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Transaminase-catalyzed continous synthesis of biogenic aldehydes

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Dedication ((optional))

Abstract: The physiological role of biogenic aldehydes such as Dopal and Dopegal 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, Shiff base, Knoevenagel condensation 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 active themselves as neurotransmitters[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]

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Synthetically, numerous attempts have been reported for the preparation of biogenic aldehydes.[9a-d] However, these methodologies required drastic reaction conditions, several steps and repeated purifications. Li *et al.*[10a-c] described for the first time the synthesis and characterization of DOPAL and DOPEGAL (metabolic intermediates deriving from dopamine and norepinephrine, respectively). These synthetic routes were based on 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 obtain the desired products in low-to-moderate yields (14-60%). Reimann *et al.*[9c] and Carosi *et al.*[9d] reported on the preparation of (3,4-dimethoxyphenyl)- and (3-methoxyphenyl)acetaldehyde respectively. Despite the satisfactory yields both the methodologies are characterized by several reaction steps with consequent purification, strong oxidizing reagents and dry reaction environment.

Enzymatically, just few examples involving whole cells of *Aspergillus Niger*[11] with over-expressed MAO, partially purified MAO fractions[12] and aromatic acetaldehyde synthases (AASs)[13] are reported. In these bio-syntheses the formed aldehydes are usually transient intermediates of other reactions (*e.g.*, Pictet-Spengler condensation), so no accumulation, purification or characterization are 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 cell.

For these reasons 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 unequivocally identify their biological functions. In addition, a 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. Aldehydes may be an index of the metabolism of newly formed catecolamines in patients with neurodegenerative diseases and of the possibility to develop alterations of the cardiovascular structures with consequent cell apoptosis. Moreover, while some of these aldehydes are commercially available, their cost is often prohibitive.

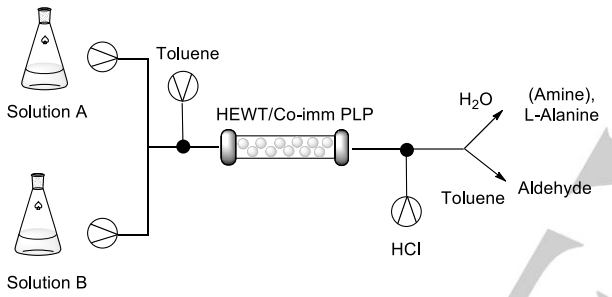
Cellular metabolism can be mimicked by assembling telescoped enzymatic reactions in flow bio-reactors which also significantly increases the efficiency and sustainability of the whole process.[14] Recirculation of aqueous media containing recycled cofactors and recovery of benign by-products allowed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

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 for 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 reaction to increase yields and longevity of the system.[19a-b]

In this work we therefore exploited the co-immobilization of PLP and 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, mimicks an *in-vivo* process, achieving excellent efficiency, sustainability and completed 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).

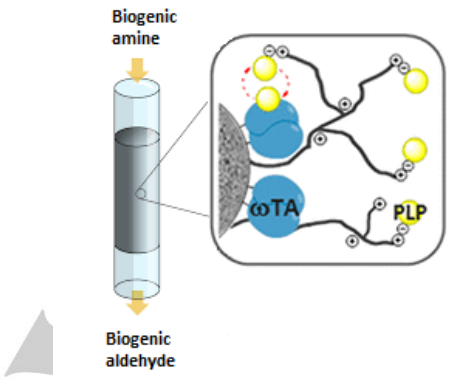


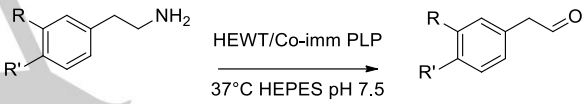
Fig. 1 Co-immobilization of HEWT and PLP

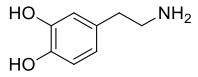
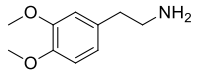
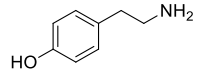
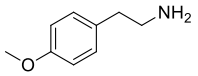
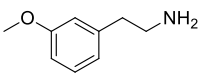
PLP here has a double role: as co-factor for the biotrasformation of amines into aldehydes and as stabilizer. High availability of PLP prevents subunit dissociation stabilizing the quaternary structure of the enzyme. A similar stabilization effect was observed when soluble PLP was incubated with soluble HEWT.[17a, 18]

Phenylacetaldehyde was obtained with 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 (0.83 μmol/min $g_{catalyst}$ - 1.9 μmol/min $g_{catalyst}$). The use of pyruvate as amino acceptor strongly favors the equilibrium reaction generating L-alanine as benign side product. A biphasic phase (20:80 toluene/HEPES buffer) was implemented to strip the newly generated aldehyde from the carrier. With the acidification of the downstream the process, 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 a reliable method had been developed, biogenic amines were tested under the same conditions (**Table 1**).

Table 1. Flow bio-synthesis of biogenic aldehydes



Entry	Substrate[a]	m.c. (%)[b]	Yield (%)	Time (min)
1		97	90	15
2		98	93	15
3		>99	94	15
4		>99	95	15
5		96	90	15

[a] 20 mM amine

[b] Determined by HPLC

The functional group interconversion was achieved with excellent reaction time (15 min), while in batch mode the prevalent side condensation reaction between amines and highly reactive aldehydes did not allow for the detection or characterization of any reasonable quantities of aldehydes. The use of flow reactors on the other hand, minimized cross-condensation and led to excellent process yields (90-95%).

Isolated enzymes compared with partially purified fractions or whole cells biotransformations avoid side reactions and permeability problems. In particular transaminases, with respect to MAO or AASs, offer a better control of the deamination step without the production of NH_3 or H_2O_2.These intermediates can cause increase of the pH as well as strong oxidizing reaction environment, which could eventually inactivate the enzyme involved in the biotransformations. 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 these working conditions (liquid-liquid phase 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 thanks 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 recovery of the pure biogenic aldehydes in the toluene stream and L-alanine/ traces of unreacted amines 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]

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 0.7 g of imm-HEWT/Co-immobilized PLP (5 $mg/_{gresin}$ 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. The flow rate was varied and optimized. 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. 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:

Equation 1.

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

where [η_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) .

Acknowledgements

This project was supported by the European Union's Horizon 2020 research and innovation programme under the Marie

Skłodowska-Curie grant agreement N. 792804_AROMAs-FLOW (M.L.C.). Thanks to Resindion S.r.l. for the Sepabeads® EC-EP/S. The authors wish to thank UK Biotechnology and Biological Sciences Research Council (BBSRC; BB/P002536/1) for financial support (F.P., M.L.C).

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

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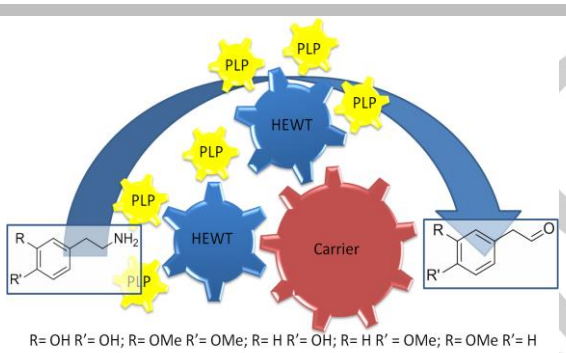

*M. L. Contente, F. Paradisi**

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

Layout 2:

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