Babeș-Bolyai University

Cluj-Napoca

Faculty of Chemistry and Chemical Engineering

Doctoral School of Chemistry

Novel Biocatalytic Routes to Optically Active Amino Alcohols and α, β-Amino Acids

PhD Thesis Abstract

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Scientific advisor: Prof. Habil. Dr. Monica Ioana TOȘA

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1. Introduction

Chiral nitrogen-containing molecules and their derivatives have gained importance in fine chemical, pharmaceutical, agrochemical industries and also in electronics and optics, since N-containing structures frequently occur in many biologically active small molecules and peptidomimetics. Amongst them, amino alcohols are extensively used as chiral auxiliaries of many biologically active compounds and natural products, whereas protected and non-protected amino acids play an important role in artificial peptide design. Therefore, synthesis of enantiopure nitrogen-containing compounds is an attractive and prominent research field providing direct applicability on both laboratory and industrial scales.

Additionally, enormous effort has also been devoted to the development of N-containing ligands to suit organometallic and coordination chemistry. Once integrated into metal complexes, amino moieties should provide important structural benefits. Transition metal complexes with nitrogen-containing ligands proved to be stable, easily separable and recyclable catalysts for asymmetric synthesis, homogeneous and heterogeneous catalysis.[1]

Numerous chemical and biochemical processes have been developed as practical route to enantiomerically pure amino-containing compounds. Biocatalysis often offers several advantages over chemical synthesis, e.g. high stereo-, regio- and chemoselectivity and mild reaction conditions (ambient temperature, atmospheric pressure), allowing the transformation of sensitive substrates. Moreover, it is a green, sustainable alternative for some transformations impossible to perform *via* traditional organic synthesis.

In chemo-enzymatic processes biocatalysts are used either as whole microorganisms or isolated enzymes. When whole cell systems are used, there is no need for enzyme isolation or purification, thus the enzyme stability can be enhanced. Regarding the cofactor dependent enzymes, cofactor regeneration is typically provided by the cell's own metabolic machinery. Although cellular systems provide all these benefits, they usually have several drawbacks: lower selectivities could be achieved (due to the competition from other similar enzymes present in the cell) and the control and maximization of the synthetic processes could be difficult. In contrast, in vitro approaches are less complex and, therefore, reaction conditions are easier to improve and optimize by enzyme or substrate concentration, co-solvents, pH or temperature.

Isolated wild-type enzymes are often highly selective catalysts of a certain transformation, but are very sensitive and generally not tolerant enough to process conditions or not sufficient active to ensure a high productivity. Protein engineering is the most important method to overcome the limitation of natural enzymes as biocatalysts. Directed evolution technologies and new developments in genomics offer many possibilities for the manufacture of tailor-made biocatalysts. Today we are able to tailor the

enzyme structure, in order to fit the process specifications. Beside protein engineering techniques, enzyme immobilization is a frequently used approach toward enzyme stabilization and reuse, thereby lowering the biocatalysts cost contribution to the process. Normally, immobilization involves attaching the enzyme to an inert and usually insoluble support. The result is a recoverable, stable and specific biocatalyst.

Amongst all enzymes, hydrolases are frequently used for the resolution processes where one of the enantiomers in a racemic mixture is selectively modified to afford a separable derivative. They catalyse many organic reactions including esterification, transesterification, acylation and hydrolysis. Popularity of hydrolases in general and lipases in particular relies on their selectivity, mild reaction conditions, ability to utilize wide range of substrates and high stability towards extreme temperature and pH. Lipases are the most important biocatalysts frequently employed to perform new reactions both in non-aqueous, aqueous media and in the synthesis of various biologically active and pharmaceutically important molecules.

Ammonia-lyases catalyse the reversible addition of ammonia and amines to double bonds of the corresponding α,β-unsaturated carboxylic acids, which are often cheap substrates. In the past decade, a wide range of biocatalytic and therapeutic applications of ammonia-lyases have been emerged. Ammonia-lyase catalysed reactions can be used for the stereoselective production of valuable synthetic amino acids.

This thesis is divided into two separate parts.

The first part describes two chemo-enzymatic approaches for the total synthesis of bufuralol enantiomers, a widely used *β*-adrenoceptor antagonist. One procedure involves the baker's yeast whole cells-catalysed enantiotope selective transformation of α-hydroxy and α-acetoxymethyl ketones, with opposite stereopreference, into the corresponding diol enantiomers. In the second approach, stereoselectivity relies on the lipase-mediated enantioselective *O*-acylation of the corresponding bromo-alcoholic intermediates.

The second part of the thesis deals with the preparation of *α*- and *β*-amino acids through biocatalytic processes. First, a microfluidic, continuous-flow system filled with phenylalanine ammonia-lyase from *Petroselinum crispum* immobilized on magnetic nanoparticles (*Pc*PAL-MNP) was developed and tested for the efficient kinetic resolution of L-phenylalanine and five unnatural α-amino acids. Reliability and reproducibility assessments of the measurements were also performed in order to validate the designed system. Finally, a lipase-catalysed stereoselective hydrolytic procedure was developed which enables the efficient kinetic resolution of poorly soluble and highly unstable *β*-amino acid ethyl esters containing a phenylfuran skeleton. These compounds were resolved, for the

first time, in their hydrochloric salt forms, which adumbrating possible applicability of this method to the manipulation of unstable amino acids and amino esters.

2. PART I. Synthesis of bufuralol

2.1. Importance of vicinal amino alcohols as *β*-blockers

Enantiomerically pure 1,2-amino alcohols, also named as vicinal amino alcohols, are important and versatile building blocks of biologically important compounds, natural products and chiral auxiliaries for asymmetric synthesis [2]. A large number of asymmetric synthetic methods have been developed, relying on the amino acids manipulation [3], introduction of the amino alcohol moiety on a pre-existing carbon skeleton [4], or the coupling of two molecules [5]. Besides of the organocatalytic processes, the importance of biocatalysis as an efficient, environmentally friendly and sustainable alternative has been growing rapidly. Lipases [6] and transaminases [7] have successfully been used for the asymmetric synthesis of 2-amino-1-arylethanol derivatives.

2.1.1. Bufuralol

Bufuralol is a chiral benzofuran-based 1,2-aminoalcohol, first described by Fothergill and developed by Roche.[8] It is a potent, non-selective *β*-adrenoceptor antagonist closely resembles propranolol but having also *β*-adrenoceptor agonistic properties.[9] It is successfully used for the treatment of hypertension [10] and presents inhibitory effect toward testosterone 6*β*-hydroxylase in bacterial membranes.[11] The metabolism of bufuralol is very complex; it is mediated by cytochrome P-450 system in human liver microsomes [12] and undergoes a series of transformations to alcohol and ketone metabolites also possessing *β*-receptor blocking activities.[13] Therefore it is also important and widely used for polymorphism studies of these enzyme complexes.[14]

Although the *β*-blocking potency of the (*S*)-bufuralol is significantly higher than that of the (*R*)-enantiomer, the (*R*)-bufuralol is a generally used as marker of hepatic CYP2D6 activity.[15] Thus, there is a high demand for the stereoselective synthesis of both isomer of bufuralol in enantiopure form.

2.2. Aim of the study

The aim of the study includes the enantioselective synthesis of both (*R*)- and (*S*)-enantiomers of bufuralol by chemo-enzymatic procedures including lipase- or baker's yeast catalysed reaction as the key stereoselective step. The first approach is based on the baker's yeast-mediated enantioselective transformation of 2-(7-ethylbenzofuran-2-yl)-2-oxoethyl acetate **5** and 1-(7-ethylbenzofuran-2-yl)-2-hydroxyethanone **6**, whereas in the second procedure, stereoselectivity was achieved by lipase-catalysed acylation of *rac*-2-bromo-1-(7-ethylbenzofuran-2-yl)ethanol *rac*-**9**. The obtained

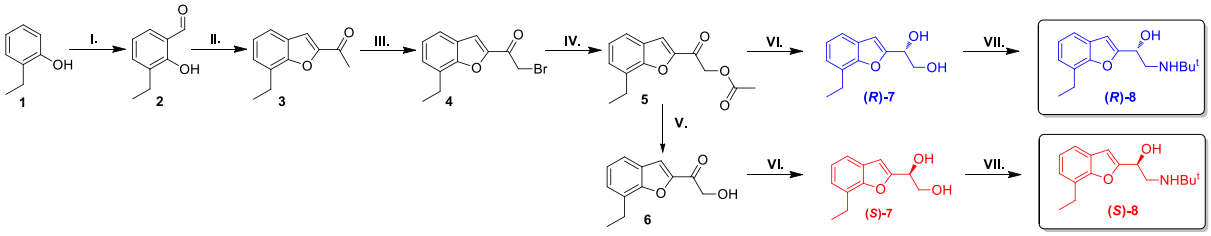
enantiomerically enriched intermediates were further transformed into the desired enantiomers of bufuralol.

2.3. Synthesis of (*R*)- and (*S*)-bufuralol via baker's yeast-mediated biotransformation

The use of baker's yeast in biocatalytic processes is widespread due to its low cost, mild reaction conditions and availability of the whole-cell system. Nevertheless, oxido-reductases and hydrolases present in *Saccharomyces cerevisiae* cells offer high stereoselectivity and broad substrate acceptability.

Our group has previously demonstrated that *Saccharomyces cerevisiae* cells transform α-hydroxy and α-acetoxymethyl ketones with opposite stereopreference into the corresponding diol enantiomers.[16] The bioreduction of the α-acetoxymethyl ketones by oxidoreductases from baker's yeast is usually followed by a subsequent enzymatic hydrolysis of the formed hydroxy-monoacetate into 1,2-ethanediol by the hydrolases also present in baker's yeast.

The synthesis starts with the preparation of racemic 2-(7-ethylbenzofuran-2-yl)-2-oxoethyl acetate **5** and 1-(7-ethylbenzofuran-2-yl)-2-hydroxyethanone **6** used as substrates for the enzymatic step (Scheme 1). 2-Ethylphenol **1** was *ortho*-formylated with paraformaldehyde in the presence of magnesium chloride and triethylamine. The obtained 3-ethyl-salicylaldehyde **2** was transformed into 7-ethyl-benzofuran-2yl-ethanone **3** which was further α-brominated using pyridinium tribromide. The bromo ketone **4** was further transformed with sodium acetate under anhydrous conditions into the corresponding α-acetoxymethyl-ketone **5**. Subsequent lipase-assisted ethanolysis of compound **5** afforded the desired hydroxymethyl-ketone **6**.



I. Paraformaldehyde, TEA, $MgCl_2$, CH_3CN, reflux; **II.** Chloroacetone, K_2SO_4,CH_3CN, reflux; **III.** $PyrBr_3$, CH_3COOH, reflux; **IV.** CH_3COONa, 18-C-6/ 1,4-dioxane, reflux; **V.** Cal-B/ EtOH, 300 rpm, r.t; **VI.** Baker's yeast, water/methanol, r.t; **VII. a)** *p*-TsCl, Bu_2SnO, TEA, CH_2Cl_2, r.t; **b)** $^tBuNH_2$, ethanol, reflux

Scheme 1. Chemo-enzymatic synthesis of bufuralol enantiomers using baker's yeast-mediated biotransformations

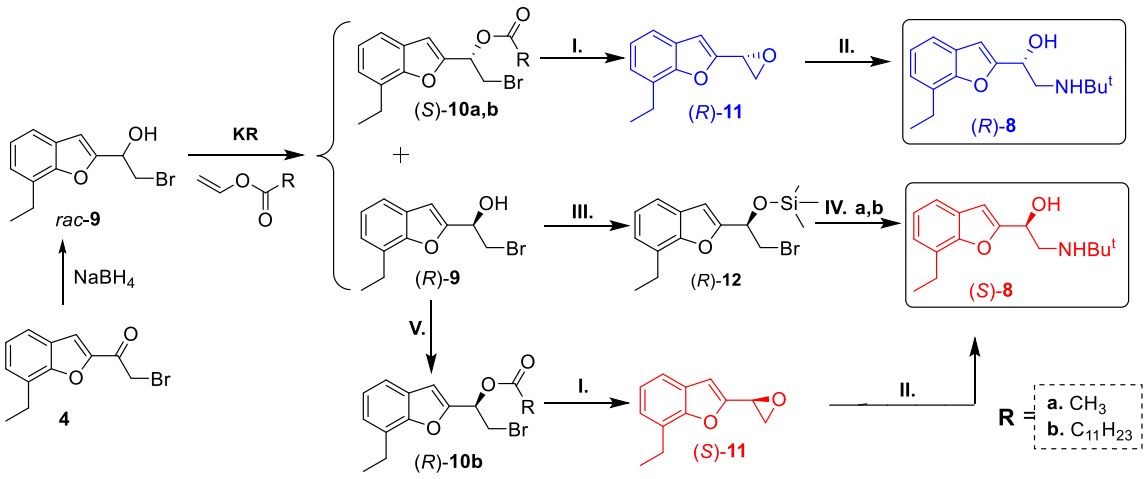
The aforementioned enantiotope selectivity of the *Saccharomyces cerevisiae* cells proved valid also for bufuralol intermediates. The analytical-scale enzymatic reactions were performed under fermenting and non-fermenting conditions. The influence of various additives upon the enzyme selectivity was also tested in order to enhance the enantiopurity of the products. Stereochemical

progress of the biotransformations was monitored by a chiral analytical HPLC method elaborated for the racemic diol *rac*-**7** (obtained by reduction of **6** with $NaBH_4$ in methanol).

The isolated (*R*)- and (*S*)-1-(7-ethylbenzofuran-2-yl)ethane-1,2-diols ((*R*)- and (*S*)-**7**) were transformed into the corresponding enantiomer of bufuralol by regioselective tosylation with *para*-toluenesulfonyl chloride in the presence of dibutyltin(IV) oxide [17] and a subsequent replace of the tosyl group with *tert*-butylamine (Scheme 1).

2.4. Lipase-catalysed kinetic resolution of 2-bromo-1-(7-ethylbenzofuran-2-yl)-ethanol for the synthesis of (*R*)- and (*S*)-bufuralol

The second approach for the synthesis of (*R*)- and (*S*)-bufuralol involves the lipase-catalysed stereoselective *O*-acylation of racemic 2-bromo-1-(7-ethylbenzofuran-2-yl)ethanol *rac*-**9** (Scheme 2) obtained through reduction of bromo-ketone **4** with sodium borohydride.



KR. Enzyme, acyl donor, r.t., 800 rpm; **I.** LiOH, EtOH, r.t.; **II.** *tert*-Butylamine, reflux; **III.** DMCTMS, Et_2O, r.t.; **IV.** a) *tert*-Butylamine, MeOH, r.t; b) HF, MeOH; **V.** Vinyl dodecanoate, CaL-A, DIPE

Scheme 2. Synthesis of (*R*)- and (*S*)- bufuralol from the lipase catalysed resolution products of the racemic bromoethanol *rac*-**9**

Commercially available immobilized lipases, such as lipases A and B from *Candida antarctica* (CaL-A on Celite and CaL-B imobilized on hydrophobic acrylic resin commercialized as Novozyme 435), lipases from *Pseudomonas* species (LAK and LPS), and *Candida rugosa* lipase (CRL) were tested for the analytical-scale *O*-acylation of *rac*-**9** using vinyl acetate and vinyl laurate as acyl donors in various organic solvents. Lipase A from *Candida antarctica* showed excellent reactivity but poor selectivity, whereas LAK, CRL and LPS displayed reduced activity but good selectivity in all tested solvents.

CaL-B proved to be the most suitable biocatalyst. Both enantiomerically pure compounds ((*R*)-**9** and (*S*)-**10**) were isolated in excellent yields and used for further transformations. In order to prevent

the undesired secondary reactions and also the racemization of the enantiomers, two particular chemical route were developed for the efficient synthesis of (*R*)- and (*S*)- bufuralol (Scheme 2).

2.5. Conclusions

We developed two chemo-enzymatic approaches which involve different biocatalytic steps, i.e. baker's yeast-catalysed biotransformation or lipase-catalysed enantioselective *O*-acylation, for the synthesis of (*S*) and (*R*)-2-(*tert*-butylamino)-1-(7-ethylbenzofuran-2-yl)ethanol ((*S*) and (*R*)-bufuralol). The desired compounds were obtained with high enantipurity (*ee* 98%) and good overall yield (53%).

3. PART II. Synthesis of *α*-and *β*-amino acids

3.1. Importance of *α*-and *β*-amino acids

Natural and unnatural *α*-amino acids are widely used in food, feed, agrochemical and pharmaceutical industries. For instance, many proteinogenic amino acids are used as infusion solutions. Aspartame, a low-calorie sweetener is formed by L-aspartic acid and L-phenylalanine methyl ester.[18] Synthetic amino acids are valuable intermediates and building blocks of many pharmaceutically important compounds, e.g. the synthesis of semisynthetic antibiotics ampicillin and amoxicillin,[19] or HIV-protease inhibitor Atazanavir.[20] They can also be used as chiral precursors for natural product total synthesis, or as chiral auxiliaries.

In contrast to *α*-amino acids, *β*-amino acids only occur in secondary metabolites.[21] Possessing an extra carbon atom between the amino and carboxylic groups, they have a higher potential for structural diversity than their *α*-analogs. *β*-Amino acids are present in peptides, glycopeptides, alkaloids and terpenes. The presence of *β*-amino acids in peptides enhances the stability of these molecules towards degradation by peptidases. Thus, *β*-amino acids have an important role in pharmaceutical chemistry and drug development.[22]

3.2. Synthesis of *α*-amino acids

3.2.1. Synthesis of *α*-amino acids using ammonia-lyases

For the production of chiral amino acids and amines, traditional biocatalytic methods have relied upon hydrolytic enzymes such as lipases, acylases and hydantoinases.[23] However, new approaches have recently been developed, based on deracemisation of racemic amines using amine oxidases and also *trans*-aminases.[24]

In an analogous way, ammonia-lyases represent attractive targets for the asymmetric synthesis of amino acids and amines and provide an additional benefit that they use readily available cinnamic acid derivatives as substrates. It was shown that when the ammonia concentration is raised to 5M, phenylalanine ammonia-lyase (PAL) can catalyse also the enantioselective addition of ammonia to arylacrylic acids.[25] In this way, enantiopure L-arylalanines containing variously substituted phenyl rings, polycyclic aromatic rings or even heterocycles, were synthesized. The method was extended to the enantioselective synthesis of 5-pyrimidinylalanine and several fluoro- and chloro-phenylalanines.[26] A sequential chemo-enzymatic process, including an esterase from porcine liver and PAL from parsley (*Pc*PAL), was developed in order to produce the enantiopure L-arylalanines from the corresponding aromatic aldehydes.[27] The *Pc*PAL was applied to produce the D-enantiomers of arylalanines in high enantiomeric purity by enantiomer selective destruction of the L-

enantiomers from their racemates.[28] It should be noted that PAL can operate also in monophasic organic solvents as *n*-octanol.[29]

3.2.2. Microfluidic systems in flow chemistry

Recent developments in the area of microfabrication techniques have been contributed to the wide spread of chip-sized laboratories (LoC-Lab-on-a-Chip). Microstructured reactors are widely recognised as important engineering tools for chemical process research and development.[30] Compared to conventional reactors, microreactors have several benefits, such as higher automation, smaller dead volume, shorter analysis time, and portability.[31] Moreover, the reaction time can be reduced due to the large surface to volume *ratio*.[32] The combination of these microsystems with continuous flow techniques can be particularly useful, because such microfluidic systems need lower reagent amount, the reaction time speeds up considerably (short molecular diffusion lengths) and the yield is enhanced by continuous discharge of the product formed and addition of new amount of substrate.[33]

The high performance of the isolated enzymes in biocatalytic processes is frequently impeded by their sensibility and low stability under non-physiological conditions. This issue can be overcome through immobilization strategies, which entail the reversible- or irreversible, covalent- or non-covalent binding of the biocatalyst to a suitable support. Proper immobilization of an enzyme can improve its physical, structural and catalytic properties affording a biocatalyst with high thermal and operational stability, enabling its recovery and reusability, and retaining its activity. The loss of biocatalyst during the process can also be minimalized.[34]

Regarding the immobilization methods, there are two generally used alternatives: the enzyme direct immobilization on the microchannel wall [35] or immobilization on a solid support, such as microbeads [36], nanoporous scaffolds [37] or magnetic nanoparticles.[38] The importance of magnetic nanoparticles as biomolecule carriers is continuously growing in biotechnology and biomedicine due to their large reactive surface, high dispersion, low diffusion limits and last, but not the least, the possibility to control them using an external magnetic field.[39]

3.2.2.1. Magnetic microreactor chip (Magne-Chip)

Magne-Chip platform contains all the necessary fluid actuators (e.g., pumps, valves, and thermostat) and sensors (e.g., pressure sensor), a microfluidic chip having microliter volume reaction chambers and a flow controller. The biomolecule is immobilized on the surface of the MNP. A suspension of the particles flows through the microchip, while magnetic particles are accumulating in the microchannels. Accumulation is selectively controlled by a directed magnetic field. In the next step, the reagents are passing through the chip, while reaction is taking place inside the microchambers and the product is collected and quantified outside the chip.

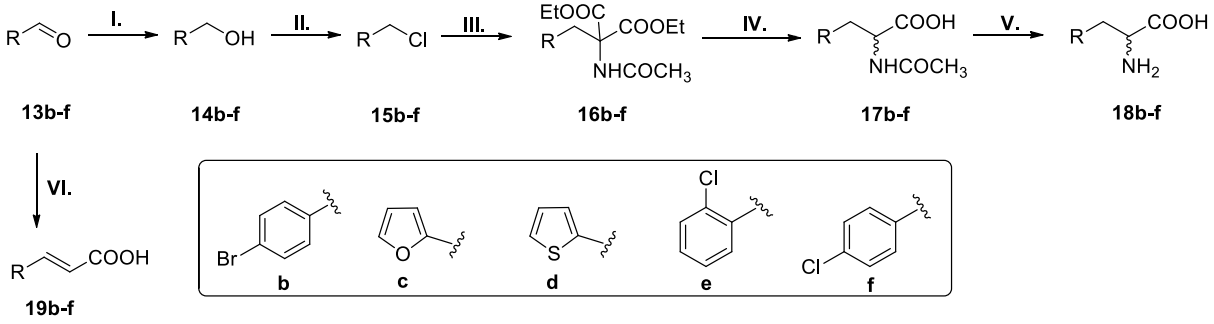
3.2.3. Aim of the work

The main objective was to study and evaluate the properties and parameter settings of the Magne-Chip system in PAL-catalysed biotransformations of L-phenylalanine (L-**18a**) and five unnatural *α*-amino acids (*rac*-**18b–f**), including *rac*-2-amino-3-(4-bromophenyl)propanoic acid (*rac*-**18b**) which has never been tested for *Pc*PAL.

3.2.4. Chemical synthesis of racemic 2-amino-3-(hetero)aryl propionic acids and the corresponding (*E*)-acrylic acids

The chemical synthesis of aryl- and heteroaryl α-alanines [28] and their acrylic acid counterparts is outlined in Scheme 3. The aldehydes **13b-f** were reduced with $NaBH_4$ to the corresponding alcohols **14b-f**, which were further transformed into chloromethylene derivatives **15b-f** using thionyl chloride and benzotriazole in CH_2Cl_2. The use of 1*H*-benzotriazole overcame the destruction of acid-sensitive compounds. The coupling of the halogenated compounds with diethyl-2-acetamido-malonate afforded diethyl-2-acetamido-2-((aryl)methyl)-malonates 1**6b-f**. By mild basic hydrolysis of the diethyl esters, followed by decarboxylation in boiling toluene, racemic 2-acetamido-3-(hetero)aryl-propionic acids *rac*-1**7b-f** were obtained. The *N*-protected α-amino acids were deprotected by acidic hydrolysis in 1,4-dioxane.

(*E*)-Acrylic acids (*E*)-**19b-f** were obtained *via* slightly modified Knoevenagel condensation of aldehydes **13b-f** and malonic acid in pyridine (Scheme 3).



I. $NaBH_4$, MeOH, r.t; **II.** $SOCl_2$, 1*H*-benzotriazole, CH_2Cl_2, r.t; **III.** NaH, $CH_3CONH(CO_2Et)_2$, DMF, 60°C; **IV.** a)10% NaOH, MeOH, reflux; b) toluene, reflux; **V.** 18% HCl, 1,4-Dioxane, reflux; **VI.** $CH_3(COOH)_2$, pyridine, reflux, 80°C

Scheme 3. Chemical synthesis of (±)-2-amino-3-(hetero)aryl propionic acids (±)-**18b-f** and (*E*)-3-(hetero)aryl acrylic acids (*E*)-**19b-f**

3.2.5. Biotransformations in Magne-Chip

Biotransformations were studied in a MNP reactor microchip containing four reaction chambers (Figure 1).

The microfluidic test bench is equipped with two precision syringe pumps (for substrate solution and for washing buffer), stereo zoom microscope with digital camera for the optical inspection of the chip, deuterium-halogen light source, spectrometer, Z-flow absorbance cell and circulating thermostat. Overpressure was measured by MPX4250 pressure sensor mounted on the syringe (Figure 1). The system was operated by the µFLU Studio software (developed for the Magne-Chip platform), which controlled the pumps, valves, and the thermostat, inspected the camera picture of the chip, acquired the data of the chip sensors (e.g., inlet pressure), and collected the data of the spectrometer.

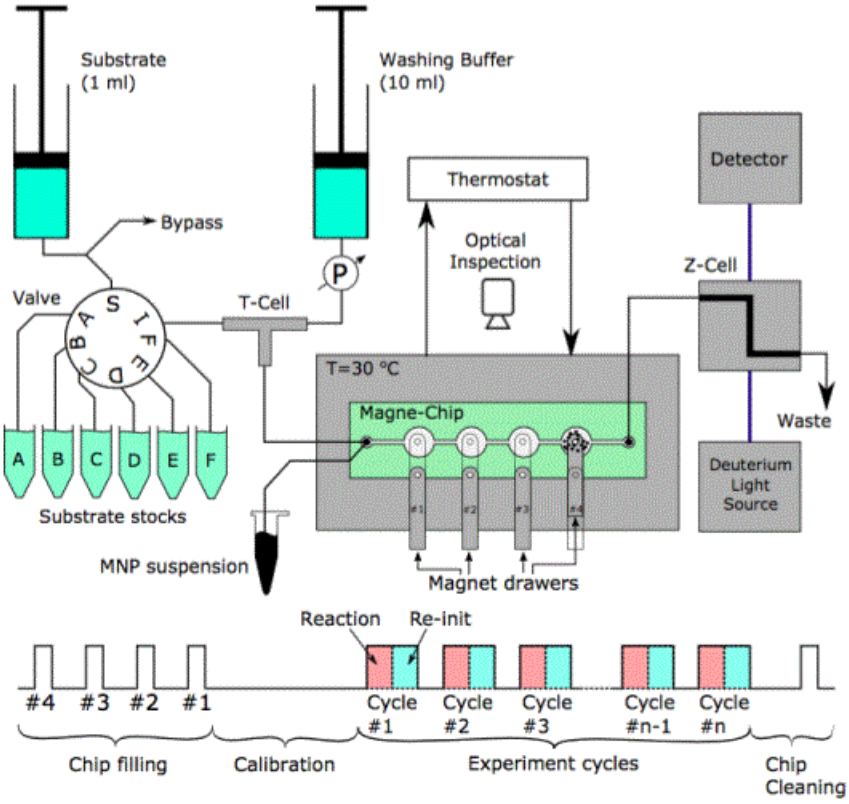
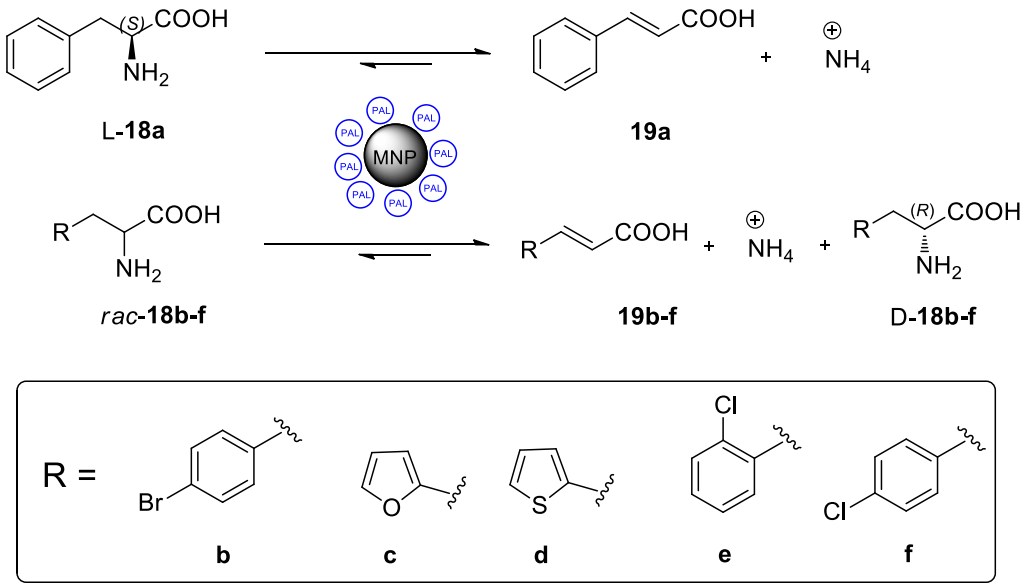


Figure 1. Schematic diagram of the microfluidic system

The experiments in Magne-Chip reactor include four steps: (**1**) Filling up the chip with magnetic nanoparticles (MNPs); (**2**) Absorbance calibration; (**3**) Experiment cycles; and (**4**) Chip cleaning. Each experiment cycle (Figure 1, "Experiment cycles") involves a reaction step and a reinitialization step. Cycles are repeated several times according to the predefined measurement sequence.

As the accessible information regarding Magne-Chip system is quite incomprehensive so far, the reliability and reproducibility assessment of the measurements was performed on the natural substrate of *Pc*PAL, L-phenylalanine (L-**18a**). Substrate concentration and flow rate were studied and optimized in the *Pc*PAL-MNP-catalysed ammonia elimination reaction using L-**18a** as model compound.

The conditions obtained herein were expanded to the biotransformation of five unnatural phenylalanine analogues (*rac*-**18b–f**, Scheme 4) and the biocatalytic activity was compared to the biocatalytic activity of L-**18a**.



Scheme 4. Ammonia elimination from different amino acids catalysed by *Pc*PAL immobilized on MNPs

3.2.5.1. Reliability assessment of the measurements

A series of subsequent measurements fulfilled by the system can be considered reliable if all the following conditions are valid:

- the product of the enzymatic reaction can be measured selectively in the ultraviolet–visible (UV–vis) range;
- the product and substrate can be completely removed during the washing steps;
- the enzymatic activity of the *Pc*PAL-MNP conjugate is preserved during the measurement;
- the MNP layer in the magnetic reactors remain unimpaired during the measurement cycles.

Therefore, a control measurement was carried out after each series of experiments, i.e., the first step of the sequence was repeated in the last step under the same conditions and the specific activity of the biocatalyst (U_B) at saturation concentrations of L-**18a** was compared. During the experiments, the chip was optically monitored by a zooming microscope and a monochrome high-speed smart camera. The plan view of the chip was used as a reference (Π_{ref}) before assessing the measurement sequence. At the end of the step *i*, the plan view of the chip was recorded again ($\Pi_{seq,i}$) and compared to the reference as follows:

$$\Pi_{diff}(j,k) = \begin{cases} \Pi_{ref}(j,k), & \Pi_{ref}(j,k) - \Pi_{seq,i}(j,k) < 0 \\ 0, & \Pi_{ref}(j,k) - \Pi_{seq,i}(j,k) \geq 0 \end{cases}$$

where (*j*, *k*) are the pixel coordinates of the plan view image.

Thus, the changes compared to the reference image are indicated by white pixels. The total sum of white pixels defines the cell difference score (SC) used as an indicator for describing the changes of

the nanoparticle layer arrangement. Hence, the changes compared to the reference image (that of the first cycle) were highlighted by white areas during the consecutive cycles.

The 3D model of a single reaction cell was also analysed. In a well-defined fraction of volume, porous cell zone conditions were applied to model the space occupied by MNPs. Pressure drop values were monitored at flow rates between 10 µL/min and 80 µL/min with and without MNPs in the chambers. Flow specific pressure drop values were 0.086 kPa/µL/min and 0.059 kPa/µL/min, respectively. As the flow in the channels is laminar, the measured pressure is proportional to the velocity:

$$grad\ p = -\mu D v$$

where p is the measured pressure, μ is the dynamic viscosity of the fluid, v is the flow rate (at the inlet) and D is the viscous resistance of the MNP layer. Using the parameters of water from the software's material database,[40] it was found that the viscous resistance $R_D=2.071\times10^{10}/m^2$. Computational fluid dynamics (CFD) results are presented in Figure 2c and d. In the first example, the chamber is fully filled with the nanoparticle layer, while, in the second case, the MNP layer is partially destroyed by the passage of an air bubble.

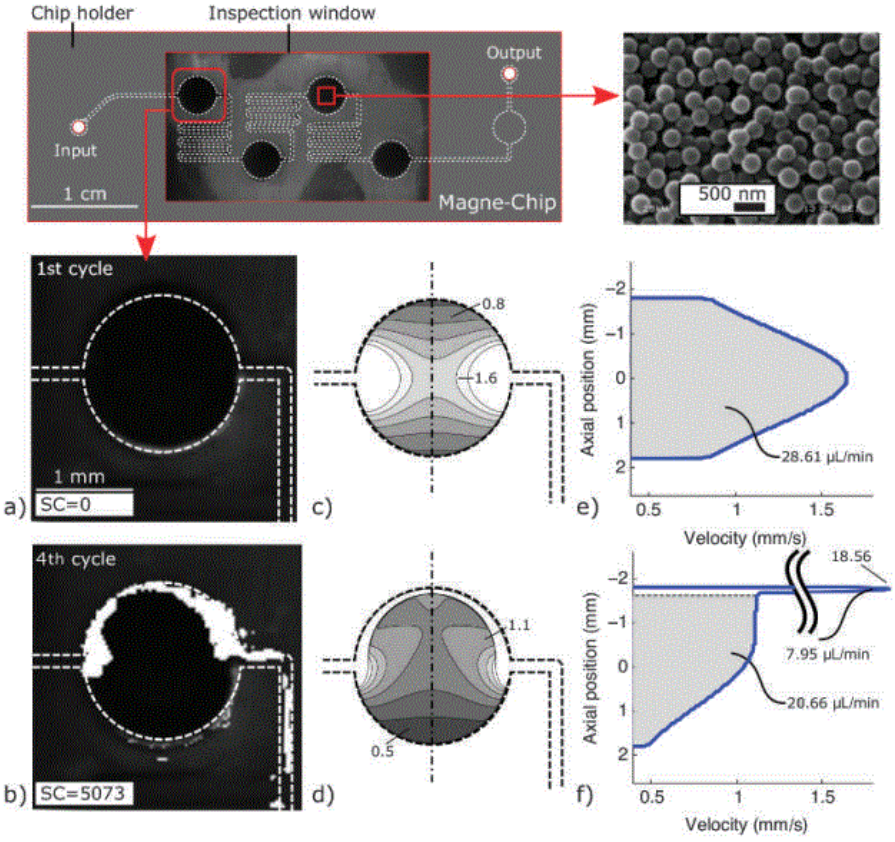


Figure 2. Magne-Chip device with four MNP-filled and external magnet-equipped microchambers (top left) and SEM image of the MNP layer (top right). a)–f): The effect of air bubble passage through the reaction cell: a) photograph, before passage; b) difference image (difference score SC= 5073), after passage; c) calculated flow velocity field before and d) after the passage; e) velocity profile in the middle cross section of the chamber before and f) after the passage

3.2.5.2. Reproducibility assessment of the measurements

Firstly, suspension homogeneity and the effect of filling the cells were determined. Biotransformation of L-phenylalanine (L-**18a**) to (E)-cinnamic acid (**19a**) by MNP biocatalyst (Scheme 4) was carried out in shake vial as three parallel reactions insuring that the homogeneity of the MNP suspension was adequate. The first chamber of the Magne-Chip reactor was filled with MNP suspension. Biotransformation of L-**18a** to **19a** was carried out in flow-through mode and monitored by on-line UV–vis. After reaching the stationary state, the magnet of the chamber was removed and the MNPs were fixed in the next chamber. The experiments were performed in three consecutive chambers (repeated three times in each chamber).

As far as the reinitialization of the chip and reusability of the biocatalyst are concerned, biotranformation of L-**18a** to **19a** was performed in 7 consecutive cycles while the chip was reinitialized during each step by washing out the substrate and the product. The concentration change of the cinnamic acid monitored at the specific wavelength (290 nm, Figure 3) demonstrated that the chip was successfully reinitialized in every cycle and the same level of conversion was reached throughout the experiments. The product quantity (*P*) was calculated in each cycle.

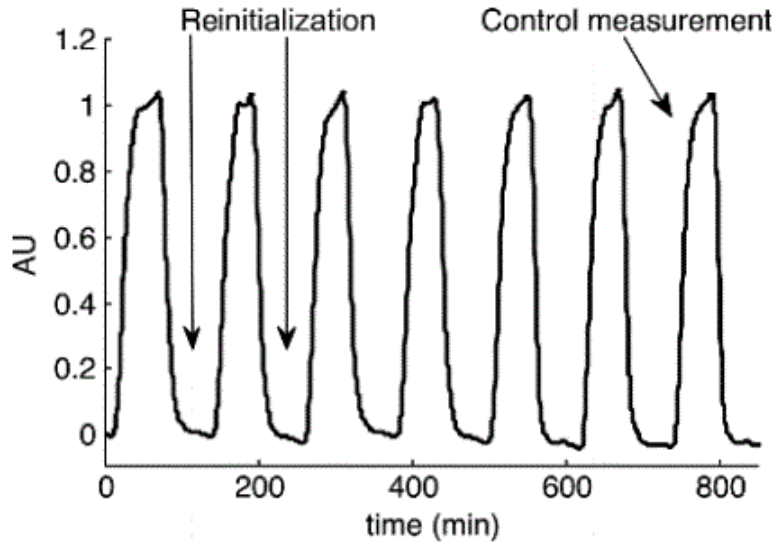


Figure 3. Time plot of the periodic absorbance change during the measurements. Reinitialization was performed between reaction steps (zero absorbance)

*3.2.5.3. Influence of the flow rate and substrate concentration on the biotransformation of L-**18a***

Biotransformations of L-**18a** at various flow rates were performed in Magne-Chip filled with *Pc*PAL-MNP in 7 consecutive cycles, while the chip was reinitialized after each cycle and a new substrate flow rate was set (started from 3.6 μL/min and increased up to 28.6 μL/min). The first measurement was repeated in the last cycle as a control.

The biotransformation of L-**18a** was also performed at various concentrations of L-**18a** [S_0] in 10 consecutive cycles, while the chip was reinitialized after each cycle and a new substrate concentration was set (Figure 4A). The linear fitting method proposed by Lilly, et al.[41] was used for the calculation of the kinetic constants (Figure 4B).

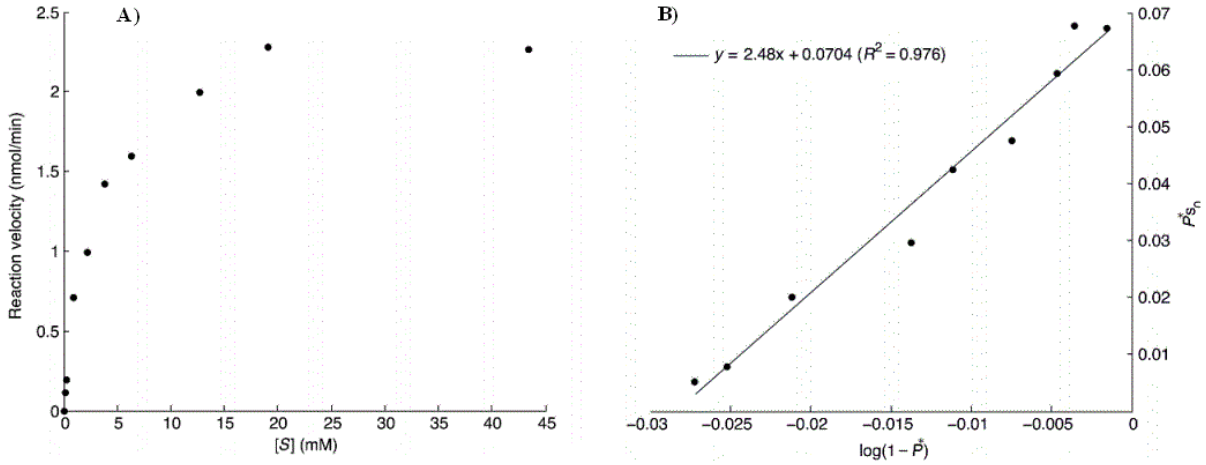


Figure 4. A) Dependency of reaction velocity on the substrate concentration on reaction velocity in Magne-Chip for the transformation of L-**18a** by MNP biocatalyst; **B)** Linear fit based on the Lilly–Hornby model to determine K_m (resulting in K_m=2.5 mM)

3.2.6. Substrate Screening with MNP Biocatalyst in the Magne-Chip System

The *Pc*PAL-MNP catalysed biocatalytic procedure previously elaborated using L-phenylalanine (L-**18a**) as model compound was expanded to five different phenylalanine analogues (*rac*-**18b–f**, Scheme 4). For the substrate screening, the same stock of biocatalyst was used as for the previous experiments. First, the extinction coefficients (ε) of the elimination products **19a–f** were determined at wavelengths where the absorbance is the highest, while that of the amino acid *rac*-**18b–f** is minimal (Table 1).

Table 1. Extinction coefficients of acrylic acids **19a-f**

Acrylic acid	Wavelength (nm)	Extinction coefficient (/M/cm)	Linear regression coefficient
19a	290	8800	0.991
19b	300	10200	0.998
19c	300	7919	0.988
19d	280	14721	0.993
19e	280	9172	0.991
19f	280	15327	0.998

During the substrate screening experiments, solutions of tested amino acids (L-**18a** and *rac*-**18b–f**) were passed through the chip (in single chip loading mode), according to a predefined sequence. The

washing procedure insured complete removal of the substrate or product from the previous cycle (reaction). Ammonia elimination from L-**18a** was chosen as reference for comparison to the other elimination reactions.

3.2.7. Conclusion

A microfluidic device equipped with four microliter volume reaction chambers and filled with *Pc*PAL-coated magnetic nanoparticles as biocatalyst was characterized. In chip cells, the magnetic nanoparticles can be manipulated with the aid of moveable external permanent magnets. The Magne-Chip device could be operated cyclically. The filling procedure and the reactions in the chambers were found highly reproducible. Saturation flow rate and substrate concentration of the deamination of L-**18a** to (*E*)-**19a** were found to be 25 μL/min and 20 mM, respectively. The Magne-Chip system equipped with on-line UV–vis was applied for the automated activity screening of five unnatural substrate of *Pc*PAL (*rac*-**18b–f**) using the same MNP filling. The specific biocatalytic activity of the catalyst with four unnatural substrates (*rac*-**18b,c,e,f**) was higher than with the natural substrate, unlike the similar reactions catalysed by native *Pc*PAL where only the heterocyclic thiophen-2-yl derivative (*rac*-**18d**) had higher specific activity. These results demonstrated that the Magne-Chip device is a reliable, efficient and reproducible tool which was capable of fast and fully automated screening of *Pc*PAL substrates using minimal biocatalyst (~1 mg MNP) and solvent (~500 μL) amounts for a test compound. The volumetric productivity of the *Pc*PAL-MNP biocatalyst in the chip was 3-fold higher than in the shake vial.

3.3. Synthesis of *β*-amino acids

3.3.1. Introduction

Natural and unnatural *β*-amino acids are valuable building blocks of bioactive natural products and pharmaceutically important compounds,[42] thus, their importance for drug research and development is continuously increasing.[43] Optically pure *β*-amino acids are present in the structure of anticancer drugs taxol[44] and bleomycin[45], dolastatins and many others.[46] Furthermore, they are key structural elements of *β*-peptides and peptidomimetic foldamers [21],[22][47] since peptides containing *β*-amino acids in their structure show higher stability against protease-type hydrolases.[48]

A huge variety of chemical and biochemical procedures have been developed for the stereocontrolled, asymmetric synthesis of *β*-amino acids.[49] Ammonia-lyases and aminomutases,[50] hydantoinases,[51] pyrimidine-catabolism enzymes,[52] *β*-transaminases,[53] and *ω*-transaminases[54] have proved efficient biocatalysts for the preparation of enantiopure *β*-amino acids. Lipases, however, became the most widely used biocatalysts due to their stability in organic solvents and their ability to accept a wide range of substrates.[55] Indeed, they catalyse the enantioselective

ring cleavage of *β*-lactams,[56] hydrolysis of *N*-protected- or non-protected *β*-amino esters,[57] *N*-acylation [58] or transesterification reactions of *β*-amino acid derivatives.[59]

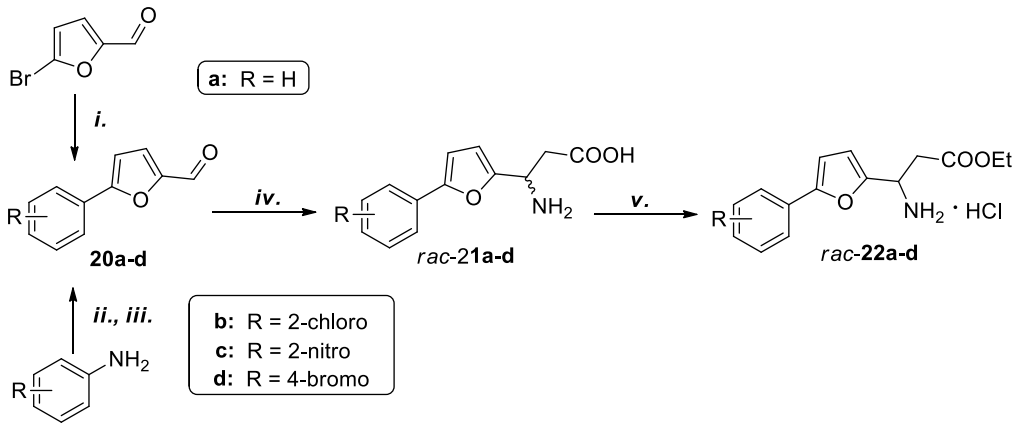
Phenylfuran-2-yl moieties are incorporated as building blocks in the structure of mPGES-1 inhibitors based on dihydropyrimidin-2(1*H*)-one,[60] anthrax lethal factor inhibitors,[61] and CXCR2/CXCR1 receptor antagonists.[62] They also exhibit cytoprotective effects and radical-scavenging activity against oxygenases.[63] These heteroaromatic structures are well-known to our research group. We have already developed highly stereoselective, baker's yeast- or lipase-mediated procedures for the kinetic resolution of racemic 1-(5-phenylfuran-2-yl)ethanols, ethanones and ethane-1,2-diols.[64] More recently, L-(5-phenylfuran-2-yl)-*α*-alanines were also obtained through a sequential multienzyme process.[65]

3.3.2. Aim of the study

The objective of this work comprises the lipase-catalysed resolution of several new, substituted phenylfuran-based *β*-amino esters (*rac*-**22a-d·HCl**) through enantioselective hydrolysis.

3.3.3. Synthesis of 3-amino-3-(5-phenylfuran-2-yl)propionic acid ethyl ester hydrochlorides *rac*-22a-d·HCl

The synthesis of racemic 3-amino-3-(5-phenylfuran-2-yl)propionic acid ethyl ester hydrochlorides (*rac*-**22a-d**·HCl**)** is presented in Scheme 5. 5-Phenylfuran-2-carbaldehyde **20a** was prepared by Suzuki-Miyaura coupling reaction between 5-bromofuran-2-carbaldehyde and phenylboronic acid in the presence of tetrakis(triphenylphosphine)palladium(0). Substituted phenylfuran-derivatives **20b-d**, in turn, were synthesized through Meerwein arylation, coupling furan-2-carbaldehyde with the corresponding aryl-diazonium halides in the presence of catalytic amount of $CuCl_2$. The *β*-amino acids *rac*-**21a-d** were obtained starting from the corresponding aldehydes by modified Rodionov-type reaction. Esterification of the latter compounds was performed with ethanol and thionyl chloride yielding the desired *rac*-**22a-d**·HCl as solid materials.

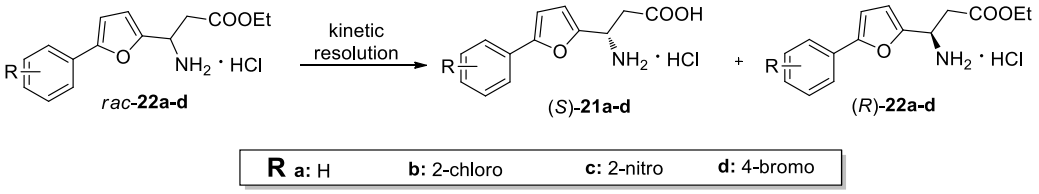


i: Phenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, Toluene/EtOH; *ii:* $NaNO_2$, HCl, 0-5°C; *iii:* 2-Furaldehyde, $CuCl_2$, r.t., overnight; *iv:* Ammonium acetate, $CH_2(COOH)_2$, AcOH, reflux; *v:* $SOCl_2$, EtOH, -10°C.

Scheme 5. Synthesis of racemic 3-amino-3-(5-phenylfuran-2-yl)propionic acid ethyl ester hydrochlorides

3.3.4. Enzymatic kinetic resolution *via* enantioselective hydrolysis of *rac*-22a-d·HCl

It is important to note that the liberation of *rac*-**22a-d** from the synthesized *rac*-**22a-d**·HCl resulted in the complete degradation of compounds, impairing their further chemical transformation or enzymatic kinetic resolution. Therefore, we turned our attention to the lipase-catalysed biotransformation of the more stable racemic phenylfuran-2-yl-β-alanine hydrochlorides *rac*-**15a-d**·HCl. (Scheme 6). To the best of our knowledge, there is no such an approach of lipase-mediated biocatalytic procedures described in the literature.



Scheme 6. Lipase-catalysed enantioselective hydrolysis of (±)-**22a-d**

First, an enzyme screening in toluene containing 0.5 equiv. of water was performed, using *rac*-**22a**·HCl as model compound. Triethylamine (TEA, 2-10 equiv.) was also added into the reaction mixture for the *in-situ* deprotonation of *rac*-**22a**·HCl. Various enzymes such as lipase PS from *Burkholderia cepacia* immobilized on diatomaceous earth (LPS-Diat), lipase AK from *Pseudomonas fluorescens* immobilized on Celite (LAK-Cel), lipase A from *Candida antarctica* immobilized on Celite, lipase B from *Candida antarctica* immobilized by adsorption on hydrophobic acrylic resin (Novozyme 435), lipase from *Mucor miehei*, pancreatic porcine lipase PPL, and lipase from *Candida rugosa* (CRL) were tested at 45 °C for the kinetic resolution of the *in-situ* released *rac*-**22a**. While lipase A from *Candida antarctica* and lipase from *Mucor miehei* proved to be inactive, PPL, Novozyme 435 and CRL showed some activities but very low enantioselectivities. LAK-Cel (4.8 % w/w protein content) displayed satisfactory activity and moderate enantioselectivity (Table 2, Entry 2),

but excellent enantioselectivities (*E* » 200) were observed for LPS-Diat (6.7 % w/w protein content, Table 2, Entry 1).

Table 2. Preliminary results for the enzymatic hydrolysis of β-amino esters (±)-**22a-d**

Entry	Substrate[a]	Enzyme[a]	Solvent[a]	T (°C)	Time (h)	ee_s[c] (%)	ee_p[c] (%)	Conversion (%)	*E*	Specific enzyme activity $\mu mol_{substrate}/(mg_{protein} \times min)$
1	*rac*-**22a**	LPS-Diat	toluene[b]	45	7	90	99	48	»200	8.6×10^{-3}
2	*rac*-**22a**	LAK-Cel	toluene[b]	45	11	25	99	20	254	3.2×10^{-3}
3	*rac*-**22a**	LPS-Diat	MTBE[b]	45	1.5	99	27	78	7	65×10^{-3}
4	*rac*-**22a**	LPS-Diat	DIIPE[b]	45	1.5	99	45	69	12	57.5×10^{-3}
5	*rac*-**22a**	LPS-Diat	H_2O	45	1	95	95	50	146	62.5×10^{-3}
6	*rac*-**22a**	LPS-Diat	H_2O	30	2	96	99	49	»200	30.6×10^{-3}
7	*rac*-**22b**	LAK-Cel	H_2O	30	4	83	96	47	102	20.4×10^{-3}
8	*rac*-**22c**	LAK-Cel	H_2O	30	8	40	78	34	12	7.4×10^{-3}
9	*rac*-**15d**	LAK-Cel	H_2O	30	8	25	85	22	16	4.8×10^{-3}

[a] 0.015 mmol/mL substrate, 30 mg/mL enzyme preparation, 1 mL solvent. [b] 5 equiv. TEA and 0.5 equiv. H_2O as reactant, [c] Determined through chiral HPLC

Bidistilled water and several organic solvents such as diisopropyl ether (DIPE), methyl *tert*-butyl ether (MTBE), 2-methyltetrahydrofuran and 1,4-dioxane were investigated as potential reaction media. While excellent reaction rates and very low enantioselectivities were observed in acyclic ethers (MTBE, DIPE, Table 2, entries 3 and 4), no catalytic activities were found in cyclic ethers. Although the biocatalytically formed (*S*)-**21a** precipitated as hydrochloride (*S*)-**21a**·HCl, due to the multiple heterogeneity of the reaction mixture, the reproducibility of the analytical-scale enzymatic reactions was unsatisfactory. Promisisng results were obtained in water. Nevertheless, it should be noted that LPS-Diat and LAK-Cel displayed poor and continually decreasing activities towards substituted derivatives (*rac*)-**22b-d**·HCl. This significant diminution in the enzyme activity can be attributed to the structural instability of the enzyme -preparations. LAK and LPS adsorbed physically on the surface of celite or diatomite are susceptible to desorption in aqueous media. Water-soluble proteins were spectrophotometric detected in the reaction mixtures (Bradford method). Moreover, free LAK and LPS tested in small-scale experiments proved to be completely inactive.

In order to improve the stability, LPS and LAK were covalently immobilized on Immobead IB-150P (IM). This is a hydrophobic methacrylate copolymers support bearing oxirane groups on its surface with an average particle size of 0.15-0.30 mm. Multipoint covalent attachment of the enzyme

free amino groups on the epoxy support groups ensures high stability of the enzyme preparation. The obtained LPS-IM and LAK-IM biocatalysts (both with 9.4% protein content) proved to be highly stereoselective and stable. Small-scale reusability assessments revealed that both biocatalysts retained more than 90% of their initial activity even after 12 reaction cycles.

It is well-known that enzyme activity, selectivity and stability are strongly influenced by the pH and the ionic strength of the solution.[66] Therefore, in order to ameliorate the operational parameters of LPS-IM and LAK-IM various phosphate-citrate and acetate buffers at different concentration (20-500 mM) and pH range (4-6) were tested. It is important to remark that above pH 6, deprotonation of (*rac*)-**22a-d**·HCl and the decomposition of the released (*rac*)-**22a-d** were observed.

Table 3. LPS- and AK-catalysed hydrolysis of substituted 5-phenylfuran-*β*-amino esters (*rac*)-**22a-d**

Entry	Substrate	Enzyme[b]	Time (h)	ee_s[b] (%)	ee_p[b] (%)	Conversion (%)	*E*	Specific enzyme activity $\mu mol_{substrate}/(mg_{protein} \times min)$
1	*rac*-**22a**	LPS-IM	6	99	95	51	206	75.4×10^{-3}
2	*rac*-**22b**	LAK-IM	6	99	93	51	145	75.4×10^{-3}
3	*rac*-**22b**	LPS-IM	2	99	94	51	170	226.1×10^{-3}
4	*rac*-**22c**	LAK-IM	6	65	93	41	54	60.6×10^{-3}
5	*rac*-**22c**	LPS-IM	6	72	88	45	34	66.5×10^{-3}
6	*rac*-**22d**	LAK-IM	9	99	95	51	206	50.2×10^{-3}
7	*rac*-**22d**	LPS-IM	6	99	95	51	206	75.4×10^{-3}

[a] 0.015 mmol/mL substrate, 3 mg enzyme preparation/mL, 20 mM NH_4OAc pH 5.8, 30 °C

[b] Determined through chiral HPLC

Enzymatic reaction rates calculated as $\mu mol_{substrate}/(mg_{protein} \times min)$[67] indicated a slightly increase of enzymatic activity for covalently immobilized enzyme preparations (LPS-IM and LAK-IM) compared to physically adsorbed biocatalysts (LPS-IM and LAK-Cel), as shown in Tables 2 and 3. Nevertheless, the low stability of LAK-Cel and LPS-Diat in water diminished their efficacy for preparative scale biotransformation of (*rac*)-**22a-d**. Excepting the *ortho*-nitro derivative (*rac*)-**22c**, LAK-IM provides high selectivities, enantiomer excesses and conversions for all tested compounds (Table 3, entries 2, 4 and 6 *vs* Table 2, entries 2, 7, 8 and 9). LPS-IM also afforded excellent activities towards substituted 5-phenylfuran-2-yl *β*-amino esters (*rac*)-**22b-d**·HCl in comparison with the LPS-Diat.

3.3.5. Preparative-scale biotransformations of compounds (*rac)*-22a-d·HCl

Preparative-scale hydrolysis of (*rac*)-**22a-d**·HCl were performed under the optimal conditions developed earlier for small scale biotransformation. The reactions were stopped by removing the biocatalyst through filtration. The unreacted (*R*)-**22a-d**·HCl and the formed (*S*)-**21a-d**·HCl were separated using preparative C18 HPLC achiral column and characterized as hydrochloride salt. As an exception, the isolation of compound (*R*)-**21a** failed. For stability reasons, (*R*)-**22a-d**·HCl esters were hydrolysed by heating in aqueous hydrochloric acid solution and characterized as amino acid hydrochloride salts (*R*)-**21a-d**.

3.3.6. The absolute configuration of the resolution products

The absolute configuration of the novel *β*-amino ester enantiomer (+)-**22d** was determined by ^{1}H NMR study of the diastereomers formed with (*S*)- and (*R*)-Mosher acids (MTPA). Therefore, the unreacted ethyl 3-amino-3-[5-(4-bromophenyl)furan-2-yl]-propanoate (+)-**22d** was *N*-acylated with (*S*)- and (*R*)-MTPA chloride and the resulting diastereomers were distinguished by their ^{1}H NMR spectra.

3.3.7. Conclusions

A chemo-enzymatic method for the preparation of four novel, exotic phenylfuran-2-yl-*β*-alanine ethyl esters was developed. The high instability of the esters and their poor solubility in organic solvents was overcome by the lipase-catalysed enantioselective hydrolysis in aqueous media of their hydrochloride salt. The covalently immobilized lipases LPS-IM and LAK-IM proved to be excellent, (*S*)-selective biocatalysts for the enantioselective hydrolysis of (*rac*)-**22a-d**·HCl in ammonium acetate buffer (20 mM, pH 5.8) at 30 °C with high enantioselectivities (E >145), leading to (*R*)-**22b-d**·HCl (*ee* > 99%) and (*S*)-**21a-d**·HCl (*ee* = 93%). The stable hydrochloride salts of (*rac*)-**22a-d** were reliable substrates for stereoselective biocatalytic hydrolysis in aqueous media, offering new possibilities for enzymatic kinetic resolution of other unstable racemates.

4. General conclusions

The present thesis deals with the development of new chemo-enzymatic procedures for the synthesis of some valuable, pharmaceutically important aryl- and heteroaryl- amino-containing compounds, both in batch mode and continuous-flow reactor. Lipase-catalysed and phenylalanine ammonia-lyase-mediated biotransformations combined with conventional synthetic strategies led to the preparation of the *β*-adrenoceptor antagonist bufuralol and several (hetero)aromatic *α*- and *β*-amino acids used as building blocks of biologically active products.

Both enantiomer of 2-(*tert*-butylamino)-1-(7-ethylbenzofuran-2-yl)ethanol ((*R*)- and (*S*)-bufuralol) were also obtained in enantiomerically enriched forms *via* baker's yeast-mediated chemo-enzymatic procedure. By selecting the appropriate prochiral substrate for the stereoselective step, both enantiomers of the corresponding 1,2-diol intermediate can be prepared separately and transformed, ultimately, into the corresponding bufuralol stereoisomer.

Another different biocatalytic approach was developed for the synthesis of (*R*)- and (*S*)-bufuralol, involving as key stereoselective step a lipase-catalysed enantioselective *O*-acylation. Both *O*-acylated and unreacted enantiomer can be used further for the synthesis of the appropriate bufuralol isomer.

The phenylalanine ammonia-lyase-mediated biotransformation of L-phenylalanine and its five unnatural analogues was performed in a microfluidic Magne-Chip system. The enzyme immobilized on magnetic nanoparticles proved to be a highly stable biocatalyst which can be easily handled with an external magnet. It was demonstrated that the Magne-Chip device is an efficient and reproducible platform for the stereoselective kinetic resolution of *α*-amino acids.

Newly synthesized phenylfuran-2-yl-*β*-alanine ethyl esters were resolved, for the first time, in their hydrochloric salt forms through enantioselective hydrolysis mediated by lipases from *Pseudomonas* species, affording the enantiopure *β*-amino acids and *β*-amino esters as resolution products. Covalent immobilization of these enzymes significantly enhances their activity and stability in aqueous media.

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7. List of publication

The present thesis is based on the following publications:

1. **B. Nagy**, N. Dima, C. Paizs, J. Brem, F.D. Irimie, M.I. Toșa: New chemo-enzymatic approaches for the synthesis of (*R)- a*nd (*S)* -bufuralol, *Tetrahedron:Asymmetry* **2014**, 25, 1316-1322, I.F. = 2.155

2. F. Ender, D. Weiser, **B. Nagy**, C.L. Bencze, C. Paizs, P. Pálovics, L. Poppe: Microfluidic multiple cell chip reactor filled with enzyme-coated magnetic nanoparticles – An efficient and flexible novel tool for enzyme catalyzed biotransformations, *Journal of Flow Chemistry*, **2016**, 6(1), 43-52, I.F. = 1.942

3. **B. Nagy**, Z. Galla, L.C. Bencze, M.I. Toșa, C. Paizs, E. Forró, F. Fülöp, Covalently immobilized lipases are efficient stereoselective catalysts for the kinetic resolution of novel *rac*-(5-phenylfuran-2-yl)-β-alanine-ethyl ester hydrochlorides, *European Journal of Organic Chemistry*, **2017**, 20, 2878-2882, I.F. = 3.068

Conference presentations

1. **B. Nagy**, Z. Galla, C. Paizs, E. Forró, F. Fülöp, Synthesis and enzymatic kinetic resolution of novel 3-amino-3-(5-phenylfuran-2-yl)propanoic acid ethyl esters, *International Symposium on Synthesis and Catalysis*, September 2^{nd} – 4^{th}, 2015, Evora, Portugal

2. **B. Nagy**, N. Dima, M.I. Toșa, C. Paizs, F.D. Irimie, Alternative Approaches for the Enantioselective Synthesis of (*S*)- and (*R*)-bufuralol, 13^{th} International Symposium and Summer School on Bioanalysis, June 27^{th} – July 7^{th}, 2013, Debrecen, Hungary

Scientific activities unrelated to the thesis

Scientific publications

- B. Nagy , M.E. Moisă, A. Filip, L.C. Bencze, M.I. Toșa, Lipase catalyzed parallel kinetic resolution of ibuprofen, *Studia Universitatis Babes Bolyai Chemia*, LXI, **2016**, 4, 255-266
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Conference presentations

- COST Action CM1303 "SysBiocat" Training School, **2016**, Certosa di Pontignano, Italy, poster
- 29th *Annual Symposium of The Protein Society*, **2015**, Barcelona, Spain, poster
- 14th *International Symposium and Summer School on Bioanalysis*, **2014**, Bratislava-Smolenice, Slovakia, poster
- COST Action CM1303 "SysBiocat" Training School, **2014**, Certosa di Pontignano, Italy, poster
- 4th *International Conference on Novel Enzymes*, **2014**, Ghent, Belgium, poster
- *PhD Conference*, **2014**, Szeged, Hungary, oral presentation
- *19th. International Conference on Chemistry*, **2013**, Baia Mare, Romania, oral presentation
- Biostruct course on basics in protein crystallization and crystallography,**2013**, Budapest, Hungary, oral presentation

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