EurJOC
European Journal of Organic Chemistry

A Journal of
ChemPubSoc Europe

Accepted Article

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To be cited as: *Eur. J. Org. Chem.* 10.1002/ejoc.201800323

Link to VoR: http://dx.doi.org/10.1002/ejoc.201800323

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COMMUNICATION

Development of an *in situ*-Product Crystallization (ISPC)-Concept to Shift the Reaction Equilibria of Selected Amine Transaminase-Catalyzed Reactions

Dennis Hülsewede,[a] Marco Tänzler,[a] Philipp Süss,[b] Andrea Mildner,[a,c] Ulf Menyes[b] and Jan von Langermann*[a]

Abstract: The synthesis of enantiopure amines via amine transaminases involves several challenges including unfavorable reaction equilibria and product inhibition. Described here is a non-catalytic approach to overcome such problems by using an *in situ*-product crystallization (ISPC) to selectively remove a targeted product amine from an amine transaminase-catalyzed reaction. The continuous removal of the product amine from its reaction solution as a barely soluble salt effectively yields a displacement of the reaction equilibrium towards the products and facilitates a simple downstream processing approach via filtration. The targeted product amine is eventually obtained from the salt, while the counter ion compound can be easily recycled.

Biotransformations became over the past decades a powerful technique for the synthesis of valuable compounds on laboratory and industrial scale.[1-7] Herein pyridoxal 5'-phosphate (PLP)-dependent transaminases (TAs) and especially amine transaminases (ATAs) have gained in recent years a significant impact in the synthesis of optically pure amines, which are valuable building blocks for various agrochemicals and active pharmaceutical ingredients, e.g. sitagliptin.[8-14] These enzymes basically catalyze the deamination of a primary amine (amine donor) with a simultaneous amination of an aldehyde or ketone (amine acceptor). The transamination-reaction can be carried out as a kinetic resolution of a racemic amine or an asymmetric synthesis from the respective prochiral ketone.[15] Due to a maximum yield of 100% the asymmetric synthesis is in theory preferred, especially if a catalyst with high enantioselectivity is used.[16] Unfortunately, thermodynamic limitations and certain product inhibitions tend to limit the applicability of transaminases in asymmetric synthesis, which needs to be overcome for synthetic purposes.[17-21] Aside using an uneconomic excess of amine donor, complex (co)product removal techniques are currently considered, e.g. enzymatic cascades, membrane processes and non-catalytic side reactions.[22-30] Such techniques unfortunately increase (in general) overall complexity of biocatalytic reaction systems, require additional or tailor-made co-substrates and generate further by-products.[31-34]

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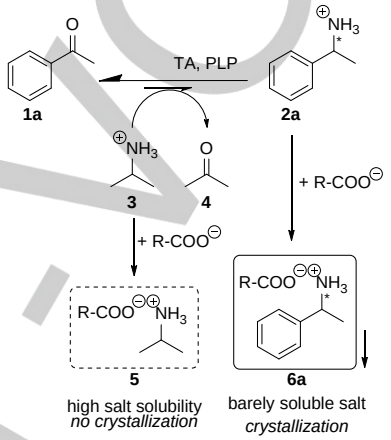
With this study we present a novel crystallization-based approach for the direct removal of a desired product amine from an amine transaminase-catalyzed reaction (Scheme 1).



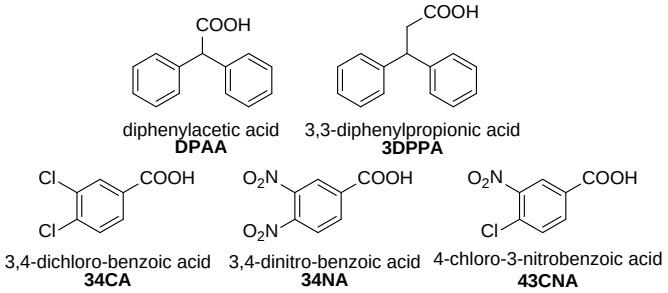
Scheme 1. Combination of an *in situ*-product crystallization (ISPC) with an amine transaminase-catalyzed reaction to yield a barely soluble 1-phenylethylamine salt

The product amine **2** is herein selectively crystallized from solution as a barely soluble amine salt **6**, while all other reactants, especially the applied donor amine **3**, remain in solution. This *in situ*-product crystallization (ISPC) continuously removes the desired product amine from solution and thus yields an equilibrium displacement towards the products.[35-37] The counter ion (here shown as a carboxylate) is added directly to the reaction solution and can be isolated for reuse from the formed solid salt. A stoichiometric use of the carboxylate in comparison to all applied amines is not required (see below).

First, the main requirement of this concept is the choice of a specific counter ion, which generates a barely soluble salt with the target amine for its crystallization, while the donor amine salt is not crystallized. Unfortunately, most commonly used amine salts, especially amine halides salts, show very high solubilities in aqueous solutions and thus are not applicable for such an ISPC concept. Moreover, simple and reliable prediction approaches for amine salt solubilities are not available. Second, the used transaminase has to tolerate the required concentration of the chosen counter ion. Third, the formed salt has to be stable under process conditions and should be easily recovered from the reaction mixture (filtration).

Consequently, commercially readily available aliphatic, aromatic and heteroaromatic carboxylic acids were selected and screened as their respective carboxylate salts towards common amines from an amine transaminase-catalyzed reaction. 1-Phenylethylamine and substituted derivatives **2a-f** thereof served as model product amines and were compared with typical donor

amines such as isopropylamine **3**, racemic 2-butylamine, DL-alanine and L-alanine (see Supporting Information for further details). Here the salt of the product amine needs to exhibit a significant lower solubility then its donor amine salt counterpart since the donor amine is still applied with an excess. This straightforward screening approach resulted in two benzylbenzene-based acids and three benzoic acid derivatives that matched the above mentioned criteria (as the respective carboxylate ions at pH 7.5) (Scheme 2). Noteworthy, all three benzoic acid derivatives show a 3,4-substitution pattern, which seems to be beneficial for the crystallization of enantiopure 1-phenylethylamine.



Scheme 2. Investigated carboxylic acids for an *in situ*-product crystallization of amines from an amine transaminase-catalyzed reaction

For example, the isopropylamine salt of 4-chloro-3-nitrobenzoic acid (43CNA) **5** shows a very high solubility of 993 mmol·L^{-1}, while the 1-phenylethylamine salt of 43CNA **6a** is considerable less soluble with 22 mmol·L^{-1}. As given by LE CHATELIER's principle, the solubility of such amine salts can be further reduced if an over-stoichiometric amount of carboxylate is added to the mother liquor, which pushes the equilibrium from the dissociated forms in solution towards its non-dissociated solid salt form (precipitated).

The applicability of the three best acids was tested with 7 exemplary amine transaminases from *Aspergillus fumigates* (*Af*ATA), *Gibberella zeae* (*Gz*ATA), *Neosartorya fischeri* (*Nf*ATA), *Aspergillus oryzae* (*Ao*ATA), *Aspergillus terreus* (*At*ATA), *Mycobacterium vanbaalenii* (*Mv*ATA) and *Silicibacter pomeroyi* (*Sp*ATA) (Figure 1).

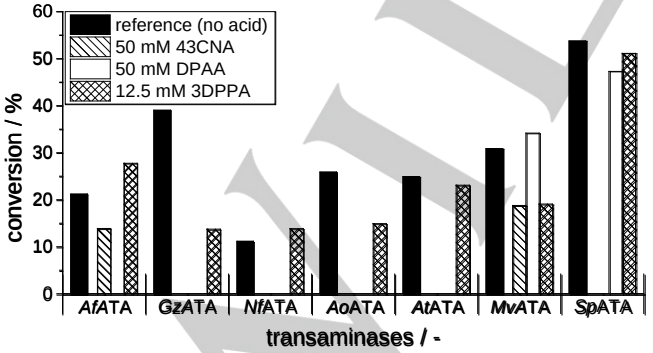


Figure 1. Effects of 43CNA, DPAA and 3DPPA on selected amine transaminases; Conditions: 200 mM phosphate buffer pH 7.5, 10 mM acetophenone **1**, 500 mM (43CNA and DPAA) or 250 mM (3DDPA) isopropylamine, 5 mg·mL^{-1} lyophilized cell extract, 30 °C, 22 h; the obtained concentrations of **2** were intentionally held below the solubility limit of product salt **6** to prevent an undesired *in situ*-product crystallization; 43CNA was chosen as a representative of the identified benzoic acid derivatives

The results show that almost all investigated amine transaminases are already strongly inhibited by 50 mM of the more soluble acids 43CNA and DPAA. Herein *Mv*ATA and *Sp*ATA were identified as the most stable enzymes. A noticeable exception from these results is the acid 3DPPA, which is only sparingly soluble in buffered solutions. This effectively limits the carboxylate concentration in an aqueous solution to a maximum of ≤25 mM, depending on temperature and pH. The excess of solid 3DPPA remains suspended in the reaction mixture. Such a low 3DPPA-concentration also does not significantly inhibit the investigated ATAs. Fortunately, the investigated 3DPPA-salts exhibit the lowest solubility of product amine salt **6**, which fits perfectly into the above mentioned ISPC-requirements (Table S3 and S4, supporting information). Consequently, 3DPPA was identified as the most valuable acid for the application in a crystallization-based *in situ*-product removal (ISPR) of amine **2** from an amine transaminase-catalyzed reaction.

The use of 3DPPA in combination with an exemplary *Sp*ATA-catalyzed conversion of 100 mM acetophenone **1a** to (*S*)-1-phenylethylamine **2a** shows clearly the synthetic advantage of an acid-based ISPC (Figure 2). The classical reaction approach with a low donor amine concentration of only 250 mM isopropylamine yields a non-sufficient conversion of 19%. A simple addition of 1.25 eq. solid 3DPPA improved the overall conversion directly to ca. 75%, regardless of the use as a whole cell biocatalyst or partially purified cell extract.

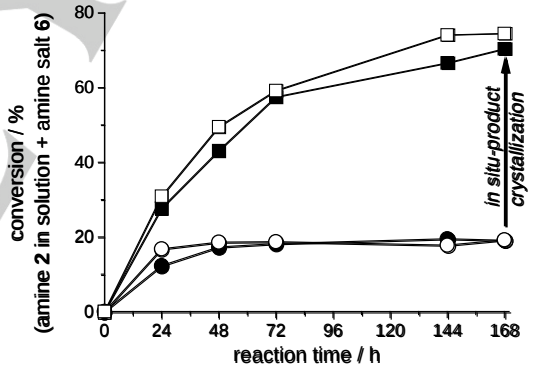


Figure 2. Time progression curves for a *Sp*ATA-catalyzed synthesis of (*S*)-1-phenylethyl-amine with and without *in situ*-product crystallization (ISPC); □-whole cells or ■-cell extract with 125 mM 3DPPA, ○-whole cells or ●-cell extract without 3DPPA; Conditions: 200 mM phosphate buffer pH 7.5, 100 mM acetophenone, 250 mM isopropylamine, 15 mg·mL^{-1} lyophilized cell extract or whole cells, 30 °C

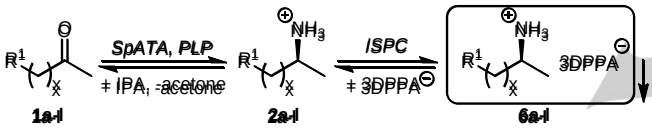
The majority of product **2** is afterward present as solid salt **6**, which can be almost quantitatively recovered by filtration after cooling the reaction mixture to 0 °C. Thus this ISPC-concept with 3DPPA translates to a more atom-efficient synthesis since less donor amine is required and a simplified downstream processing-approach is facilitated (see below). Noteworthy, the low solubility of 3DPPA does not limit the crystallization of product amine salt since the constant removal of 3DPPA from aqueous solution is continuously compensated back to its original solubility limit by a simultaneous dissolution of 3DPPA (from excess solid).

The shown ISPC-concept with acid 3DPPA was also successfully used for the *Sp*ATA-catalyzed conversion of 100 mM

10.1002/ejoc.201800323

of selected acetophenone derivatives **1b-g** and five non-aromatic substrates **1h-l** (Table 1). As expected, for all substrates low conversions were obtained without ISPC due to the low, but still over-stoichiometric, use of 250 mM isopropylamine. A simple addition of 3DPPA increases product formation significantly for almost all investigated substrates. Improvements range between 2 and 8.1-fold with yields of up to 96 % for **1l**, while the products are selectively crystallized as its 3DPPA-salts. Noteworthy, the obtained high enantiomeric excesses of the product amines are solely based on the high enantioselectivity of the applied enzyme and are not affected by the crystallization of the respective amine salt.[38] The remaining mother liquor, including excess isopropylamine, can be directly reused for a further increase of atom efficiency of the transaminase-catalyzed reaction. Even higher donor amine concentrations will yield a further increase in conversion, but include the risk of an undesired crystallization of donor amine salt, which eventually yields a decrease in product formation. A noticeable entry is also the conversion of 2-pentanone **1i**, which represents the lower limit of the shown concept. Herein the respective product amine salt is too similar to its donor amine salt and thus both salts crystallize simultaneously, lowering the apparent conversion.

Table 1. ISPC-supported *Sp*ATA-catalyzed synthesis of chiral amines



substrate	R^1	x	conversion reference / %	conversion ISPC / %	e.e.(*S*) / %[a]
1a	Ph	0	19	75	>99.5
1b	m-F-Ph	0	21	69	>99.5
1c	p-F-Ph	0	11	61	>99.5
1d	m-Cl-Ph	0	8	46	>99.5
1e	p-Cl-Ph	0	8	65	>99.5
1f	m-MeO-Ph	0	10	37	>99.5
1g	p-MeO-Ph	0	4	8	>99.5
1h	Cy	0	0	8	n.d.
1i	Me	2	61	53	n.d.
1j	Me	3	37	72	>99.5
1k	Me	4	20	78	98.7
1l	iPr	1	36	96	n.d.

[a] Values are given for the ISPC-supported reaction; Conditions: 200 mM phosphate buffer pH 7.5, 100 mM substrate, 250 mM isopropylamine, 15 mg·mL^{-1} lyophilized whole cells, 30 °C; 125 mM 3DPPA for ISPC; n.d. – not determined

Isolation of product amine **2** is easily realized by dissolving product salt **6** in an aqueous solution at high pH, followed by extraction with MTBE and a subsequent solvent evaporation. Alternatively, the respective hydrochloride salt can be directly crystallized from the ether phase by a careful addition of HCl. In addition, the spent acid 3DPPA can also be precipitated from the remaining aqueous phase by acidification with concentrated HCl, due to its low solubility at low pH.

Summarizing, the presented *in situ*-product crystallization of an amine from an amine transaminase-catalyzed reaction by addition of a selected acid/carboxylate presents a powerful synthetic alternative to the use of tailor-made donor amines and complex cascade reaction systems. The main advantages of this ISPC are a more atom-efficient use of donor amines and a simplified downstream processing-approach by filtration. The targeted product amine can be afterwards extracted from its salt and the applied 3DPPA acid easily recycled.

Experimental Section

General semi-preparative procedure for the amine transaminase-catalyzed synthesis of 2a-m in combination with an *in situ*-product crystallization. To 25 mL 200 mM phosphate buffer pH 7.5 532 µL isopropylamine (≙ 250 mM) and 707 mg 3DPPA (≙ 125 mM) were given and the resulting suspension adjusted to pH 7.5 with aqueous H_3PO_4-solution. Afterwards PLP, substrate (≙ 100 mM) and biocatalyst were added and the resulting mixture shaken at 200 rpm. After completion of the reaction the resulting mixture was filtered to obtain the formed product amine salt. (This solid will also contain the remaining biocatalyst and excess 3DPPA.) Afterwards the solid fraction was washed with 10 mL MTBE to remove remaining substrate and parts of excess 3DPPA. The solid was then given into 5 mL water, 0.5 ml conc. NaOH was added to increase pH and formed product **2** extracted with 5 mL MTBE. After phase separation the product was obtained as its hydrochloride by a slow addition of conc. HCl to the ether phase. 3DPPA can be precipitated from the remaining aqueous solution by adding conc. HCl, e.g. for recycling (isolated yield 71%).

General reaction control procedure. Samples (500 µL) were taken periodically and thoroughly mixed by a vortex mixer with 50 µl conc. NaOH to quench the reaction and increase pH. Afterwards 500 µL MTBE were added, mixed again by a vortex mixer and centrifuged (2 min, 3000 rpm) to improve phase separation. 200 µl were taken from the organic layer, combined with 50 µl of a 25 mM *n*-decane-solution in MTBE (internal standard) and subsequently analyzed by gas chromatography (column: CP-Chirasil-Dex CB; 25 m, 0.25 mm, 0.25 µm by Agilent, USA). Enantiomeric excesses of the obtained amines were measured by High Performance Liquid Chromatography (HPLC) using a Chiralcel OD-H column with a n-heptane/ethanol eluent (see also supporting information).

Acknowledgements

The authors thank Prof. Dr. Udo Kragl for his ongoing support & fruitful discussions, Jennifer Kuziola & Jan-Niklas Dohm for their help with the acid screening procedure, Dr. Dirk Michalik for his assistance with NMR-measurments and Dr. Christine Fischer and Eleonora Gerova-Martin from the analytical department of Leibniz Institute for Catalysis, Rostock for measuring the enantiomeric excesses of amine samples. Funding by German Federal Ministry of Education and Research (BMBF – Bundesministerium für Bildung und Forschung; project number: 031A123) and German Research Foundation (DFG – Deutsche

Forschungsgemeinschaft; LA 4183/1-1) is gratefully acknowledged.

Keywords: biocatalysis • amine synthesis • *in situ*-product removal • crystallization • chiral

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10.1002/ejoc.201800323

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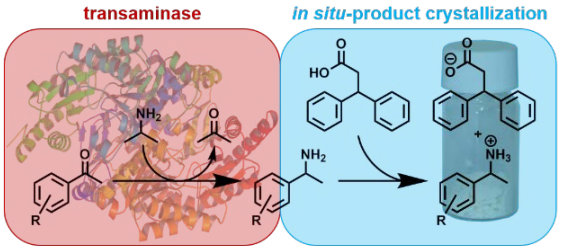

Crystallization vs. equilibrium: A novel *in situ*-product crystallization method has been developed to overcome the unfavorable equilibrium of transaminase-catalyzed reactions. A specific carboxylic acid is added to the aqueous reaction mixture to form a barely soluble salt with the targeted product amine, which pushes the reaction to the product side and simplifies downstream-processing.

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