Transaminase-catalyzed continous synthesis of biogenic aldehydes

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Dedication ((optional))

Abstract: The physiological role of biogenic aldehydes such as DOPAL has been associated with cardiovascular and neurodegenerative disorders. The availability of these substrates is limited and robust synthetic methodologies would greatly facilitate further biological studies. Here we report on transaminase mediated single-step process in continuous mode which leads to excellent product yields (90-95%). Co-immoblization of the PLP cofactor eliminated the need of exogenous addition of this reagent without affecting the longevity of the system, delivering a truly self-sufficient process.

Aldehydes species are continuously generated in biological systems from a wide range of endogenous and exogenous precursors.[1a] Biogenic aldehydes are defined as those formed endogenously through enzyme-dependent or spontaneous oxidation of lipids, sugars and primary amines.[1b-e] Thanks to the strong electrophilicity of the terminal carbonyl group they are considered the most reactive compounds between biomolecules.[2] If not metabolized, biogenic aldehydes can become cytotoxic due to covalent modifications or adduct formation with proteins (typically Cys, Lys, His, and Arg residues),[3] nucleic acids (involving amino groups of both purines and pyrimidines)[4] and coenzymes[5] with subsequent inactivation *via* Michael addition, Knoevenagel condensation, Shiff base, and free radical formation. Moreover aldehydes are relatively long-lived so they not only react with targets in their proximity but can also diffuse or be transported to remote sites.[6] While, originally, biogenic aldehydes were believed to be innocuous intermediates predominantly formed by oxidative amination *via* monoamine oxidase (MAO), recent studies demonstrated that not only they are neurotransmitters themselves,[7] but are also involved in the development of cardiovascular[8] and neurodegenerative diseases (such as Alzheimer's and Parkinson's disease)[7] and play a key role in alcohol-related disorders.[2]

Synthetically, numerous attempts have been reported for the preparation of biogenic aldehydes.[9a-d] However, these methodologies require several steps, drastic reaction conditions, and repeated purifications. Li *et al.*[10a-c] described for the first time the synthesis and characterization of DOPAL (a metabolic intermediate deriving from dopamine) which required the use of restricted starting materials, (*e.g.*, piperonal belonging to the list of the controlled substances under drug legislation), environmentally unfriendly conditions (strong acids or bases, toxic solvents and reagents such as benzene, $POCl_3$ and mercury salts) to yield the desired products in low-to-moderate amounts (14-60%). More recently, Reimann *et al.*[9c] and Carosi *et al.*[9d] reported on the preparation of (3,4-dimethoxyphenyl)- and (3-methoxyphenyl)acetaldehyde respectively. Despite achieving better yields, both methodologies still require several reaction steps followed by purification, strong oxidizing reagents, and dry reaction environment.

Enzymatically, just a few examples involving whole cells of *Aspergillus niger*[11] with over-expressed MAO, partially purified MAO,[12] and aromatic acetaldehyde synthases (AASs)[13] are reported. In these cases, the formed aldehydes were usually transient intermediates of other reactions (*e.g.*, Pictet-Spengler condensation), therefore no accumulation, purification or characterization were described. Moreover, the use of AASs, able to catalyze the decarboxylation-oxidative deamination of aromatic amino acids, lead to the production of additional byproducts such as NH_3 and H_2O_2, which are generally toxic to the enzymes (increase in the pH, strong oxidizing reaction environment).

Therefore, an alternative, reliable strategy for the preparation of biogenic aldehydes in reasonable quantities and purity, would enable tailored *in-vitro* and *in-vivo* studies to further probe their biological functions. Aldehydes may in fact be an index of the metabolism of newly formed catecolamines in patients with neurodegenerative diseases and could be linked to alterations of cardiovascular structures with consequent cell apoptosis. In addition, their availability as pure standard will allow accurate measurements in human and animal tissues and a better understanding of sources, reactivity, and pathological mechanism under conditions of compromised aldehydes detoxification. Some of these aldehydes are commercially available, however they can be expensive, especially if needed in larger amounts, and a simple synthesis from readily available starting materials would allow more competitive pricing.

Cellular metabolism can be mimicked by assembling telescoped enzymatic reactions in flow bio-reactors which also significantly increases the efficiency and sustainability of the whole process.[14] Recirculation of aqueous media containing recycled cofactors and recovery of benign by-products allowed for the development of ultra-efficient closed-loop flow-systems with excellent atom efficiency and automation.[15] Within this work

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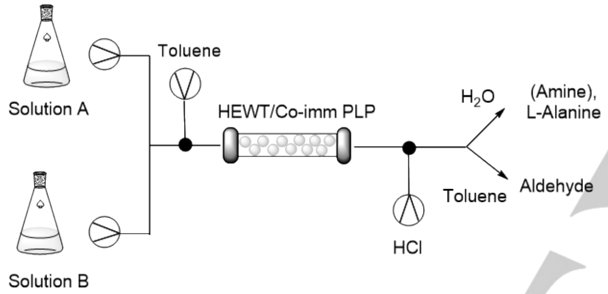
Supporting information for this article is given via a link at the end of the document.((Please delete this text if not appropriate)) biogenic aldehydes were produced as intermediates in the preparation of the corresponding alcohols from amine starting materials, but were never accumulated, isolated or characterized.

Key to the success of enzymatic reactions is the robustness and the reusability of the biocatalyst.[16] Immobilization of ω-transaminases has proven to be very efficient in increasing their operational stability and in the intensification of the productivity under flow conditions, including the preparation of a range of aldehydes from amines.[17a-b] In a further development, co-immobilization of the cofactor PLP (pyridoxal phosphate) has shown to eliminate completely the requirement of exogenous addition of this reagent [18] which was otherwise routinely added to all transaminase mediated reactions to increase yields and longevity of the system.[19a-b]

In this work we therefore exploited the co-immobilization of PLP with an ω-transaminase from the haloadapted bacterium *Halomonas elongata* (HEWT) for the synthesis of high value biogenic aldehydes in flow reactors. The system is self-sufficient, mimics an *in-vivo* process, and it achieves excellent efficiency, sustainability and complete automation.

The strategy (**Scheme 1**) was first optimized for the preparation of phenylacetaldehyde under flow conditions.



Scheme 1. Solution A: 40 mM amine in HEPES buffer (10 mM pH 7.5) Solution B: 40 mM pyruvate in HEPES buffer (10 mM pH 7.5). T= 37 °C. P= atm. Toluene (20%) is added upstream.

In the co-immobilized system, HEWT (5 mg/g_{resin}) was oriented and irreversibly bound through a multivalent attachment, while PLP (1 mM) through ionic bridges and reversible imine bonds was free to shuttle between the enzyme active sites without leaving the pore microenvironment of the resin (Figure 1).

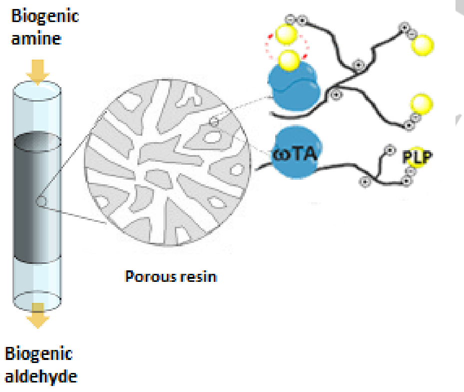


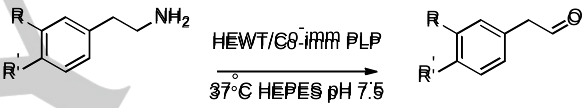
Fig. 1 Co-immobilization of HEWT and PLP

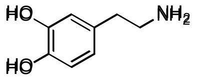
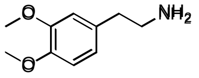
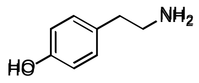
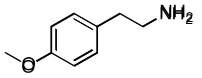
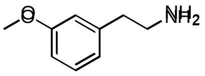
PLP here is acting not only as co-factor for the biotrasformation of amines into aldehydes, but also as stabilizer for the enzyme structure. High availability of PLP prevents subunit dissociation stabilizing the quaternary structure of the enzyme. A similar stabilization effect was observed when soluble PLP was incubated with soluble HEWT.[17a, 18]

Phenylacetaldehyde was obtained in high yield (>99%) and excellent residence time (15 min) when compared to batch methods (240 min, >99% of m.c.). The continuous synthesis showed a 2.3-fold increase in reaction rate with respect to batch strategies (batch mode: 0.83 μmol/min $g_{catalyst}$ - flow mode:1.9 μmol/min $g_{catalyst}$). The use of pyruvate as amino acceptor strongly favors the equilibrium of the reaction generating L-alanine as benign side product.[15,17b,20] A segmented flow (20:80 toluene/HEPES buffer) was implemented to strip the newly generated aldehyde from the carrier and, through acidification downstream of the reactor, the product was extracted in-line and recovered as pure compound without any further manipulation. The presence of toluene had no effect on the catalytic efficiency of the co-immobilized system which was extensively used over several days.

Once optimal reaction conditions had been established, the system was tested with biogenic amines (**Table 1**).

Table 1. Flow bio-synthesis of biogenic aldehydes



Entry	Substrate[a]	m.c. (%)[b]	Yield (%)
1		97	90
2		98	93
3		>99	94
4		>99	95
5		96	90

[a] 20 mM amine
[b] Determined by HPLC
Residence Time = 15 min

The functional group interconversion was consistently achieved with excellent reaction time (15 min). Interestingly, these set of amines did not behave like phenylacetaldehyde when tested in batch mode; here the aldehydes appeared to readily react with unreacted amines preventing the detection or characterization of any reasonable quantities of aldehydes. The addition of toluene in batch set up did not show any increase in the aldehyde detection. The flow approach in this case, resolves completely this drawback as it minimizes cross-condensation of product and starting material, affording excellent process yields (90-95%).

Isolated enzymes, when compared with partially purified fractions or whole cells biotransformations, behave more similarly to traditional catalysts and generally do not cause side reactions, and of course never encounter permeability problems. In particular transaminases, with respect to MAO or AASs, offer a better control of the deamination step which does not generate NH_3 or H_2O_2 as the amino group is simply transferred onto a sacrificial acceptor. Both NH_3 or H_2O_2, if let to accumulate in the reaction vessel, can cause pH increase as well as strong oxidizing reaction environment, which could eventually inactivate the enzyme involved in the biotransformations (even under flow conditions). HEWT, when pyruvate is the amino acceptor, forms L-alanine that can be easily recovered *via* scavenging columns as previously reported[15]. Notably HEWT is completely stable under segmented flow (buffer/toluene), and the same packed bed reactor was used to perform all the experiments without any loss of the activity.

In summary a new strategy for the synthesis of aromatic biogenic aldehydes was developed. HEWT was further stabilized due to the co-immobilization with PLP producing a self-sufficient heterogeneous biocatalyst for deamination reaction without exogenous addition of cofactors. In addition, with respect to previously reported data on amine conversion,[17b] here the concentration of the starting material was doubled and still yields of 90-95% were achieved. Side condensation reactions typically present in the batch methods were never observed.

The in-line extraction step allowed for the separation and recovery of the pure biogenic aldehydes in the toluene stream, while L-alanine/ traces of unreacted amines remained in the water phase, with no further work-up procedure making the process fully automated.

The combination of continuous mode and biocatalysis not only leads to significant reduction of the reaction times (15 min) and increased productivity but also has established a highly sustainable routes to an array of valuable products.

Experimental Section

Expression, purification, and immobilization of HEWT in *E. coli*

Protein expression and purification was performed following previously reported protocols in Cerioli *et al.*[20] Immobilization was conducted according to the procedure reported by Benítez-Mateo *et al.*[18] The retained activity observed for the co-immobilized HEWT/PLP system was >50% with respect to the free form with exogenously added cofactor (free enzyme: 5 U/mg, immobilized system 2.6 U/mg).

Batch reactions with immobilized HEWT

Batch reactions using the imm-HEWT with co-immobilized PLP were performed in 1.5 mL micro centrifuge tubes; 500 µL reaction mixture in 10 mM HEPES buffer pH 7.5, containing 20 mM pyruvate, 20 mM amino donor substrate and 50 mg of imm-HEWT with co-immobilized PLP (5 mg/g_{resin}, 1 mM PLP) was left under gentile shaking at 37 °C. 10 µL aliquots were quenched with a 50:50 mixture of hydrochloric acid (HCl) 0.2%:acetonitrile solution every hour and then analyzed by LC-MS equipped with a Waters X-Bridge C18 (3.5 µm, 2.1 mm x 30.0 mm). The compounds were detected using a DAD detector at 250 nm after a 5 min gradient run (A: 0.1% Ammonia in water, B: Acetonitrile. Gradient: 0 min 5% A 95% B; 3.10 min 0% A 100% B; 3.50 min 0% A 100% B; 3.51 min 95% A 5% B; 4.50 min 95% A 5% B. Injection volume 2 µL) at 40 °C with a flow rate of 0.8 mL/min. The retention times in minutes were: 2-phenylethylamine (2.0 min), 2-phenylacetaldehyde (2.3 min).

Flow reactions with immobilized HEWT and co-immobilized PLP

Continuous flow biotransformations were performed using a R2+/R4 Vapourtec flow reactor equipped with an Omnifit glass column (6.6 mm i.d. × 100 mm length) filled with 1.3 g of imm-HEWT/Co-immobilized PLP (5 mg/g_{resin} 1 mM PLP). 40 mM sodium pyruvate in HEPES buffer (10 mM, pH 7.5) and 40 mM amino donor solutions were prepared. The two solutions were mixed in a T-piece. A second junction for additional supplement of toluene was installed before the packed enzyme column. The resulting segmented flow stream (80:20 buffer/toluene) was directed to the imm-HEWT/co-imm PLP (reactor volume: 1.8 mL, flow rate: 0.12 mL/min). 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 T-junction. After an in-line extraction using a Zaiput liquid-liquid separator, the organic and aqueous phases were analyzed by HPLC exploiting a calibration curve and the toluene containing the desired product was evaporated to yield the aldehydes.

Analysis of biogenic aldehydes reactions

The flow biotransformations were analyzed by a Thermo Ultimate 300s HPLC equipped with a Accucore™ C18 LC Column (2.6 µm, 4.6 mm x 150 mm). The mobile phase was composed by A: 0.05% formic acid in water, B: Acetonitrile. The compounds were detected using a DAD detector at 280 nm after a gradient run increasing the concentration of B as follows: 0-5 min 10%, 5-10 min 50%, 10-11 min 10% (Injection volume 10 µL) at 40 °C with and flow rate of 1 mL/min. The retention times in minutes were: dopamine (2.7 min); DOPAL (3.4 min); 3,4-methoxyphenetilamine (3.4 min); 3,4-methoxyphenylacetaldehyde (4.2 min); 4-hydroxyphenetilamine (2.9 min); 4-hydroxyphenylacetaldehyde (3.5 min); 4-methoxyphenetilamine (3.2 min); 4-methoxyphenylacetaldehyde (4.0 min); 3-methoxyphenetilamine (3.0 min); 3-methoxyphenylacetaldehyde (3.8 min). The isolated aldehydes were further characterized by ^{1}H-NMR spectra which corresponded to those previously reported in literature.

Reaction rate comparison between batch and flow mode

Specific reaction rates in batch and continuous-flow systems were calculated using the following equations:[21]

Equation 1.

$$r_{batch} = \eta_p / t\ m_b\ (\mu mol / min\ g)$$

where $[\eta_p]$ is the amount of product (expressed as µmol), t is the reaction time (expressed as min), and m_b [g] is the amount of biocatalyst employed.

Equation 2.

$$r_{flow} = [P] x\ f / m_b\ (\mu mol / min\ g)$$

where $[P]$ is the product concentration flowing out of the reactor (expressed as µmol mL^{-1}), f is the flow rate (expressed as mL min^{-1}), and m_b [g] is the amount of biocatalyst loaded in the column.

Characterization of the products

The purity of aldehydes was assessed by HPLC and ^{1}H NMR. ^{1}H NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are expressed in Hz.

3,4-Dihydroxyphenylacetaldehyde (DOPAL): NMR (300 MHz, DMSO-d_6) δ (ppm): 9.58 (t, J = 2.33 Hz, 1H), 6.70 (d, J = 7.99 Hz, 1H), 6.61 (d, J = 2.13 Hz, 1H), 6.47 (dd, J = 7.99, 2.13 Hz, 1H), 3.52 (d, J = 2.40 Hz, 2H), 2.85 (t, J = 7.3 Hz, 2H), 1.93 (s, 3H).

3,4-Dimethoxyphenylacetaldehyde:NMR (300 MHz, $CDCl_3$) δ (ppm): 9.75 (t, J = 2.45 Hz, 1H), 6.89 (d, J = 8.15 Hz, 1H), 6.81 (dd, J = 8.15, 2.05 Hz, 1H), 6.73 (d, J = 2.0 Hz, 1H), 3.98 (s, 6H), 3.65 (d, J = 2.48 Hz, 2H).

4-Hydroxyphenylacetaldehyde: NMR (300 MHz, DMSO-d_6) δ (ppm): 9.62 (t, J = 2.18 Hz, 1H), 9.34 (s, 1H), 7.03 (m, 2H), 6.74 (m, 2H), 6.73 (d, J = 2.0 Hz, 1H), 3.98 (s, 6H), 3.61 (d, J = 2.20 Hz, 2H).

4-Methoxyphenylacetaldehyde: NMR (300 MHz, DMSO-d_6) δ (ppm): 9.65 (t, J = 2.03 Hz, 1H), 7.16 (m, 2H), 6.92 (m, 2H), 6.73 (d, J = 2.0 Hz, 1H), 3.74 (s, 3H), 3.68 (d, J = 2.09 Hz, 2H).

3-Methoxyphenylacetaldehyde: NMR (300 MHz, $CDCl_3$) δ (ppm): 9.73 (t, J = 2.40 Hz, 1H), 7.31-7.26 (m, 1H), 6.85-6.74 (m, 3H), 3.82 (s, 3H), 3.65 (d, J = 2.48 Hz, 2H) .

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Keywords: Biocatalysis • Biogenic amine • Biogenic aldehydes • Flow reactors • Transaminase

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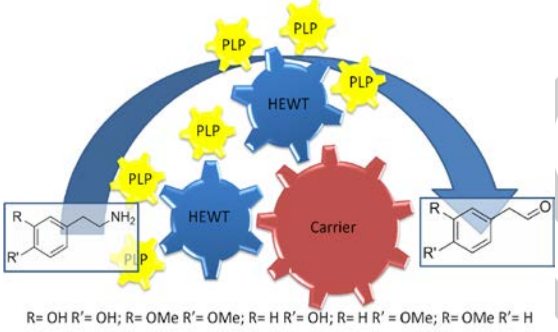

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Transaminase-catalyzed direct synthesis of biogenic amines into aldehydes using flow reactors

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