



# Use of Immobilized Amine Transaminase from *Vibrio fluvialis* under Flow Conditions for the Synthesis of (S)-1-(5-Fluoropyrimidin-2-yl)-ethanamine

Riccardo Semproli<sup>+</sup>,<sup>[a]</sup> Gianmarco Vaccaro<sup>+</sup>,<sup>[a, b]</sup> Erica E. Ferrandi,<sup>[c]</sup> Marta Vanoni,<sup>[c]</sup> Teodora Bavaro,<sup>[a]</sup> Giorgio Marrubini,<sup>[a]</sup> Francesca Annunziata,<sup>[b]</sup> Paola Conti,<sup>[b]</sup> Giovanna Speranza,<sup>[d]</sup> Daniela Monti,<sup>\*[c]</sup> Lucia Tamborini,<sup>\*[b]</sup> and Daniela Ubiali<sup>\*[a]</sup>

We report on the covalent immobilization of the (S)-selective amine transaminase from *Vibrio fluvialis* (Vf-ATA) and its use in the synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine, a key intermediate of the JAK2 kinase inhibitor AZD1480. Immobilized Vf-ATA on glyoxyl-agarose (activity recovery: 30%) was used in a packed-bed reactor to set-up a continuous flow biotransfor-

mation coupled with a straightforward in-line purification to circumvent the 2-step process described in literature for the batch reaction. The newly developed biotransformation was run in a homogeneous system including dimethyl carbonate as a green co-solvent. Optically pure (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (ee > 99%) was isolated in 35% yield.

## Introduction

Amine transaminases (ATAs, EC 2.6.1.x) catalyze the enantioselective amination of ketones by utilizing simple amines as amino donors and the vitamin B<sub>6</sub>-based pyridoxal 5-phosphate (PLP) cofactor as a molecular shuttle.<sup>[1-4]</sup> Since the first characterization of these enzymatic activities in the late 90s,<sup>[5]</sup> ATAs have been intensively investigated for the synthesis of chiral amines. These molecules are valuable building blocks for the preparation of APIs including antiarrhythmics (e.g. vernakalant), antibiotics (e.g. moxifloxacin), anti-epilepsy agents (e.g. levetiracetam and pregabalin), and antidiabetics (e.g. sitagliptin).<sup>[6]</sup> Among the examples of ATAs application on an

industrial scale,<sup>[7-12]</sup> a milestone was placed by Merck and Codexis 10 years ago with the synthesis by transamination of sitagliptin (Januvia<sup>®</sup>), the first marketed oral antihyperglycemic drug belonging to the gliptin family. This bioprocess allowed the replacement of a high-pressure rhodium-catalyzed asymmetric enamine hydrogenation step by applying a highly engineered ATA variant.<sup>[7]</sup> Later on, a number of reports about ATA immobilization for the synthesis of this API were released.<sup>[13-16]</sup> These outstanding achievements have demonstrated that, if optimized, ATA-based enzymatic platforms can be competitive with the established manufacture processes, since align selectivity, efficiency and sustainability.<sup>[17]</sup>

Another example of a pre-industrial ATA-catalyzed process was developed by AstraZeneca for the production of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**, Scheme 1A), a key intermediate in the synthesis of the JAK2 kinase inhibitor AZD1480, a molecule targeting idiopathic myelofibrosis and polycythaemia rubra vera.<sup>[9,18]</sup> The well-known ATA from *Vibrio fluvialis* (Vf-ATA)<sup>[19-22]</sup> was selected among 60 transaminases; process conditions were optimized on a gram scale starting from the fluorinated ketone (**1**) and using (S)- $\alpha$ -methylbenzylamine (**2**, (S)-MBA) as the amino donor.

The reaction was performed in a biphasic system by using toluene to pull out the co-product acetophenone (**4**). The continuous extraction was necessary to shift reaction equilibrium and to avoid product inhibition effects.<sup>[18]</sup> Since the synthesized amine is water soluble, the purification protocol relies on the conversion of the amine to the corresponding Boc-derivative followed by extraction and precipitation of the amine hydrochloride with HCl in *iso*-propanol. Besides co-product removal through the use of a biphasic system as described above, unfavorable reaction equilibrium of ATA-catalyzed amine synthesis as well as product inhibition suffered by these enzymes have been managed in different ways. The use of amino donors in large excess combined with the *in situ* removal of amine-derived by-products have been pursued, either

[a] R. Semproli,<sup>+</sup> G. Vaccaro,<sup>+</sup> Dr. T. Bavaro, Dr. G. Marrubini, Prof. D. Ubiali  
Department of Drug Sciences  
University of Pavia  
Viale Taramelli 12  
Pavia I-27100 (Italy)  
E-mail: daniela.ubiali@unipv.it

[b] G. Vaccaro,<sup>+</sup> F. Annunziata, Prof. P. Conti, Prof. L. Tamborini  
Department of Pharmaceutical Sciences  
University of Milano  
Via Mangiagalli 25  
Milano I-20133 (Italy)  
E-mail: lucia.tamborini@unimi.it

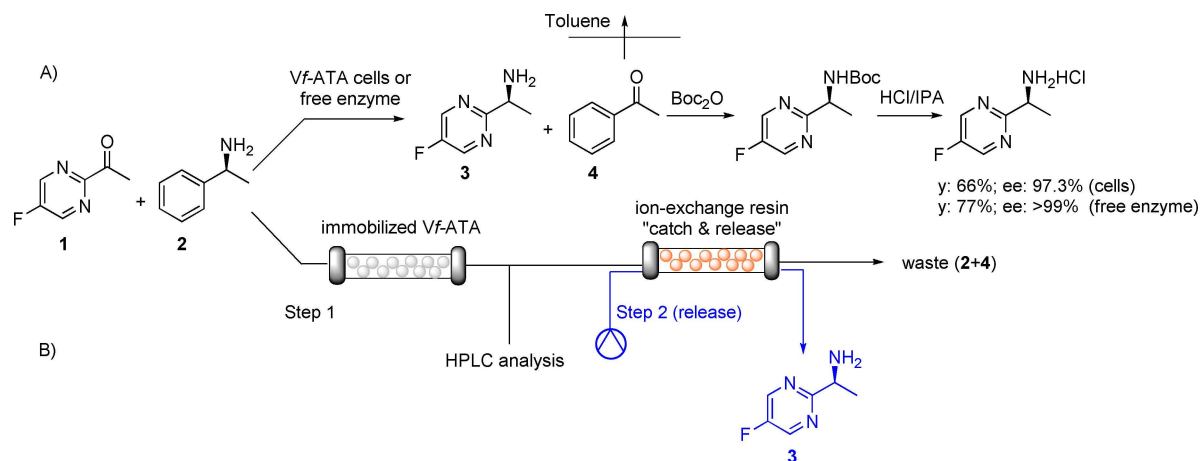
[c] Dr. E. E. Ferrandi, M. Vanoni, Dr. D. Monti  
Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC) - CNR  
Via Bianco 9  
Milano I-20131 (Italy)  
E-mail: daniela.monti@scitec.cnr.it

[d] Prof. G. Speranza  
Department of Chemistry  
University of Milano  
Via Golgi 19  
Milano I-20133 (Italy)

[<sup>+</sup>] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cctc.201902080>

This publication is part of a joint Special Collection with ChemBioChem on "Excellence in Biocatalysis Research". Please follow the link for more articles in the collection.



**Scheme 1.** Synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) catalyzed by Vf-ATA. A) Synthetic route as reported in Ref. [18]. B) Synthetic route investigated in the present work.

through an ancillary enzymatic reaction, or by exploiting specific diamines generating by-products that spontaneously undergo cyclization/tautomerization.<sup>[23–24]</sup>

Given the increasing evidences supporting the use of biocatalysis for industrial scale synthesis of APIs, a number of innovative technologies have been considered as complementary tools for the development of intensified and industrially relevant biocatalytic processes. In particular, there is a growing interest in performing biocatalyzed transformations in continuous flow reactors.<sup>[25–28]</sup> Both biocatalysis and continuous processing do have the features that make these technologies as the foremost key green research areas for sustainable manufacturing of APIs and fine chemicals. Biocatalysts, either enzymes or whole cells, are typically immobilized onto the reactor wall or on particles of a carrier material, which is then packed into a column to form a packed-bed reactor with a plug-flow behavior.<sup>[29]</sup> Among the various methods for enzyme immobilization, covalent immobilization is probably the most successful approach, allowing a robust attachment of the enzyme. Powdery materials can be used as carriers and packed in a fixed bed reactor as well as porous monoliths, which have been successfully used for ATA immobilization.<sup>[30–31]</sup> Flow chemistry, along with enzyme immobilization, can circumvent some critical issues of biocatalytic reactions, thus positively influencing the outcome of the biotransformation.<sup>[32]</sup> In particular, whereas enzyme immobilization can improve enzyme stability, operating under flow-conditions can significantly reduce substrate/product inhibition effects and push forward unfavorable reaction equilibria through the continuous removal of the product from the reaction site. Moreover, a better process control makes the reaction more efficient and minimizes waste generation.

Based on these premises, we developed a new protocol integrating flow biocatalysis with downstream processing for the end-to-end synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**), using immobilized Vf-ATA as the biocatalyst.

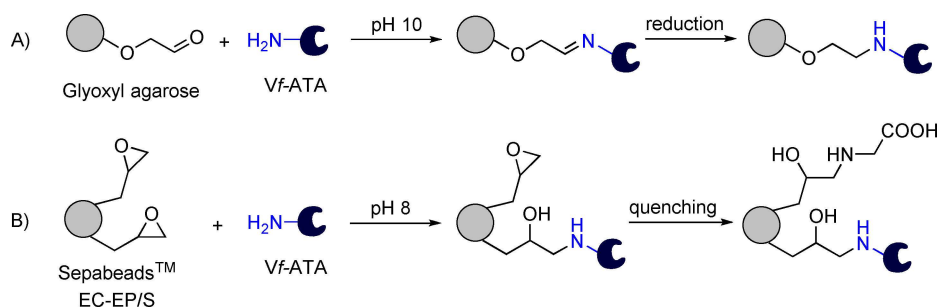
## Results and Discussion

The amine transaminase from *Vibrio fluvialis* (Vf-ATA) was successfully produced by recombinant expression in *E. coli* BL21 (DE3) cells from the corresponding codon-optimized synthetic gene (GenBank AEA39183.1). Subsequent purification by nickel-nitrilotriacetic acid (Ni-NTA) chromatography afforded 80 mg of pure protein from 1 L culture (Supporting Information, Figure S1). The specific activity of the purified enzyme was determined spectrophotometrically in the transamination reaction between the benchmark substrates (*S*)-MBA (**2**) and pyruvate (Supporting Information, Scheme S1). Two different carriers were chosen for the immobilization: glyoxyl-agarose and Sepabeads™ EC-EP/S. Both carriers rely on the covalent interaction enzyme-support and have been demonstrated to be versatile for the immobilization of many enzymes.<sup>[33–34]</sup> Moreover, both glyoxyl-agarose and Sepabeads™ EC-EP/S have been successfully applied to biotransformations under flow conditions.<sup>[35–36]</sup>

### Vf-ATA immobilization on glyoxyl-agarose

The immobilization of Vf-ATA on glyoxyl-agarose was performed at pH 10 to obtain a “multi-point” interaction through the lysine residues of the enzyme and the aldehyde groups of the carrier. The resulting enzyme-carrier bonds are weak Schiff bases and are thus reversible. Chemical reduction of the immobilized enzyme (usually by NaBH<sub>4</sub> or NaCNBH<sub>3</sub>) is frequently applied<sup>[37–38]</sup> to convert enzyme-carrier bonds into stable secondary amino groups (Scheme 2A).

This immobilization protocol gave quantitative yields in terms of immobilized protein and immobilized activity: at the endpoint (3 h) neither residual protein nor activity were found in the supernatant, but the activity recovery was very poor (3%).

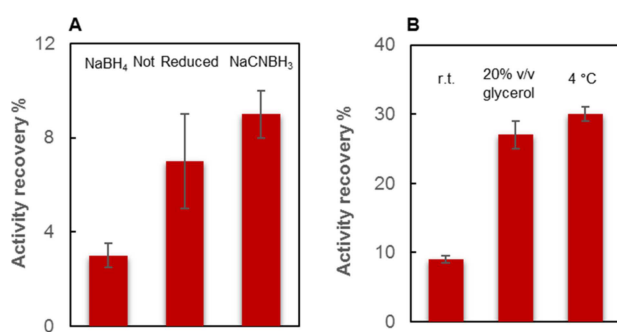


**Scheme 2.** Vf-ATA immobilization. A) Immobilization on glyoxyl-agarose was performed in sodium bicarbonate (pH 10, 50 mM) under mechanical stirring (protein loading: 2 mg g<sup>-1</sup>; carrier/total volume: 1 g/10 mL). Experiments were performed at 4 °C or r.t. for 3 h, with or without glycerol (20% v/v). NaBH<sub>4</sub> or NaCNBH<sub>3</sub> (1 mg mL<sup>-1</sup>) was used as reducing agent. B) Immobilization on Sepabeads™ EC-EP/S was performed in phosphate buffer (pH 8.0, 50 mM) under mechanical stirring (protein loading: 1 mg g<sup>-1</sup>; carrier/total volume: 1 g/10 mL). Experiments were performed at r.t. for 24 h. Quenching was performed with 3 M glycine in phosphate buffer (pH 8.5, 50 mM, 4 mL) under mechanical stirring at r.t. for 20 h.

However, the activity assay performed on the immobilization suspension when the supernatant was already non-active, but before adding the reducing agent, showed activity (data not shown), thus suggesting that the reduction step might be the cause of enzyme inactivation. To confirm whether the reduction was the critical step, three immobilization trials were performed in parallel by following the protocol described in the Experimental Section. When both protein content and activity were negligible in the supernatant (3 h), two aliquots of the same immobilized biocatalyst were reduced with either NaBH<sub>4</sub> or NaCNBH<sub>3</sub>, respectively, whereas the third aliquot was not reduced (Figure 1A). The presence of the cyano group in NaCNBH<sub>3</sub> makes this reducing agent milder than NaBH<sub>4</sub>; in fact, NaCNBH<sub>3</sub> is generally used to selectively reduce imines even in presence of aldehydes and ketones under neutral or weakly acid conditions. Although in all cases the activity recovery was < 10%, the highest activity recovery was obtained for the non-reduced biocatalyst and when the reduction was performed by using NaCNBH<sub>3</sub>. Since the reversibility of the imine groups could be a limitation in the use of the immobilized enzyme, NaCNBH<sub>3</sub> was selected as the reducing agent for the following

immobilization trials. To further increase the activity recovery of the immobilized Vf-ATA, the effect of glycerol and temperature were evaluated (Figure 1B).

In fact, glycerol is routinely used to preserve proteins under storage.<sup>[39]</sup> For this reason, glycerol has been used as an additive in some immobilization procedures; typically, the amount of glycerol does not exceed 20% v/v.<sup>[40]</sup> Furthermore, a recent report on Vf-ATA thermostability<sup>[41]</sup> showed that this enzyme retains 100% activity at 4 °C for more than 50 h, and 80% activity when it is incubated at 25–37 °C. In the presence of glycerol (20% v/v) and at low temperature (4 °C), the yields in terms of immobilized protein and activity remained quantitative and both the immobilization experiments showed a remarkable increase of the activity recovery (around 30%, Table S1). From these results, 4 °C and NaCNBH<sub>3</sub> as the reducing agent were selected for the following immobilization experiments. The activity recovery (30%) was considered suitable to switch from batch to flow applications, in agreement with previous reports.<sup>[36]</sup> Immobilization results for glyoxyl-agarose are summarized in Figure 1.

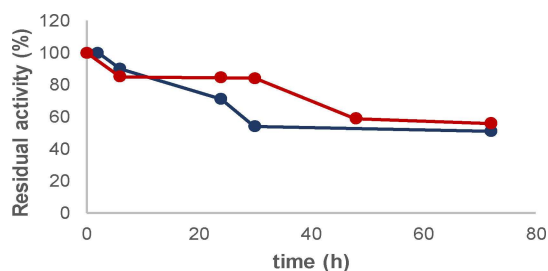


**Figure 1.** A) Effect of the reducing agent on the activity recovery (observed activity/starting activity) × 100. B) Effect of glycerol and temperature on the activity recovery (NaCNBH<sub>3</sub>). These immobilization experiments were performed with 0.5 g of glyoxyl-agarose in a final volume of 5 mL by using the same protein loading (2 mg g<sup>-1</sup>), activity loading (4.58 U g<sup>-1</sup>) and run time (3 h). For the reduction step, 5 mg of the reducing agent were added and the mixture was stirred for further 30 min.

#### Stability assay of Vf-ATA immobilized on glyoxyl-agarose

The immobilized enzyme must be active at least for the time required by the reaction to be completed. However, it is desirable that the biocatalyst maintains its activity for a longer period, thus allowing its reuse for more reaction cycles. Vf-ATA immobilized on glyoxyl-agarose (at 4 °C and reduced with NaCNBH<sub>3</sub>) was incubated with the reaction mixture containing all the reactants with the exception of the prochiral ketone (1, amino acceptor) due to its high cost. The immobilized biocatalyst maintained about 50% of the starting activity after 72 h (Figure 2).

Although a striking difference of stability between native and immobilized enzyme did not emerge, the clear advantage of the immobilized biocatalyst is its easier separation from the reaction medium and application for in continuum biotransformations.



**Figure 2.** Stability of Vf-ATA immobilized on glyoxyl-agarose (blue) compared to free Vf-ATA (red). Experimental conditions: phosphate buffer (pH 8.0, 100 mM) containing DMSO (2% v/v) in the presence of 10 mM (S)-MBA and 1 mM PLP. Immobilized Vf-ATA: 50 mg, final volume: 1 mL; free Vf-ATA: 2.6 mg, final volume: 3 mL. The residual activity was determined by the spectrophotometric assay (see Experimental Section).

### Vf-ATA immobilization on Sepabeads™ EC-EP/S

Besides glyoxyl-agarose, epoxy carriers are probably the most used supports to immobilize enzymes. These resins do not need to be pre-activated since epoxides act as the reactive groups.<sup>[34]</sup> Thus, immobilization of Vf-ATA was also performed on Sepabeads™ EC-EP/S. Enzymes bind covalently to this support by exploiting the nucleophilic features of lysine  $\epsilon$ -amino groups, thus generating a multi-point interaction (as for glyoxyl-agarose). In this case, a stable secondary amine bond is formed without the need for any further step (Scheme 2B). However, epoxides are much more reactive than aldehydes and may still react either with the protein, even after the immobilization, or with substrates and/or products of the target reaction. For this reason, unreacted epoxy groups of Sepabeads™ EC-EP/S must be quenched in order to avoid side-reactions.<sup>[42–43]</sup> Glycine was chosen for the post-immobilization quenching according to a literature protocol in which a derivatized Sepabeads™ EC-EP/S was used to immobilize the transaminase from *Halomonas elongata* (HEWT).<sup>[36]</sup> The efficient immobilization of an engineered ATA mutant from *Chromobacterium violaceum* on a properly designed epoxy-functionalized carrier has been also recently described for continuous-flow applications.<sup>[44]</sup>

Vf-ATA immobilization on Sepabeads™ EC-EP/S gave quantitative yields in terms of immobilized protein and immobilized activity after 24 h incubation. The activity of this biocatalyst was determined by a discontinuous assay because, unlike glyoxyl-agarose, epoxy-acrylic carriers are bead-like materials that interfere with the spectrophotometric analysis. Although the activity of Vf-ATA immobilized on Sepabeads™ EC-EP/S was remarkable (activity recovery:  $\approx 50\%$ , Table S2), it was observed that acetophenone (4), i.e. the product of the investigated biotransformation, was adsorbed by the carrier, thus affecting its accurate quantification. The quenching with glycine increased the hydrophilicity of the carrier, thus decreasing (but not eliminating) the acetophenone adsorption. This adsorption phenomenon was confirmed by incubating Sepabeads™ EC-EP/S (without the enzyme) with a solution of acetophenone at a

known concentration and monitoring the supernatant absorbance at 245 nm over time (data not shown).

Taking in consideration this phenomenon, glyoxyl-agarose appeared a more convenient carrier due to the acceptable activity recovery and stability. Moreover, this carrier was already shown to be suitable for flow applications.<sup>[35]</sup>

### Batch synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

The enzymatic synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3) was performed by using Vf-ATA immobilized on glyoxyl-agarose in view of transferring the bioconversion from batch to a flow system. As a preliminary approach, the amination of the non-fluorinated ketone was carried out with the aim of exploring the reaction conditions, testing the feasibility of the bioconversion with the newly immobilized enzyme, and setting-up a downstream process that might be more straightforward than that described in literature.<sup>[18]</sup> (S)-MBA (2) was selected as the amino donor because it was also used in the activity assay and it is commonly used as benchmark substrate with (S)-selective transaminases (both in batch and in flow systems).<sup>[20,36,45]</sup> Moreover, (S)-MBA is the amino donor used in the synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine reported by Meadows *et al.*<sup>[18]</sup> The reaction was performed at pH 8, according to the enzymatic assay conditions (see also Figure S2). In contrast to literature,<sup>[18]</sup> in this work a monophasic system containing DMSO (2%) as the co-solvent was used. As reported in Table 1, a 30% conversion was observed which was comparable with the results obtained with the non-fluorinated ketone (data not shown). Conversions (%) were calculated by HPLC, using the acetophenone calibration curve.

After the reaction, the biocatalyst was easily separated from the reaction mixture by filtration and showed a residual activity of 50%. This result was consistent with the stability assay of the immobilized Vf-ATA. Furthermore, no protein release was detected in the reaction (Bradford assay),<sup>[46]</sup> as a consequence of the covalent immobilization.

The data obtained in the batch reaction were considered suitable to switch the bioconversion to a flow system and thus to run the study both of the reaction and the downstream in such a set-up.

**Table 1.** Batch synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3) catalyzed by Vf-ATA immobilized on glyoxyl-agarose.

| Substrate [mM] | Vf-ATA [U] <sup>[a]</sup> | Ratio [acceptor: donor] | Conversion [%] <sup>[b]</sup> | Time [h] |
|----------------|---------------------------|-------------------------|-------------------------------|----------|
| 20             | 1.80                      | 1:1                     | 30                            | 31       |

[a] The biocatalyst was immobilized at 4 °C and NaCNBH<sub>3</sub> was used as the reducing agent. Protein loading: 2 mg g<sup>-1</sup>; activity recovery: 30%. The specific activity, determined by spectrophotometric assay, was 2.76 U g<sup>-1</sup>, [b] Conversion was calculated by HPLC. The reaction was performed in duplicate.

### Flow synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

A packed-bed reactor with immobilized *Vf*-ATA was prepared for the continuous flow application. First, the effect of residence time and temperature on the reaction outcome was evaluated using a 0.65 mL volume bioreactor (immobilized *Vf*-ATA = 0.65 g) and flowing through it a solution of 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (1, 20 mM), (*S*)-MBA (2, 20 mM) and PLP (1 mM) in phosphate buffer (100 mM, pH 8) containing DMSO (2% v/v). Conversions (%) were calculated by HPLC, using the acetophenone calibration curve. As reported in Table 2, a 40% conversion was achieved in 10 minutes of residence time (entry 2); a further increase of the residence time was not beneficial (entries 3 and 4). Keeping constant the residence time (10 min), concentration of the substrates in the feeding flow was varied, but the conversion was dramatically reduced (entry 6). Also, an increase of the temperature up to 38 °C did not speed up the reaction (entry 5).

Under a similar degree of conversion (i.e.  $c=30\%$  after 31 h for the batch biotransformation and  $c=26\%$  in 5 minutes for the flow biotransformation), the specific reaction rate ( $r$ ) of the flow reaction resulted to be about 22-fold higher than the batch reaction ( $r_{\text{flow}}=0.52 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ;  $r_{\text{batch}}=0.024 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ), probably due to the high local concentration of the biocatalyst in the packed-bed reactor.<sup>[47]</sup>

To increase the sustainability of the proposed protocol, we tested dimethyl carbonate as the co-solvent (5% v/v) for the flow reaction and evaluated the reaction outcome under continuous work.

To this aim, a 2 mL bioreactor was prepared and 100 mL of the substrate solution was flowed through it under the following conditions: residence time = 10 min,  $T=28^\circ\text{C}$ ,  $P=\text{atm}$ . Overall, a 38% conversion was obtained. Finally, an in-line purification procedure for product isolation was developed (Scheme 1B).

A first attempt to extract the produced amine using an inlet of an organic solvent (i.e. EtOAc) and a membrane phase separator was performed. However, only a low amount of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3) was recovered in the organic phase (<5%). Therefore, we exploited a catch-and-release strategy, using a column packed with a strongly acidic

resin (Dowex Marathon C) to trap any unreacted (*S*)-MBA and the desired amine. Then, after a washing step with distilled water, an inlet of aqueous ammonia (1 M) was used to release the amine product that, due to the different pKa compared to (*S*)-MBA, was collected separately and isolated in 35% yield.

### Determination of the enantiomeric excess of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

Upon in-line purification, the target amine was submitted to a HPLC chiral analysis to assess the enantiomeric excess. The absolute configuration of a chiral amino donor has to match the stereospecificity of the ATAs in order to be accepted: this feature explains why transaminases can be employed in the kinetic resolution of racemic amines.<sup>[48]</sup> For this reason, *Vf*-ATA, which is a (*S*)-selective ATA, should not be able to produce amines with (*R*)-configuration. Surprisingly, the chiral HPLC analysis of the amine synthesized enzymatically did result in a 90% enantiomeric excess for the *S*-enantiomer which was totally unexpected. To shed light on this result, new experiments were performed. Taking into account that both immobilization and the presence of organic co-solvents can alter enzyme selectivity,<sup>[49–50]</sup> the reaction was repeated both by using the non-immobilized enzyme and either DMSO or dimethyl carbonate as co-solvent. However, it is worth to underline that, to the best of our knowledge, decrease and/or inversion of enantioselectivity have never been reported for transaminases.

The biotransformation performed in batch by using the soluble *Vf*-ATA under the same conditions of the flow reaction (donor and acceptor: 200  $\mu\text{mol}$ ; PLP: 20  $\mu\text{mol}$ ; medium: 100 mM phosphate buffer, pH 8.0, 5% v/v dimethyl carbonate) gave the same ee% (90%): this result thus ruled out the influence of immobilization on the incomplete (*S*)-enantioselectivity of *Vf*-ATA.

The same result was obtained upon analyzing the reaction mixtures containing two different co-solvents (DMSO and dimethyl carbonate, respectively), thus excluding a role of these solvents, too.

Our attention was then focused on PLP, the co-factor of ATAs. PLP is an activated aldehyde and the formation of imines is its natural role. It has been reported that PLP, upon the formation of a Schiff base with  $\alpha$ -amino acid esters or amines, enhances racemization through a reversible proton migration.<sup>[51]</sup> This phenomenon has been exploited for the dynamic resolution of a number of racemic substrates.<sup>[52–54]</sup> The hypothesized mechanism of PLP toward (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3) is reported in Scheme S2 (Supporting Information).

The chiral analysis of a batch biotransformation which was performed without adding PLP showed a complete ee%, thus supporting our hypothesis concerning the role of PLP in the amine racemization. In this case, the (*S*)-amine formation catalyzed by *Vf*-ATA relies exclusively on the PLP intrinsically bound to *Vf*-ATA. We concluded, indeed, that the driving force of the side-reaction was the concentration of PLP used in the

**Table 2.** Flow synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3) catalyzed by immobilized *Vf*-ATA.<sup>[a]</sup>

| Entry | Substrate [mM] | Residence time [min] | Temperature [°C] | Conversion [%] <sup>[b]</sup> |
|-------|----------------|----------------------|------------------|-------------------------------|
| 1     | 20             | 5                    | 28               | 26                            |
| 2     | 20             | 10                   | 28               | 40                            |
| 3     | 20             | 15                   | 28               | 39                            |
| 4     | 20             | 30                   | 28               | 39                            |
| 5     | 20             | 10                   | 38               | 38                            |
| 6     | 40             | 10                   | 28               | 17                            |

[a] Immobilized *Vf*-ATA: 0.65 g; reactor volume: 0.65 mL; ratio acceptor/donor 1:1; PLP 1 mM; flow stream: phosphate buffer (100 mM, pH 8.0) containing DMSO (2% v/v); atmospheric pressure, [b] Conversion was calculated by HPLC.

reactions (1–2 mM) which is probably uselessly high for the biotransformation, although consistent with other reports on transaminases.<sup>[55–58]</sup> Furthermore, the hypothesized mechanism might be enhanced by the alkaline pH of the reaction mixture: at this pH value (around 8–9), ATAs show the highest activity, but amines are predominant in their neutral form. Thus, the nucleophilic reaction of amines with the PLP aldehyde group is plausible to occur. On the other hand, although the absence of PLP in the reaction mixture allowed to restore the complete enantioselectivity of Vf-ATA and to achieve a ee > 99%, this condition was detrimental for the biocatalyst. After this reaction, immobilized Vf-ATA lost almost completely its activity. The concentration of PLP is therefore a crucial parameter which deserves to be further investigated.

In this context, we tested the reaction without PLP under continuous flow using the conditions reported above. However, a very low conversion (< 5%) was obtained. Therefore, increasing amounts of PLP (i.e., 0.1 mM and 0.25 mM) were added to the substrate solution that was flowed through the bioreactor and the performance of the biotransformation was evaluated. Using 0.1 mM PLP, again, a very low conversion in 10 minutes of residence time was observed, whereas, using 0.25 mM PLP, a conversion comparable to the previous experiments (38%) was achieved. The amine was isolated as reported above and the enantiomeric excess was assessed by chiral HPLC, resulting to be > 99%.

It is worth reporting that the use of tailor-made carriers which can enable the co-immobilization of transaminase and PLP has been recently described.<sup>[38]</sup> Through this strategy, the exogenous addition of cofactor is avoided, since a self-sufficient source of PLP is ensured by the co-immobilized preparation for an efficient catalysis.

## Conclusions

A new protocol for the synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3), which integrates flow biocatalysis with an in-line downstream processing, was successfully developed.

The main benefits of the set-up herein described include the increased specific reaction rate in comparison with the batch protocol and a significantly reduced time of the overall process (reaction and product recovery). Interestingly, a possible role played by the cofactor PLP in transaminase-catalyzed amination of ketones was highlighted; albeit essential for the catalysis, PLP seemed to affect the enantioselectivity of the transamination reaction and, indeed, its concentration needs to be finely tuned. These results demonstrated that the combination of biocatalysis and flow chemistry should be regarded as a truly competitive approach with established fully chemical protocols.

## Experimental Section

### General

All reagents and solvents were purchased from Sigma-Aldrich® Srl (Milan, Italy) unless otherwise stated. 1-(5-Fluoropyrimidin-2-yl)-ethan-1-one (1) was purchased from Fluorochem (Hadfield, UK). For the production, purification and characterization of the enzyme, the following instruments were used: Thermoshaker INNOVA 42 (New Brunswick); Sorvall RC6 Plus (Thermo) centrifuge, A.L.C. 4226 centrifuge, Omni-Ruptor 250 sonicator, UV-VIS V-530 (Jasco) spectrophotometer or a UV 1601 spectrophotometer (Shimadzu).

Agarose gel 6B-CL was purchased from Amersham Biosciences (Uppsala, Sweden) and activated to glyoxyl-agarose as previously reported.<sup>[59]</sup> Sepabeads™ EC-EP/S was kindly supplied by Resindion srl (Binasco, Italy).

The flow experiments were performed by using a R2 +/R4 flow reactor (Vapourtec).

### Immobilization yields

The immobilization yields (%) were determined as: immobilized protein, immobilized activity and activity recovery as reported by Sheldon *et al.*<sup>[60]</sup> according to the following equations:

Immobilized protein (%): (immobilized protein/loaded protein) × 100; immobilized activity (%): (immobilized activity/loaded activity) × 100; activity recovery (%): (observed activity of the immobilized enzyme/starting activity) × 100.

### Specific reaction rate

The specific reaction rate for batch ( $r_{\text{batch}}$ ) was calculated from the amount of the product (P, μmol), the reaction time (t, min), and the mass of biocatalyst employed ( $m_{\text{E}}$ , mg):  $r_{\text{batch}} = nP/t m_{\text{E}}$  (μmol min<sup>-1</sup> mg<sup>-1</sup>). The specific reaction rate for flow biotransformation ( $r_{\text{flow}}$ ) was calculated from the concentration of the product ([P], μmol mL<sup>-1</sup>), the flow rate (f, mL min<sup>-1</sup>), and the mass of protein employed ( $m_{\text{E}}$ , mg):  $r_{\text{flow}} = [P] \times f/m_{\text{E}}$  (μmol min<sup>-1</sup> mg<sup>-1</sup>).

### Vf-ATA expression and purification

The amine transaminase from *Vibrio fluvialis* (Vf-ATA) was produced as previously reported.<sup>[57]</sup> Briefly, *E. coli* BL21(DE3) cells carrying the expression plasmid for Vf-ATA were streaked on LB-Agar plates containing kanamycin 30 μg mL<sup>-1</sup> (LB<sub>KAN30</sub>) and incubated overnight at 37 °C. Colonies were aseptically removed, inoculated in 50 mL of LB<sub>KAN30</sub> medium and incubated overnight at 37 °C and 220 rpm. The pre-cultures were used for the inoculation of 500 mL of LB<sub>KAN30</sub> medium, then incubated at 37 °C and 220 rpm. When the OD<sub>600</sub> value was between 0.5 and 1, IPTG (0.5 M stock solution) was added till a final concentration of 1 mM to induce the expression. The Vf-ATA cultures were incubated at 30 °C and 220 rpm for 24 h. At the endpoint of the expression, the cells were recovered by centrifugation at 5000 rpm and 4 °C for 30 min and resuspended in 20 mL of washing buffer (500 mM NaCl, 20 mM imidazole, 20 mM potassium phosphate buffer, pH 7.0). Cells were lysed by three sonication cycles at 0 °C and the cell lysate was centrifuged at 10000 rpm and 4 °C for 30 min. The transaminase was purified by IMAC using Ni-NTA Sepharose 6 Fast Flow resin (GE Healthcare, Italy) as the stationary phase according to the manufacturer's instruction. The eluates containing the target protein were collected and dialyzed for 16 h at 4 °C in 50 mM phosphate buffer pH 9. After the dialysis, samples were stored at –80 °C. Protein content was measured

using the Bio-Rad Protein Assay according to the method of Bradford; bovine serum albumin (BSA) was used as standard protein. Protein purity was verified by SDS-PAGE analysis (10% T, 2.6% C). The molecular weight protein standard mixture from BioRad (Karlsruhe, Germany) was used as reference. Gels were stained for protein detection with Coomassie Brilliant Blue.

### Glyoxyl-agarose preparation

Glyoxyl-agarose was prepared as reported by Guisan.<sup>[59]</sup> Briefly, agarose (10 g) was suspended in deionized water (2.8 mL) at 4 °C under mechanical stirring. A 1.7 M solution of NaOH (4.8 mL) containing NaBH<sub>4</sub> (14.3 mg mL<sup>-1</sup>) was added. Etherification was carried out in an ice bath by adding dropwise glycidol (3.4 mL). The reaction was carried out for 18 h. After the incubation period, the suspension was filtered and the carrier was washed with deionized water. Oxidation was initiated by adding 100 mM NaO<sub>4</sub> (68 mL). The reaction was carried out for 2 h at room temperature, then the carrier was filtered under reduced pressure and washed thoroughly with deionized water and stored at 4 °C.

### Vf-ATA immobilization on glyoxyl-agarose

Vf-ATA (2 mg; 9.3 U) was incubated with glyoxyl-agarose (1 g) in NaHCO<sub>3</sub> buffer (pH 10, 50 mM, 10 mL) at 4 °C for 3 h under mechanical stirring. Chemical reduction of imines was carried out over 30 min by adding NaCNBH<sub>3</sub> (10 mg) to the mixture. The immobilized enzyme was then filtered under vacuum and washed thoroughly with deionized water and K<sub>2</sub>HPO<sub>4</sub> buffer (pH 8, 50 mM) and stored at 4 °C. The immobilization procedure was monitored by evaluating the residual protein and the residual activity of the supernatant by Bradford<sup>[46]</sup> and activity assay, respectively. For the flow reactions, a protein loading of 5 mg/g was used.

### Vf-ATA immobilization on Sepabeads™ EC-EP/S

Vf-ATA (1 mg; 1.23 U) was incubated with Sepabeads™ EC-EP/S (1 g), previously hydrated with deionized water (10 mL) under gentle shaking for 1 h in phosphate buffer (pH 8, 50 mM, 5 mL), at room temperature for 24 h under mechanical stirring. The immobilization procedure was monitored by evaluating the residual protein and the residual activity of the supernatant by Bradford assay<sup>[46]</sup> and activity assay, respectively. The immobilized enzyme was then filtered, washed with deionized water and re-suspended in K<sub>2</sub>HPO<sub>4</sub> buffer (pH 8.5, 50 mM, 4 mL) containing glycine (3 M) and stirred for further 20 h to quench the unreacted epoxides.<sup>[36,43]</sup> The biocatalyst was eventually filtered under vacuum, washed thoroughly with deionized water and K<sub>2</sub>HPO<sub>4</sub> buffer (pH 8, 50 mM) and stored at 4 °C.

### Vf-ATA activity assay

Enzyme specific activity was determined by a spectrophotometric assay (Scheme S1) as previously reported.<sup>[45]</sup> Briefly, phosphate buffer (pH 8, 50 mM, 3 mL) containing sodium pyruvate (2.5 mM), (S)- $\alpha$ -methyl benzylamine ((S)-MBA, 2.5 mM) and DMSO (0.25% v/v) was poured in a quartz cuvette. Soluble Vf-ATA (6  $\mu$ L) was added and the absorbance of acetophenone ( $\epsilon = 12 \text{ mM}^{-1} \text{ cm}^{-1}$ ) at 245 nm was detected for 60 s under the kinetic mode. One unit (U) is defined as the amount of enzyme which produces one  $\mu$ mol of acetophenone per minute under the above conditions. For the immobilized Vf-ATA, the same assay was performed by using 10–15 mg of the immobilized biocatalyst under magnetic stirring for

180 s. Enzymatic activity was determined according to Equation (1). The measurements were performed at least in duplicate.

$$\frac{U}{\text{mL or g}} = \frac{\Delta A / \text{min} \times V_s}{\epsilon \times E} \quad (1)$$

**Equation 1.**  $\Delta A / \text{min}$  = slope of Abs vs time curve ( $\text{mAU min}^{-1}$ ),  $V_s$  = total assay volume (mL),  $\epsilon$  = acetophenone molar absorptivity ( $12 \text{ mM}^{-1} \text{ cm}^{-1}$ ),  $E$  = amount of soluble enzyme (mL) or immobilized enzyme (g)

The activity of Vf-ATA immobilized on Sepabeads™ EC-EP/S was determined by a discontinuous assay. Three aliquots of immobilized Vf-ATA (15 mg) were placed in plastic tubes and the assay solution (2 mL) was added. The samples were kept under gentle stirring (rotary shaking); every two minutes, a sample was quenched with HCl (2 M, 2 mL) and the absorbance at 245 nm was registered, as a single reading. Acetophenone ( $\mu$ mol) was quantified by a calibration curve and used to determine the enzyme activity.

### Residual activity of Vf-ATA immobilized on glyoxyl-agarose

A stock solution containing (S)-MBA (64.5  $\mu$ L, 500  $\mu$ mol), DMSO (1 mL) in phosphate buffer (pH 8, 100 mM, 49 mL) and PLP (12.4 mg, 50  $\mu$ mol) was prepared. Aliquots (50 mg) of immobilized Vf-ATA were placed in Eppendorf micro tubes and 1 mL of the stock solution was added to each Eppendorf. The samples were kept under stirring (rotary shaker) at 25 °C. At fixed times, each sample was filtered under vacuum, rinsed thoroughly with distilled water, conditioned with phosphate buffer (pH 8, 50 mM) and dried for 5 min. Residual activity ( $\text{U g}^{-1}$ ) was measured by submitting the filtered immobilized enzyme (10–15 mg) to the spectrophotometric assay as reported above and compared with the activity ( $\text{U g}^{-1}$ ) of the enzyme before incubation, which was considered as 100%.

### Batch synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

1-(5-Fluoropyrimidin-2-yl)-ethanone **1** (28.0 mg, 200  $\mu$ mol) was dissolved in DMSO (200  $\mu$ L) and added to phosphate buffer (pH 8, 100 mM, 9.8 mL) containing PLP (4.9 mg, 20  $\mu$ mol) and (S)-MBA **2** (25.8  $\mu$ L, 200  $\mu$ mol) in a plastic flask. Immobilized Vf-ATA (652 mg, 1.80 U) was added and the mixture was kept under mechanical stirring. Samples were taken, diluted with bidistilled water (1:10) and analyzed by HPLC. Once the highest conversion was achieved, the biocatalyst was recovered by filtration under vacuum.

### Flow synthesis of (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

Immobilized Vf-ATA (2.0 g) was packed into a glass column (Omnifit, 6.6 mm i.d. x 100 mm length). The packed-bed reactor was washed with a 100 mM phosphate buffer pH 8 at 100  $\mu$ L min<sup>-1</sup> for 10 min. The obtained reactor volume was 2.0 mL. A solution (100 mL) of 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**, 20 mM), (S)-MBA (**2**, 20 mM) and PLP (1 mM) in phosphate buffer (pH 8, 100 mM) with 5% v/v dimethyl carbonate was prepared. The solution was pumped at 0.20 mL min<sup>-1</sup> (residence time = 10 min) through the bioreactor maintained at 28 °C. The exiting flow stream was directed into a column packed with Dowex Marathon C resin (reactor volume: 5 mL) that was previously activated with 2 M HCl and washed with H<sub>2</sub>O. The desired amine was trapped by the resin. At the end of the reaction, the column packed with Dowex Marathon C resin was washed by flowing H<sub>2</sub>O at 0.25 mL min<sup>-1</sup> for

20 min. (S)-1-(5-Fluoropyrimidin-2-yl)-ethanamine **3** was then released using a 1 M solution of  $\text{NH}_4\text{OH}$  at  $0.25 \text{ mL min}^{-1}$  for 30 min. The flow stream was collected in fractions of 2.0 mL; TLC assays were performed to select the samples containing the pure product. After evaporation of the solvent, the pure amine was recovered in 35% isolated yield (99 mg).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$  8.60 (s, 2H), 4.65 (br s, 2H), 4.07–4.15 (m, 1H), 1.20 (d, 3H).

### Chemical synthesis of racemic

#### 1-(5-fluoropyrimidin-2-yl)-ethanamine

The chemical synthesis of racemic 1-(5-fluoropyrimidin-2-yl) ethanamine was performed slightly modifying a previously reported procedure.<sup>[61]</sup> i) 1-(5-Fluoropyrimidin-2-yl)-ethan-1-one **1** (200 mg, 1.43 mmol) was dissolved in MeOH (5 mL) at room temperature. The solution was cooled at  $0^\circ\text{C}$  and  $\text{NaBH}_4$  (54 mg) was added. The resulting mixture was stirred at room temperature for 1 h. Water (2 mL) was added and the organic solvent was removed under reduced pressure; the aqueous phase was extracted with EtOAc (3 x 3 mL). The organic extracts were dried over  $\text{Na}_2\text{SO}_4$  and, after evaporation of the volatiles under reduced pressure, 1-(5-fluoropyrimidin-2-yl)ethan-1-ol was obtained as a red oil (75% yield). ii) The alcohol obtained from the previous step (152 mg, 1.07 mmol) was dissolved in  $\text{CH}_2\text{Cl}_2$  (2 mL), and triethylamine (297  $\mu\text{L}$ , 2.14 mmol) was added at room temperature. The solution was cooled to  $0^\circ\text{C}$ , and methanesulfonyl chloride (100  $\mu\text{L}$ , 1.28 mmol) was added dropwise. The resulting mixture was allowed to stir at r.t. for 2 h. The mixture was washed with 1 M HCl (2 x 2 mL), the organic phase was dried over  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure to give 1-(5-fluoropyrimidin-2-yl)ethyl methanesulfonate (90% yield). iii) The residue (212 mg, 0.96 mmol) was dissolved in DMF (5 mL) and treated with sodium azide (62 mg, 0.96 mmol). The resulting mixture was stirred at r.t. for 72 h and was then partitioned between EtOAc (5 mL) and brine (10 mL). The organic layer was collected, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated under reduced pressure. The crude material was purified by flash column chromatography as a colorless oil (55% yield). iv) 2-(1-Azidoethyl)-5-fluoropyrimidine (88 mg, 0.53 mmol) was dissolved in MeOH (5 mL) and submitted to a hydrogenation reaction in a Thales-Nano H-Cube Mini (cartridge: Pd/C 10%, flow rate:  $1 \text{ mL min}^{-1}$ ). The exiting solution was evaporated under reduced pressure to afford racemic 1-(5-fluoropyrimidin-2-yl) ethanamine as a pale yellow oil in quantitative yield. HPLC chiral analysis retention times ( $t_R$ ): (S)-MBA (**2**) = 3.8 min, (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) = 5.9 min; (R)-1-(5-fluoropyrimidin-2-yl)-ethanamine = 6.1 min; 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**) = 8.0 min.

### Analytical methods

HPLC analyses of batch reactions were performed on a HPLC Chromaster 600 bar System, Merck Hitachi VWR, equipped with a Purospher® STAR RP-18 endcapped (3  $\mu\text{m}$ ), Hibar HR 100–2.1, UHPLC column, Sorbent Lot No. TA 1822868 (Merck KGaA, Darmstadt, Germany). Injection volume: 2  $\mu\text{L}$ ; mobile phase:  $\text{H}_2\text{O} + \text{TFA}$  0.05% (A)/ACN + TFA 0.05% (B); gradient conditions: 0–5 min 100% A; 5–20 min 50% (A)/50% (B); flow rate:  $0.3 \text{ mL min}^{-1}$ ;  $\lambda$ : 245–254 nm. Retention times ( $t_R$ ): (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) = 3.60 min, 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**) = 13.40 min, (S)-MBA (**2**) = 14.37 min, acetophenone (**4**) = 20.90 min.

HPLC analyses of the flow reactions were performed on a Breeze 2 HPLC System, Waters, equipped with a Waters 2489 UV–vis detector (Waters, Milford, MA) and a Phenomenex Synergi column (150 mm x 4.6 mm, 4  $\mu\text{m}$ ). Injection volume: 10  $\mu\text{L}$ ; mobile phase:  $\text{H}_2\text{O} + 0.05\% \text{ TFA}$  (A)/ACN + 0.05% TFA (B); gradient conditions: 0–9 min

90% (A)/10% (B), 9–14 min 50% (A)/50% (B); flow rate:  $1.0 \text{ mL min}^{-1}$ ;  $\lambda$ : 254 nm. Reaction samples were diluted with a solution 1:10  $\text{H}_2\text{O}/\text{ACN} + 0.05\% \text{ TFA}$ . Retention times ( $t_R$ ): (S)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) = 2.7 min, (S)-MBA (**2**) = 5.6 min, 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**) = 7.0 min, acetophenone (**4**) = 16.4 min.

HPLC chiral analyses were performed using a Jasco PU-980 pump (PerkinElmer, Inc., Waltham, MA) equipped with a UV–vis detector Jasco UV-975 and a Phenomenex Lux Amylose 2 column (150 mm x 4.6 mm, 3  $\mu\text{m}$ ). Injection volume: 10  $\mu\text{L}$ ; mobile phase: hexane + 0.5% diethylamine/iso-propanol + 0.5% diethylamine, isocratic elution 80:20; flow rate:  $1.0 \text{ mL min}^{-1}$ .

### Acknowledgements

This work was financially supported by Cariplo Foundation (Italy) (Call: “Integrated research on industrial biotechnologies and bioeconomy 2016”, project BIOFLOW, ID 2016–0731).

**Keywords:** biocatalysis · flow chemistry · chiral amines · transaminase · immobilization

- [1] S. Mathew, H. Yun, *ACS Catal.* **2012**, *2*, 993–1001.
- [2] E. E. Ferrandi, D. Monti, *World J. Microbiol. Biotechnol.* **2018**, *34*, 13.
- [3] F. Steffen-Munsberg, C. Vickers, H. Kohls, H. Land, H. Mallin, A. Nobili, L. Skalden, T. van den Bergh, H.-J. Joosten, P. Berglund, M. Hohne, U. T. Bornscheuer, *Biotechnol. Adv.* **2015**, *33*, 566–604.
- [4] F. Guo, P. Berglund, *Green Chem.* **2017**, *19*, 333–360.
- [5] J. S. Shin, B. G. Kim, *Biotechnol. Bioeng.* **1997**, *55*, 348–358.
- [6] P. Domínguez de María, G. de Gonzalo, A. R. Alcántara, *Catalysts* **2019**, *9*, 802.
- [7] C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman, G. J. Hughes, *Science* **2010**, *329*, 305–309.
- [8] M. Girardin, S. G. Ouellet, D. Gauvreau, J. C. Moore, G. Hughes, P. N. Devine, P. D. O’Shea, L. C. Campeau, *Org. Process Res. Dev.* **2013**, *17*, 61–68.
- [9] L. Frodsham, M. Golden, S. Hard, M. N. Kenworthy, D. J. Klauber, K. Leslie, C. Macleod, R. E. Meadows, K. R. Mulholland, J. Reilly, C. Squire, S. Tomasi, D. Watt, A. S. Wells, *Org. Process Res. Dev.* **2013**, *17*, 1123–1130.
- [10] Y. Feng, Z. Luo, G. Sun, M. Chen, J. Lai, W. Lin, S. Goldmann, L. Zhang, Z. Wang, *Org. Process Res. Dev.* **2017**, *21*, 648–654.
- [11] Z. Peng, J. W. Wong, E. C. Hansen, A. L. A. Puchlopek-Dermenci, H. J. Clarke, *Org. Lett.* **2014**, *16*, 860–863.
- [12] J. Limanto, E. R. Ashley, J. Yin, G. L. Beutner, B. T. Grau, A. M. Kassim, M. M. Kim, A. Klapars, Z. Liu, H. R. Strotman, M. D. Truppo, *Org. Lett.* **2014**, *16*, 2716–2719.
- [13] M. D. Truppo, H. Strotman, G. Hughes, *ChemCatChem* **2012**, *4*, 1071–1074.
- [14] X.-J. Zhang, H.-H. Fan, N. Liu, X.-X. Wang, F. Cheng, Z.-Q. Liu, Y.-G. Zheng, *Enzyme Microb. Technol.* **2019**, *130*, 109362.
- [15] M. D. Truppo, J. M. Janey, G. Hughes, WO2012177527A1 **2012**.
- [16] K. S. Midelfort, M. Journet, H. Strotman, J. P. McMullen, S. Grosser, WO2014133928 A1 **2014**.
- [17] S. A. Kelly, S. Pohle, S. Wharry, S. Mix, C. C. R. Allen, T. S. Moody, B. F. Gilmore, *Chem. Rev.* **2018**, *118*, 349–367.
- [18] R. E. Meadows, K. R. Mulholland, M. Schürmann, M. Golden, H. Kierkels, E. Meulenbroeks, D. Mink, O. May, C. Squire, H. Straatman, A. S. Wells, *Org. Process Res. Dev.* **2013**, *17*, 1117–1122.
- [19] J.-S. Shin, B.-G. Kim, *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1782–1788.
- [20] K. S. Midelfort, R. Kumar, S. Han, M. J. Karmilowicz, K. McConnell, D. K. Gehlhaar, A. Mistry, J. S. Chang, M. Anderson, A. Villalobos, J. Minshall, S. Govindarajan, J. W. Wong, *Protein Eng. Des. Sel.* **2013**, *26*, 25–33.
- [21] A. Nobili, F. Steffen-Munsberg, H. Kohls, I. Trentin, C. Schulzke, M. Hohne, U. T. Bornscheuer, *ChemCatChem* **2015**, *7*, 757–760.



- [22] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, *Trends Biotechnol.* **2010**, *28*, 324–332.
- [23] A. P. Green, N. J. Turner, E. O'Reilly, *Angew. Chem. Int. Ed.* **2014**, *53*, 10714–10717; *Angew. Chem.* **2014**, *126*, 10890–10893.
- [24] M. D. Truppo, J. D. Rozzell, N. J. Turner, *Org. Process Res. Dev.* **2010**, *14*, 234–237.
- [25] L. Tamborini, P. Fernandes, F. Paradisi, F. Molinari, *Trends Biotechnol.* **2018**, *36*, 73–88.
- [26] P. Žnidaršič-Plazl, *Biotechnol. J.* **2019**, *14*, 1800580.
- [27] J. M. Woodley, *Curr. Op. Green Sus. Chem.* **2020**, *21*, 22–26.
- [28] J. M. Woodley, *Appl. Microbiol. Biotechnol.* **2019**, *103*, 4733–4739.
- [29] Z. Molnár, E. Farkas, Á. Lakó, B. Erdélyi, W. Kroutil, B. G. Vértessy, C. Paizs, L. Poppe, *Catalysts*, **2019**, *9*, 438.
- [30] L. van den Biggelaar, P. Soumillion, D. P. Debecker, *Catalysts* **2017**, *7*, 54.
- [31] L. van den Biggelaar, P. Soumillion, D. P. Debecker, *RSC Adv.* **2019**, *9*, 18538–18546.
- [32] J. Britton, S. Majumdar, G. A. Weiss, *Chem. Soc. Rev.* **2018**, *47*, 5891–5918.
- [33] C. Mateo, J. M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. Lopez-Gallego, B. C. C. Pessela, A. Hidalgo, G. Fernandez-Lorente, R. Fernandez-Lafuente, J. M. Guisan, *Enzyme Microb. Technol.* **2006**, *7*, 274–280.
- [34] E. Katchalski-Katzir, D. M. Kraemer, *J. Mol. Catal. B* **2000**, *10*, 157–176.
- [35] F. Dall'Oglio F, M. L. Contente, P. Conti, F. Molinari, D. Monfredi, A. Pinto, D. Romano, D. Ubiali, L. Tamborini, I. Serra, *Catal. Commun.* **2017**, *93*, 29–32.
- [36] M. Planchestainer, M. L. Contente, J. Cassidy, F. Molinari, L. Tamborini, F. Paradisi, *Green Chem.* **2017**, *19*, 372–375.
- [37] O. Barbosa, R. Torres, C. Ortiz, Á. Berenguer-Murcia, R. C. Rodrigues, R. Fernandez-Lafuente, *Biomacromolecules* **2013**, *14*, 2433–2462.
- [38] S. Velasco-Lozano, A. I. Benítez-Mateos, F. López-Gallego, *Angew. Chem. Int. Ed.* **2017**, *56*, 771–775; *Angew. Chem.* **2017**, *129*, 789–793.
- [39] V. V. Mozhaev, in *Stability and Stabilization of Biocatalysts. Progress in Biotechnology*, Vol. 15, (Eds.: A. Ballesteros, F. J. Plou, J. L. Ibor, P. J. Halling), Elsevier Science B. V., Amsterdam, **1998**, pp 355–363.
- [40] I. Serra, S. Daly, A. R. Alcantara, D. Bianchi, M. Terreni, D. Ubiali, *RSC Adv.* **2015**, *5*, 23569–23577.
- [41] S. Chen, J. C. Campillo-Brocal, P. Berglund, M. S. Humble, *J. Biotechnol.* **2018**, *282*, 10–17.
- [42] S. Cantone, V. Ferrario, L. Corici, C. Ebert, D. Fattor, P. Spizzo, L. Gardossi, *Chem. Soc. Rev.* **2013**, *42*, 6262–6276.
- [43] C. Mateo, V. Grazu, J. M. Palomo, F. Lopez-Gallego, R. Fernández-Lafuente, J. M. Guisán, *Nat. Protoc.* **2007**, *2*, 1022–1033.
- [44] E. Abaházi, P. Sátorhelyi, B. Erdélyi, B. G. Vértessy, H. Land, C. Paizs, P. Berglund, L. Poppe, *Biochem. Engin. J.* **2018**, *132*, 270–278.
- [45] S. Schätzle, M. Höhne, E. Redestad, K. Robins, U. T. Bornscheuer, *Anal. Chem.* **2009**, *81*, 8244–8248.
- [46] M. M. Bradford, *Anal. Biochem.* **1976**, *27*, 248–254.
- [47] C. Csajági, G. Szatzker, E. R. Tóke, L. Üрге, F. Darvas, L. Poppe, *Tetrahedron: Asymmetry* **2008**, *19*, 237–246.
- [48] M. Höhne, K. Robins, U. T. Bornscheuer, *Adv. Synth. Catal.* **2008**, *350*, 807–812.
- [49] G. Ottolina, S. Riva, in *Methods Non-Aqueous Enzymol.* (Ed.: M. N. Gupta), Birkhäuser Basel, Basel, **2000**, pp. 133–145.
- [50] Y. Mao, M. Krischke, C. Hengst, U. Kulozik, *Food Chem.* **2018**, *253*, 194–202.
- [51] K. Faber, *Biotransformations in Organic Chemistry: A Textbook*, Springer, Berlin, **2011**.
- [52] P. Jacob, *J. Org. Chem.* **1996**, *61*, 2916–2917.
- [53] S.-T. Chen, W.-H. Huang, K.-T. Wang, *J. Org. Chem.* **1994**, *59*, 7580–7581.
- [54] S. Shen, K. Soda, *J. Chem Soc, Chem. Commun.* **1983**, 82–83.
- [55] A. Basso, W. Neto, S. Serban, B. D. Summers, *Chim. Oggi – Chem. Today* **2018**, 40–42.
- [56] D. Monti, M. C. Forchin, M. Crotti, F. Parmeggiani, F. G. Gatti, E. Brenna, S. Riva, *ChemCatChem* **2015**, *7*, 3106–3109.
- [57] E. E. Ferrandi, A. Previdi, I. Bassanini, S. Riva, X. Peng, D. Monti, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 4963–4979.
- [58] D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell, W. Kroutil, *Adv. Synth. Catal.* **2008**, *350*, 2761–2766.
- [59] J. M. Guisán, *Enzyme Microb. Technol.* **1988**, *10*, 375–382.
- [60] R. A. Sheldon, S. van Pelt, *Chem. Soc. Rev.* **2013**, *42*, 6223–6235.
- [61] S. Ioannidis, M. L. Lamb, T. Wang, L. Almeida, M. H. Block, A. M. Davies, B. Peng, M. Su, H.-J. Zhang, E. Hoffmann, C. Rivard, I. Green, T. Howard, H. Pollard, J. Read, M. Alimzhanov, G. Bebernitz, K. Bell, M. Ye, D. Huszar, M. Zinda, *J. Med. Chem.* **2011**, *54*, 262–276.

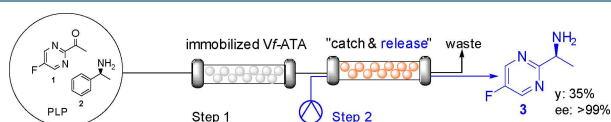
Manuscript received: November 2, 2019

Revised manuscript received: December 13, 2019

Accepted manuscript online: December 18, 2019

Version of record online: ■■■, ■■■■

## FULL PAPERS



**All-in-One:** The amine transaminase from *Vibrio fluvialis* (Vf-ATA) was covalently immobilized and used in the synthesis of (S)-1-(5-fluoropyrimidin-2-yl)ethanamine (**3**) in a flow system.

Coupling of the continuous flow biotransformation with an in-line purification step resulted in a highly productive and straightforward bioprocess.

R. Semproli, G. Vaccaro, Dr. E. E. Ferrandi, M. Vanoni, Dr. T. Bavaro, Dr. G. Marrubini, F. Annunziata, Prof. P. Conti, Prof. G. Speranza, Dr. D. Monti\*, Prof. L. Tamborini\*, Prof. D. Ubiali\*

1 – 10

**Use of Immobilized Amine Transaminase from *Vibrio fluvialis* under Flow Conditions for the Synthesis of (S)-1-(5-Fluoropyrimidin-2-yl)ethanamine**

