Self-sustaining closed-loop multienzyme mediated conversion of amines into alcohols in continuous reactions

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ABSTRACT

The synthesis of alcohols from amines starting material is an excellent, yet challenging, strategy for the preparation of pharmaceuticals and polymers. Here, we developed a versatile, self-sustaining closed-loop multienzymatic platform for the biocatalytic synthesis of a large range of non-commercially available products in continuous flow with excellent yields (80->99%), reaction times and optical purity of secondary alcohols (>99 e.e.). This process was also extended to the conversion of biogenic amines into high value alcohols, such as the powerful anti-oxidant hydroxytyrosol, and the synthesis of enantiopure 2-arylpropanols *via* dynamic kinetic resolution of commercially affordable racemic amines. The system exploits the *in situ* immobilisation of transaminases and redox enzymes which were combined to cater for a fully automated, ultra-efficient synthetic platform with cofactor recycling, in-line recovery of benign by-products and recirculation of the aqueous media containing the recycled cofactors in catalytic amount, which increases the efficiency of the system by over 20-fold.

MAIN

Rapid functional group interconversion with potential retention or inversion of the stereochemistry significantly broadens the range of molecules that can be produced through an established chemical route. Aromatic primary and secondary alcohols are an important class of compounds for synthetic purpose as they are key targets in the manufacturing of a wide range of chemicals such as agrochemicals, food additives, fragrances and pharmaceuticals.[1,2] Among other synthetic methods, they can be obtained from the corresponding aromatic amines which are often more easily accessible (*e.g.,* from natural sources), commercially available, and in many cases surprisingly less expensive.[3,4] Notably, various methods for the preparation of primary amines by amination of alcohols have been developed,[5-7] but the reverse reaction, formally a simple substitution of the amino group with a different nucleophile, remains a challenging transformation.[8] The importance of a one-shot amine-alcohol transformation has been already highlighted in the total synthesis of Scopadulin, an antiviral tetracyclic diterpenoid,[9] or the preparation of xylene glycols, precursors of synthetic fibers and resins starting from the corresponding diamines.[10] A strategy for the bio-deracemisation of racemic aromatic amines leading to chiral alcohols was patented in 2014.[11] A microbial cell factory approach was used in that case to overcome limitations due to cofactor recycling, however, whole-cell biocatalysis may suffer from side-product contamination, substrate permeability, and generation of debris. Synthetically, Khusnutdinova *et al.* achieved direct deamination of primary amines to the corresponding alcohols in aqueous media employing a Ru catalyst, but conversion yields were extremely variable (from 55% to 90%) and reaction times could extend to 72 h.[12] The authors also reported moderate isolated yields (50-60%) due to difficult purification and formation of secondary condensation products. More importantly, efficient strategies to prepare secondary

aromatic alcohols, with retention of the configuration starting from the corresponding amines are almost non-existent. A reported methodology, *via* diazotisation, requires strong acids and low temperature for the formation of the diazonium intermediate, followed by high temperatures for the hydroxy substitution of the diazo group. Such process is energetically expensive and yields are low due to competitive reactions (*e.g.* nucleophilic attacks, elimination and molecule rearrangement).[13,14]

In vivo, biogenic amines such as norepinephrine and dopamine undergo metabolic degradation to the corresponding alcohols *via* monoamine oxidase, and subsequent reduction of the aldehyde to alcohol by NADPH-dependent aldehydes reductase.[15,16]

Mimicking of cellular enzymatic cascades can be achieved through the assembly of multi-step enzymatic reactions in a continuous flow bioreactor, which also significantly increases the reaction efficiency and sustainability of the process.[17] Furthermore, while the scalability of flow reactions strongly facilitates technology transfer from lab to industry,[18] the complexity of multi-enzymatic processes in the absence of cellular regulation, has limited their applications to some chemo-enzymatic synthesis,[19] and just few fully enzymatic processes.[20-22] Challenges involved in setting up integrated multistep enzymatic flow processes include the number of reaction steps, cost of the biocatalysts, flow rate control (affecting the individual reaction residence time), solvent requirements and compatibility of the different steps (especially for biphasic processes), need for intermediate work-up, dilution effects, and cofactors costs for redox enzymes.[17] In batch reactions, *in situ* recycling of the cofactor allows for an enzymatic system to be self-sustaining achieving total substrate conversion with a catalytic amount of cofactors. However, in continuous systems involving redox enzymes, the cofactor, even if regenerated, is lost downstream and the benefit of an apparent sustainable set up is purely limited to the use of catalytic amounts of expensive reagents which must be

constantly added upstream for the duration of the process. The concept of ultra-efficiency in reaction design reconciles efficiency and effectiveness where resources are processed with minimal loss or negative impact on the environment, ideally achieving a closed-loop system where materials can be reused (Fig. 1).[23]

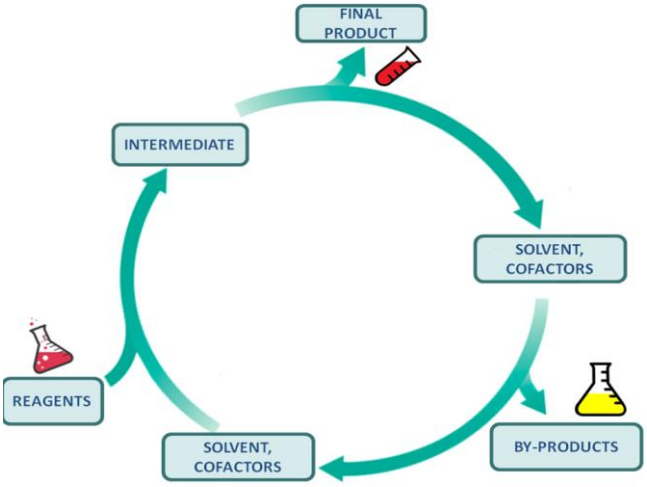


Figure 1. Ultra-efficient closed-loop concept

Reusability of the solvent (and cofactors) allows for extensive improvement of the efficiency of a continuous system.

Essential to successful enzymatic systems, especially for industrial applications, is the robustness and longevity of the catalyst, which can be dramatically enhanced by immobilisation.[24,25] Aminotransferases and oxidoreductases, among other enzymes, have been heavily investigated and applied in pharmaceutical manufacturing in recent years.[26-29] Redox enzymes, as mentioned, naturally require exogenous cofactors which must be recycled, often limiting their competitiveness on the market.[30] On the other hand, while aminotransferases benefit from a bound cofactor (pyridoxal phosphate) this routinely has to

be added in the reaction medium in moderate to significant concentrations to maintain catalytic efficiency adding to the overall cost of the process.[31,32]

Here, we present an extremely versatile multienzymatic cascade reaction which combines an ω-transaminase (HEWT) from *Halomonas elongata*[33] with oxidoreductases such as horse liver alcohol dehydrogenase (HLADH) or a ketoreductase from *Pichia glucozyma* (KRED1-Pglu),[34,35] for the preparation of aromatic alcohols from the corresponding amines in continuous flow. Specifically, this strategy could be applied to the synthesis, among others, of high value alcohols from inexpensive biogenic amines, such as hydroxytyrosol (one of the most powerful anti-oxidant derived from dopamine),[36] tryptophol (from tryptamine, a key intermediate for a number of bioactive compounds),[37] and histaminol (from histamine) for which efficient syntheses are lacking (reported yields to date between 5 and 13.5%).[38, 39] This strategy mimics an *in-vivo* system and leads to an ultra-efficient zero-waste and closed-loop process with excellent atom efficiency and automation.

RESULTS

Preparation of primary alcohols

The immobilisation of HEWT and HLADH (imm-HEWT, imm-HLADH) onto epoxy resin, so far reported in batch,[34,40] was optimised and directly performed on the cell lysate in flow yielding >35% of recovered activity for both enzymes, avoiding pre-purification, mass loss and long immobilisation time. Single step reactions were optimised (see Methods and Supplementary Table 1 and 2), before assembling the multi-enzymatic process to test the system, in the first instance, for the interconversion of simple primary aromatic amines to the corresponding alcohols (Fig. 2).

The reduced nicotinamide adenine dinucleotide cofactor (NADH) required in the second step was kept catalytic (0.1% mol eq.) and regenerated *in situ* by co-substrate coupling (ethanol). The tunability of the flow system allowed for the use of different optimal temperatures in the two bioreactors (37 °C for the oxidation step catalysed by HEWT, and 28 °C for the subsequent reduction catalysed by HLADH) which minimised residence times with excellent conversions (>99% for the aldehydes, ≥90% for the alcohols). The system was integrated with an in-line purification step (QP-BZA column) to trap any trace of unreacted aldehydes. The alcohol products were then extracted in a stream of EtOAc and recovered by simple evaporation of the organic solvent (from 86 to 97% isolated yield), significantly simplifying and accelerating the work-up procedures.

The use of pyruvate as amino acceptor strongly favours the equilibrium reaction of the first step, and the generated by-product, L-alanine, was fully recovered downstream (Fig. 2).

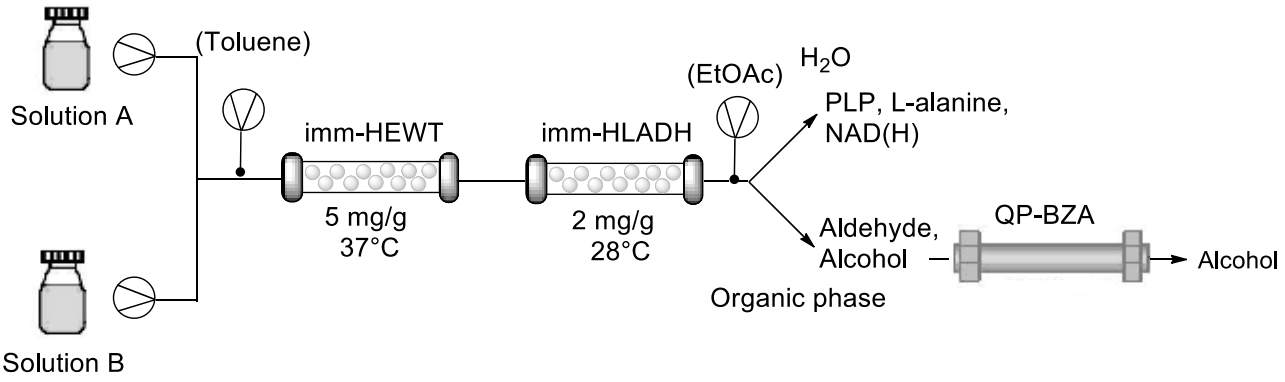
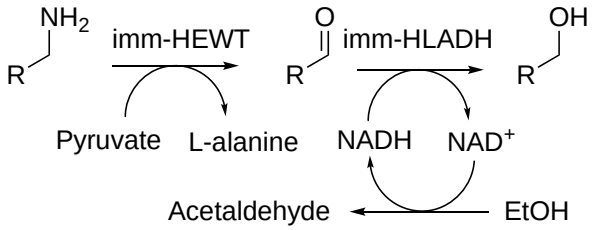


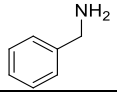
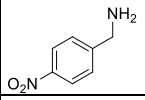
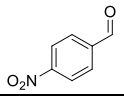
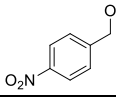
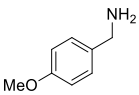
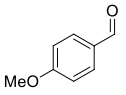
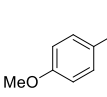
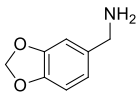
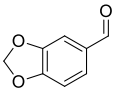
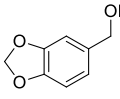
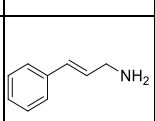
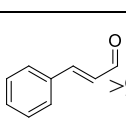
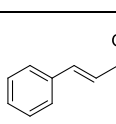
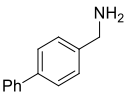
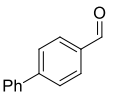
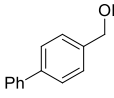
Figure 2. Flow system assembly for the synthesis of primary alcohols

Solution A: 10 mM amine in phosphate buffer (50 mM pH 8) containing 2 M EtOH and 0.02 mM NADH Solution B: 10-20 mM solution pyruvate in phosphate buffer (50 mM pH 8) containing 0.2 mM PLP. T= 37° C for HEWT and 28 °C for HLADH. P=atm. If required, EtOAc was used for downstream inline extraction (entries 1-3, Table 1), alternatively toluene (15%) is added upstream (entries 4-6, Table 1).

The functional group interconversion was achieved with excellent reaction times when compared to traditional batch methods, 30/45 minutes for the whole process (15 min for the oxidising reactions, 15 or 30 min for the reduction ones) and common condensation side-products[12] were never observed. Furthermore, the use of isolated enzymes avoids secondary reactions which can be at times observed with whole cells biotransformations. The optimised bio-flow-cascade was applied to different aromatic amines for their bioconversion to aromatic alcohols (Table 1).

Table 1. Preparation of primary aromatic alcohols from aromatic amines



Entry	Amine	r. t. (min)	Aldehyde *m.c.%	r. t. (min)	Alcohol *m.c.%	Isolated yield (%)
1		15	 >99	30	 90	86
2		15	 >99	15	 >99	95
3		15	 >99	30	 90	86
4		15	 >99[a]	30	 95[c]	90
5		15	 >99[a,b]	30	 >99[c]	97
6		15	 >99[a]	30	 96[c]	90

Reactions were performed in the presence of 5 mM substrates and pyruvate, 0.1 mM PLP, 1 M EtOH and 0.01 mM NADH at 37 °C for the first step and 28 °C for the second one. HEWT: 5 mg/ $g_{resin,}$ HLADH: 2 mg/ $g_{resin.}$ *m.c.: molar conversion. For analytical details see Supplementary Methods. [a]Reaction performed at 45 °C. [b]10 mM Pyruvate. [c]Liquid-liquid-phase flow stream (see procedure summarized in Fig. 2).

Piperonylamine, *trans*-cinnamylamine and *p*-phenylbenzylamine (entries 4-6) required higher temperature (45 °C) in the deaminating step, as well as a toluene inlet (generating a liquid-liquid phase 15:85 toluene buffer) to minimise adsorbance of the corresponding aldehydes on the resin. In these two cases, downstream EtOAc extraction was unnecessary as the products separated in the toluene stream. Notably both enzymes were completely stable under harsher working conditions and were reused more than 20 times without any loss of activity.

To exemplify the value of this strategy, we applied it to the synthesis of significantly more relevant products, such as the alcohols derived from biogenic amines, like the aromatic antioxidant hydroxytyrosol as well as the heteroaromatic tryptophol and histaminol (Fig. 3).

Previous attempts to convert biogenic amines into the corresponding aldehydes have utilised monoamine oxidases (MAO) but the highly reactive aldehydes readily generate condensation products with the unreacted amines.[41] Transaminases however, offer a better control of the deamination reaction and the use of flow reactors minimise cross-condensation products.

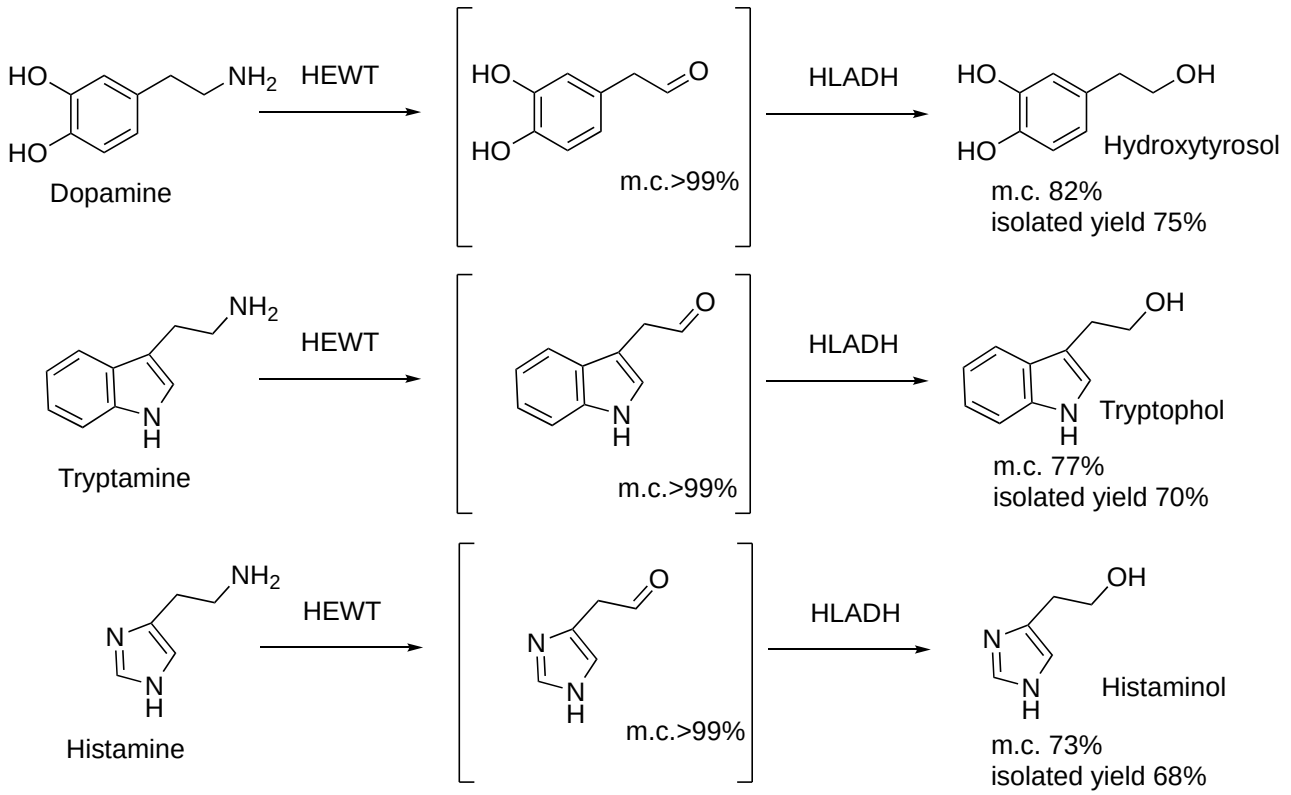


Figure 3. Synthesis of high value alcohols from biogenic amines

Hydroxytyrosol, tryptophol, and histaminol were easily synthesised *via* telescoped reactions without the isolation of the aldehyde intermediates (for analytical details see Supplementary Methods).

Chemical processes for the preparation of hydroxytyrosol, one of the most powerful and expensive anti-oxidant on the market (200£ for 25 mg, see Supplementary Notes), are unsuitable for large scale production due to the cost of the reagents, while the isolation from olive mill wastewaters is very low yielding.[42] Employing the procedure schematised in Figure 2 (biphasic stream toluene/HEPES buffer 50 mM pH 7.5 15:85) dopamine was fully converted into the alcohol in 45 min of total residence time. Following in-line purification, pure hydroxytyrosol was recovered (75% isolated yield) without any further work-up step.

Tryptophol and histaminol were also successfully obtained under the same conditions confirming the versatility of the system with different types of aryl rings. No undesirable side reactions between the amines and aldehydes were observed resulting in significantly enhanced process yields (70% and 68% respectively). For hydroxytyrosol, and histaminol in particular, this is a powerful, highly competitive synthesis, which does not suffer from drawbacks such as low yield and long reaction times commonly associated with their synthesis.

Flow dynamic kinetic resolution

Chemoenzymatic dynamic kinetic resolution (DKR), which combines resolution and *in situ* racemisation of the unreacted enantiomer was also successfully achieved with this system. A DKR process mediated by HLADH has been previously reported for the batch conversion of racemic arylpropionic aldehydes to the (*S*)-alcohols, valuable intermediates in the synthesis of profenic non-steroidal anti-inflammatory drugs (NSAIDs).[43] We recently reported that both (*S*) and (*R*)-2-pheny-1-propylamine (Fig. 4) are equally accepted as substrates of HEWT achieving 99% of molar conversion to the corresponding aldehydes.[33] The racemic amines are significantly cheaper and easier to handle than the corresponding aldehydes making this process economically sound (see Supplementary Notes). The combination of a 2-step continuous enzymatic process, including a DKR in the absence of built in chemical racemisation steps[44] has never been reported (Fig. 4) and required extensive optimisation of the system described in Figure 2.

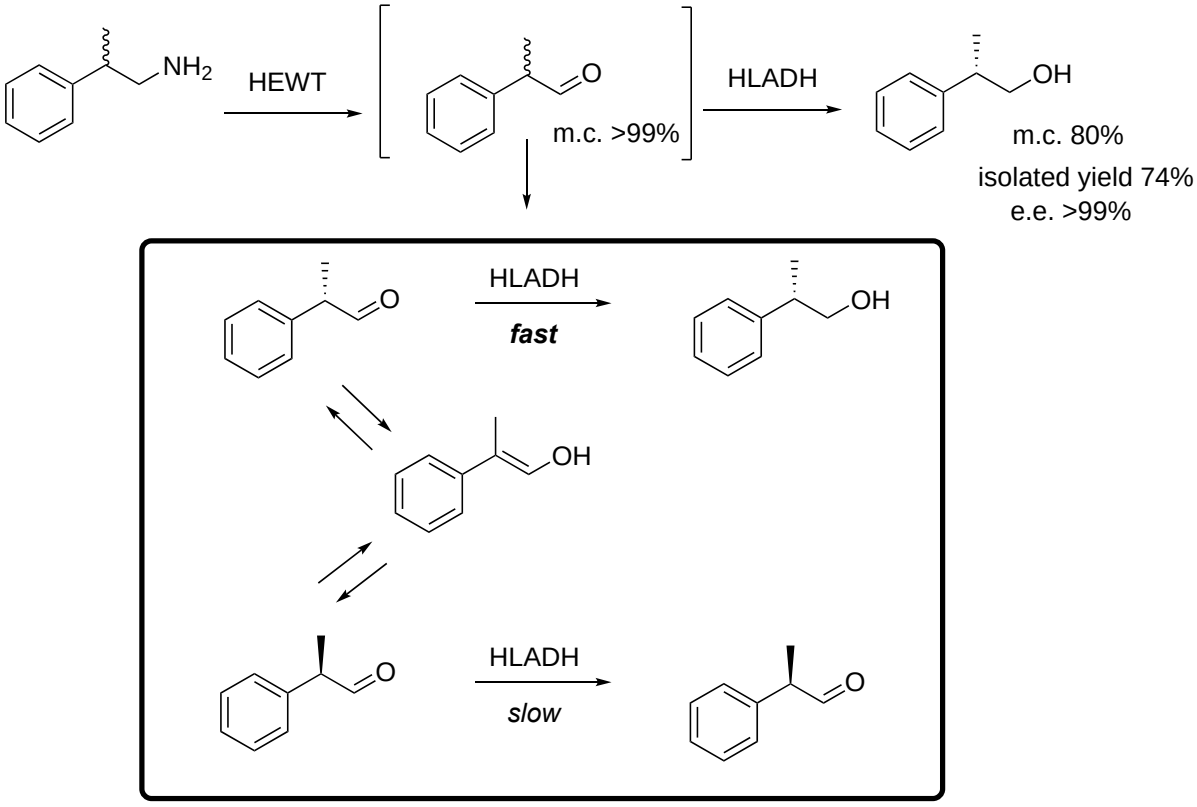


Figure 4. Preparation of (*S*)-2-phenylpropanol from (*R,S*)-2-phenylpropylamine

(*S*)-2-phenylpropanol could be obtained optically pure in 74% isolated yield exploiting spontaneous racemisation of the aldehyde intermediate.

Different flow reaction conditions were trialed (substrate concentrations, residence times, temperature, biphasic reaction with both toluene and *n*-hexane in different ratio) and the best conversion to the (*S*)-2-phenylpropanol (m.c. 80%, isolated yield 74%, e.e. >99%, see Supplementary Methods) was achieved using 2 mM of racemic amine and pyruvate, 0.1 mM PLP, 1 M EtOH and 0.01 mM NADH, 15 min of residence time at 37 °C for the oxidising step and 30 min at 30 °C for the reduction step. The flow cascade reaction was performed with Tris HCl buffer pH 9 and HLADH immobilisation loading was significantly reduced to

0.1 mg/g_{resin} to favour the concurrent racemisation reaction. Remarkably the new working conditions had no effect on the catalytic efficiency of the enzymes which were continuously utilised over several cycles. In-line extraction with EtOAc was followed by a QP-BZA column to trap any trace of unreacted aldehydes yielding the (*S*)-alcohol with high chemical purity (>99%). This continuous synthesis showed a 10-fold increase in reaction rate (see Supplementary Equations 1 and 2) with respect to previously reported batch strategies (0.019 μmol/min $g_{catalyst}$ in batch,[43] 0.15 μmol/min $g_{catalyst}$ under flow condition) and offers an excellent alternative for the preparation of these building blocks.

Preparation of pure secondary alcohols

A set of chiral amines was then investigated to further expand the applicability of this system for the synthesis of non-commercially available chiral 1-phenylethanols (see Supplementary Notes). Many aromatic amines are readily accessible, affordable, and generally sold with a high degree of purity, while the corresponding alcohols, when commercially available, are orders of magnitude more expensive, therefore this biocatalysed process is highly competitive. As a proof of concept, we used an (*S*)-selective ketoreductase to retain the stereochemistry of the original amine. A pre-mixed bed system composed of aldehyde-agarose immobilised ketoreductase from *Pichia glucozyma* (KRED1-Pglu, 4.3 g) and a glucose dehydrogenase from *Bacillus megaterium* (*Bm*GDH, 4.3 g) was used as reported in the literature[45] for the stereoselective reduction of different acetophenones under flow conditions (Fig. 5). Before assembling the bio-flow cascade single step reactions were optimised as reported in Methods.

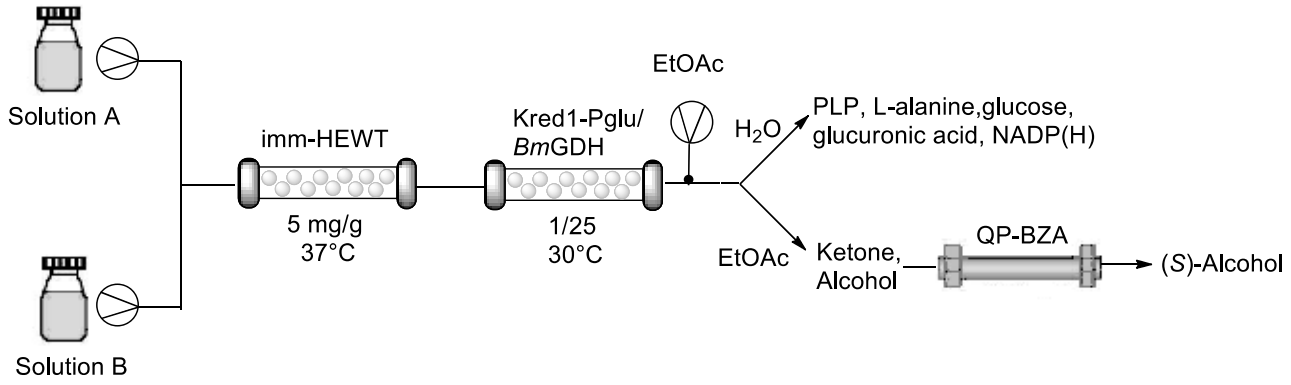
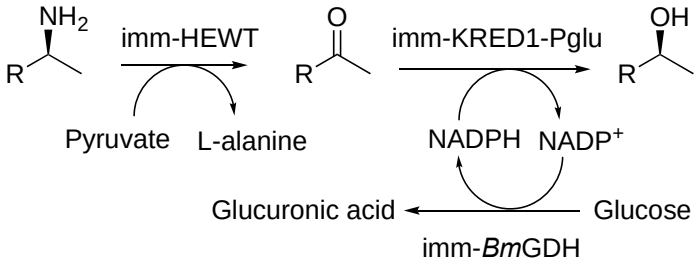


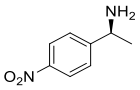
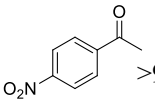
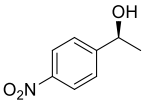
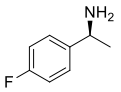
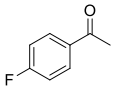
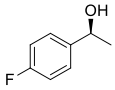
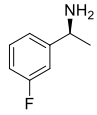
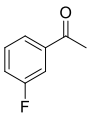
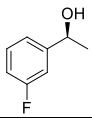
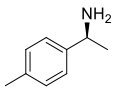
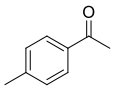
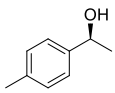
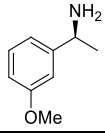
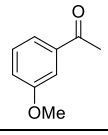
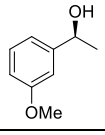
Figure 5. Multi-enzymatic cascade synthesis of enantiopure (*S*)-1-phenylethanols

Solution A: 6 mM (*S*)-methybenzylamine, in Tris-HCl buffer (50 mM pH 8) containing 20% DMSO, 24 mM glucose and 0.2 mM $NADP^+$. Solution B: 6 mM solution pyruvate in Tris-HCl buffer (50 mM pH 8) containing 0.2 mM PLP. T= 37 °C for HEWT and 30 °C for KRED1-Pglu/*Bm*GDH. P=atm.

To maximise the solubility of the substrates in Tris-HCl buffer (50 mM, pH 8.0), 10% of DMSO was used as co-solvent. HEWT catalytic efficiency was unaffected by the different reaction conditions, and yielded consistently >99% of the corresponding acetophenones (Table 2). The deamination step was performed at atmospheric pressure under optimised conditions (37 °C, 15 min of residence time) with just one equivalent of pyruvate. Imm-KRED1-Pglu (14 mg/g_{resin}) and imm-*Bm*GDH (5 mg/g_{resin}) were blended together in an optimised ratio (KRED1-Pglu/*Bm*GDH 1:25) to obtain a homogeneous agarose gel system suitable for the stereoselective reduction of acetophenones at 30 °C with a highly efficient *in situ* regeneration of the cofactor system NADPH/$NADP^+$.

Table 2. Synthesis of optically pure alcohols starting from the corresponding amines



Entry	(*S*)-Amine	r. t. (min)	Ketone *m.c.%	r. t. (min)	(*S*)-Alcohol *m.c.%	Isolated yield (%) e.e. (%)
7		15	 >99	180	 96	90 >99
8		15	 >99	180	 90	85 >99
9		15	 >99	180	 90	84 >99
10		15	 >99	180	 95	90 >99
11		15	 >99	180	 85	73 >99
12		15	 >99	180	 90	82 >99

Reactions were performed in the presence of 3 mM substrates and pyruvate, 0.1 mM PLP, 0.1 mM $NADP^+$, 12 mM glucose at 37 °C for the first step and 30 °C for the second one. 10% of DMSO was used as co-solvent. *m.c.: molar conversion. For analytical details see Supplementary Methods.

The best molar conversion for the reductive step in the synthesis of the different (*S*)-alcohols (isolated yield 73- 90%, ee >99%) was achieved after 180 min of residence time significantly improving on both yields and reaction times achievable in batch (see Supplementary Table 3, 4) confirming the greater efficiency of the developed bio-flow cascade. In-line extraction followed by a purification step was again implemented to trap the unreacted ketone and obtain the enantiopure (*S*)-alcohols with high chemical purity.

Finally, two additional reactions were carried out starting from racemic amines (*p*-fluoro-methylbenzylamine and *m*-methoxy-methylbenzylamine) in a deracemisation process (see Supplementary Figure 1).

HEWT is highly enantioselective and exclusively converts the (*S*)-enantiomer achieving the maximum 50% conversion. The pure, unreacted (*R*)-methylbenzylamines were isolated with a catch and release strategy using an A15 resin, and recovered as methylbenzylamine hydrochloride by flushing the column with a diluted solution of HCl (isolated yield of 47% for (*R*)-*p*-fluoro-methylbenzylamine and 45% for (*R*)-*m*-methoxy-methylbenzylamine). The ketone containing solution, required pH adjustment before progressing to the second reactor (reduction step). This system could therefore be used for the selective and complete conversion of the (*S*)-amine allowing for excellent recovery of the unreacted (*R*)-amine and the synthesised (*S*)-alcohol ((*S*)-*p*-fluoro-phenylethanol 45% and (*S*)-*m*-methoxy-phenylethanol 43%, isolated yields).

Ultra-efficient closed-loop multi-enzyme conversion of amines into alcohols

Despite the benefits of biocatalytic processes, their application on large scale pose a significant challenge because the recovery of water by standard methodologies (distillation,

ultrafiltration) is economically non-viable.[46] Furthermore, in flow reactors, the reaction medium downstream still contains traces of reagents, by-products or catalysts that are discarded, deeply influencing the economy of the system. Here, although cofactors were already used catalytically and recycled *in situ* with a significant improvement to the efficiency of the reactions in batch, a further step towards a closed-loop zero-waste reaction was designed to recover by-products and reuse the cofactors. Standard reactions were used to prove the feasibility and the efficiency of the process in each of the two steps. L-alanine and glucuronic acid, benign by-products deriving from the deamination and reduction steps, respectively, were recovered in excellent yields exploiting catch and release strategies (97% for L-alanine, A15 resin, and 95% for glucuronic acid, Ambersept 900 OH resin, Supplementary Figure 2, 3), while the aqueous phase containing the cofactors (PLP for the transaminase and NAD(P)H for the redox enzymes) was subjected to extensive reutilization. After 20 cycles, no loss of enzymatic performance was noted for HEWT while minimal reduction (<10%) was observed for the imm-KRED1-Pglu/imm-*Bm*GDH system. The recirculation of the water stream was subsequently applied to the combined cascade system using (*S*)-*p*-nitromethylbenzylamine to (*S*)-*p*-nitrophenylethanol conversion as test reaction. The trapping of both side-products (L-alanine and glucuronic acid) was achieved using a single column packed with Ambersept 900 OH (Fig. 6). The recovered waste waters, containing the cofactors were recirculated in the system by simple addition of fresh amine starting material. After 5 working days more than 80% of the alcohol was recovered showing the ultra-efficiency of the system. With water recirculation, the ratio cofactors:substrate decreased from 1:100 to 1:2000.

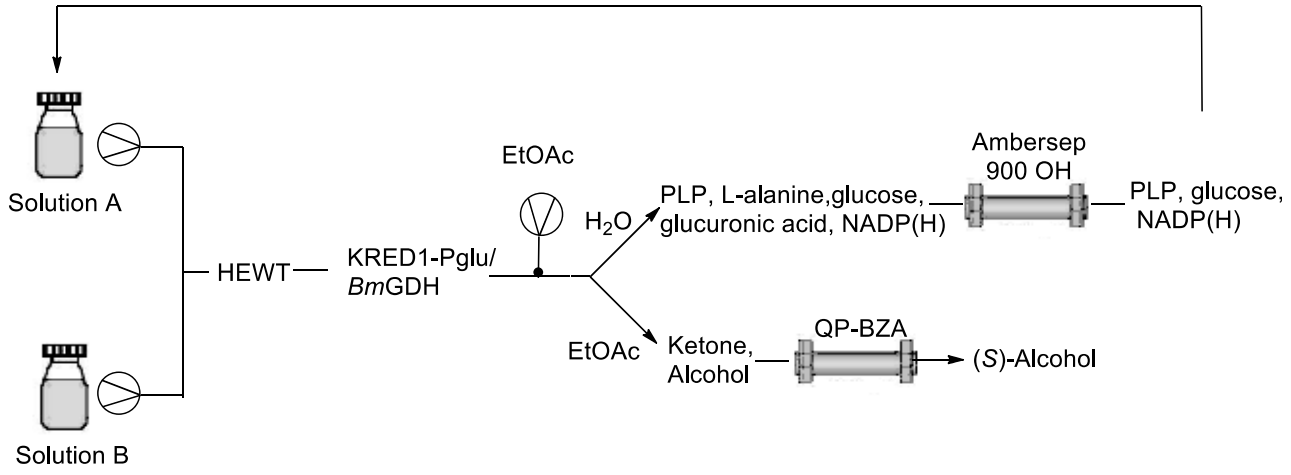


Figure 6. Ultra-efficient multi-enzyme interconversion of amines into alcohols

Solution A: 6 mM (*S*)-*p*-nitromethylbenzylamine, in Tris-HCl buffer (50 mM pH 8) containing 20% DMSO, 24 mM glucose and 0.2 mM $NADP^+$. Solution B: 6 mM solution pyruvate in Tris-HCl buffer (50 mM pH 8) containing 0.2 mM PLP. T= 37 °C for HEWT and 30 °C for KRED1-Pglu/*Bm*GDH. P=atm.

CONCLUSIONS

We report here on the development of a versatile multi-enzymatic platform for the continuous and completely automated interconversion of amines to the corresponding alcohols with excellent yield and reaction times. High value molecules such as hydroxytyrosol, tryptophol and histaminol deriving from inexpensive biogenic amines have been effortlessly prepared demonstrating the applicability of this process to reactions which in batch had very limited success. By eliminating amine/aldehyde condensations commonly happening in batch, the overall process was significantly enhanced. The system was fine-tuned to achieve a dynamic kinetic resolution for the synthesis of valuable enantiopure 2-arylpropanols, key intermediates in the total synthesis of sesquiterpens and profens, offering a valid alternative to existing methods and starting from commercially affordable racemic amines. Absolute optical purity

is achieved in the preparation of non-commercially available secondary alcohols; here we employed an (*S*)-selective KRED for the retention of the stereochemistry but the process could easily be expanded to the synthesis of (*R*)-alcohols if an anti-Prelog ADH (*e.g., Lactobacillus brevis* ADH)[47] was to be used. In comparison to traditional methods this continuous synthesis offers an excellent route for the efficient and cost-effective preparation of these important building blocks. Ultra-efficiency was achieved by using simple trapping columns downstream of the process, which can be easily regenerated, to separate from the efflux pure products and by-products, and extensively recirculating the aqueous phase in a closed-loop system. Here, the concept of waste waters can be significantly revised, reducing to virtually zero the costs associated with cofactors which is a major leap forward for enzyme-mediated processes.

METHODS

Materials

All the substrates, solvents, scavengers and reference products used for this project were bought from Sigma-Aldrich. Sepabeads EC-EP/S were kindly donated by Resindion S.R.L. The R-series modular flow chemistry system was purchased from Vapourtec® equipped with Omnifit® glass columns (6.6 mm i.d × 100 mm length or 10 mm i.d × 150 mm length).

Enzyme preparation and immobilisation

Expression of HEWT and HLADH was performed following previously reported protocols in Cerioli *et al.*[48] and Quaglia *et al.*[34] Their immobilisation was performed directly in flow exploiting the following procedure: an Omnifit® glass column (6.6 mm i.d. × 100 mm length)

was filled with 1 g of dry Resindion Sepabeads EC-EP/S (final volume 0.96 mL). The resin was washed with distilled water for 30 min (flow 0.5 mL/min). The epoxy resin was firstly reacted with modification buffer (0.1 M sodium borate, 2 M iminodiacetic acid in phosphate buffer 50 mM, pH 8.5, 2 h, 0.1 mL/min) and subsequently washed with distilled water (30 min, 0.5 mL/min). It was then treated with metal buffer (1 M NaCl, 5 mg/mL CoCl2 in acetate buffer 50 mM, pH 6) and washed again with distilled water (30 min, 0.5 mL/min). The cell lysate containing the desired amount of protein (5 mg/gresin for HEWT and 2 mg/ gresin for HLADH) was loaded and kept in contact with the resin without any flow. After 4 h the resin was washed with distilled water and the metal ion was then removed by washing with desorption buffer (50 mM EDTA, 0.5 M NaCl in phosphate buffer 20 mM, pH 7.2, 20 min, 0.2 mL/min). To ensure that no reactive epoxide remained, the beads were finally treated with glycine buffer as capping agent over night without flowing (3 M glycine in phosphate buffe 50 mM, pH 8.5). The column was subsequently washed with phosphate buffer 50 mM, pH 8. The recovered activity assay was performed as described in *Planchestainer et al.*[40] for the HEWT and as described in *Quaglia et al.*[34] for the HLADH. KRED1-Pglu and *Bm*GDH were expressed and purified using the protocols reported in *Contente et al*.[35] while their immobilisation was performed following the procedure reported in *Dall'Oglio et al.*[45]

Single step flow reactions for the preparation of aldehydes from amines

An Omnifit® glass column (6.6 mm i.d × 100 mm length) filled with 1.2 g of imm-HEWT (5 mg/g) was set up. A 10 mM amino acceptor substrate in phosphate buffer (50 mM, pH 8.0) containing 0.2 mM PLP, and 10 mM amine solution with 2 M ethanol were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 1.7 mL). The flow rate was varied

and optimised. An in-line acidification was performed by using an inlet of 1 N HCl aqueous solution (flow rate: 0.17 mL/min) that was mixed to the exiting reaction flow stream using a tee junction. The resulting aqueous phase was extracted in-line using a stream of EtOAc (flow rate: 0.17 mL/min). Both organic and aqueous phases were analysed by HPLC using the conditions reported Supplementary Methods. The organic phase, containing the aldehyde, was evaporated to yield the desired product.

For entry 4-6 in Table 1, 10 or 20 mM pyruvate in phosphate buffer (50 mM, pH 8.0) containing 0.2 mM PLP, and 10 mM amine solution were prepared. The two solutions were mixed in a T-piece. A second junction for additional supplement of toluene (20 µL/min) was installed before the packed enzyme column. The resulting segmented flow stream was directed to the imm-HEWT. The flow rate was varied and optimised. After an in-line acidification step, as previously reported, the exiting flow stream was separated and the organic and aqueous phases analyzed by HPLC exploiting a calibration curve (see Supplementary Methods) and the toluene, containing the desired product, was evaporated to yield the aldehydes.

Single step reactions for the preparation of primary alcohols from aldehydes

An Omnifit® glass column (6.6 mm i.d × 100 mm length) filled with 1.2 or 2.4 g of imm-HLADH (2 mg/g or 0.1 mg/g in the case of the DKR) was set up. A 10 mM or 4 mM aldehyde as substrate in phosphate buffer (50 mM, pH 8.0) or Tris-HCl (50 mM, pH 9) with 2 M ethanol and 0.02 mM NADH solution were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 1.7 mL or 3.4 mL). The flow rate was varied and optimised. An in-line

extraction was performed by using an inlet of EtOAc (flow rate: 0.17 mL/min) that was mixed to the exiting reaction flow stream using a tee junction. Both organic and aqueous phases were analysed by HPLC using the conditions reported in Supplementary Methods. The organic phase, containing the alcohol and possible traces of unreacted aldehyde, was passed through a column packed with a polymer supported benzylamine (QP-BZA). The organic solvent was evaporated to yield the desired alcohol.

For entry 4-6 in Table 1, a 10 mM aldehyde solution in phosphate buffer (50 mM, pH 8.0) containing 2 M ethanol, and 0.002 mM NADH solution were prepared. The two solutions were mixed in a T-piece. A second junction for additional supplement of toluene (20 μL/min) was installed before the packed enzyme column. The resulting segmented flow stream was directed to the imm-HLADH. The flow rate was varied and optimised. As previously reported, the exiting flow stream was separated and the organic and aqueous phases analysed by HPLC exploiting a calibration curve (see Supplementary Methods) and the toluene, containing the alcohol and possible traces of unreacted aldehyde, was passed through a column packed with a polymer supported benzylamine (QP-BZA). The organic solvent was evaporated to yield the desired alcohol.

Single step reactions for the preparation of acetophenones from amines

An Omnifit® glass column (6.6 mm i.d. × 100 mm length) filled with 0.7 g of imm-HEWT (5 mg/g) was set up. A 6 mM amino acceptor substrate in Tris-HCl (50 mM, pH 8.0) containing 0.2 mM PLP, and 6 mM amine solution with 20% DMSO were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 1 mL). The flow rate was varied and

optimised. An in-line acidification was performed by using an inlet of 1 N HCl aqueous solution (flow rate: 0.1 mL/min) that was mixed to the exiting reaction flow stream using a tee junction. The resulting aqueous phase was extracted in-line using a stream of EtOAc (flow rate: 0.1 mL/min). Both organic and aqueous phases were analysed by HPLC using the conditions reported in Supplementary Methods. The organic phase, containing the acetophenone, was evaporated to yield the desired product.

Single step reactions for the preparation of (*S*)-1-phenylethanols from ketones

An Omnifit® glass column (10 mm i.d × 150 mm length) was packed with pre-mixed imm-KRED1-Pglu (4.3 g) and *Bm*GDH (4.3 g) in order to obtain a KRED1-Pglu/*Bm*GDH ratio of 1/25. A 6 mM ketone solution in Tris-HCl buffer (50 mM, pH 8.0 or pH 9) with 20% DMSO, and 0.2 mM $NADP^+$ with 24 mM glucose solution were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 12.2 mL). The flow rate was varied and optimised. An in-line extraction was performed by using an inlet of EtOAc (flow rate: 0.07 mL/min) that was mixed to the exiting reaction flow stream using a tee junction. Both organic and aqueous phases were analysed by HPLC using the conditions reported in Supplementary Methods. The organic phase, containing the alcohol and possible traces of unreacted ketone, was passed through a column packed with a polymer supported benzylamine (QP-BZA). The organic solvent was evaporated to yield the desired alcohol.

Typical flow set up for the interconversion of amines into alcohols

Continuous tandem flow biotransformations were performed using two Omnifit® glass columns (6.6 mm i.d × 100 mm length or 10 mm i.d × 150 mm length) in line. The first biocatalyst, imm-HEWT (5 mg/g) was employed for the transformation of aromatic amines into carbonyls, while imm-HLADH (2 mg/g - 0.1 mg/g) or pre-mixed imm-KRED1-Pglu/imm-*Bm*GDH (ratio of 1/25) for the preparation of primary or secondary (*S*)-alcohols, respectively. Sodium pyruvate (4, 6, 10, 20 mM) in phosphate, HEPES or Tris HCl (pH 8.0, 7.5 or 9) buffer containing catalytic amount of cofactors (0.2 mM PLP, 0.02 mM NADH or 0.2 mM $NADP^+$), and 4, 6, 10 mM amino donor solution with different concentration of co-solvents (2 M EtOH or 20% DMSO) were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the columns packed with the biocatalysts. The flow rate was varied and optimised. The flow stream was extracted in-line using EtOAc and the resulting organic phase subsequently purified using the QP-BZA scavenger. The reactions were analysed by HPLC using the conditions reported in Supplementary Methods, and the solvent evaporated to obtain the final pure alcohols. The aqueous phase was passed through an Ambersep 900 OH resin to trap the side-products (L-alanine, glucuronic acid) and recirculate the cofactors (PLP, NAD(P)H/$NAD(P)^+$) and glucose.

In the case of biphasic reactions, a second inlet for the addition of toluene (15:85 ratio toluene/buffer) was installed before the packed enzyme columns. The resulting segmented flow stream was directed to the imm-biocatalysts and subsequently in-line extracted and purified as reported above. The toluene, containing the desired product, was evaporated to yield the alcohols.

Data availability. All data are available from the corresponding author upon reasonable request.

Contributions

M.L.C. performed the experimental work and analysed the results; M.L.C. and F.P. conceived and designed the experiments. M.L.C. and F.P. co-wrote the manuscript.

Competing interests

The authors declare no competing financial interests.

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Acknowledgments

This work was supported by the Biotechnology and Biological Sciences Research Council [grant number BB/P002536/1]; and the authors wish to thank Resindion S.R.L. for donating the Sepabeads® EC-EP/S.