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XXXI CICLO

DEVELOPMENT OF CONTINUOUS-FLOW PROCESSES FOR REDOX BIOCATALYSIS AND APPLICATION IN THE CHEMO-ENZYMATIC SYNTHESIS OF ACTIVE PHARMACEUTICAL INGREDIENTS

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ABSTRACT

The present thesis has the aim of develop and find new and more environmental friendly synthetic routes for the synthesis of active pharmaceutical ingredients (APIs) and pharmaceutically interesting intermediates, exploiting the advantages of the combination between flow chemistry and biocatalysis. Indeed, biocatalytic processes in continuous flow reactors have attracted attention in recent years for carrying out continuous manufacturing systems with high level of intensification. Flow processing has the potential to accelerate heterogeneous biotransformations due to biocatalyst high local concentration and enhanced mass transfer, making large-scale production more economically feasible in significantly smaller equipment with a substantial decrease in reaction time, from hours to a few minutes, and improvement in space–time yield, with increases of up to 650-fold as compared to batch processes. Moreover, biocatalyst stability is enhanced by working in an environment where harsh mixing is avoided. Overall, these features result in reduced inventory, waste and energy requirements of the flow biocatalytic process, as compared to the conventional batch mode.

In particular, I focused my attention on redox reactions, since for these the traditional chemical procedures and reagents are far from being sustainable and environmental friendly. For example, for oxidative reactions the most used chemical reactives are Chromium VI (a well known cancerogenic agent), Dess-Martin periodinane (a potential explosive reagent) and the Swern reagent, a thiol based compound that produces dimethyl sulphide as co-product. Moreover, the traditional chemical methods are not able to reach the selectivity and specificity that is possible to achieve with biocatalytical systems.

Briefly, the projects I was involved in during my PhD and that are present in the thesis are:

1. Development of a new synthetic route to obtain Captopril, using both chemical and biocatalyzed reactions and exploiting the advantages of flow chemistry, that allows to perform continuous synthesis;
2. Development of a flow based biocatalyzed oxidation with immobilized whole cells of *Acetobacter aceti* in order to obtain enantiomerically pure mono-carboxylic acids, starting from the corresponding diols;
3. Stereoselective reduction of ketones and di-ketones, in order to obtain enantiomerically pure mono-alcohol products, using together two enzymes (ketoreductase from *Pichia glucozyma* and a glucodehydrogenase from *Bacillus megaterium*) in a Flow Chemistry pcked bed reactor;
4. Stereoselective reduction of 2,2-disubstituted 1,3-cyclopenta- and 1,3 cyclohexanediones using both whole cells and a purified ketoreductase from *Pichia glucozyma*, to obtain enantiomerically pure mono-alcohols products, that can be important intermediates in the synthesis of various steroids.
5. Use of an immobilized transaminase from *Halomonas elongata* able to perform transaminations in both directions (from amine to aldehydes, and from aldehydes to amine) with the Flow Reactor technology;

To reach the goal, I both used whole cells and purified enzymes as biocatalysts, either in a free or immobilized form, and I exploited many advantages of continuous flow technology, as for example

downstream processes (*i.e.*, in-line acidifications, extractions and purifications) that allowed me to in-line purify the products, thus avoiding the traditional work-up procedures, reducing the operational times and the amount of organic solvents used.
In almost all cases, important results were achieved, as faster kinetics, cleaner procedures that required less purification steps, complete stereo- and regioselectivity, higher conversions and productivities compared to batch procedures, increased stability of the biocatalyst, that could be used for several cycles, thus reducing the waste.

INTRODUCTION

Enabling techniques

In the next years, the entire world will face environmental, social and economic challenges. The demand of new healthy products will increase all over the world. Indeed, poor countries will need more and more drugs to win mortal diseases, the entire population will increase and the sustainability of our resources will be under danger. Consequently, many efforts are currently directed to the development of innovative, solid and, in view of an industrial application, easily scalable routes to produce commodities, high-value chemicals and active pharmaceutical ingredients (APIs), in a safer, more economic and more sustainable way. For these reasons, synthetic chemists are asked to find different and innovative solutions that can be positive answers to these problems. In particular, pharmaceutical chemists are trying to find different synthetic ways that can be interesting for the industries, easily scalable and environmentally friendly to produce and deliver active pharmaceutical ingredients in a faster way[1]. In this context, an answer to these problems has been the introduction of enabling techniques to speed up reactions and to simplify work-up and purification procedures. Enabling techniques include various traditional but also new techniques, as:

- Catalysis – is a technique, not always considered an enabling one, which modifies the rate of a reaction, usually accelerating it, by the addition of a substance not consumed during the reaction itself. Since the catalyst is not consumed, each catalyst induces the transformation of many molecules of reactant. There are several types of catalysis, such as biocatalysis (that is already an established field in terms of industrial applications and I will particularly focus on it later), organocatalysis, in both cases also in the form of solid supported catalysis. Solid-supported biocatalysts can be used for asymmetric and stereoselective transformations and the product can be easily separated from the catalyst by simple filtration.
- Solid phase assisted synthesis – is commonly used for automated peptide synthesis and greatly simplifies purification procedures. An alternative is the solid-phase assisted solution-phase synthesis using immobilized reagents or catalysts that allow, in addition to the simple purification, to drive the solution-phase reactions to completion thanks to the use of immobilized reagents in excess.
- New solvent systems – for example ionic liquids, have been created to separate the reagent from the catalyst, so that the reagent stays in a solvent, and the catalyst in another one. This can allow multiphase reactions, with the possibility to easily isolate and recover the used catalysts. There are also indications that switching from a normal organic solvent to an ionic liquid can lead to novel and unusual chemical reactivity.
- Microwave assisted organic synthesis – generally, provides a considerable increase of the reaction rates, promoted by focused heating, associated with higher yields and purities.
- New reactor systems such as flow reactor technology (which will be deeply discussed later).

It is possible to combine two or more enabling techniques to get advantages by both. Anyway, each reaction has its best combination, which must be determined and optimized[2] (Figure 1).

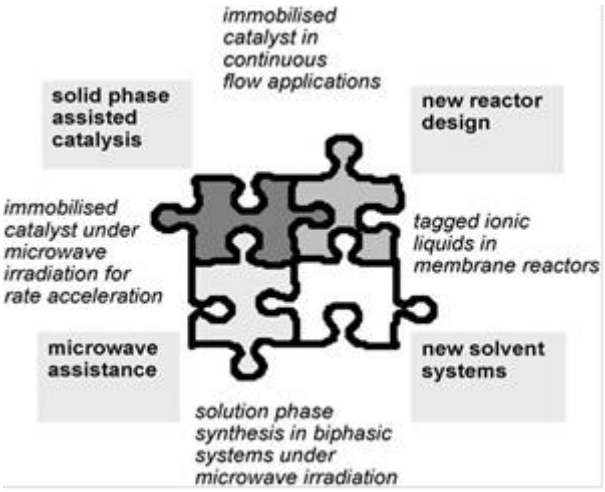


Figure 1. Enabling techniques and some proposed combinations (adapted from ref. 2)

Green chemistry

Chemistry has a great impact on earth and human's health. Chemists should keep in mind that row materials are not unlimited: more than 98% of the organic chemicals currently used are derived from petroleum. In addition, it was estimated that between 25 to 100 kg of waste result from every kilogram of pharmaceutical synthesized.[3] It is not possible for the chemical industry to ignore the devastating impact on the environment that is caused by the traditional organic synthesis. In this context, in the recent years, the concept of "green chemistry" has been introduced. Green chemistry consists in the design of chemical products and processes that reduce or eliminate the generation of hazardous and polluting substances. The introduction of this revolutionary and diverse discipline will lead to significant environmental benefits, innovation and a strengthened economy[3]. Developed by Paul Anastas and John Warner[4], the following list (Figure 2) outlines an early conception of what would make a greener chemical, process, or product

Figure 2. The twelve principles of green chemistry.

In the last fifteen years the concept of green chemistry has been widely embraced in both industrial and academic circles. The essence of the green chemistry concept can be summarized in this sentence: "*Green chemistry efficiently utilises (preferably renewable) raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products"*.[5] It is important to underline that there is no absolute greenness, one process is greener than another process, but appropriate green metrics are a prerequisite for a meaningful comparison of greenness. The most widely accepted measures of the environmental impact of chemical processes are the two most simple metrics: the E factor, defined as the mass ratio of waste to desired product[5], and the atom economy, defined as the molecular weight of the desired product divided by the sum of the molecular weights of all substances produced in the stoichiometric equation, expressed as a percentage.[5] So, a knowledge of the stoichiometric equation allows one to predict, without performing any experiments, the theoretical amount of waste that can be expected. Atom economy (AE), introduced by Trost in 1991 has become the widely accepted terminology and it is a theoretical number that is based on a chemical yield of 100% of theoretical and assumes that reactants are used in exactly stoichiometric amounts. It disregards substances, such as solvent and acids or bases used in work-up, which do not appear in the stoichiometric equation. The E factor, in contrast, is the actual amount of waste produced in the process, defined as everything but the desired product. It takes the chemical yield into account and includes all reagents, solvents losses, all process aids and, in principle, even the energy required as this generates waste in the form of carbon dioxide. A higher E factor means more waste and, consequently, greater negative environmental impact. The ideal E factor is zero. It can be easily calculated from a knowledge of the number of tons of raw materials purchased and the number of tons of product sold, for a particular product or a production site or even a whole company (Figure 3). [5]

$$\mathrm{E-factor} = \frac{\text{total waste}}{\text{product}}$$

Figure 3. Equation of E-factor: it is the ratio of the mass of waste per mass of product.[5]

It is clear that the E factor increases substantially on-going downstream from bulk chemicals to fine chemicals and pharmaceuticals. In fact, firstly there is a more widespread use of stoichiometric reagents in these industry segments, and secondly it is a consequence of the fact that the target pharmaceuticals, for example, are more complicated molecules compared to bulk chemicals and, hence, their production involves multi-step syntheses which can be expected to generate more waste.

Until this point, only the amount of the waste has been taken into account. However, the environmental impact of waste is not only determined by its amount but also by its nature. In order to consider this issue, the term environmental quotient, EQ, was introduced by Sheldon et al, and it is obtained by multiplying the E factor with an arbitrarily assigned value, Q, called environmental hazardous quotient.[5] For example, one could arbitrarily assign a Q value of 1 to NaCl and, say, 100–1000 to a heavy metal salt, such as chromium, depending on its toxicity, ease of recycling, etc. The magnitude of Q is obviously debatable and difficult to quantify but, importantly, 'quantitative assessment' of the environmental impact of chemical processes is, in principle, possible. Also, the Q value of a particular waste should take into account the ease of disposal or recycling a certain

waste. Ironically, the waste generated in the manufacture of organic compounds consists primarily of inorganic salts. This is a direct consequence of the use of stoichiometric inorganic reagents in organic synthesis, particularly in fine chemicals and pharmaceuticals manufacture.[5]

Another major source of waste is solvent losses, which end up in the atmosphere or in ground water. Indeed, solvent losses are a major contributor to the high E factors of pharmaceutical manufacturing processes. Furthermore, health and/or safety issues associated with many traditional organic solvents have led to their use being severely curtailed. Solvents are divided into four classes[5]:

- Class 1: solvents should not be used in the manufacture of drug substances because of their unacceptable toxicity or deleterious environmental effects. They include benzene and various chlorinated hydrocarbons.
- Class 2: solvents should be used only sparingly in pharmaceutical processes because of inherent toxicity and include acetonitrile, dimethyl formamide, methanol and dichloromethane.
- Class 3: solvents may be regarded as less toxic and of lower risk to human health. They include many lower alcohols, esters, ethers and ketones.
- Class 4: solvents, for which no adequate data are available, include di-isopropyl ether, methyl tetrahydrofuran and isooctane.

Many pharmaceutical companies are focusing their attention on minimizing solvent use and on replacing many traditional organic solvents, such as chlorinated and aromatic hydrocarbons, by more environmentally friendly alternatives such as lower alcohols, esters and some ethers such as methyl *tert* butyl ether (MTBE).[5] Pfizer scientists, for example, have produced a solvent selection guide for medicinal chemists, dividing solvents into three categories: undesirable (red), usable (yellow) and preferred (green) as shown in Fig. 4.[6]

Preferred	Usable	Undesirable
Water	Cyclohexane	Pentane
Acetone	Toluene	Hexane(s)
Ethanol	Methylcyclohexane	Di-isopropyl ether
2-Propanol	TBME	Diethyl ether
1-Propanol	Isooctane	Dichloromethane
Heptane	Acetonitrile	Dichloroethane
Ethyl Acetate	2-MeTHF	Chloroform
Isopropyl acetate	THF	NMP
Methanol	Xylenes	DMF
MEK	DMSO	Pyridine
1-Butanol	Acetic Acid	DMAc
t-Butanol	Ethylene Glycol	Dioxane
		Dimethoxyethane
		Benzene
		Carbon Tetrachloride

Figure 4. List of undesirable, usable and preferred solvents made by Pfizer scientists (adapted from reference 6).

Since the major sources of waste in chemicals manufacture are clearly stoichiometric reagents and solvent losses, the solution to the waste problem is evident: catalytic reactions in alternative

reaction media. With regard to the latter, the best solvent is no solvent but if a solvent is needed it should be safe to use and there should be provisions for its efficient separation from the product and reuse. The use of water and supercritical carbon dioxide as reaction media fits well with the current trend towards the use of renewable, biomass-based raw materials, which are ultimately derived from carbon dioxide and water.

Biocatalysis

One of the principles of Green Chemistry involves reaction catalysis, and this is also one of the enabling techniques mentioned above (Figure 5).

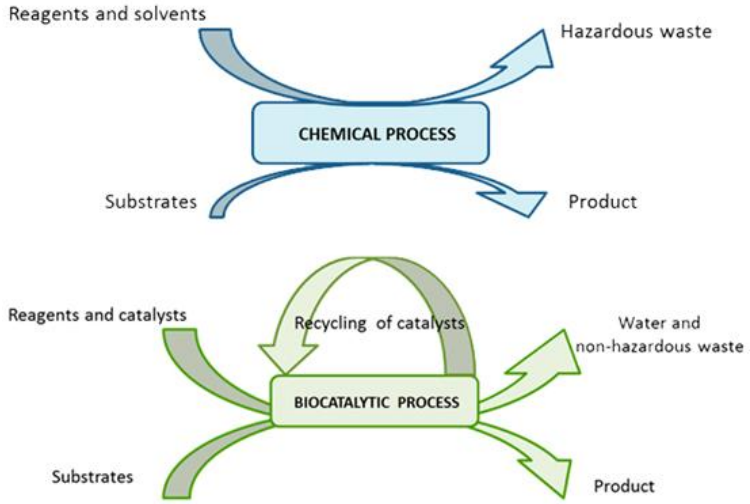


Figure 5: Differences between biocatalytic and chemical process (adapted from reference 7a).

In a biocatalytic process occurs a biotransformation, *i.e.*, a chemical reaction, catalyzed by biological catalysts, mostly enzymes. Biocatalysts can be used as isolated proteins, crude cell extract or as whole cell, free or immobilized on a solid support. The substrates could be different molecules, often synthetic but also natural compounds.
A general scheme for the enzymatic mechanism of catalysis is showed in Figure 6:

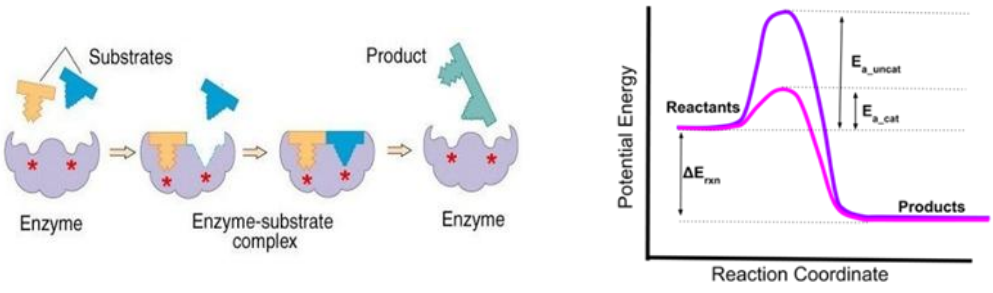


Figure 6: Enzymatic mechanism of catalysis and effect on Activation energy during reaction process.

Biocatalysis posseses some unique features:[7b,8]

- Selectivity
  - Chemo-specificity: it means that an enzyme acts on a single type of functional group, whereas other sensitive functionalities, which would normally react to a certain extent

under chemical catalysis, survive. As a result, biocatalytic reactions tend to be cleaner and laborious purifications of product(s) from impurities emerging through side-reactions are not required anymore;

- Regio-specificity: considering their complex three-dimensional structure, enzymes can distinguish between functional groups which are situated in different regions of the substrate;
- Stereo-specificity: since almost all enzymes are made from L-aminoacids, enzymes are chiral catalysts. Consequently, any type of chirality present in the substrate molecule is recognized upon the formation of the enzyme-substrate complex. Thus, the two enantiomers of a racemic substrate may react at different rates (kinetic resolution); moreover, a prochiral substrate can be transformed into an optically active product due to a stereospecific interaction with the reaction intermediate;

- Mild reaction conditions;
- High reaction rates, enzymes can be very efficient.

Other advantages of biocatalyzed reactions involve less production costs and less process passages, with consequent less waste of time, solvents and substrates. In addition, biotransformations represent a more sustainable alternative to chemical synthesis, with less use of toxic reagents and metals. With enzymes, chemistry becomes greener, with less impact on the environment, also because they can be easily re-used.

Even if biocatalysis has gained importance in the latter half of the 20th century, enzymes as catalysts however suffer from some limitations:

- Many enzymes are not accessible in enough large quantities for practical applications
- Enzyme stability is affected by several factors such as temperature, pH, surfactants, etc.
- Some enzymes show a narrow substrate scope.

The recombinant DNA technology has solved some problems and the number of enzyme is now virtually infinite thanks to techniques as high throughput microbial screening, genomic and metagenomic screening.[9] The enzyme stability, on the other hand, has been improved with different approaches: selecting new stable enzymes from peculiar natural habitats, using protein engineering or immobilization. This technique limits heat and mass transfer and minimizes access of destabilizing agents to the enzyme. Moreover, immobilization onto a surface introduces additional interactions that stabilize the tertiary structure of the enzyme. In batch operations, immobilized enzymes could be recovered and reused.[10]

Biocatalysts immobilization

In the first Enzyme Engineering Conference that was held at Henniker, NH, USA, in 1971, it was defined that immobilized biocatalysts, enzymes, or cells, are physically fixed in a defined region in order to catalyze a specific reaction. The desiderable aim of immobilization should be the lower loss of catalytic activity of the immobilized biocatalyst, and so the possibility to re-use it several times. To face up the necessity of increasing productivity, reducing time and the total cost of the whole process at once, immobilization became one of the most promising technique. The recovery of the biocatalysts during downstream processes and the improvement of the operational stabilities are the

greatest problems working with biocatalysts. Immobilization allows to overcome most of the process limitations (Figure 7) as:

- recovery of biocatalysts for reuse
- stability, activity and selectivity
- resistance against inhibition
- separation and purification steps.

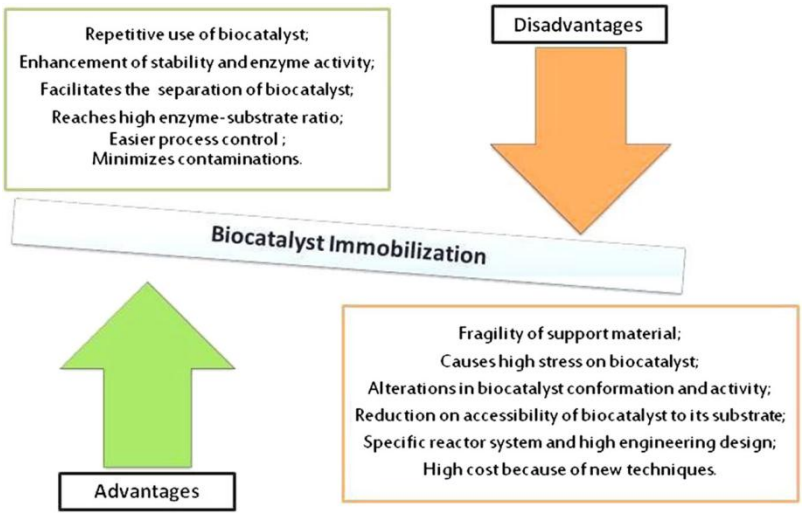


Figure 7: Advantages and disadvantages of biocatalysts immobilization (adapted from reference 11)

Immobilized biocatalysts are employed in various industries such as food processing, biomedical production, wastewater treatment, textile, detergent industry, and even biodiesel production.
The immobilization consists in confining a biocatalyst, cell or isolated enzyme, on the surface or within a solid or semi-solid support to preserve its activity but also to prevent its mobility. It is essential for an immobilized enzyme that two functions are comprised:

1. The non-catalytic functions, which provide an easier separation and a consequent re-use of the catalyst;
2. The catalytic functions, which are fundamental to convert the substrates into the product.

However, immobilization of an enzyme can modify its chemical and physical properties.[11]

Immobilization techniques

The interaction between the enzyme and the support could be by a strong chemical bond, but generally, the linkages are not covalent. In Figure 8, some methods of immobilization are shown.

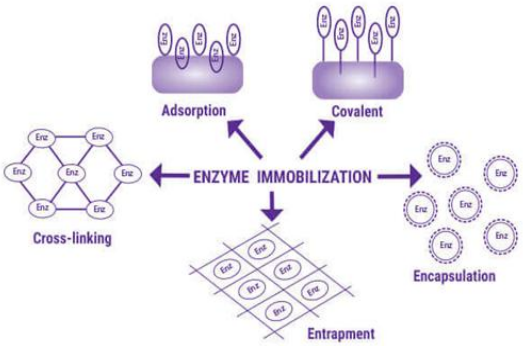


Figure 8: Immobilization techniques (adapted from reference 12a)

1 Physical adsorption: it is a simple method of immobilization. The enzyme is adsorbed on the external surface of the support. This type of immobilization requires no permanent bonds, but only weak bonds such as ionic interactions, hydrogen bonds, Van der Waals forces. Its main advantage is the minimal distortion to the protein that can avoid enzyme denaturation. However, its efficiency and its lifetime are low compared to the covalent technique. Some of the materials used for the adsorption immobilization are, for example, coconut fibers, cellulose, kaolin and micro and mesoporous materials;[12b]

2 Encapsulation: in this method, the enzyme is put into a capsule. The capsule has a semi-permeable membrane. In this case, the enzyme activity depends on its stability inside the capsule. This is a cheap method, however, the main limitation is the difficulty for the substrate to overcome membrane.

3 Entrapment: it consists of trapping the biocatalyst into a caged network with covalent and no-covalent interactions with the immobilization support. The supports that can be used for this technique can be, for example, alginate–gelatin hybrids, nano-materials such as nanowires, chitosan, mesoporous silica, calix[n]arene polymers, and k-carrageenan.[12b] It is a fast method of immobilization, but it has pore diffusion limitation.

4 Crosslinking method: it is also called copolymerization. Enzymes are directly linked by covalent bond, there is not support or matrix in this method. This method is simple and cheap, but there is the risk that enzymes can lose their activity because of covalent bond between them.

5 Covalent binding: it involves the formation of covalent bonds between a surface-exposed reactive group of the enzyme or cell and a reactive group of the support or carrier. Generally, surface-exposed functional groups include guanidines, carboxylic acids, imidazoles, indoles, phenols, hydroxyl groups, and thiols. Immobilization surfaces used for covalent immobilization include peptide-modified surfaces for high specific activity and controlled protein orientation, cyanogens bromine-infused agarose and sepharose, glutaraldehyde-modified glass surfaces, silica, chitosan, epoxides, and nanowires amongst others. This technique is able to improve catalyst lifetime due to decreased leaching.

In Table 1 are summarized the advantages and disadvantages of each technique.

Immobilization technique	Advantages	Disadvantages
Physical Adsorption	Simple and cheap	Low stability
	High catalytic activity	Possible loss of biomolecules
	No conformational change of the biocatalyst	Weak bonds might cause desorption of biocatalyst
	No need to use reagents	
	Reuse of expensive material	
Encapsulation and Entrapment	Protection of biocatalyst	Limitations on mass transfer
	Allows the transport of low molecular weight compounds	Low enzyme loading
	Enables continuous operation due to maintained cell density	
	Facilitates cell separation and simplified downstream process	
	Allows controlled release of product	
Cross-linking	Strong biocatalyst binding	Might cause alteration in active site
	Prevents leakage	Diffusion limitations
	Decreases desorption	Loss of enzyme activity
	Increases the stability of biocatalyst	
Covalent Binding	Strong binding	Limited enzyme mobility causes decreased enzyme activity
	High heat stability	Less effective for immobilization of cells
	Facilitates the enzyme contacts with its substrate	Support materials are not renewable
	Prevents elution of biocatalysts	
	Flexibility in design of support material and method	

Table 1: Advantages and disadvantages of most commonly known immobilization techniques (adapted from reference 11).

Type of supports

The choice of the most suitable support material is a very important issue. The ideal support material should have the following properties: large surface area, sufficient functional groups for attachment, hydrophilic character, water insolubility, chemical and thermal stability, mechanical strength, high rigidity, resistance to microbial degradation, and ease of regeneration. Ideally, resins will also be non-toxic, and have a low price.[12b] In reality, many immobilization supports have several of these qualities, but not all. There are a lot of support material, each one made of a compound that must or has been approved for food and pharmaceutical applications and, depending on the circumstance, the best one is chosen. Some of them are:

- Collagen: is the main protein of the connective tissues of the animal bodies. In fact, tendon, skin, bone and cartilage are generous sources for its isolation and, therefore, collagen is naturally available. This polymer is largely used because of its biocompatibility and ability to attach cells. The main issue is the expensive purification process.
- Alginate: is a polysaccharide which contains L-glucuronic acid and D-mannuronic acid. It is soluble in water as a sodium salt, and it becomes a gel in presence of bi- or tri-valent cations, like calcium. The size of the internal pore depends on the proportions of the two acids.[13]
- K-Carrageenan: sometimes this polysaccharide is used as an alternative to alginate, thanks to its stronger resistance to chelating agents. The rigidity of the carrageenan is related to high potassium concentration.

• Chitosan: is a polysaccharide obtained by deacetylation of chitin, a structural component found in crustacean, shells, fungi, insects, and mollusks. Chitosan can form hydrogels by ionic or chemical crosslinking with glutaraldehyde and can be hydrolyzed enzymatically. Conversely, chitosan has weak mechanical properties compared to other support materials.[11]

• Agar and Agarose: agar is extracted from the cell walls of some red seaweeds, belonging to *Rhodophyceae* class, widespread all along the world (including Japan, Korea, Spain, Portugal, some African countries, Mexico, Chile, and India). Agar is formed by two components: agaropectine and agarose. Agarose-based beads are highly porous, mechanically resistant, chemically and physically inert, and sharply hydrophilic (Figure 9).[14] Agarose gel contains pentagonal pores with proportional size to allow diffusional access of many proteins. On the other hand, agarose gel does not have a strong mechanical structure.[11]

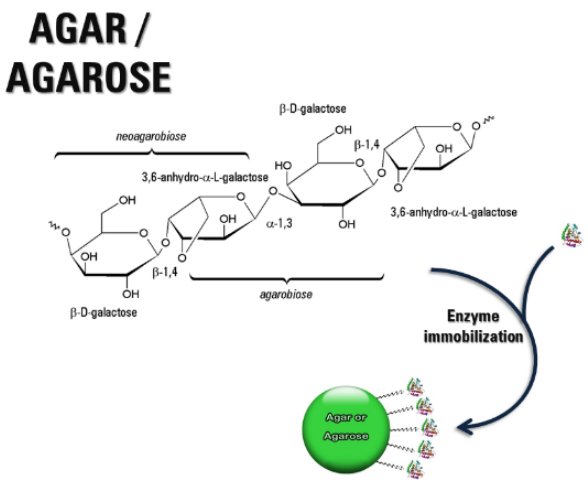


Figure 9: Agarose as support material.

Whole cells versus isolated enzymes

Whole-cell biocatalysis employs recombinant or native enzymes produced by cellular metabolism to perform interesting reactions. Generally, it is necessary to consider the type of reaction, the cofactor recycling and the biotransformation scale to decide what to employ as catalysts, if whole cells or purified enzymes. In fact, using isolated enzymes allows to obtain high specific activity and high selectivity, but it is necessary to supply specific cofactors (especially in redox reactions), it is expensive and there could be problems in the recovery of the biocatalyst. On the other hand, whole cells are less expensive, easily recoverable and own cofactors and systems for their regeneration. However, they could show competing enzymatic activities, low specific activity and require facilities for fermentations.

Table 2 and 3 summarize some advantages and disadvantages of these two different approaches.

ISOLATED ENZYMES		
FORM	ADVANTAGES	DISADVANTAGES
Dissolved in water	• Simple apparatus • Simple work-up • Better productivity • High substrate concentration tolerance • High catalytic activity	• Cofactor recycling necessary • Side reaction possible • Lipophilic substrates insoluble • Workup requires extraction
Suspended in organic solvents	• Easy to perform • Simple workup • Lipophilic substrates soluble • Easy enzyme recovery	• Reduced catalytic activity
Immobilized	• Easy enzyme recovery • Enzymatic recycling • Possibility to use continuous flow reactors	• Loss of activity during immobilization

Table 2: Advantages and disadvantages of isolated enzymes.

WHOLE CELLS		
FORM	ADVANTAGES	DISADVANTAGES
Growing cultures or resting cells	• No cofactor recycling necessary • High activity • Simple workup • Fewer by-products	• Expensive equipment • Tedious workup • Low productivity • Low substrate concentration tolerance • Low organic solvents tolerance • Uncontrolled side reactions
Immobilized cells	• Possible cell re-use • Use in continuous flow reactors	• Loss of activity during immobilization

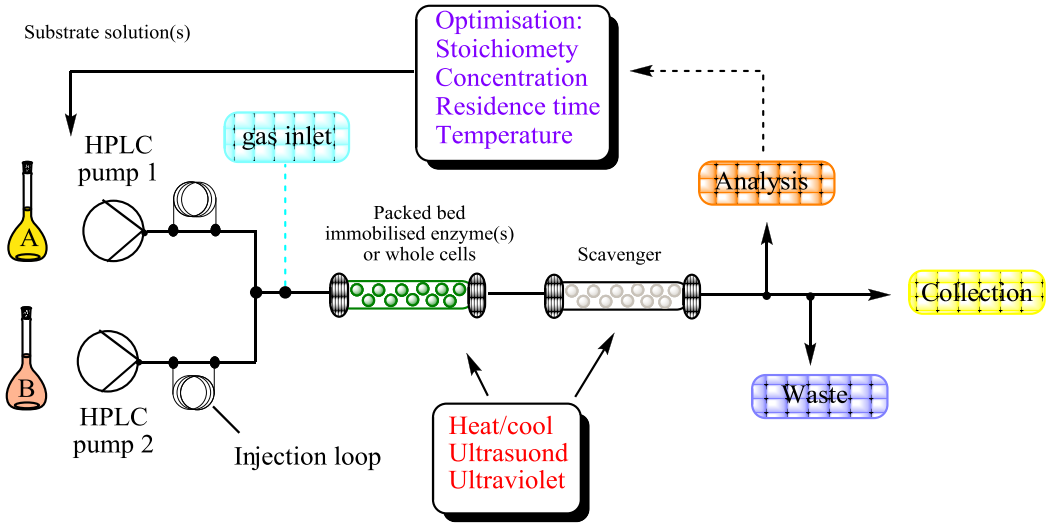
Table 3: Advantages and disadvantages of whole cells.

Flow reactor technology

One of the enabling techniques introduced in the recent years is flow chemistry. This technology has been developed in order to overcome some problems related to the traditional batch synthesis and to provide a more sustainable and environmental friendly chemistry. A flow chemistry reactor is composed by two or more pumps that move fluids into small channels and where the channels join each other, the fluids meet each other. If these fluids are reactive, a reaction takes place in a reactor, which can be of different types, for example a glass column packed with immobilized reagents (catalysts and biocatalysts) for heterogenous reactions solid/liquid or a coil for homogenous reactions liquid/liquid (Figure 10).

Figure 10. Different types of reactors.

After the reactor, the product can be collected and directly analyzed, if systems like UV or infrared detectors are linked to the flow device. This would provide a rapid optimization of the reaction parameters (*e.g.*, stoichiometry, concentration, residence time and temperature). The product can be also directly purified-in line with solid resins or scavengers, avoiding in this way traditional work-up or purification procedures (Scheme 1).[15]



Scheme 1. Schematic representation of a flow reactor configuration.

As shown in the scheme above, in some reactors is possible to have two injection loops after the pumps, that can be used to bypass the pumps if corrosive reagents are used.
Flow reactors have been classified depending on reactor dimensions in micro-, meso- and macro reactors. Microchannels have diameters between 10 and 1000 µg and can produce until hundred

milligrams of product. They are divided into microfluidic (i.d: 10-500 µm) and minifluidic reactors (i.d: 500 µm-several mm). They both have advantages and disadvantages depending on their dimensions (Table 1). Meso reactors have channels with diameters from 1 to 100 mm and they can produce from few grams to ten grams of product. Macro or large scale reactors can produce more than 100 mega tones each year. They are used in industrial plants, while micro and meso ones are commonly used in research laboratories. Channels are generally made of polymers (*e.g.*, PFE, PTFE), glass, stainless steel or silicon (Figure 11).[1,16a]

Figure 11: Flow reactors for homogeneous liquid synthesis (adapted from reference 16b).

A scientific debate is currently open on which, between micro or mini reactors, are better (Table 4). As it is possible to note in table 3, the major advantage of microfluidic reactors is the high heat transfer, due to the high ratio surface/volume. On the other hand, this peculiarity could create high pressure drops that could limit flow capacity and block the whole system.[16a]

	Advantages	**Disadvantages**
Microfluidic reactor (10-500 µm i.d)	• high heat transfer surface to product volume ratios; • good heat transfer capabilities ideally suited for optimizing reactions conditions; • efficient mixing.	• micro channels suffer from restricted flow capacity; • high pressure drop; • tendency to block.
Minifluidic reactor (500 µm - several mm i.d)	• improved flow capacity; • lower pressure drop; • no blocking of channels; • preparation of multigram to multikilogram quantities; • possibility to work with packed bed reactors.	• lower heat transfer surface; • poorer heat transfer capabilities.

Table 4. Advantages and disadvantages of microfluidic and minifluidic reactors.

Flow chemistry has demonstrated to be perfectly in line with the principles of green chemistry, because it results in high yields, increased safety, possibility for automation, less usage of solvent, re-use of supported reagents or catalyst (which is connected with less waste), more efficient reactions.[7] Also, flow reactor technology allows another way to achieve atom economy, *i.e.*, reaction telescoping. It means that one-pot synthesis is conducted in a continuous reactor, in which reagents are added one at a time and work up is not necessary.[3]

Principles of Flow Chemistry

As mentioned above, organic chemistry is a changing world: nowadays, chemists need more time to design and discover new drugs and, consequently, they have less time to apply standard procedures and recipes. For these reasons, a common think now is that a machine-assisted approach can help chemists in their researches. It is possible to state that traditional chemistry follows the concept of "quick and dirty chemistry", for which HPLC systems have been invented. The main goal of flow reactor technology is to achieve a clearer and faster chemistry, allowing a dramatic reduction of waste.[17,18]

In a batch reaction, stoichiometry is determined by the concentration of reagents and their volumetric ratio, whereas in flow it is dependent on concentration of reagents and/or the ratio of their flow rates. Flow rate and reactor volume determine the residence time, which is the time during whom the reagents stay in contact in the reactor. Reaction time corresponds or is lower than the residence time.

Other parameters that must be considered are temperature and pressure. Heat transfer in flow systems is better than in batch: in fact, the small dimensions of the channels determine a high contact surface for heat exchange. The result is that reagents are homogeneously heated and the temperature can be precisely controlled. This is a very important issue because a non-homogeneous heating, as in batch systems, can cause reagent and product degradation or the formation of secondary products. A consequence is obviously the need of performing extensive purifications (*e.g.*, aqueous work-ups, column chromatography, crystallizations) by which ensue increased costs in terms of time, manpower and environment impact (resources, also manufacturing ones). The reagent degradation could be also due to their high reactivity that leads to degradation. Also, the high surface/volume ratio allows to perform exothermic reactions, that are very hazardous in batch, in a safer and more controlled way. The increased safety is an important advance of flow chemistry that can have an important impact in industry. The safer conditions depend on the fact that the used volumes are smaller than batch ones, and this allows to have a better control on reactions and to use hazardous reagents and solvents. Some examples are the fluorination reaction using DAST and the nitration reaction. This peculiarity can be also exploited for photochemical reactions, in which it is important to well control the energy. These reactions are currently receiving increased attention[16]. In flow reactors, there is not only a better heat transfer, but also a better mass transfer, that can result in a better mixing (if compared with batch), short reaction times, and improved safety[19]. In fact, in batch, mass transfer is made by convection, which results in chaotic and turbulence mixing. Only a minimal portion of materials is indeed efficiently mixed. These idle portions lead to poor heat-transfer, concentrate reagents, and to the formation of "heat spots"; so, in this way, reactions are often inefficient. In flow, microchannels have a volume/surface ratio of about 30.000 m^2/m^3.

This peculiarity and the continuous flow allow complete and homogeneous mixing, that is achieved in milliseconds, usually in T o Y piece mixer (Figure 12).[3]

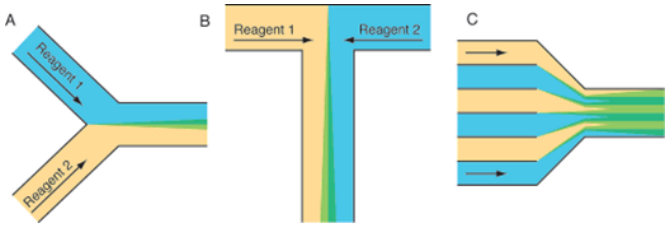


Figure 12: Types of mixing in flow reactors (adapted from reference 3)

As mentioned above, reaction parameters, such as stoichiometry, residence time, pressure and temperature, can be easily changed and optimized. The optimization can be quick and precise, and allows flow reactor technology to be used for high-throughput screening, and, so, for preparing libraries of candidates. This is also due to the small volumes of reagent and solvent that are used which can result in time and cost reductions. The easier *in-line* monitoring of the reactions allows more control and[19] safer conditions, also for handling hazardous reagents[3]. This is a very important issue because, at present, a lot of time is spent for optimization and manipulation of reactions, and too much energies and economic resources are involved in these activities. Figure 13 sums up briefly the advantages of using continuous flow reactors[20].

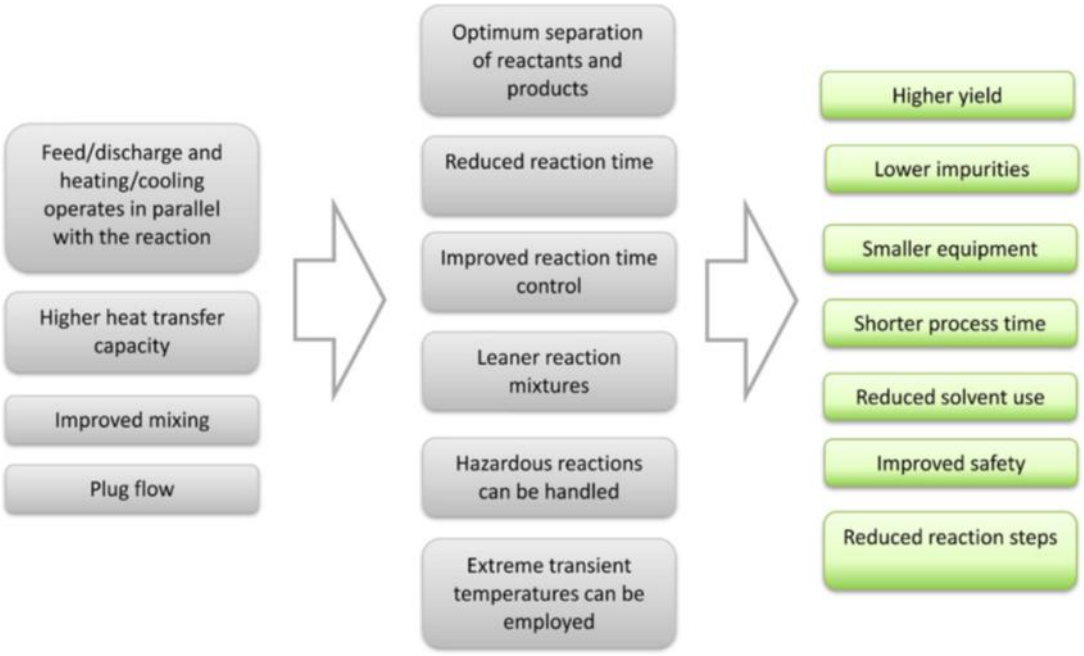


Figure 13: How the characteristics of a continuous flow reactor can affect the process parameters

Another important issue concerns multistep reactions. This type of reactions is an interesting strategy to reduce solvent waste, especially if it is possible to re-use it. In batch, every intermediate of each step has to be isolated and purified to perform the following synthetic step. In a flow reactor, this is not necessary, if the chemist is able to build a continuous system where an intermediate is the reagent of the next reaction. It is also possible to converge two different synthetic flows into one flow device, which allows to produce products with complex structure[16].

The biggest problem in multistep reactions is the necessity of switching the solvent. In fact, in flow reactors, it is fundamental that the reagents and products are in solution; otherwise, there could be the formation of a precipitate with a blockage and a consequent increment of pressure. For this reason, it is necessary that each step is conducted with the appropriate solvent. Therefore, the solvent should be the same in all the synthetic steps, in order to make a continuous process, or it has to be available a mechanism to switch solvents in continuous[18]. To overcome this issue, a prototype in-line evaporation system (Figure 14) has been developed by Ley's group at the University of Cambridge. It was constructed using a glass column and Swagelok fittings, common equipment in flow chemistry laboratories; the device acts as a spray drier for an incoming liquid stream. A fine dispersion of solution is nebulized by a gas, usually N_2, at high temperature. In this way, the solvent is partially evaporated and the solution is concentrated. The exhausted gas passes through a condenser to recover and recycle solvent. Using this prototype, it was possible to switch solvent in continuous, by taking advantage of the differences in their volatilities[17].

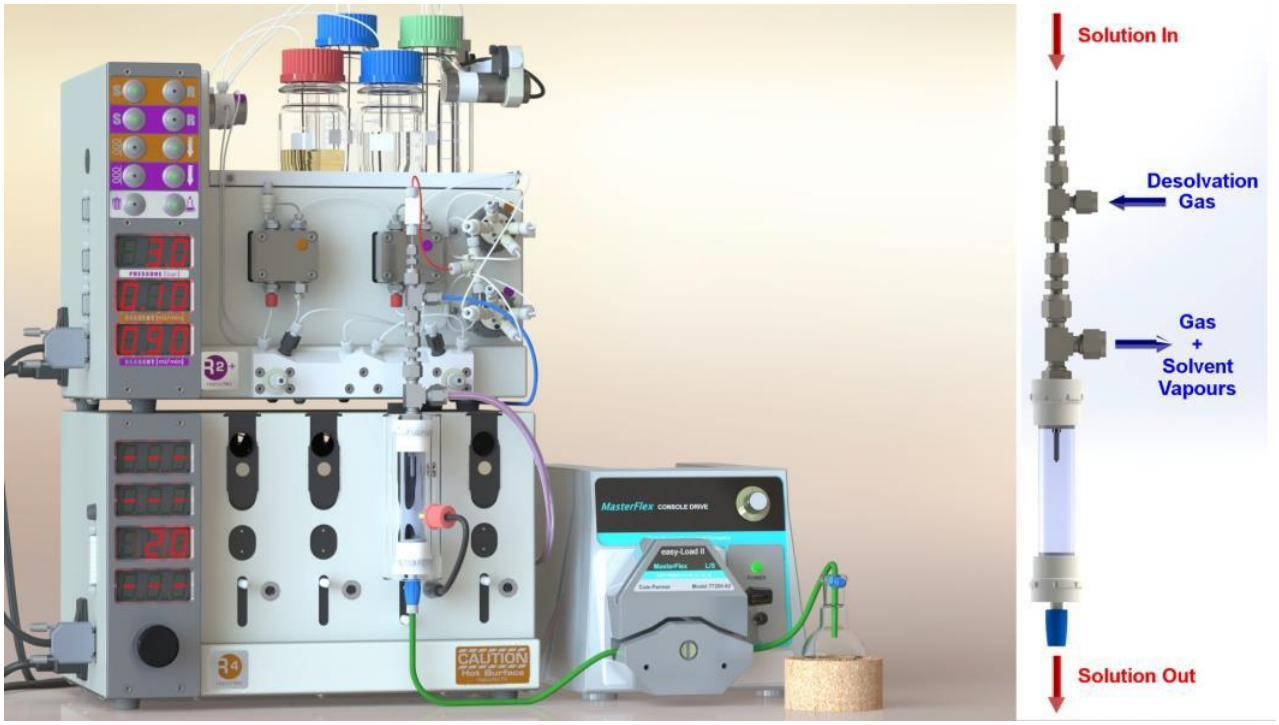


Figure 14. In-line evaporation system.

Flow can be also combined with immobilized reagents or catalysts. As opposed to what happens in batch conditions, in flow, supported reagents or catalysts can permanently remain in the reactor and can be easily recovered and re-use. Reaction and filtrations are simultaneous. As mentioned above, catalysis offers many advantages, such as selectivity and, in the case of immobilized catalysts, the possibility to well separate products from reagents, in order to make the work up easier. The choice of the support is decisive in determining the reaction set-up and in dictating the recovery and the recycle techniques. In addition, in a flow reactor, the immobilized reagent or catalyst is packed into a glass column (Figure 15 and 16) and, therefore, there is not mechanical stirring or agitation. Consequently, supported materials do not degrade, and this can extend the material lifetimes[19].

Figure 15: Glass columns for continuous flow.

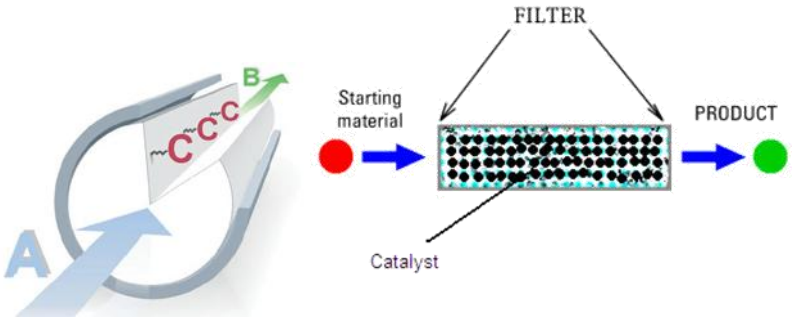


Figure 16: 1. A starting material; B product; C immobilized reagent. 2. The picture shows the case of an immobilized catalyst (adapted from reference 18).

Today, also continuous purification systems have been developed, and they can be linked in-line in continuous flow systems, to reduce times and chemist manual work (for examples, HPLC, electrophoresis, etc).

Flow chemistry: a process intensification technology

Flow chemistry is the result of a combination between process intensification and process optimization, and, so, it is fundamental to have chemical and engineering knowledge (Figure 17)[18]. It also requires an adjustment in how the chemists plan and conduct the chemical development. In general, process intensification has four main goals: i) to maximize the effectiveness of intra- and inter-molecular events, ii) to provide the same processing experience for each molecule, iii) to optimize the driving forces and maximize specific interfacial areas, and iv) to maximize the synergistic effects of partial processes. So, it aims at reducing waste, energy consumption, costs, time and material usage. The main goal is to "produce much more with much less".

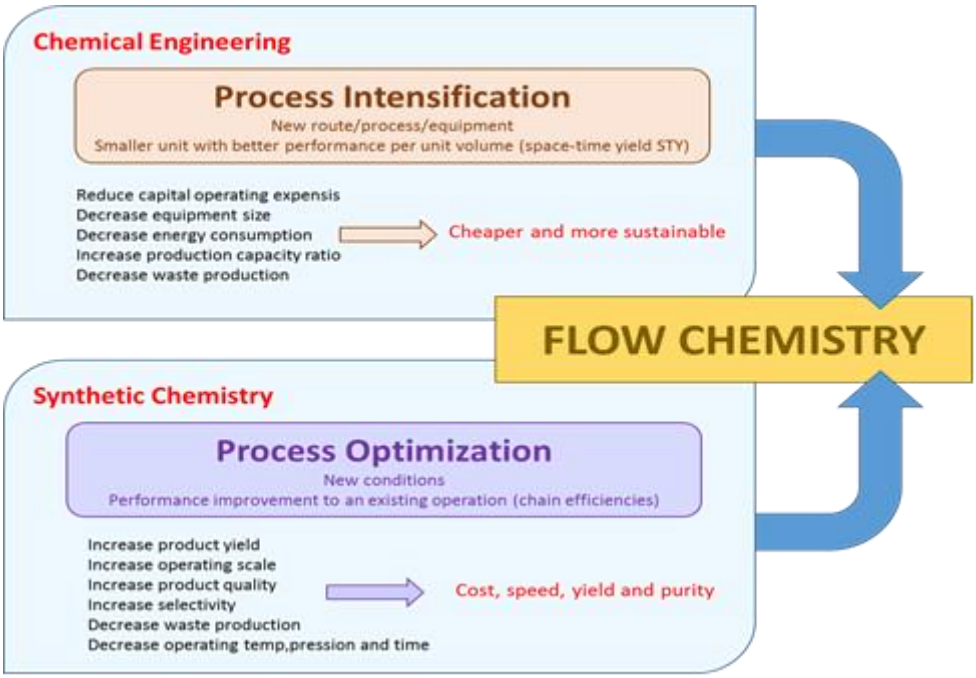


Figure 17. Process intensification and process optimization in flow chemistry.

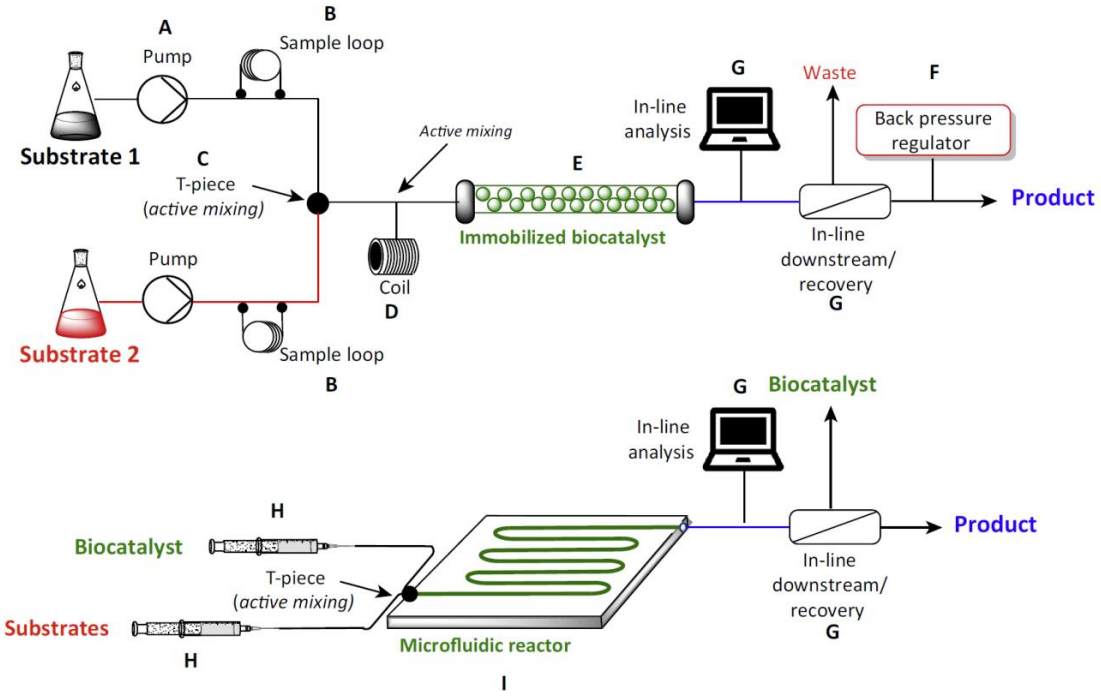
In flow, it is possible to work on three parameters to increase the production: more channels or more devices that work in parallel, longer operational times and higher flow rates[21]. It is easy to understand that also scale up in flow is simplified. In fact, optimized reaction parameters on a small scale can be used unchanged also in a larger scale[16]. Clearly, flow chemistry has much to offer to pharmaceutical industry, for the easy scale up, easy optimization and process intensification. Most of all, a peculiarity is the ability of producing large quantities of product in a short time, and this is possible when the synthetic pathway is clean, simple, efficient, safe, reliable and reproducible. There is a continuous balance between speed and optimization, and flow reactor technology has all the qualities to be inserted in these transitions. All the industrial departments can benefit from flow chemistry.

Biocatalysis in flow reactors

In response to the challenges of the pharmaceutical industry and medicinal chemistry, biocatalytic flow reactors have been increasingly developed. The use of biocatalysts (both whole cells and isolated enzymes) in flow reactors allows to further improve the productivity of the process, without forgetting the green aspect of the synthetic chemistry. Flow processing has the potential to accelerate biotransformations due to enhanced mass transfer, making large-scale production more economically feasible in significantly smaller equipment with a substantial decrease in reaction time (from hours to few minutes), and improvement in space-time yield.[10] As mentioned above, flow reactors could be of different dimensions, materials and shapes and they could be used for heterogeneous or homogeneous reactions. The whole system can be coupled to in-line monitoring systems in addition to mixing and heating devices. In-line monitoring is desirable to quickly react to fluctuations of the key parameters together with tracking the reaction's progress.
Another important issue of flow processes concerns their application on a large scale. For batch reactions, the scale has implications on the mass and heat transfer within the system, so the process

conditions must often be reoptimized. Although scaling up microreactors for produc-ing compounds at least at the gram scale seems simple on paper, the cost of individual microchip type reactors and the challenge of pumping liquid throughout the microreactors limit this approach. Larger mesoreactors can overcome these limitations, ultimately allowing for throughputs from g/h to tons/year. They may consist of scaled-up versions of the planar chip-type microreactor, single tubular reactors or parallel capillary reactors. The amount of product generated is determined by the duration over which the entire flow regime is operated, once flow rates and reactor volumes are defined.[10]

All these features combined with biocatalysts are shown in Scheme 2. Among biocatalytic flow reactors the mesoreactors and minireactors are mostly used. Two types of microfluidic chemical reactors were reported to date, the first one is chamber type and the second one is the continuous flow type. The micro chamber type has the static reagent in the micro chamber and the continuous flow type has the continuous flow of reagent through the overall microchannel[22]. The micro chamber type can be problematic when is necessary to increase the production up to gram scale. In fact, even if is a modular system, this device has a great impact on the cost of the process.



Scheme 2: Schematic representations of main components of biocatalytic flow reactors: A pumps; B reaction loops; C T-piece; D coil reactor; E column reactor; F back pressure regulator; G downstream unit; H syringe pumps; I microfluidic reactor (adapted from reference 10).

Both cell-free enzymes and whole cells could be used ad biocatalysts. Immobilized enzyme reactors and free enzyme reactors are the most common because cell-free systems offer advantages versus the whole cell approach, such as[10]:

- Generally faster flow
- Less barriers between the substrate and the catalyst
- No need to maintain the cell wall integrity
- Less side reactions.

Figure 18 shows different configurations of biocatalytic flow reactors, both for free and immobilized enzymes.

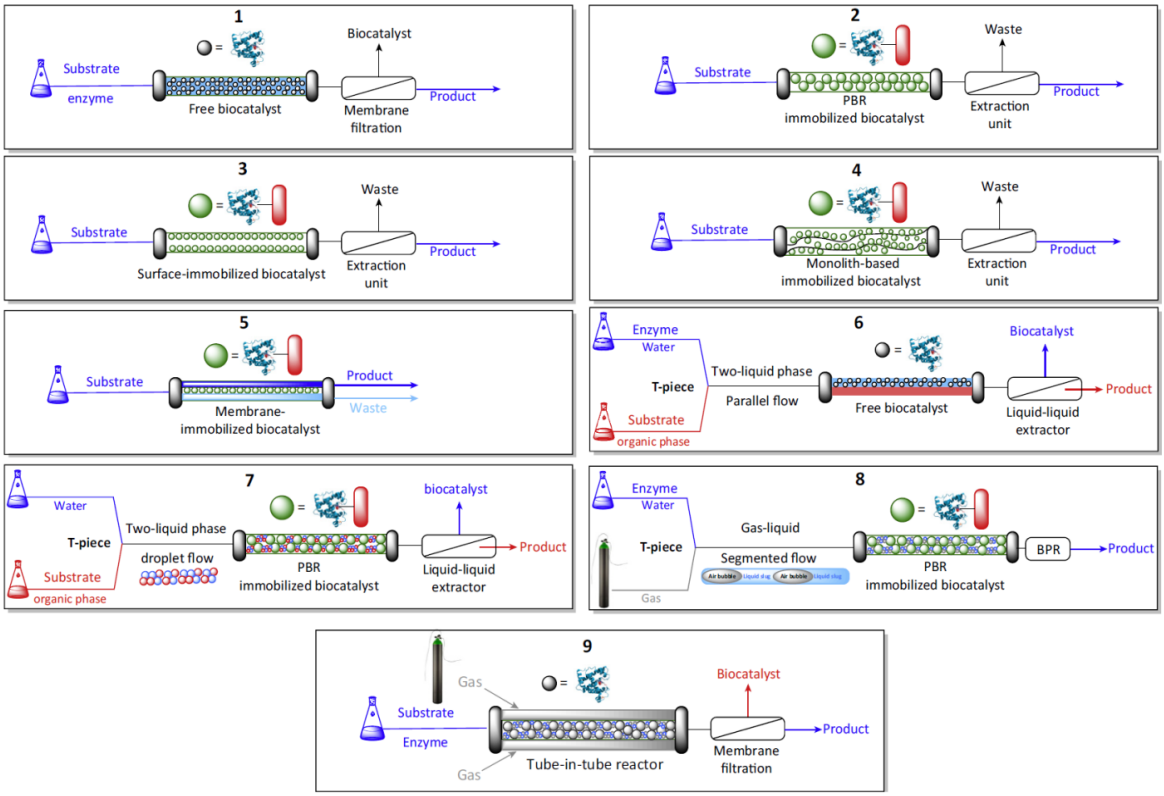


Figure 18: 1 Free biocatalyst; 2 immobilized biocatalyst in a packed bed reactor; 3 biocatalyst immobilized on the inner surface of the channel; 4 biocatalyst immobilized on a monolith; 5 biocatalyst immobilized on a membrane; 6/7 free biocatalyst/immobilized biocatalyst in a l/l biphasic parallel flow stream; 8 immobilized biocatalyst in a g/l biphasic flow stream; 9 free biocatalyst in a tube-in-tube reactor (adapted from reference 10).

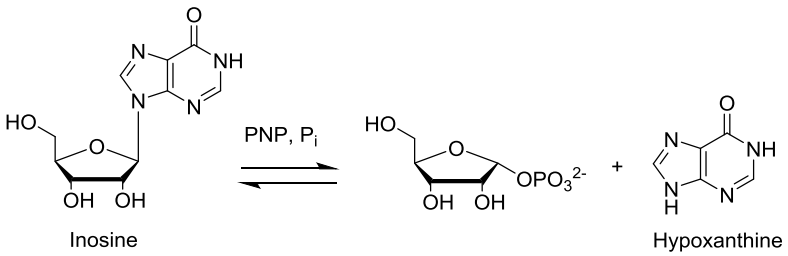
However, the stability of th biocatalyst can be a challenge in flow chemistry, which has to be studied very deeply. Enzyme stability is typically affected by several factors, such as temperature, pH, surfactants, etc., which can disrupt the catalyst structural interactions. Immobilization is often used to enhance enzyme stability: it limits heat and mass transfer, minimizing access of destabilizing agents to the enzyme; moreover, immobilization onto a surface introduces additional interactions that stabilize the tertiary structure of the enzyme; and loss of quaternary structure can be minimized by crosslinking unbound subunits to those already bound to the support. This is not, unfortunately, true for all the examples: in some cases, immobilization can be a problem, for both activity and stability, if not correct domains are touched. Indeed, in these cases, a deep study must be done concerning the best immolization technique to be used. Also, in flow a particular attention must be paied for the flowstream that passes through the column with the immobilized reagents: indeed, if the stability is not great, the biocatalyst can be flowed away from the support, and therefore a release in the exiting flowstream will be observed, concerning the nature of the solvent used.[10]

Biocatalysis in flow reactors: selected examples

The following list shall include some examples from the recent literature of biocatalysis in flow reactors:

1 Hydrolases: catalyze the hydrolysis of many functional groups as esters, amides, glycosides, nitriles and epoxydes. Hydrolyses do not require organic coenzyme. There are many examples in literature, mainly lipase, such as the dynamic kinetic resolution of a protected amino acid derivative (N-Boc-phenylalanine thioethyl estert) in continuous flow mediated by an alcalase.[10]

2 Transferases: catalyze the transfer of a functional group from a molecule to another. In this group are included transaminases, which are really relevant in the pharmaceutical industry but require cofactors, tranketolases and purine nucleoside phosphorylase (PNP). One example is PNP from *Aeromonas hydrophyla* (*Ah*PNP) immobilized in a packed stainless steel column and used for bioconversion on analytical scale coupled with product purification (Scheme 3)[23].



Scheme 3: PNP catalysed reaction.

3 Lyases: add or remove a chemical group with a different mechanism from hydrolases or oxidoreductases. As much as they would be a powerful instrument in the synthesis of non-natural amino acid, there are limitations due to the immobilization of the free enzyme.

REDOX reactions

A reduction-oxidation reaction is a chemical reaction in which the oxidation states of atoms are changed. Any such reaction involves both a reduction process and a complementary oxidation process, two key concepts involved with electron transfer processes. Redox reactions include all chemical reactions in which atoms have their oxidation state changed; in general, redox reactions involve the transfer of electrons between chemical species. The chemical species from which the electron is stripped is said to have been oxidized, while the chemical species to which the electron is added is said to have been reduced. The processes of oxidation and reduction occur simultaneously and cannot happen independently of one another. The oxidation alone and the reduction alone are each called a half-reaction, because two half-reactions always occur together to form a whole reaction. Familiar examples of redox reactions are, for example:

- Combustion reactions: oxidation-reduction reactions that occur when oxygen oxidizes another material and are accompanied by the release of heat and sometimes flames;

- Corrosion reactions: limited to the oxidation of metals and usually occur when moisture is present. They occur most rapidly when metals are strained and bent; the metals rapidly oxidize in the strained regions;
- Respiration: this is one of the key ways a cell releases chemical energy to fuel cellular activity, where large molecules (*i.e*, sugars, amino acids, fatty acids) are broken into smaller ones. The formed chemical energy is stored in ATP molecules, that can be used in processes requiring energy. Cellular respiration is considered an exothermic redox reaction which releases heat. The overall reaction occurs in a series of biochemical steps, most of which are redox reactions themselves.

Moreover, redox reactions are the foundation of the electrochemical cells, which can generate electricity from chemical energy.

In organic chemistry oxidations and reductions are different from ordinary redox reactions because many reactions carry the name but do not actually involve electron transfer in the electrochemical sense of the word. Instead the relevant criterion for organic oxidation is gain of oxygen and/or loss of hydrogen. Classical reductions include alkene or alkyne reduction to alkanes and classical oxidations include oxidation of alcohols to aldehydes/ketones/carboxylic acids. In oxidations electrons are removed and the electron density of the organic compound is reduced. In reductions electron density increases when electrons are added to the organic compound.

Oxidations

Oxidation is a key transformation in organic synthesis. Most of oxidative procedures are far from being sustainable, because of the generation of copious amounts of toxic waste, and the employment of environmentally undesirable solvents, typically chlorinated hydrocarbons. In fact, organic oxidations can occur with different mechanisms and different oxidizing agents, for example chromium (VI), Dess-Martin periodinane and the Swern reagent. A very useful and interesting alternative to convert alcohols into their corresponding carbonyl compounds is to use *N*-oxoammonium salts as oxidants that can satisfy the principles of green chemistry. TEMPO (2,2,6,6-tetramethylpiperidin- 1-yl) oxidanyl) or derivatives, when used in catalytic amounts, exploit cheap sodium hypochlorite as a stoichiometric oxidant and generate in situ oxoammonium salt. TEMPO is still very expensive, but cheaper derivatives have been introduced recently.

However, many of the oxidative procedures that nowadays are mostly used are far from being sustainable. For example, the use of stoichiometric amounts of carcinogenic chromium (VI) on an industrial scale would raise serious issues, while Dess-Martine periodinane is an unstable and potentially explosive reagent. The use of the Swern reagent produces dimethyl sulfide as co-product, which has a very disagreeable odor even at very low concentrations. Both economic and environmental critical issues cause an urgent demand for greener, more atom efficient scalable oxidation methods that employ clean oxidants, such as molecular oxygen or hydrogen peroxide, and a recyclable catalyst for synthetic applications in the fine chemical industry[24].

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Reductions

For reductive reactions, the most employed reagents are the one made of heavy metals, as for example $LiAlH_4$, $NaBH_4$ or just Na, Mg, Zn or Fe as reductive catalysts. The main problem connected with these reagents is their removal, because is really important that no traces of them are present in the final product, in particular if it is a pharmaceutical one. This leads to the formation of

big amounts of waste, that must be selectively and carefully disposed of, thus using a lot of energies, money and time. Also, classical hydrogenation must be performed in order to reduce alkyne or alkene groups into alkanes and it is well known that these reactions are far from being safe and clean.

Biocatalyzed redox reactions: an overview of the biocatalysts employed in the present thesis

A possible answer to the demands illustrated before can be biocatalysis, with whom is possible to perform greener oxidations and reactions, helping in finding new synthetic routes that can avoid the formation of huge amounts of waste, thus reducing costs and saving the operational time, and the use of hazardous reagents [24].
In this thesis, the different chapters (one for each project) are organized as articles, therefore each one of them has their introduction, discussion and conclusion. In each introduction, an enlarged discussion is made for the specific reactions and biocatalysts employed in the correspondent project. In this paragraph, I just want to make a briefly overview about them:

- Oxidation of alcohols are among the most important reactions in organic synthesis. Biocatalytic oxidations are attractive reactions, since they often occur with high chemo- regio- and stereo-selectivity, under mild conditions of temperature, pressure and pH, using environmentally benign oxidants (*i.e.*, O_2).[26-27] They are mostly performed by dehydrogenases and oxidases, that can be found in different microorganisms, as for example *Acetobacter*. They possess a PQQ-dependent periplasmic alcohol dehydrogenase (ADH III), which catalyzes the oxidation into aldehydes, which are not accumulated into the cells but further oxidized to carboxylic acids;
- The use of alcohol dehydrogenases (ADH, named also ketoreductases -KREDs - or carbonyl reductases -CR-) is a well established method for the enantioselective reduction of prochiral ketones into stereo-defined chiral alcohols.[28a)] The reduction of these substrates frequently occurred with activity and enantioselectivity strongly dependent on the conditions of growth and biotransformation, suggesting the possible occurrence of different ketoreductases acting on aromatic ketones. Enantiomerically pure mono-alcohols can be important building blocks in the synthesis of various steroids, employed as hormonal contraceptives;
- Aromatic aldehydes are key intermediates in a number of synthetic processes and have a prominent role as flavor and fragrance components. Among other synthetic methods,[28b)] they can be obtained from the corresponding primary aromatic amines, which are readily available substrates. Transaminases are a family of enzymes with high potential in biotechnological applications, that can be used to obtain aromatic aldehydes. They can displayed high stability, high turnover rate, broad substrate specificity and no requirement for external cofactors

AIM OF THE THESIS

Nowadays scientists and industries talk a lot about sustainability, reduction of waste and processes with a lower impact on the environment, even if, in practice, we are far from the so-called "Green Chemistry". There is an urgency in finding different methods, resources and, also, different ways of thinking, to be able to change direction now.
In this context, in the present thesis I worked on the combination between flow chemistry and biocatalysis to develop innovative, robust and versatile protocols for performing biocatalyzed redox reactions using a combination of biocatalysis and flow-reactor technology for the obtainment of high value chemicals of pharmaceutical interest. The focus on redox reactions was chosen since traditional chemical redox processes are polluting and toxic, and they are usually not selective. On the other hands, biocatalyzed redox reactions often suffer of low productivities and scalability issues.
The thesis is divided into different chapters, and all them have the intent to perform traditional reactions in a new and more sustainable way to synthesize active pharmaceutical ingredients or pharmaceutically interesting intermediates.
The projects presented in this thesis are summarized below:

1. Biocatalyzed regio- and stereo-selective oxidation reaction of a cheap commercially available prochiral diols to chiral mono carboxylic acids: application in the continuous flow synthesis of Captopril: this project had the aim to synthesize important building blocks for the synthesis of various pharmaceutical ingredients by using an innovative biocatalyzed oxidation performed in a flow chemistry reactor. Also, one of these reactions was used in order to obtain Captopril, with a completely continuous-flow synthesis. I woul like to thank Fondazione Cariplo for the fundings of this project;

2. Stereoselective reduction of ketones and di-ketones, in order to obtain the corresponding enantiomerically pure mono-alcohols, using an immobilized ketoreductase from *Pichia glucozyma* and a glucodehydrogenase from *Bacillus megaterium* mixed bed reactor: the project had the aim to obtain stereo-and regioselective mono-alcohols, some of them important intermediates in the synthesis of various hormonal contraceptives, with an innovative flow-biocatalyzed approach, using two different purified and immobilized enzymes (a ketoreductase from *Pichia glucozyma* and a glucodehydrogenase from *Bacillus megaterium*). The flow approach was chosen in order to enhance productivity, reduce time reactions and increase stability, compared to the batch reactions.

3. Stereoselective reduction of 2,2-disubstituted 1,3-cyclopenta- and 1,3 cyclohexanediones using both whole cells and a purified ketoreductase from *Pichia glucozyma*, to obtain enantiomerically pure mono-alcohol products, that can be important intermediates in the synthesis of various steroids: the project, made in collaboration with the group of Prof. Dörte Rother of the Forschungszentrum Jülich (Germany), had the aim to synthesize enantiomerically pure mono-alcohols starting from cyclic diones. These can be important building block in the synthesis of various steroids. The batch reactions were deeply studied with different biocatalysts in order to understand the differences between them;

4. Oxidation of amines to aldehydes using an immobilized form of pure transaminase from *Halomonas elongata*: the project was made in collaboration with the group of Prof. Francesca Paradisi, of the University of Nottingham. The aim was to use an immobilized transaminase from Halomonas elongate in a flow reactor to enhance productivity, reduce time reactions and increase stability, compared to the batch reactions. The produced aldehydes are flavours and fragrances used in food, beverage, cosmetics and pharmaceutical. The project followed a study in which the same enzyme was used to perform the opposite reaction, demonstrating the versatility of the process.

Biocatalyzed regio- and stereo-selective oxidation reaction of a cheap commercially available prochiral diols to chiral mono carboxylic acids: application in the continuous flow synthesis of Captopril.

Articles involved:

1. "Chemoenzymatic Synthesis in Flow Reactors: A Rapid and Convenient Preparation of Captopril", Valerio De Vitis,[a] Federica Dall'Oglio,[b] Andrea Pinto,[a] Carlo De Micheli,[b] Francesco Molinari,[a] Paola Conti,[b] Diego Romano,[a] and Lucia Tamborini[b], *ChemistryOpen*, **2017**, 6(5), 668-673;
2. "Bioprocess intensification using flow reactors: stereoselective oxidation of achiral 1,3-diols with immobilized *Acetobacter aceti*" manuscript in preparation

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Authors contribution

The discover of the microorganism, its production and immobilization and the batch biocatalyzed synthesis of the different mono carboxylic acids was made by the group of Dr. Valerio De Vitis, Dr. Diego Romano and Prof. Francesco Molinari.

The optimization of the flow set-up for all the reactions involved in the project and the implementation of them in the continuous flow process was performed by me and my group, which includes Dr. Lucia Tamborini, Prof. Andrea Pint, Prof. Paola Conti and Prof. Carlo De Micheli, with the collaboration of Dr Valerio De Vitis for the optimization of the flow set-up of the biocatalyzed reaction. I would like to thank all the people involved in this project, and a special thank to **Fondazione Cariplo** for funds.

Introduction

Oxidation of alcohols are among the most important reactions in organic synthesis. Primary and secondary alcohols can be oxidized to their corresponding aldehydes or carboxylic acids and ketones, respectively. The oxidation occurs through the transfer of two-hydrogens, and can be achieved chemically using metal oxides, as Ag-, Mn-, or Cr oxides (e.g. Jones, Sarett, Collins, and Cornforth reagents). However, these reactions have some potential disadvantages in terms of their environmental impact (*i.e.* energy use, toxic byproducts, etc.) and are still often poorly stereoselective.

Biocatalytic oxidations are attractive reactions, since they often occur with high chemo- regio- and stereo-selectivity, under mild conditions of temperature, pressure and pH, using environmentally benign oxidants (*i.e.*, O_2).[26-27] The impact of enzyme-mediated oxidations in organic chemistry has been reviewed recently.[28c)]

Alcohol oxidations are mostly performed by dehydrogenases and oxidases, which mediate the dehydrogenation of the substrate, although, chloroperoxidases, laccases and monooxygenases are also known to catalyze these reactions. The most frequently used cofactors are nicotinamide adenine dinucleotide (NAD^+, $NADP^+$), flavin adenine dinucleotide (FAD) and pyrroloquinoline quinone (PQQ) and they are necessary to perform the reaction. Their regeneration is a key point because it is necessary to make the reaction proceeding. Surely, the relative amount of reduced/oxidized cofactor plays a crucial role for the equilibrium of the reaction and, in addition, their use in stoichiometric amounts is too expensive.[26]

The use of whole cells (either wild type or engineered) from aerobic microorganisms allows to use their respiratory metabolism as co-factor regeneration system. This guarantees the continuous regeneration of cofactors and shifts the equilibrium towards oxidation.

Different aerobic bacteria (including *Actinomycetes*), molds and yeasts have been used as sources of oxidative enzymes, but their use as whole cells is hampered by degradative reactions that can further metabolize the carbonyl compounds. Metabolic engineering allows for the selective knock-out of enzymes involved in further metabolization of the desired products. Thus, whole cells can be used as efficient systems. Examples are the selective inactivation of alcohol dehydrogenases for accumulating aldehydes or of CoA-synthases for accumulating carboxylic acids.[26]

A well known exception to this are acetic acid bacteria. These microorganisms have a respiratory mechanism with incomplete oxidation where partially oxidized organic compounds are excreted as final products. So, these compounds are not further metabolized and the accumulation of, for example, carboxylic acids and ketones occurs. The group of Adachi showed most of the mechanism of the oxidative enzymes in acetic acid bacteria, as *Acetobacter* and *Gluconobacter*.[29] Both of them possess a PQQ-dependent periplasmic alcohol dehydrogenase (ADH III), which performs a rapid oxidation that is very useful in industrial applications. Indeed, the main application of these bacteria is the production of vinegar. In addition, they are important for the production of several sugar derivatives, such as D-xylulose, L-ribulose and D-fructose.[29] ADH III catalyzes the oxidation into aldehydes, which are not accumulated into the cells but further oxidized to carboxylic acids. Acetic acid bacteria have an incomplete oxidative metabolism and, therefore, can efficiently perform chemo-, regio- and stereo-selective oxidations, affording good yields of the corresponding carboxylic acids[26]. Dehydrogenases of acetic acid bacteria are versatile enzymes for alcohol oxidation.[30-26] Production of structurally diverse aldehydes,[26-31] aldoximes,[32] lactones,[33] and

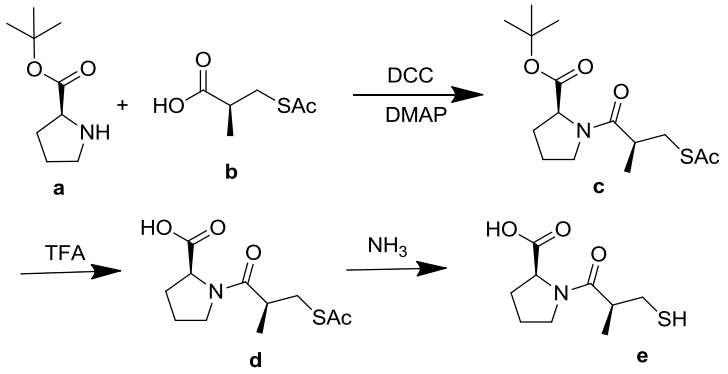
carboxylic acids[34-36] has been achieved using whole cells of acetic acid bacteria, often with high enantioselectivity.[37-38]

Different strategies can be exploited to improve the productivity and selectivity of a biotransformation carried out with whole cells. For example, they can be immobilized for enhancing stability and easy recovery;[39] moreover, immobilization may dramatically change substrate and product diffusion, thus affecting reaction rates and inhibition effects.[40] Importantly, immobilized cells can be used in packed bed flow micro- and meso-reactors, which ensure high surface-to-volume ratios, thus providing high heat and mass transfer rates. Flow packed bed reactors generally ensure that the substrate stream flows at the same speed through all the reactor volume without orbital shaking or stirring.[41-10]

Gas transfer (air or pure O_2-enriched air) to the liquid phase is often the factor limiting the efficiency of liquid phase bio-oxidations.[42-44] A solution to increase the reaction rate is to facilitate mass transfer by increasing the interfacial area by applying a segmented gas-liquid flow regime.[45] It is characterized by gas bubbles alternating with short liquid slugs and recirculation occurs within segments of the two-phase segmented flow, providing an efficient mass transfer between the gaseous and the liquid phase. [41]

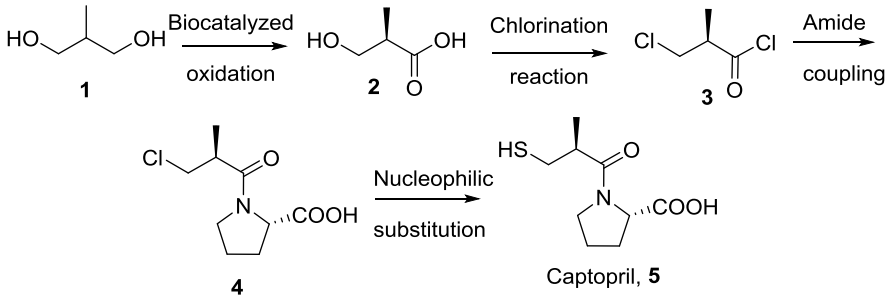
Results and discussion

Chiral 3-hydroxy acids are interesting building blocks for the synthesis of a number of active pharmaceutical ingredients, such as Captopril, Scopolamine (a tropane alkaloid drug) and the antidiarrheal drug Racecadotril.[46] Therefore, in this part of the project, I focused my attention to the development of a versatile biocatalyzed protocol for the obtainment of differently 2-substituted chiral hydroxy acid in flow. Among them, 1,3-propane diol **1** (Scheme 2) can be used as starting material for the obtainment of the antihypertensive drug Captopril [*i.e.*, (*S*)-1-((*S*)-3-mercapto-2-methylpropanoyl) pyrrolidine-2-carboxylic acid **5**], using a chemo-enzymatic approach in continuous flow. Captopril is an angiotensin-converting enzyme (ACE) inhibitor used for the treatment of hypertension and heart failure, discovered at E. R. Squibb & Sons Pharmaceuticals in the 1970s. It was the first ACE inhibitor developed, followed by other drugs of this class, that were developed in order to overcome some problems of Captopril, such as the short half-life and the adverse effects. The short half-life necessitates two/three times dosing per day, which can decrease the patient compliance. Moreover, Captopril has some side effects as rash and taste which are not characteristic of others ACE inhibitors. However, it is still a widely used drug for the hypertension.
Captopril has been synthesized in different ways. A first possible synthetic pathway was described by Ondetti at al. in the 1977[47] (Scheme 1).



Scheme 1. Synthetic pathway reported by Ondetti et al.[47]

As reported in Scheme 1, the first step of this synthesis is the formation of compound **c** starting from proline *tert*-butyl ester **a** and (*S*)-3-acetylthio-2-methylpropionic acid **b** with DCC in dichloromethane. Then, after removal of the *tert*-butyl ester group and the acetyl group, compound **e** (Captopril) was obtained in 50% yiled. (*S*)-3-Acetylthio-2-methylproprionic acid **b** was prepared following a procedure reported by Holmberg at al.[48] in which the preparation of this intermediate started from the addition of thiolacetic acid to the substituted acrylic acid. Enantiomerically pure **b** was obtained with a resolution using a chiral amine, such as 1,2-diphenylethylamine or 2-amino-1-butanol.
A second possible chemical synthesis is the one reported by Shimazaki et al.[49] This synthetic pathway is the one I decided to developed under flow conditions, and, therefore, a detailed description will be reported in the next pages. The flow protocol I developed is made of 4 steps and it is represented in Scheme 2. Shimazaki et al started from the microbiologically derived enantiomerically pure carboxylic acid **2,** but they didn't specify how exactly it was obtained.

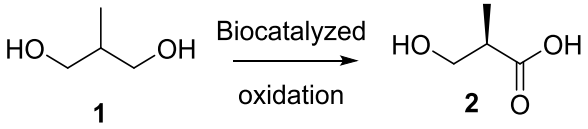


Scheme 2. Four-step chemoenzymatic flow synthesis of enantiomerically pure Captopril.

Carboxylic acid **2** can be obtained through a biocatalyzed oxidation reaction from the cheap, commercially available, prochiral diol. Importantly, using whole cells of *A. aceti*, just one hydroxyl group can be oxidized to carboxylic acid and just one of the two possible enantiomers can be obtained. Compound **2** was isolated and then three chemical steps were performed; each step was first optimized separately and then the steps were linked together, in order to start from compound **2** obtaining directly, in a continuous flow way, Captopril as final product. In particular, the second step, as represented in Scheme 2, is a chlorination reaction on both the 3-hydroxyl group and on the carboxylic acid. The next step is an amide coupling using L-proline, while the last reaction is a nucleophilic substitution of the chlorine in position 3 with a thiol group.

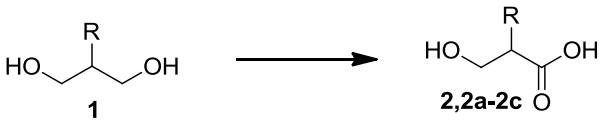
First step: biocatalyzed oxidation

The first step is, as mentioned above, the biocalyzed oxidation reaction shown in Scheme 3:



Scheme 3. Schematic representation of the first step in the synthesis of Captopril.

This oxidative reaction is challenging using traditional oxidative methods that are toxic and polluting agents and are not able to oxidize the substrate in a regio and stereoselective way. This skill is typical of biocatalysts, either whole cells and enzymes.
In this case, I performed the oxidative reaction using whole cells of *Acetobacter aceti* MIM 2000/28, a strain that belongs to the University of Milan. This microorganism was chosen among others (*i.e.*, *Asaia bogorensis* SF2.1 and *Gluconobacter oxydans* DSM 2343) by previous batch studies performed by the group of Dr. Diego Romano. They tested different achiral 2-substituted 1,3 diols **1, 1a-1c** with crude extract of *Acetobacter aceti* (Scheme 4).[50]



R	Sub
Me	**1**
Et	**1a**
n-Bu	**1b**
n-Pent	**1c**

Scheme 4. Oxidation of achiral diols **1,1a-1c** to obtain the corresponding enantiomerically pure mono-carboxylic acids (**2, 2a-2c**).
Results are reported in Table 1:

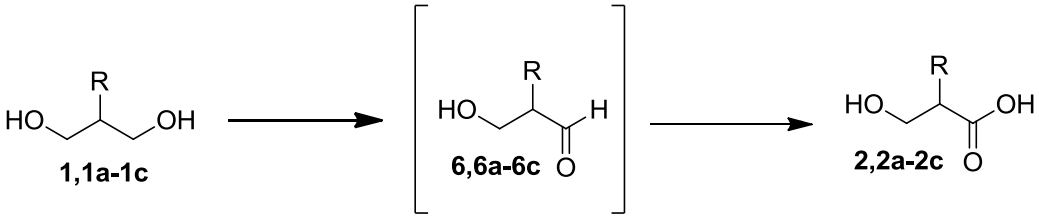
Entry	Substrate	Conversion[a] (%)	*ee*[b] (%)
1	**1**	95	94 (*R*)
2	**1a**	33	54 (*R*)
3	**1b**	88	80 (*S*)
4	**1c**	98	88 (*S*)

Table 1. Oxidation of achiral 2-substituted 1,3-diols **1,1a–1c** (12 mM) with free cells of *Acetobacter aceti* 2000/28 in conventional shaken flasks (batch mode). [a]Conversions calculated by chiral GC or HPLC. [b]Enantiomeric excesses measured by chiral GC or HPLC analysis after treatment with CH_2N_2.

Under these conditions, quantitative conversions were obtained with diols **1** (95%, entry **1**) and **1c** (98%, entry **4**), having a methyl and a *n*-pentyl substituent in position 2, respectively, while the ethyl-substituted diol **1a** afforded the lowest conversion value (33%, entry **2**). It was also observed the formation of reaction by-products, *i.e.*, α-methylenic alkanoic acid and α-methyl alkanoic acid, in different ratio depending by the substrate. For example, for compound **1a**, a 20% formation of the corresponding α-methylenic alkanoic acid and 47% of the corresponding α-methyl alkanoic acid were obtained.[50]
For what concern the enantiomeric excess, it was observed that the (*R*)-enantiomer of compounds **2** and **2a** were obtained with decreasing *ee* values as the length of the alkyl chain at position 2 increased from methyl to ethyl (entries **1** and **2**, Table 1). An inversion of stereoselectivity was probably promoted by the reduced flexibility of the three-carbon chain of the 2-alkyl derivatives **1b** and **1c**, affording (*S*) *ee* values (80 and 88 %, entries **3** and **4**, respectively). A possible explanation of the different enantioselectivity was reported[50]: since the reaction is known to occur according to a two-step sequence with an intermediate aldehyde, the enantioselectivity is controlled by the PPQ-ADH acting during the first step. A tentative explanation for the observed inversion of enantioselectivity can be proposed by comparing the steric hindrance of the alkyl substituent R in position 2 and that of the CH_2OH, which is left unreacted. If R is small (*i.e.*, Me and Et), the (*R*)-enantiomer of compound **2** and **2a** is favoured. When the bulkiness of R increases (*i.e.*, *n*Bu and *n*-Pent), a switch to (*S*) selectivity is observed.[50]

Starting from these results with free cells of *Acetobacter aceti*, I investigated this oxidative reaction using immobilized whole cells firstly in a batch mode and then in a continuous flow environment. To this aim, I chose as immobilization form dried alginates beads, which are cheap, easy to prepare and easy to use, also in a flow bioreactor. This immobilization technique was previously used by our group for the flow preparation of fructooligosaccharides (FOS)[35] and they are an example of immobilization based on physics entrapment of the whole cells. They showed excellent stability under continuous work. Details of the procedure are reported in the Experimental section of this chapter.

2-Methyl-1,3-propandiol (**1**) was used as substrate for optimizing the biotransformation, since it is also the starting material for the synthesis of Captopril. Substrate concentration, buffer pH, and immobilized cells concentration were used as control parameters and optimization was carried out using a Multisimplex approach;[51] conversion and enantiomeric excess of the product were the response variables. Optimized batch conditions (substrate 12 mM in acetate buffer 20 mM pH 6.0, 40 mg/mL of alginate beads containing 10 $mg_{dry\ weight}$ of cells) gave (*R*)-3-hydroxy-2-methylpropanoic acid **2** with >95% molar conversion and *ee* = 94% after 120 minutes. The analysis, carried out with chiral GC after derivatization with diazomethane, showed traces of the intermediate aldehyde **6**, before being completely oxidized to the corresponding carboxylic acid **2** (Scheme 5).



Scheme 5. Oxidation of the prochiral diols to carboxylic acid through formation of the aldehyde intermediate **6**, **6a-6c**.

Considering the good results obtained with diol **1**, I used the immobilized alginate beads of *Acetobacter aceti* also on the other compounds (**1a-1c**) under previously optimized conditions. Obtained results are shown in Table 2.

Entry	Substrate	**R**	**6,6a-6c** (%)[a]	**2-2c** (%)[a]	*ee* (**2,2a-2c**) (%)[b]	Time (h)
1	**1**	Me	5	86	94 (*R*)	1
2	**1**	Me	-	> 97	94 (*R*)	2
3	**1a**	Et	8	56	59 (*R*)	3
4	**1a**	Et	-	> 97	59 (*R*)	5
5	**1b**	*n*-Bu	-	> 97	80 (*S*)	5
6	**1c**	*n*-Pent	12	45	88 (*S*)	3
7	**1c**	*n*-Pent	-	> 97	88 (*S*)	6

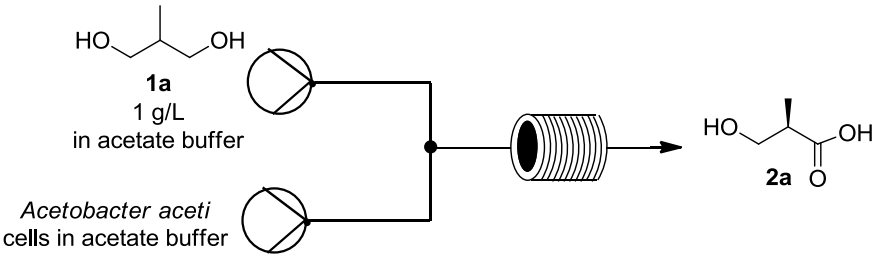
Table 2. Oxidation of achiral 2-substituted 1,3-diols **1–1c** (12 mM) with immobilized cells of *Acetobacter aceti* 2000/28 in conventional shaken flasks (batch mode). [a]Conversions calculated by chiral GC or HPLC and confirmed after work-up by ^{1}H-NMR of the crude mixture after the

indicated reaction time. [b]Enantiomeric excesses measured by chiral GC or HPLC analysis after treatment with CH_2N_2.

All the tested substrates gave the desired chiral 2-hydroxymethyl alkanoic acid as major product and only small amounts of the intermediate aldehydes (**6, 6a-6c**) were observed during the reaction; enantioselectivity was not affected by immobilization.[50] Reaction rates show a strong dependence on the steric hindrance at C2 position; interestingly, dried immobilized cells were much more selective than free cells.[50] The lack of side reactions can be justified considering that cells entrapment alters substrate and product diffusion through the solid support, as already observed in literature using similar systems.[49, 40]

With the batch results in hands, I then transferred the reaction in flow. As well as before, I optimized the reaction using compound **1**.

At the beginning, I used growing free cells in a tubular coil flow reactor to perform the oxidation, in order to evaluate if the oxygen dissolved in the aqueous flow stream was enough to make the reaction running. One flow stream was made by the free whole cells in acetate buffer (20 mM pH 6), while the other one was a 1 gr/L solution of substrate **1** in the same buffer. The two flow streams were mixed in a T-piece and directed in a 10 mL coil maintained at 28 °C (Scheme 6).



Scheme 6. Schematic representation of flow configuration with *Acetobacter aceti* free cells.

Despite different reactions times were tested, no product was observed under this condition. This is probably due to the lack of oxygen that is required by the biocatalyst. In fact, as reported in the Introduction (pag. 33), *A. aceti* is characterized by different membrane dehydrogenases which are associated to pyrroloquinoline quinone (PQQ) that during the reaction is reduced. So, it must be re-oxidized to let the reaction proceeding, and this happens in presence of oxygen. Probably, with the set-up explained above, the oxygen present in the system was not enough for the reaction. Therefore, I used in flow the immobilized dried alginate beads, in order to exploit the advantages of the immobilized biocatalysts, and I developed a suitable system to guarantee the oxygen supply.

To this aim, a segmented gas-liquid flow stream was used, in which the gas was compressed air (Figure 1).

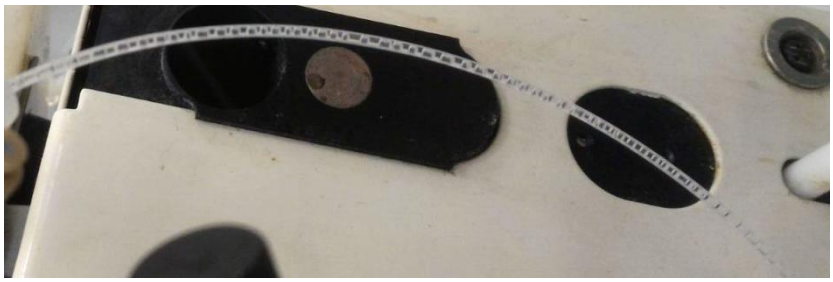


Figure 1. Air-liquid segmented flow stream.

The internal circulation in both phase A and phase B segments allows the passage of components between the two parts, and therefore oxygen can pass from the air stream to the liquid one (buffer), in this way getting in touch with the cell beads that have been packed into a reactor column.

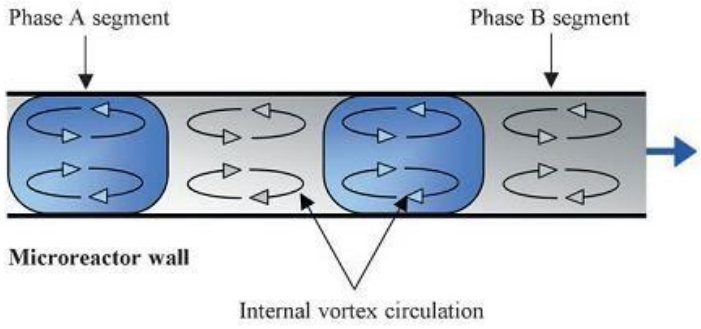
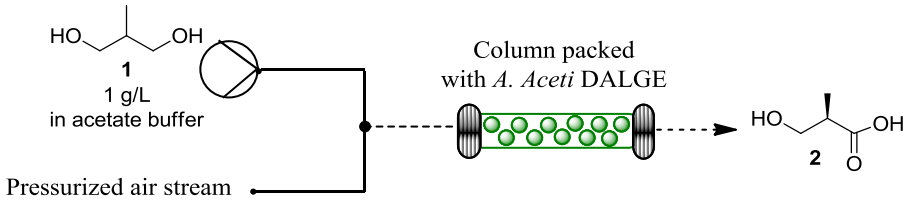


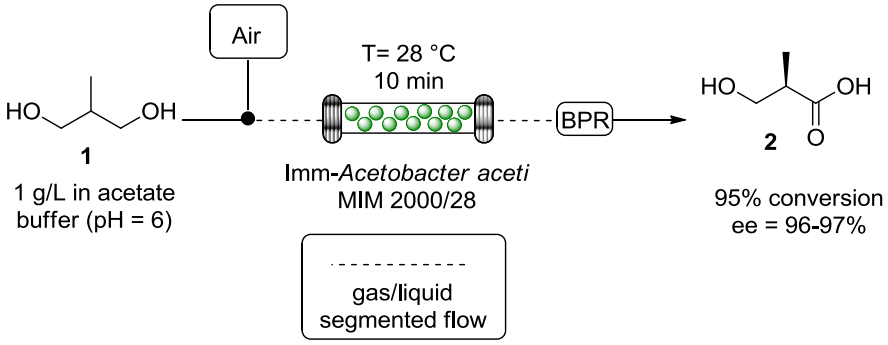
Figure 2. Segmented gas-liquid flow stream.

For the reaction, I first tried the simplest possible set-up (Scheme 7). One HPLC pump pumped 1 g/L 2-methyl 1,3 propandiol **1** solution in sodium acetate buffer (20 mM pH 6) and at a T-junction it met a pressurized air flow stream. Then the segmented stream entered an Omnifit glass column (i.d. 15 mm) packed with 400 mg of alginates (containing 100 mg of whole cells). The alginate beads were swelled before starting the reaction by flowing acetate buffer (20 mM pH 6) for 60 minutes at a flow rate of 400 µL/min. After this time, the alginates triplicated their volume and reached a packed volume of 5.3 mL. Importantly, no catalyst release was observed in the exiting flow stream. Temperature was kept at 28 °C.



Scheme 7. Representation of the flow set-up with segmented flow stream.

With this flow set-up, the segmented flow stream was not stable. Indeed, it was not possible to ensure a constant flow stream and this led to a very small conversion.
To obtain a constant flow of air, I used a mass flow regulator, that guarantees a constant flow rate once the parameters are set (Scheme 8). It was then connected to the T junction and it merged with the buffer solution of compound **1**. After the column with alginates, a backpressure of 5 PSI was applied to the system in order to stabilize the exiting flow stream.



Scheme 8. Biocatalyzed heterogeneous oxidation of prochiral 2-methyl-1,3-propandiol **1**.

With this configuration, I tried different residence times and different concentrations of compound **1**, keeping constant the other parameters (as reported above). Flow outcomes were analyzed by chiral GC after derivatization with diazomethane and then confirmed by ^{1}H-NMR. For each condition, I also calculated the specific reaction rate according to equation 1. In this thesis, the flow specific reaction rate will be always calculated according to the Eq.1.

$$r_{\text{flow}} = \frac{[P] \times f}{m_{\text{E}}} \ (\mu\text{mol/min g})$$

Eq. 1. r_{flow}: [P] (μmol/mL) concentration of the formed product, *f:* flow rate of the liquid phase (mL/min), m_{E}: mass of the biocatalyst expressed as dry weight of the cells employed (g dry weight).

Results are reported in the table below (Table 3):

Entry	[**1**] (mM)	Flow rate (mL/min)	r_{flow}[a] (mol/min gdry cells)	Molar conversion (%)[b]
1	12	0.015	1.80	> 97
2	12	0.030	3.60	> 97
3	12	0.060	4.68	65
4	24	0.015	3.60	> 97
5	24	0.030	7.20	>97
6	24	0.060	7.34	51
7	48	0.015	4,32	60
8	48	0.030	5.09	41
9	48	0.060	8.07	28

Table 3. Results obtained with different residence times and different concentrations of compound **1** at 28 °C. [a] Specific reaction rates were calculated according to Eq.1. [b] Conversions were determined by chiral GC or HPLC and confirmed with ^{1}H-NMR.

As it is possible to see from the Table 2, total conversion was obtained with substrate concentration of 12/24 mM (entries **2** and **5**) with both flow rates 15 and 30 μL/min, whereas at higher substrate concentrations and lower flow rates (entry **7**) the conversion was far from being complete. The best results were the ones obtained with 12 mM and 24 mM concentration of **1** at the flow rate of 30

µL/min. With this flow rate, the residence time inside the column was 10 minutes. For all the entries, one enantiomer ((*R*)-3-hydroxy-2-methylpropanoic acid **2**) was detected by chiral GC with an excellent *ee* = 97%.
This set-up was then tried on the other achiral 2-subtituted 1,3 diols (**1a-1c**), under optimized conditions found with compound **1**, in order to demonstrate the versatility of the flow protocol, using the diols already tested under batch conditions (Table 4).

Entry	Substrate	Conv. (%)	*ee* (%)
1	**1**	> 97	94 (*R*)
2	**1°**	> 97	59 (*R*)
3	**1b**	> 97	80 (*S*)
4	**1c**	> 97	88 (*S*)

Table 4. Oxidation of achiral 2-substituted 1,3-diols **1, 1a-1c** (24 mM) with immobilized *Acetobacter aceti* MIM 2000/28 in flow reactor. Liquid flow stream: 30 µL/min.

The continuous flow biotransformation of diols **1a-c** occurred with high rates and total conversion of the substrate into the desired chiral hydroxy acid, proving that the experimental protocol optimized in this work could be generally applied. The enantioselectivity remained the same obtained in batch.
I then tested the stability of the system and of the bioreactor under continuous work, using compound **1** as reference compound. Therefore, using the last shown set-up, I flowed 50 mL of 1g/L solution of compound **1** in acetate buffer through a glass column packed with 400 mg of alginates. The conditions in which I tested the stability were the ones reported in table 1, entry **2**. Here below a graphic summarizes the stability results (Figure 3).

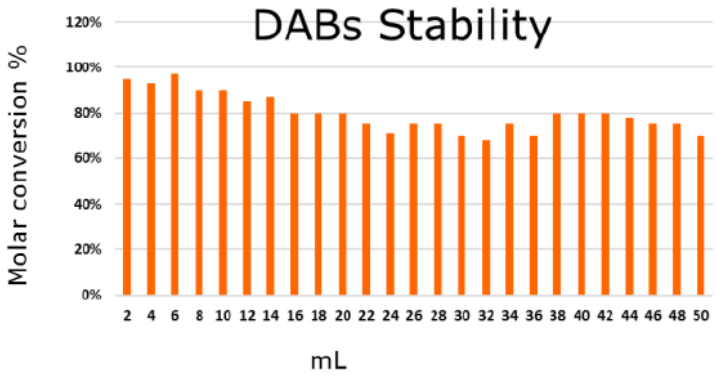
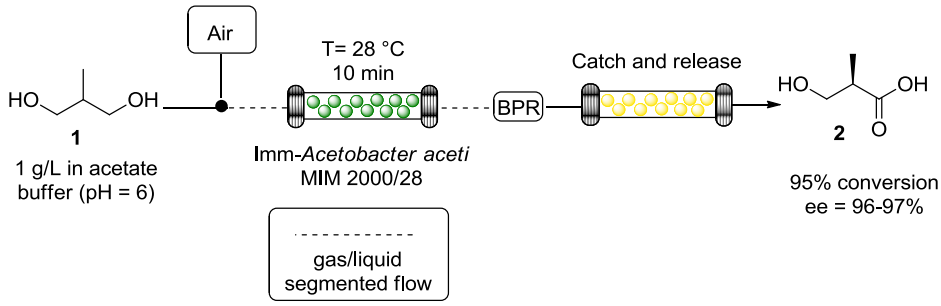


Figure 3. Stability of flow packed bed reactor with 400 mg of alginate beads under continuous work (1 g/L solution of compound **1**) in 10 minutes of residence time.

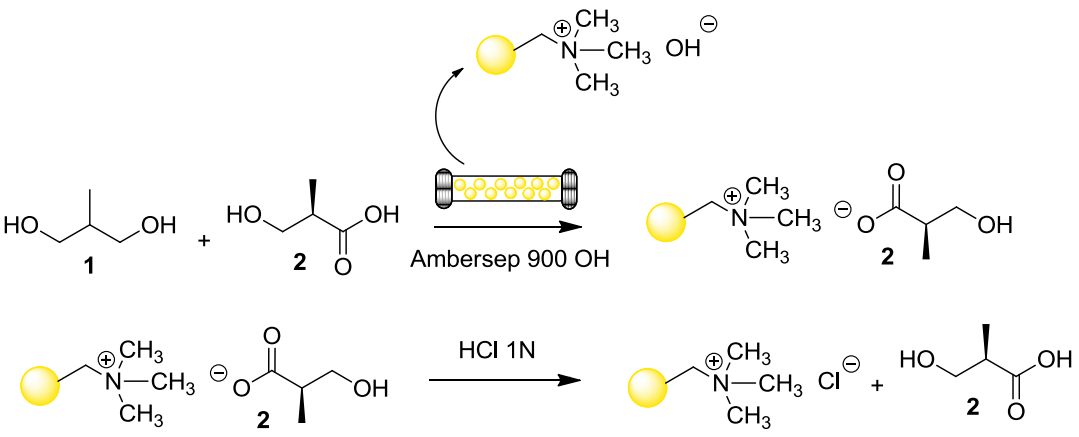
As it is possible to see from the graphic, the flow outcome was constant under 12h of continuous monitoring. Also the enantiomeric excess of product **2** was constant (97%).
Then I performed, after the reaction, the purification and isolation of product **2**. Firstly, I tried an in-line acidification and extraction with ethyl acetate, in order to obtain the desired product in the organic phase. So, one HPLC pump pumped the acidified exiting flow stream containing **2**, while the other pump pumped ethyl acetate. I tried different ratios between the two flow rates, trying to

force the extraction of the carboxylic acid, maintaining the pH of the aqueous solution around 1, but, unfortunately, all the attempts were not successful due to the high hydrophilicity of the product. Indeed, 10/12 extractions were required during normal work up in order to completely obtain **2** in the organic phase. Therefore, I used a catch and release strategy in order to isolate it from the water phase and to purify it from the any unreacted diol **1**. In order to do so, I inserted another Omnifit glass column packed with a strong basic resin, Ambersep 900 OH, at the exiting of the bioreactor in which the oxidation takes place, (Scheme 9).



Scheme 9. Representation of the final flow set-up including the catch and release column.

Different resins, as for example Ambersep 400 OH and Cl, or Ambersep 900 Cl, have been first tested in batch for the ability to trap the carboxylic acid, and, finally, 900 OH resulted to be the only one which was able to completely catch the carboxylic acid and to leave the diol in the buffer stream. Scheme 10 shows the used catch and release strategy.



Scheme 10. Catch and release of carboxylic acid **2** using Ambersep 900 OH.

After trapping the acid through the formation of an ionic interaction with the positively charged quaternary amine reactive group of the resin, the release was achieved using 1N HCl to recover the desired product **2**, in an aqueous solution. After lyophilization, product **2** was obtained as a pale yellow oil in 91% yield.

I also applied the same catch and release strategy on the others carboxylic acids synthesized (**2a-2c**).

Second step: chlorination reaction

The isolated mono carboxylic acid was then submitted to chemical reactions to synthesize Captopril. The second step of the protocol is the chlorination reaction using thionyl chloride reported in Figure 4.

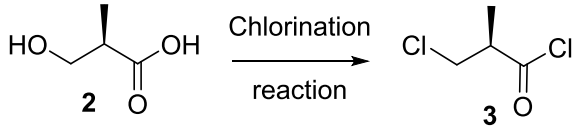
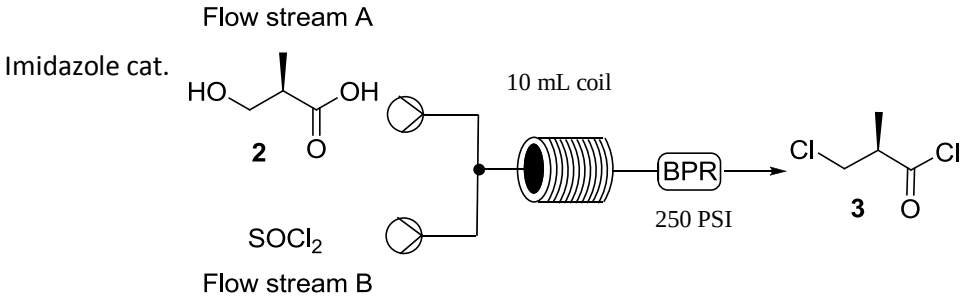


Figure 4. Second step of synthesis protocol.

This reaction meant to insert a chlorine atom in position 3 and to transform carboxylic acid into an acyl chloride. As mentioned above, to perform this reaction I considered the protocol developed by Shimazaki et al.[49] In this work, the chlorination reaction is performed using 2.5 equivalents of thionyl chloride in CH_2Cl_2 containing imidazole in a catalytic amount. Thionyl chloride was added dropwise at a temperature between 0 and 15 °C over a period of 30 minutes. Then, the reaction was stirred at 85 °C for about 2 h.

I first repeated this reaction in batch and I obtained, after removal of the solvent, compound **3** in a yield of 50 %. Remarkably, compound **3** was unstable and particular attention is required to handle it.

I then performed the reaction in flow, using the configuration reported in Scheme 11.



Scheme 11. Flow configuration of chlorination reaction.

A HPLC pump pumped a 1 M solution of compound **2** containing a catalytic amount of imidazole. Different solvents have been tested to prepare the initial solution. The second flow stream was made of a solution of $SOCl_2$. The equivalents of $SOCl_2$ have been considered during reaction optimization. The two flow streams met each other in a T-junction and entered a 10 mL coil heated at different temperatures. A 250 PSI backpressure regulator (BPR) was applied to the system to avoid the evaporation of the solvent at elevated temperatures. Using this set-up, I changed and optimized different parameters. Firstly, I selected the organic solvent to use. I tried dichloromethane (as in the batch procedure), THF and toluene. All the solvents were freshly distilled or dry. In all the cases, an addition of 15% v/v of DMF was required to better solubilize compound **2** and the imidazole. In fact, in flow, using HPLC pumps, it is really important to work with clear solutions, in order to avoid precipitates that can damage the pumps and block the entire system. Also, DMF acted as second catalyst, accelerating the reaction, because it forms the Vilsmeier reagent with a chloride atom coming from thionyl chloride. Among the solvents tried, dichloromethane was the

worst one for flow applications. In addition, according to what reported by Alfonsi et al.,[6] it is one of the most polluted and toxic solvents (it was also inserted in the Group 2A by the World Health Organization as probable carcinogenic to humans). For all these reasons, I decided to avoid its use. THF and toluene are both solvents which were considered as "usable".[6] The best between them was toluene because it allowed us to obtain a cleaner reaction profile and better results.
Secondly, I optimized the equivalents of thionyl chloride. I started with the same of the batch procedure (2.5 eq) and I then increased them up to 3.5 eq. This last amount was the necessary one, in order to have the chlorination in both the desired positions. In fact, the main problem of this reaction is not the formation of the acyl chloride (which is rapidly formed) but the substitution of the hydroxyl group with the chloride atom. It is important to underline that, exploiting the advantages of flow chemistry, the potential danger of this exothermic reaction and gas (HCl) releasing was mitigated in continuous, because each time only a small amount of thionyl chloride passes through the channels and reacts with a solution of compound **2**. Indeed, no refrigeration neither slow addition of $SOCl_2$ were required in flow, as necessary in batch procedure. So, even if a higher use of thionyl chloride is required in flow, this does not represent a problem in terms of safety.
I also tried different temperatures and different reaction times. The results are reported in Table 5.

Entry	*T* [°C]	t_r [min]	Conversion **3**[a] [%]
1	85	60	80
2	100	60	100
3	110	30	100
4	125	15	70
5	150	15	70
6	150	20	80
7[b]	100	30	100
8[b]	100	15	85

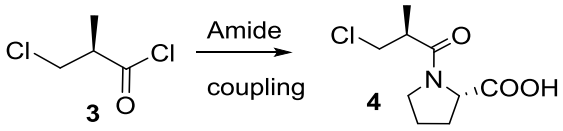
Table 5. Results of the chlorination reaction in flow changing temperatures and residence times. Conditions (entries **1**-**6**): flow stream A: 1M solution of compound **2** in anhydrous toluene with a catalytic amount of imidazole and 15% DMF v/v; flow stream B: 3.5 eq of $SOCl_2$ in anhydrous toluene. [a] Conversions were determined by ^{1}H NMR after removal of the solvent. [b] For these two entries, 1M solution of compound **2** and 1% imidazole were used in DMF; flow stream B was neat $SOCl_2$.

As it is possible to see from the Table 4, a total conversion was achieved in two cases (entries **2** and **3**) if anhydrous toluene was used as organic solvent to prepare the solutions. A 100% conversion was also reached if DMF was used to prepare solution of compound **2** and neat thionyl chloride acted as second flow stream (entry **7**). Due to the easier product recovery, toluene was chosen as the reaction solvent.

After removal of the solvent, the pure product was collected (purity > 95% determined by ^{1}H NMR) as an oil with an 80% isolated yield. Importantly, the (*R*) configuration of the methyl group in position 2 was maintained, in batch as well as in flow.

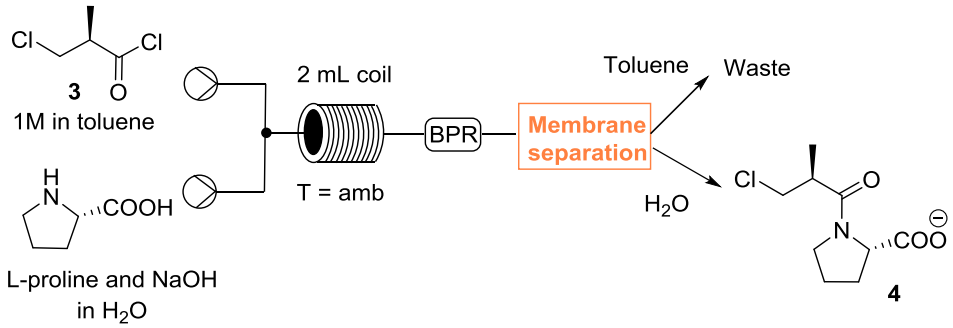
Third step: amide coupling

The next step of the synthesis is an amide bond formation represented in Scheme 12.



Scheme 12. Schematic representation of amide coupling step.

Following the procedure reported in the article of Shimazaki et al[49], I first performed the reaction in batch, where a solution of L-proline was prepared in NaOH 2N and compound **3** (1 eq) was added at 0 °C; the mixture was stirred for 1h at this temperature. Then, the reaction was stirred for another hour at room temperature. After acidification and extraction with ethyl acetate, the solvent was removed, a chromatographic column was made and compound **4** was obtained in 50% yield.
Then, I moved the reaction to flow (Scheme 13).



Scheme 13. Schematic representation of flow configuration for amide coupling.

In view of a continuous flow protocol, I decided to prepare the solution of compound **3** in toluene in a 1M concentration. Therefore, a biphasic liquid-liquid system made by water (a solution of L-proline in aqueous NaOH) and toluene was formed. One HPLC pump pumped a 1M solution of compound **3** in toluene, while the second pump pumped a water solution at different concentrations of L-proline and different concentrations of NaOH. The two solutions entered a 2 mL coil maintained at room temperature. Again in view of a continuous protocol, a 250 PSI backpressure was applied to the system.
For this reaction, I focused the attention mostly on the concentration of L-proline and NaOH and the residence time. Therefore, first I tried with the same equivalents used in the batch procedure (1 eq. of L-proline and 2 eq. of NaOH), but unfortunately I did not obtain full conversion. So, I decided to push the reaction by using 2 equivalents of L-proline and 3 equivalents of NaOH. Again, the advantages of flow chemistry allowed us to better and safer handling these reagents, even at

higher concentrations. Remarkably, there was no need of cooling the system when the two flow streams met at the T-junction, as happened for the batch procedure. The increase of equivalents was successful. Two equivalents of sodium hydroxide were necessary to deprotonated the L-proline, while the left equivalent was necessary to neutralize the HCl formed during the reaction.
For what concerns the residence time, using the optimized equivalents, I first tried 30 minutes. I observed that the reaction was complete and, consequently, I decreased the residence time. The reaction turned out to be complete in only 1 minute.
In order to separate in line the water phase (containing the desired product **4**, which is de-protonated for the basic pH of the water solution) from the organic phase (Scheme 6), I used a continuous Zaiput liquid-liquid separator (Figure 13).

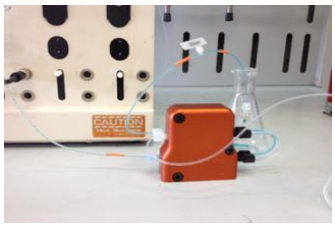
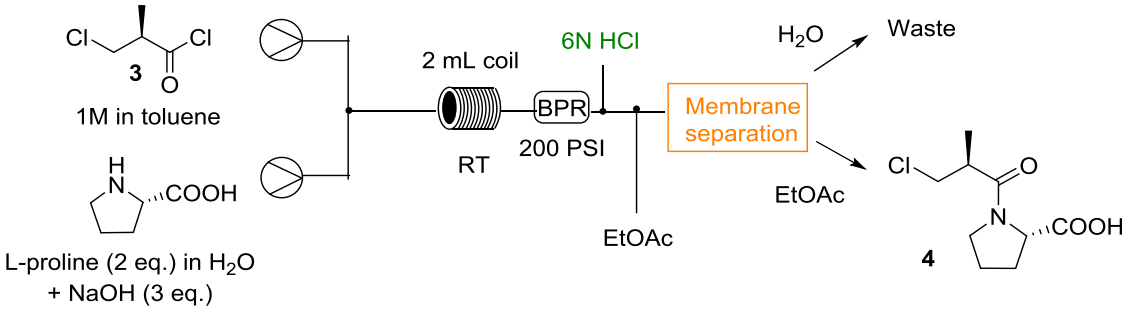


Figure 13. Zaiput liquid-liquid separator.

This separator is commercially available and it works thanks to a hydrophobic membrane inside the device. The reaction flow stream can enter the separator, and, here, the organic phase passes through the hydrophobic membrane, while the aqueous one is retained. In this way, the two phases can be separated and collected in two different flasks.
So, I obtained the desired product **4** in the water phase. I then tried to perform an in-line acidification and extraction with ethyl acetate of compound **4** (Scheme 14).

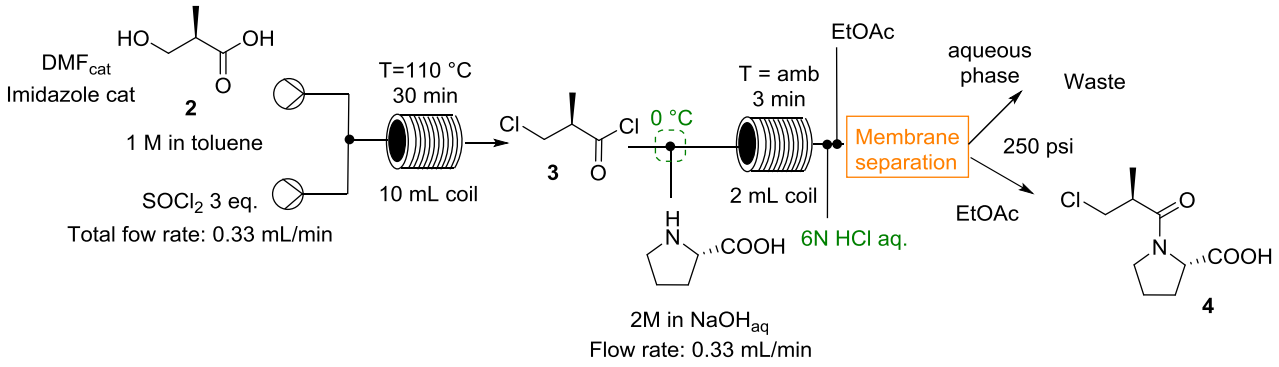


Scheme 14. In-line acidification and extraction with ethyl acetate of compound **4.**

In this way, it was possible to avoid the traditional work-up procedures that normally require more solvents and produce more waste. Therefore, I inserted an inlet of HCl 6N to the water phase line and this acidified flow stream met at a T junction an ethyl acetate flow. The mixture entered a 2 mL coil and then the two phases were separated again using a Zaiput liquid-liquid separator. In the end, I completely collected product **4** in the acetate phase, and, after removal of the solvent, I obtained it in a yield of 80%. Remarkably, no chromatographic column was required to purify compound **4**. The configuration at position 2 was retained.

Two sequential steps: second and third steps in continuous

After the optimization of the first two steps, I afforded to link them together, starting from compound **2** and obtaining in a continuous way compound **4** (Scheme 15).



Scheme 15. Schematic representation of the first two sequential steps.

For the chlorination reaction, I used the conditions reported in Table 5 entry **3** (100 % conversion in 30 minutes at 110 °C), because, compared to entry **4**, the residence time was lower and compound **3** was equally pure, even if the temperature was higher. In order to link the steps together, some modifications were required. Indeed, in a continuous way, everything has to be designed so that the excess of reagents and reaction byproducts from each reaction are compatible with the downstream reactions. Therefore:

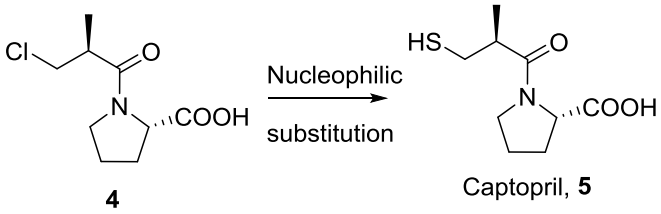
- The T-junction between the first and the second step was maintained at 0 °C; this was necessary in order to be completely safe, since at this point thionyl chloride meets water, and in this way the uncontrolled exothermic neutralization and the release of large quantities of HCl vapors were easily controlled. Of course, this adverse situation was also mitigated thank to the advantages of flow chemistry, as discussed above;
- A modification of the equivalents of NaOH was required. Indeed, sodium hydroxide was necessary in order not only to de-protonate L-proline and to neutralize the formed HCl, but now it must neutralize also the un-reacted $SOCl_2$. If the pH of the water solution is acid, no coupling reaction can occur. If it was neutral or slightly basic, the reaction runs very well. So, after many attempts, I found that 7 equivalents were the minimum amount of NaOH that must be used;
- The residence time of the second step increased from 1 to 3 minutes. This was an automatically modification considering the flow rate coming from the first step.

After the chlorination step, compound **3** met directly, without any isolation or purification, the flow stream composed by L-proline and NaOH in water (Scheme 8). In the end, the liquid-liquid separator was used again in order to separate toluene from the water phase, and for the acidification and extraction with ethyl acetate. In this way, product **4** was collected in the organic phase and separated from all the impurities and neutralized reagents coming from the two steps, that are in water phase. A 250 PSI backpressure regulator was applied to the entire system. After removal of ethyl acetate, compound **4** was obtained as a pale yellow oil in a yield of 70%. Again, no

chromatographic column was required to purify the desired product. A very important and interesting thing to underline is that, with this protocol, no isolation of the unstable intermediate **3** was required, and, therefore, the yield was increase, since it was not manipulated and it was retained inside system and directly reacted in the next coupling step. Despite the required modifications, the absolute configuration of the two stereocenters was retained, since optical rotation values remained the same as before.

Fourth step: nucleophilic substitution

The last reaction for the obtainment of Captopril is the nucleophilic substitution of the chlorine with a thiol group (Scheme 16).



Scheme 16. Representation of the nucleophilic substitution reaction.

Following the reported procedure,[49] the batch reaction was performed preparing a DMF solution of compound **4** and 3 equivalent of NaHS. The mixture was stirred for 4 h at 50 °C under nitrogen, and after a work-up and a chromatographic column, Captopril was obtained as a white solid in 40% yield. The main impurities that were detected during this reaction were the possible oxidative products, obtained through the formation of a disulfide bond or a thiolether. These dimers were formed due to the presence of the free thiol group (Figure 14).

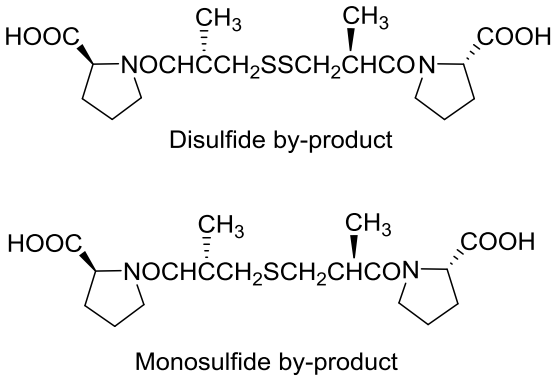
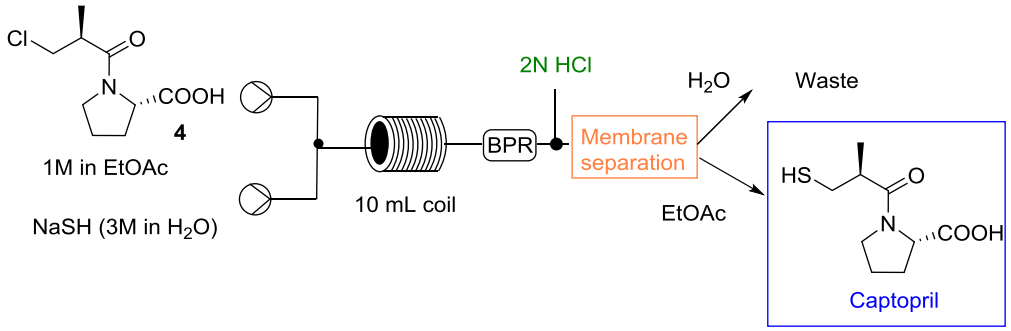


Figure 14. By-products formed during the nucleophilic substitution.[49]

Then, I transferred the reaction in flow (Scheme 17).



Scheme 17. Schematic representation of the flow configuration used for the nucleophilic substitution with NaHS.

A HPLC pump pumped a 1M solution of compound **4** in ethyl acetate. I decided to try first this solvent because, in view of a continuous protocol, compound **4** would be in an ethyl acetate solution. The second flow stream is composed by a solution of 3 equivalents of NaHS in degassed water, therefore, a biphasic liquid-liquid system was formed. Water was degassed in order to limit the presence of oxygen in the solution and therefore to avoid the formation of the oxidative by-products observed in batch. After mixing in a T-piece, the two flow streams entered a 10 mL coil reactor. In view of a continuous protocol, a 250 PSI backpressure regulator was applied to the system. Different temperatures and residence times were tested in order to achieve total conversion (Table 6).

Entry	*T* [°C]	*Tr* (min)	**5**[a] (%)
1	50	60	70
2	80	60	90
3	100	60	93
4	125	30	100
5	125	15	75

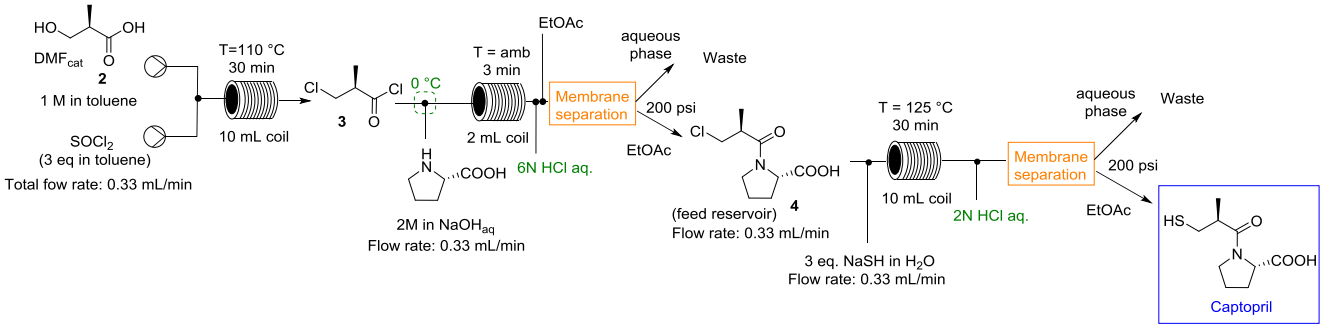
Table 6. Reported conversions obtained for compound **5** (Captopril) with different temperatures and residence times.[a] Conversions were determined by ^{1}H-NMR.

As it is possible to see from Table 6, a complete conversion was achieved in 30 minutes at 125 °C (entry **4**).

For this reaction as well, I decided to perform an in-line acidification and extraction of Captopril using the Zaiput liquid-liquid separator (Scheme 17). So, the flow outcome was acidified with HCl 2N in order to protonate the carboxylic acid and to extract Captopril from the water phase. The organic phase was ethyl acetate, since it was already the solvent used for the reaction. The extraction was successful and the desired product was completely collected in the organic phase, while some of the other impurities remained in the water phase. After removal of the solvent, a chromatographic column was necessary at this point to completely purify the product (in fact, some oxidative by-products have been formed during the reaction). After the column and the crystallization, Captopril was obtained as a white solid with a yield of 80%. ^{1}H NMR and the optical rotation values match the ones reported in literature.[52]

Continuous flow-synthesis of Captopril

Finally, I linked the last reaction (nucleophilic substitution with NaSH) to the continuous process. In the Scheme 18, it is possible to see the entire synthesis developed.



Scheme 18. Schematic representation of the continuous flow synthesis of Captopril.

In the continuous protocol, after the amide coupling reaction and its acidification and extraction with in-line liquid-liquid separator, compound **4** in acetate acted as feed reservoir and it was used without handling or modification for the next and last step. A backpressure regulator of 250 PSI was applied to the system. The optical rotation confirmed that the absolute configuration of the two stereocenters was retained along all the steps. In the end of the protocol, after crystallization, Captopril was obtained with an overall yield of 55-60% over 2h of work. As said before, only one final chromatographic column was required to obtain the pure product. This continuous flow protocol was robust and could run for multiple hours without interruption. Indeed, I was able to scale-up the system until the gram scale (1-2 grams of product was recovered by running the system for 1-3 days).

Conclusions

The multistep flow synthesis presented here enabled Captopril to be obtained in an overall yield of 55-60% after crystallization in an overall time of about 2h, while with the batch procedure an overall yield of 45% was achieved after 3 days of work, which include 2 work-ups and two chromatographic columns. In contrast, by exploiting the flow protocol, only one final purification column was necessary to obtain pure Captopril. This underlines some of the most important advantages of flow chemistry, which are the possibility to perform in line quenching and work ups to obtain cleaner synthesis and cleaner products, thus reducing solvents and time spent in the purification procedures. Simultaneously, yields are higher, compared to traditional protocols. The biocatalytic oxidation allowed the conversion of a prochiral substrate into a chiral intermediate with high enantiomeric excess. Three chemical transformations were performed without isolation of the intermediates. The separation of co-products, by-products, and excess reagents was achieved in-line. In this way, compared to the traditional strategies, it was possible to reduce solvent waste and time consuming. These results clearly highlight the benefits of performing multistep chemical synthesis in a flow environment. As a further advantage, our synthetic protocol benefits from the use of an environmentally friendly biocatalytic oxidation, avoiding the use of toxic chemical oxidants. In this context, desymmetrisation of achiral 1,3-diols with *Acetobater aceti* was studied

and developed in a flow chemistry reactor, taking advantage of the previous batch results that showed an efficient procedure for the preparation of enantiomerically enriched hydroxymethyl alkanoic acids.[50] Batch reaction with free cells carried out in conventional shake flasks showed also formation of the corresponding α-methylenic alkanoic and α-methyl alkanoic acids. Immobilization in Ca-alginate, beyond the advantages of easier work up and potential catalyst reusability, was found to be an excellent tool for improving the selectivity of the reactions with respect to the use of cell free systems. As further improvement, the oxidation of diols **1, 1a-1c** was tested under continuous-flow conditions, exploiting the application of alginate beads in a packed-bed reactor. A segmented air-water flow regime (which ensured high mass transfer between the gaseous and the liquid phase) was applied allowing for high rates.

The bioprocess might be further linked to the chemical transformations by optimizing the extraction of acid **2** with organic solvents, for example, using specific organic-acid-complexing carriers.[53] In view of a future scale-up, whereas none of the chemical steps have foreseen limitations, further efforts should be directed toward increasing the productivity of the first biocatalyzed step, which at present represents a bottleneck in the process. The new proposed protocol for the synthesis of Captopril represents a powerful integration of biocatalysis and flow-chemistry technology.

Material and methods

General

All reagents and solvents were purchased from Sigma–Aldrich. The continuous flow reactions were performed using a commercial R2C/R4 flow reactor (Vapourtec, Bury St. Edmunds, Suffolk, UK) equipped with Omnifit glass columns (15 mm i.d. V 100 mm length) and PFA reactor coils (2 and 10 mL, respectively). The R2C unit is the pumping unit that contains two adapted Knauer pumps, which are able to pump highly concentrated and corrosive acids. R4 is the heating unit with four heating positions. The additional HPLC pumps necessary to perform the overall synthesis were provided by another R2+/R4 flow reactor (Vapourtec) and by two external pumps (ThalesNano). The temperature sensor sits on the wall of the PFA tubing. The pressure was controlled by using two 100 psi BPRs. In-line liquid–liquid extractions were performed using a Zaiput separator. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (d) are expressed in ppm, and coupling constants (J) are expressed in Hz. The molar conversion of the biotransformation was determined by HPLC analysis using a Luna NH_2 100 Ä column (250 mmV4.6 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) and a Biorad refractor index detector with an acidic aqueous KH_2PO_4 buffer (20 mM, pH 2.7) as the mobile phase (flow rate 0.2 $mLmin^{-1}$). The samples (40 mL) were injected as soon as collected and without further treatment. The enantiomeric composition of **2** was determined by gas chromatographic analysis of the corresponding methyl ester [methyl (*R*)-3- hydroxy-2-methylpropionate: tr=3.6 min], obtained after treatment with diazomethane, using a chiral capillary column (diameter 0.25 mm, length 25 m, DMePeBeta-CDX-PS086, MEGA, Legnano, Italy). Optical rotation determinations were performed using a Jasco P-1010 spectropolarimeter coupled with a Haake N3-B thermostat. MS analyses were performed on a Varian 320-MS triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. Microanalyses (C, H, N) were within: 0.4% of theoretical values.

Strain preparation

The *A. aceti* MIM 2000/28 strain was routinely maintained on GYC agar plates [glucose (50 gL^{-1}), yeast extract (10 gL^{-1}), CaCO3 (30 gL^{-1}), agar (15 gL^{-1}), pH 6.3] at 28 °C. Strain was inoculated into 100 mL Erlenmeyer baffled flask containing GLY medium [yeast extract (10 gL^{-1}) and glycerol (25 gL^{-1}), pH 5; 20 mL]. After growth for 24 h (shaking at 150 rpm, 28 °C), the liquid culture was entirely used to inoculate a 1 L Erlenmeyer baffled flask containing GLY medium (150 mL). Flasks were grown for 24 h at 28 °C, with shaking at 150 rpm.

Preparation of Dry Alginate Beads

Gel beads were prepared by ionotropic gelation, by following a protocol previously developed by us[10]: a 4% (w/v) sodium alginate solution was prepared in distilled water and stirred until a homogeneous clear solution was formed. The solution was allowed to settle for 2 h in order to eliminate the air bubbles. The alginate solution was then gently mixed in a 1:1 (w/w) ratio with a suspension of *A. aceti* cells (40 $ODmL^{-1}$) in sodium acetate buffer (20 mm, pH 6). The resulting

mixture was then pumped dropwise into a slightly agitated $CaCl_2$ solution (0.2m). Calcium alginate beads were agitated for 20 min, then filtered, washed with deionized water and dried at 25 °C for 16 h.

General batch procedure for the preparation of mono-carboxylic acids (2,2a-2c)

A 12 mM solution of compounds **1-1c** was prepared in acetate buffer pH 6.0. A concentration of 40 mg/mL of alginates was used (containing 10 mg of whole cells). The reaction was maintained at 28 °C. The reaction was followed by HPLC. After 120 minutes, the mixture was acidified until pH 2 and it was extracted with ethyl acetate (10/12 times). The organic phase was dried on Na_2SO4 and the solvent was evaporated under reduced pressure. Enantiomeric excesses were detected by chiral GC after derivatization with diazomethane or by chiral HPLC.

General flow procedure for the preparation of mono-carboxylic acids (2,2a-2c)

Dry alginate beads (400 mg, containing 100 $mg_{dry\ weight}$ of cells of *Acetobacter aceti*) were packed into a glass column (i.d. 15 mm X 100 mm lenght) and swelled until their volume tripled by flowing acetate buffer (20 mm, pH 6) through the column (flow rate: 400 $mLmin^{-1}$, 60 min). The final volume of the bed was 5.1 mL. Air was delivered at 17 psi; its flow was measured and controlled thank to a mass flow controller device, that inlet into the system a continuous air flow stream at 6 mL/min. A buffer solution of **1-1c** (24 mM in acetate buffer 20 mM pH 6.0) was pumped at 30 $mLmin^{-1}$, joining the airflow at the T-junction, before entering the column in which the oxidation occurs in approximately 10 min. A BPR (5 psi) ensured a constant and controlled biphasic air-liquid flow. The exiting flow stream was directed into a column filled with Ambersep 900 OH resin (2 g) and, after washing the column with water (20 mL, 0.5 $mLmin^{-1}$), the trapped acid was released by flowing HCl (1N, 5 mL). Reactions were followed by HPLC and *ee* were calcualated with chiral GC after derivatization with diazomethane or with chiral HPLC.

(*R*)-(Hydroxymethyl) propanoic acid (2): $[\alpha]_D^{20}$ = -11.55 (c=1.00 in EtOH); *ee*=94%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22 (d, *J*=7.4 Hz, 3H), 2.10 (s, 1H), 2.68–2.80 (m, 1H), 3.75 (d, *J*=6.0 Hz, 2H), 5.70 ppm (br s, 1H); ^{13}C NMR (75 MHz, CDCl3): δ = 13.2, 41.6, 64.0, 180.1 ppm.[54] HPLC analysis: 1, t_r=22 min; 2, t_r=18 min.
(*R*)-2-(Hydroxymethyl) butanoic acid (2a): $[\alpha]_D^{20}$=−2.8 (*c*=0.50, CHCl3), *ee*=54%; ^{1}H NMR (300 MHz, $CDCl_3$): *δ*=3.90–3.70 (2H, m, CH_2OH), 2.56 (1H, m, *CH*COOH), 1.80–1.55 (2H, m, CHCH_2), 1.00 ppm (3H, t, *J*=7.6 Hz, CH_2CH_3); 13C NMR (75 MHz, $CDCl_3$): *δ*=180.4, 62.7, 49.0, 21.6, 11.8 ppm.[55]
(*S*)-2-(Hydroxymethyl) hexanoic acid (2b): [$\alpha]_D^{20}$=−5.1 (*c*=0.75, CH_3OH), *ee*=81%; ^{1}H NMR (300 MHz, $CDCl_3$): *δ*=3.85–3.75 (2H, m, CH_2OH), 2.61 (1H, quint., *J*=5.9 Hz, C*H*COOH), 1.75–1.65 (1H, m, CHC*H*HCH$_2$), 1.60–1.45 (1H, m, CHC*H*HCH$_2$), 1.40–1.28 (4H, m, CHCH$_2$(CH_2)$_2$), 0.91 ppm (3H, t, *J*=6.4 Hz, CH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): *δ*=180.1, 63.2, 47.9, 29.8, 28.4, 22.7, 14.0 ppm.[56] Chiral GC: DAcTBSil BetaCDX, 60 °C/0.8 °C min^{-1}/90 °C/30 °C min^{-1}/220 °C (2 min), *t*R (*R*)-**2b**=29.9 min, *t*R (*S*)-**2b**=30.6 min.

(S)-2-(Hydroxymethyl) heptanoic acid (2c): $[\alpha]_D^{20}$= -2.1 (*c*=2.3, $CHCl_3$), *ee*=92%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ=3.85–3.70 (2H, m, CH_2OH), 2.59 (1H, quint, *J*=5.9 Hz, CHCOOH), 1.70–1.57 (1H, m, $CHCHHCH_2$), 1.55–1.42 (1H, m, $CHCHHCH_2$), 1.40–1.20 (6H, m, $CHCH_2(CH_2)_3$), 0.88 ppm (3H, t, *J*=6.5 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$, 75.0 MHz): δ=179.6, 63.2, 47.9, 31.8, 28.4, 26.9, 22.5, 14.0 ppm.

Synthesis of (*R*)-3-Chloro-2-methylpropanoyl Chloride (3)

Batch procedure[49]

Thionyl chloride (1.42 gr, 12 mmol) was added dropwise to a solution of **2** (500 mg, 4.80 mmol) in CH_2Cl_2 (480 µL) containing imidazole (25 mg, 0.37 mmol) as a catalyst with stirring at 0-15 °C over a period of 30 minutes. The reaction mixture was then warmed to 80 °C. Stirring was continued until gaseous evolution of hydrogen chloride and sulfur dioxide subsided. The reaction was followed by TLC (1:1 CHX/EA; R_f of **3** = 0.86). After removal of the solvent and excess of thionyl chloride on a rotary rotavapor at 40 °C, (*R*) 3-Chloro-2-methylpropanoyl Chloride **3** was obtained as a pale yellow oil. Yield=50%; $[\alpha]^{20}_D$= - 5.63 (c=2.0, CH_2Cl_2) ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.42 (d, 3H), 3.20–3.31 (m, 1H), 3.66–3.80 ppm (m, 2H).

Flow procedure

A solution of **2** (52 mg, 0.5 mmol) was prepared in anhydrous toluene (410 mL). Imidazole (3.5 mg, 0.1 equiv) and DMF (50 mL) were added to the solution. A second solution of thionyl chloride (125 mL, 3.5 equiv) was prepared in anhydrous toluene (375 mL). The two solutions were mixed into a T-piece and flowed through a 10 mL reactor coil according to the conditions reported in Table 2. Reactions were followed by TLC (1:1 CHX/EA, R_f of **3** = 0.86). A 250 psi backpressure regulator was applied to the system. The exiting solution was collected, the solvent was evaporated under reduced pressure to yield **3** as a crude oil, which was analyzed by ^{1}H NMR spectroscopy. Yield=50%; $[\alpha]^{20}_D$= - 5.63 (c=2.0, CH_2Cl_2); ^{1}H NMR (300 MHz, CDCl3): δ= 1.42 (d, 3H), 3.20–3.31 (m, 1H), 3.66–3.80 ppm (m, 2H).

Synthesis of (*S*)-1-[(*S*)-3-Chloro-2-methylpropanoyl] pyrrolidine-2-carboxylic Acid (4)

Batch procedure[49]

Compound **3** (350 mg, 2.48 mmol) was added in one portion to a cold solution of L-proline (285.5 mg, 2.48 mmol) in 2N NaOH (2.5 mL) at 0 °C, and the resulting mixture was stirring at the same temperature for 1h, then allowed to room temperature for another hour. A TLC was made to follow the reaction (95:5 DCM/MeOH + 4 drops of acetic acid; R_f of **4** = 0.43). The reaction mixture was adjusted to pH 1 with HCl 6N, extracted with ethyl acetate (3 times) and dried over anhydrous Na_2SO_4. The solvent was reduced under vacuum at 40 °C and a chromatographic column was made in order to yield compound **4** as a crude solid (99:1 DCM/MeOH). Yield= 50%; $[\alpha]^{20}_D$ = -103.6 (c=1 in EtOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.24 (d, 3H, J=7.0), 2.02–2.18 (m, 4H), 2.96–3.08 (m, 1H), 3.44–3.51 (m, 1H), 3.57–3.72 (m, 2H), 3.81 (t, 1H, J=10.4), 4.62–4.69 (m, 1H), 11.30 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=15.8, 24.9, 27.7, 41.3, 45.8, 47.7, 59.6, 173, 175 ppm; MS (ESI): m/z: 217.9 $[M–H]^-$; elemental analysis calcd (%) for $C_9H_{14}ClNO_3$: C 49.21, H 6.42, N 6.38; found: C 49.00, H 6.31, N 6.50.

Flow procedure

A solution of compound **3** (70 mg, 0.5 mmol) in toluene (0.5 mL) was prepared. A second solution of L-proline (115 mg, 2 equiv) and NaOH (60 mg, 3 equiv) was prepared in water (0.5 mL). The two solutions were flowed at a total rate of 2 $mLmin^{-1}$ through a 2 mL reactor coil, that had been washed with a mixture of toluene and water, at room temperature. The aqueous phase was acidified to pH 2 by introducing a flow of HCl (6N, 1 $mLmin^{-1}$) and the resulting biphasic system was mixed using a T-junction into an ethyl acetate stream (1 $mLmin^{-1}$) to perform in-line extraction. The two phases were separated in-line using a Zaiput liquid–liquid separator. A 250 psi backpressure was applied to the system. The organic solvent was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield **4** as a crude solid, which was analyzed by ^1H NMR spectroscopy. Reactions were followed with TCL (95:5 DCM/MeOH + 4 drops of acetic acid; R_f of **4** = 0.43) Yield=80% $[\alpha]^{20}_D$ = -103.6 (c=1 in EtOH); ^1H NMR (300 MHz, $CDCl_3$): δ=1.24 (d, 3H, *J*=7.0), 2.02–2.18 (m, 4H), 2.96–3.08 (m, 1H), 3.44–3.51 (m, 1H), 3.57–3.72 (m, 2H), 3.81 (t, 1H, *J*=10.4), 4.62–4.69 (m, 1H), 11.30 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=15.8, 24.9, 27.7, 41.3, 45.8, 47.7, 59.6, 173, 175 ppm; MS (ESI): m/z: 217.9 $[M–H]^-$; elemental analysis calcd (%) for $C_9H_{14}ClNO_3$: C 49.21, H 6.42, N 6.38; found: C 49.00, H 6.31, N 6.50.

Synthesis of (*S*)-1-[(*S*)-3-Chloro-2-methylpropanoyl] pyrrolidine-2-carboxylic Acid (Captopril, 5)

Batch procedure[49]

A mixture of **4** (130 mg, 0.59 mmol) and NaHS (134 mg, 1.77 mmol) in DMF (1.2 mL) was stirred for 4 h at 50 °C under nitrogen. The reaction was followed by TLC (9:1 DCM/MeOH + 4 drops of acetic acid, R_f of **5** = 0.69). The reaction was then cooled and acidified with HCl 6N until pH 1 and the desired product was then extracted with ethyl acetate (3 times). The organic phase was then dried over anhydrous Na_2SO_4 and evaporated under vacuum at 40 °C. After a chromatographic column (98:2 DCM/MeOH), compound **5** was obtained as a white solid with a yield of 40%. $[\alpha]^{20}_D$ = -128.5 (c=1 in EtOH); lit= - 129.4 (c=1.35 in EtOH at 22 °C)[12] ^1H NMR (300 MHz, $CDCl_3$): δ=1.24 (d, *J*=7.0 Hz, 3H), 1.58 (t, *J*=9.2 Hz, 1H), 2.02–2.18 (m, 4H), 2.43–2.51 (m, 1H), 2.79–2.90 (m, 2H), 3.55–3.71 (m, 2H), 4.62–4.69 (m, 1H), 11.30 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=17.0, 20.8, 24.7, 27.4, 42.5, 47.4, 59.2, 173.0, 175.0 ppm; MS (ESI): m/z: 215.9 $[M–H]^-$; elemental analysis calcd (%) for $C_9H_{15}NO_3S$: C 49.75, H 6.96, N 6.45; found: C 49.60, H 6.89, N 6.52.

Flow procedure

A solution of compound **4** (22 mg, 0.1 mmol) in toluene (0.1 mL) was prepared. A second solution of NaSH (21 mg, 3 equiv) was prepared using degassed water (0.1 mL). The two solutions were flowed through a 10 mL reactor coil. A 250 psi backpressure was applied to the system. Reaction time and temperature were optimized, as reported in Table A. Reactions were followed by TLC (9:1 DCM/MeOH + 4 drops of acetic acid, R_f of **5** = 0.69). The aqueous phase was acidified at pH 2 by introducing a flow of HCl (2N) and the resulting biphasic system was mixed using a T-junction into an EtOAc stream to perform in-line extraction. The two phases were separated in-line using a Zaiput liquid–liquid separator. The organic solvent was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. After a chromatographic column (98:2 DCM/MeOH), **5** was obtained as a white solid, which was analyzed by ^1H NMR spectroscopy. Yield=80%; $[\alpha]^{20}_D$ = -

128.5 (c=1 in EtOH); lit= - 129.4 (c=1.35 in EtOH at 22 °C)[12] ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.24 (d, *J*=7.0 Hz, 3H), 1.58 (t, *J*=9.2 Hz, 1H), 2.02–2.18 (m, 4H), 2.43–2.51 (m, 1H), 2.79–2.90 (m, 2H), 3.55–3.71 (m, 2H), 4.62–4.69 (m, 1H), 11.30 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=17.0, 20.8, 24.7, 27.4, 42.5, 47.4, 59.2, 173.0, 175.0 ppm; MS (ESI): m/z: 215.9 $[M–H]^-$; elemental analysis calcd (%) for $C_9H_{15}NO_3S$: C 49.75, H 6.96, N 6.45; found: C 49.60, H 6.89, N 6.52.

Three-Step Continuous-Flow Synthesis of Captopril (5)

A solution of **2** (156 mg, 1.5 mmol) in anhydrous toluene (1.23 mL) was prepared. Imidazole (10.5 mg, 0.1 equiv) and DMF (150 mL) were added to the solution. A second solution of thionyl chloride (375 mL, 3.5 equiv) was prepared using anhydrous toluene (1.12 mL). The two solutions were flowed through a 10 mL coil reactor that was maintained at 110 °C, with a residence time of 30 min (total flow rate 0.33 $mLmin^{-1}$). Then, the exiting flow was merged at a T-junction with an aqueous solution (1.5 mL) of L-proline (2 equiv) and NaOH (7 equiv). This then entered a 2 mL reactor coil, maintained at room temperature with a residence time of 3 min (total flow rate 0.66 $mLmin^{-1}$). The aqueous phase was acidified to pH 2 by adding a flow stream of HCl (6N, 0.33 $mLmin^{-1}$) and the resulting biphasic system was mixed using a T-junction into an EtOAc stream (0.33 $mLmin^{-1}$) to perform an in-line extraction. The two phases were separated in-line using a Zaiput liquid–liquid separator. The organic phase was collected in a vial acting as a substrate reservoir for the final nucleophilic substitution reaction. The organic phase was pumped and mixed with a solution of NaSH (c=1.5 M, 3 equiv) in degassed water, and entered a 10 mL coil reactor maintained at 125 °C for 30 min (total flow rate 0.66 $mLmin^{-1}$). A 250 psi backpressure was added to the whole system. The exiting flow was then acidified to pH 1 with HCl (2N) and a continuous extraction with the Zaiput liquid–liquid separator was performed. The organic solvent was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield a crude material that was purified by column chromatography (dichloromethane/methanol 98:2) to yield Captopril, which was crystallized from ethyl acetate/hexane (1:1, total volume 1 mL; 160 mg, 50%).

Flow-based stereoselective reduction of ketones using an immobilized ketoreductase/glucose dehydrogenase mixed bed system.

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Authors contribution

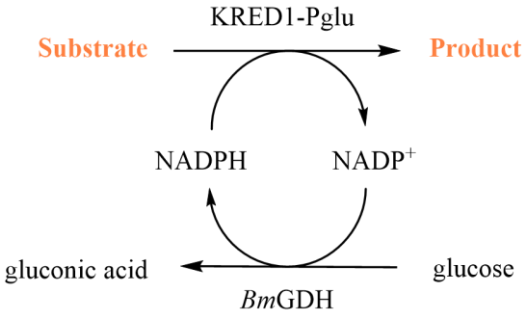
The discover of the enzyme, it production and purification and the different batch reaction were performed by the group of Dr. Immacolata Serra, Dr Martina Letizia Contente, Dr Danila Manfredi, Dr. Diego Romano, Prof. Francesco Molinari and by the group of Prof. Daniela Ubiali. The flow adaptation and reaction optimization were performed by me and my group, that includes Dr. Lucia Tamborini, Prof. Andrea Pinto and Prof. Paola Conti. I woul like to that all the people involved in this project.

Introduction

The use of alcohol dehydrogenases (ADH, named also ketoreductases -KREDs - or carbonyl reductases -CR-) is a well established method for the enantioselective reduction of prochiral ketones into stereo-defined chiral alcohols.[57] Reduction of aromatic ketones has been studied with different reductases, with particular focus on the reduction of sterically hindered (bulky) substrates, not easily recognized by most of the available KREDs[58]. Commercially available or engineered KREDs can reduce structurally different ketones and their use has been also scaled-up to industrial processes[59]. Other alcohol dehydrogenases, such as the ones from *Rhodococcus ruber* (ADH-A[60]), *Ralstonia sp*. (RasADH[58]), and *Lactobacillus brevis* (LBADH[61]) have been identified as suitable biocatalysts for the reduction of different aromatic bulky substrates. Steric effects of the substrates on the activity of LBADH towards aromatic ketones (acetophenone derivatives) were accurately studied. In most cases, a tendency for higher activity with smaller substrates could be detected, but noticeable deviations from this general trend were observed due to stereoelectronic effects[62]. Whole cells of the non-conventional yeast *Ogataea glucozyma* CBS 5766 have been used for the reductive biotransformation of various aromatic ketones and ketoesters (including bulky ones), often showing interesting stereoselectivity[63]. The reduction of these substrates frequently occurred with activity and enantioselectivity strongly dependent on the conditions of growth and biotransformation, suggesting the possible occurrence of different ketoreductases acting on aromatic ketones[64].

Results and discussion

The aim of this project was to develop a biocatalytic flow system in order to reduce in a regio- and stereo-selective way ketones and di-ketones, to obtain the corresponding mono-alcohols. To achieve this aim, we used a combination of two purified enzymes, the ketoreductase1 from *Pichia glucozyma* and a glucose dehydrogenase from *Bacillus megaterium* (Scheme 1).



Scheme 1. Schematic representation of the reaction mechanism

As it is possible to see in Scheme 1, the enzyme which performs the reductive reaction from ketone to alcohol is the ketoreductase KRED1-Pglu, that is able to use only NADPH as cofactor. The NADPH regeneration, which allows to use catalytic amounts of cofactor, is assured by a second enzyme, glucose dehydrogenase, which oxidize the co-substrate glucose to gluconic acid and NADPH.

This system as free biocatalysts was previously used in the group of Dr. Diego Romano on aromatic ketones[64]. Good results were achieved obtaining mono-alcohols in a stereoselective way. On the basis of this preliminary good results, the enzymes were immobilized, in order to exploit them in a packed bed reactor, always in collaboration with the group of Dr. Diego Romano, at the University of Milan. This strategy could lead to some advantages, for example higher and longer stability of the biocatalysts, re-usability (with consequent less waste), higher enzyme-substrate ratio.

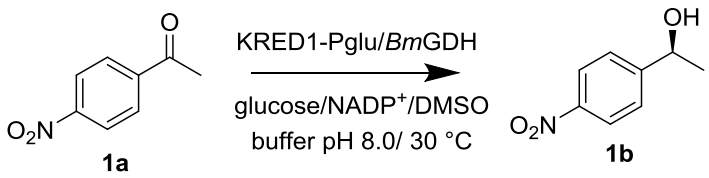
Ketoreductase from *Pichia glucozyma* (KRED1-Pglu) and glucose dehydrogenase from *Bacillus megaterium* (*Bm*GDH) were prepared as previously reported[64].

Different supports were taken into account for the immobilization of the enzymes. The enzymes were immobilized separately. The first support tried was the commercially available epoxy-activated Relizyme 403/S. Although KRED1-Pglu was completely bound to the support, the resulting biocatalyst was poorly active (4% of recovered activity). Moreover, on the same support only 2% of the offered *Bm*GDH was bound to the matrix. The second support we tried was aldehyde activated agarose, that is a suitable support for the immobilization/stabilization of several enzymes[65]. Immobilization on aldehyde agarose relies on the reaction between not protonated ε-amino groups of surface lysines and aldehyde groups on the support. Since alkaline conditions (pH ≥ 10) are required to ensure that the ε-amino groups are not protonated, stability studies of both soluble enzymes were carried out at this pH. *Bm*GDH lost more than 50% of the initial activity after 1 h of incubation at pH 10.0 both at 25 °C and 4 °C. The addition of stabilizing agents (glycerol or PEG600) allowed to maintain 100% and 70% of the initial activity after 3 h of incubation at pH 10. For what concerns KRED1-Pglu, it retained 100% of its initial activity over 3 h at pH 10.0 under all the conditions tested (25 °C or 4 °C with 20% glycerol as additive). On the basis of these stability

tests, different conditions were tried for the immobilization, changing loading, temperature and the presence of additive:

- For KRED1-Pglu, the best results were obtained at 4 °C without additives and they were independent from the protein loading;
- For *Bm*GDH an addition of 20% of glycerol allowed to retain 32% and 28% of activity upon immobilization at 25 °C, while the addition of PEG600 was not beneficial to the immobilization outcome.

In order to understand if, in this case, there was, through immobilization, an increase of the stability of the enzymes, I decided to compare the stability between free and immobilized enzymes in the presence of DMSO, which is an organic solvent (water miscible) widely used to solubilize hydrophobic substrates. Immobilization remarkably improved the enzymatic stability towards DMSO: 60–65% residual activity was found for immobilized KRED1-Pglu after 24 h in the presence of 5–20% DMSO, and only 4–6% of the original activity was lost in the case of immobilized *Bm*GDH. It should be noted that both the free enzymes were mostly inactivated after 6 h even at the lowest DMSO concentration.
Next step was to try the immobilized enzymes in batch reactions. A homogenous agarose gel system was obtained by mixing the two immobilized enzymes together (1/50 KRED1-Pglu/*Bm*GDH), and the system shown in Scheme 1 was employed for the co-factor regeneration system, adding $NADP^+$ (0.1 mM) and glucose (4 eq x eq of substrate). 4-nitroacetophenone **1** was used as a "model" substrate, which was reduced to the corresponding (*S*)-1-(4-nitrophenyl) ethanol (**2**) (Scheme 2).



Scheme 2. Schematic representation of the "model" reaction

The system, maintained at 30 °C, was able to convert **1a** (3.0 mM) with a conversion between 78-83% depending on the amount of DMSO used (from 4 to 8%). The enantiomeric excess of **1b** was always >98%. With free enzymes, the conversions were sluggish with DMSO > 4%. The re-usability of the immobilized system was investigated in a sequence of reaction cycles performed in the presence of 5% DMSO, where the biocatalysts were recovered by filtration after every cycle and used for a new batch reaction (Figure 2).

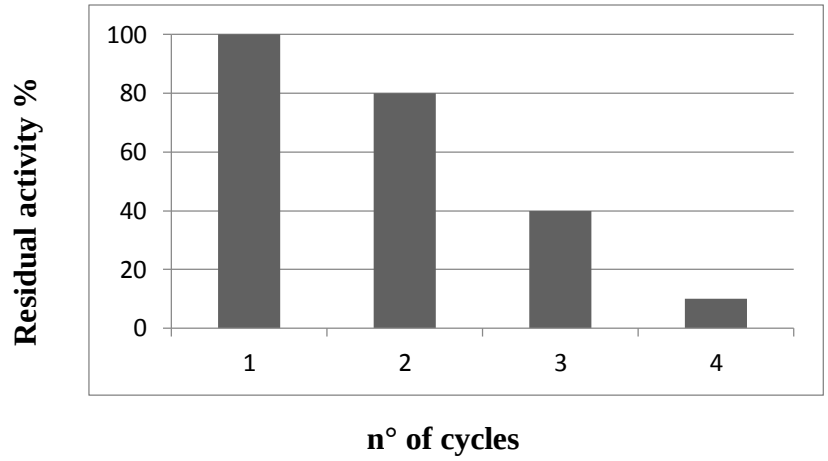
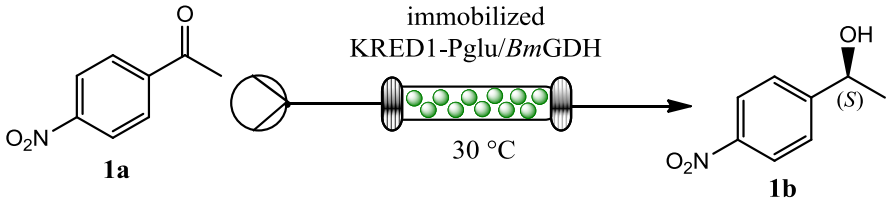


Figure 2. Assay of the immobilized KRED1-Pglu/*Bm*GDH system in repeated-batch reductions of **1a**. After each reaction cycle (24 h), the biocatalyst was filtered from the reaction medium, washed with fresh buffer and used for a new reaction cycle.

The immobilized biocatalysts lost 20% of the original activity after one cycle and were totally inactive after 4 reaction cycles.

I then transferred the same reaction in flow (Scheme 3):



Scheme 3. Schematic representation of the flow configuration.

One HPLC pump pumped a 3.0 mM solution of compound **1a**, containing $NADP^+$ (0.1 mM) and glucose (4 eq x eq of substrate), in TRIS HCl buffer pH 8.0 (50 mM) through a column (i.d.: 6.6 mm) packed with the two immobilized enzymes. To this solution I added 5% of DMSO. The column was maintained at 30 °C during the reaction. Different ratios between the enzymes and residence times were tried in order to obtain the product **1b**. The *ee* was always > 97%.

In Figure 3 are summarized the results of these tests:

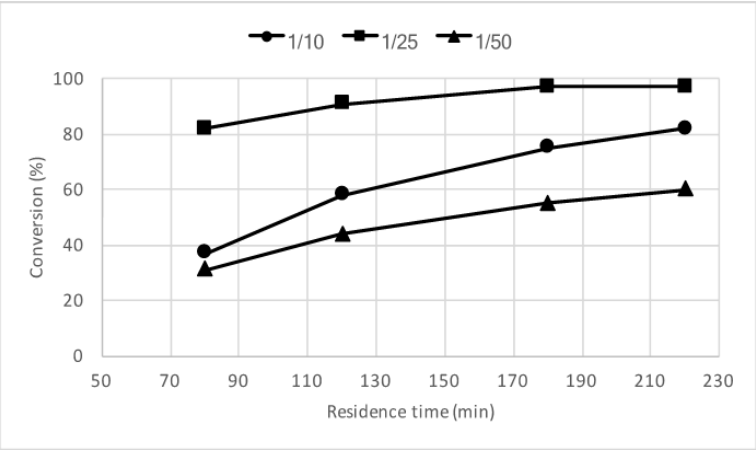


Figure 3. Summary of the results obtained by changing residence times and enzymatic ratio.

As it is possible to see from the graphic above, three enzymatic ratios (1/10; 1/25; 1/50) were tried with different residence times (80 min; 120 min; 180 min; 220 min). All the ratios are KRED1-Pglu/*Bm*GDH, which means that the ketoreductase was always lower (in terms of mUnits used) than the glucodehydrogenase. From the graphic is possible to see that the best results were obtained with the ratio 1/25, and this is true for all the residence times tried. With this ratio, a total conversion was achieved with a residence time of 220 min and 180 min, while a 90% and an 80% of conversion were achieved with 120 min and 80 min, respectively.
I then studied the stability of the column. I wanted to evaluate if in flow was possible to further increase the stability observed with the immobilized enzymes. The Figure 4 here below shows the results:

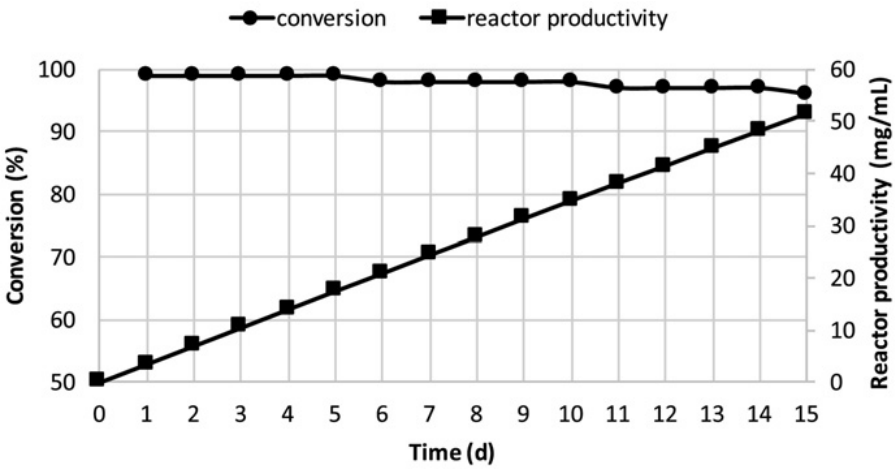


Figure 4. Representation of the stability of the column under 15 days of continuous work.

The column was tested for 15 days under continuous work, flowing through it a 3.0 mM solution of compound **1a**, with the best condition found (ratio 1/25 and 180 min of residence time). As it is possible to see from the graphic, the conversion was constant for 15 days, and the *ee* of the product always remained > 97%. Noteworthy, after 6 months of operation in flow, the reactor only lost 30-32% of the original activity. The different operational stability shown by the immobilized system in the flow reactor and in shaken flasks may be due to the reduced mechanical stress under flow conditions. It should be underlined that biotransformations with immobilized enzymes are multi-phase systems needing continuous agitation in conventional batch reactors; agitation (orbital

shaking in our case) can damage or grind the biocatalyst to fine particles. This is an evidence of one of the advantages of running biotransformations in a flow reactor.
The same system was then applied on other three substrates, which were different from **1a** since they are di-ketones. Indeed, it was nice to see the behavior of the system when two possible ketones were present in the molecule. The selected substrates were the following (Figure 5):

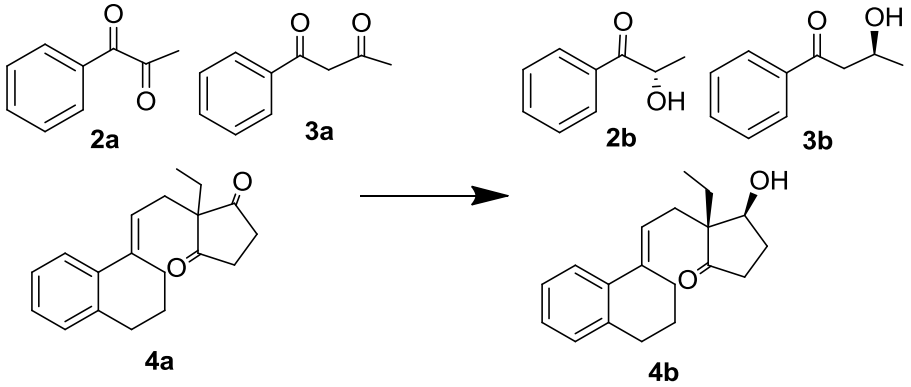


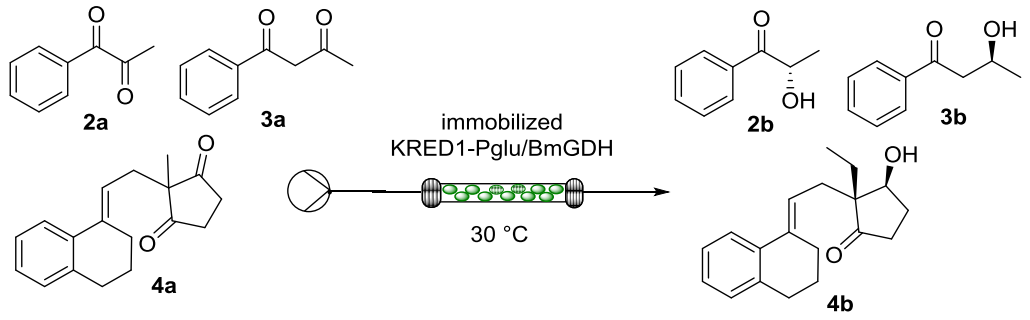
Figure 5. Di-ketones used and their products.

The reactions were first conducted in batch under the optimized conditions described above for compound **1a**, using the same co-factor regeneration system. Therefore, a solution of the substrate (**2a**-**4a**) at different concentrations was prepared in buffer TRIS HCl 50 mM pH 8.0 and 5% DMSO. KRED1-Pglu (20 mU/mL) and *Bm*GDH (1U/mL) were added to the solution, with $NADP^+$ (0.1 mM) and glucose (4 eq x eq of substrate). The system was maintained at 30 °C. Here below a table is reported with the batch results (Table 1):

Entry	Substrate	Concentration (mM)	*t* (min)	Conversion (%)	*ee* (%)
1	**2a**	3.0	60	>95%	>97%
2	**3a**	3.0	1440	60%	>97%
3	**4a**	6.5	480	65%	>97%

Table 1. Reaction conditions and conversions of batch biotransformations with immobilized KRED1-Pglu.

Starting from these results, I transferred the reactions in flow (Scheme 4). For all the substrates, a solution of the substrate (**2a**-**4a**) at different concentrations in buffer TRIS HCl 50 mM pH 8.0 and at different percentages of DMSO was prepared. $NADP^+$ (0.1 mM) and glucose (4 eq x eq of substrate) were added to the solution. This was pumped by one HPLC pump and entered an Omnifit glass column packed with the immobilized enzymes (KRED1-Pglu and *Bm*GDH), with the ratio found before for compound **1a**, which is 1/25 respectively. The exiting flow stream was then analyzed by chiral HPLC.



Scheme 4. Schematic representation of the flow system used for the reduction of substrates **2a-4a.**

The results are summarized below:

- 1-Phenylpropane-1,2-dione (**2a**): I decided to perform this reaction with the same concentration used in batch, but, in order to completely solubilize the substrate, 10% of DMSO was necessary. Remarkably, even if a much higher amount of DMSO was employed, the enzymes were able to work very well in the same way. I investigated different reaction times: I started with 120 minutes of residence time, and, once I noticed that the reaction was complete after this time, I decreased it. I finally stopped at 7 minutes, which was the lowest residence time that gave a total conversion of enantiomerically pure product **2b**;
- 1-Phenylbutane-1,3-dione (**3a**): even for this compound I decided to run the reaction with the same concentration used in batch (3 mM), but to do so it was necessary to add 10% of DMSO in order to completely solubilize the substrate. Again, I investigated different residence times in order to find the lowest reaction time with the maximum conversion. In this case, a full conversion was achieved in 120 minutes. Product **3b** was obtain with a *ee* >97%;
- ethyl secodione (**4a**): this substrate is a key intermediate in the synthesis of different hormonal contraceptives[66]. Using the same concentration as in batch (8.0 mM), even adding DMSO up to 20%, it was not possible to obtain a clear solution. So, I decreased to 3.0 mM the concentration of compound **4a**. Concentrations between 3.0 mM and 8.0 mM were tried with percentages > 20% of DMSO, but the enzymes resulted to be less active and stable. I tried different residence times: the lowest residence time which led to the highest conversion (65%) was 180 min. An increase in the residence time was no productive. A similar conversion was achieved in batch after 480 minutes with free enzymes. Interesting, in this case just one enantiomer among the four possible isomers was formed with an *ee* > 97%. The diastereoisomeric composition (% *de*) was determined by HPLC using a Phenomenex LUX Cellulose-2 (*n*-hexane/*i*-PrOH 85:15; 0.5 mL/min 254 nm): rt (13*S*, 17*S*) 18.9 min, (13*S*, 17*R*) 20.6 min, (13*R*, 17*S*) 23.3 min, (13*R*, 17*R*) 24.6 min. In the previous article published by us[66], the absolute configurations of the different possible isomers were assigned using Mosher's methodology.

For all the substrates, the bioreactor was tested under continuous work for 15 days, at the optimized conditions.

A summarizing table is reported below (Table 2):

Entry	Subst.	DMSO (%)	t_r (min)	Conversion day 1 (%)	Conversion day 15 (%)	Space time yield ($mg \cdot d^{-1}$)	Catalyst productivity ($mmol \cdot U^{-1}$)	Product recovered (mg)
1	**2a**	10	7	>98	97	81.5	150.1	1199
2	**3a**	10	120	96	94	5.0	8.4	67
3	**4a**	20	180	65	63	4.4	3.8	60

Table 2. Table reporting the conversions of substrates (**2a-4a**) under optimized conditions, as concentration, % of DMSO, residence time. Conversions after 1 and 15 days of continuous work are reported. Space time yield, catalyst productivity and product recovery were calculated for all three cases.

As it is possible to see from the table above, after 15 days of continuous work with the same bioreactor, no significant changes in the composition of the flow outcome were detected. The conversions in all cases after 15 days remained the same even if a 10% or a 20% of DMSO was used. This is a very important point because underlines the advantages of flow chemistry, as for example the higher stability of the immobilized biocatalyst probably due to the absence of mechanical stress. Enzymes were washed before and after each reaction with pure buffer TRIS HCl 50 mM pH 8.0, in order to remove any trace of DMSO and of the other component of reaction solution. The stability of the biocatalysts under these conditions enabled the obtainment of millimolar amounts of the desired products in a 0.90 mL reactor, using the same amount of enzyme that produced only micromolar quantities in batch reactions.
I also calculated the space-time yield and the catalyst productivity. The space-time yield allows to understand the reactor productivity of a reaction. It is calculated knowing the amount of product that is formed during a certain time ($mg\ d^{-1}$). This value is normalized for the reactor volume and for the amount of biocatalyst used during the reaction. As reported in Table 2, the best productivity was achieved in the case of substrate **2a** (entry **1**), because a very short residence time (7 minutes) allowed to reach total conversion. For compound **3a** (entry **2**), a residence time of 120 minutes was necessary to obtain its complete conversion. Comparing to the batch procedure, the reaction time was lower (in batch was 24 h) and higher conversion was achieved (100% vs 60%) using the same substrate concentration. Concerning compound **4a** (entry **3**), the main problem was the very low water solubility that makes me to reduce the concentration in comparison to the batch procedure (3.0 mM vs 8.0 mM). The flow reaction proceeded with a similar degree of conversion compared to batch with a slightly decrease of the reaction time, from 480 min in batch to 180 min in flow.

Conclusions

In conclusion, an immobilized system composed of a ketoreductase (KRED1-Pglu) and a glucose dehydrogenase (*Bm*GDH) was successfully used to perform the continuous stereoselective reduction of ketones in a flow reactor for weeks; the prolonged operational stability in the flow reactor makes the set-up of the biotransformation attractive for preparative (bio)catalysis.

Materials and methods

General

All reagents and solvents were obtained from Sigma-Aldrich-Fluka and used without further purification or drying. TLC was performed with Merck silica gel 60 F254 pre-coated plates. ^{1}H-NMR and ^{13}C-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. HPLC analyses were performed with a Jasco Pu-980 equipped with a UV-vis detector Jasco UV-975. Chiral HPLC columns used: Lux cellulose-3 column (4.6 mm x 150 mm, Phenomenex), Lux cellulose-2 column (4.6 mm x 150 mm, Phenomenex), Chiralcel OD-H (250 mm x 4mm, Daicel), Chiralcel OD (250 mm x 4mm, Daicel). The continuous flow biotransformations were performed using a R2 +/R4 flow reactor commercially available from Vapourtec and equipped with Omnifit glass columns (6.6 mm x 100 mm length).

Enzyme immobilization and preparation of the mixed bed system

Aldehyde agarose (1 g), prepared as previously described[63], was suspended in 50 mM potassium carbonate buffer at pH 10.0. After the addition of the desired amount of protein, the solution (14 mL) was kept under mechanical stirring for 3 h; 14 mg of $NaBH_4$ (1 mg/mL of suspension) were then added. The reduction time was 30 minutes. Finally, the immobilized enzyme was filtered and washed with 10 mM potassium phosphate buffer pH 5 and deionized water. Different amounts of the two immobilized enzymes were mixed together to form a homogeneous slurry of immobilized KRED1-Pglu/*Bm*GDH with different ratios (1/10, 1/25, 1/50).

General procedure for batch biotransformations

Reactions were carried in 2 mL volume of 50 mM Tris HCl buffer pH 8.0 containing $NADP^+$ (0.1 mM), substrate (0.5 g/L), glucose (4 eq × eq of substrate) at 30 °C. The reaction was started by the addition of KRED1-Pglu and *Bm*GDH, maintained under orbital shaking (180 rpm), and was monitored by HPLC.

General procedure for flow biotransformations

Solutions of the substrates at different concentrations were prepared in Tris HCl buffer pH 8.0 (50mM) with DMSO (variable percentages depending on the substrate, for details see Table 2), 0.1 mM $NADP^+$ and glucose (4 eq × eq of substrate). An Omnifit glass column (6.6 mm i.d. × 100 mm) was packed with pre-mixed KRED1-Pglu (540 mg) and *Bm*GDH (540 mg), in order to obtain a KRED1-Pglu/*Bm*GDH ratio of 1/25. The volume of the reactor was 0.90 mL. The packed column was pre-washed by flowing through it a solution of Tris HCl buffer pH 8.0 (0.050 mL/min for 15 min) followed by a solution of Tris HCl buffer pH 8.0 with DMSO (0.050 mL/min for 15 min). Then, the substrate solution was pumped through the column maintained at 30 °C. The exiting solution was collected and analyzed at different times.

Product characterization (2a-2d)

(*S*)-(1-(4-nitrophenyl) ethanol), 2a: ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.18 (d, *J* = 8.5 Hz, 2H), 7.51 (d, *J* = 8.0 Hz, 2H,), 4.95 (q, *J* = 6.5 Hz, 1H), 1.50 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ 153.1, 147.1, 126.1, 123.7, 69.4, 25.4 ppm. Conversion was determined by HPLC using a Purosphere RP-18e STAR column (Merck) 250 mm x 4.6 mm, 5 µm (H_2O/CH_3CN 25:75, 1 mL/min; T= 25 °C; 254 nm): rt: **1a** 4-nitroacetophenone, 4.14 min; **2a** (*S*)-(1-(4-nitrophenyl) ethanol), 3.42 min. The enantiomeric excess of **2a** (% *ee*) was determined by HPLC using a Phenomenex LUX Cellulose-3 column (*n*-hexane/*i*-PrOH 90:10, 0.5 mL/min, 254 nm): rt (*S*)-1-(4-nitrophenyl) ethanol 23.4 min, rt (*R*)-1-(4-nitrophenyl) ethanol 24.9 min.

(*S*)-2-Hydroxy-1-phenylpropan-1-one, 2b: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.87 (d, *J* = 8.2 Hz, 2H), 7.57 (t, *J* = 7.42 Hz, 1H), 7.46 (t, *J* = 7.7 Hz, 2H), 5.17 (m, 1H), 3.5 (br s, 1H, OH), 1.45 (d, *J* = 7.0 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 202.3, 133.9, 133.4, 128.7, 128.6, 69.3, 22.2. The enantiomeric excess (% *ee*) of **2b** was determined by HPLC using a Chiralcel OD column (*n*-hexane/*i*-PrOH 95:5, 0.4 mL/min, 254 nm): rt (*R*)-2-hydroxy-1-phenylpropan-1-one 17.2 min, rt (*S*)-2-hydroxy-1-phenylpropan-1-one 21.9 min.

***S*)-3-Hydroxy-1-phenyl-butan-1-one, 2c**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.97 (dd, *J* = 8.5, 2.0 Hz, 2H), 7.50 (m, 3H), 4.42 (m, 1H), 3.24 (br s, 1H, OH), 3.10 (d, *J* = 6.0 Hz, 2H) 1.30 (d, *J* = 6.4 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 200.95, 136.8, 133.6, 128.8, 128.1, 64.1, 46.5, 22.5 ppm. The enantiomeric excess (% *ee*) of **2c** was determined by HPLC using Chiralcel OD-H column (*n*-hexane/*i*-PrOH 95:5, 0.8 mL/min, 254 nm): rt (*R*)-3-hydroxy-1-phenyl-butan-1-one 12.1 min, rt (*S*)-3-hydroxy-1-phenyl-butan-1-one 13.6 min.

(13*R*,17*S*)-E-13-ethyl-3-methoxy-8,14-secogona-1,3,5(10),9(11)-tetraene-17-ol-14-one, 2d: ^{1}H NMR (300 MHz, C_3D_6O): δ 7.46 (d, *J* = 8.7 Hz, 1H), 6.72 (dd, *J*= 2.8, 8.7 Hz, 1H), 6.65 (d, *J* = 2.8 Hz, 1H), 5.86 (t, *J* = 7.5 Hz, 1H), 4.32 (q, *J* = 5.5 Hz, 1H), 4.05 (d, *J* = 4.4 Hz, 1H; OH), 3.77 (s, 3H), 2.73 (t, *J* = 6.2 Hz, 2H), 2.49 (t, *J* = 5.6 Hz, 2H), 2.13-2.40 (m, 5H), 1.88-1.97 (m, 1H), 1.78 (qui, *J* = 6.3 Hz, 2H), 1.63-1.73 (m, 2H), 0.91 (t, *J* = 7.5 Hz, 3H) ppm. ^{13}C NMR (300 MHz, C_3D_6O): δ 218.8, 159.0, 138.7, 135.9, 129.3, 125.2, 117.1, 113.2, 112.7, 75.2, 56.6, 54.8, 35.0, 30.7, 30.5, 27.9, 26.7, 23.4, 21.6, 8.0 ppm. The diastereoisomeric composition (% *de*) was determined by HPLC using a Phenomenex LUX Cellulose-2 (*n*-hexane/*i*-PrOH 85:15; 0.5 mL/min 254 nm): rt (13*S*, 17*S*) 18.9 min, (13*S*, 17*R*) 20.6 min, (13*R*, 17*S*) 23.3 min, (13*R*, 17*R*) 24.6 min.

Biocatalyzed stereo- and regioselective reduction of 2,2-disubstituted-1,3-cyclopenta- and 1,3-cyclohexanediones

Manuscript in preparation

Authors contribution

The production of KRED-Pglu enzyme and of recombinant whole cells with the same enzyme were performed at the group of Prof. Dörte Rother, with, in particular, the help of Dr. Reinhard Oeggl, at the Forschungszentrum Jülich (Jülich, Germany). At the same group, the optimization of the biotrasformation was performed. The production of wild type whole cells E. coli BL21 and the optimization of the biontrasformation with this biocatalyst were perfomed at the University of Milan, with the collaboration of Prof. Francesco Molinari, Dr. Diego Romano Dr. Martina Letizia Contente, Dr Lucia Tamborini, Prof. Paola Conti and Prof. Andrea Pinto.

I would like to thank Prof Dörte Rother, Dr Reinhard Oeggl and all the people of their group for hosting me for six months and for their precious help.

Introduction

Steroids are biologically active compounds characterized by a fixed structure. This structure is formed by four rings arranged in a typical molecular configuration, which represents the core of the structure (called cyclopentaperhydrophenanthrene or gonane). This is made by seventeen carbons that create three cyclohexane rings (A, B and C rings, Figure 1) and one cyclopentane ring (D ring, Figure 1).

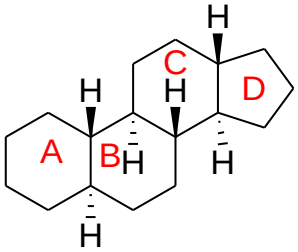


Figure 1. 5-Alpha gonane, core of the steroidal structure.

If a chain made by eight carbons is present at C17 and two methyl groups are in C10 and C18 positions, the steroid has a cholestane framework (Figure 2).

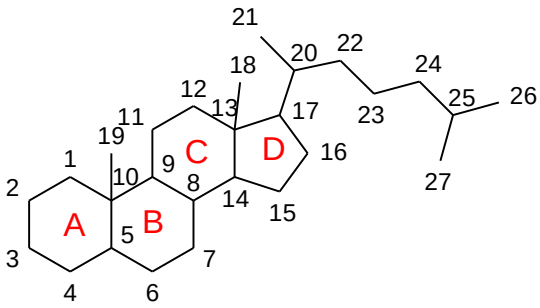


Figure 2. Cholestane structure.

A well known compound that has a cholestane structure is cholesterol: this is the precursor of two different classes of molecules, which are corticosteroids and sex steroids. These two groups have different applications and properties.

Corticosteroids can be divided into two classes: glucocorticoids and mineralcorticoids. The former group controls the metabolism of lipids, carbohydrates and proteins. They can also have anti-inflammatory properties. On the other hand, the latter group is important for the regulation of blood pressure. A general structure of corticosteroids is represented by 5-alpha pregnane reported in Figure 3.

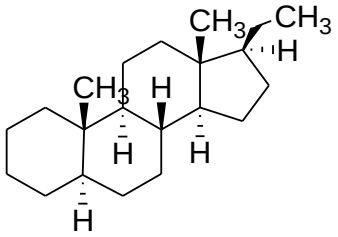


Figure 3. 5-alpha pregnane: representative structure of corticosteroids.

For what concerns sex steroids, they can be female (progesterone and estrogens) or male (androgens). The representative structure of progesterone is again 5-alpha pregnane (Figure 3), whereas androgens have as principal structure 5-alpha-androstane (Figure 4) and estrogens has 5-alpha estrange structure (Figure 5).

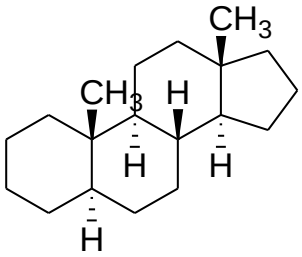


Figure 4. 5-alpha-androstane

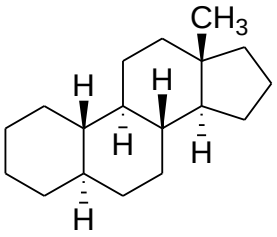


Figure 5. 5-alpha-estrange

The steroids mentioned above are all hormones. They are able to explicit their pharmacological action even if they have a very low concentration in the blood. Because of their lipophilic nature, they can reach the target cells through protein carriers. Once inside cells, steroids go into nucleus and activate receptors that interact with DNA in particular regions called Hormone Response Element (HRE).

The manufactured steroid compounds have a wide range of therapeutic purposes; in fact, they have been used:

- as anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic and contraceptive agents;
- as anti-fungal agents;
- as inhibitors of HIV integrase, in the prevention and treatment of infections by HIV and in the treatment of declared AIDS cases;
- in the prevention of coronary heart diseases and as replacement agents in the treatment of adrenal insufficiencies;
- as active ingredients in anti-obesity agents;
- in the treatment of some forms of breast and prostate cancer and osteoporosis[67].

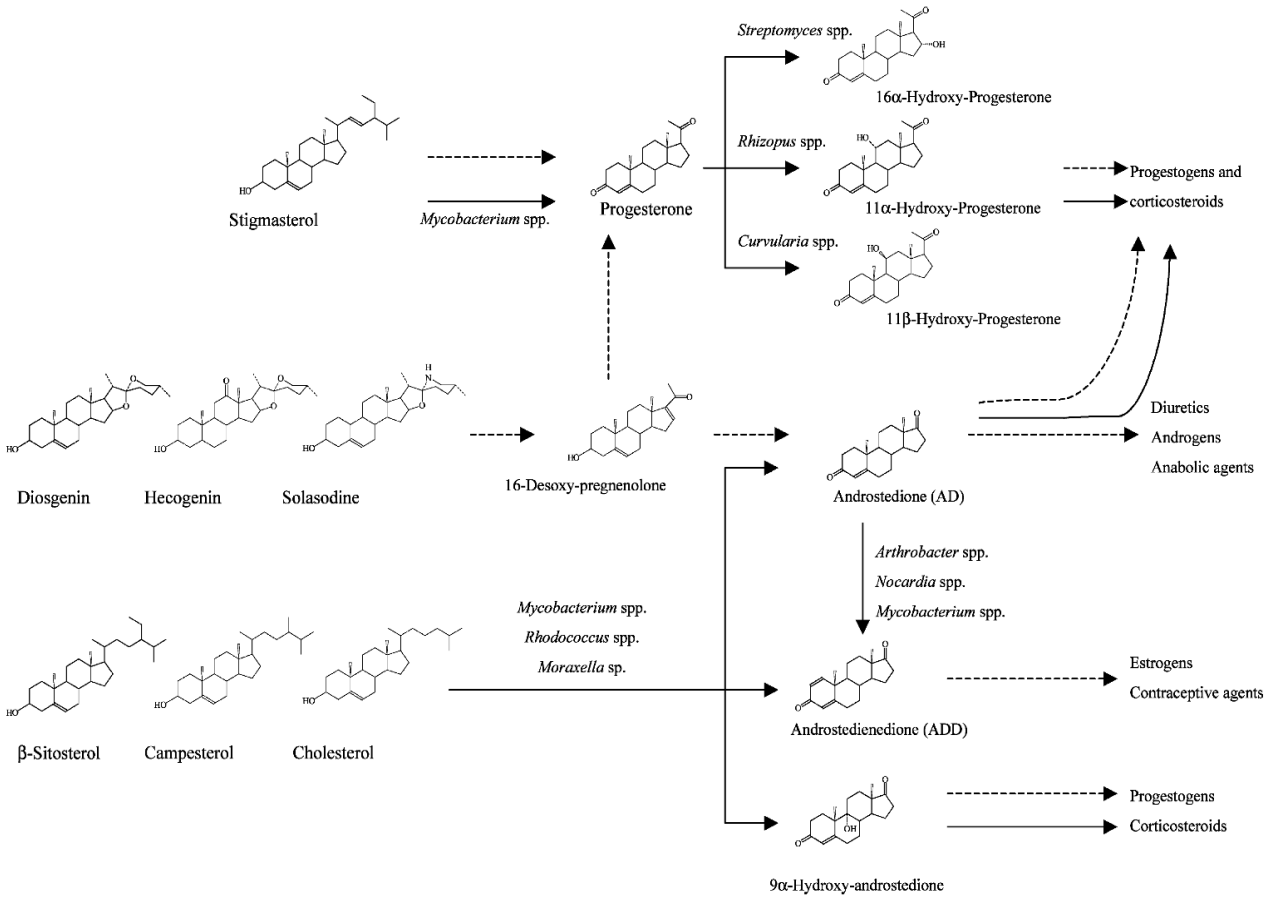
Steroids synthesis: chemical and biochemical processes

Steroid molecules, because of their complex structure and the precise stereochemistry, require complicated and multi-step schemes for the chemical synthesis. The synthesis often involves the preparation of intermediate derivatives with protected groups and their subsequent regeneration, once the intended reaction has occurred, limiting the overall process yield and making it expensive and time consuming. Furthermore, the core structure (basic ring) of some steroid derivatives is sensitive to cleavage by a wide variety of chemicals.

Chemical synthesis also requires the use of some highly-reactive reagents which are hazardous to researcher's health and constitute a serious environmental disposal problem.

Indeed, biocatalyzed steroid syntheses are performed under mild temperature and pressure conditions and can provide an efficient alternative to chemical synthesis, once the limitations often encountered of unsatisfactory productivity and/or purity levels of the products are overcome.

The research efforts in this field were triggered around 1950, with the announcement of the pharmacological effects of cortisol and progesterone and with the identification of the 11-alpha-hydroxylation activity of a *Rhizopus* species, a decisive step in the development of the practical synthesis of steroids with useful biological activity. Several microbial bioconversions of steroids have been reported until now, mainly focusing on steroid hydroxylations, delta-1-dehydrogenation and sterol side-chain cleavage. It is important to underline that these modified steroids are currently favoured when compared to their natural counterparts due to some therapeutic advantages, such as an increased potency, longer half-lives in the blood stream, simpler delivery methods and reduced side effects[67]. In Scheme 1, a general view of both chemical and biocatalyzed approaches for the synthesis of steroids is showed.



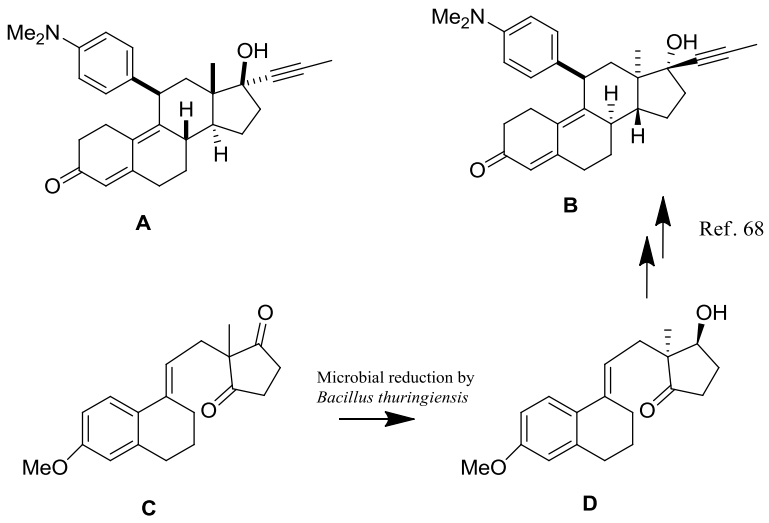
Scheme 1. An overview of steroid production, from raw materials to completed products. Full lines indicate bioconversions whereas dashed lines indicate chemical transformations.[67]

Hydroxylated steroids

Hydroxylations are probably the most applied type of steroids functionalization. Compared with their less polar non-hydroxylated analogues, steroids with hydroxyl group often show a better biological activity, which is convenient for therapeutic applications. Over 1500 species (*e.g.*, *Mycobacterium*, *Streptomyces*, *Aspergillus*) have been studied for this specific application.

11-Alpha-, 11-beta-, 15-alpha and 16-alpha-hydroxylations are currently validated processes in steroid industry, mainly for the production of adrenal cortex hormones and their analogues. 16-Alpha-hydroxylated steroids have increased glucocorticoid activity, in which the oxygen group in position C11 is regarded as essential for anti-inflammatory action. 11-Alpha-hydroxylation is also used in the production of an intermediate in the synthesis of a contraceptive drug (*i.e.*, Desogestrel)[67].

An example of the utility of hydroxylated steroids was given by Jean-Francois Biellmann who studied about enantiomerically pure steroids[68]: he confirmed the fact that the steroid biological activity is in general restricted to one enantiomer. Thus, he mentioned some examples, one of them was RU-486 (A, Scheme 2): the synthesis of the corresponding enantiomer of RU-486 (B) starts from the reduction of diketone (C) to enantiomerically pure hydroxyketone (D).



Scheme 2. Reduction of diketone (**C**) to hydroxyketone (**D**) to obtain **A**.[68]

Results and discussion

The aim of the project was to regio- and stereo-selective reduce different 2,2-disubstituted-1,3-cyclopentanediones and cyclohexanediones, that are represented in Figure 6, in order to obtain mono-alcohols as main products. These compounds represent simplified structures that can be used as model compounds to study the biocatalyzed regio- and stereo-selective reduction.

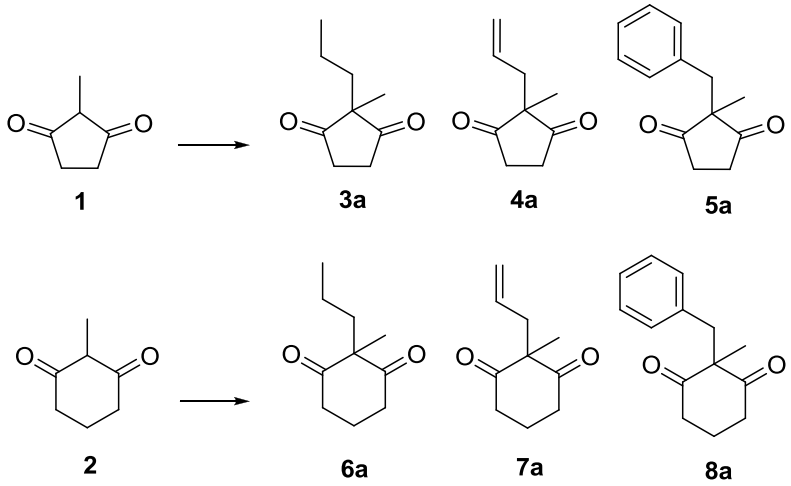


Figure 6. 2,2-disubstituted-1,3-cyclopenta- and 1,3-cyclohexanediones used as substrates.

The reductive reaction was performed using a biocatalytical approach, with both whole cells and purified enzymes. The whole cells used were empty cells of *E.coli* BL21 DE3 star and cells of the same microorganism that expressed a ketoreductase, specifically the KRED1-Pglu from *Pichia glucozyma*. As last, the same substrates were reduced with the purified free KRED1-Pglu. We decided to try different biocatalysts in order to see the differences between the various approaches.
The main goal was to obtain enantiomerically pure mono-alcohols, that can be important building blocks in the synthesis of various steroidal molecules[67,68]. However, this goal is challenging, because two stereo-centres are generated after the reduction of the prochiral substrates (**3a-8a**), thus forming four possible isomers. In addition, a reaction by-product can be the diol (**3f-8f**) (Figure 7).

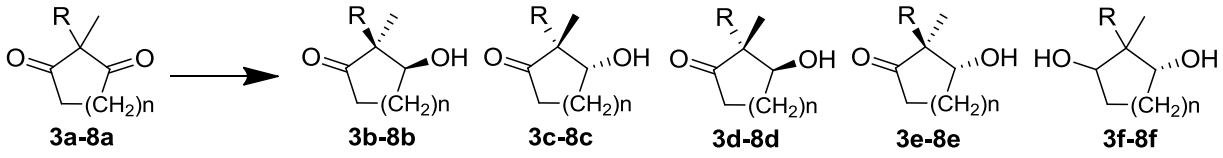


Figure 7. Substrates (**3a-8a**), their four possible mono-alcohol products and the diols.

Synthesis of 2,2-disubstituted 1,3-cyclopenta- and 1,3-cyclohexanediones

I firstly chemically synthesized the 2,2-disubstituted 1,3-cyclopenta- and 1,3-cyclohexanediones (**3a-8a**) starting from their precursors **1** and **2** (Figure 8).

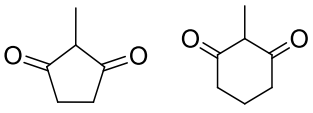


Figure 8. Precursors used for the synthesis of substrates (**3a-8a**)

Compounds **4a** and **5a** were synthesized following a reported procedures[69,70] starting from 2-methyl-1,3-cyclopentanedione (**1**), that can be easily deprotonated in position 2 under basic conditions and the resulting anion can afford a nucleophilic substitution on allyl or benzyl bromide respectively, thus producing the alkylated desired product. The same protocols were followed for compounds **7a** and **8a** starting from 2-methyl-1,3-cyclohexanedione (**2**), achieving also in this case good results.

For obtaining derivatives with propyl group as substituent in position 2 (compounds **3a** and **6a**), I reduced the double bond of the alkenes **4a** and **7a** using the H-cube reactor, a flow reactor able to perform hydrogenation reactions in a continuous way (Figure 9).

Figure 9. H-cube flow reactor for continuous hydrogenation reactions.

Hydrogenation is a very useful reaction, since can be used for reducing C-C double bonds, imines, for de-benzylation reactions or to remove Cbz groups. However, it must be run very carefully because hydrogen is a highly flammable gas and also the catalysts (*e.g.*, Pd/C 10%) used for the reaction are very hazardous.

In the H-cube reactor, many safety issues related to hydrogenation reactions have been solved. In fact, H_2 is generated in situ by electrolysis from water and directly used in the reaction. Moreover, the pyrophoric catalysts are stuck in sealed catalyst cartridges and are not in contact with the air.

Summarizing, the advantages are:

- Increased safety
- Easy to use
- No catalyst filtration
- Fast reactions.

The reactions are performed in a continuous flow stream and they can be heated up to 100 °C and easily pressurized (up to 100 atm). The steps of the hydrogenation reaction using H-cube are the following:

1. Hydrogen is generated by water electrolysis

2. The reaction parameters (temperature, pressure and residence time) are set
3. The starting material flow stream is passed through the cartridge containing the catalyst, after being in contact with the hydrogen
4. The flow stream containing the product exits from the reactor (again reaction and filtration are simultaneous, Figure 10).

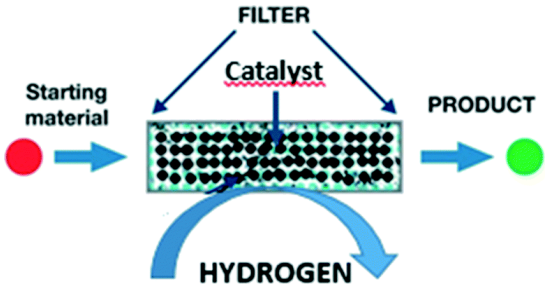
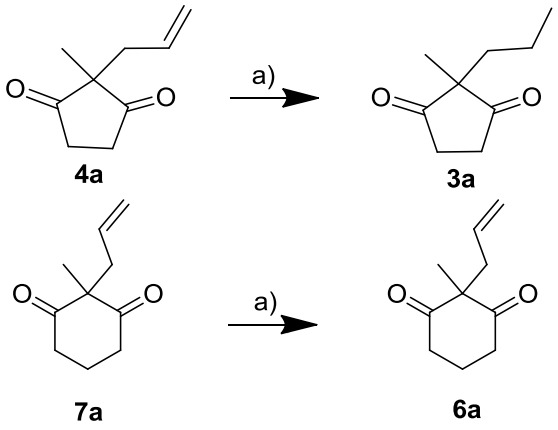


Figure 10. Schematic representation of a reaction in the H-cube reactor.[71]

To run the hydrogenation reaction on alkenes **4a** and **7a**, 5% Pd/C was selected as catalyst (Scheme 3). The reaction was run in methanol at 30 °C and 1 bar of pressure. The reaction was very fast; it was completed with both the substrates using a flow rate of 0.5 mL/min.
Since the hydrogen is produced from water, it is wet. If the reaction is moisture sensitive, a drying cartridge can be added to dry hydrogen. Then, methanol was evaporated under vacuum and the aqueous phase was extracted with CH_2Cl_2. Both the substrates were obtained with total conversion and no chromatographic column was required after the work-up procedure.



Scheme 3. Reaction conditions for the hydrogenation with H-cube: a) MeOH, Pd/C 5%, 30 °C, 1 bar, 0.5 mL/min.

In order to be able to analyze the biocatalyzed reduction reaction with, I also synthesized the standards of the mono-alcohol products for all the substrates. I made this in a chemical way with $NaBH_4$, and therefore a mixture of the four possible isomers represented in Figure 7 was obtained. I reduced with $NaBH_4$ substrates **4a-5a** and **7a-8a**, using 0.25 eq of the reducing agent in order to obtain a mono reduction. Indeed, sodium borohydride has four available hydrides and it means that 1 mole reduces 4 moles of ketone. The reaction was performed in methanol, a polar, protic solvent.

The mixture was stirred for 15 minutes, since longer reaction times will lead to the formation of the diol. Then, water was added to quench the reaction and the pH was adjusted to pH = 2 with 1N HCl. To obtain the mono-alcohols of compounds **3a** and **6a**, I used again the H-cube, starting from the corresponding unsaturated mono-alcohols of substrates **4a** and **7a**. The conditions I applied were the same that I used to obtain **3a** and **6a**. Also in this case, after the hydrogenation, I evaporated methanol (the solvent of the reaction) and I extracted the water phase with dichloromethane to afford the desired products with an almost complete yield. Importantly, all the substrates **3a-8a** and their reduced mono-alcohol products must be treated carefully, since they are quite volatile compounds and they could evaporate under low reduced pressure.

Biotransformations with whole recombinant cells expressing KRED1-Pglu

A part of this project was developed at the group of Prof. Dörte Rother at the Forschungszentrum Jülich, in Germany, where I spent six months. The synthesis of substrates **3a-8a** was performed at the University of Milan, whereas in Jülich I learnt the biotechological processes in order to cultivate cells, produce and isolate enzymes. Then, I tried compounds **3a-8a** with both whole cells expressing KRED1-Pglu and with purified free KRED1-Pglu. At the Forschungszentrum Jülich, I cultivated and grew the whole cells *E.coli* DE3 expressing the desired protein KRED1-Pglu, with the supervision of Prof. Dörte Rother and Dr. Reinhard Oeggl, following the procedure described in the Experimental Session of this Chapter. After the production, I then lyophilized the cells and directly used them for the biocatalyzed reaction.

I firstly used the whole cells on a model substrate, Acetophenone[64] in order to both check cells activity and to find the best buffer for the reaction. The four buffers tried were: HEPES, TEA, K_3PO_4 and TRIS, at pH 8.0 and at a concentration of 50 mM. Reactions were run with 1 gr/L acetophenone, 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (commercially available with > 200 U/mg) and 50 mg of lyophilized whole cells in 1 mL volume reaction. Glucose and GDH were added for the NADPH regeneration system necessary for KRED1-Pglu (as explained in Figure 11):

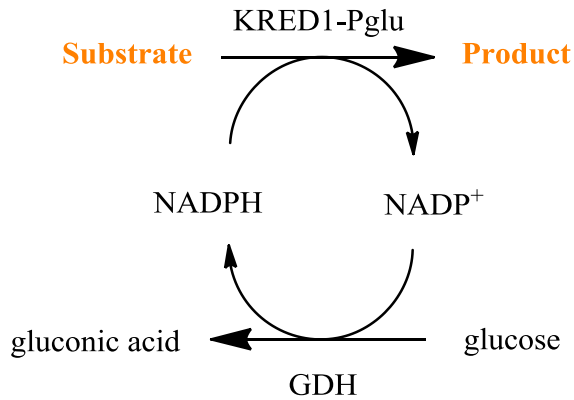


Figure 11. Schematic representation of the cofactor regeneration system mechanism used in these trials.

The higher conversion was obtained with phosphate buffer (70% after 24h). HEPES buffer was not bad, but less performing compared to phosphate one.

I then tested the biotransformation on compounds **3a-8a**. I decided to firstly evaluate the reaction conditions using substrate **3a** (2-methyl-2-propyl-1,3-cyclopentanedione). The first two reaction parameters I optimized were the amount of whole cells to be employed and if NADPH and its cofactor recycling system was actually required. Indeed, it is known that one of the advantages of whole cells is generally that no external addition of cofactor and cofactor regeneration system is required, because usually everything is already inside the cells. Therefore, I tried 50 mg, 25 mg and 5 mg of whole cells with and without cofactor. Results are reported in the following Table 1.

Entry	Cells amount (mg)	NADPH	Time (h)	**3a** (%)	**3b** (%)	**3c** (%)	**3d** (%)	**3e** (%)	**3f** (%)
1	50	Yes	1	0	14	n.d	41	n.d	45
2	50	Yes	24	0	20	n.d	45	n.d	35
3	50	No	1	0	22	n.d	43	n.d	35
4	50	No	24	0	27	n.d	44	n.d	29
5	25	Yes	1	0	20	n.d	40	n.d	40
6	25	Yes	24	0	19	n.d	44	n.d	37
7	25	No	1	10	36	n.d	41	n.d	13
8	25	No	24	0	26	n.d	46	n.d	28
9	5	Yes	1	6	34	n.d	43	n.d	17
10	5	Yes	24	0	23	n.d	52	n.d	25
11	5	No	1	86	8	n.d	6	n.d	0
12	5	No	24	39	32	n.d	25	n.d.	4

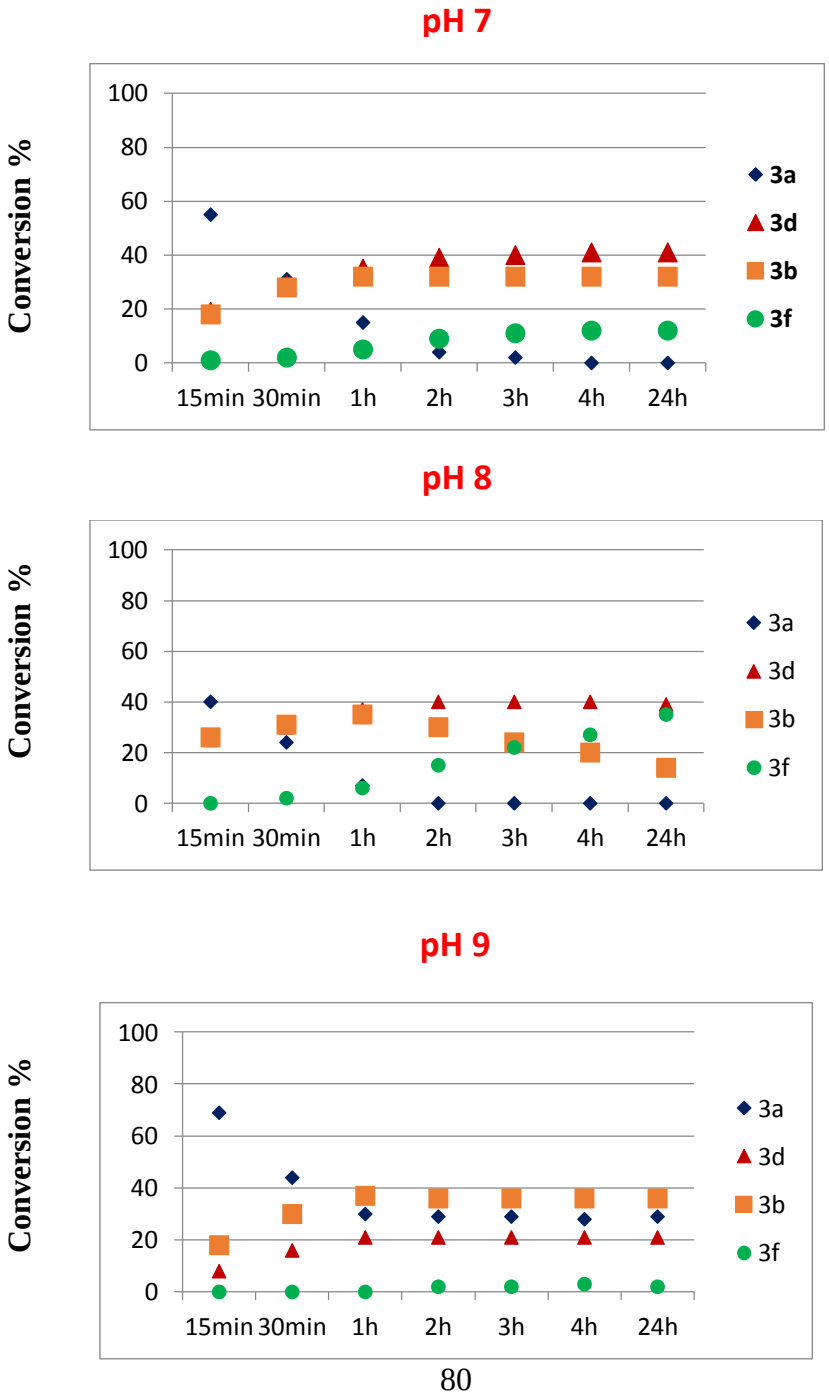
Table 1.Results of biotransformations run with a 14 mM solution of compound **3a** in a phosphate buffer solution (pH 8.0, 50 mM) with 40 mM glucose, 0.1 mM NADPH as cofactor (when added), a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg) and different amounts of lyophilized whole cells in 1 mL total volume reaction. Different samples were taken each hour and analyzed by chiral GC. Here are reported the most important results.

As it is possible to see from Table 1, important information were collected with these reactions. First, we observed that always two isomers were formed during the reactions in a significant amount, that are isomer **3b** (*2S, 3S*) and **3d** (*2R, 3S*). In the next pages, I will explain how it was possible to understand the absolute configurations of these two isomer. In addition, we saw that the diol **3f** was detected as well. The structure of diol **3f** was confirmed by ^{1}H NMR after a purification column of a reaction performed under the conditions reported in Table 1 entry **9** (5 mg whole cells and NADPH).

For what concerns the two variables investigated so far, the presence of a catalytic amount of NADPH (0.1 mM) surely speeded up the reaction. This is largely visible when a small amount of whole cells is employed (entries **9-10-11-12**), while it has a lower impact with higher amounts of cells (50 mg, entries **3** and **4**, and 25 mg, entries **6** and **8**). In general, the reductive reaction resulted to be quite fast.

Taking into considerations these results, I decided to use the condition of entries **9** and **10**, therefore to use 5 mg of whole cells and a catalytic amount of NADPH (0.1 mM), reducing of ten times the amount of biocatalyst.
Then, I modified various reaction conditions in order to find the conditions that allow us to have a mono stereoselective reduction, with the formation of only one isomer and without the diol.

Keeping constant the other parameters, I evaluated the pH effect, to see if there was an equilibrium between the oxidized part (the substrate, **3a**) and the reduced one (mono-alcohols **3b-3e** and diol **3f**). The tested pH values were 7, 8 , 9 and 10. pHs 7 and 8 were made with phosphate buffer as before, while for pH 9 and 10 I used CAPSO buffer (cyclohexylamine 1-propanesulfonic acid). The concentration of the buffers was always 50 mM. Graphics here below show the obtained results (Figure 12).



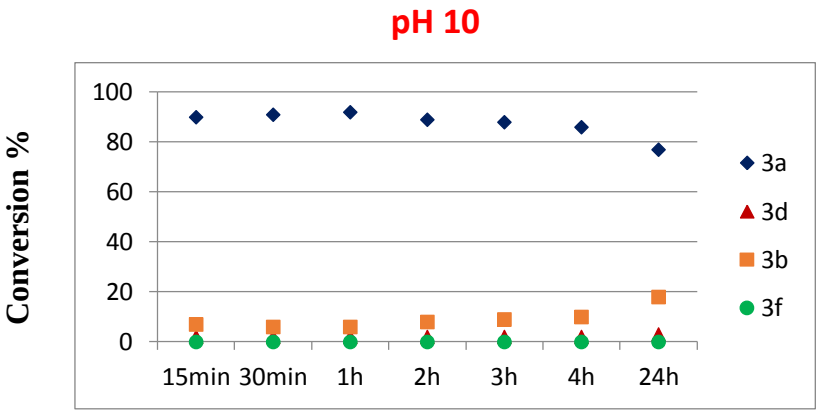


Figure 12. Four graphics that show the trend of reactions run changing the pH values. Reaction conditions: 14 mM solution of compound **3a** in a phosphate buffer solution (pH 7.0/8.0/9.0/10.0, 50 mM) with 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg) and 5 mg of lyophilized whole cells in 1 mL total volume reaction. Samples were analyzed in chiral GC.

From these biotransformations, it was observed that at higher pH values (9 and 10) the oxidized form is favorite (and the reaction was slower, at pH 9, or did not proceed, at pH 10), while at lower pH the reduced form is favorite, even if at pH 7 less diol **3f** is formed compared to pH 8. In general, the reaction is faster at pH 8, and it is possible to note that the formation of diol **3f** seems to be possible because the isomer **3b** is used by the biocatalyst. Leaving the reactions run further did not change the results. The pH value was not a parameter that allowed us to obtain a stereoselective reduction and, simultaneously, to avoid the formation of the diol **3f**. Again in these cases, isomers **3c** and **3e** were not significantly detected.

I then tried to add different co-solvent in order to reach our aim. Indeed, there are reported in literature some cases that present the potentiality of co-solvents and additives in directing stereselectivity.[72] The co-solvents I tried are: DMSO, acetone, chloroform, dichloromethane, MTBE (methyl *tert*-butyl ether) and MIBK (methyl isobutyl ketone). All the co-solvents were added in 1% v/v. I also tried in the same set of reactions the one without co-solvent, as standard reference (Table 2).

Entry	Co-solvent	**3a**(%)	**3b**(%)	**3c**(%)	**3d**(%)	**3e**(%)	**3f**(%)
1	DMSO	0	22	n.d	42	n.d	36
2	Acetone	0	28	n.d	50	n.d	22
3	DCM	14	49	n.d	33	n.d	4
4	TCM	22	75	n.d	5	n.d	0
5	MTBE	0	39	n.d	43	n.d	20
6	MIBK	40	47	n.d	13	n.d	0
7	No cosolvent	0	20	n.d	50	n.d	30

Table 2. Results of the biotransformations after 72h with different co-solvents. Reaction conditions: 14 mM solution of compound **3a** in a phosphate buffer solution (pH 8.0, 50 mM) with 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg), 5 mg of lyophilized whole cells in 1 mL total volume reaction and 1% of various co-solvents. Different samples were taken after 15 minutes, 30 minutes, 1h, 2h, 3h, 4h, 24h, 48h, 72h and analyzed by chiral GC. Here are reported only the results after 72h in order to observe the trend of the different reactions.

Actually, the stereoselectivity seems to be influenced by the co-solvent used: some of them have a preference for the mono-alcohol **3d** (as DMSO and acetone, entries **1** and **2,** respectively) and others towards the mono-alcohol **3b** (DCM, TCM, MIBK, entries **3**, **4** and **6**, respectively). MTBE (entry **5**) does not seem to have an impact on the stereselectivity. The condition without co-solvent (entry **7**) perfectly reflects the results showed in Table 1, entry **10**, showing that, if the reaction is run for 24h or more, isomer **3b** seems to be used to give diol **3f**. As before, products **3c** and **3e** were not detected even under these conditions.
Among all the results, the best one is obtained using 1% of chloroform (entry **4**): indeed, after 72h, an almost 60% of conversion was obtained, yielding only isomer **3b**, without the presence of the diol **3f**. This was the best result that was obtained so far. Leaving the reaction run for more time did not further increase the conversion.

Since the best obtained result was the one with 1% of chloroform, we decided to investigate this co-solvent, by changing the amount of it inside the reaction. Therefore, I tried 0.5, 0.75, 1, 1.5 and 2% of chloroform. The control reaction without co-solvent was always performed. Results are shown in Table 3 (as well as before, only results at 72 h are reported). I just reported results for mono-alcohols **3b** and **3d**, since the other two products were never detected during these experiments. Of course, I inserted in Table 3 results about the diol product **3f**.

Entry	TCM (%)	**3a**(%)	**3b**(%)	**3d**(%)	**3f**(%)
1	0	0	20	50	30
2	0.5	0	30	45	25
3	0.75	9	50	31	10
4	1	22	75	5	0
5	1.5	75	20	5	0
6	2	91	5	4	0

Table 3. Results of the biotransformations after 72h with different % of chloroform (TCM). Reaction conditions: 14 mM solution of compound **3a** in a phosphate buffer solution (pH 8.0, 50 mM) with 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg), 5 mg of lyophilized whole cells in 1 mL total volume reaction and various % of chloroform. Different samples were taken after 15 minutes, 30 minutes, 1h, 2h ,3h, 4h, 24h, 48h, 72h and analyzed by chiral GC. Here are reported only the results after 72h in order to see the trend of the different reactions.

As it is possible to see from Table 3, when the amount of chloroform (TCM) was increased, a "shift" in the enantioselectivity was obtained. Without the co-solvent (entry **1**), a preference for isomer **3d** can be observed, but with 0.75% (entry **3**) the situation was completely the opposite, with a higher conversion to isomer **3b**, and this preference remains with 1% of chloroform (entry **4**), when the stereoselectivity for **3b** was almost complete, without the formation of **3f**. A further increase of chloroform led to an inhibition of the biocatalytical activity (entries **5** and **6**).

With these results in hands, I changed other parameters, for example by combining % of TCM or MIBK (the two co-solvents that gave the best results, Table 2 entries **4** and **6**) and pH values, but no better results were achieved, or something that could increase our comprehension on the reaction outcome.
Looking at the results obtained with chloroform and the substantial difference between them and the one without co-solvent (Table 3), we thought that maybe, with chloroform, the activity of KRED enzyme expressed by the whole cells was reduced, while another innate reducing activity of *E.coli* whole cells remained active. Therefore, back to Milan, I started to investigate the same reaction on lyophilized whole cells of *E. coli* BL21 DE3 star, that will be discussed in the next paragraph.

Biotransformations with wild type whole cells of *E.coli* BL21

The cultivation and production of the empty whole cells of *E. coli* was carried on following the protocol used for *E.coli* whole cells expressing the KRED1-Pglu enzyme, in order to obtain cells grown up in the same way. Then they were lyophilized and used.
I first performed three experiments with 5 mg of whole cells: without co-solvent, with 1% of DMSO and with 1% of chloroform. Compound **3a** was always used for reaction optimization. Results are reported in Table 4.

Entry	Co-solvent	**3a** (%)	**3b** (%)	**3c** (%)	**3d** (%)	**3e** (%)	**3f** (%)
1	DMSO	82	18	0	0	0	0
2	TCM	25	75	0	0	0	0
3	No-cosolvent	80	20	0	0	0	0

Table 4. Results obtained from reactions conducted with 1% of DMSO or chloroform (TCM) and without co-solvent. Reaction conditions: 14 mM solution of compound **3a** in phosphate buffer (pH 8.0, 50 mM) with 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg), 5 mg of lyophilized whole cells in 1 mL total volume reaction. Different samples were taken after 15 minutes, 30 minutes, 1h, 2h, 3h, 4h, 24h, 48h, 72h and analyzed by chiral GC. Here are reported only the results after 72h in order to see the trend of the different reactions.

Actually, there was an innate activity in empty whole cells of *E.coli* BL21, and it is a stereselective activity, that does not lead to the formation of diol **3f**. Importantly, this activity is enhanced when chloroform is used (entry **2**), while if DMSO or no co-solvent was employed the conversions were much lower (entries **1** and **3**). Remarkably, the activity shown in entry **2** with chloroform is completely comparable with the one found with whole cells expressing KRED1-Pglu enzyme

(Table 2, entry **4**), while the one obtained with DMSO or without co-solvent are completely different from the ones found with the full microorganism (Table 2, entries **1** and **7**). Therefore, the KRED1-Pglu enzyme expressed by whole cells of *E. coli* works very well but it really seems to be inactivate with chloroform, and under this condition probably remains the innate activity of the empty microorganism. We still do not know why chloroform is able to make this effect on empty and full whole cells, again an hypothesis can be correlated with the enhanced permeability of the cell membrane, but at present we do not have experimental evidences of the explanation.
However, I tried to push the reaction with empty cells, using reaction conditions without the presence of chloroform, and increasing the amount of cells (5, 25 and 50 mg) or the percentage of DMSO (0, 10 and 20%). Results are summarized in Table 5. In this table, I reported the conversions to the mono-alcohol **3b**, since the other isomers were never detected with the empty cells, neither the diol. I repeated, as standard reference, the reaction without co-solvent and with 5 mg of whole cells.

Entry	Cells amount (mg)	DMSO(%)	**3a**(%)	**3b**(%)
1	5	0	80	20
2	5	10	88	12
3	5	20	88	12
4	25	0	75	25
5	25	10	89	11
6	25	20	88	12
7	50	0	72	28
8	50	10	90	10
9	50	20	90	10

Table 5. Results conducted with different amounts of cells (5, 25 or 50 mg) and various percentages of DMSO (0, 10 and 20%). Reaction conditions: 14 mM solution of compound **3a** in a phosphate buffer solution (pH 8.0, 50 mM) with 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg), different amounts of lyophilized whole cells in 1 mL total volume reaction and various % of DMSO. Different samples were taken after 15 minutes, 30 minutes, 1h, 2h, 3h, 4h, 24h, 48h, 72h and analyzed by chiral GC. Here are reported only the results after 72h in order to see the trend of the different reactions.

By changing these parameters, no better results were obtained, and, with higher amounts of DMSO, conversions seem to be worse, even when more cells are employed (entries **5, 6**, **8** and **9**). Remarkably, I tried to run the reaction without cofactor ($NADP^+$) and without the cofactor regeneration system, but, in these cases, I did not see the mono-alcohol formation. I also tried to perform the reaction only with GDH, because we thought about a secondary activity of this enzyme during the reaction, but again I did not observed any conversion.

With these results, the best one obtained so far was again the one with 1% of chloroform. Therefore, as proof of concept, I tried the other substrates (**4a-8a**) under the optimized conditions. The reactions were run for 72h and then acidified with HCl 2N until pH 1 and extracted with ethyl acetate (for three times). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under

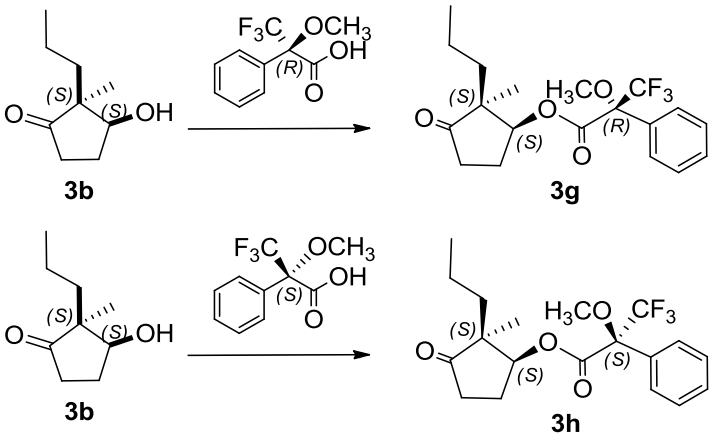
reduced pressure. After purification by column chromatography, different yields for the various substrates (**3a-8a**) were obtained according to Table 6.

Entry	Compound	Un-reacted substrate (%)	*2S, 3S* isomer **3b-8b** (%)
1	**3°**	30	70
2	**4°**	65	35
3	**5°**	70	30
4	**6°**	75	25
5	**7°**	30	70
6	**8°**	90	10

Table 6. Reaction conditions: 14 mM solution of compounds (**3a-8a)** in a phosphate buffer solution (pH 8.0, 50 mM) and 1% of TCM with 40 mM glucose, 0.1 mM NADPH as cofactor, a tip of spatula of GDH (from *Pseudomonas sp.*, commercially available with > 200 U/mg), 5 mg of lyophilized whole cells in 1 mL total volume reaction. Reactions were stopped after 72 h, worked-up and yields were determined by isolation of products and ^{1}H NMR.

All the compounds **3a-8a** were reduced under the optimized conditions, giving different isolated yields (entries **1-6**). The reactions were also controlled by chiral GC after different times (1h, 2h, 3h, 4h, 24h, 48h, 72h, 96h). Conversions did not further increase after 72h. For compounds **5a** and **8a** (with benzylic chain in position 2), I had some analytical problems, because these products could not be seen so clearly in GC. We also tried the HPLC approach but it did not give good results. For this reason, the reactions on these two compounds were stopped at 72h, submitted to work up and purification and analyzed by ^{1}H NMR. As for the other substrates, only one isomer and the un-reacted substrate were detected, with conversions reported in Table 6 (entries **3** and **6**). Leaving the reactions for more time did not further increase their conversions.
I was able to assign the absolute configurations of mono-alcohols obtained under optimized conditions by comparison between the ^{1}H NMR spectra and optical rotation values of mono-alcohols with the ones reported in literature.[73,69,72] For all, the absolute configuration was *2S, 3S*.

To be completely sure of the attribution, I prepared the Mosher ester of compound **3b**, obtained under optimized conditions, following a reported procedure[69]. To do so, I performed the reaction on compound **3a** with both (+)-(*R*)-alpha-methoxy-alpha-(trifluoromethyl) phenylacetic acid (*R*-Mosher acid) and (-)-(*S*)- alpha-methoxy-alpha-(trifluoromethyl) phenylacetic acid (*S*-Mosher acid) (Scheme 4).



Scheme 4. Schematic representation of reactions performed on mono-alcohol product **3b** with both (+)-(*R*)-Mosher acid and (-)-(*S*)-Mosher acid. Reaction conditions: 10 mg of **3b** in 2 mL of dichloromethane, with 2 equivalents of DCC and 0.2 equivalents of DMAP. Reactions were stirred overnight and controlled by TLC.

Reactions were run at room temperature for 24h with 2 equivalents of DCC and 0.2 equivalents of DMAP. After work-up with dichloromethane and evaporation of the solvent, column chromatography was made and the ^{1}H NMR spectra of the two purified esters (**3g** and **3h**) were obtained. The NMR signal of the methyl and the propyl groups in position 2 of product **3g** resulted to be more shielded if compared with the same signals of ester **3h**. Therefore, I was able to assess that the mono-alcohol **3b** obtained under optimized conditions (Table 6, entry **1**) has the absolute configuration (*2S*,*3S*).

Biotransformations with purified free KRED1-Pglu

At the Forschungszentrum Jülich, in the group of Prof. Dörte Rother, I also purified the KRED1-Pglu (ketoreductase from *Pichia glucozyma*), lyophilized it and started to use it on cyclo-diketones **3a-8a**. As done before with whole cells, I decided to use compound **3a** to firstly optimize reaction parameters. Contrary to what done before, the purified KRED1-Pglu enzyme should be dissolved in TRIS HCl buffer, as already reported in literature[64], since it is the best buffer for KRED1-Pglu enzyme. I decided to start with 5% of DMSO as co-solvent and 2 mg of lyophilized purified enzyme. Results are reported in the graphic below (Figure 13).

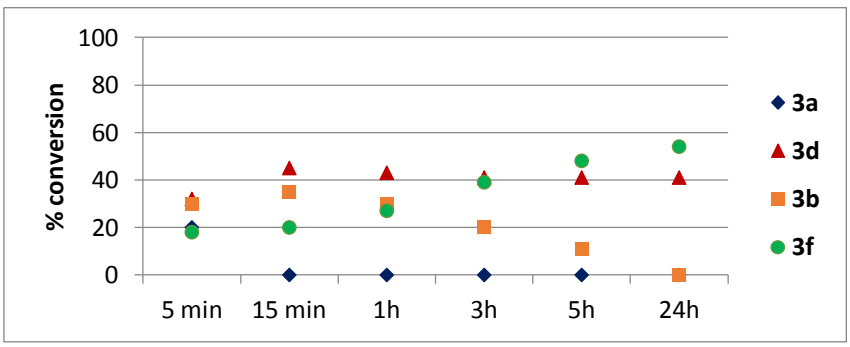


Figure 13. Graphic representation of the reaction performed with 14 mM solution of compound **3a** in buffer TRIS HCl (50mM pH 8.0) containing 5% of DMSO, glucose (40 mM), $NADP^{+}$ (0.1 mM)

and a tip of spatula of GDH from *Pseudomonas sp* (commercially available with > 200 U/mg). 2 mg of lyophilized KRED1-Pglu were used as biocatalyst. Conversions were detected by chiral GC at different times.

Under conditions reported in Figure 3, the reaction was very fast. After 15 minutes the **3a** wasn't detected anymore, but only two mono-alcohols (**3b** and **3d**) and the diol (**3f**). If the reaction was left for more time, at 24h just one mono-alcohol (**3d**) was detected and the diol **3f**, while **3b** wasn't present anymore. Leaving the reaction for more time didn't change the composition of the reaction. As observed before with whole cells, products **3c** and **3e** were never detected. Therefore, the enzyme seems to perform two reactions: the first one was the reduction from **3a** to the two mono-alcohols (**3b** and **3d**), which was very fast. The second reaction was the reduction of one mono-alcohol (**3b**) to the diol (**3f**), while the mono-alcohol **3d** was not further reduced. This was nice because only one mono-alcohol was obtained, but of course the presence of the diol was not desired. Therefore, we decided to change some reaction parameters to try to get rid of it.
Firstly, I increased the concentration of **3a** in the buffer solution, keeping constant the other parameters. I reached a 56 mM concentration, and the results are reported here below (Figure 14).

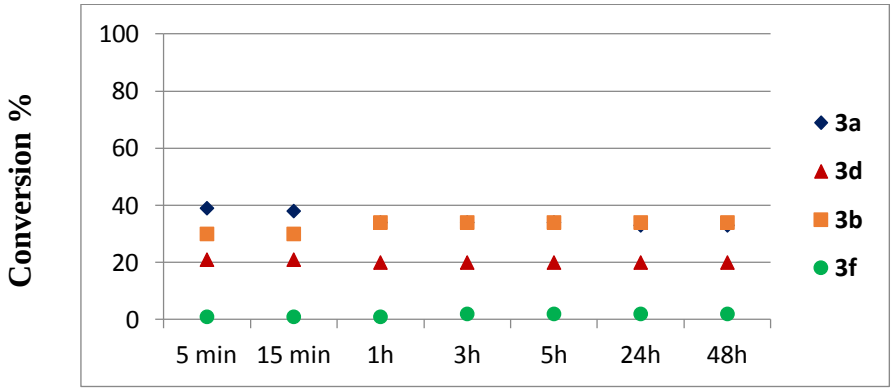


Figure 14. Graphic representation of the reaction performed with 56 mM solution of compound **3a** in buffer TRIS HCl (50 mM pH 8.0) containing 5% of DMSO, glucose (40 mM), $NADP^+$ (0.1 mM) and a tip of spatula of GDH from *Pseudomonas sp* (commercially available with > 200 U/mg). 2 mg of lyophilized KRED1-Pglu were used as biocatalyst. Conversions were detected by chiral GC at different times.

By increasing the concentration of **3a**, the reaction wasn't complete. Therefore, both the mono-alcohols **3d** and **3b** were detected. As a result, the diol formation was not seen. Even if higher reaction times were tried, the composition remained the same. It seems that, in order to obtain only one isomer, the diol formation is necessary.
Secondly, I tried to reduce the amount of enzyme used in the reaction. I tried 1 mg/mL and 0.2 mg/mL. With this last amount, the reaction didn't even start: after 48h it was present almost only substrate. With 1 mg/mL, the reaction was quite slower but the same profile of Figure 13 was seen (Figure 15).

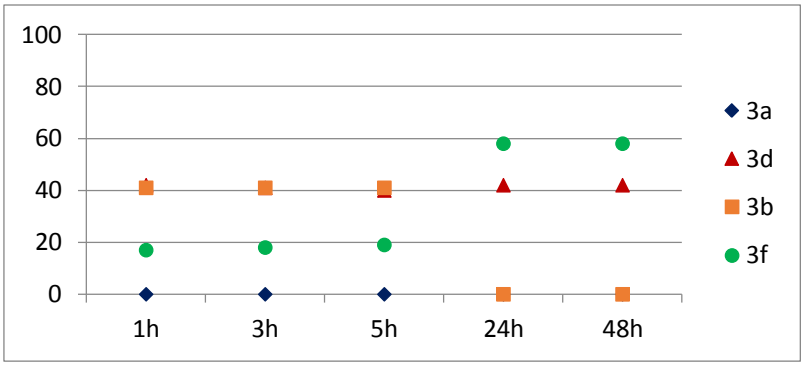


Figure 15. Graphic representation of the reaction performed with 14 mM solution of compound **3a** in buffer TRIS HCl (50 mM pH 8.0) containing 5% of DMSO, glucose (40 mM), $NADP^+$ (0.1 mM) and a tip of spatula of GDH from *Pseudomonas sp* (commercially available with > 200 U/mg). 1 mg of lyophilized KRED1-Pglu were used as biocatalyst. Conversions were detected by chiral GC at different times.

As it's possible to see in Figure 15, after 1h, **3a** was completely used but a mixture of **3b** and **3d** was formed. After 24h, only isomer **3d** and the diol **3f** were detected with a conversion of 42% of the mono-alcohol. Therefore, it seemed that the reaction could just be slowed down, since the same ratio between the products was observed.
We decided to try the same conditions found for the whole cells, therefore to use 1% of chloroform instead of 5% of DMSO. Results are reported here below (Figure 16).

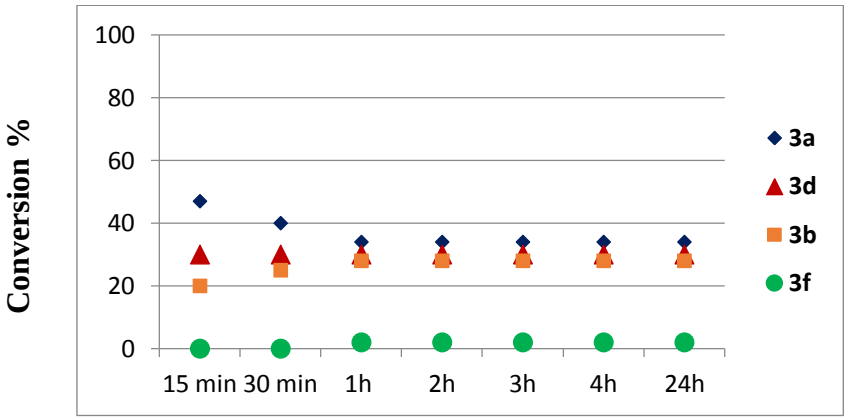


Figure 16. Graphic representation of the reaction performed with 14 mM solution of compound **3a** in buffer TRIS HCl (50 mM pH 8.0) containing 1% of TCM, glucose (40 mM), $NADP^+$ (0.1 mM) and a tip of spatula of GDH from *Pseudomonas sp* (commercially available with > 200 U/mg). 2 mg of lyophilized KRED1-Pglu were used as biocatalyst. Conversions were detected by chiral GC at different times.

Under these conditions, the diol **3f** was not formed, but I detected both **3d** and **3b** with the same conversion. So this is a different result if compared with the one obtained with whole cells because in this case chloroform didn't change the reaction outcome, but a similar reaction trend is obtained if compared with the one achieved by increasing the substrate (**3a**) concentration (Figure 14). Therefore, it could be a confirmation of a second activity in the whole cells as said before (Pag. 84).

I also tried to change the amount of chloroform in the reaction medium, but the profile was always the same. If 0.5% or 0.75% chloroform is used, the trend is more similar to the one reported in Figure 13. If a higher percentage of TCM is used (1.5% or 2%), the reaction doesn't proceed.
Last thing tried was to use a small amount of LiOH to try to stop the reaction before forming the diol. Indeed, in some chemical reactions lithium hydroxide is necessary to block the ketone and to avoid the reduction of it. Therefore, different concentrations of LiOH were used inside the reaction (0.5, 1, 2 mM), but the reaction shows always the same profile with low amounts of LiOH, while increasing it the reaction started to block and only the substrate **3a** was detected.
So, the best result that was achieved with the free KRED1-Pglu is the one represented in Figure 15, with a 14 mM concentration of **3a** and 1 mg/mL concentration of enzyme. We exploited the second reaction performed by the enzymes on the mixture of compounds **3b** and **3d** in order to achieve only the mono-alcohol **3d**, but, of course, the diol **3f** was obtained as well.
As proof of concept, the reduction of the other substrates was performed (**4a-8a**) under optimized conditions (14 mM solution of compounds **4a-8a** in buffer TRIS HCl 50 mM pH 8.0 containing 5% of DMSO, glucose (40 mM), $NADP^+$ (0.1 mM) and GDH from *Pseudomonas sp* (tip of spatula: it should be in terms of unit five times the unit of KRED1-Pglu). 1 mg of lyophilized KRED1-Pglu was used. For each substrate, the reaction profile was the same and samples were taken at different times to stop the reaction at the correct point, in order to obtain only one enantiomerically pure mono-alcohol product and the corresponding diol. Then the reactions were performed at a higher scale, ad after the correct time the buffer solutions were acidified with HCl 1N to pH 1.0 and extracted with ethyl acetate (3 times). The organic phases were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The mono-alcohol and the diol were separated with a chromatographic column and both of them were characterized with ^1H NMR and optical rotation values. In Table 7 are summarized the recovered yields of all the obtained products.

Entry	Compound	Time (h)	*2S, 3S* isomer (%)	*2R, 3S* isomer **4d-8d** (%)	diol (%)
1	**4a**	24	0	50	50
2	**5a**	36	50	0	50
3	**6a**	4	0	40	60
4	**7a**	2	0	25	75
5	**8a**	36	60	0	40

Table 7. Results of the reactions performed with 14 mM solution of compound **4a-8a** in buffer TRIS HCl (50 mM pH 8.0) containing 5% of DMSO, glucose (40 mM), $NADP^+$ (0.1 mM) and a tip of spatula of GDH from *Pseudomonas sp* (commercially available with > 200 U/mg). 1 mg of lyophilized KRED1-Pglu was used as biocatalyst. Yields were obtained after isolation and ^1H NMR of the products.

As it is possible to see from Table 7, the same profile was detected for all the substrates **3a-8a**. Indeed, in all cases the substrate was completely used, ad after a certain time (different from case to case), only one mono-alcohol product and the diol were detected. The faster compound to be reduce was **7a**, which instead gave the worst conversion and ratio between mono-alcohol and diol (entry **4**). For it, as well as for **3a**, **4a** and **6a**, the produced isomer was *2R,3S*, with various conversions

(entry **1,2,4** and **5**). The results were different for compounds **5a** and **8a**, the ones which have the benzylic chain in position 2. For these, after 36h, the isomer detected was the *2S,3S*, differently from the one obtained with the other substrates. This can be probably due to the increased steric hindrance of compounds **5a** and **8a**. For these two compounds some analytical problems were detected, because these products could not be seen so clearly in GC. Also the HPLC approach was tried but it wasn't better. For this reason, these two compounds were stopped at 36h and, as the other substrates, only one isomer and the diol were detected, with conversions reported in Table 7 (entries **2** and **5)**. The reaction composition wasn't modified if a higher reaction time was applied .

In all cases, the absolute configurations were assigned by comparison with ^{1}H NMR and optical rotation values reported in literature.[25,75] Since for the whole cells I synthesized the two Mosher esters (**3g** and **3h**) to confirm the results found in literature[69], here we just compared our NMR spectra and optical rotation values with the one reported in the same article[69].

Conclusions

A deep study on 2,2-disubstituted-1,3-cyclopenta- and 1,3-cyclohexanedione was developed with the aim to obtain only one enantiomerically pure mono-alcohol product, among the four possible isomers that can be obtained with the mono reductive reaction. Whole cells of *E.coli* expressing KRED1-Pglu enzyme, empty whole cells of *E.coli* and purified free KRED1-Pglu enzyme were used as biocatalysts to understand the trend and the potentialities of the reaction. The reaction seems very fast with two drawbacks: the formation of two mono-alcohol isomers and the presence of the diol. Optimizing the reaction parameters, two different results were achieved: with whole cells, probably, a secondary activity of *E. coli* was exploited, reached using 1% of chloroform in the reaction medium, and obtaining only one enantiomerically pure mono-alcohol, without the diol. The biotransforations did not go to completion, and the same results were obtained with both recombinant and wild type cells. Of course, the same trick wasn't successful with purified free enzyme, because here a possible second activity is not possible. Therefore, with the free enzyme the best result we obtained was to run the reaction for more time and to exploit a second activity of the biocatalyst, that seems to use only one mono-alcohol to give the diol, while the second mono-alcohol remains un-touched. In this way, also with the free enzyme only one enantiomerically pure product was obtained, but this time also the diol. We solved the absolute configuration by comparison of our NMR spectra and optical rotation values with the ones reported in literature.

Materials and methods

General

All reagents and solvents were purchased from Sigma–Aldrich. ^{1}H NMR and ^{13}C 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. The molar conversion of the biotransformation and its enantiomerical purity was determined by GC equipped with a (CP)-Chirasil-DEX-CB and a FID detector. Different methods were used for the various substrates:

- **3a-3f** → starting from 90 °C for 10 minutes, then increasing the temperature with a rate of 4 °C/min until 180 °C for 5 minutes;
- **4a-4f** → starting from 80 °C for 10 minutes, then increasing the temperature with a rate of 2 °C/min until 180 °C for 5 minutes;
- **5a-5f** → starting from 90 °C, then increasing the temperature with a rate of 3 °C/min until 180 °C for 5 minutes;
- **6a-6f** → starting from 90 °C for 10 minutes, then increasing the temperature with a rate of 2 °C/min until 180 °C for 5 minutes;
- **7a-7f** → starting from 90 °C for 10 minutes, then increasing the temperature with a rate of 3 °C/min until 180 °C for 5 minutes;
- **8a-8f** → starting from 90 °C for 10 minutes, then increasing the temperature with a rate of 2 °C/min until 180 °C for 5 minutes;

Synthesis and characterization of 2-methyl-2-(3-propenyl)-1,3-cyclopentadienone (4a)[69]

To a solution of **1** (673 mg, 6 mmol) in 1M NaOH (6 mL) was added allyl bromide (1.451 gr, 12 mmol). The reaction was stirred for 24h at room temperature. It was followed by TLC (9:1 DCM/MeOH). The mixture was extracted with dichloromethane (5 times) and the organic phase was dried over Na_2SO_4, filtered and evaporated. The crude extract was then purified with a chromatographic column (9:1 cyclohexane/ethyl acetate) to obtain compound **4a** as a pale yellow oil with a yield of 50% (470 mg, 3 mmol): bp 65 °C (2 mm); ^{1}H NMR ($CDCI_3$, 300 MHz) δ 1.1 (3H, s, CH_3), 2.3 (2 H, d, J = 8 Hz), 2.7 (4 H, br s), 4.8-5.2 (2 H, m), 5.3-5.9 (1 H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 18.7 (CH_3), 35.4 ($2CH_2$), 40.1 (CH_2), 56.6 (C), 119.7 (=CH_2), 131.7 (CH=), 215.9 (2 C=O), MS, *m/e* M^+ 152. Anal. Calcd for $C_9H_{12}0_2$: C, 71.03; H, 7.95. Found: C, 71.12; H, 7.83. Chiral GC retention time: 26.179 minutes.

Synthesis and characterization of 2-methyl-2-propyl-1,3-cyclopentanedione (3a)[69]

A solution of compound **4a** (700 mg, 4.6 mmol) was prepared in MeOH (140 mL) and it was pumped by the HPLC pump of H-cube mini ThalesNano. A CatCart of Pd/C 5% was used, at a temperature of 30 °C and a pressure of 1 bar. A flow rate of 0.5 mL/min was used and a DryCart was applied to the system, to remove the major part of water formed for the in-situ production of H_2. The reaction was controlled by TLC (7:3 CHX/EA). The exiting flow was collected and MeOH was evaporated. The low amount of water left was extracted with dichloromethane (3 times), the organic phase was then dried on Na_2SO_4 and evaporated. Compound **3a** was obtained with a yield

of 95% (674 mg, 4.37 mmol): bp 65 °C (2 mm); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.84 (3 H, t, *J* = 7 Hz, CH_3), 1.08 (3 H, s, CH_3), 1.15-1.75 (4 H, m), 2.73 (4H, br s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3 (CH_3), 18.1 (CH_2), 19.1 (CH_3), 35.3 ($2CH_2$), 38.2 (CH_2), 56.8 (C), 216.6 (2 C=O); MS, *m/e* M^+ 154. Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found C, 69.95; H, 9.25. Chiral GC retention time: 18.105 minutes.

Synthesis and characterization of 2-methyl-2-benzyl-1,3-cyclopentanedione (5a)[70]

To a solution of 1 (650 mg, 5.8 mmol) in 1M NaOH (6.5 mL) was added benzyl bromide (1.978 gr, 11.57 mmol). The reaction was stirred for 36h at room temperature and it was followed by TLC (7:3 CHX/EA). The mixture was then extracted with ethyl acetate (3 times) and the organic phase was dried on Na_2SO_4 and evaporated. The crude extract was purified with a chromatographic column (9:1 CHX/EA). Compound **5a** was obtained as a white solid with a yield of 50% (587 mg, 2.9 mmol): mp 50 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.20 (3H, s), 2.01-2.08 (2H, m), 2.51-2.57 (2H, m), 2.95 (2H, s), 7.02-7.04 (2H, m), 7.19-7.24 (3H, m); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 20.1, 35.9, 43.2, 58.4, 127.3, 128.7, 129.7, 135.9, 217.9. Chiral GC retention time: 20.065 minutes.

Synthesis and characterization of 2-methyl-2-(3-propenyl)-1,3-cyclohexanedione (7a)[69]

To a solution of **2** (757 mg, 6 mmol) in 1M NaOH (6 mL) was added allyl bromide (1.451 gr, 12 mmol). The reaction was stirred for 24h at room temperature. It was followed by TLC (9:1 DCM/MeOH). The mixture was extracted with dichloromethane (3 times) and the organic phase was dried over Na_2SO_4, filtered and evaporated. The crude extract was then purified with a chromatographic column (8:2 CHX/EA) to obtain compound **7a** as a pale yellow oil with a yield of 50% (500 mg, 3 mmol): ^{1}H NMR ($CDCl_3$, 300 MHz): δ=5.63–5.54 (m, 1H), 5.10–5.05 (m, 2H), 2.68-2.64 (m, 4H), 2.54 (d, 2H, J=8 Hz), 2.05–1.87 (m, 2H), 1.25 (s, 3H); ^{13}C NMR ($CDC1_3$, 75 MHz) δ 14.0 (CH_3), 15.6 (CH_2), 30.48 ($2CH_2$), 33.0 (CH_2), 51.8 (C), 94.5 (=CH_2), 105.2 (CH=), 209.0 (2 C=O); MS, *m/e* M^+ 166. Anal. Calcd for $C_{10}H_{14}0_2$: C, 72.26; H, 8.49. Found: C, 72.34; H, 8.29. Chiral GC retention time: 24.698 minutes.

Synthesis and characterization of 2-methyl-2-propyl-1,3-cyclohexanedione (6a)[69]

A solution of compound **7a** (700 mg, 4.2 mmol) was prepared in MeOH (140 mL) and it was pumped by the HPLC pump of H-cube mini ThalesNano. A CatCart of Pd/C 5% was used, at a temperature of 30 °C and a pressure of 1 bar. A flow rate of 0.5 mL/min was used and a DryCart was applied to the system, to remove the major part of water formed for the in-situ production of H_2. The reaction was controlled by TLC (7:3 CHX/EA). The exiting flow was collected and MeOH was evaporated. The low amount of water left was extracted with dichloromethane (3 times), the organic phase was then dried on Na_2SO_4 and evaporated. Compound **6a** was obtained with a yield of 95% (671 mg, 3.9 mmol): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.9 (3H, t, *J* = 7 Hz), 1.0-1.3 (2H, m), 1.2 (3H, s, CH_3), 1.7-2.2 (4H, m), 2.6-2.8 (4H, m); 13C NMR ($CDCl_3$, 75 MHz) δ 14.4 (CH_3), 17.8 (CH_3), 18.1 (CH_2), 18.8 (CH_2), 38.0 ($2CH_2$), 40.0 (CH_2), 65.9 (C), 210.3 (2C=O); MS, *m/e* M^+ 168. Anal. Calcd for $C_{10}H_{16}O_2$: C, 71.39; H, 9.59. Found C, 71.42; H, 9.48. Chiral GC retention time: 27.780 minutes.

Synthesis and characterization of 2-methyl-2-benzyl-1,3-cyclohexanedione (8a)[70]

To a solution of 1 (650 mg, 5.2 mmol) in 1M NaOH (6.5 mL) was added benzyl bromide (1.978 gr, 11.57 mmol). The reaction was stirred for 36h at room temperature and it was followed by TLC (7:3 CHX/EA). The mixture was then extracted with ethyl acetate (3 times) and the organic phase was dried on Na_2SO_4 and evaporated. The crude extract was purified with a chromatographic column (9:1 CHX/EA). Compound **5a** was obtained as a white solid with a yield of 50% (562 mg, 2.6 mmol): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.28 (3H, s), 1.55-1.42 (1H, m), 1.80-1.67 (1H, m), 2.35-2.23 (2H, m), 2.60-2.48 (2H, m), 3.11 (2H, s), 7.04-6.99 (2H, m), 7.28-7.16 (3H, m), 13C NMR ($CDCl_3$, 75 MHz,) 17.0, 22.5, 39.7, 44.3, 65.7, 127.4, 128.8, 130.3, 137.1, 211.8. Chiral GC retention time: 48.694 minutes.

Preparation of recombinant *E. coli* BL21 DE3 cells expressing KRED1-Pglu

Cultures of *E. coli BL21* (DE3) containing Chaperone III transformed with the plasmid pET26 KRED1-Pglu were pr-inoculated in 100 mL Erlenmeyer baffled flask containing 20 mL of LB medium [yeast extract (5 gL-1), tryptone (10 gL-1) and NaCl (10 gL-1)] supplemented with 25 μg/mL kanamycin and 25 μg/mL chloramphenicol. After growth for 24h (37 °C at 150 rpm) the OD concentration was checked and a calculated volume was picked up, in order to have in the main culture a 0.1 OD concentration. The inoculation was made in 200 mL of TB medium [yeast extract (24 gL-1), tryptone (12 gL-1), glycerol (4 gL-1)], 10% of buffer phosphate pH 7.0 0.1M, 25 μg/mL kanamycin, 25 μg/mL chloramphenicol and 0.5 mg/mL L-arabinose. The shaking flasks were leaved at 37 °C and 90 rpm until the culture reached an OD value of 0.5. At this point, cells were induced for 72h with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, at a temperature of 10 °C and a shaking of 90 rpm. Cells were then harvested by centrifugation at 8000 rpm and 4 °C for 45 min, washed once with 20 mM phosphate buffer at pH 7.0, lyophilized for 24/48h and stored at −20 °C.

Preparation of wild type *E. coli* BL21 DE3 star cells

The preparation of the wild type cells was performed following perfectly the protocol reported above for the *E. coli* cells expressing KRED1-Pglu enzyme. The only difference is that, in case of empty cells, no induction with IPTG was performed.

Expression and purification of KRED1-Pglu enzyme

For the expression of KRED1-Pglu enzyme, the hosting cells of E. coli BL21 DE3 were prepared as described above. Cells were suspended in 50 mM phosphate buffer, 300 mM NaCl, pH 8.0. Proteins were extracted by sonication (10 cycles of 1 min each, in ice, with 1 min interval) and cell debris were harvested by centrifugation at 21000 rpm for 30 min at 4 °C. The enzyme was purified by affinity chromatography with HIS-Select® Nickel Affinity Gel using an AKTÄ system. Briefly, the column was equilibrated with 50 mM phosphate buffer, 300 mM NaCl, pH 8.0 and the crude extract loaded; column was then washed with 50 mM phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0; finally, the adsorbed enzyme was eluted with 50 mM phosphate buffer, 300 mM NaCl, 250

mM imidazole, pH 8.0. Pellets, crude extracts and collected fractions were analysed by SDS-PAGE. The fractions showing the presence of a band of the expected size (27 kDa) were pooled and stored at −20 °C. After 24h, we lyophilized the enzyme for a 24/48h. From 4 g of whole cells we obtained 600 mg of lyophilized enzyme.

KRED1-Pglu activity test

Activity measurements were performed following a protocol previously reported[74]. It was made spectrophotometrically at 340 nm by determining the consumption of NAD(P)H at 25 °C in a half-microcuvette (total volume 1 mL) for 5 min. One unit (U) of activity is defined as the amount of enzyme which catalyses the consumption of 1 μmol of NAD(P)H per minute under reference conditions, namely with 0.25 mM NAD(P)H and 0.47mM benzil as substrate (added as concentrated DMSO solution; final DMSO concentration in cuvette amounts to 0.1 %), in 50 mM Tris–HCl buffer, pH 8.0.

General procedure for the biotransformation with *E. coli* cells (both recombinant and wild type)

A solution of the substrate (**3a-8a**) (14 mM) was prepared in 20 mL of phosphate buffer 50 mM pH 8.0 with 1% of TCM. In the same solution were added glucose (40 mM), $NADP^+$ (0.1 mM) and a tip of spatula of GDH from *Pseudomonas sp* (commercially available from Sigma Aldrich, > 200 U/mg). As last, lyophilized wild type E.coli cells BL21 DE3 star were added to the buffer solution (5mg/mL). After 72h, the reactions were then acidified, extracted with ethyl acetate (3 times) and the organic phase was dried with sodium sulphate. After removal of the solvent, a chromatographic column was performed to separate the un-reacted substrate from the products (8:2 cyclohexane/ethyl acetate). Both the un-reacted substrates and the obtained mono-alcohols were confirmed with ^{1}H NMR and ^{13}C NMR. The absolute configuration of products (**3b-8b**) were assigned by comparison with reported NMR spectra and reported optical rotation values.[73,69,72]

Products characterization (3b-8b):

(*2S,3S*)-3-hydroxy-2-methyl-2-propylcyclopentan-1-one (**3b**)[69]: α_D^{20}= +61.0 ° **(**c 1.94, $CHC1_3$,); IR (neat) 3400 (br s), 1720 cm^{-1} (s); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.94 (3 H, t, *J* = 7.2 Hz, CH,), 1.00 (3 H, s, CH,), 1.29 (1 H, m), 1.42 (1 H, m), 1.50 (2 H, m), 1.61 (1 H, br s, OH), 1.95 (1 H, m), 2.16-2.32 (2 H, m), 2.46 (1 H, m), 4.11 (1 H, t, *J* = 4.5 Hz); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 14.8 (CH_3), 17.1 (CH_2), 19.2 (CH_3), 27.8 (CH_2), 32.3 (CH_2), 34.0 (CH_2), 53.2 (C), 77.6 (CHOH), 221.0 (C=O); MS, *m/e* M^+ 156. Anal. Calcd for $C_9H_{16}0_2$: C, 69.19; H, 10.32. Found: C, 68.97; H, 10.59. Chiral GC retention time: 26.590 minutes.

(*2S,3S*)-2-allyl-3-hydroxy-2-methylcyclopentan-1-one (**4b**)[69]: α_D^{20}= + 54.0 ° ***(***c 0.4, $CHCl_3$); IR (neat) 3380 (br s), 3010 (w), 1710 cm^{-1} (s); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.01 (3 H, s, CH,), 1.78 (1 H, d, *J* = 3 Hz), 1.97 (1 H, m), 2.16-2.52 (5 H, m), 4.13 (1 H, dd, *J* = 4, 3 Hz), 5.16 (2 H, m), 5.89 (1 H, m); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 19.7 (CH_3), 27.8 (CH2), 34.0 (CH2), 35.4 (CH2),

53.2 (C), 77.2 (CHOH), 118.1 (=CH2), 134.4 (CH=), 220.6 (C=O); MS , *m/ e* M^+ 154**.** Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found: C, 70.40; H, 9.43.

(*2S,3S*)-2-benzyl-3-hydroxy-2-methylcyclopentan-1-one (**5b**)[76]: α_D^{20}= + 74.88 ° (*c* 1.0, CHCl3); IR (film) 3503, 2975, 2954, 2905, 1725, 1636, 1558, 1458, 1069, 1027, 741, 703 cm-1; ^{1}H NMR (300 MHz, CDCl3) δ 0.87 (3H, s), 1.93-1.85 (2H, m); 2.18 (1H, m), 2.38 (1H, m), 2.51 (1H, m), 2.73 (1H, d, *J* =14.0 Hz) 3.06 (1H, d, *J* = 14.0 Hz), 4.05 (1H, t, *J* = 3.0 Hz), 7.21 (1H, m), 7.28-7.25 (4H, m), ^{13}C NMR (125 MHz, CDCl3) δ 220.5, 137.9, 130.4, 128.1, 126.2, 76.4, 54.8, 35.7, 33.7, 28.2, 19.6; mp 88-90 °C.

(*2S,3S*)-3-hydroxy-2-methyl-2-propylcyclohexan-1-one (**6b**)[69]: α_D^{20}= + 65 ° (*c* 0.57, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.97 (3H, t, *J* = 7.5 Hz, CH3), 1.02 (1H, m), 1.18 (3H, s, CH3), 1.29 (1H, m), 1.60 (4H, m), 1.70 (1H, m), 1.97 (2H, m), 2.37 (2H, m), 3.69 (1H, dd, *J* = 8.5,4 Hz); ^{13}C NMR ($CDCI_3$, 300 MHz) δ 14.9 (CH_3), 16.7 (CH_2), 19.0 (CH_3), 20.7 (CH_2), 28.8 (CH_2), 33.8 (CH_2), 37.7 (CH_2), 54.9 (C), 77.6 (CHOH), 215.4 (C=O); MS, *m/e* M^+ 170. Anal. Calcd for $C_{I0}H_{l8}O_2$: C, 70.55; H, 10.66. Found: C, 70.67; H, 10.56.

(*2S,3S*)-2-allyl-3-hydroxy-2-methylcyclohexan-1-one (**7b**)[69]: α_D^{20}= + 27.5 ° (*c* 0.42, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.19 (3H, s, CH_3), 1.72 (1H, m), 1.76 (1H, br s, OH), 1.90 (1H, m), 2.05 (2H, m), 2.41 (4H, m), 3.81 (1H, m), 5.10 (2H, m), 5.78 (1H, m); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 20.0 (CH_3), 20.6 (CH_2), 28.5 (CH_2), 36.9 (CH_2), 37.7 (CH_2), 54.0 (C), 76.7 (CHOH), 118.0(=CH_2), 134.2 (CH=), 214.4 (C=O); MS, *m/e* M^+ 168. Anal. Calcd for $C_{10}H_{16}0_2$: C, 71.39; H, 9.59. Found: C, 71.41; H, 9.45.

(*2S,3S*)-2-benzyl-3-hydroxy-2-methylcyclohexan-1-one (**8b**)[77]: α_D^{20}= + 10.5 ° (*c* 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.06 (3H, s, CH_3), 1.72 (1H, m), 1.76 (1H, m, OH), 1.86 (1H, m), 2.05 (1H, m), 2.12 (1H, m), 2.55 (2H, t, *J* = 6.9 Hz), 2.96 (1H, d, *J* = 13.7 Hz), 3.10 (1H, d, *J* = 13.7 Hz), 3.75 (1H, m), 7.13-7.29 (5H, m). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 20.5, 20.9, 28.7, 37.6, 38.0, 54.8, 75.9, 126.6, 128.3, 130.7, 137.8, 214.4.

General procedure for biotransformation with purified KRED1-Pglu enzyme

A solution of the substrates (**3a-8a**) (14 mM) was prepared in 20 mL of buffer TRIS HCl 50mM pH 8.0 containing 5% of DMSO. In the same solution were added glucose (40 mM), $NADP^+$ (0.1 mM) and a tip of spatula of GDH from *Pseudomonas sp* (commercially available at Sigma Aldrich with > 200 U/mg). 1 mg of lyophilized KRED1-Pglu was used as biocatalyst. After different times (according with Table 7, pag 89) the reactions were acidified, extracted with ethyl acetate (3 times) and the organic phases were dried with sodium sulphate. After removal of the solvent, a chromatographic column was performed to separate the mono-alcohols and the diols (8:2 cyclohexane/ethyl acetate). Both were then confirmed with ^{1}H NMR. The absolute configuration of products were assigned by comparison with reported NMR spectra and reported optical rotation values.[73,69,72]

Products characterization (3d-8d):

(*2R,3S*)-3-hydroxy-2-methyl-2-propylcyclopentan-1-one (**3d**)[69]: α_D^{20}= -110 ° **(**c 1.5, $CHC1_3$,); ^1H NMR ($CDCl_3$, 300 MHz) δ 0.89 (3 H, t, *J* = 7.2 Hz, CH_3), 1.00 (3 H, s, CH_3), 1.20-1.40 (4H, m), 1.93 (1H, m), 2.05-2.15 (2H, m), 2.42 (1H, m), 4.20 (1H, t, *J* = 6.0 Hz) Chiral GC retention time: 26.072 minutes.

(*2R,3S*)-2-allyl-3-hydroxy-2-methylcyclopentan-1-one (**4d**)[69]: α_D^{20}= - 73 ° **(**c 0.26, $CHCl_3$); IR (neat) 3400 (br s), 3050 cm^{-1} (w), 1730 cm^{-1} (s); ^1H NMR ($CDCl_3$, 300 MHz) δ 1.02 (3H, s, CH_3), 1.69 (1H, br s OH), 1.87 (1H, m), 2.14-2.30 (4H, m), 2.48 (1H, m), 4.23 (1H, t, *J* = 6.4 Hz), 5.11 (2H, m), 5.76 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 15.0 (CH_3), 27.5 (CH_2), 34.9 (CH_2), 39.8 (CH_2), 53.0 (C), 75.4 (CHOH), 118.7 (=CH2), 133.5 (C=), 219.9 (C=O); MS , *m/ e* M^+ 154**.** Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found: C, 70.06; H, 9.40.

(*2S,3S*)-2-benzyl-3-hydroxy-2-methylcyclopentan-1-one (**5b**)[76]: α_D^{20}= + 74.88 ° (*c* 1.0, CHCl3); IR (film) 3503, 2975, 2954, 2905, 1725, 1636, 1558, 1458, 1069, 1027, 741, 703 cm-1; ^1H NMR (300 MHz, CDCl3) δ 0.87 (3H, s), 1.93-1.85 (2H, m); 2.18 (1H, m), 2.38 (1H, m), 2.51 (1H, m), 2.73 (1H, d, *J* =14.0 Hz) 3.06 (1H, d, *J* = 14.0 Hz), 4.05 (1H, t, *J* = 3.0 Hz), 7.21 (1H, m), 7.28-7.25 (4H, m), ^{13}C NMR (125 MHz, CDCl3) δ 220.5, 137.9, 130.4, 128.1, 126.2, 76.4, 54.8, 35.7, 33.7, 28.2, 19.6; mp 88-90 °C.

(*2R,3S*)-3-hydroxy-2-methyl-2-propylcyclohexan-1-one (**6d**)[69]: α_D^{20}= - 40.85 ° (*c* 0.54, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 0.92 (3H, t, *J* = 7.5 Hz, CH_3), 1.10 (1H, m), 1.12 (3H, s, CH_3), 1.34 (1H, m), 1.57 (3H, m), 1.80 (2H, m), 2.11 (2H, m), 2.34 (1H, m), 2.42 (1H, m), 3.91 (1H, dd, *J* = 5.5, 2.5 Hz); ^{13}C NMR ($CDCI_3$, 75 MHz) δ 14.8 (CH_3), 17.3 (CH_2), 17.5 (CH_3), 20.7 (CH_2), 28.2 (CH_2), 37.9 (CH_2), 38.6 (CH_2), 54.5 (C), 76.4 (CHOH), 214.9 (C=O); MS, *m/e* M^+ 170. Anal. Calcd for $C_{I0}H_{l8}O_2$: C, 70.55; H, 10.66. Found: C, 70.48; H, 10.71.

(*2R,3S*)-2-allyl-3-hydroxy-2-methylcyclohexan-1-one (**7d**)[69]: α_D^{20}= - 9.4 ° (*c* 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 1.17 (3H, s, CH_3), 1.68 (2H, m), 1.88 (1H, m), 2.05 (2H, m), 2.20 (4H, m), 3.90 (1H, m), 5.10 (2H, m), 5.78 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.6 (CH_3), 20.4 (CH_2), 28.5 (CH_2), 37.6 (CH_2), 40.2 (CH_2), 54.5 (C), 75.1 (CHOH), 118.0(=CH_2), 134.0 (CH=), 214.4 (C=O); MS, *m/e* M^+ 168. Anal. Calcd for $C_{10}H_{16}0_2$: C, 71.39; H, 9.59. Found: C, 71.43; H, 9.48.

(*2S,3S*)-2-benzyl-3-hydroxy-2-methylcyclohexan-1-one (**8b**)[77]: α_D^{20}= + 10.5 ° (*c* 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 1.06 (3H, s, CH_3), 1.72 (1H, m), 1.76 (1H, m, OH), 1.86 (1H, m), 2.05 (1H, m), 2.12 (1H, m), 2.55 (2H, t, *J* = 6.9 Hz), 2.96 (1H, d, *J* = 13.7 Hz), 3.10 (1H, d, *J* = 13.7 Hz), 3.75 (1H, m), 7.13-7.29 (5H, m). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 20.5, 20.9, 28.7, 37.6, 38.0, 54.8, 75.9, 126.6, 128.3, 130.7, 137.8, 214.4.

Diols characterization (3f-8f)

2-methyl-2-propylcyclopentane-1,3-diol (**3f**): α_D^{20}= + 44.6 ° (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.92 (3H, s, CH_3), 0.96 (3H, t, *J* = 7.2), 1.22-1.42 (4H, m), 1.42-1.59 (2H, m), 2.17 (2H, m), 3.90 (1H, t, *J* = 4.5 Hz), 4.03 (1H, t, *J* = 6.0).

2-allyl-2-methylcyclopentane-1,3-diol (**4f**): α_D^{20}= + 69.68 ° (*c* 2.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.89 (3H, s, CH_3), 1.42-1.60 (2H, m), 2.09-2.22 (2H, m), 2.22-2.35 (2H, m), 3.90 (1H, dd, *J* = 4.3 Hz), 4.11 (1H, t, *J* = 6.4 Hz), 5.11 (2H, m), 5.95 (1H, m).

2-benzyl-2-methylcyclopentane-1,3-diol (**5f**): α_D^{20}= + 91.00 ° (*c* 1.5, CHCl3); ^{1}H NMR (300 MHz, CDCl3) δ 0.80 (3H, s), 1.45-1.62 (3H, m); 2.10-2.36 (2H, m), 2.67 (1H, d, *J* = 14.0), 2.91 (1H, d, *J* = 14.0), 3.89 (1H, dd, *J* = 1.5 Hz), 4.26 (1H, t, *J* = 3.0 Hz), 7.21 (1H, m), 7.28-7.25 (4H, m).

2-methyl-2-propylcyclohexane-1,3-diol (**6f**): α_D^{20}= + 40.63 ° (*c* 0.95, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.92 (3H, s), 0.95 (3H, t, *J* = 7.5 Hz), 1.20-1.35 (2H, m), 1.35-1.45 (2H, m), 1.35-1.76 (6H, m), 3.71 (1H, dd, *J* = 8.5,4 Hz), 3.77 (1H, dd, *J* = 5.5, 2.5 Hz).

2-allyl-2-methylcyclohexane-1,3-diol (**7f**): α_D^{20}= + 42.0 ° (*c* 2.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.94 (3H, s, CH_3), 1.44-1.57 (6H, m), 2.32 (2H, d, *J* = 4.8), 3.71 (1H, m), 3.78 (1H, m), 5.11 (2H, m), 5.95 (1H, m).

2-benzyl-2-methylcyclohexane-1,3-diol (**8f**): α_D^{20}= + 36.0 ° (*c* 3.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.86 (3H, s, CH_3), 1.46-1.91 (6H, m), 2.84 (1H, d, *J* = 13.7 Hz), 2.99 (1H, d, *J* = 13.7 Hz), 3.60 (1H, m), 3.88 (1H, dd, *J* = 3.0 Hz), 7.13-7.29 (5H, m).

TRANSAMINATION REACTIONS WITH TRANSAMINASE FROM HALOMONAS ELONGATA IN A FLOW REACTOR

Article involved: "Highly efficient oxidation of amines to aldehydes via flow-based biocatalysis" Martina L. Contente,[a,b,d] Federica Dall'Oglio,[c] Lucia Tamborini,[c] Francesco Molinari,[a] Francesca Paradisi[b,d]

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Authors contribution

The discover of the enzyme, its production and purification and the different batch reaction were performed by the group of Prof. Francesca Paradisi and Dr. Martina Letizia Contente. The flow reactions and the continuous downstream processes were made by both the group of Prof. Francesca Paradisi and Dr. Martina Letizia Contente and by me and by my group, with the collaboration of Dr. Lucia Tamborini and Prof. Francesco Molinari.

I would like to thank all the people involved in this work.

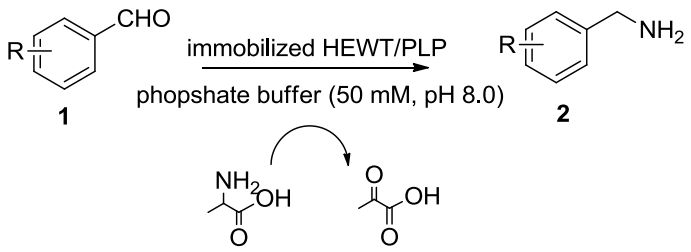
Introduction

Aromatic aldehydes are key intermediates in a number of synthetic processes and have a prominent role as flavor and fragrance components. Among other synthetic methods,[78] they can be obtained from the corresponding primary aromatic amines, which are readily available substrates. Methods for the oxidation of amines to carbonyl compounds have received significant attention, but these approaches are often poorly sustainable, because they require drastic reaction conditions, produce waste and by-products difficult to recycle and often proceed with poor selectivity.[79] Therefore, biocatalytic processes are interesting alternatives for amine oxidations under mild and benign conditions. For example, copper amine oxidases (CAOs) have been used to catalyze the oxidation of primary amines to aldehydes (while O_2 is simultaneously reduced to H_2O_2).[80] Vanillin has been prepared by oxidation of vanillylamine using an amine oxidase (AO) from *Aspergillus niger*.[81] Recently, selective oxidation of amines to aldehydes has been obtained using a laccase with TEMPO (2,2,6,6-tetramethylpiperidine *N*-oxide) as mediator and O_2 as oxidant.[82] Aromatic aldehydes can also be enzymatically prepared using other approaches, such as oxidation of primary alcohols[83,26,34] and reduction of carboxylic acids.[84]
Also, the preparation of enantiopure aromatic amines can be challenging with traditional synthesis. Indeed, three main strategies can be used: (1) the resolution of racemic β-amino acids derivatives, (2) the use of naturally occurring chiral α-amino acids as starting material, and (3) asymmetric synthesis. All these strategies have their limitations when applied in an industrial scale (*i.e.*, resolutions of complex and time-consuming racemic mixtures, the limited chiral pool of natural α-amino acids and the high costs of catalysts or chiral auxiliaries).[85] Thus, biocatalytic paths are a promising alternative for the preparation of chiral β-amino acids and enantiopure amines and they are receiving increased attention in the last years for their application in the manufacture of pharmaceutical intermediates on a large scale.[86]

In this context, transaminases are a family of enzymes with high potential in biotechnological applications. They can be useful for the enantioselective production of a series of compounds with high pharmaceutical value such as chiral amines and enantiopure amino alcohols.[87] Some transaminases displayed high stability, high turnover rate, broad substrate specificity and no requirement for external cofactors.[88] Problems such as inability to accept larger molecules, unfavourable equilibrium constant, substrate and product inhibition and reduced stability at extreme pHs, temperature and organic solvents are often challenging but by protein engineering several aspects can be successfully addressed.
Halophilic organisms and their enzymes have a remarkable tolerance towards organic solvents.[89] They are a type of extremophile organisms, that usually thrive in high salt concentrations, as for example in the Great Salt Lake in Utah and the Dead Sea, which have a salt concentration five times greater that the salt concentration in oceans.
A moderate halophilic organism which evolved an organic-osmolyte strategy to overcome the high osmotic pressure of its natural environment is *Halomonas elongata*.[90] In addition, *H. elongata* preserves an appropriate cytoplasm osmotic pressure by accumulation and/or biosynthesis of organic solute. Since *H. elongata* is a haloadapted bacterium, it allows for heterologous expression of its proteins inside a common mesophilic host like *Escherichia coli*, thus avoiding less conventional expression systems required for true halophilic proteins.[8]

Results and discussion

This project was developed with the group of Prof. Francesca Paradisi, School of Chemistry, University of Nottingham.
The aim of this project was to perform biocatalyzed transamination using an immobilized form of ω-transaminase from *Halomonas elongata* in a flow chemistry reactor. The enzyme was isolated and immobilized by prof. Paradisi's research group.[86a/87a] The versatility of this enzyme is that the reaction can be performed in both the directions: from aldehydes to amines and from amines to aldehydes.
In order to study the biotrasformation and its adaptability in continuous flow reactors, we started from aromatic aldehydes as substrates, in order to obtain the corresponding enantiomerically pure amines (Scheme 1).



Scheme 1. General representation of transamination reaction from aromatic aldehydes substrates to enantiomerically pure corresponding amines.

As it is possible to see from Scheme 1, the transaminase used needs a second co-substrate in order to run the reaction. In this case, we used L-alanine which is transformed during the reaction in pyruvate. Also, the enzyme needs as co-factor the pyridossal phosphate (PLP), also known as vitamin B6. PLP acts as co-factor not only in transamination reactions, but also in some decarboxylation, deamination and racemization reactions of amino acids. The mechanism of transamination reaction is reported in Figure 1.

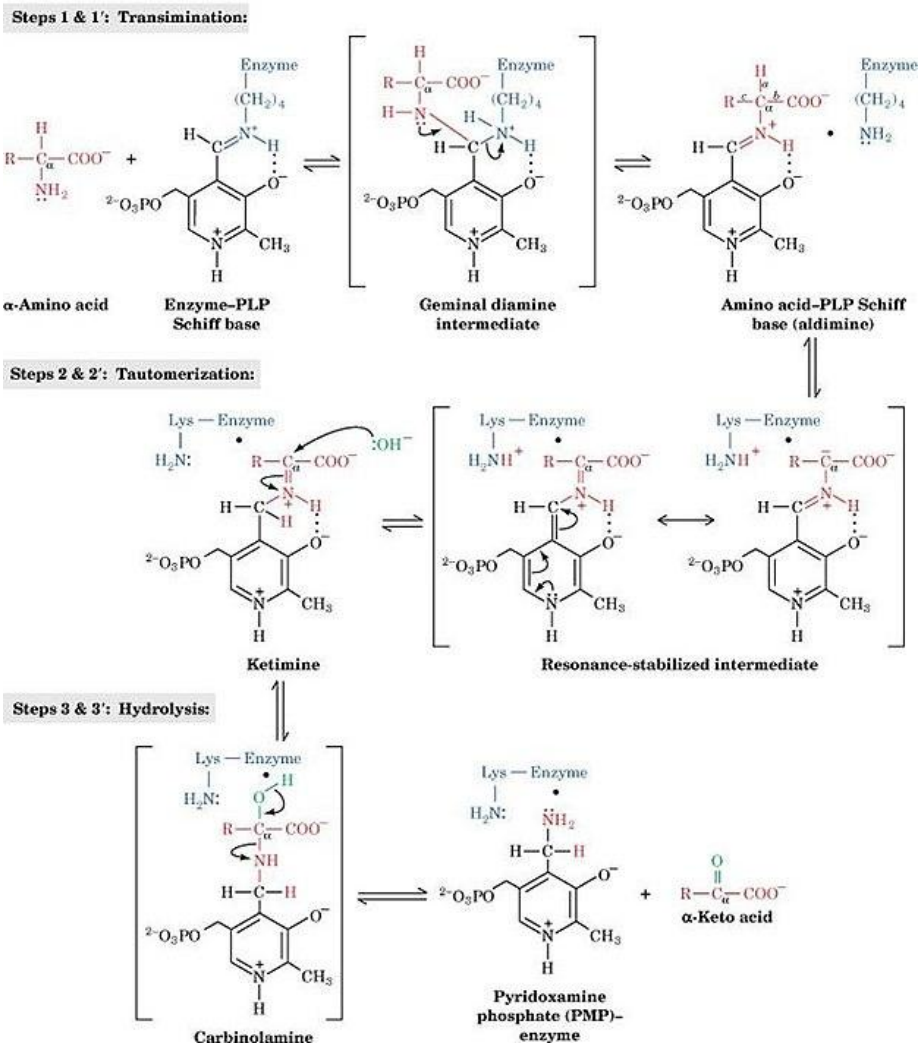


Figure 1. Mechanism of transamination reaction.

The proposed mechanism is referred to amino acids, but it can be applied also for transamination reaction with amines and aldehydes, or amines and ketones.

The process starts with the formation of the Shiff base between PLP and the ε-amino group of the active-site lysine residue. The amine group of the substrate displaces the ε-amino group of the enzyme with a process called transaldimination, and an aldimmine group is formed. After loss of one hydrogen, the aldimmine group becomes a ketimine and the carbonyl moiety is able to break off the amino group attached to PLP. In this way, the corresponding aldehyde is able to exit and the pyridoxal phosphate becomes pyridoxamine. Then, in order to re-convert pyridoxamine to pyridoxal phosphate a second substrate (an aldehyde) is necessary in order to take the amino group from the co-factor.

The strategy used for the immobilization of ω-transaminase from *Halomonas elongata* exploits the poly-His-tag fused to the enzyme for a selective interaction with a metal derivatized epoxy-resin, in order to obtain a multi-covalent system of interactions[91]. Cobalt (II) resulted to be the best metal ion for complexation with the resin.[91]

The immobilized transaminase was used in a packed bed reactor to perform the desired biotransformation over three aromatic aldehydes, that are shown in Figure 2 (*p*-NO_2-benzaldehyde, cinnamaldehyde and vanillin, respectively):

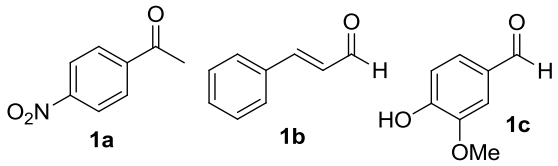
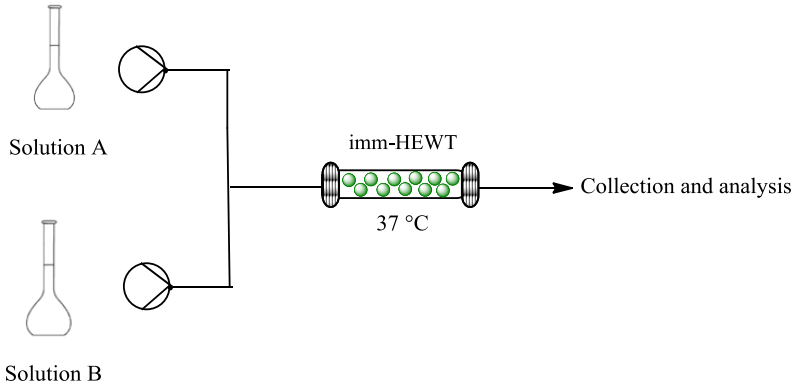


Figure 2. Aromatic aldehydes used as substrates.

The flow reactor configuration used for optimization of the reaction parameters (*i.e.*, aldehyde concentration, L-alanine concentration, % of DMSO, residence time) is the following (Scheme 2):



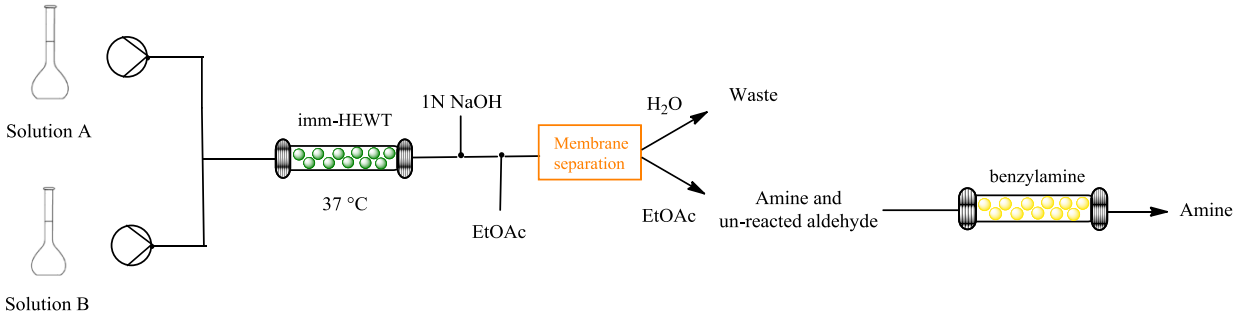
Scheme 2. Schematic representation of flow set-up used for the transamination reactions with substrates (**1a-1c**).

Solution A is the solution of aldehyde (**1a-1c**) at different concentrations in phosphate buffer (50 mM pH 8.0) with different percentages of DMSO. Solution B is a 500 mM solution of L-alanine containing 0.1 mM PLP. The two flow streams entered the column packed with immobilized enzymes, and the exiting flow was collected and analyzed by chiral HPLC. The bioreactor was maintained at 37 °C and atmospheric pressure.
The results for each compound are the following[86a]:

- *p*-NO_2-benzaldehyde (**1a**): after different attempts, to completely solubilize this substrate, a 10 mM substrate concentration in phosphate buffer containing 10% of DMSO was prepared. Indeed, it is really important to have a solution in flow if HPLC pumps are used, otherwise the pumps and the system can be blocked. Secondly, different reaction times were tried, obtaining a maximum of conversion (>99%) in only 2 minutes. In batch, the same conversion was achieved after 210 minutes of reaction;
- Cinnamaldehyde (**1b**): the corresponding amine of this substrate represents an important intermediate in the synthesis of biologically active molecules.[92] It was obtained using a 20 mM solution of substrate **1b** in a mixture phosphate buffer with 10% of DMSO. Different residence times were tested, obtaining 90% of molar conversion in 2 minutes. The same conversion in batch was achieved after 24 h.

For the compounds **1a** and **1b**, an in-line purification procedure was developed to isolate the pure amines without any manipulation. Therefore, after the biotransformation, an in-line basification and

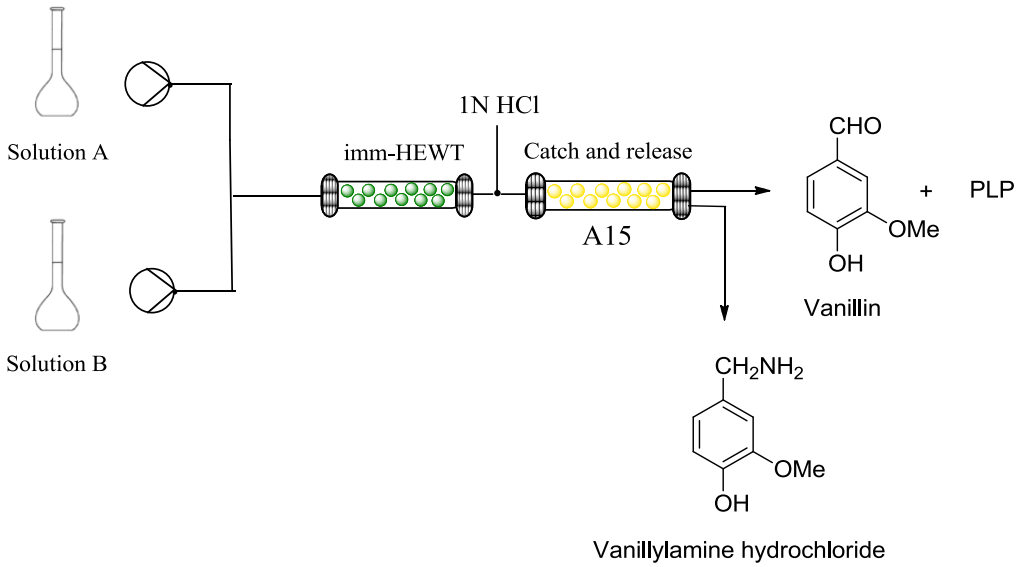
followed by an extraction with ethyl acetate were performed. The in-line extraction was performed using a commercially available Zaiput liquid-liquid separator.
In details, since the exiting flow stream buffer has a pH of 8.0 that was too low to allow the extraction of the amines, we added an inlet of 1N NaOH to reach a pH of 10/11 and to extract the amine in the organic phase. The set-up used is depicted in Scheme 3:[86a]



Scheme 3. Schematic representation of the flow set-up used for the transamination reaction, with in-line basification and extraction with ethyl acetate and purification with polymer supported benzylamine.

After the in-line basification and extraction with ethyl acetate both the amine product and any un-reacted aldehyde were collected in the organic phase (Scheme 3). Therefore, in order to obtain only the desired product amine in the organic phase, an in-line purification with polymer supported benzylamine was performed, that selectively links the un-reacted aldehyde, while the amine product remains in the flow stream. After the collection, only evaporation of the solvent under reduced pressure is required in order to obtain the pure amine. With this system, for compound **1a**, the isolated yield was 95%, while for substrate **1b** was 86%[86a]

- Vanillin (**1c**): this was the most challenging reaction. Indeed, in batch a conversion of only 18% was achieved after 24 h of reaction time without significant improvement over longer incubation times. In flow, the same conversion was obtained after 2 minutes of residence time, using a 20 mM substrate concentration in phosphate buffer containing 10% DMSO. Even if different reaction parameters were changed (*e.g.*, concentration, residence time, temperature), it was not possible to increase the conversion. Therefore, a recycling system of un-reacted vanillin was tried, followed by a removal of the product exploiting an acidic resin (A15) (Scheme 4). In this way, a conversion of 50% was achieved after 5 cycles. Increasing the number of cycles did not further increase the conversion. The vanillylamine, an important building block for the synthesis of natural products, such as capsaicinoids,[93] was recovered by flowing through the immobilized acidic resin a diluted HCl flow stream. The isolated yield was 46%.[86a]



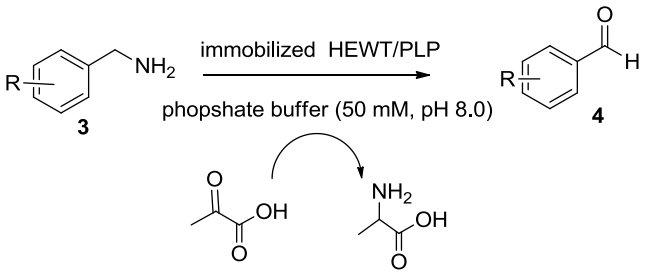
Scheme 4. Solution A: 20 mM vanillin solution in phosphate buffer (50 mM, pH 8.0) and 10% DMSO containing 0.1 mM PLP. Solution B: 1 M alanine solution in phosphate buffer (50 mM, pH 8.0). T = 37 °C, P = atm. The un-reacted vanillin is then recycled into the system after separation with A15 resin.

A summarizing table is reported here below (Table 1):

			Batch			Flow		
Entry	Sub. (10 mM)	Amino donor	Time	Conv.	*r*(µmol $min^{-1}g^{-1}$)[a]	Time	Conv.	*r*(µmol $min^{-1}g^{-1}$)[a]
1	*p*-NO_2-benzaldehyde	L-alanine (500 mM)	3.5 h	>99%	0.24	2 min	>99%	1.9
2	Cinnamaldehyde	L-alanine (500mM)	24 h	92%	0.06	2 min	90%	3.5
3	Vanillin	L-alanine (500 mM)	24 h	18%	0.012	2 min	18%	0.7
4	Vanillin	L-alanine (500 mM)	24 h	18%	0.012	5·2 min	50%	0.4

Table 1. Residence times, conversions and specific reaction rates for substrates (**1a-1c**) for both batch and flow procedures are reported. For *p*-NO_2-benzaldehyde the concentration used was 5 mM. For all the reactions, 10% of DMSO was added to the substrate solution. The bioreactor was stable after several weeks of continuous work. [a]Specific reaction rates for batch and flow calculated as reported in reference [94].

To prove the versatility of the bioreactor, we secondly tried to perform the opposite reaction, starting from benzylamine derivatives and obtaining aldehydes as products. In order to do so, pyruvate as second substrate was necessary, and it was transformed during the reaction in L-alanine (Scheme 5).



Scheme 5. Schematic representation of transamination reaction from benzylamine derivatives to aldehyde products.

The selected substrates are reported in Figure 3.

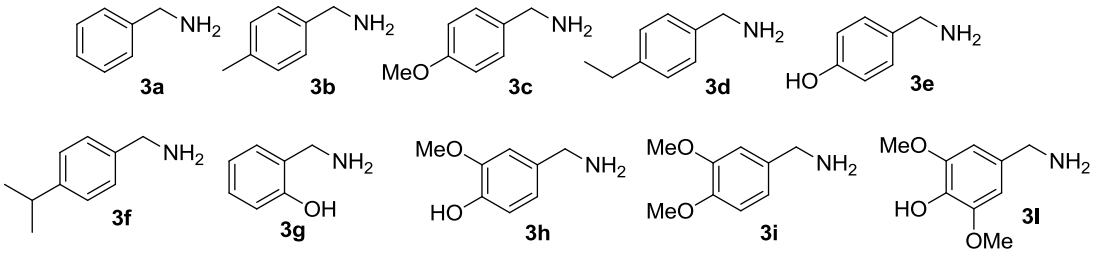


Figure 3. Benzylamine derivates used as substrates for the biotransformation.

The corresponding aldehyde products are normally used as fragrance and flavor components in food, beverage, cosmetics and also pharmaceutical. For examples, among them, there are the bitter almond aroma (benzaldehyde, **4a**), anice aroma (**4c**), cumin aroma (**4f**), vanillin (**4h**), woody aromas (**4i** and **4l**) (Figure 4).

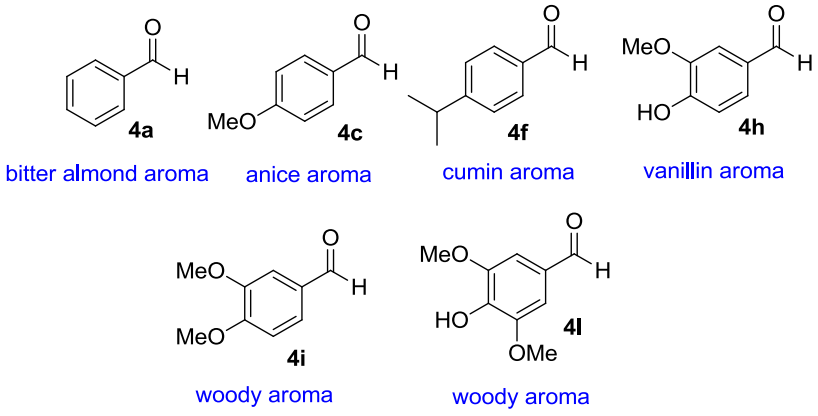
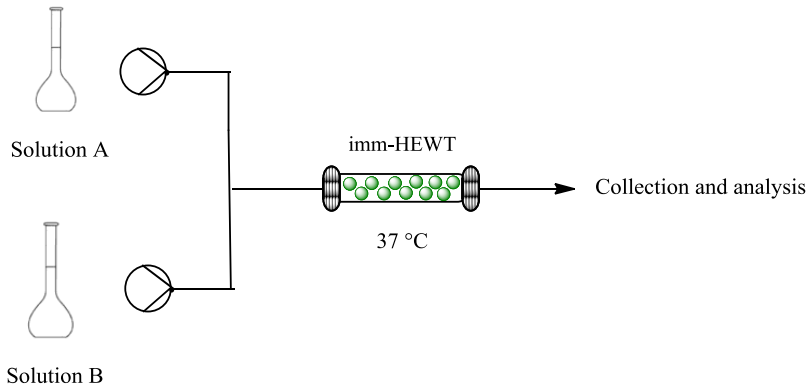


Figure 4. Examples of the synthesized aromas.

The flow set-up we used for the reaction is reported in Scheme 6:



Scheme 6. Schematic representation of flow set-up used for the transamination reaction from amines to aldehydes. Solution A: 20 mM solution of amines (**3a-3l**) in phosphate buffer (50 mM, pH 8.0) with 10% DMSO. Solution B: 20 mM solution of pyruvate containing 0.1 mM PLP.

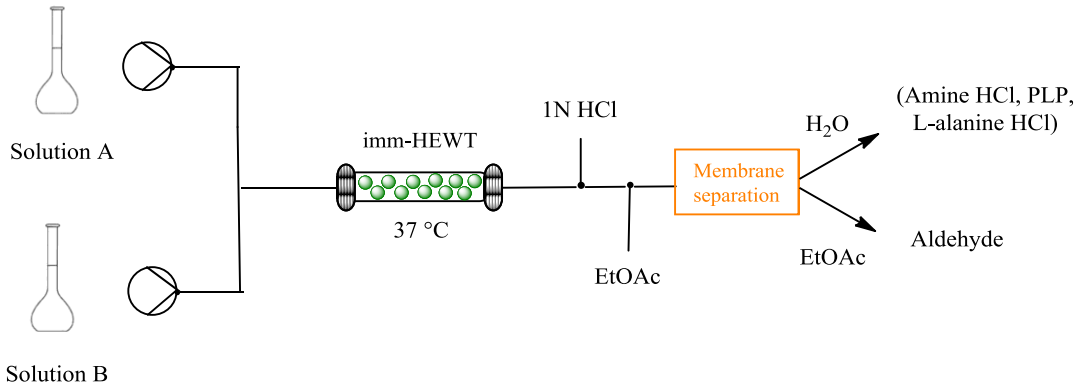
Solution A and solution B were pumped into the reactor, mixed in a T piece and the resulting flow stream was directed into an Omnifit glass column packed with the immobilized transaminase, maintained at 37 °C. Residence time was varied and the exiting flow stream was collected and analyzed. In all the cases, the reactions were completed in 15 minutes and therefore the residence time was decreased, in order to find the minimum time that allows us to obtain the higher conversion, as reported in Table 2:

Entry	Subs.	Batch reaction time (min)	M. c. (%)	r_{batch}[a] (μmol/min g)	Flow residence time (min)	M. c. (%)	r_{flow}[a] (μmol/min g)
1	**3a**	120	> 99	0.83	3	> 99	4.24
2	**3b**	120	> 99	0.83	3	> 99	4.24
3	**3c**	120	> 99	0.83	3	> 99	4.24
4	**3d**	120	> 99	0.83	3	> 99	4.24
5	**3e**	120	> 99	0.33	10	> 99	1.41
6	**3f**	300	> 99	0.33	10	90	1.29
7	**3g**	300	> 99	0.33	10	90	1.29
8	**3h**	120	> 99	0.83	3	95	4.07
9	**3i**	300	> 99	0.33	10	> 99[b]	1.41
10	**3l**	300	> 99	0.33	10	> 99[b]	1.41

Table 2. Reaction times, conversions and specific reaction rates for batch and flow procedures are reported. All the conversions were determined by HPLC. [a]Specific reaction rates for batch and flow calculated as reported in reference [94]

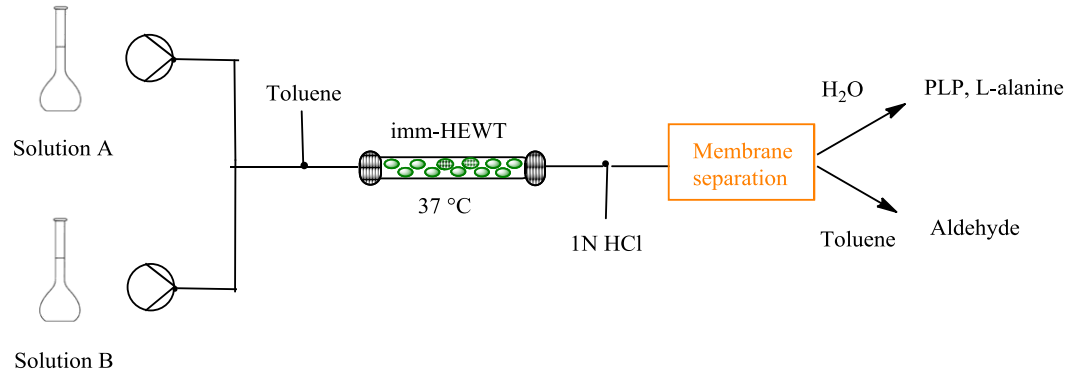
As it is possible to see in Table 2, we obtained conversions comparable to the batch ones with residence times between 3 and 10 minutes. Specific reaction rates (normalized to the amount of biocatalyst employed for the reaction) were higher in flow, compared to the batch procedures.

In order to exploit the advantages of flow chemistry and to avoid the traditional work-up procedures, the downstream process was automated, by performing an acidification of the exiting flow stream with 1N HCl and inserting an inlet of ethyl acetate for the extraction. Using a membrane separator the two phases were separated and, in this way, the pure aldehyde product was collected in the organic phase (Scheme 7).



Scheme 7. Schematic representation of flow configuration for biotransformation and in-line product isolation.

The protocol described above was used for the ten selected substrates (**3a-3l**). However, it was successful for the first eight compounds (**3a-3h**), while for the last two (**3i** and **3l**) the formed aldehyde probably remained attached to the support used for the immobilization of the enzyme. In order to solve this problem, we thought about an inlet of an organic solvent before entering the column packed with the immobilized enzymes. In this way, a biphasic liquid-liquid biphasic system was obtained and this led to avoid the attachment of the product on the support. Different organic solvents were tried and, in the end, we selected toluene. Exploiting this strategy, the desired aldehydes **3i** and **3l** were obtained (Scheme 8).



Scheme 8. Schematic representation of flow configuration used for substrates **3i** and **3l**.

Again, an in-line acidification with 1M HCl and extraction with toluene was performed, and only the pure aldehyde was collected in the organic phase. After removal of the solvent under reduced pressure, we obtained the products. The residence times, conversions and specific reaction rates for these two substrates are reported in Table 2 (entries **9** and **10**). In these cases, DMSO was not added to solution A.

Conclusions

New biocatalytic method for the synthesis of aldehydes with extensive applications as components of flavours and fragrances was developed. This is the first example of a transaminase exploited in a flow chemistry reactor under highly favourable oxidizing conditions for the preparation of aromatic aldehydes, showing excellent adaptability and stability during the processes. The use of a flow-based approach allowed for dramatic accelerations of the reactions, with all the reaction tested occurring with isolated yields above 80% and very short residence times (3-15 min) of the substrates. This system required in the majority of cases only one equivalent of pyruvate as the amino acceptor, generating alanine as by-product. A successful implementation was achieved with an in-line extraction step, which permitted the recovery of the desired pure aldehydes in the organic stream and alanine in the aqueous one, with an extremely simplified work-up procedure and almost no manipulation. Due to the high local concentration of the (bio)catalyst and to the enhanced heat and mass transfer,[95,7] the combination between biocatalysis and flow chemistry reactors not only leads to significant reductions of reaction times and increased productivity, but it can be also considered a sustainable technology for the production of aldehydes commonly used in food, cosmetic, and pharmaceutical industry.

Materials and methods

General

All reagents and solvents were purchased from Sigma–Aldrich. The continuous flow reactions were performed using a commercial R2+/R4 flow reactor (Vapourtec, Bury St. Edmunds, Suffolk, UK) equipped with Omnifit glass columns (6.6 mm i.d. X 100 mm length). The R2+ unit is the pumping unit that contains two adapted Knauer pumps, which are able to pump highly concentrated and corrosive acids. R4 is the heating unit with four heating positions. The additional HPLC pumps necessary to perform the overall synthesis were provided by another R2+/R4 flow reactor (Vapourtec) and by two external pumps (ThalesNano). The temperature sensor sits on the wall of the PFA tubing. The pressure was controlled using BPRs with different psi values. In-line liquid–liquid extractions were performed using a Zaiput separator. ^{1}H NMR and ^{13}C 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. The molar conversion of the biotransformation was determined by HPLC equipped with a Supelcosil LC-18-T column (250 mm x 4.6 mm, 5 µm particle size; Supelco, Sigma-Aldrich, Germany). The compounds were detected using an UV detector at 210 nm, 250 nm or 280 nm after an isocratic run with 25% acetonitrile/75% water with TFA 0.1% v/v at 25 °C with a flow rate of 1 mL/min.

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

Protein expression and purification were performed following previously reported protocols in Cerioli *et al.*;[87a] immobilization was carried out according to the procedure reported by Planchestainer *et al.*[86a]

Imm-HEWT catalysed batch reactions[86a]

The batch reactions using the imm-HEWT were performed in 1.5 mL micro centrifuge tubes; 500 µL reaction mixture in 50 mM phosphate buffer pH 8.0, containing 10 mM amino acceptor substrate, amino donor substrate (concentration reported in Tab 1), 0.1 mM PLP, and 50 mg of imm-HEWT (5 mg/g) was left under gentile shaking at 37 °C. For reactions starting from amine compounds, rections were performed using the same volumes and buffer as before, but adding10 mM pyruvate, 10 mM amino donor substrate, 0.1 mM PLP, and 50 mg of imm-HEWT (5 mg/g). for both, 10 µL aliquots were quenched with trifluoroacetic acid (TFA) 0.2% every hour and then analysed by HPLC equipped with a Supelcosil LC-18-T column (250 mm x 4.6 mm, 5 µm particle size; Supelco, Sigma-Aldrich, Germany). The compounds were detected using an UV detector at 210 nm, or 250 nm after an isocratic run with 25% acetonitrile/75% water with TFA 0.1% v/v at 25 °C with a flow rate of 1 mL/min. The retention times in minutes are: *p*-nitrobenzaldehyde (12.2 min), (*p*-nitrophenyl)methanamine (4.4 min), vanillin (5.7 min), vanillylamine (3.7 min), cinnamaldehyde (16.9 min), cinnamylamine (6.6 min), benzylamine (4.1 min), benzaldehyde (9.4 min), *p*-methylbenzylmine (5.2 min), *p*-tolualdehyde (16.4 min), *p*-methoxylbenzylmine (4.4 min), *p*-anisaldehyde (10.3 min), *p*-ethylbenzylamine (5.0 min), *p*-ethylbenzaldehyde (16.5 min), *p*-hydroxybenzykamine (3.8 min), *p*-hydroxybenzaldehyde (10.5 min), *p*-isopropylbenzylmine (10.0

min), cuminaldehyde (35.0 min), 2-(aminomethyl)-phenol (3.7 min), salicilaldehyde (10.3 min), veratrylamine (4.1 min), veratraldehyde (8.0 min), 4-(aminomethyl)-2,6-dimethoxyphenol (3.5 min), syringaldehyde (5.4 min), confirmed by comparison with commercially available compounds.

Imm-HEWT catalysed flow reactions, in-line purification and product isolation for compounds 2a and 2b[86a]

The continuous flow biotransformations were performed using a R2+/R4 flow reactor commercially available from Vapourtec® equipped with an Omnifit® glass column (6.6 mm i.d × 100 mm length) filled with 1.0 g of imm-HEWT (5 mg/g). A 20 mM amino acceptor substrate solution (solution A, for *p*-NO_2-benzaldehyde 10 mM solution, Scheme 2) in phosphate buffer (50 mM, pH 8.0) containing 0.1 mM PLP and a 1 M amino donor solution (solution B, Scheme 2) were prepared. The percentage of cosolvent (*i.e.,* DMSO) is reported in the text (Table 1). 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: 0.78 mL, Scheme 2). The flow rate was varied and optimised. The exiting flow stream was analysed by HPLC using the conditions reported in the paragraph above.
An in-line basification to pH 11 was performed by using an inlet of 1N NaOH aqueous solution (flow rate: 0.20 mL/min) that was mixed to the exiting reaction flow stream using a T junction. The resulting aqueous phase was extracted in-line using a stream of EtOAc (flow rate: 0.59 mL/min) and a Zaiput liquid/liquid separator. The organic phase, containing the amine and possible traces of unreacted aldehyde, was passed through a column packed with a polymer supported benzylamine (PS-BZA) (Scheme 3). The organic solvent was evaporated to yield the desired amine. The purity of the amine was assessed by HPLC and ^{1}H NMR, compared with the one found in literature. ^{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.

Characterization of products 2a and 2b:

- **(*p*-nitrophenyl) methanamine (2a)**[96]: yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): 4.06 (2H, s), 7.55 (2H, d, J = 8.4), 8.20 (2H, d, J = 8.4)
- **(E)-3-phenylprop-2-en-1-amine (cinnamylamine)** (**2b**)[97]: slightly yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): 3.48 (2H, d, J = 4.8), 6.25 (1H, dt, J = 4.8, 15.6), 6.52 (1H, d, J = 15.6), 7.20–7.40 (5H, m)

Flow synthesis and characterization of vanillylamine (2c)[86a]

An Omnifit® glass column (6.6 mm i.d × 100 mm length) was packed with 1.0 g of imm-HEWT (5 mg/g). A 20 mM vanillin solution (solution A, Scheme 2) in phosphate buffer (50 mM, pH 8.0) and 10% DMSO containing 0.1 mM PLP and a 1 M alanine solution (solution B, Scheme 2) 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: 0.78 mL, residence time: 2 min, T = 37 °C). The exiting flow stream was directed into a column (10 mm i.d × 100 mm length) packed with A-15 resin (packed bed reactor volume: 3.8 mL) and then collected into a separated

flask from which was newly pumped through the imm-HEWT packed column after mixing with the amine donor solution. After 5 cycles a 50% conversion was achieved. The vanillylamine was then recovered as the salt from the A15 resin by flowing through the A-15 column 1N HCl.

(4-hydroxy-3-methoxyphenyl) methanaminium chloride (vanillylamine hydrochloride, 2c)[98]: ^{1}H NMR (300 MHz, DMSO-*d6*): 3.75 (s, 3H), 3.85 (m, 2H), 6.75 (1H, d, J = 8.1), 6.82 (1H, dd, J = 1.9, 8.1), 7.10 (1H, d, J = 1.9), 8.20 (3H, bs), 9.18 (1H, s).

Flow reactions with immobilized HEWT (compounds 4a-4h)

Continuous flow biotransformations were performed using a R2+/R4 Vapourtec® flow reactor equipped with an Omnifit® glass column (0.3421 mm i.d × 100 mm length) filled with 0.7 g of imm-HEWT (5 mg/g). A 20 mM sodium pyruvate in phosphate buffer (50 mM, pH 8.0) containing 0.1 mM PLP, and 20 mM amino donor (**3a-3h**, Figure 3) solution with 10% of 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: 1.0 mL). The flow rate was varied and optimized. An in-line acidification was performed by using an inlet of 1N HCl aqueous solution (flow rate: 0.1 mL/min) that was mixed to the exiting reaction flow stream using a T-junction. The resulting aqueous phase was extracted in-line using a stream of EtOAc (flow rate: 0.2 mL/min) and a Zaiput liquid/liquid separator. Both the organic and aqueous phase were analyzed by HPLC using the above reported conditions. The amount of substrate and product was evaluated by exploiting a previously prepared calibration curve. For the optimization procedure, the reactions have been performed by injecting 250 µL of each starting solutions (volume of EtOAc used for the in-line extraction: 1 mL). To isolate the product, 10 mL of each starting solutions have been used (volume of EtOAc used for the in-line extraction: 40 mL). The organic phase, containing the aldehyde, was evaporated to yield the desired product.

Flow reactions in two-liquid-phase systems with immobilized HEWT (compounds 4i and 4l)

A 20, 40 or 100 mM pyruvate in phosphate buffer (50 mM, pH 8.0) containing 0.1 mM PLP, and 20 mM amino donor (**3i** and **3l**, Figure 3) solution were prepared. The two solution were mixed in a T-piece. A second junction for additional supplement of toluene at the same flow rate was installed before the packed enzyme column. The resulting segmented flow stream was directed to the imm-HEWT. The flow rate was varied and optimized. After an in-line acidification step, as previously reported, the exiting flow stream was separated by a Zaiput liquid/liquid separator, the organic and aqueous phases analyzed by HPLC exploiting a calibration curve (see conditions above) and the toluene, containing the desired product, was evaporated to yield the aldehydes.

Characterization of products (4a-4h)

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:

- **Benzaldehyde (4a)**: colourless oil; yield 95%; ^{1}H NMR ($CDCl_3$) δ 10.00 (s, 1H), 8.15-8.12 (m, 2H), 7.67-7.51 (m, 3H) ppm.
- ***p*-Tolualdehyde (4b)**: yellow oil; yield 96%; ^{1}H NMR ($CDCl_3$) δ 9.95 (s, 1H), 7.74 (d, *J* = 7.5 Hz, 2H), 7.32 (d, *J* = 7.5 Hz, 2H), 2.40 (s, 3H) ppm.
- ***p*-Anisaldehyde (4c)**: colourless oil; yield 94%; ^{1}H NMR ($CDCl_3$) δ 9.85 (s, 1H), 7.80 (d, *J* = 8.0 Hz, 2H), 6.96 (d, *J* = 8.0 Hz, 2H), 3.90 (s, 3H) ppm.
- ***p*-Ethyl benzaldhyde (4d)**: yellow oil; yield 94%; ^{1}H NMR ($CDCl_3$) δ 9.98 (s, 1H), 7.81 (d, *J* = 8.1 Hz, 2H), 7.36 (d, *J* = 8.1 Hz, 2H), 2.74 (q, *J* = 7.5 Hz, 2H), 1.27 (t, *J* = 7.5 Hz, 3H) ppm.
- ***p*-Hydroxybenzaldehyde (4e)**: yellow solid; yield 92%; ^{1}H NMR ($CDCl_3$): δ 9.61 (s, 1 H), 7.60 (d, *J* = 8.3 Hz, 2 H), 6.73 (d, *J* = 8.3 Hz, 2 H) ppm.
- **Cuminaldehyde (4f)**: colourless oil; yield 84%; ^{1}H NMR ($CDCl_3$) δ 9.98 (s, 1H), 7.84 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 3.00 (septet, *J* = 6.9 Hz, 1H), 1.30 (d, *J* = 6.9 Hz, 6H) ppm.
- **Salicilaldehyde (4g):** yellow oil; yield 82%; ^{1}H NMR ($CDCl_3$) δ 11.00 (bs, 1H, OH), 9.85 (s, 1H), 7.46-7.54 (m, 2H), 6.94-7.00 (m, 2H) ppm.
- **Vanillin (4h)**: white solid; yield 90%; ^{1}H NMR ($CDCl_3$) *δ* 9.78 (s, 1H), 7.37–7.40 (m, 2H), 7.02 (d, *J* = 8.5 Hz, 1H), 6.72 (bs, 1H, OH), 3.90 (s, 3H) ppm.
- **Veratrylaldehyde (4i)**: yellow solid; yield 96%; ^{1}H NMR ($CDCl_3$) δ 9.85 (s, 1H), 6.70-7.65 (m, 3H), 3.98 (s, 3H), 3.95 (s, 3H) ppm.
- **Syringaldehyde (4j)**: yellow solid; yield 94%; ^{1}H NMR ($CDCl_3$) *δ* 9.83 (s, 1H), 7.15 (s, 2H), 6.10 (s, 1H), 3.98 (s, 6H) ppm.

GENERAL CONCLUSIONS AND PERSPECTIVES

The present thesis had the aim to develop innovative, robust and versatile protocols for performing biocatalyzed redox reactions using a combination of biocatalysis and flow-reactor technology for the obtainment of high value chemicals of pharmaceutical interest. This goal crossed all the projects presented in this PhD work, and for all interesting results were achieved, underlining the importance of flow chemistry, as a novel chemical methodology, and of biochemistry, as an alternative way to perform reactions in a more environmental friendly way. The possibility to apply this combination to the pharmaceutical chemistry can be a new perspective for also the industrial point of view, if processes demonstrate to have advantages in terms of productivity, costs, waste produced. For this reason, during my thesis I tried to optimize the flow processes keeping in mind this corollary aim, even if sometimes it was hard to find more adavantageous processes, compared to the ones already existent. I think that, in the future, both flow chemistry and biocatalysis will be more and more present in the industrial reality, and that, maybe, there will be a cooperation between these systems and the traditional chemistry. Indeed, some synthesis can benefit if a flow processes is applied, while for others is better to remain with "batch" processes, when disadvantages can be higher that advantages. In any case, is mandatory for companies (pharmaceutical and others) to keep in mind the environmental problems of our world. This thesis wants to remember this, since "green" chemical solutions are used.

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$$r_{batch} = \frac{\eta_p}{t \times m_B} \text{ (mol/min g)}$$

Where [n_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.

$$r_{flow} = \frac{[P] \times f}{m_B} \text{ (mol/min g)}$$

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. Comparison of the kinetics of the same reaction in a batch or flow-mode was made at similar degrees of conversion

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