obtained in a yield of 95 % with an *ee* of 70 %.[4, 11] The subsequent reductive amination of 10 mM (*S*)-PAC with 15 mM (*S*)-α-MBA or (*R*)-α-MBA, respectively, was carried out with *At*-(*R*)TA (0.5 $mg_{protein}$ mL^{-1} – lyophilized crude cell extract) or *Cv*-(*S*)TA (1 $mg_{protein}$ mL^{-1} – purified protein) in 100 mM HEPES (pH 7.5) containing 200 µM PLP. The reactions (reaction volume: 1.5 mL, 2 mL glass vials, 25 °C, 300 rpm) were analyzed regarding conversion (see ESI chapter 2.2) and optical purity (see ESI chapter 2.2) of the product N(P)E after 16 h reaction time.

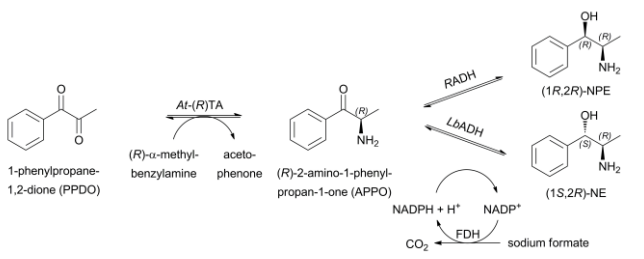
3.3) Synthetic strategy B-1 and B-2 (transaminase-ADH):

The two strategies of the 1-pot 2-step reaction combining either *Cv*-(*S*)TA (synthesis strategy B-1) or *At*-(*R*)TA (synthesis strategy B-2) in the first cascade step and the *R*ADH or *Lb*ADH in the second cascade step were performed using the same method. For both cascade steps the application of purified enzymes and lyophilized whole cells (LWC) was investigated (exception: reaction with *At*-(*R*)TA, which was only available as lyophilized crude cell extract; see ESI chapter 1.4). As an example, the reductive amination of 10 mM 1,2-PPDO (1-phenylpropane-1,2-dione) with *Cv*-(*S*)TA containing LWC was optimized with respect to different concentrations of the amine donor (*S*)-α-MBA (see ESI chapter 3.3.1). The reduction reaction was performed using a previously published protocol.[12]

synthesis strategy B-1:
combining (*S*)-transaminase and (*R*)- or (*S*)-alcohol dehydrogenase



synthesis strategy B-2:
combining (*R*)-transaminase and (*R*)- or (*S*)-alcohol dehydrogenase



3.3.1) Optimization of the amine donor ratio for the reductive amination of 1,2-PPDO with *Cv*-(*S*)TA containing lyophilized whole cells

The reductive amination of 10 mM 1,2-PPDO with LWC containing *Cv*-(*S*)TA (10 mg_{LWC} mL^{-1}) was carried out with different concentrations of the amine donor (*S*)-α-MBA (10-20 mM, see Fig. 3). The reaction was performed in 100 mM HEPES (pH 7.5) containing 200 µM PLP at 25 °C in 1.5 mL reaction volume in 2 mL closed glass vials (CS-Chromatographie Service GmbH, Germany) and 800 rpm shaking speed in a Eppendorf Thermomixer comfort with a glass vial adapter (Eppendorf AG, Germany). The reaction was analyzed after 16 h regarding concentration of acetophenone and the substrate 1,2-PPDO (see ESI chapter 2.1).

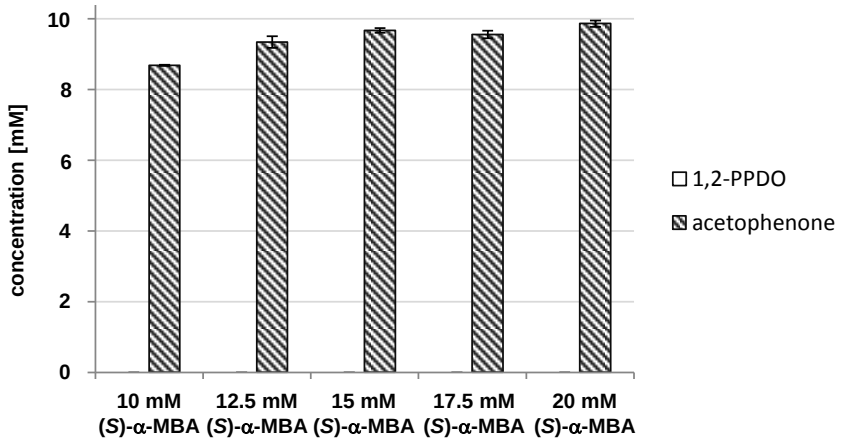


Figure 3 *Cv*-(*S*)TA catalyzed reductive amination of 10 mM 1,2-PPDO with different concentrations of (*S*)-α-MBA and 10 mg_{LWC} mL^{-1} in 100 mM HEPES (pH 7.5) containing 200 µM PLP at 25 °C and 800 rpm.

3.3.2) Synthetic strategy B - first cascade step: reductive amination of 1,2-PPDO with *At*-(*R*)TA or *Cv*-(*S*)TA

The reductive amination of 1,2-PPDO was carried out in case of the *Cv*-(*S*)TA with 15 mM (*S*)-α-MBA and either 1 $mg_{protein}$ mL^{-1} purified enzyme or 10 mg_{LWC} mL^{-1} LWC. Reactions with *At*-(*R*)TA were performed with 1 $mg_{protein}$ mL^{-1} lyophilized crude cell extract and 15 mM (*R*)-α-MBA. As a reaction buffer 100 mM HEPES (pH 7.5) containing 200 µM PLP was used. The reaction volumes were added up to ~10 mL (in 15 mL closed glass vials) and were shaken at room temperature (21 °C) at 500 rpm in a Barloworld Scientific Ltd. (UK) vortex shaker. At different time points (see Figure 2-A, 3-A, 4-A and 5-A) the reactions were analyzed regarding their concentration of the co-product acetophenone and the substrate 1,2-PPDO (see ESI chapter 2.1)

3.3.3) Termination of the transaminase reaction

As indicated (see Tab. 1 – main article), two general strategies for the termination of the transaminase reaction (chapter 3.3.2) were applied: ultrafiltration and pH-shift. In the ultrafiltration method, the TA was removed from the reaction solution (volume ~10 mL, see ESI chapter 3.3.2) by centrifugation (4 °C, 30 min, 4.000 rpm, universal centrifuge 32-R – Hettrich GmbH & Co.KG (Germany)) in a VivaSpin-20 (Vivascience AG, Germany) with a membrane cut-off of 10 kDa. For the pH-shift method, the solution was titrated with 20 % (v/v) HCl to pH 2 and then re-titrated with 10 M NaOH to pH 7.5. This solution was then used in the reduction reaction (see ESI chapter 3.3.4). Here, the transaminase was deactivated by titration to pH 2 with 20 % (v/v) HCl. The reaction was incubated for 1 min at room temperature (21 °C) before re-titration with 10 M NaOH to pH 7.5. The denatured enzymes (visible as precipitate) were removed by centrifugation in closed glass vials (4 °C, 5 min, 4.000 rpm, universal centrifuge 32-R-Hettrich GmbH (Germany)). The supernatant was used for the reduction reaction (see ESI chapter 3.3.4).

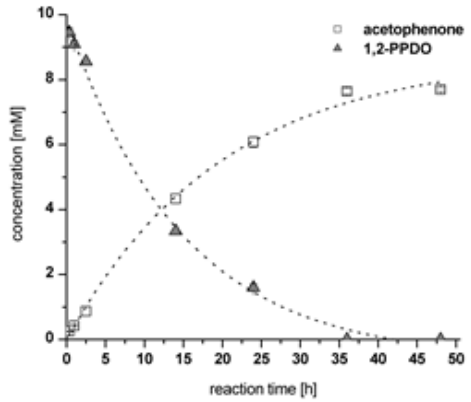
3.3.4) Synthetic strategy B - second cascade step: reductive reaction catalyzed by *R*ADH or *Lb*ADH

The reductive reaction was performed as specified (see Tab. 1 – main article) either directly with the reaction solutions from the TA reaction (see ESI chapter 3.3.2), or with the permeate/supernatant from the termination step (see ESI chapter 3.3.3). Reaction solutions performed in the first cascade step with *Cv*-(*S*)$TA_{purified\ enzyme}$, *Cv*-(*S*)TA_{LWC} or *At*-(*R*)TA_{CCE} were split as indicated for the corresponding reductive step (see main article, Tab. 1). In general 0.5 mM $NADP^+$, 150 mM sodium formate, 10 µL mL^{-1} FDH (Jülich Fine Chemicals, Jülich, now Codexis, no. 25.10) and either purified enzyme (1 $mg_{protein}$ mL^{-1}) or lyophilized whole cells (10 mg_{LWC} mL^{-1}) of the respective alcohol dehydrogenases (*Lb*ADH *or R*ADH) were added to the corresponding solution. Without changing any further reaction conditions (like buffer, shaking rate and reaction temperature) the 1-pot 2-step cascades were continued in ~4 mL reaction volume (in 5 mL closed glass vials) as in the first reaction step (see ESI chapter 3.3.2). At different time points, the concentration of the intermediate 1-amino-1-phenyl-propan-2-one and the N(P)Es were quantified (see chapter 2.2). Moreover, at the endpoint of the reactions the absolute configuration (*de*, *ee*) of the products N(P)E were determined (see ESI chapter 2.3).

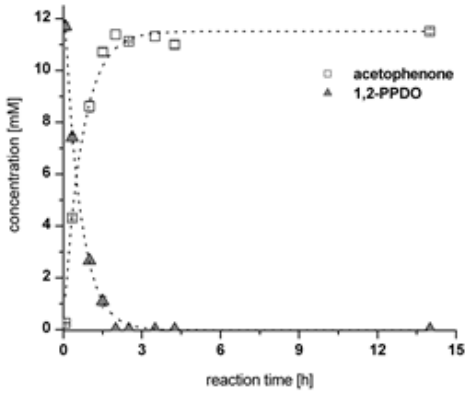
3.3.5) Time-dependent reaction curves for the synthesis strategy B

The time-dependent reaction curves for all 1-pot 2-step cascade reactions are shown in Fig. 2-4 and summarized in Tab. 1 of the main article.

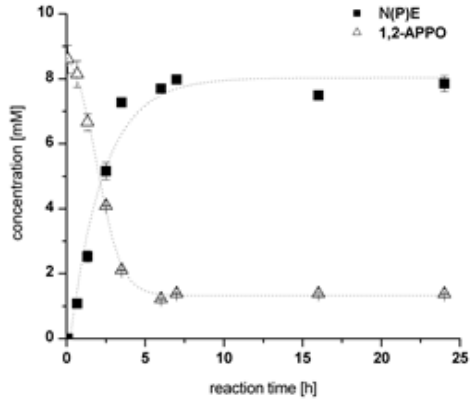
A: 1st step: 1,2-PPDO converted with *Cv*-(*S*)TA purified enzyme



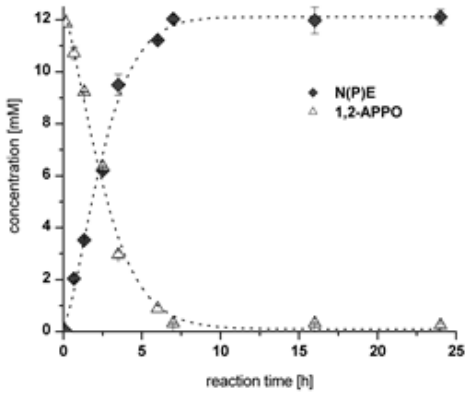
A: 1st step: 1,2-PPDO converted with *At*-(*R*)TA as CCE



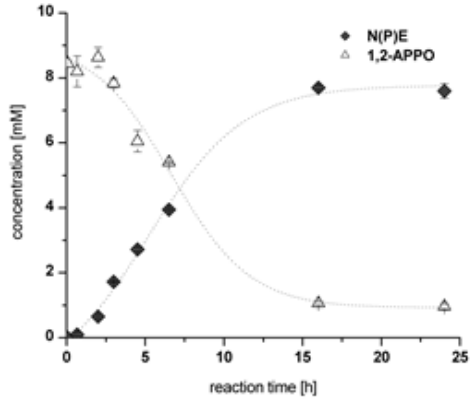
B: 2nd step: APPO converted with *R*ADH purified enzyme



B: 2nd step: APPO converted with *R*ADH purified enzyme



C: 2nd step: APPO converted with *Lb*ADH purified enzyme



C: 2nd step: APPO converted with *Lb*ADH purified enzyme

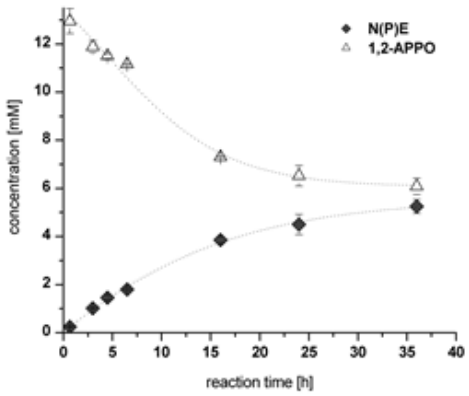


Figure 3 1-Pot 2-step sequential cascade combining the *Cv*-(*S*)TA$_{\text{purified enzeme}}$ (left) or the *At*-(*R*)TA$_{\text{CCE}}$ (right) in the first reaction step (A: reductive amination of 1,2-PPDO) with either the *R*ADH$_{\text{purified enzyme}}$ (B) *or Lb*ADH$_{\text{purified enzyme}}$ (C) in the second step (reductive hydrogenation)

A: The reductive amination was carried out with 1 $mg_{protein}\,mL^{-1}$ *Cv*-(*S*)TA (purified enzyme) or 1 $mg_{protein}\,mL^{-1}$ *At*-(*R*)TA (crude cell extract - CCE) in 100 mM HEPES (pH 7.5), 200 µM PLP, with ~10 mM 1,2-PPDO (*At*-(*R*)TA: 12 mM) and 15 mM (*R*)- or (*S*)-□-MBA, respectively, at room temperature (21 °C). The reactions were terminated in this case by a pH-shift (titrated with 20 % (v/v) HCl to pH 2 then re-titrated with 10 M NaOH to pH 7.5).

B, C: For the reductive hydrogenation the corresponding solutions (A) were split and 0.5 mM $NADP^+$, 150 mM sodium formate, 10 µL mL^{-1} FDH and either 1 $mg_{protein}$ mL^{-1} purified enzyme of the *R*ADH (B) or *Lb*ADH (C) were added and subsequently further incubated at room temperature (21 °C). The concentration of N(P)E is given as a sum of all N(P)E isomers.

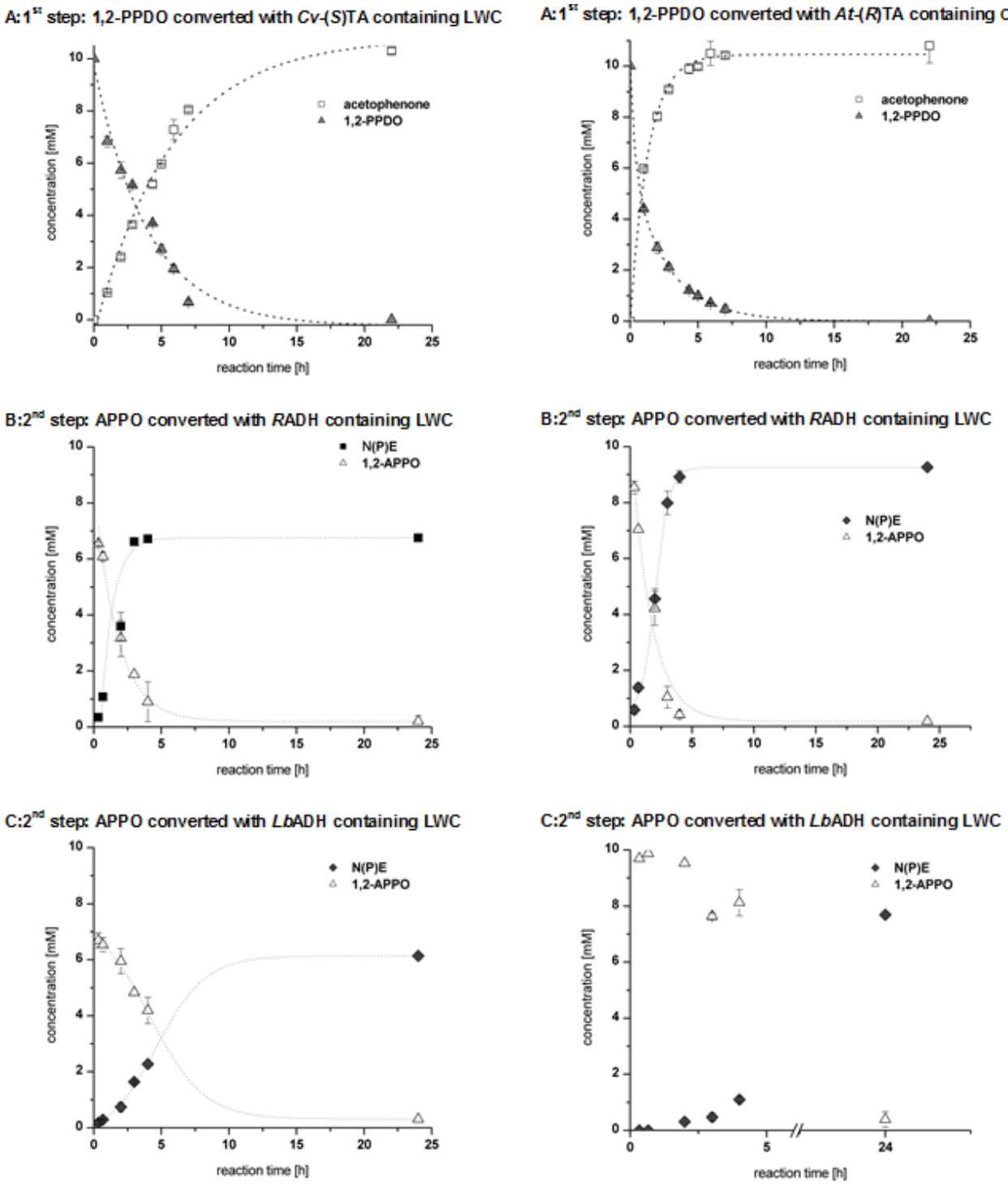


Figure 4 1-Pot 2-step sequential cascade combining the *Cv*-(*S*)TA_{LWC} (left) or the *At*-(*R*)TA_{CCE} (right) in the first reaction step (A: reductive amination of 1,2-PPDO) with either the *R*ADH_{LWC} (B) *or Lb*ADH_{LWC} (C) in the second step (reductive hydrogenation)

A: The reductive amination was carried out with 10 mg_{cells} mL^{-1} *Cv*-(*S*)TA (lyophilized whole cells - LWC) or 1 $mg_{protein}$ mL^{-1} *At*-(*R*)TA (crude cell extract - CCE) in 100 mM HEPES (pH 7.5), 200 µM PLP, with ~10 mM 1,2-PPDO and 15 mM (*R*)- or (*S*)-α-MBA, respectively, at room temperature (21 °C). The reactions were terminated in this case by a pH-shift (titrated with 20 % (v/v) HCl to pH 2 then re-titrated with 10 M NaOH to pH 7.5)

B, C: For the reductive hydrogenation the corresponding solutions (A) were split and 0.5 mM $NADP^+$, 150 mM sodium formate, 10 µL mL^{-1} FDH and either 10 mg_{yells} mL^{-1} lyophilized whole cells of the *R*ADH (B) or *Lb*ADH (C) were added and subsequently further incubated at room temperature (21 °C). The concentration of N(P)E is given as a sum of all N(P)E isomers.

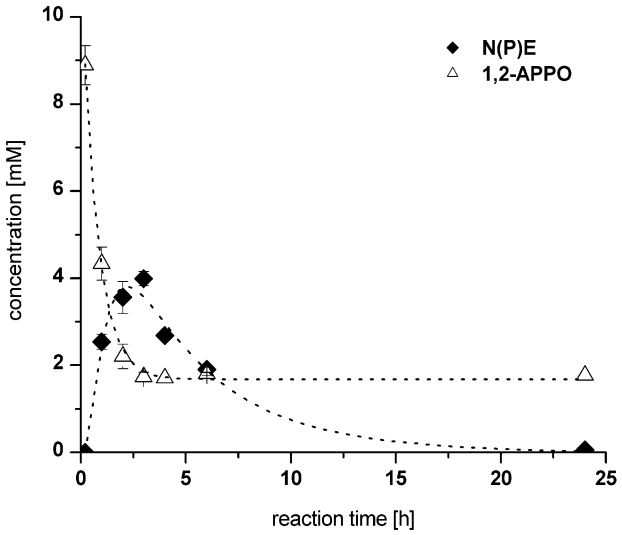


Figure 5 Second reaction step (reductive hydrogenation) of the 1-pot 2-step sequential cascade combining the *Cv*-(*S*)TA/*Lb*ADH (each as purified enzyme) without inactivation of the TA after the first reaction step.

The reductive amination (data not shown) had been carried out with 1 $mg_{protein}\,mL^{-1}$ *Cv*-(*S*)TA (purified enzyme) in 100 mM HEPES (pH 7.5), 200 µM PLP, with ~10 mM 1,2-PPDO and 15 mM (*S*)-α-MBA at room temperature (21 °C). The reactions were not terminated in this case.

For the reductive hydrogenation 0.5 mM $NADP^+$, 150 mM sodium formate, 10 µL mL^{-1} FDH and 1 $mg_{protein}\,mL^{-1}$ purified enzyme of the *Lb*ADH was added and subsequently further incubated at room temperature (21 °C). The concentration of N(P)E is given as a sum of all N(P)E isomers.

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