Biocatalysis Engineering
Developing Enzymatic Systems

Cherando Pereira, Pedro

DOI
10.4233/uuid:0d956b69-5b39-4e43-b62a-431a122e4588

Publication date
2016

Document Version
Final published version

Citation (APA)
Cherando Pereira, P. (2016). *Biocatalysis Engineering: Developing Enzymatic Systems*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:0d956b69-5b39-4e43-b62a-431a122e4588

Biocatalysis Engineering

Developing Enzymatic Systems

Pedro A. Cherando Pereira

Cover: Concept by Pedro A. C. Pereira; Design by Gerjanne Beumer

Biocatalysis Engineering

Developing Enzymatic Systems

Proefschrift

Ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van Rector Magnificus prof. ir. K.C.A.M. Luyben,
voorzitter van het College van Promoties,
in het openbaar te verdedigen op dinsdag 26 april 2016 om 12:30 uur

door

Pedro Alexandre Cherando Pereira
Engenheiro Químico, Biotecnologia, Instituto Superior Técnico, Lisboa, Portugal
geboren te Lissabon

This thesis has been approved by promotor:
Prof. dr. I.W.C.E. Arends

Samenstellingen promotiecommissie:

Rector Magnificus,	Technische Universiteit Delft, voorzitter
Prof. dr. I.W.C.E Arends	Technische Universiteit Delft, Promotor

Onafhankelijke leden:

Dr. Ir. A.J.J. Straathof	Technische Universiteit Delft
Prof. dr. W.R. Hagen	Technische Universiteit Delft
Dr. Linqiu Cao	Friesland Campina
em Prof. dr. Ir. Herman van Bekkum	Technische Universiteit Delft
Prof. dr. Vytas K. Svedas	Lomonosov Moscow State University
Prof. dr. Ulf Hanefeld	Reservelid

Other Member:

em Prof. dr. R.A. Sheldon	Technische Universiteit Delft

This thesis was started and for a large part conducted under the supervision of Prof. dr. Roger A. Sheldon.

ISBN:

Ao meu pai, que já não estando estará para sempre

À minha mãe

“A likely impossibility is always preferable to an unconvincing possibility”

Aristotle (384 BC – 322 BC), Rhetoric

Table of contents

Introduction

1

Part I

Biocatalysis

1. Overview

Biocatalysis is the use of enzymes, or enzyme containing cells, in chemical transformations. Enzymes are proteins synthesized by living organisms, which proficiently catalyze very specific chemical reactions. Biocatalysis is therefore a part of biotechnology, which is the technology that deals with the practical application of living organisms, or components of living organisms [1,2].

The use of enzymes is today a well established tool in the chemical industry. Biocatalysis has undergone an explosive growth over the past years, playing increasingly key roles in chemical manufacturing and in enabling environmentally friendly green chemistries [3,4,5,6].

The global market for enzymes in 2010 was estimated to be around 2500 million € and was divided in the areas of feed (9%), food (22%), detergents (34%) and others (35%) [7]. The growth of this market was significant in the past decades. For example, the turnover of Novo Industri (now Novozymes) increased from US $ 50 million in 1969 to an estimate of US $ 1400 million in 2009. The global specialty enzymes market is projected to reach the $ 4700 million in 2018 [8].

Biocatalysis is seen today as a promising route in the quest for sustainability and improvement of reaction efficiency [9,10]. Furthermore biocatalysis offers growth opportunities, both for established chemical industries as well as emerging enterprises [11].

2. Historical overview

Man has used the catalytic potential of enzymes for millennia, often by using enzyme-producing microorganisms, without even the knowledge of their existence. Classic examples of the use of biological agents can be found in food processing, namely, beer brewing, bread rising, wine making and milk curdling for cheese production. In the early 19th century, developments in biochemistry and organic chemistry provided an important contribution to the clarification and understanding of enzyme catalyzed phenomena [12,13].

a. The discovery of enzymes

In 1833 Payen and Persoz prepared an extract of malted barley to which they called “soluble ferment” and with which they were able to hydrolyze starch. In this way they showed this activity could be dissolved out of the organized structures of the malted barley or yeast. They called this activity *diastase* [14].

However, the organic processes in the living tissues were far from being understood. In 1837 Schwann, Kuntzig and Cagniard-Latour showed yeast is a living organism. The cause of phenomena such as alcoholic fermentation was the motive subject of controversy, with some purporting that the theory that this was a result of living organisms and others defending they were caused by simple chemical substances [15].

Berzelius and Liebig proscribed to the latter theory. In contrast, Pasteur believed that such activity was a “vital physiological act of the living yeast cell” and in 1860 showed fermentation indeed required the presence of living yeast. It would later be seen that on one hand the processes are in fact caused by chemical compounds, i.e. proteins but on the other hand these are formed by living organisms [16].

The controversy was resolved in 1897, when Eduard Buchner showed degradation of glucose into ethyl alcohol and carbon dioxide to be possible using a cell extract, in the absence of living cells [17]. This meant although enzymes are produced by living organisms, they can retain their activity *in vitro* and their activity is independent of living cells.

In 1907 he was awarded the Nobel prize for his studies on “cell-less fermentation” and the agents present in cell extracts which catalyze these reactions were called “en zyme”, which means “in yeast” [18].

b. Enzyme sources for production

The first standardized enzyme preparation was commercialized in 1874. It was a rennet containing extract from dried calves´ stomachs, which is used in cheese production [19]. In 1890 a product composed of a mixture of amylolytic and proteolytic enzymes, produced by the fungus *Aspergillus oryzae*, was commercialized in the USA.

This product was named *takadiastase*, after its developer J. Takamine, and is considered to be the first commercialized extracellular microbial enzyme as well as the first enzyme preparation to be patented for industrial use [20, 21]. In 1907 the company Röhm and Haas was founded, based on commercializing a mixture containing pancreatic extract, to replace dung in the leather industry [22]. In 1913 pancreatic extract enzymes started being sold as washing aids for laundry cleaning [23].

Although enzymes can be obtained from any life form, microorganisms were found to be an attractive source of enzymes because of the much higher rate of reproduction leading to lower costs. In the 1950´s the number of commercial microbial enzymes was still limited [24]. After the 1960´s microbial enzymes gradually and progressively replaced those from other sources. For example, pancreatic extracts were replaced in the laundry industry in the 1980´s, by alkaline proteases produced by *Bacillus licheniformis* commercialized by Novo Industri A/S [25].

The enzyme production from animal and plant sources is estimated to have decreased from 70% in the 1960´s to 15% in 2008. The production of microbial enzymes is more reliable, simpler and cheaper and, consequently, easier to be industrialized [26]. Fungi and bacteria dominate the market, particularly the *Aspergillus* family (see Figure 1).

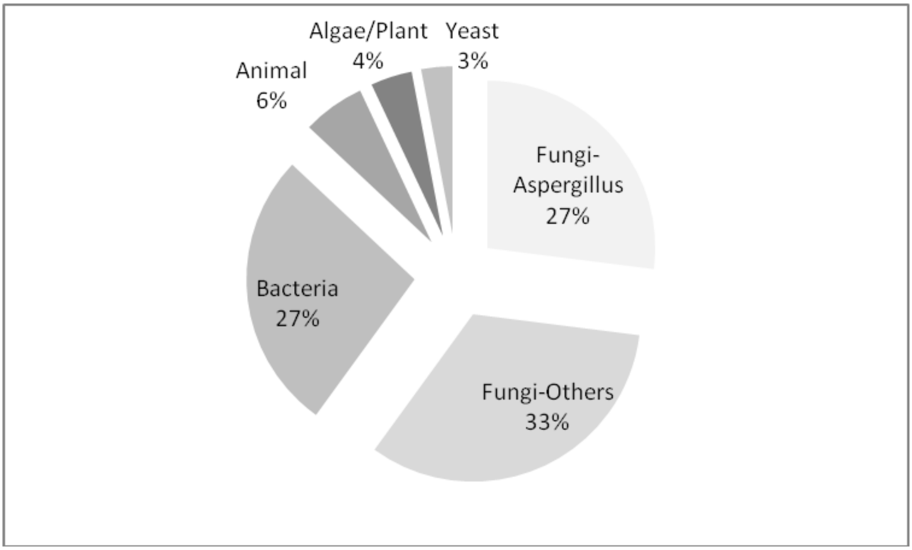


Figure 1: Donor organisms for industrial enzymes (adapted from [27]).

c. Recombinant DNA technology and protein engineering

A big contribution to enzyme production was given by the advances in genetic engineering, since the late 1970´s. The production of an enzyme from any source in a suitable host became possible, which in its turn allowed to increase the amount of enzymes produced.

Recombinant DNA is a form of DNA which is created by introducing a fragment of DNA into the DNA of another organism [28]. Later it was shown that inserting recombinant DNA in a host could be used to produce a biologically functional protein, including DNA from animal cells [29,30]. A recombinant protein, or enzyme, is one derived from an organism with recombinant DNA and with this technology microorganisms are currently used to produce specific proteins [31].

The first recombinant enzyme produced on an industrial scale was an α-galactosidase in 1982, by Boeringer Mannheim in Germany. Overexpressing genes in fast growing hosts with high protein productivity allowed for cheap enzyme production on an industrial scale. With it, the number and quality of enzymes produced increased [22].

It was estimated that by 1992 the recombinant enzyme production market reached 50 % of the market share. With the exception of the food processing area, most of the enzymes used today in biocatalytic processes are recombinant [32,7].

The possibility to modify the amino acid sequence of enzymes was another milestone in the improvement of biocatalysis. This protein engineering provides new enzymes with altered properties, such as substrate specificity, activity or stability. A common technique is called directed evolution, which will be further addressed in part II of this chapter [33].

3. Biocatalysis in industry

The areas of food, animal feed and detergents occupy more than 60% of the total enzyme market (see Figure 2). This market was estimated to be 1000 million $ in 1995, 1500 million $ in 2000 and 2500 million € in 2010.

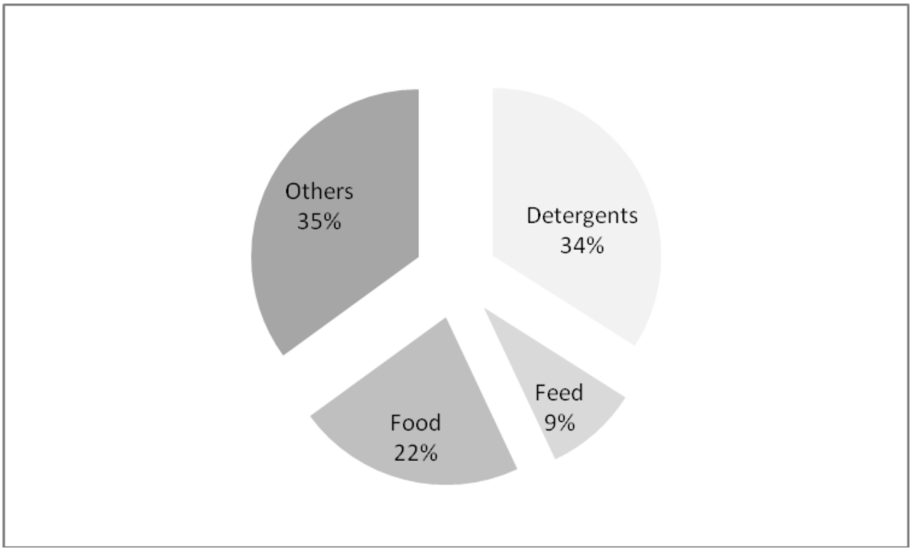


Figure 2: Market for enzymes used as biocatalysts in 2010 (adapted from [7])

The remainder of the market is mainly comprised of the starch, leather, pulp and paper industries (see Table 1 for a more detailed description) [34,7].

Table 1: Industrial applications of enzymes [34,35]

Application	*Enzyme*	*Description*
Detergents	Protease	Remove protein-based stains
	Lipase	Digest oils and fats
	Amylase	Remove starch residues
	Cellulase	Anti-redeposition
Starch Industry	Amylase, amyloglucosidases and glucoamylases	Converts starch into glucose and sugar syrups
	Glucose Isomerase	Converts glucose syrups into fructose syrups
Dairy Industry	Rennin	Cheese manufacture
	Lipases	Enhances ripening of blue-mold cheeses
	Lactases	Break down lactose into glucose and galactose
Textile Industry	Amylases	Remove starch from woven fabrics
Brewing Industry	Amylases, glucanases, proteinases	Splits polysaccharides and proteins in the malt
	Proteinases	
	Proteinases	Reduces clouding of beers
	Amyloglucosidase	Low calory beer production
	Β-glucanase	Improves filtration characteristics
Baking Industry	α-amylase	Catalyzes the breakdown of starch in flours, manufacture of bread

	B-xylanases	Rising of bread
	Proteinases	Biscuit manufactures
Leather Industry	Proteinase (trypsin)	"Bating" – making leather pliable
Pulp and paper Industry	B-xylanases	Pulp bleaching
	Lipases	Reduces "pitch" which causes paper to tear

Enzyme prices vary over a broad range. This can be illustrated by examples found in the food industry (see Table 2).

Table 2: Commercial enzymes used in the food industry (adapted from [36])

Enzyme activity	*Application*	*Price ($ / Kg)*
Protease	Cheese, Meat, Beverages	3 - 30
Pullulanase	Cereal, Beverages	15 - 30
Xylanase	Cereal, Bakery	10 - 80
α-Acetolactate decarboxylase	Beverages	50 - 60
Glucose oxidase	Egg, Beverages, Bakery	180 - 190
Lipase	Fats, Bakery	200 - 210
Chymosin	Cheese	460 - 500
Catalase	Milk, Eggs	1000 – 10 000

The three highest tonnage value enzymes used in industry are amylases, proteases and glucose isomerase (D-Glucose/xylose isomerase) [37].

Amylases occupy approximately 25% of the world enzyme market. α-Amylase catalyses the hydrolysis of α-1,4-glycosidic linkages in starch into glucose, maltose and maltotriose units. It is a very thermostable enzyme as it hydrolyses starch at 105 ºC [38,39].

Proteases are hydrolytic enzymes and account for a large share of sales in industrial sectors. Their traditional applications are in detergents, leather industry, milk coagulant in cheese production and meat tenderization. In 2002 it was estimated they accounted for 40% of the enzyme market [37,40,41].

Glucose isomerase is used in the production of high fructose corn syrup (HFCS). This enzyme catalyzes the isomerization of D-glucose to D-fructose and the isomerization of D-xylose to D-xylulose. Industrially GI is used immobilized and retains its activity for more than 2 years. The GI syrup production is above 15 million tons / year [37,38].

Part of the industrial enzymes is used in the synthesis of chemicals - a few examples are given in Table 3. Two cases which deserve special attention are acrylamide and 6-aminopenicillanic acid (6-APA).

Acrylamide is a commodity chemical used in the production of polymers, for which there is a chemical route as well as an enzymatic. The biocatalytic step is the conversion of acrylonitrile into acrylamide with a nitrile hydratase and its production is currently above 100 000 tons / year. It is a relevant example because it helped establishing the use of biocatalysts in the synthesis of commodity chemicals. Its production increased from 30 000 tons / year in 1999 to more than 100 000 tons / year in 2012 [42,43,7]. The enzymatic process is more cost effective than the chemical and its contribution in the overall acrylamide production is expected to grow.

6-APA is the penicillin nucleus which is used to make semi-synthetic penicillins and is obtained via penicillin acylase catalyzed hydrolysis of penicillin-G. It was discovered in the 1950´s and today its production is above 10 000 tons / year.

It is a relevant example because it is a pharmaceutical intermediate produced in large amounts and because it is a long established industrial process. Interestingly, the industrial production of 6-APA was originally biocatalytic in the 1960´s, then changed to fully chemical, but in order to reduce the high energy costs associated with the chemical process which is performed at -40 ºC and avoid the environmental issues associated with the use of dichloromethane solvent, it later reverted to an enzymatic process [44,45].

Table 3: Examples of industrial products using biocatalysis (from [7])

Product	*Scale (tons / year)*	*Enzyme*
Acrylamide	**$> 10^5$**	Nitrilase
6-Aminopenicillanic acid (6-APA)	**$> 10^4$**	Penicillin acylase
(*S*) – Aspartic acid	**$> 10^3$**	Aspartase
Aspartame (sweetener)	**$> 10^3$**	Thermolysin
Amoxicillin (antibiotic)	**$10 - 10^3$**	Penicillin acylase
Cephalexin (antibiotic)	**$10 - 10^3$**	Penicillin acylase
Enantiopure alcohols and amines	**$10 - 10^3$**	Lipase
(*R*) – Mandelic acid	**$10 - 10^3$**	Nitrilase

The search for enantiopure compounds grew in recent decades. Simultaneously biocatalysis became well established as a viable technology in the production of fine-chemicals, most of which are chiral compounds [46,47].

Some examples include *L*-Menthol, an optically pure alcohol used in confectionary goods and pharmaceuticals; (2*S*,3*R*)-3-Phenylglycidate methyl ester, which is an intermediate in the production of the anti-cancer drug Taxol®; optically pure styrene oxides, intermediates in pharmaceuticals production; optically pure aryl glycidyl ethers, important precursors of ß-blocker drugs; (*R*)-o-chloromandelic acid, a precursor of the anti platelet aggregation drug Plavix®; in the production of (*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, which is the antidiabetic drug Sitagliptin [48,49].

The exquisite enantioselectivity and regioselectivity of enzymes offered attractive solutions for the production of pharmaceutical compounds and the applications of biocatalysis in pharma increased with a growing pace in the last decades [50,51,52].

Therefore the known enzymatic processes for the synthesis of chiral pharmaceutical intermediate are numerous. The majority of industrial biocatalysis products are used in the pharma segment, although with lower production volumes [53,54,55].

4. Growth potential of biocatalysis

The numerous examples of enzyme applications previously shown and the constant growth of enzyme use in the last decades, is a clear evidence that biocatalysis offers advantages. These originate from enzyme properties, such as their selectivity, high catalytic efficiency or ability to function at ambient temperatures.

Complicated synthesis become therefore feasible, often avoiding protection and deprotection steps. In addition to this biocatalysis offers cost saving processes because it allows energy saving and waste reduction [56,57,58,59,60,61,62,63].

The possibility of reducing waste is particularly interesting in the pharma and the fine-chemicals industry. The production volumes are lower in these segments but the waste produced relative to product is considerably higher (see Table 4) [64].

Table 4: E factors (kg of waste per Kg of product) in chemical industry [64]

Industry segment	**Product (tons / year)**	**E factor (Kg waste / Kg product)**
Bulk chemicals	**10^4 - 10^6**	**< 1 - 5**
Fine chemicals	**10^2 - 10^4**	**5 - > 50**
Pharmaceuticals	**10 - 10^3**	**25 - > 100**

Regardless of the expected continuous growth of biocatalysis in all segments, the pharmaceutical and fine-chemicals areas are possibly the ones with highest growth potential. Adding to the ample room for improvement concerning waste reduction, there is a continuous need for new compounds and synthetic routes, which differs from bulk applications where processes show little change over time and make use of a limited collection of enzymes. The added value in a biocatalytic process brought by the development of the biocatalyst is potentially higher in the areas where the production volumes and productivities are lower, but where products have a higher value [65,55,66].

In spite of the positive prospects for biocatalysis in the fine chemistry industry, the application of enzymes in new synthetic processes is not obvious. Limited stability, selectivity and rate of the biocatalyst for the transformation of interest are recurrent issues in the proper evaluation of an enzymatic route [67].

There is a continuous need for biocatalysis improvement techniques that can be evaluated for every new case. These improvement techniques include both enzyme and process improvement and will be outlined in the coming paragraphs.

Part II

Biocatalysis improvement

Like any chemical process, a biocatalytic process comprises many variables, of which the biocatalyst is merely one part. Consequently, the improvement of a biocatalytic process does not necessarily depend on the exclusive development of the biocatalyst or transformation in question.

In this work, we shall describe methods of improvement that relate directly to the biocatalyst and are specifically directed towards the optimization of an existing transformation or the creation of a new one (Table 5). Therefore "Biocatalysis engineering" concerns the maximization of the practical use of enzymes as catalysts, either by modifying the enzyme or the reaction system.

The methods that concern modifying the reaction system comprise optimization via the choice of substrates (substrate engineering) and tailoring the reaction medium in which the transformation takes place (solvent engineering).

A special case of improving the reaction system is the combination of different enzymes, with the purpose of performing several enzymatic reactions in one pot. For convenience we shall refer to this methodology as multiple enzyme engineering.

The optimization of the biocatalyst itself mainly consists of two methodologies. One relies on altering the amino acid sequence of the enzyme (protein engineering), which is achieved by genetic techniques.

The second approach is the post-transcription chemical or physical modification of the enzyme formulation, creating a new biocatalyst without altering the native structure of the enzyme. The immobilization of enzymes is the most relevant example of this approach, which we will call biocatalyst engineering.

Table 5: Methods of biocatalysis improvement

Designation	*Variable*	**Description**
Substrate engineering	Reacting compounds	Design of reaction systems with the use of substrates
Solvent engineering	Reaction medium	Tailoring the reaction medium
Protein engineering	Catalyst structure	Obtaining a new biocatalyst by altering the protein structure
Biocatalyst engineering	Catalyst formulation	Obtaining a new biocatalyst without altering the original structure
Multiple enzyme engineering	Number of biocatalysts	Combining multiple biocatalytic steps in one pot

A strategy towards a given target often combines several of these methods. An immobilized enzyme can be applied to enable a reaction in a water-free organic medium or several enzymes can be co-immobilized to catalyze one pot reaction systems.

1. Substrate engineering

Substrate engineering is to create a new or optimized transformation by varying the substrates. Enzymes have the ability to accept different substrates. As a result, a reaction step, synthetic route or biocatalytic process can be improved by choosing an adequate substrate system.

Enzymes naturally catalyze reactions for which there are equally natural substrates. Nonetheless, enzymes can accept different compounds with greater or less structural similarity to the natural substrates. The structural change can either be on the reactive region of the molecule or somewhere else, e.g. in the periphery.

In other cases the substrate difference is not structural but fundamental. For example, a hydrolase can accept different nucleophiles than water. This originates a different product following the same mechanism but the reaction is an acyl transfer, not a hydrolysis reaction. These species are often called unnatural substrates and this phenomenon is called substrate promiscuity.

There are many examples of improvement through substrate engineering. The applications can range from protective chemistry to enantiopure synthesis. The improvement of a resolution or the optimization of a work-up can integrate screening of structurally similar or related substrates, as well as unnatural.

Lipase properties and applications make them suited biocatalysts to illustrate substrate engineering strategies. These enzymes are serine hydrolases and these often have a large substrate scope. Lipases naturally hydrolyze triglycerides (see Figure 3) but can accommodate other acyl donors, such as aliphatic, alicyclic, bicyclic and aromatic esters [68,69,70,71].

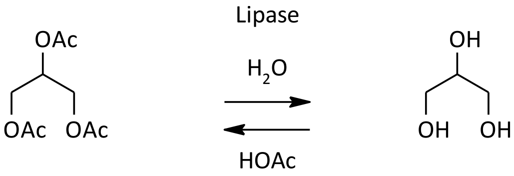


Figure 3: Lipase hydrolysis of triglycerides

The “synthetic portfolio” of an enzyme can be increased by altering reaction conditions, in order to allow the use of different substrates. For example, using a lipase with an alcohol or another ester as solvent, allows trans and interesterifications (see Figure 4). This is possible because lipases are stable in organic media [72].

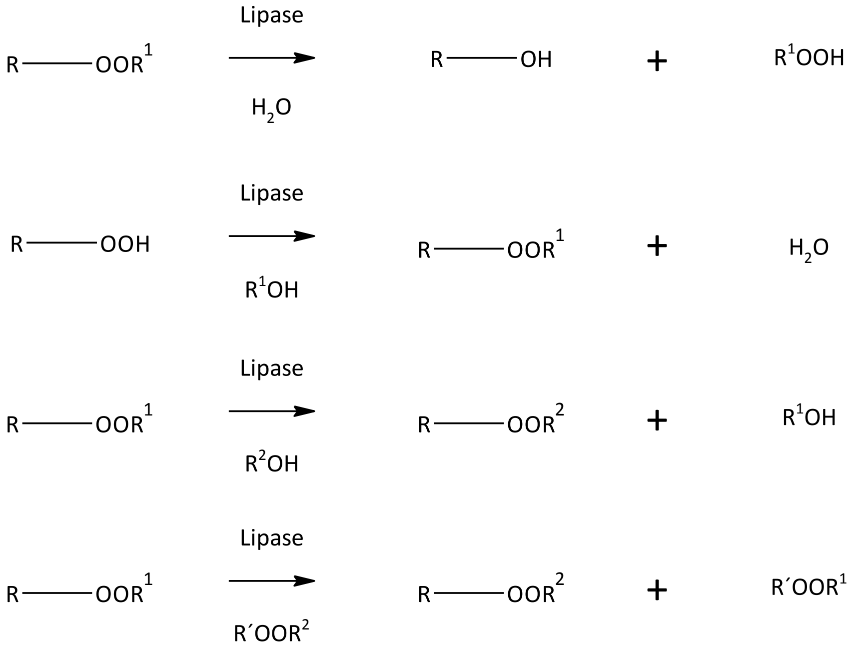


Figure 4: Lipase catalyzed ester hydrolysis, acid esterification, ester alcoholysis (transesterification) and interesterification

As a result possible products of enzymatic reaction can be increased by using so called unnatural nucleophiles. Lipases can accept a large variety of these, such as ammonia, amines, hydrazine, hydroxylamine and hydrogen peroxide. Because of the different functional groups that are introduced, these compounds are particularly interesting in organic synthesis (see Figure 5) [73,74,75].

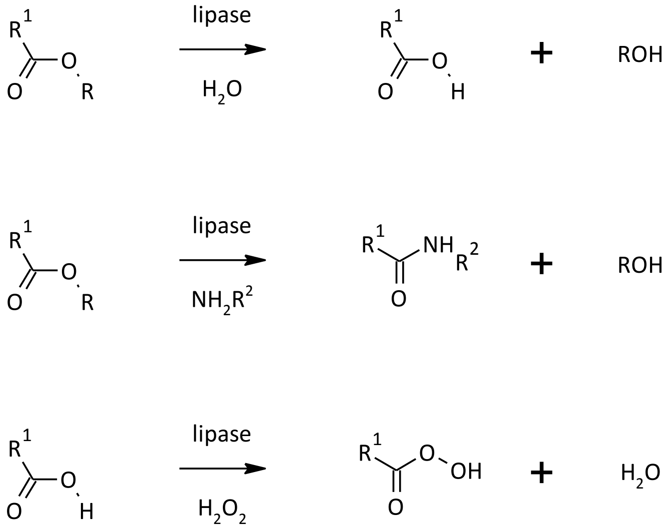


Figure 5: Lipase catalyzed natural ester hydrolysis; unnatural amide and peroxide formation

The concept “one enzyme, many substrates” can be applied in sequential steps, in the same route. A halohydrin dehalogenase (HHDH) is used in the synthesis of Atorvastatin, catalyzing the sequential enantioselective epoxide ring formation and opening (Figure 6) [76].

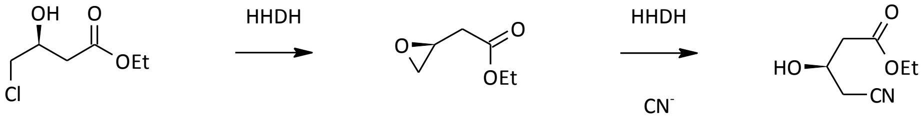


Figure 6: Halohydrin dehalogenase catalyzed enantioselective epoxide formation and enantioselective ring-opening with cyanide insertion

The possibility for HHDH to accept an unnatural nucleophile, cyanide, pulls the equilibrium in the direction of the desired compound, avoiding isolation of the epoxide. Furthermore cyanide is a relevant group in synthesis, because it adds a carbon unit to the skeleton and allows further functionalization.

Another example of sequential steps with the same enzyme is the selective coupling of aldehydes with DERA, an aldolase (Figure 7). Interestingly this example is also taken from the synthesis of Atorvastatin, the active ingredient in the cholesterol lowering drug Lipitor [77].

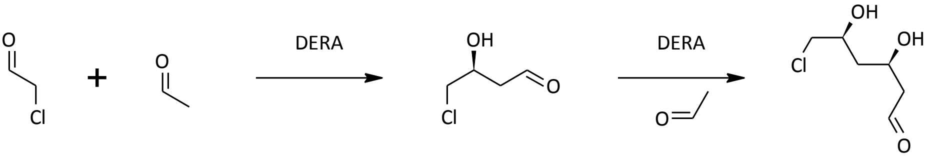


Figure 7: DERA catalyzed selective sequential aldehydes coupling

A kinetic resolution is a widely used method to obtain enantiopure compounds, particularly alcohols and amines [78,79,80,56]. A racemic mixture is resolved by performing a reaction where the conversion rates of both enantiomers are different enough to allow discrimination.

However, the applications of kinetic resolutions transcend isolation of an enantiopure compound which does not react or the enantiopure synthesis of a product. Moreover, kinetic resolutions are a fertile field for substrate engineering examples.

The works of *Youshko et al* show how using substrates that are structurally similar, but different in a non reactive part of the molecule, can lead to improved results in a resolution. In this case, the hydrolysis rates of esters and amides of non-natural amino-acids were higher for some of the *N*-acetyl derivatives than for the traditional substrates [81].

Another example is the mild protection and deprotection of an amine via a so-called easy on easy off resolution, which was elegantly performed in two consecutive steps using one acylase (Figure 8). The use of D-phenylglycine amide instead of phenylacetamide as acylating agent resulted in a remarkable improvement in both selectivity and synthesis / hydrolysis ratio [82].

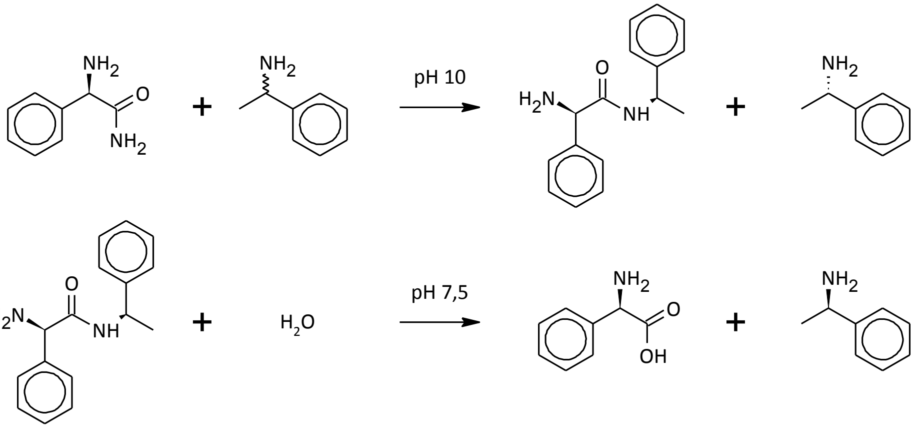


Figure 8: Optimized "easy on – easy off" protection of phenylethyl amine with (D)-phenylglycine amide, via kinetic resolution, catalyzed by penicillin acylase from *Alcaligenes faecalis*

Kinetic resolutions can equally be used to perform new enantiopure synthesis, such as the lipase catalyzed synthesis of (D)-Phenylglycine amide via ammonolysis, under mild conditions (Figure 9). This synthesis comprises both the use of an unnatural nucleophile and acyl donor screening [83].

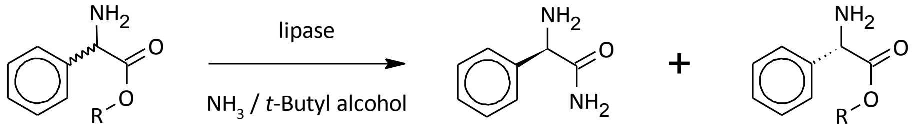


Figure 9: Lipase catalyzed enantioselective ammonolysis of phenylglycine methyl, ethyl and butyl esters via kinetic resolution

Depending on the goal, there might be no need for candidate substrates to be structurally similar. Full conversion was achieved in the resolution of a nitro-alcohol by replacing vinyl acetate with succinic anhydride (Figure 10) [84].

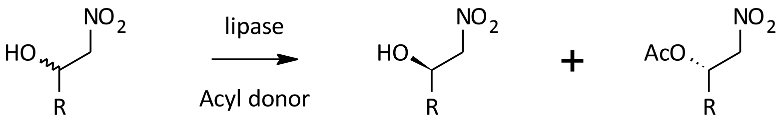


Figure 10: Optimization of a lipase catalyzed kinetic resolution of a nitro alcohol, by screening different acyl donors

In addition to obtaining full conversion, the product obtained by reaction with succinic anhydride can be readily extracted from the reaction mixture, which contrasts with the laborious work up when using vinyl acetate. Similar cases of substrate engineering in downstream optimization were reported [85, 86].

2. Solvent engineering

Solvent engineering in biocatalysis is to use non-aqueous systems in order to improve a biocatalytic transformation. Enzymes are naturally suited to function in water but they are active in organic solvents [87].

Synthetic organic chemistry is generally performed in organic solvents rather than water because substrates are more soluble in the former than water.

Compound solubility is higher, product recovery is easier and there are no side reactions or decomposition caused by water [88,89,90].

The possibility of enzymatic use in organic solvents is therefore equivalent to maximizing the synthetic potential of biocatalysis. A solvent can furthermore enhance, or revert, the enantioselectivity of an enzyme (Table 6 and Table 7). These modifications are normally only achievable by protein engineering [91,92].

Table 6: Solvent effect on enantioselectivity, in the lipase catalyzed kinetic resolution of *trans*-sobrerol (from [91])

Solvent	*E*
t-Amyl alcohol	518
3-Pentanone	212
Dioxane	178
Acetone	142
Vinyl acetate	89
Tetrahydrofuran	69

Table 7: Solvent influence on stereochemical preference in the lipase catalyzed asymmetric hydrolysis of two dihydropyridine diesters (from [91])

Solvent	*Product configuration*	*Product e.e. (%)*
Cyclohexane	*R*	89
Iso-Propyl ether	*S*	99
Cyclohexane	*R*	91
Iso-Propyl ether	*S*	68

Biocatalysis in organic media became interesting to industry and currently several industrial processes are performed in organic media. These include chiral resolutions and selective polymerizations. Lipases are particularly stable in organic media and can accommodate a large variety of substrates, accounting for their wide use in resolutions [56,93].

Organic media do not necessarily hinder the enzyme´s stability or conformation. However, this is often the case, resulting in a decrease in reaction rates [94,95]. In some cases the use of lyophilized enzyme suspensions in pure organic media afforded comparable or even improved rates [96].

Apart from the tendency to lower enzymatic reaction rates, organic solvents conventionally used in laboratory or industry are often volatile and liquid in only a small temperature window. A large contribution for the relatively high waste generated in the pharmaceutical industry (see above) derives from solvents. High amounts are unloaded into ground water or evaporate into the atmosphere [97].

Alternatives such as ionic liquids have emerged as greener options to conventional solvents, in biocatalysis. Ionic liquids are salts, which are liquid at room temperature. They are therefore fluids of a different nature than organic solvents [98,99,100].

Ionic liquids first appeared in 1914, stating the existence of a new class of liquids. Following this, in 1934 the first patent for ionic liquid industrial application is published. Ionic liquids are applicable in synthesis and were found an acceptable non-volatile alternative to organic solvents for biocatalysis, concerning efficiency and product solubility [101,102,103,104,105].

One example of ionic liquid rate improvement compared to an organic solvent, is the lipase resolution of 1-Phenyl ethanol, with ionic liquids as different as the ones belonging to the methylimidazolium family and ionic liquids prepared from tertiary amines and carboxylic acids. The latter are more recent and inexpensive [106,107].

In the above mentioned experiments, both free as well as immobilized enzyme formulations were used. Immobilization was furthermore shown to grant stabilization, when the ionic liquid would compromise the stability of the native enzyme [108].

A particular feature of these solvents is the dependency of their polarity, hydrophobicity and solvent miscibility, according to the cation and anion used. Putatively this allows the ionic liquid to be designed according to the purpose, hence being called "designer solvents" [101].

A third type of solvent is supercritical fluids. Supercritical fluids (SCFs) combine the low viscosity of a gas with the solubilizing capacity of a liquid. Several SCFs were shown to be applicable for biocatalysis. Supercritical carbon dioxide (ScCO2) in particular is also a green solvent. It is furthermore non-flammable, non-toxic and highly available. Product recovery simply involves depressurization, which makes it a simple procedure [109,110].

$ScCO_2$ has induced asymmetry where conventional organic solvents did not. Furthermore, the enantioselectivity can be influenced by the pressure and temperature used. One example of the latter effect was observed with several lipases, in alcohol resolution. $ScCO_2$ has therefore the potential to tailor a given reaction. It is inconclusive whether $SCCO_2$ enhances or reduces reaction rates when compared with organic solvents, because both cases have been reported [111,112,113,114].

The lysine groups present in the enzyme can react with CO_2 to afford carbamate. On the other hand, CO_2 can react with water to give carbonic acid. The latter generates pH drop. In both cases there is the possibility of enzymatic activity loss [109].

Immobilization techniques, such as cross-linking, add stability to the enzyme in direct contact with $ScCO_2$. $ScCO_2$ can equally be used with ionic liquids and in this case it acts as an extracting agent, while the biocatalyst remains in the ionic liquid. Because of its independence of volatile compounds, this technology has been classified as “the clean and green reaction technology of the future” [115,116,117,118,119].

3. Protein engineering

Protein engineering is to alter amino acid sequences of enzymes, in order to obtain enzymes with improved properties. Available enzymes often fail to meet the requirements for large-scale implementation. These can be related to selectivity, particularly enantioselectivity, or stability and activity under certain conditions. Stability is particularly important in industry [120,121].

Because of this, protein engineering is often found as a part of an integrated strategy. For example, it can be applied within a substrate or solvent engineering strategy or in the optimization of an already well established industrial process [122,123,124].

In nature, enzymes have changed in the course of time becoming a highly specific catalyst. *In vitro* this can be mimicked by performing sequences of mutation and screening, without requiring previous knowledge of which amino acid sequence is changed or its function.

This technique is called directed evolution. The independence of previous information makes directed evolution widely applicable but it leads to a cumbersome screening effort, which might exceed the practical range [125].

An alternative is to operate precise changes in the amino acid sequence, based on recognition of relevant sequences, or regions, of the protein. The challenge for this semi-rational design lies on the fact that one modification at a region might not produce the predicted effect and excluding other amino acid sequences might exclude unpredicted but nonetheless wanted changes, in addition that it requires considerably more information. With directed evolution information about the tertiary structure or even the reaction mechanism is not a pre-requisite [126].

The conclusion to be withdrawn is that these two methods are complementary, rather than opposing. The former leads to larger libraries with fewer hits and bigger focus on screening, whereas the second affords higher quality but smaller libraries.

A particularly successful case of how protein engineering can be harnessed was the improvement in wild-type HHDH promiscuous hydrocyanation activity in the synthesis of ethyl (*R*)-4-cyano-3-hydroxy-butyrate, which is an important intermediate in the synthesis of atorvastatin. This led to an increase in volume productivity by a factor of 4000 [127,128].

Another challenge faced by protein engineering is how to introduce a new catalytic activity, not present in the native enzyme. The introduction of a novel activity is perhaps an ambitious goal, as to optimize an existing function is easier than creating a new one. Nonetheless, this constitutes an important step towards having a fully designed catalyst at disposal [129,130].

The possibility to enhance, or create, an enzyme property, naturally grants protein engineering an undeniable potential. Several examples, such as the case of cholesterol decreasing drugs, show protein engineering is already a very powerful tool in the improvement of industrial biocatalysis [131,132,133,134,135].

Protein engineering is a very particular method, which differs in one essential feature from all others described here. Other methods have the purpose to adapt an enzyme to new conditions, or to perform changes to it. By modifying an existing enzyme, protein engineering has the specific purpose of making an enzyme that did not previously exist.

A synergy between different methods is often needed, in the quest for the best solution. In this case, all other methods can be developed *ab initio* for an already modified enzyme, making this method particularly suited for combined strategies.

4. Biocatalyst engineering

Biocatalyst engineering is to modify a native biocatalyst, to improve a particular aspect of a transformation. Contrary to protein engineering, the protein structure is not altered. The result is a different biocatalyst formulation with the same native enzyme.

Enzyme immobilization is a fundamental method, or group of methods, in biocatalyst engineering. Immobilization appeared in industry in the 1950´s. The main goal is to achieve enhanced operational stability, reusability and isolation of the biocatalyst.

This also means preventing product contamination with the enzyme. Some activity is usually lost. Stability is particularly important in industry and, together with reusability, it is a factor that can drastically reduce cost [136,137].

The applications in penicillin hydrolysis and glucose isomerisation are often referred as the landmarks in establishing the industrial use of immobilized enzymes. The productivities are 600 Kg of product per kg of enzyme in the former and 11,000 in the latter. Immobilized Glucose isomerase is still a benchmark in industrial uses of immobilized enzymes [22,32,138].

There are three main enzyme immobilization categories: immobilization to a solid support; encapsulation, or entrapment in a physical matrix; and carrier-free or self-immobilization. For each of these there are different techniques, which can be compared. Notwithstanding, optimization usually requires a tailored solution for each enzyme and process [139,140].

For example, an enzyme can be covalently bound to a solid support or be adsorbed on its surface. Enzymes can be self-immobilized as cross-linked aggregates (CLEA) or crystals (CLEC) [141].

The cross-linking concept is to bind enzyme molecules to each other. Cross-linking therefore avoids the use of a carrier and the costs associated. Carriers are often expensive. Because the biocatalyst is simple protein without a solid support, the biocatalyst productivities are high (Kg product / Kg biocatalyst). Cross-linking allows for easy preparation and multiple enzyme co-immobilization [142].

Whereas immobilization to a carrier involves binding the enzyme to a particle, encapsulation is the inclusion or integration of the enzyme into a polymeric network, forming a matrix. This can be organic or inorganic. The binding is usually weak, which often leads to leaching [141].

An interesting application of encapsulation is in cosmetics. Encapsulated lipases are used as active ingredients in cosmetic products, because they grant improved properties to the final formulation [143].

The nature of the bond between enzyme and a solid support can be ionic, covalent or physical. Depending on the application, this is a relevant factor. An enzyme bound by a weak interaction to a support can leach out under industrial conditions. On the other hand, immobilization with a covalent bond might yield a less leaching prone biocatalyst but if there is inactivation, enzyme and support are unusable.

The support can be a synthetic resin, a biopolymer or an inorganic solid. The carrier can be porous or non-porous. One interesting example is provided by mesoporous silicates, which allows functionalizing the carrier, and consequently tailoring it, according to the enzyme used [141,144,145].

Although immobilization usually involves activity loss, both selectivity and stability can be improved by it. This variation in performance is associated with the structural modifications, including conformational changes that happen during immobilization. An immobilized enzyme conformation can be even more active than the native [146].

Another major goal of enzyme immobilization is to allow the use in a media other than aqueous. Lipases are, again, representative catalysts, due to their stability in non-aqueous media and in some cases activity increased after immobilization and use in organic media.

Another way of allowing the use in non-aqueous media is to coat an enzyme with a surfactant, which in this way becomes soluble. It is not so much an immobilization method as a solubilizing method, although the enzyme is to some extent fixed as a micelle [147].

Lyophilization is another technique for enzymatic use in pure organic solvents. This consists of freeze-drying an enzyme containing formulation and obtaining a dry powder, which retains some activity. This can then be used in a solvent, isolated and reused. This is often used with additives, such as polyethylene glycol (PEG) or salts, as stabilizing agents [148].

5. Multiple enzyme engineering

Multiple enzyme engineering is to use more than one enzyme in a one pot synthesis, with the purpose of maximizing chemical or catalyst potential. In cells, metabolic pathways occur with high efficiency, involving multiple enzymes. *In vitro*, multi-step enzymatic synthesis can occur in isolated separate biocatalytic steps or in "one pot", which means the whole sequence of reactions takes place in one reactor [149].

From a chemist's perspective, combining several steps in one pot is a reasonable approach for optimizing a system. A multi-step synthesis avoids solvent shifts and circumvents the isolation and purification of intermediary compounds, which are time costly and yield reducing. An unfavorable equilibrium can be driven to completion by combining multiple steps [150,151].

Industrially this translates into an overall efficiency increase, with less unit operations waste reduction and higher productivities. Biocatalytic processes often take place without large temperature and pressure changes, which makes them suited for cascade processes [149,152].

The feasibility of multi-enzyme cascade synthesis is demonstrated by cases such as the (two-step) synthesis of cephalexin, combining penicillin acylase and a nitrile hydratase [153], or the (four-step) synthesis of carbohydrates, starting from glycerol and an aldehyde [154].

The latter case is particularly interesting, because the activities of the different enzymes are controlled by pH switch. In addition to successfully combining four enzymes, it requires only cheap and readily available starting materials.

One pot cascade systems can be sequential or cyclic. The above cases are sequential reactions, because the reactions take place step-wise. Cyclic reactions are often seen in co-factor recycling systems, such as oxidation-reduction [155].

Combining both types of reactions is equally possible in multi-enzyme systems. The conversion of D-Methionine to L-Methionine is one example (Figure 11), which was furthermore reported to produce quantitative yields [156].

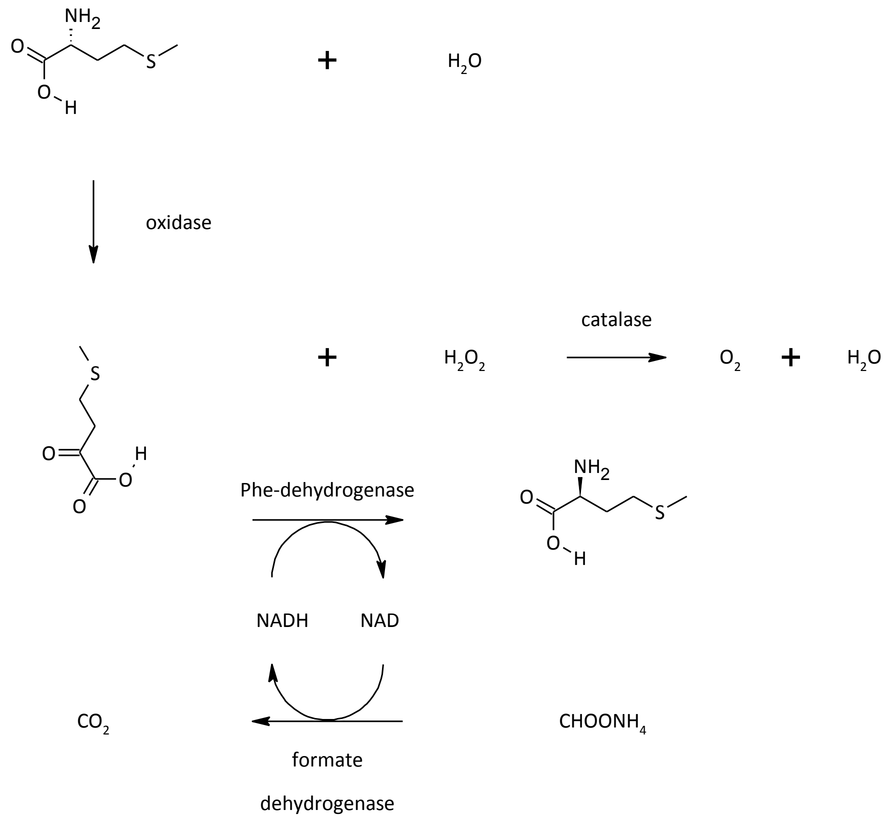


Figure 11: One pot, four enzyme conversion of D-Methionine to L-Methionine, with *in situ* co-factor regeneration

This is a two-step cascade, which makes use of four enzymes. A D-amino acid oxidase and L-phenylalanine dehydrogenase catalyze the synthesis reactions. The co-factor (NADH), which is consumed in the latter step, is regenerated by a formate dehydrogenase. A fourth enzyme (catalase) prevents accumulation of the first step side product hydrogen peroxide.

Multi-enzymatic systems have been combined with immobilization. In these cases the enzymes are co-immobilized, either in a support or cross-linked [157,158].

Notwithstanding, it should equally be possible to use multi-enzymatic systems of enzymes immobilized independently [149].

Generically, it is not possible to conclude whether cascade systems will fulfill their promises (see above). Therefore a distinction can be made between cases in which multi-enzyme systems offer putative advantages and cases in which this approach is an obvious solution for a concrete problem.

For example, an enzyme can be used to remove a poisonous side product, not interfering with the synthesis. In the above mentioned case, catalase is used to destroy hydrogen peroxide, which is toxic for most enzymes.

Another such case is when one intermediate compound is too unstable and must therefore be consumed rapidly. A final example is *in situ* co-factor regeneration. Co-factors are expensive and the possibility of reusing them reduces costs significantly [149].

Most of the examples of multi-enzymatic systems are at a laboratory scale. The production of L-methionine, L-norleucine and L-2-aminobutyric acid are at kilogram scale [151].

Part III

Scope of the thesis

This thesis shows three distinct cases of biocatalysis improvement, applying the various concepts of biocatalysis engineering. The first case concerns the development of new synthetic routes with an already developed biocatalyst. The second case concerns the design and development of a biocatalyst. Finally, the third case concerns a preliminary screening on the applicability of biocatalysis engineering techniques, on an enzyme with interesting properties.

Chapters 2 and 3 describe how substrate engineering was applied in the development of new chemo-enzymatic syntheses of chiral diketopiperazines, using immobilized penicillin acylase. The scope of this green synthetic route was significantly broadened, by allowing the use of racemic mixtures as starting reagents.

Chapters 4 and 5 address the development of a tandem co-immobilized enzyme biocatalyst, to be used in a water-solvent mixture. Chloroperoxidase and glucose oxidase were co-immobilized as combi-CLEAs (Cross-Linked Enzyme Aggregates) and applied in the oxidation of thioanisole. The tandem free enzyme system was studied and the implications and challenges of this particular two enzyme cascade were discussed.

Chapter 6 addresses the preliminary exploration of the biocatalytic potential of an atypical epoxide hydrolase. Several approaches were followed and discussed, namely substrate and solvent engineering as well as immobilization. For this study we used limonene epoxide hydrolase.

References

---

1 Neidleman, S.L.; *Biotechnology and Genetic Reviews* **1984**; 1, 1 - 38

2 Zhang, X.; Houk, K.N.; *Accounts of Chemical Research* **2005**; 38, 5, 379 - 385

3 Schmid, A.; Hollman, F.; Park, J.B.; Bühler, B.; *Current Opinion in Biotechnology* **2002**, 13, 359 - 366

4 Fox, R.J.; Huisman, G.W.; *Trends in Biotechnology* **2008**; 26, 3, 132 - 138

5 Wells, A.; Meyer, H.-P.; *ChemCatChem* **2014**; 6, 918 - 920

---

6 Roschangar, F.; Sheldon, R.A.; Senanayake, C.H.; *Green Chemistry* **2015**; 17. 752 - 768

7 Buchholz, K.; Kasche, V.; Bornsheuer, U.T.; *Biocatalysis and Enzyme Technology* **2012**; Wiley-Blackwell

8 http://www.companiesandmarkets.com/Market/Food-and-Drink/Market-Research/Specialty-Enzymes-A-Global-Strategic-Business-Report/RPT794043

9 Sanderson, K.; *Nature* **2011**; 471, 397 - 398

10 Gupta, P.; Mahajan, A.; *RSC Advances* **2015**; 5; 26686 - 26705

11 Schneider, B.W.; *Journal of Business Chemistry* **2009**; 6, 3, 108 - 110

12 Vasic-Racki, D.; *History of industrial biotransformations – Dreams and realities* in : Liese, A.; Seebald, S.; Wandrey, C.; *Industrial Biotransformations* **2006**;Weinheim: Wiley-VCH; 1 - 37

13 Fernandes, P.; *Enzyme Research* **2010**; 1 - 19

14 Roberts, S.M.; Turner, N.J.; Willets, A.J.; Turner, M.K.; *Introduction to Biocatalysis Using Enzymes and Micro-Organisms* **1995**; Cambridge University Press

15 Barnett, J.A.; *Microbiology* **2003**; 149, 557 - 567

16 Kohler, R.; *Journal of the History of Biology* **1971**; 4, 1, 35 - 61

17 Buchner, E.; “Alcoholische Gärung ohne Hefezellen (Vorläufige Mitteilung)”; *Berichte der Deutschen Chemischen Gesellschaft* **1897**; 30, 117 - 124

18 Rosenberg, E.; *The Prokaryotes – Prokaryotic Biology and Symbiotic Associations* **2013**; E. Rosenberg et al. (eds.); Springer-Verlag Berlin Heidelberg; 315 - 328

19 Poulsen, P.B.; Buchholz, K.; *Handbook of Food Enzymology* **2002**; Whitaker, J.R.; Voragen, A.G.J.; Wong, D.W.S. (eds); CRC Press

20 Headon, D.R.; Walsh, G.; *Biotechnology Advances* **1994**; 12, 635 - 646

21 Jr, T.J.; Oshima, K.; *Journal of the American Chemical Society* **1920**; 46, 6, 1261 – 1265.

22 Buchholz, K.; Collins, J.; *Applied Microbiology and Biotechnology* **2013**; 1 - 16

23 Chandel, A.K.; Rudravaram, R.; Rao, L.V.; Ravindra, P.; Narasu, M.L.; *Journal of Commercial Biotechnology* **2007**; 13, 4, 283 - 291

24 Smythe, C.V.; *Economic Botany* **1951**; 5, 2, 126 - 144

25 Maurer, K.-H.; *Current Opinion in Biotechnology* **2004**; 15, 330 - 334

26 Illanes, A.; *Enzyme Biocatalysis Principles and Applications* **2008**; Springer Science + Business Media B.V.

27 Østergaard, L.H.; Olsen, H.S.; “Industrial applications of fungal enzymes”. *Industrial Applications* **2011**; Springer Berlin Heidelberg; 269 - 290

28 Jackson, D.A.; Symons, R.H.; Berg, P.; *Proceedings of the National Academy of Sciences USA* **1972**; 69; 10; 2904 - 2909

29 Cohen, S.N.; Chang, A.Y.; Boyer, H.W.; Helling, R.B.; *Proceedings of the National Academy of Sciences USA* **1973**; 70; 11; 3240 - 3244

30 Chang, A.C.Y.; Nunberg, J.H.; Kaufman, R.J.; Erlich, H.A.; Schimke, R.T.; Cohen, S.N.; *Nature* **1978**; 275; 617 - 624

31 Cohen, S.N.; *Proceedings of the National Academy of Sciences USA* **2013**; 110; 39; 15521 - 15529

32 Bornscheuer, U.T.; Buchholz, K.; *Engineering in Life Sciences* **2005**; 5, 4, 309 - 323

33 Bloom, J.D.; Meyer, M.M.; Meinhold, P.; Otey, C.R.; MacMillan, D.; Arnold, F.H.; *Current Opinion in Structural Biology* **2005**; 15, 447 - 452

34 Kirk, O.; Borchert, T.V.; Fuglsang, C.C.; *Current Opinion in Biotechnology* **2002**; 13, 345 - 351

35 Choi, J.-M.; Han, S.-S.; Kim, H.-S.; *Biotechnology Advances* **2015**; (in press)

36 Sarrouh, B.; Santos. T.M.; Miyoshi, A.; Dias, R.; Azevedo, V.; *Journal of Bioprocessing and Biotechniques* **2012**; 4, 2

37 Bhosale, S.H.; Rao, M.B.; Deshpande, V.V.; *Microbiology and Molecular Biology Reviews* **1996**; 60, 2, 280 - 300

38 Soetaert, W.; Vandamme, E.; *Biotechnology Journal* **2006**; 1, 756 - 769

39 de Souza, P.M.; Oliveira e Magalhães, P.; *Brazilian Journal of Microbiology* **2010**; 41, 850 - 861

40 Gupta, R.; Beg, Q.K.; Lorenz, P.; *Applied Microbiology and Biotechnology* **2002**; 59, 15 - 32

41 Li, Q.; Yi, L.; Marek, P.; Ivserson, B.L.; *Federation of European Biochemical Societies Letters* **2013**; 587, 1155 - 1163

42 Kobayashi, M.; Nagasawa, T.; Yamada, H.; *Trends in Biotechnology* **1992**; 10, 402 - 408

---

43 Liese, A.; Filho, M.V.; *Current Opinion in Biotechnology* **1999**; 10, 595 - 603

44 Elander, R.P.; *Applied microbiology and Biotechnology* **2003**; 61, 385 - 392

45 Bornscheuer, U.T.; Huisman, G.W.; Kazlauskas, R.J.; Lutz, S.; Moore, J.C.; Robins, K.; *Nature* **2012**; 485, 185 - 194

46 Sheldon, R.A.; *Journal of Chemical Technology and Biotechnology* **1996**; 67, 1 - 14

47 Breuer, M.; Ditrich, K.; Habicher, T.; Hauer, B. ; Keßler, M. ; Stürmer, R. ; Zelinski, T ; *Angewandte Chemie International Edition* **2004**; 43, 788 - 824

48 Zhang, Z.-J.; Pan, J.; Ma, B.-D. ; Xu, J.-H. ; *Adv Bioechem Eng Biotechnol* **2014**; Springer-Verlag Berlin Heidelberg

49 Nestl, B.M.; Hammer, S.C.; Nebel, B.A.; Hauer, B.; *Angewandte Chemie Int. Ed.* **2014**; 53; 3070 - 3095

50 Lundemo, M.T.; Woodley, J.M.; *Applied Microbiology and Biotechnology* **2015**; 99; 2465 - 2483

51 Narancic, T.; Davis, R.; Nikodinovic-Runic, J.; O´Connor, K.E.; *Biotechnology Letters* **2015**; 37; 943 - 954

52 Dunn, P.J.; *Chemical Society Reviews* **2012**; 41; 1452 - 1461

53 Patel, R.N.; *Coordination Chemistry Reviews* **2008**; 252, 659 - 701

54 Patel, R.N.; *ACS Catalysis* **2011**; 1, 1056 - 1074

55 Straathof, A.J.J.; Panke, S.; Schmid, A.; *Current Opinion in Biotechnology* **2002**; 13, 548 - 556

56 Schmid, A.; Dordick, J.S.; Hauer, B.; Kieners, A.; Wubbolts, M.; Witholt, B.; *Nature* **2001**; 409, 258 - 268

57 Jegannathan, K.R.; Nielsen, P.H.; *Journal of Cleaner Production* **2013**; 42, 228 - 240

58 Di Salle, A.; Calarco, A.; Petillo, O.; Margarucci, S.; D´Apolito; M.; Galderisi, U.; Peluso, G.; *Biomolecular Research & Therapeutics* **2015**; 4; 1 - 10

59 Xu, G.-C.; Yu, H.-L.; Shang, Y.-P. ; Xu, J.-H. ; *RSC Advances* **2015**; 5 ; 222703 - 222711

60 Fait, M.E.; Garrote, G.L.; Clapés, P. ; Tanco, S. ; Lorenzo, J. ; Morcelle, S.R. ; *Amino Acids* **2015** (in press)

---

61 Serra, I.; Daly, S.; Alcantara, A.R.; Bianchi, D.; Terreni, M.; Ubiali, D.; *RSC Advances* **2015**; 5; 23569 - 23577

62 Wells, A.S.; Finch, G.L.; Michales, P.C.; Wong, J.W.; *Organic Process Research & Development* **2015**; 16; 1986 - 1993

63 Wagner, N.; Bosshart, A.; Failmezger, J.; Bechtold, M.; Panke, S.; *Angewandte Chemie* **2015**; 127; 4256 - 4260

64 Sheldon, R.A.; *Green Chemistry* **2007**; 9, 12, 1261 - 1384

65 Pollard, D.J.; Woodley, J.M.; *Trends in Biotechnology* **2006**; 25, 2, 66 - 73

66 Tufvesson, P.; Lima-Ramos, J.; Nordblad, M.; Woodley, J.M.; *Organic Process Research & Development* **2011**; 15; 266 - 274

67 Bornscheuer, U.T.; “Trends and challenges in enzyme technology”; *Biotechnology for the future*; **2005**; Springer Berlin Heidelberg; 181 - 203

68 Khersonsky, O.; Tawfik, D.S.; *Annual Review of Biochemistry* **2010**; 79, 471 - 505

69 Busto, E.; Gotor-Fernández, V.; Gotor, V.; *Chemical Society Reviews* **2010**; 39, 4504 - 4523

70 Soumanou, M.M.; Pérignon, M.; Villeneuve, P. ; *European Journal of Lipid Science and Technology* **2013** ; 115, 270 - 285

71 Schmid, R.D.; Verger, R.; *Angewandte Chemie International Edition* **1998**; 37, 1608 - 1633

72 De Zoete, M.C.; van Rantwijk, F.; Sheldon, R.A.; *Catalysis Today* **1994**; 22, 563 - 590

73 Hacking, M.A.P.J.; van Rantwijk, F.; Sheldon, R.A.; *Journal of Molecular Catalysis B: Enzymatic* **2000**; 9, 183 - 191

74 Hacking, M.A.P.J.; van Rantwijk, F.; Sheldon, R.A.; *Journal of Molecular Catalysis B: Enzymatic* **2001**; 11, 315 - 321

75 US Pat. 5541092; **1996**

76 Ma, S.K.; Gruber, J.; Davis, C.; Newman, L.; Gray, D. ; Wang, A. ; Grate, J.; Huisman, G.W.; Sheldon, R.A.; *Green Chemistry* **2010**; 12, 81 - 86

77 Jennewein, S.; Schürmann, M.; Wolberg, M.; Hilker, I.; Luiten, R.; Wubbolts, M.; Mink, D.; *Biotechnology Journal* **2006**; 1, 537 - 548

78 van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron* **2004**; 60, 501 - 519

79 Guranda, D.T.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A.; Švedas, V.K.; *Tetrahedron: Asymmetry* **2001**; 12, 1645 - 1650

80 Gill, I.I.; Das, J.; Patel, R.N.; *Tetrahedron: Asymmetry* **2007**; 18, 1330 - 1337

81 Youshko, M.I.; van Langen, L.M.; Sheldon, R.A.; Švedas, V.K.; *Tetrahedron: Asymmetry* **2004**; 15, 1933 - 1936

82 Guranda, D.T.; Khimiuk, A.I.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A.; Švedas, V.K.; *Tetrahedron: Asymmetry* **2004**; 15, 2901 - 2906

83 Wegman, M.A.; Hacking, M.A.P.J.; Rops, J.; Pereira, P.; van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron Asymmetry* **1999**; 10, 1739 - 1750

84 Sorgedrager, M.J.; Malpique, R.; van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron: Asymmetry* **2004**; 15, 1295 - 1299

85 Gutman, A.L.; Brenner, D.; Boltanski, A. ; *Tetrahedron: Asymmetry* **1993**; 4, 5, 839 - 844

86 Bouzemi, N.; Debbeche, H.; Aribi-Zouioueche, L.; Fiaud, J.-C. ; *Tetrahedron Letters* **2004**; 45, 627 - 630

87 Zaks, A.; Klibanov; A.M.; *Science* **1984**; 224, 1249 - 1251

88 Zaks, A.; Klibanov, A.M.; *Proceedings of the National Academy of Sciences* **1985**; 82, 3192 - 3196

89 Therisod, M.; Klibanov, A.M.; *Journal of the American Chemical Society* **1986**; 108, 5638 - 5640

90 Klibanov, A.M.; *Accounts of Chemical Research* **1990**; 23, 114 - 120

91 Carrea, G.; Ottolina, G.; Riva, S.; *Trends in Biotechnology* **1995**; 13; 2; 63 - 70

92 Wescott, C.R.; Klibanov, A.M.; *Biochimica et Biophysica Acta* **1994**; 1206, 1 - 9

93 Park, O-J.; Kim, D-Y.; Dordick, J.S.; *Biotechnology and Bioengineering*, **2000**; 70, 2, 208 - 216

94 Klibanov, A.M.; *Trends in Biotechnology* **1997**; 15, 97 - 101

95 Doukyu, N.; Ogino, H.; *Biochemical Engineering Journal* **2010**; 48, 270 - 282

96 Ru, M.T.; Dordick, J.S.; Reimer, J.A.; Clark, D.S.; *Biotechnology and Bioengineering* **1999**; 63, 2, 233 - 241

97 Sheldon, R.A.; *Chemical Society Reviews* **2012**; 41, 1437 - 1451

98 Deive, F.J.; Ruivo, D.; Rodrigues, J.V.; Gomes, C.M.; Sanromán, M.A.; Rebelo, L.P.N.; Esperança, J.M.S.S.; Rodriguez, A.; *RSC Advances* **2014**; 5; 3386 - 3389

---

99 Liu, Y.; Wang, Y.; Jiang, Y.; Hu, M.; Li, S.; Zhai, Q.; *Biotechnology Progress* **2015**; (in press)

100 Bi, Y.-H.; Duan, Z.-Q.; Li, X.-Q. ; Wang, Z.-Y. ; Zhao, X.-R.; *Journal of Agricultural and Food Chemistry* **2015**; 63; 1558 - 1561

101 Plechkova, N.V.; Seddon, K.R.; *Chemical Society Reviews* **2008**; 37, 123 - 150

102 Sheldon, R.A.; *Chemical Communications* **2001**; 2399 - 2407

103 Sheldon, R.A.; Lau, R.M.; Sorgedrager, M.J.; van Rantwijk, F.; Seddon, K.R.; *Green Chemistry* **2002**; 4, 147 - 151

104 Nara, S.J.; Harjani, J.R.; Salunkhe, M.M.; *Tetrahedron Letters* **2002**; 43, 2979 - 2982

105 Hernández-Fernández, F.J.; de los Rios, A.P.; Lozano-Blanco, L.J.; Godinez, C.; *Journal of Chemical Technology and Biotechnology* **2010**, 85, 11, 1423 - 1435

106 Schöfer, S.H.; Kaftzik, N.; Wasserscheid, P.; Kragl, U.; *Chemical Communications* **2001**; 425 - 426

107 de los Rios, A.P.; van Rantwijk, F.; Sheldon, R.A.; *Green Chemistry* **2012**; 14, 1584 - 1588

108 Toral, A.R.; de los Rios, A.P.; Hernández, F.J.; Janssen, M.H.A.; Schoevaart, R.; van Rantwijk, F.; Sheldon, R.A.; *Enzyme and Microbial Technology* **2007**; 40, 1095 - 1099

109 Hobbs, H.R.; Thomas, N.R.; *Chemical Reviews* **2007**; 107, 2786 - 2820

110 Matsuda, T.; *Journal of Bioscience and Bioengineering* **2013**; 115, 233 - 241

111 Mase, N.; Sako, T.; Horikawa, Y.; Takabe, k.; *Tetrahedron Letters* **2003**; 44, 5175 - 5178

112 Matsuda, T.; Kanamaru, R.; Watanabe, K.; Kamitanaka, T.; Harada, T.; Nakamura, K.; *Tetrahedron: Asymmetry* **2003**; 14, 2087 - 2091

113 Paizs, C.; Toşa, M.; Bódai, V.; Szakács, G.; Kmecz, I.; Simándi, B.; Majdik, C.; Novák, L.; Irime, F-D.; Poppe, L.; *Tetrahedron: Asymmetry* **2003**; 14, 1943 - 1949

114 Catoni, E.; Cernia, E.; Palocci, C.; *Journal of Molecular Catalysis A: Chemical* **1996** ; 105, 79 - 86

115 Hobbs, H.R.; Kondor, B.; Stephenson, P.; Sheldon, R.A.; Thomas, N.R.; Poliakoff, M.; *Green Chemistry* **2006**; 6, 816 - 821

116 van Rantwijk, F.; Sheldon, R.A.; *Chemical Reviews* **2007**; 107, 6, 2757 - 2785

117 Hoang, H.N.; Matsuda, T.; *Tetrahedron Letters* **2015**; 56; 639 - 641

118 Bubalo, M.C.; Vidovic, S.; Redovnikovic, I.R.; Jokić, S.; *Journal of Chemical Technology and Biotechnology* **2015** (in press)

119 Comim, S.R.R.; Veneral, J.; de Oliveira, D.; Ferreira, S.R.S.; Oliveira, J.V.; *The Journal of Supercritical Fluids* **2014**; 96; 334 - 348

120 Böttcher, D.; Bornscheuer, U.T.; *Current Opinion in Microbiology* **2010**; 13, 274 - 282

121 Eijsink, V.G.H.; Gåseindes, S.; Borchert, T.V.; van den Burg, B.; *Biomolecular Engineering* **2005**; 22, 21 - 30

122 Engström, K.; Nyhlén, J.; Sandström, A.G.; Bäckvall, J-E.; *Journal of the American Chemical Society* **2010**; 132, 20, 7038 - 7042

123 Reetz, M.T.; Soni, P.; Fernández, L.; Gumulya, Y.; Carballeira, J.D.; *Chemical Communications* **2010**; 46, 8657 - 8658

124 Hlima, H.B.; Aghajari, N.; Ali, M.B.; Haser, R.; Bejar, S.; *Journal of Industrial Microbiology and Biotechnology* **2012**; 39, 537 - 546

125 Otten, L.G.; Hollman, F.; Arends, I.W.C.E.; *Trends in Biotechnology* **2009**; 28, 1, 46 - 54

126 Chen, R.; *Trends in Biotechnology* **2001**; 19, 1, 13 - 14

127 Renata, H.; Wang, Z.J.; Arnold, F.H.; *Angewandte Chemie International Edition* **2015**; 54; 3351 - 3367

128 Fox, R.J.; Davis, S.C.; Mundorff, E.C.; Newman, L.M.; Gavrilovic, V.; Ma, S.K.; Chung, L.M.; Ching, C.; Tam, S.; Muley, S.; Grate, J.; Gruber, J.; Whitman, J.C.; Sheldon, R.A.; Huisman, G.W.; *Nature Biotechnology* **2007**; 25; 3; 338 - 344

129 Jestin, J.-L.; Vichier-Guerre, S.; *Research in Microbiology* **2005** ; 156, 961 - 966

130 Brustad, E.M.; Arnold, F.H.; *Current Opinion in Chemical Biology* **2011**; 15, 201 - 210

131 Nestl, B.M.; Nebel, B.A.; Hauer, B.; *Current Opinion in Chemical Biology* **2011**; 15, 187 - 193

132 Huisman, G.W.; Collier, S.; *Current Opinion in Chemical Biology* **2013**; 17, 284 - 292

133 Turner, N.J.; *Nature Chemical Biology* **2009**; 5, 8, 567 - 573

134 Wang, M.; Si, T.; Zhao, H.; *Bioresource Technology* **2012**; 115, 117 - 125

---

135 Bommarius, A.S.; Blum, J.K.; Abrahamson, M.J.; *Current Opinion in Chemical Biology* **2011**; 15, 194 - 200

136 Bommarius, A.S.; Paye, M.F.; *Chemical Society Reviews* **2013**; 42, 6534 - 6565

137 DiCosimo, R.; McAuliffe, J.; Poulose, J.; Bohlmann, G.; *Chemical Society Reviews* **2013**

138 Sheldon, R.A.; *Advanced Synthesis & Catalysis* **2007**; 349, 1289 - 1307

139 Hanefeld, U.; *Chemical Society Reviews* **2013**; 42, 6308 - 6321

140 Cantone, S.; Ferrario, V.; Corici, L.; Ebert, C.; Fattor, D.; Spizzo, P.; Gardossi, L.; *Chemical Society Reviews* **2013**; 42, 6262 - 6276

141 Sheldon, R.A.; van Pelt, S.; *Chemical Society Reviews* **2013**; 42, 6223 - 6235

142 Sheldon, R.A.; *Applied Microbiology and Biotechnology* **2011**; 92, 467 - 477

143 Ansorge-Schumacher, M.B.; Thum, O.; *Chemical Society Reviews* **2013**; 42, 6475 - 6490

144 Hartman, M.; Kostrov, X.; *Chemical Society Reviews* **2013**; 42, 6277 - 6289

145 Magner, E.; *Chemical Society Reviews* **2013**; 42, 6213 - 6222

146 Rodrigues, R.F.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernández-Lafuente, R.; *Chemical Society Reviews* **2012**; 42, 6290 - 6307

147 Adlercreutz, P.; *Chemical Society Reviews* **2013**; 42, 6406 - 6436

148 Klibanov, A.M.; *Nature* **2001**; 409, 241 - 246

149 Santacoloma, P.A.; Sin, G.; Gernaey, K.V.; Woodley, J.M.; *Organic Process Research & Development* **2011**; 15, 203 - 212

150 Lopez-Gallego, F.; Schmid-Dannert, C.; *Current Opinion in Chemical Biology* **2010**; 14, 174 - 183

151 Bruggink, A.; Schoevaart, R.; Kieboom, T.; *Organic Process Research Development* **2003**; 7, 622 - 640

152 Sheldon, R.A.; *Applied Microbiology and Biotechnology* **2011**; 92, 3, 467 - 477

153 Wegman, M.A.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A.; *Biotechnology and Bioengineering* **2002**; 79, 3, 356 - 361

154 Schoevaart, R.; van Rantwijk, F.; Sheldon, R.A.; *Journal of Organic Chemistry* **2000**; 65, 6940 - 6943

155 Schrittwieser, J.H.; Sattler, J.; Resch, V.; Mutti, F.G.; Kroutil, W.; *Current Opinion in Chemical Biology* **2011**; 15, 249 - 256

---

156 Findrik, Z.; Vasić-Rački, D.; *Biotechnology & Bioengineering* **2007**; 98, 5, 956 - 967

157 Mateo, C.; Chmura, A.; Rustler, S.; van Rantwijk, F.; Stolz, A.; Sheldon, R.A.; *Tetrahedron: Asymmetry* **2006**; 17, 320 - 323

158 El-Zahab, B.; Jia, H.; Wang, P.; *Biotechnology and Bioengineering* **2004**; 87, 2, 178 - 183

Robust and Straightforward Chemo-Enzymatic Enantiopure Dipeptides Syntheses and Diketopiperazines Thereof

2

The contents of this chapter have been published in Pereira, P.C.; Arends, I.W.C.E.; Sheldon, R.A.; *Tet: Asymm* **2014**; 25, 825 - 832

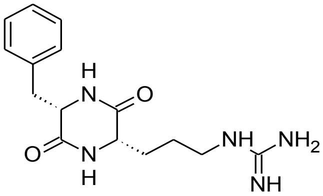
Chapter 2

Introduction

Diketopiperazines are cyclic dipeptides and constitute an important family of biologically active compounds that have attracted growing interest in several areas of research [1,2]. They are regarded as useful synthons for active pharmaceutical ingredients while histidine containing diketopiperazines are studied in the development of therapeutic agents [3,4,5,6]. Moreover, these dipeptides have been shown to possess a wide range of biological properties (antitumor, antiviral, antifungal and antibacterial), and have been linked with prebiotic activity while displaying enantioselective catalytic activity (Figure 1) [7,8,9,10,11].

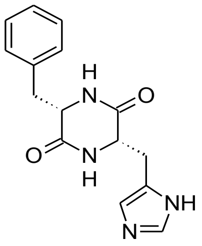
The goal of mimicking enzymatic activity with amino acids and peptides is not recent and important progress has been made in the development of asymmetric reactions with amino acid and peptide-based catalysts [12,13,14]. Another relevant example of both the growing interest in diketopiperazines as well as in developing bio mimetic peptides is the peptide-catalyzed synthesis of diketopiperazines and dipeptides. This makes diketopiperazines interesting for research, as well as showing the need for development of new synthetic methods and strategies to enable access to a larger variety of such compounds [15,16].

The use of biocatalysts in organic synthesis is widespread, mainly due to its main features: high selectivity and mild conditions. Unsurprisingly, steps towards environmentally benign syntheses of peptides have been reported. Penicillin G acylase, for example, is an efficient biocatalyst used in the synthesis of ß-lactam antibiotics and was also shown to be effective in the synthesis of chiral dipeptides [17,18,19].



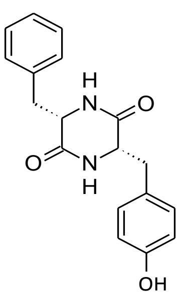
1-{3-[(2*S*,5*S*)-5-benzyl-3,6-dioxo piperazin-2-yl]propyl}guanidine

Catalyst



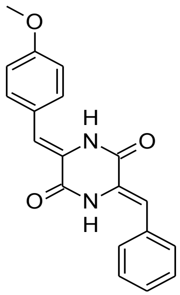
(3*S*,6*S*)-3-benzyl-6-(1*H*-imidazol -5-ylmethyl)piperazine-2,5-dione

Catalyst, Anti tumor



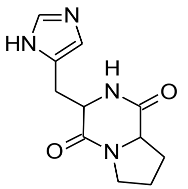
(3*S*,6*S*)-3-benzyl-6-(4-hydroxy benzyl)piperazine-2,5-dione

Anti tumor



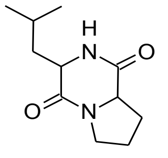
(3*Z*,6*Z*)-3-benzylidene-6-(4-methoxy benzylidene)piperazine-2,5-dione

PAI-1 Inhibitor



3-(1*H*-imidazol-5-ylmethyl)hexahydropyrrolo [1,2-*a*]pyrazine-1,4-dione

Anti hyperglycemic



3-(2-methylpropyl)hexahydropyrrolo[1,2-*a*] pyrazine-1,4-dione

Anti microbial

Figure 1: Examples of diketopiperazines and functions

Chemo-enzymatic routes to diketopiperazines containing the D-phenylglycine moiety have been described, either starting from enantiopure natural amino acids, or from the considerably cheaper racemic mixture in the case of unnatural amino acids [20,21]. In the latter case it was shown that when using a racemate, there was no interference with either the formation or isolation of the desired pure compound. In addition, expanding the scope of the procedure resulted in a green, efficient and inexpensive way of obtaining diketopiperazines containing unnatural amino acids with known anti-viral activity on a preparative scale [20,22].

The coupling of D-phenylglycine amide with the L-enantiomers of glycine, alanine, valine, leucine, isoleucine, phenylalanine, tryptophan and histidine was previously successfully performed [20]. The route using penicillin G acylase provides a green and straightforward approach to the synthesis of dipeptides comprising D-phenylglycine as well as their cyclic diketopiperazines derivatives.

The yields described fall within the range of 34-70%, which combined with room temperature, normal pressure and an aqueous system, make it a powerful tool in the synthesis of enantiopure dipeptides. In order to obtain the enantiopure diketopiperazine, the linear dipeptide undergoes esterification by treatment with thionyl chloride in methanol, followed by alkaline cyclization at room temperature, after which the formed product precipitates from solution.

To explore the full potential of the penicillin G route towards the synthesis of enantiopure diketopiperazines, we decided to screen all natural amino acids that were not yet reported (Figure 2). Here, we report the results obtained with the remaining amino acids, which comprise the interesting remainder of the polar and the charged amino acids. Proline was not tested, because secondary amines are not accepted as a nucleophile by penicillin G acylase. Bearing in mind the known broad selectivity of the enzyme towards nucleophiles and the reactivity of tryptophan and histidine in previous work, neither the volume nor the presence of a basic group in the residue was expected to constitute a hindrance for the procedure [20,23].

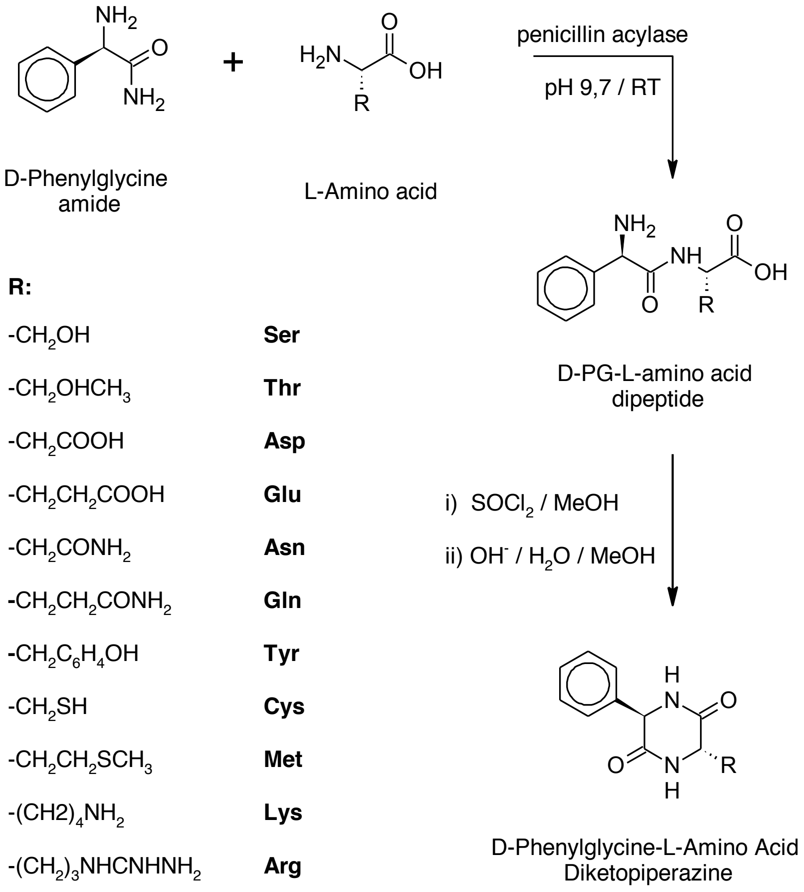


Figure 2: Proposed penicillin G acylase catalyzed peptide synthesis with natural amino acids possessing chemical functionalities

Results and Discussion

Enzymatic Coupling Reactions

The proposed penicillin G acylase coupling method was successful in the coupling of the L-amino acids serine, threonine, glutamic acid, glutamine and methionine (Table 1). The variation in isolated yields is greater than that observed with amino acids with non-functionalized side-chains [20]. This suggests that the presence of a functional group in the side chain of one of the reactants can interfere with the reaction.

Table 1: Coupling reaction isolated yields and amino acid initial concentrations used (in the successful cases).

Dipeptide	*Isolated Yield (%)*	*Initial Concentration (mM)*
D-PG-L-Ser	10	260
D-PG-L-Thr	41	300
D-PG-L-Glu	73	200
D-PG-L-Gln	33	500
D-PG-L-Met	53	300
D-PG-L-Asp	traces	-
D-PG-L-Asn	traces	-
D-PG-L-Cys	traces	-
D-PG-L-Arg	-	-
D-PG-L-Lys	-	-
D-PG-L-Tyr	-	-

We observed that an increase in the initial amino acid concentration, starting from 200 mM, led to an improvement in purity with serine, threonine, glutamine and methionine. In these cases this lead to a decreasing amount of the side product, D-phenylglycine. This was confirmed through NMR analysis of the obtained crude products. We concluded that, in these cases, hydrolysis of phenylglycine amide to phenylglycine decreases with increasing concentrations of the starting amino acid.

This was most notable in the case of the glutamine containing dipeptide, as we could only obtain pure dipeptide when performing the reaction with an initial concentration of 500 mM. On the other hand, we have also observed that in the cases of L-serine, L-threonine and L-methionine, an increase in initial concentration to 500 mM afforded an impure product. Further studies and optimization were not carried out.

The amino acids aspartic acid, asparagine and cysteine were tested without success. It was however possible to detect traces of the formed dipeptide through mass spectrometry in these three cases. This indicates the coupling reaction did occur to some extent, although not making it feasible for synthetic purposes without further studies.

In the case of cysteine, trace amounts of phenylglycine were found with mass spectrometry, together with data fitting cystine, the corresponding disulfide. However, this could not be fully confirmed by the NMR data. The NMR profile obtained matched that described but the chemical shifts did not [24].

Previous work indicated the affinity of L-aspartic acid, L-glutamic acid and L-asparagine with the enzyme to be comparable, in acyl transfer reactions where these species act as acyl acceptors [23]. However, we were able to obtain a dipeptide with both L-glutamic acid and L-glutamine but not with L-aspartic acid and L-asparagine.

In the trials with L-arginine, L-lysine and L-tyrosine, no evidence of the desired dipeptide was found, although the specificity of the enzyme to L-arginine was reported to be higher than the one of L-glutamic acid [23]. We did detect the side product D-phenylglycine, which indicates there was no enzyme inactivation.

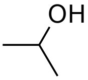
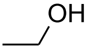
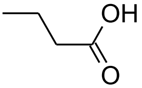
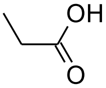
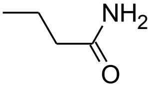
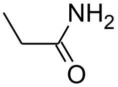
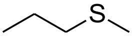
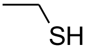
The high solubility of L-lysine and L-arginine might have made it impossible to determine if there was product formation, because no precipitation occurred at the dipeptide's isoelectric point. D-phenylglycine was obtained when lowering the pH.

The yields obtained varied considerably according to the side chain of the starting amino acid, not necessarily showing a dependency on the chemical function (Table 2). Previously, the nature of the side chain was found to influence the enzyme selectivity equally, that is, non-branched alkyl side-chains produced nucleophiles that are more effective [20,21,23].

We successfully reproduced the results obtained by *Khimiuk et al* [20] in the case of phenylalanine and tryptophan, where the residue is bulky. This suggests that the volume of the residue should not be an influential variable. We were, however, not able to convert L-tyrosine into a dipeptide. This might have been due to tyrosine's low solubility.

The data regarding the pair aspartic or glutamic acid and the pair asparagine or glutamine, suggests amino acids with two methylene groups in the residue are preferred (Table 2), whereas the equivalent amino acid with one methylene group less is not accepted, or only formed in very little amounts.

Table 2: Comparative Apparent Residue Preference

Side-chain	*Yield (%)*		*Side-chain*	*Yield (%)*
	42	>		10
	73	>		traces
	33	>		traces
	53	>		traces

The isolated yield strongly depends on the dipeptide's solubility, not necessarily reflecting the real yield, as already observed [20]. However, due to the chemical similarity between this pair, one can consider aspartic acid and glutamic acid dipeptides to have comparable solubilities and the same applies for L-glutamine and L-asparagine. This difference in results could then be exclusively attributed to the enzyme's substrate preference.

The obtained yield for L-threonine was four fold higher than the one obtained with L-serine. This also suggests the decisive factor is again not solubility but compatibility with the catalyst, as the solubility of both amino acids is very high.

The data regarding the sulfur containing amino acids L-methionine and L-cysteine, although suggestive, cannot be used to draw conclusions in this matter because both compounds differ not only in side-chain length but also in chemical functionality.

Dipeptides were obtained within a period of 2 days, either by single or sequential precipitation, with the exception of the L-serine containing dipeptide, which was obtained after 4 days. In this and in the D-phenylglycyl-L-glutamic acid cases, significant amounts of product could still be obtained by precipitation after 50 and 10 days respectively. These steps may be optimized by reducing the crystallization temperature, combined with either an earlier second isolation step or with a single isolation step, as a first isolation will necessarily translate into a larger second crystallization period. In these reactions only fresh enzyme was used. However, due to the known stability of this industrial catalyst, we think the effective re-use should be feasible [25].

Esterification and Condensation reactions

The subsequent steps in the chemo-enzymatic diketopiperazine syntheses are esterification with thionyl chloride in methanol, followed by in-situ aqueous alkaline cyclization. The resulting dipeptide was isolated by precipitation from solution. This procedure allowed the diketopiperazine to be obtained pure and was successful in the cases of L-serine, L-threonine and L-methionine (Table 3).

Table 3: Esterification and Condensation Reactions Isolated Yields

Diketopiperazine	*Isolated Yield (%)*
Cyclo (D-PG, L-Ser)	43
Cyclo (D- PG, L-Thr)	48
Cyclo (D- PG, L-Met)	35
Cyclo (D- PG, L-Glu/Gln-OMe)	68

Both L-glutamic acid and L-glutamine containing dipeptide esters, afforded the same product. This was confirmed by infrared analysis and was consistently obtained in several trials (Figure 3). This compound was obtained pure and was positively identified by NMR and mass spectrometry as the corresponding diketopiperazine methyl ester. Rather than having the acid or amide functional group, corresponding to L-glutamic acid and L-glutamine, the compound obtained had the same ester moiety in the side chain. Isolated yields varied between 66% and 69%.

With L-glutamic acid, a possible explanation for this result is that the ring closes before hydrolysis of the side chain's carboxylate ester, which then precipitates thus preventing any further reaction. An amine intra-molecular attack on the residue's ester group and not at the C terminus of the dipeptide could occur, which would yield an eight membered ring compound. However, we found only one of these two possible products (Figure 4).

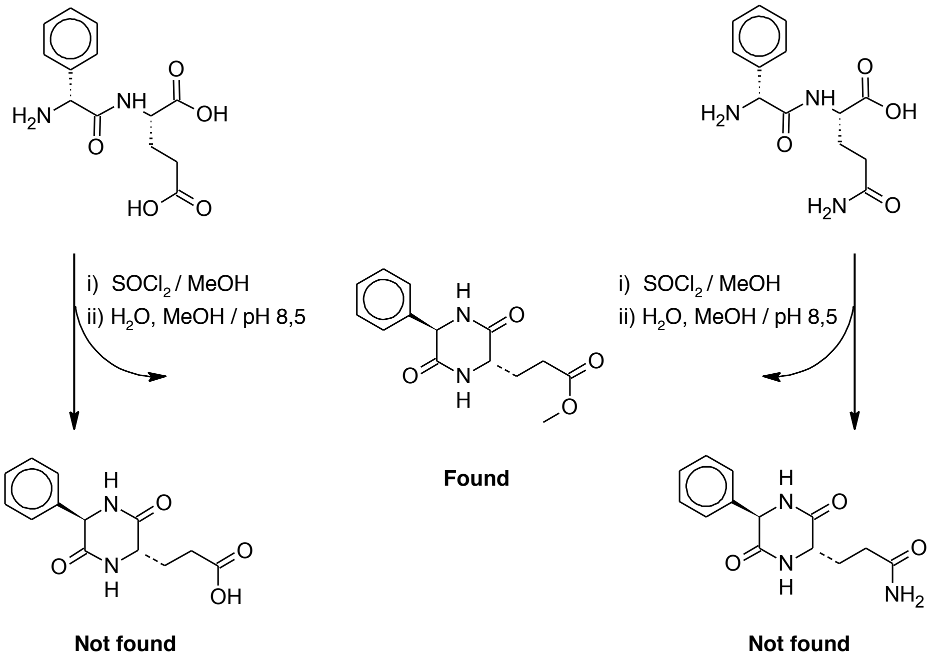


Figure 3: Same final product obtained in both cases is the corresponding diketopiperazine methyl ester and not the two expected corresponding diketopiperazines.

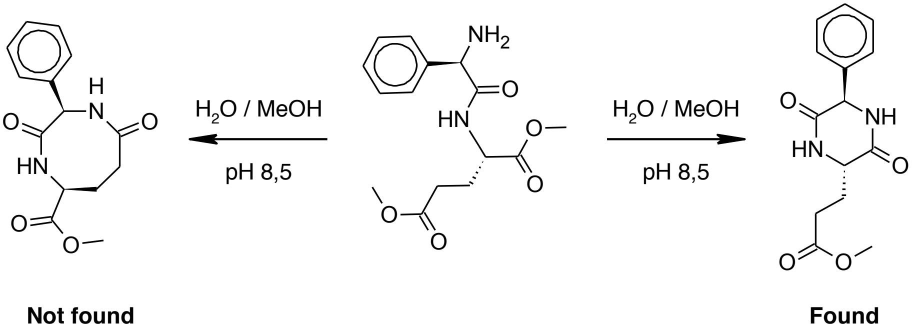


Figure 4: Amine intramolecular attack affords cyclic dipeptide methyl ester and not eight member ring product

The explanation is not so clear in the case of glutamine because one must account for the loss of an amide group. One possible explanation is that the amide group is subjected to alcoholysis during esterification (Figure 5). Although amides are stable bonds, the low pH at which esterification occurs might cause substitution of the amide group by the ester. However, the yields of diketopiperazine methyl ester found in both L-glutamic acid and L-glutamine cases are similar, although one equivalent of thionyl chloride is used over the course of the esterification of the latter and two equivalents in the former. This would presumably lead to a lower yield in the case of L-glutamine, suggesting amide alcoholysis was not the cause for our results.

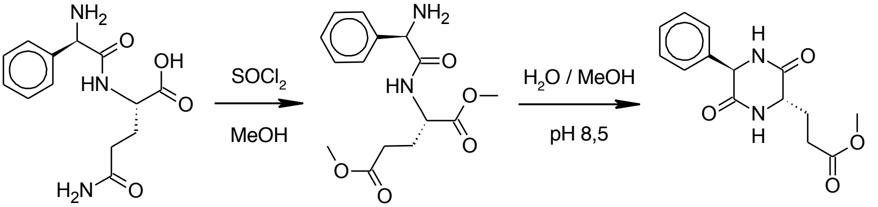


Figure 5: Putative formation of D-PG-L-Gln methyl ester via amide esterification

An alternative, perhaps more likely explanation, involves a neighboring group participation mechanism to account for the conversion of the amide into an ester moiety. In this hypothesis this transformation takes place after ring-closure and not during the esterification (Figure 6).

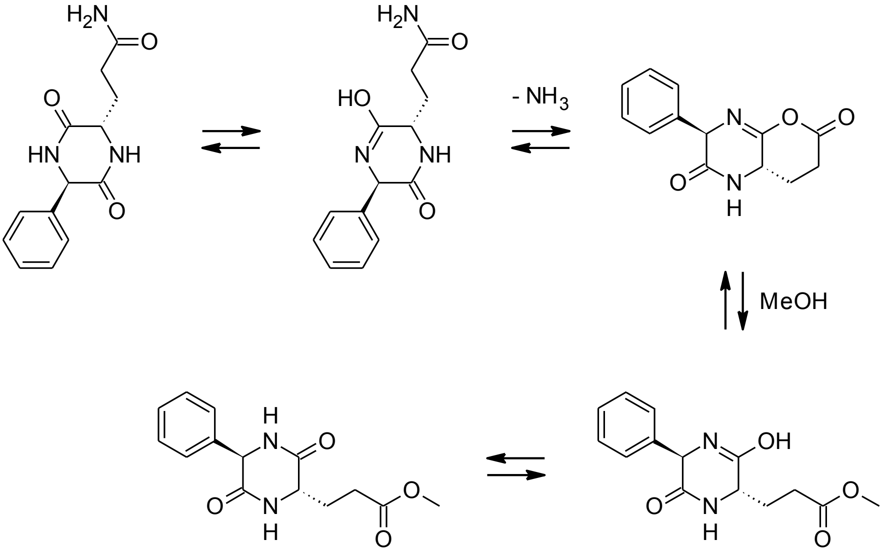


Figure 6: Proposed mechanism for the diketopiperazine ester formation with enol intramolecular attack

The hydroxyl group adjacent to the imine attacks the terminal amide, affording a six membered lactone with a loss of ammonia. Subsequent attack of methanol on the lactone carbonyl regenerates the open ring configuration, affording a methyl ester as indeed we have obtained.

Attack of a water molecule and not of a methanol molecule is unlikely, since no evidence of the formation of the diketopiperazine acid was found. In accordance with our results, and as observed in the case of the glutamic acid containing dipeptide, the ester presumably precipitates before hydrolysis of the side chain ester can take place. In addition to this, we observed that the time required for the precipitation during the ring-closure step was faster in the case of the L-glutamine containing dipeptide than in the case of the L-glutamic acid containing dipeptide, thus suggesting a different mechanism in the two cases.

Conclusions

The coupling procedure is applicable in the case of L-serine, L-threonine, L-methionine and L-glutamine, thus leading to a pure product without further purification. Data suggest there is room for optimization. In some cases it was possible to improve the product purity, simply by altering the initial concentration of amino acid.

The complete procedure is valid in the synthesis of the corresponding diketopiperazines, except in the case of L-glutamine and L-glutamic acid. In these cases, the same compound was obtained pure and it was identified as the corresponding ester of the desired products. Two mechanisms have been proposed in order to explain this result.

A green and straightforward procedure was therefore established for the synthesis of dipeptides and diketopiperazines, containing both the D-phenylglycyl and chemically functionalized amino acids moieties, which are compounds of growing interest regarding catalytic and biological activity.

Experimental Part

Materials and Methods

L-amino acids and D-phenylglycine amide were obtained from commercial suppliers. Immobilized penicillin acylase from *Escherichia coli,* Assemblase 7500 ®, was kindly donated by DSM Anti-Infectives (Delft, The Netherlands). Mass spectrometry analysis gave coherent results, M_w or $M_w +1$, according to the technique used. The melting points were measured with a Büchi 510 and are not corrected. Optical rotations were measured with either a Perkin Elmer 241

Polarimeter or a Perkin Elmer 343 Polarimeter and concentrations were converted to the standard value of *c* 1.

Synthesis of D-phenylglycyl-L-amino acid dipeptides

Synthesis of D-phenylglycyl-L-serine

At first 6.30 g (60.0 mmol) of L-serine and 2.25 g (15.0 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.70 with NH_4OH 25%, making an overall volume of 230 mL. Next 6.01 g of immobilized penicillin acylase was added at room temperature. Three further portions of D-PGA (2.25 g, 15.0 mmol) were added, with a 1.5-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was adjusted to 5.6 with H_2SO_4 (6M). The mixture was left stirring for 4 days, after which precipitation occurred. The formed solid was filtered, washed with petroleum ether 40-60, dried, and 200 mg were obtained. The mixture was kept stirring for a period of 50 days and more precipitate was formed. This was collected, following the same procedure, after which 1.17 g was obtained in the second fraction, affording a total yield of 10%. The product was isolated pure.

m.p. 224-226 °C (d). $[\alpha]_D^{20}$ = -68.9 (*c* 1, 2.5M HCl). ^{1}H-NMR (300 MHz, D_2O + DCl): δ = 7.49 - 7.51 (m, aromatic protons), δ = 5.22 (s, C*H*NH_2), δ = 4.54 (t, *J* = 4.5 Hz, C*H*CH_2OH), δ = 3.86 (dd, J_1 = 5.1 Hz, J_2 = 11.7 Hz, CHCH_2OH), δ = 3.73 (dd, J_1 = 5.1 Hz, J_2 = 11.7 Hz CHCH_2OH); ^{13}C-NMR (75 MHz, D_2O + DCl): δ = 174.5, 170.3 (*C*=O), δ = 133.3, 132.0, 131.3, 129.6 (Ar); δ = 62.26 (*C*H_2OH), δ = 58.08, 56.55 (C_α).

Synthesis of D-phenylglycyl-L-threonine

At first, 7.16 g (60.4 mmol) of L-threonine and 2.25 g (15.0 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.80 with NH_4OH 25%, making an overall volume of 200 mL. 6.27 g of immobilized penicillin acylase were added at room temperature. Three further portions of D-PGA (2.25 g, 15 mmol) were added, with a 1.5-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was then lowered to 6.4 with H_2SO_4 6M. The mixture was left stirring overnight, after which precipitation occurred. The solid was filtered, washed with petroleum ether 40-60 and dried. The yield was 41% (6.25 g) and the product was isolated NMR pure.

m.p. 250-253 °C (d). $[\alpha]_D^{24}$ = -27.8 (*c* 1, 2.5M HCl). ^1H-NMR (300 MHz, D_2O + DCl): δ = 7.5 (m, aromatic protons), δ = 5.26 (s, C*H*NH_2), δ = 4.5 (s, CON*H*), δ = 4.3 (m, C*H*$OHCH_3$), δ = 0.85 (d, *J* = 6.3 Hz, CHOHC*H_3*); ^{13}C-NMR (75 MHz, D_2O + DCl): δ = 174.8, 170.7 (*C*=O), δ = 133.5, 132.0, 131.2, 129.5 (Ar); δ = 68.51 (*C*HOH), δ = 59.57, 58.10 (C_α), δ = 20.05 (CHOH*C*H_3).

Synthesis of D-phenylglycyl-L-glutamic acid

At first, 14.7 g (100 mmol) of L-glutamic acid and 5.52 g (36.2 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.7, making an overall volume of 500 mL. To this, 12.2 g of immobilized penicillin acylase were added at room temperature. Two further portions of D-PGA (4.74 g, 31.6 mmol) were added, with a 2-hour interval each, at which the pH was adjusted to 9.9 with the NH_4OH. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was then lowered to 3.3 with approximately 30 mL H_2SO_4 6M. The mixture was left stirring for a period of two days, after which the formed precipitate was filtered, washed with petroleum ether 40-60, dried and 13.2 g was obtained. The mixture was kept stirring for an extra period of 10 days. More precipitate was formed and collected, following the same procedure, affording 7.28 g, which corresponds to an overall yield of 73%. The product was isolated NMR pure.

m.p. 217-219 °C. $[\alpha]_D^{24}$ = -87.5 (*c* 1, 2.5M HCl). ^{1}H-NMR (300 MHz, D_2O + DCl): δ = 7.49 (m, aromatic protons), δ = 5.16 (s, C*H*NH_2), δ = 4.68 (s, CON*H*), δ = 4.49 (dd, J_1 = 3.9 Hz, J_2 = 9.6 Hz C*H*CH_2CH_2COOH), δ = 2.1 - 1.8 (m, CHCH_2CH_2COOH); ^{13}C-NMR (75 MHz, D_2O + DCl): δ = 178.1, 176.0 , 170.3 (*C*=O), δ = 133.4, 132.0, 131.3, 129.5 (Ar); δ = 58.14, 53.44 (C_α), δ = 30.99, 26.91 (CHCH_2CH_2COOH).

Synthesis of D-phenylglycyl-L-glutamine

At first, 15.1 g (104 mmol) of L-glutamine and 5.21 g (34.7 mmol) of D-PGA was suspended in 200 mL of water and the pH was adjusted to 9.7 with NH_4OH 25%. To this, 4.12 g of immobilized penicillin G acylase were added at room temperature. Two further portions of D-PGA (5.20 g, 34.7 mmol) were added, with a 2-hour interval each, at which the pH was readjusted to 9.7 with the same base. After 6 hours of reaction time the enzyme was filtered and the pH was then lowered to 5 with H_2SO_4 (6M); precipitation was immediate. The mixture was left stirring overnight, after which the precipitate was filtered, washed with petroleum ether 40-60, dried and 8.59g (29.7%) were obtained. A second precipitate fraction was filtered from the mother liquor the day after and a third fraction 2 days after, affording 840 mg and 1.68 g, respectively. The first fraction was NMR pure and the second with minor impurities.

m.p. 238-240 °C. $[\alpha]_D^{24}$ = -89.5 (*c* 1, 2.5M HCl). ^{1}H-NMR (300 MHz, D_2O + DCl): δ = 7.48 (m, aromatic protons), δ = 5.16 (s, C*H*NH_2), = 4.71 (s, CON*H*), δ = 4.42 (dd, J_1 = 4.5 Hz, J_2 = 9.3 Hz, C*H*$CH_2CH_2CONH_2$), δ = 2.8 – 2.1 (m, CHCH_2CH_2COOH);

^{13}C-NMR (75 MHz, D_2O + DCl): δ = 177.6, 174.5 , 168.9 (*C*=O), δ = 132.0, 130.7, 129.9, 128.2 (Ar), δ = 56.74, 52.47 (C_α), δ = 30.89, 26.1 (CH*C*H_2*C*H_2CONH_2).

Synthesis of D-phenylglycyl-L-methionine

At first, 8.98 g (60.2 mmol) of L-methionine and 2.25 g (15 mmol) of D-PGA was suspended in water and the pH was adjusted to 9.80 with of NH_4OH 25%, making an overall volume of 200 mL. To this, 6.05 g of immobilized penicillin G acylase was added at room temperature. Three further portions of D-PGA (2.25 g, 15 mmol) were added, with a 1.5-hour interval each, at which point the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was then lowered to 5.8 with H_2SO_4 (6M). The mixture was left stirring overnight, after which precipitation occurred and the formed solid was filtered, washed with petroleum ether 40-60 and dried under vacuum and P_2O_5 for a period of 9 days, affording 9,05 g of product corresponding to a yield of 53%. The product was isolated NMR pure.

m.p. 235-237 °C. $[\alpha]_D^{24}$ = -97.9 (*c* 1, 2.5M HCl). ^{1}H-NMR (300 MHz, D_2O + DCl): δ = 7.5 (m, aromatic protons), δ = 5.16 (s, C*H*NH_2), δ = 4.59 (dd, J_1 = 3.9 Hz, J_2 = 10.2 Hz, (C*H*CH_2CH_2SCH_3), δ = 2.2 - 2.02 (m, CHCH_2CH_2SCH_3), δ = 1.86 (s, CHCH_2CH_2SCH_3); ^{13}C-NMR (75 MHz, D_2O + DCl): δ = 176.4, 170.3 (*C*=O), δ = 133.5, 132.0, 131.3, 129.5 (Ar), δ = 58.14, 52.86 (C_α), δ = 30.97, 30.71 (CH*C*H_2*C*H_2SCH_3), δ = 15.48 (CHCH_2CH_2S*C*H_3).

Synthesis of D-phenylglycyl-L-serine diketopiperazine

At first, 1.00 g (4.20 mmol) of D-phenylglycyl-L-serine was suspended in 44 mL of dry methanol. A white suspension was obtained which was cooled. To this suspension 370 µL of $SOCl_2$ (5.09 mmol, 1.21 equivalent) were added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 168 mL of water were added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40-60 and dried. The product was isolated and 400 mg was obtained, which corresponds to a yield of 43%.

m.p. 263-265 °C. $[\alpha]_D^{24}$ = -59,9 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.45 (s, N*H*), δ = 8.0 (s, N*H*), δ = 7.35 (m, aromatic protons), δ = 5.08 (t, *J* = 4,9 Hz, C*H*CH$_2$OH), δ = 4.9 (d, *J* = 1.2 Hz, C*H*Ph), δ = 3.9 (s, CHCH$_2$O*H*), δ = 3.8 – 3.6 (m, CHC*H*$_2$OH); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 167.6, 167.5 (*C*=O), δ = 140.0, 129.0, 128.5, 128.2 (Ar), δ = 63.27 (*C*H$_2$OH), δ = 59.45 (*C*HPh), δ = 57.62 (*C*HCH$_2$OH).

Synthesis of D-phenylglycyl-L-threonine diketopiperazine

At first, 1.00 g (3.97 mmol) of D-phenylglycyl-L-threonine was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled. To this suspension 350 µL of $SOCl_2$ (4.82 mmol, 1.2 equivalent) was added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 40 mL of water were added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40-60 and dried. The product was isolated and 450 mg was obtained, which corresponds to a yield of 43%.

m.p. 274-276 °C. $[\alpha]_D^{24}$ = -71.4 (*c* 1, DMSO). ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 8.39 (s, N*H*), δ = 8.13 (d, *J* = 1.6 Hz, N*H*), δ = 7.3 (m, aromatic protons), δ = 5.1 (d, *J* = 5.6 Hz, C*H*CHOHCH$_3$), δ = 4.94 (s, C*H*Ph), δ = 4.12 (m, C*H*OHCH$_3$), δ = 3.64 (s, CHCH$_2$O*H*), δ = 1.14 (d, *J* = 6.4 Hz, CHOHC*H*$_3$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 167.8, 167.7 (*C*=O), δ = 138.9, 128.0, 127.9, 127.6 (Ar), δ = 67.95 (*C*HOH), δ = 60.56 (*C*HPh), δ = 58.28 (*C*HCHOHCH$_3$), δ = 19.79 (CHCH$_2$OH*C*H$_3$).

Synthesis of D-phenylglycyl-L-methionine diketopiperazine

At first, 1.04 g (3.69 mmol) of D-phenylglycyl-L-methionine was suspended in 15 mL of dry methanol. A white suspension was obtained which was cooled. To this suspension 325 µL of $SOCl_2$ (4.48 mmol, 1.2 equivalent) were added dropwise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 30 mL of water were added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 10 days, the formed precipitate was filtered. This was washed with petroleum ether 40-60 and dried. The product was isolated and 590 mg was obtained, which corresponds to a yield of 35%.

m.p. 253-255°C. $[\alpha]_D^{24}$ = -47.6 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.62 (d, *J* = 2.7 Hz, N*H*), δ = 8.29 (s, N*H*), δ = 7.35 (m, aromatic protons), δ = 4.93 (d, *J* = 2.4 Hz, C*H*Ph), δ = 4.08 (t, *J* = 5.0 Hz $C\mathit{H}CH_2CH_2SCH_3$), δ = 3.32 – 2.0 (m, $C\mathit{H_2}C\mathit{H_2}SCH_3$); δ = 2.05 (s, $CH_2CH_2SC\mathit{H_3}$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 168.3, 167.4 (*C*=O), δ = 139.4, 129.2, 128.6, 127.7 (Ar), δ = 59.40 (*C*HPh), δ = 53.32 ($\mathit{C}HCH_2CH_2SCH_3$), δ = 32.21 ($CH\mathit{C}H_2CH_2SCH_3$), δ = 29.14 ($CHCH_2\mathit{C}H_2SCH_3$), δ = 15.21 ($CHCH_2CH_2S\mathit{C}H_3$).

Attempted synthesis of D-phenylglycyl-L-glutamic acid diketopiperazine

At first, 1.05 g (3.75 mmol) of D-phenylglycyl-L-glutamic acid was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled to 0-5 °C. To this suspension 570 µL of $SOCl_2$ (7.86 mmol, 2.1equivalent) was added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 40 mL of water were added. The pH was adjusted to 8.7 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40-60 and dried. The product was isolated and 680 mg was obtained, which corresponds to a yield of 66 %. The product was identified as the corresponding methyl ester.

m.p. 236-238°C. $[\alpha]_D^{24}$ = -43.8 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.62 (d, *J* = 2.4 Hz, N*H*), δ = 8.24 (s, N*H*), δ = 7.35 (m, aromatic protons), δ = 4.92 (d, *J* = 2.7 Hz, C*H*Ph), δ = 4.05 (m, C*H*$CH_2CH_2CO_2CH_3$), δ = 3.6 (s, $CH_2CH_2CO_2CH_3$), δ = 2.5 – 2.0 (m, $CH_2CH_2CO_2CH_3$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 173.6, 168.2, 167.5 (*C*=O), δ = 139.4, 129.2, 128.6, 127.7 (Ar), δ = 59.40 ($CHCO_2CH_3$), δ = 53.3 (*C*HPh), δ = 52.1 ($CHCH_2CH_2CO_2CH_3$), δ = 29.5 ($CHCH_2CH_2CO_2CH_3$), δ = 27.9 ($CHCH_2CH_2CO_2CH_3$).

Attempted synthesis of D-phenylglycyl-L-glutamine diketopiperazine

At first, 1.02 g (3.66 mmol) of D-phenylglycyl-L-glutamine was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled to 0-5 °C. To this suspension 320 µL of $SOCl_2$ (4.41 mmol, 1.2 equivalent) were added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 40 mL of water were added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40-60 and dried. The product was isolated and 700 mg was obtained, which corresponds to a yield of 69%. This was identified as the same methyl ester that was obtained from the phenlyl glycine glutamic acid dipeptide (see above).

m.p. 236-238°C. $[\alpha]_D^{24}$ = -44,9 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.65 (d, *J* = 2.7 Hz, N*H*), δ = 8.27 (s, N*H*), δ = 7.4 - 7,3 (m, aromatic protons), δ = 4.94 (d, *J* = 2.7 Hz, C*H*Ph), δ = 4.07 (m, C*H*$CH_2CH_2CO_2CH_3$), δ = 3.60 (s, $CH_2CH_2CO_2CH_3$), δ = 2.5 – 2.03 (m, $CH_2CH_2CO_2CH_3$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 173.6, 168.2, 167.5 (*C*=O), δ = 139.3, 129.2, 128.6, 127.7 (Ar), δ = 59.4

(CO_2CH_3), δ = 53.3 ($CHPh$), δ = 52.1 ($CHCH_2CH_2CO_2CH_3$), δ = 32.21 ($CHCH_2CH_2CO_2CH_3$), δ = 29.5 ($CHCH_2CH_2CO_2CH_3$), δ = 27.9 ($CHCH_2CH_2 CO_2CH_3$).

References

---

1 Ressurreição, A:S:M.; Delatouche, R.; Gennare, C.; Piarulli, U.; *European Journal of Organic Chemistry* **2011**; 217 - 228

2 Fischer, P.M.; *Journal of Peptide Science* **2003**; 9, 9 - 35

3 Avendaño, C.; de la Cuesta, E.; *Current Organic Synthesis* **2009**; 6, 143 - 168

4 Borthwick, A:D:; Liddle, J.; *Medicinal Research Reviews* **2011**; 31, 576 - 604

5 McCleland, K.; Milne, P.J.; Lucieto, F.R.; Frost, C.; Brauns, S.C.; Van De Venter, M.; Du Plessis, J.; Dyason, K.; *Journal of Pharmacy and Pharmacology* **2004**; 56, 1143 - 1153

6 Song, M.K.; Hwang, I.K.; Rosenthal, M.J.; Harris, D.M.; Yamaguchi, D.T.; Yip, I.; Go, V.L.W.; *Experimental Biology and Medicine* **2003**; 228, 1338 - 1345

7 Martins, M.B.; Carvalho, I.; *Tetrahedron* **2007**; 63, 9923 - 9938

8 Carrea, G.; Colonna, S.; Kelly, D.R.; Lazcano, A.; Ottolina, G.; Roberts, S.M.; *Trends in Biotechnology* **2005**; 23, 507 - 513

9 Kowalski, J. ; Lipton, M.A. ; *Tetrahedron Letters* **1996**; 37, 33, 5839 - 5840

10 Jackson, W.R.; Jayatilake, G.S.; Matthews, B.R.; Wilshire, C.; *Australian Journal of Chemistry* **1988**; 41, 2, 203 - 213.

11 Noe, C.R.; Weigand, A.; Pirker, A.; *Monatshefte für Chemie* **127** (1996), 1081-1097.

12 Jarvo, E.R.; Miller, S.J.M.; *Tetrahedron* **2002**; 58, 2481 - 2495

13 List, B.; *Tetrahedron* **2002**; 58, 5573 - 5590

14 Wennemers, H.; *Chemical Communications* **2011**; 47, 12036 - 12041

15 Huang, Z-Z.; Leman, L.J.; Ghadiri, M.R.; *Angewandte Chemie International Edition* **2008**; 1758 - 1761

16 Pichowicz, M.; Simpkins, N.S.; Blake, A. J.; Wilson, C.; *Tetrahedron* **2008**; 64, 3713 - 3755

17 Datta, S.; Sood, A.; Török, M; *Current Organic Synthesis* **2011**; 8, 262 - 280

---

18 Kallenberg, A.I.; van Rantwijk, F.; Sheldon, R.A.; *Advanced Synthesis & Catalysis* **2005**; 347, 905-926

19 van Langen, L.M.; van Rantwijk, F.; Švedas, V.K.; Sheldon, R.A.; *Tetrahedron: Asymmetry* **2000**; 11, 1077 – 1083

20 Khimiuk, A.Y.; Korennykh, A.V.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A.; Svedas, V.K.; *Tetrahedron: Asymmetry* **2003**; 14, 3123 - 3128

21 Pereira, P.C.; Sheldon, R.A.; Arends, I.W.C.E.; *Tetrahedron Letters* **2014**; 55, 4991 - 4993

22 Vanžura, J.; Palát, K.; Čeladník, M.; Hradec, K ; Klimes, J.; František, S.; *CS 210383* **1983**

23 Didžiapetris, R.J.; Švedas, V.K.; *Biomedica Biochimica Acta* **50** (1991), 10-11, S237-S242.

24 Pouchert, C. J.; Behnke, J.; *The Aldrich Library of 13C and 1H FT NMR Spectra* **1**; Aldrich Chemical Co, 1993; pp 894

25 Schroën, C.G.P.H.;Mohy Eldin, M.S.; Janssen, A.E.M.; Mita, G.D.; Tramper, J.; *Journal of Molecular Catalysis B: Enzymatic* **2001**; 15, 163 - 172

A Green and Expedient Synthesis of Enantiopure Diketopiperazines via Enzymatic Resolution of Unnatural Amino Acids

3

The contents of this chapter have been published in Pereira, P.C.; Arends, I.W.C.E.; Sheldon, R.A.; *Tet Lett* **2014**; 55, 4991 - 4993

Chapter 3

Introduction

Piperazine-2,5-diones represent a rich source of biologically interesting compounds [1]. The 2,5 diketopiperazine motif has been the subject of significant attention due to its biological properties [2, 3]. These include alteration of blood clotting functions [2], antitumor [4], antiviral including HIV-I antagonists [5,6,7], antifungal [8], antibacterial [9], antihyperglaecimic [10] and analgesic [11], amongst others [12]. Diketopiperazines are now seen as ideal compounds for the rational design of new therapeutic agents [13,14].

The applicability of these cyclic dipeptides extends beyond this, as they are also known for possessing catalytic activity in the hydrocyanation of aldehydes [15] and in the Strecker synthesis of amino acids [16,17]. In the former case, it was shown that several diketopiperazines have enantioselective catalytic activity [18], with enantiomeric excesses reaching 90%. In addition to this there is great potential displayed by simple peptides in asymmetric catalysis [19], which has even been considered of prebiotic relevance [20] The versatile scope of these compounds, together with the recent growth in research developed around them, suggests their full potential is yet to be explored [21].

Most biologically active diketopiperazines have been obtained from natural sources [12]. One representative example is the case of the (*R*,*R*)-diketopiperazines obtained from marine bacteria. These show inhibitory activity against *V. anguillarum*, a pathogenic bacterium responsible for Vibriosis, suffered by wild and farmed bivalves, crustaceans and fishes, which in turn gives rise to severe economic losses, as it is a limitation in the development of aquaculture [9].

Diketopiperazines can also be synthesized. Although they have frequently been deemed as unwanted side products, research regarding cyclic dipeptides has actually contributed to peptide synthesis in general [22]. This goal could be achieved by solid-phase strategies or solution-phase syntheses. Representative examples of solution-phase syntheses are thermal condensation of a dipeptide ester [23], coupling of protected amino acids followed by deprotection and condensation [24,25], a multi component approach (Ugi reaction) [26] and an innovative microwave heated procedure has also been reported [27]. Solid-phase strategies are more widely used and have been frequently used in building libraries of these compounds [12].

Biocatalysis is today a widely accepted method for the production of chemicals in general and enantiopure compounds in particular [28,29,30,31]. Enzyme catalyzed transformations proceed under mild conditions of temperature and pressure at physiological pH in water as solvent and are often highly chemo-, regio-, and enantioselective in addition to being atom and step economic [32]. An illustrative example is provided by the industrial synthesis of the artificial sweetener Aspartame, a dipeptide, catalyzed by the metalloprotease, thermolysin [33].

We have previously described a chemo-enzymatic route for the synthesis of enantiopure diketopiperazines [34]. The key enzymatic step involved coupling of two enantiopure species, namely D-phenylglycine amide and an L-amino acid. This step was catalyzed by an immobilized form (Assemblase 7500 ®) of penicillin G acylase (penicillin amidohydrolase EC 3.5.1.11) from *Escherichia coli,* which is used in the manufacture of ß-lactam antibiotics [35,36]. The resulting dipeptide was subsequently esterified followed by reaction with sodium hydroxide in methanol, to afford the corresponding diketopiperazine (Figure 1**).** We have recently broadened the scope of this procedure by testing eleven new enantiopure amino acids, all with chemically functionalized side-chains [37].

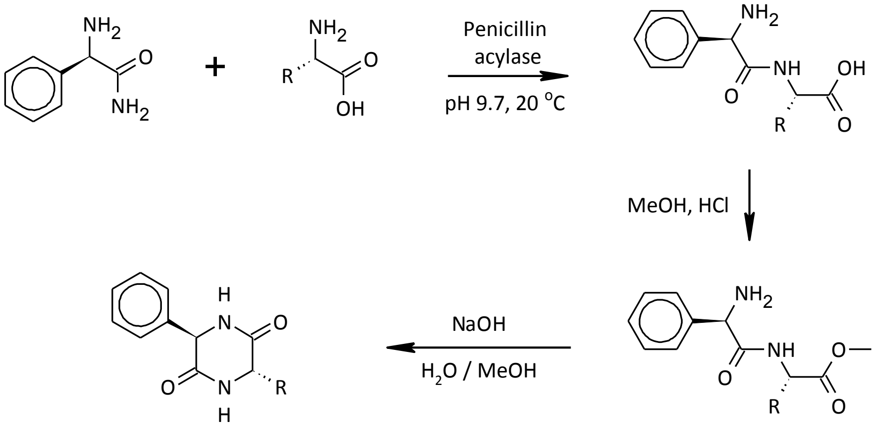


Figure 1: Penicillin acylase catalyzed peptide synthesis with enantiopure natural amino acids

This work describes a further expansion of the scope and synthetic utility of this concise and practical methodology but uses a different approach. This consists of starting with a racemic mixture rather than a pure enantiomer, thereby integrating a kinetic resolution into the procedure. To this end, we have proceeded by ascertaining the viability of this route with substrates that had not been previously tested in this route and could afford interesting products, for example, new compounds or compounds with specific biological activity [5].

Here we report the results of these studies, in which we tested the racemates of unnatural amino acids as one of the reactants. In addition to fulfilling the above mentioned pre-requisites, these racemic mixtures are significantly less expensive than the corresponding pure enantiomer (Figure 2).

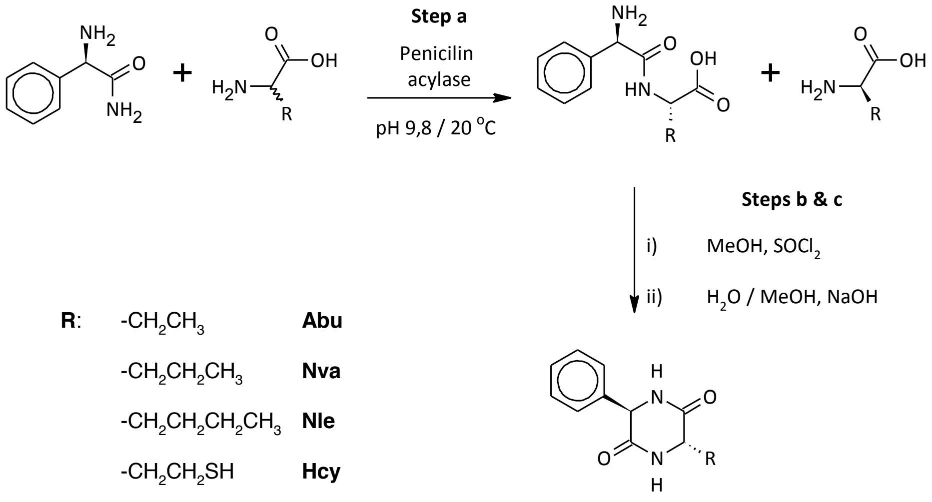


Figure 2: Proposed penicillin G acylase catalyzed peptide synthesis with racemic unnatural amino acids

Results and Discussion

The enzymatic coupling reaction was performed at pH 9.8, in order to maximize the concentration of reactive amine nucleophile. The acyl donor was added in portions, in order to prevent D-phenylglycine amide induced inactivation. This is known to occur at high concentrations and high pH [38]. Only one coupling product was obtained in all coupling reactions, consistent with the known selectivity of the enzyme catalyst and demonstrating the feasibility of the starting hypothesis (Table 1) [39].

The dipeptide derived from D-phenylglycine amide and L-norvaline was obtained in pure form according to NMR spectroscopy, with no need for any further purification. The other three linear dipeptides were obtained with minor impurities, though they were easily purified. NMR analysis showed 10-20% impurity for D-phenylglycyl-L-norleucine, 10-11% for D-phenylglycyl-L-homocysteine and not quantifiable for the case of D-phenylglycyl-L-amino-butyric acid. The impurities were identified by NMR as unreacted amino acid in the first three cases and mass spectra indicated traces of phenylglycine in the last example. Nevertheless, this was not reflected in the melting points. The isolated yields obtained (Table 1) fit within the window already observed previously [34].

Table 1: Isolated yields of step a and steps b & c (see Figure 2) [a]

Dipeptide	Residue	Linear Dipeptide	Diketopiperazine
D-PG-L-Abu	$-CH_2CH_3$	36%	62%
D-PG-L-Nva	$-CH_2CH_2CH_3$	46%	64%
D-PG-L-Nle	$-CH_2CH_2CH_2CH_3$	52%	63%
D-PG-L-Hcy	$-CH_2CH_2SH$	32%	not determined

[a]: *Step a* isolated yield was calculated as η=100x (product/substrate) x2; *Steps b&c* isolated yield was calculated as η=100x (product/substrate)

Although the trend in our experiments suggests that nucleophiles with more carbon atoms seem to be preferred, the isolated yields depend strongly on solubility. It was previously found that the yield after crystallization is frequently lower than the observed HPLC yield and that this varies with the amino acid residue [34,35].

These reactions were carried out on a relatively small scale and were not optimized, suggesting there is ample room for further improvement in yields and product purity. The thiol function showed no hindering effect, as already observed with other chemical functionalities [34,35].

Both steps *b* and *c* (Figure 2) afforded the desired compounds, as was shown previously with similar compounds [34,35,39]. The methyl ester intermediates were not isolated and were used directly in the condensation step, without any further purification or characterization. The final product precipitated from solution. All the expected diketopiperazines were obtained and three of the proposed four were obtained in pure form according to ^{1}H-NMR spectroscopy. The diketopiperazine containing the L-homocysteine residue could not be dissolved for an NMR analysis but was positively identified through mass spectrometry.

Conclusions

We have developed a green, cost-effective and chemo-enzymatic route for the synthesis of enantiopure 2,5-diketopiparazines containing unnatural amino acids, starting from the corresponding racemic amino acids. This was demonstrated in the synthesis of four dipeptides which were subsequently converted into the corresponding 2,5-diketopiparazines, all eight targeted compounds, some of which have anti-viral activity [5], were isolated and/or identified. This suggests that the approach may be applicable to other cases where the route is already feasible with enantiopure amino acids.

Of the four coupling products, one was obtained pure and three were obtained with minor impurities. Three of four possible diketopiperazines were obtained in pure state.

The presence of the D-enantiomer of the nucleophilic species did not seem to hinder the formation of the desired enantiopure linear dipeptide, allowing for a synthetic strategy involving a kinetic resolution. The dipeptide D-PG-L-Abu and its diketopiperazine were not previously known compounds.

Experimental Part

Materials and Methods

L-Amino acids and D-phenylglycine amide were obtained from commercial suppliers. Immobilized penicillin acylase from *E. coli*, Assemblase 7500 ®, was kindly donated by DSM Anti-Infectives (Delft, The Netherlands). Mass spectrometry analysis gave coherent results, M_w or M_w *+1*, according to the technique used. The melting points were measured with a Büchi 510 and were not corrected. Optical rotations were measured with either a Perkin Elmer 241 Polarimeter or a Perkin Elmer 343 Polarimeter. The dipeptides derived from D-phenylglycine amide and L-amino-butyric acid, L-norleucine and L-homocysteine were recrystallized from water, in order to measure the optical rotations and elemental analysis. Concentrations of all compounds used for the measuring of optical rotations varied between 1.68 and 125 mg/L and were converted to the standard value of *c* 1.

Synthesis of D-phenylglycyl-L-amino butyric acid

4.12 g (40 mmol) of D,L-amino-butyric acid and 1.16 g (7.7 mmol) of D-phenylglycine amide were suspended in 90 mL of water. The pH was adjusted to 9.80 with approximately 10 mL of NH_4OH 25% and 2.8 g of immobilized penicillin acylase were added at room temperature. Two further portions of D-phenylglycine amide (0.93 g, 6.2 mmol) were added, with a 2-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a clear solution was obtained. The pH was then lowered to 6.0 with 6M H_2SO_4. The mixture was left stirring for a period of 3 days during which time precipitation occurred. The solid was filtered, washed with petroleum ether 40-60 and dried to afford 1.3 g (36% yield) of product . The product was recrystallized in water (by adjusting the pH change), for analytical purposes.

m.p. 243°C (dec). $[\alpha]_D^{24}$ = -120 (*c* 1, 2.5M HCl). ^{1}H-NMR (300 MHz, D_2O + DCl): δ = 7.5 (m, aromatic protons); δ = 5.21 (s, $\mathrm{C}\mathit{H}\mathrm{NH_2}$); δ = 4.29 (dd, *J* = 5.10 Hz, $\mathrm{C}\mathit{H}\mathrm{CH_2CH_3}$); δ = 1.78-1.58 (m, $\mathrm{CHC}\mathit{H_2}\mathrm{CH_3}$); δ = 0.65 (t, $\mathrm{CHCH_2C}\mathit{H_3}$); ^{13}C-NMR (75 MHz, D_2O + DCl): δ = 176.8, 170.2 (*C*=O); δ= 133.4, 131.9, 131.3, 129.6 (Ar), δ = 58.0, 56.0 (C_α); δ = 25.5 ($\mathit{C}\mathrm{H_2CH_3}$), δ= 10.89 ($\mathrm{CH_2}\mathit{C}\mathrm{H_3}$). Anal calcd for $C_{12}H_{16}N_2O_3$: 11.85 N; 60.94, C; 6.77, H. Found: 11.32, N; 60.3, C; 6.62 H.

Synthesis of D-phenylglycyl-L-norvaline

4.71 g (40 mmol) of D,L-norvaline and 1.16 g (7.7 mmol) of D-phenylglycine amide were suspended in 94 mL of water. The pH was adjusted to 9.80 with approximately 6 mL of NH_4OH (25) % and 3.1 g of immobilized penicillin acylase were added at room temperature. Two further portions of D-phenylglycine amide (0.93 g, 6.2 mmol) were added, with a 2-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a slightly turbid solution was obtained. The pH was then lowered to 6.0 with 6M H_2SO_4 . The mixture was left stirring overnight and the formed solid was filtered, washed with petroleum ether 40-60 and dried to afford 2.3 g (46%yield) of D-phenylglycyl-L-norvaline.

m.p. 253°C (dec). $[\alpha]_D^{24}$ = -82.4 (*c* 1, 2.5M HCl) ^{1}H NMR (300 MHz, D_2O + DCl): δ = 7.50 (m, aromatic protons), δ = 5.17 (s, $\mathrm{C}\mathit{H}\mathrm{NH_2}$), δ = 4.36 (dd, *J* = 5.10 Hz, $\mathrm{C}\mathit{H}\mathrm{CH_2CH_2CH_3}$), δ = 1.7 (m, $\mathrm{CHC}\mathit{H_2}\mathrm{CH_2CH_3}$), δ = 0.96 (m, $\mathrm{CHCH_2C}\mathit{H_2}\mathrm{CH_3}$), δ = 0.67 (t, $\mathrm{CHCH_2CH_2C}\mathit{H_3}$); ^{13}C NMR (75 MHz, D_2O + DCl): δ = 177.1, 170.2 (*C*=O), δ = 133.4, 132.0, 131.2, 129.5 (Ar), δ= 58.1, 54.2 (C_α), δ = 33.8 ($\mathrm{CH}\mathit{C}\mathrm{H_2\ CH_2\ CH_3}$), δ = 19.7 ($\mathrm{CHCH_2}\mathit{C}\mathrm{H_2\ CH_3}$), δ= 13.9 ($\mathrm{CHCH_2\ CH_2}\ \mathit{C}\mathrm{H_3}$). Anal calcd for $C_{13}H_{18}N_2O_3$: 11.19 N; 62.33, C; 7.19, H. Found: 11.65, N; 58.60, C; 7.27, H.

Synthesis of D-phenylglycyl-L-norleucine

5.24 g (40 mmol) of D,L-norleucine and 1.0 g (7.7 mmol) of D-phenylglycine amide were suspended in 100 mL of water, the pH was adjusted to 9.80 with 25% NH_4OH and 3.1 g of immobilized penicillin acylase were added at room temperature. Two further portions of D-Phenylglycine amide (0.95 g, 6.3 mmol) were added, with a 2-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a slightly turbid solution was obtained. The pH was then lowered to 6.0 with 6M H_2SO_4 . The mixture was left stirring overnight and the formed solid was filtered, washed with petroleum ether 40-60 and dried to afford 2.8 g (52% yield) of D-phenylglycyl-L-norleucine. The product was further recrystalized from water, by pH change, for analytic purposes.

m.p. 258°C (dec). $[\alpha]_D^{20}$ = -79.7 (*c* 1, 2.5M HCl). ^{1}H-NMR (300 MHz, D_2O + DCl): δ = 7.50 (m, aromatic protons); δ = 5.15 (s, $\mathrm{C}\mathit{H}\mathrm{NH2}$), δ = 4.38 (dd, *J* = 4.20 Hz, $\mathrm{C}\mathit{H}\mathrm{CH_2CH_2CH_2CH_3}$), δ = 1.78 - 1.57 (m, $\mathrm{CHC}\mathit{H_2}\mathrm{CH_2CH_2CH_3}$), δ = 1.07 – 0.96 (m, $\mathrm{CHCH_2C}\mathit{H_4}\mathrm{CH_3}$), δ = 0.65 (t, $\mathrm{CHCH_6CH_3}$) ; ^{13}C NMR (75 MHz, D_2O + DCl): δ= 177.2, 170.2 (C=O), δ = 133.5, 132.0, 131.2, 129.5 (Ar), δ= 58.1, 54.3 (C_α), δ= 31.6 ($\mathrm{CH}\mathit{C}\mathrm{H_2CH_2CH_2CH_3}$), δ = 28.5 ($\mathrm{CHCH_2}\mathit{C}\mathrm{H_2CH_2CH_3}$), δ = 22.7 ($\mathrm{CHCH_2CH_2}\mathit{C}\mathrm{H_2CH_3}$), δ = 14.5 ($\mathrm{CHCH_2CH_2CH_2}\mathit{C}\mathrm{H_3}$). Anal calcd for $C_{14}H_{20}N_2O_3$: 10.59 N; 63.56, C; 7.57, H. Found: 10.65, N; 60.62, C; 7.60, H.

Synthesis of D-Phenylglycyl-L-homocysteine

1.02 g (7.4 mmol) of D,L-homocysteine and 190 mg (1.4 mmol) of D-Phenylglycine amide were suspended in 20 mL of water, the pH was adjusted to 9.80 with NH_4OH 25% and 550 mg of immobilized penicillin acylase were added at room temperature. Two further portions of D-phenylglycine amide (190 mg, 1.6 mmol) were added, with a 2-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and the pH was then lowered to 5.4 with 6M H_2SO_4. The mixture was left stirring overnight and the formed solid was filtered, washed with petroleum ether 40-60 and dried to afford 0.32 g (32% yield) of D-phenylglycyl-L-homocysteine

m.p. 228-230°C (dec). $[\alpha]_D^{24}$ = -144 (*c* 1, 2.5M HCl) δ = 7.50 (m, aromatic protons); δ = 5.18 (s, C*H*NH2), δ = 4.62 (dd, C*H*CH_2CH_2SH), δ = 2.02 (m, CHCH_4SH); ^{13}C NMR (75 MHz, D_2O + DCl): δ= 176.4, 170.3 (*C*=O), δ = 133.5, 132.1, 131.3, 129.5 (Ar), δ= 58.1, 52.7 (C_α), δ= 31.6 (CH*C*H_2CH_2SH), δ = 21.5 (CHCH_2*C*H_2SH). Anal calcd for $C_{12}H_{16}N_2O_3S$: 10.44, N; 53.71 C; 5.79, H; 11.95 S. Found: 10.20, N; 51.14, C; 6.01, H; 11.82, S.

Synthesis of D-phenylglycyl-L-amino butyric acid diketopiperazine

1.01 g (4.28 mmol) of D-Phenylglycyl-L-amino butyric acid was suspended in 19 mL of dry methanol. A white suspension was obtained which was cooled to approximately 5°C. To this suspension 345 µL of $SOCl_2$ (4.75 mmol, 1.11 equivalent) were added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 19 mL of water were added. The pH was adjusted to 8.4 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 6 days, the formed precipitate was filtered, washed with petroleum ether 40-60 and dried to afford 0.58 g (62% yield) of 3*S*,6*R*)-3-ethyl-6-phenylpiperazine-2,5-dione .

m.p. 244- 246 °C. $[\alpha]_D^{20}$ = -63.3 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.55 (s, CHN*H*CO), δ = 8.19 (s, CHN*H*CO), δ = 7.35 (m, aromatic protons), δ = 4.91 (d, *J* = 3.0 Hz, C*H*Ph), δ = 3.99 (t, C*H*CH_2CH3), δ = 1.78 (m, CHC$H_2$$CH_3$), δ = 0.87 (t, $CHCH_2$CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 168.4, 167.5 (*C*=O), δ = 139.7, 129.2, 128.6, 127.7 (Ar), δ = 59.5 (*C*HPh), δ = 55.0 (*C*HCH_2CH_3), δ = 25.6

(CH*C*H$_2$CH$_3$), δ= 8.99 (CHCH$_2$*C*H$_3$). Anal calcd for $C_{12}H_{14}N_2O_2$: 12.83, N; 65.96, C; 6.41, H. Found: 12.15, N; 65.12, C; 6.31, H.

Synthesis of D-phenylglycyl-L-norvaline diketopiperazine

1.04 g (4.16 mmol) of D-phenylglycyl-L-norvaline was suspended in 17 mL of dry methanol. A white suspension was obtained which was cooled to approximately 5°C. To this suspension 320 µL of $SOCl_2$ (4.41 mmol, 1.06 equivalent) were added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 10 mL of water were added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days the formed precipitate was filtered, washed with petroleum ether 40-60 and dried to afford 0.62 g (64% yield) of (3*R*,6*S*)-3-phenyl-6-propylpiperazine-2,5-dione.

m.p. 264- 266 °C. $[\alpha]_D^{24}$ = -55.2 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-*d$_6$*): δ = 8.52 (d, *J* = 2.1 Hz, CHN*H*CO), δ = 8.20 (s, 1H, CHN*H*CO), δ = 7.36 (m, aromatic protons), δ = 4.91 (d, *J* = 2.4 Hz, C*H$_a$*Ph); δ = 3.96 (t, C*H*CH$_2$CH$_2$CH$_3$), δ = 1.72 (m, CH*H$_2$*CH$_2$CH$_3$), δ = 1.36 (m, CHCH$_2$C*H$_2$*CH$_3$), δ = 0.88 (t, CHCH$_2$CH$_2$C*H$_3$*); ^{13}C-NMR (75 MHz, DMSO-*d$_6$*): δ=168.6, 167.4 (*C*=O), δ = 139.6, 129.2, 128.6, 127.8 (Ar), δ = 59.4, 54.3 (*Ca*), δ = 34.9 (CH*C*H$_2$CH$_2$CH$_3$), δ = 17.7 (CHCH$_2$*C*H$_2$CH$_3$), δ = 14.5 (CHCH$_2$CH$_2$*C*H$_3$). Anal calcd for $C_{13}H_{16}N_2O_2$: 12.05, N; 67.15, C; 6.89, H. Found: 11.92, N; 67.00, C; 6.86, H.

Synthesis of D-phenylglycyl-L-norleucine diketopiperazine

1.20 g (4.545 mmol) of D-phenylglycyl-L-norvaline was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled to approximately 5°C. To this suspension 365 µL of $SOCl_2$ (5.03 mmol, 1.1 equivalent) were added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 15 mL of water were added. The pH was adjusted to 8.5 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 3 days, the formed precipitate was filtered, washed with petroleum ether 40-60 and dried to afford 0.42 g of (3*S*,6*R*)-3-butyl-6-phenylpiperazine-2,5-dione. A further 0.28 g was obtained when the filtrate was left stirring for another 13 days. These amounts combined correspond to an overall yield of 63%.

m.p. 265- 267 °C. $[\alpha]_D^{24}$ = -52.8 (*c* 1, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.52 (d, *J* = 2.1 Hz, CHN*H*CO), δ = 8.19 (s, CHN*H*CO), δ = 7.36 (m, aromatic protons), δ = 4.91 (d, *J*=2.4 Hz, C*H*Ph), δ = 3.98 (t, $\mathrm{C}\mathit{H_a}\mathrm{CH_2CH_2CH_2CH_3}$), δ = 1.73 (m, $\mathrm{CHC}\mathit{H_2}\mathrm{CH_2CH_3}$), δ = 1.29 (m, CHCH2C$\mathit{H_4}$CH3), δ = 0.88 (t, $\mathrm{CHCH_2CH_2CH_2C}\mathit{H_3}$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 167.6, 166.4 (*C*=O), δ = 138.7, 128.2, 127.6, 126.8 (Ar), δ = 58.5, 53.4 (*Ca*), δ = 31.5 ($\mathit{C}\mathrm{H_2CH_2CH_2CH_3}$), δ = 25.5 ($\mathrm{CH_2}\mathit{C}\mathrm{H_2CH_2CH_3}$), δ= 21.8 ($\mathrm{CH_2CH_2}\mathit{C}\mathrm{H_2CH_3}$); δ= 13.6 ($\mathrm{CH_2CH_2CH_2}\mathit{C}\mathrm{H_3}$). Anal calcd for $C_{14}H_{18}N_2O_2$: 11.37, N; 68.21, C; 7.31, H. Found: 11.26, N; 67.50, C; 7.32, H.

Synthesis of D-phenylglycyl-L-homocysteine diketopiperazine

0.21 g (783 µmol) of D-phenylglycyl-L-homocysteine was suspended in 3.5 mL of dry methanol. A white suspension was obtained which was cooled to approximately 5° C. To this suspension 100 µL of $SOCl_2$ (1.38 mmol, 1.8 equivalent) were added drop-wise. The mixture was allowed to reflux for 5 hours and after cooling to room temperature, 7.0 mL of water were added. The pH was adjusted to 8.6 with concentrated sodium hydroxide. The mixture was left stirring and after 30 days, the formed precipitate was filtered. This was washed with petroleum ether 40-60 and dried. The product was isolated and 180 mg were obtained of a crude product with NMR impurities.

Anal calcd for $C_{12}H_{14}N_2O_2S$: 11.19, N; 57.53, C; 5.59, H; 12.78, S. Found: 11.02, N; 55.57, C; 5.16, H; 12.75, S.

References

---

1 Li, W.-R.; Yang, J. H.; *Journal of Combinatorial Chemistry* **2002**; 4, 106 - 108

2 McCleland, K.; Milne, P.J.; Lucieto, F.R.; Frost, C.; Brauns, S.C.; Van De Venter, M.; Du Plessis, J.; Dyason, K.; *Journal of Pharmacy and Pharmacology* **2004**; 56, 1143 - 1153

3 Ressurreição, A:S:M.; Delatouche, R.; Gennare, C.; Piarulli, U.; *European Journal of Organic Chemistry* **2011**; 217 - 228

4 Nicholson, B.; Lloyd, G.K.; Miller, B.R.; Palladino, M.A.; Kiso, Y.; Hayashi, Y; Neuteboom, S.T.C.; *Anti-Cancer Drugs* **2006** ; 17, 25 - 31

5 Vanžura, J.; Palát, K.; Čeladník, M.; Hradec, K ; Klimes, J.; František, S.; *CS 210383* **1983**

6 Sinha, S.; Srivastava, R.; De Clercq, E.; Singh, R.K.; *Nucleosides, Nucleotides and Nucleic Acids* **2004**; 23, 1815 - 1824

7 McCombie, S.W.; Tagat, J.R.; Vice, S.F.; Lin, S.-I. ; Steensma, R.;Palani, A.; Neustadt, B.R.; Baroudy. B.M.; Strizki, J.M.; Endres, M.; Cox, K.; Dan, N.; Chou, C.-C. ; *Bioorganic & Medicinal Chemistry Letters* **2003**; 13, 567 - 571

8 Houston, D.R.; Synstad, B.; Eijsink, V.G.H.; Stark, M.J.R.; Eggleston, I.M.; van Aalten, D.M.F.; *Journal of Medicinal Chemistry* **2004**; 47, 5713 - 5720

9 Fdhila, F.; Vásquez, V.; Sánchez, J. L.; Riguera, R.; *Journal of Natural Products* **2003**, 66, 1299 - 1301

10 Song, M.K.; Hwang, I.K.; Rosenthal, M.J.; Harris, D.M.; Yamaguchi, D.T.; Yip, I.; Go, V.L.W.; *Experimental Biology and Medicine* **2003**; 228, 1338 - 1345

11 Sasaki, Y.; Akutsu, Y.; Suzuki, K.; Sakurada, S.; Kisara, K.; *Chemical & Pharmaceutical Bull*etin **1981**; 29, 11, 3403 - 3406

12 Martins, M.B.; Carvalho, I.; *Tetrahedron* **2007**; 63, 9923 - 9938.

13 Avendaño, C.; de la Cuesta, E.; *Current Organic Synthesis* **2009** ; 6, 143 - 168

14 Borthwick, A:D:; Liddle, J.; *Medicinal Research Reviews* **2011**; 31, 576 - 604

15 Tanaka, K.; Atsunori, M.; Inoue, S.; *Journal of Organic Chemistry* **1989**;55, 181 - 185

16 Iyer, M. S.; Gigstad, K. M.; Namdev, M. D.; Lipton, M. ; *Journal of the American Chemical Society* **1996**, 118, 4910 - 4911

17 Kowalski, J.; Lipton, M. A.; *Tetrahedron Letters* **1996**, *37*, *33*, 5839 - 5840

18 Jackson, W.R.; Jayatilake, G.S.; Matthews, B.R.; Wilshire, C.; *Australian Journal of Chemistry* **1988**; 41, 2, 203 - 213

19 Gruttadauria, M.; Giacalone, F.; Noto, R.; *Advanced Synthesis & Cataysis.* **2008**, *351*, 33 - 57

20 Carrea, G.; Colonna S.; Kelly, D. R.; Lazcano, A.; Ottolina, G.; Roberts, S. M. *Trends in Biotechnology* **2005**, 23, 507 - 513

21 Wennemers, H.; *Chemical Communications* **2011**, *47*, 12036 - 12041

22 Fischer, P.M.; *Journal of Peptide Science* **2003**; 9, 9 - 35

23 Kanoh, K.; Kohno, S.; Katada, J.; Takahashi, J.; Uno, I.; Hayashi, Y.; *Bioorganic & Medicinal Chemistry* **1999**, 7, 1451 - 1457

24 Zeng, Y.; Li, Q.; Hanzlik, R.P.; Aubé, J.; *Bioorganic & Medicinal Chemistry Letters* **2005**, 15, 3034 - 3038

25 Asada, S.; Kobayashi, Y.; Inoue S.; *Macromolecular Chemistry and Physics* **1985**; 186, 1755 - 1762.

26 Cho, S.; Keum, G.; Kang, S.B.; Han, S.-Y.; Kim, Y.; *Moecular Diversity* **2003**, 6, 283 - 286

27 Giraud, M.; Bernad, N.; Martinez, J.; Cavelier, F.; *Tetrahedron Letters* **2001**, 42, 1895 - 1897

28 Straathof, A.J.J.; Panke, S.; Schmid, A.; *Current Opinion in Biotechnology* **2002**; 13, 6, 548 - 556

29 Adamczak, M.; Krishna, S.H.; *Food Technology and Biotechnology* **2004**; 42, 4, 251 - 264

30 Huisman, G. W.; Collier, S. J.; *Current Opinion in Chemical Biology* **2013**; *17*, 284 – 292

31 Patel, R. N.; *ACS Cataysis.* **2011**; *1*, 1056 - 1074

32 Tramper, J.; *Biotechnology and Bioengineering* **1996**; 52, 290 - 295

33 Cheetham, P.S.J.Z.; Straathof, A. J. J.; Adlercreutz, P. *Applied Biocatalysis* (2nd Edition), Harwood, Amsterdam (2000), 93-152.

34 Khimiuk, A.Y.; Korennykh, A.V.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A.; Švedas, V.K.; *Tetrahedron: Asymmetry* **2003**; 14, 3123 - 3128

35 Bruggink, A.; Roos, E.C.; de Vroom, E.; *Organic Process Research & Development* **1998**; 2, 128 - 133

36 Kallenberg, A.I.; van Rantwijk, F.; Sheldon, R.A.; *Advanced Synthesis & Catalysis* **2005**; 347, 905-926

37 Pereira, P. C.; Sheldon, R. A.; Arends, I. W. C. E.; *Tetrahedron: Asymmetry* **2014**; 25, 825 - 832

38 Shcherbakova, T.A.; Korennykh, A.V.; van Langen, L.M.; Sheldon, R.A.; Švedas, V.K.; *Journal of Molecular Catalysis B: Enzymatic* **2004**; 31, 63 - 65

39 van Langen, L.M.; van Rantwijk, F.; Švedas, V.K.; Sheldon, R.A.; *Tetrahedron: Asymmetry* **2000**; 11, 1077 - 1083

Application of Co-Immobilized Cross-Linked Enzyme Aggregates in the Chloroperoxidase - Glucose Oxidase System

4

Introduction

Cascade biocatalysis

Industrial biotechnology, the application of biotechnology in industrial processes, is today well established. With the use of enzymes or microorganisms instead of traditional chemical synthesis, higher process efficiencies have been reached resulting in lower energy and water consumption as well as reduction of toxic waste [1].

Biocatalytic processes can be divided into two types: those using isolated enzymes (or resting cells) and those using growing cells (fermentations). Fermentations can be cost-effective but they are limited by the microorganism´s metabolic pathways [2] and efficiency of transport mechanisms [3].

Industrial production of penicillin-G and cephalosporin-C are representative examples of well established industrial fermentations for fine chemicals manufacture [4]. On the other hand, the use of isolated enzymes allows for a better control, as unwanted side-reactions are avoided, but enzymes must be isolated from an organism and might need to be immobilized for economic viability [5]. Representative examples of industrial production with isolated enzymes are the semi-synthetic production of ß-lactam antibiotics with immobilized penicillin acylase [6,7] and liquefaction of starch by native α-amylase [8].

One way to improve fermentation production is the use of genetic techniques to insert a specific desired activity in the cell. For example, DSM Nutritional Products replaced the company’s traditional six-step process for producing vitamin B2 (riboflavin) with a one-step fermentation process that was made feasible by engineering the bacterium, to increase vitamin yield by 300.000-fold compared to what could be achieved with the wild-type strain. The one-step fermentation process reduced cost of production by 50% relative to the conventional process [9].

Chemistry as it is performed in the cells of organisms involves coupled conversions without intermediate recovery steps. The use of convenient combinations of isolated enzymes to enable difficult reactions has been receiving growing attention [10,11]. Isolation of intermediates becomes unnecessary, by replacing the traditional stepwise syntheses with cascade processes comprising several steps in one pot. Such a strategy enables, for example, pathways that involve unstable or thermodynamically unfavourable intermediates [12]. Eliminating individual steps saves on energy costs, labour, reagents and waste is reduced, making the system economically and environmentally more attractive [13].

One example of an oxidation cascade system is shown in Figure 1, in which chloroperoxidase catalyses the asymmetric oxidation of Thioanisole with *in situ* generated hydrogen peroxide.

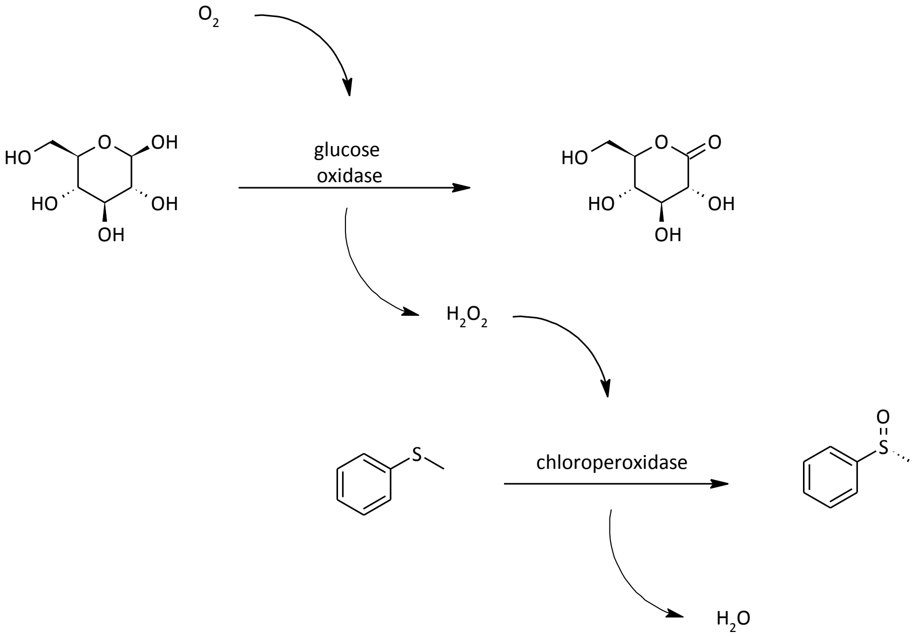


Figure 1: Oxidation of thioanisole and ß-D-glucose with no hydrogen peroxide accumulation, using a tandem chloroperoxidase and glucose oxidase, affording (*R*)-methyl-phenyl-sulfoxide and D-gluconolactone

Given the nature of the S-O bond, stabilized by resonance, a lone pair of electrons resides in the sulfur atom giving it a tetrahedral geometry as in a carbon sp^3. The energy required to invert the chiral centre is sufficiently high to allow for racemization to be slow at room temperature, making chiral sulfoxides optically stable [14].

This system is advantageous in many respects. Glucose oxidase (beta-D-glucose:oxygen-1-oxireductase, EC 1.1.3.4) is a relatively cheap biocatalyst, the applications of which in industry are well established [15]. Known examples are its use in oxygen removal [16] and glucose detection [17]. It uses (D) - glucose as substrate, which is a readily available, non toxic and cheap substrate.

The other reagent required is molecular oxygen at atmospheric pressure, so it can simply be taken from air avoiding handling of oxygen under pressure. It generates hydrogen peroxide which is then used as oxidant in the oxidation catalyzed by chloroperoxidase (CPO), and consequently generates water as co-product.

Because hydrogen peroxide is generated *in situ* and directly consumed, this system avoids its direct handling, or mechanically controlled addition, as well as its accumulation. Gluconolactone, the oxidation product of glucose, is a non toxic, biodegradable compound used in the food industry [18].

The chloroperoxidase from *Caldariomyces fumago* (CPO, E.C. 1.11.1.10) is an enzyme that catalyzes enantioselective sulfoxidation, hydroxylation and epoxidation reactions [19]. In addition CPO does not require the use of expensive cofactors, and uses hydrogen peroxide as oxidant, which makes it an attractive catalyst for preparative organic chemistry [20].

The high synthetic potential of this enzyme is however severely hindered by a poor operational stability caused by inactivation with hydrogen peroxide [21]. Other reported drawbacks of using chloroperoxidase oxidation systems are substrate low water solubility and competition with the chemical background reaction [22].

Several strategies have been adopted to circumvent CPOs low stability, namely controlled addition [23] or *in situ* generation of hydrogen peroxide. The latter approach has been achieved enzymatically with glucose oxidase [24], electrochemically [25], photochemically [20], or with a metal catalyst [26]. The use of polyethylene glycol as an additive [27] was found to improve chloroperoxidase´s stability and another approach found to improve peroxide tolerance was immobilization [28].

Immobilization of enzymes

An immobilized enzyme is physically confined or localized in a certain defined region of space with retention of its catalytic activity and can be used repeatedly and continuously [29]. Immobilization often improves enzyme stability [30] but the main technical advantage is the possibility to separate the enzyme from the process stream.

This allows for long term use, enhancing the economic viability of a biocatalytic process. The ability to efficiently recover and reuse a costly enzyme can define the economic viability of a specific operation [31]. Moreover, carry-through of protein or activity in subsequent processes is in this way prevented [32].

Enzyme immobilization can be generically classified in three different types: Carrier bound, Cross-linking and Entrapment (see Figure 2). Carrier based immobilizations involve binding the enzyme to a physical support.

This binding can be of different nature, such as adsorption or covalent binding. Cross linking techniques, or self-immobilization, consist in binding enzymes to each other, using bifunctional cross-linkers such as glutaraldehyde, without resorting to a support. Entrapment is the inclusion of the enzyme in a polymeric matrix, such as an organic polymer, silica sol-gel, or membrane.

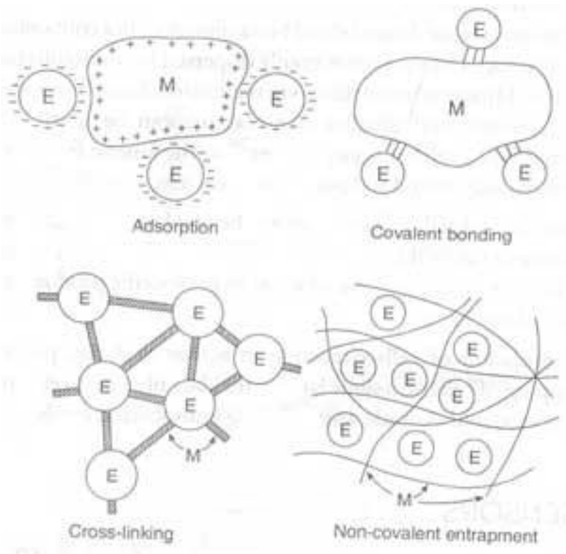


Figure 2: Enzyme immobilization resorting to carrier-binding (adsorpion and covalent binding), cross-linking and entrapment

Table 1 shows the generic advantages and disadvantages of the different methods. It should be pointed out that it is difficult to make direct comparisons of the different methodologies as most authors compare the performance of the immobilized enzyme, prepared using a particular technique, with that of the free enzyme but do not compare different methods of immobilization. The choice for a certain immobilization method will depend heavily on the intended purpose of the biocatalyst.

Table 1: Generic advantages and disadvantages of the different immobilization methodologies

Type of Immobilization	Advantages	Disadvantages
Adsorption (carrier)	Simplicity	Leaching in aqueous media
Covalent bonding (carrier)	Good applicability in aqueous systems	Carrier cost
Entrapment	Mild procedure	Leaching
Cross-Linking (carrier free)	High volumetric and catalytic productivity no leaching	Small particle size

Immobilization to a prefabricated support can provide a high degree of control over the physical properties of the resulting biocatalyst, such as particle size and mechanical stability. Carriers are expensive and usually not recycled [33], so the lifetime of the carrier is therefore defined by the operational lifetime of the enzyme attached to it. Support based techniques can vary in the nature of the interaction between enzyme and support.

Adsorption is relatively cheap, simple and does not modify the enzyme chemically. The binding can be based on, *e.g.*, ionic or hydrophobic interactions. Common polymeric carriers can be used, such as microporous polypropylene beads (Accurel) [34], or ion exchange resins [35].

A drawback of this technique is facile leaching of the enzyme from the carrier, particularly under aqueous conditions. Consequently this method is best suited for applications in organic media. An example is the resolution of chiral amines using adsorbed CALB (*Candida antartica* Lipase B) in a water-free medium [36].

Carriers for covalent binding have reactive groups that can react with nucleophilic groups, usually lysine residues, at the surface of the enzyme. Often applied reactive groups on the carriers are the epoxide and amine, which latter group needs activation by a cross-linking agent prior to the immobilization procedure.

Covalent bound immobilizations grant an irreversible binding, therefore minimizing leaching. An industrial example is the biocatalyst Assemblase ®, a biopolymer gel matrix to which *E. coli* penicillin acylase is covalently bound [37]. This biocatalyst is used in the production of semi-synthetic ß-lactam antibiotics [6]. However, the use of a carrier can account for 90-99% of the overall biocatalyst mass, leading to dilution of volumetric activity, which is a serious drawback.

Enzyme entrapment is the physical entrapment of enzymes in a cage-like matrix. This can be accomplished by the formation of a matrix from a solution containing the enzyme. Immobilization into calcium alginate and polyvinyl-alcohol are typical examples of entrapment [38]. Entrapment protects enzymes by preventing direct contact with the environment and it is arguably the best means to avoid any negative influence on the enzyme structure [39]. The challenge with this procedure is to find the optimum matrix density, as a higher density will minimize leaching but will also limit mass transfer. Therefore, this method is very effective for immobilization of larger structures, such as whole cells [40].

Due to the intrinsic drawbacks of enzyme immobilization on or in carriers, such as activity dilution and activity loss [41], there is a growing interest in innovative self-immobilization methods, such as cross-linking [42], where the enzyme acts as its own carrier. The immediate advantages of such methodologies are high concentration of activity in the catalyst and low production cost, as carriers are excluded.

Several types of cross-linking methodologies can be defined. A cross-linked enzyme (CLE) is the product obtained by addition of a cross-linking agent to an enzyme solution, leading to spontaneous flocculation of cross-linked enzyme. However, this results in physically weak and difficult to handle structures [41].

Physically stronger cross-linked biocatalysts can be produced when the enzymes are already in close proximity at the time of cross-linking. Subjecting the cross-linking to enzyme crystals or aggregates will result in the formation of Cross-Linked Enzymes Crystals (CLECs) [43] or Cross-Linked Enzyme Aggregates (CLEAs) [44], respectively. The production of CLECs is limited by the need to crystallize the enzyme, which is not always possible, and increases the cost of the biocatalyst [45].

Aggregating the enzyme by precipitation and then cross-linking it, is a simpler and more robust technique to obtain highly concentrated cross-linked biocatalysts (see Figure 3). Typical precipitating agents are water miscible organic solvents or salts. Glutaraldehyde is often used to cross-link the amine groups present at the surface of the enzyme [46].

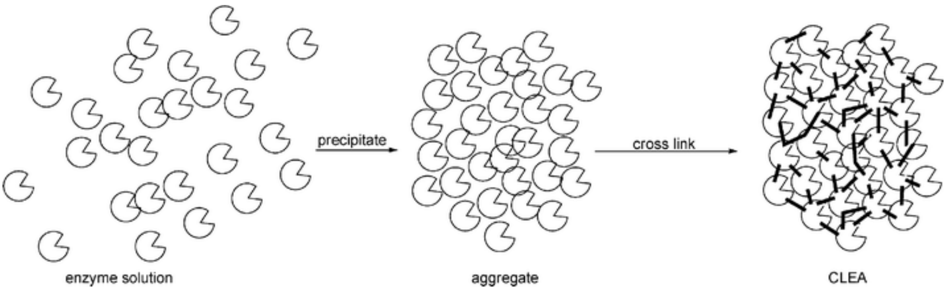


Figure 3: CLEA preparation – a soluble enzyme is aggregated by precipitation followed by chemical cross-linking

Application of Co-Immobilized Cross-Linked Enzyme Aggregates in the Chloroperoxidase - Glucose Oxidase System

Cross Linked Enzyme Aggregates (CLEAs) are a proven method for immobilizing a wide variety of enzymes. CLEAs were shown to be stable, recyclable and to have high productivities [47].

The CLEA procedure allows for the use of crude cell free extracts as well as the combination of multiple enzymes. The co-immobilization of multiple enzymes permits multiple transformations, cascade processes, with only one biocatalyst [48].

A COMBICLEA is a CLEA where more than one enzyme is co-immobilized. It was shown that a COMBICLEA of oxynitrilase and nitrilase [49] resulted in better performance in the synthesis of (*S*)-2-hydroxyacids than the use of two separate CLEAs of oxynitrilases and nitrilases, which the authors suggest could be caused by the proximity of both enzymes in the biocatalyst [50]. Another example of a successful COMBICLEA is the recently reported co-immobilization of the peroxidase from *Bjerkandera adusta* and glucose oxidase [51].

Co-immobilization of chloroperoxidase and glucose-oxidase as a COMBICLEA seems, therefore, an attractive approach to obtain a viable, green and selective oxidation biocatalyst. The successful co-immobilization of chloroperoxidase and glucose-oxidase, using different methodologies such as sol-gel as well as polyurethane foam, was already previously reported [24,53]. This chapter describes the results of a study towards the use of a glucose-oxidase and chloroperoxidase COMBICLEA, using the selective oxidation of thioanisole as a model reaction.

Results and Discussion

COMBICLEAs of CPO and GOX were prepared by precipitation and subsequent cross-linking. In addition, single-enzyme CLEAs of GOX and CPO were prepared using the same procedure (see Table 2).

Table 2: CLEA preparations used in this work

Immobilized enzyme preparation	*Enzyme Immobilized (unit basis)*	*Precipitant*	*Cross-Linker*
COMBICLEA CPO : GOX 1:1	CPO:GOX (1:1)	1,2-Dimethoxyethane	Glutaraldehyde
COMBICLEA CPO : GOX 1:5	CPO:GOX (1:5)	1,2-Dimethoxyethane	Glutaraldehyde
CLEA CPO	CPO	1,2-Dimethoxyethane	Glutaraldehyde
CLEA GOX	GOX	1,2-Dimethoxyethane	Glutaraldehyde

The resulting COMBICLEAs were used as catalyst in the oxidation of thioanisole (Figure 4). As a control experiment, the bi-enzymatic oxidation of thioanisole was studied using combinations of a single-CLEA and the free enzyme (Figure 6).

Figure 4 shows the conversations obtained with the COMBICLEAS under various conditions were disappointing compared to the earlier potential of this system, where quantitative oxidation of several substrates were reached with high selectivity (Table 3).

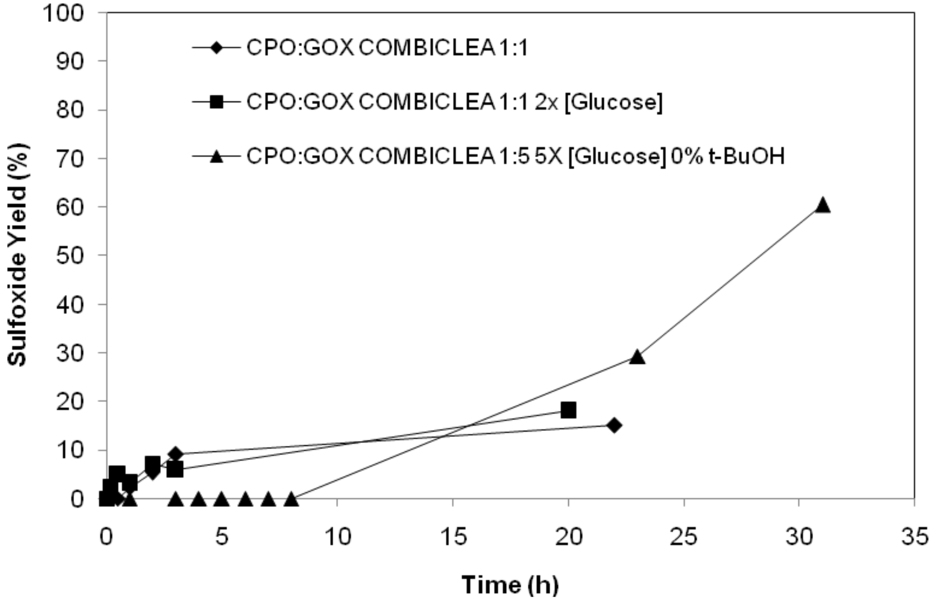


Figure 4: Oxidation of thioanisole using different COMBI-CLEA preparations under different conditions. Standard conditions as described in the experimental part.

Table 3: CPO – catalyzed sulfoxidation of thioanisole with different H_2O_2 generation / addition modes

Method	*Yield (%)*	*e.e. (%)*
Controlled H_2O_2 addition [52][a]	73	99
Co-immobilization with glucose oxidase in PUF [53][b]	100	99
Photochemical H_2O_2 generation [20][c]	100	> 98

a - thioanisole 50 mM, 50% *t*-BuOH / H_2O (v / v) ; *b* - thioanisole 15 mM , 50% *t*-BuOH / H2O (v / v), 1 atm O_2; *c* - thioanisole 50 mM, 25% *t*-BuOH / H2O (v / v)

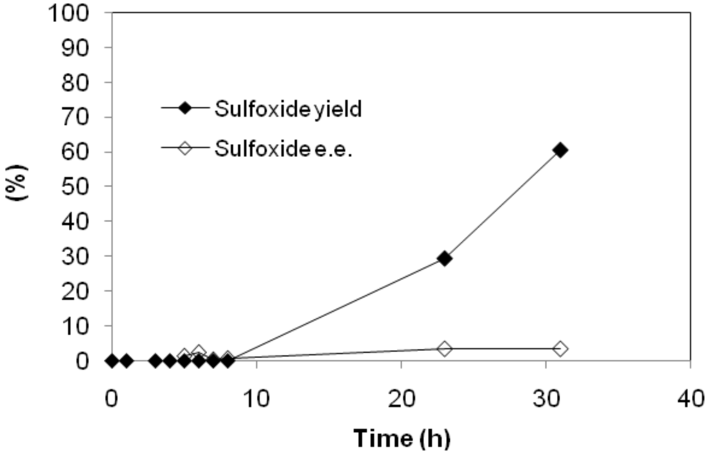


Figure 5: Product formation and selectivity progress curves in the oxidation of thioanisole using a CPO / GOX - COMBI-CLEA preparation with an enzyme ratio of 1:5, 5 equivalents of glucose and no co-solvent. Remaining conditions as described in experimental part.

From analysis of the enantiomeric excess of the oxidized reaction product obtained in the reaction leading to the highest conversion (Figure 5), it became clear that the majority of the conversion could be attributed to the non-enzymatic background reaction.

The experiments performed with single-immobilized enzymes (Figure 6 and Figure 7) remarkably showed that in the case of native chloroperoxidase and a glucose oxidase CLEA a fast and quantitative oxidation of thioanisole was achieved. The high enantiomeric excess of the reaction product (Figure 7) indicates that the oxidation is enzyme-mediated. Furthermore, it can also be concluded from this experiment that the glucose oxidase CLEA is a viable catalyst.

Using, on the other hand, a chloroperoxidase CLEA and native glucose oxidase, led to a slower and incomplete oxidation of Thioanisole. The obtained result, however, appears better than the observed reactions with the COMBICLEAs, as the enantiomeric excess of the product from this reaction was found to be high, indicating an enzyme mediated conversion. This observation is corroborated by Perez *et al.*, who described the successful immobilization of chloroperoxidase as a cross-linked enzyme aggregate.

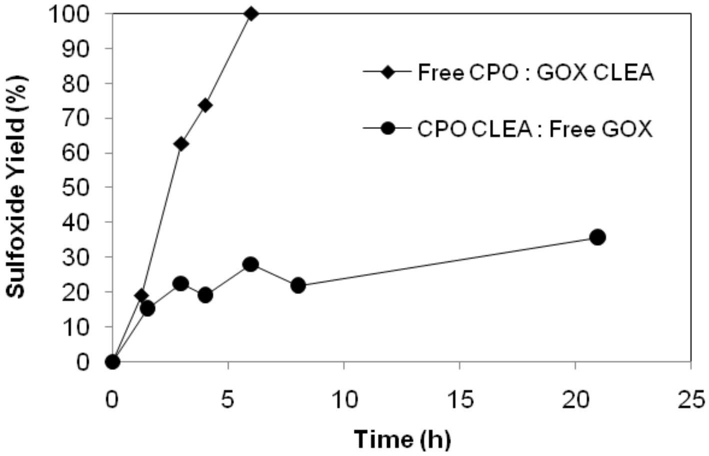


Figure 6: Comparison of thioanisole oxidation using free CPO / GOX - CLEA and CPO - CLEA / Free GOX using an enzyme ratio of 1:5, 5 equivalents of Glucose and no co-solvent. Remaining conditions as described in experimental part.

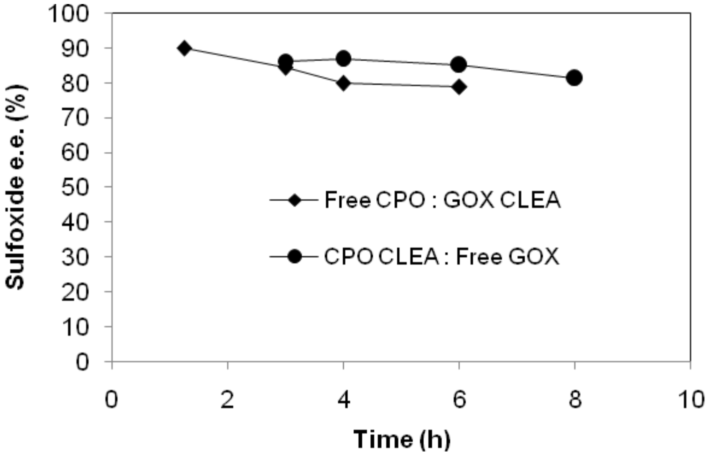


Figure 7: Product selectivity progress curve in the oxidation of thioanisole using CPO - Free / GOX - CLEA and CPO - CLEA / Free - GOX using an enzyme ratio of 1:5 and no co-solvent. Remaining conditions as described in experimental part.

Considering the immobilization procedure subjected to both enzymes separately results in active biocatalysts, we conclude that in a newly prepared COMBICLEA of glucose oxidase and chloroperoxidase both enzymes are active. However, on the basis of the experiments described in this Chapter, we also conclude that the co-immobilization of glucose oxidase and chloroperoxidase in a COMBICLEA does not lead to better performance in selective oxidation of thioanisole.

We suspect that chloroperoxidase in a COMBI-CLEA with glucose oxidase is prone to inactivation. This might be caused by an excessively high local hydrogen peroxide concentration, as CPO is easily deactivated by it. This suggests that the CPO-catalyzed step is rate-determining and that the H_2O_2 is produced too quickly, leading to a concentration build-up resulting in inactivation of the CPO. In this case a possible solution to this problem is to use a higher CPO:GOX rate in the COMBICLEA.

In order to understand the cause for the low performance of the COMBICLEA, a better insight in the two-enzyme system is needed. In view of the earlier reported good performance of co-immobilized glucose oxidase and CPO in polyurethane foam [53], further improvement of the applicability of the COMBICLEA CPO:GOX seems feasible. In the following Chapter 5, the oxidation of thioanisole using native glucose oxidase and chloroperoxidase is further studied.

Conclusions

Co-immobilized glucose oxidase and chloroperoxidase as cross-linked enzyme aggregate (COMBICLEA) does not show improved catalytic activity in the oxidation of thioanisole compared with the separately cleated enzymes, which are shown to be viable biocatalysts.

The limiting factor for the performance of the COMBICLEA of chloroperoxidase and glucose oxidase is suggested to be the instability of chloroperoxidase, possibly as a result of high local concentration of H_2O_2.

Improvement of the performance of the COMBICLEA as an oxidation catalyst requires a better understanding of the role of the two separate enzymes in the cascade reaction.

Experimental Part

Oxidation Reactions

In a 50 mL round bottom flask containing 12.5 mL of *t*-BuOH and 12.5 mL of phosfate buffer pH 5 100 mM, 1.25 mmol of thioanisole (1.47 µL) and 227 mg of (D) – glucose (1 equivalent) were added. To this 610U (33 µL) of chloroperoxidase

(Fluka) were added and the mixture was stirred for 5 minutes. Reaction was started with the addition of 610U (3.3 mg) of glucose oxidase (Sigma).

In the cases where a CPO/GOX – COMBI-CLEA 1:1 was used, 1.32 mL of a COMBICLEA solution was added. In the cases where a CPO / GOX–COMBI-CLEA 1:5 was used, 2 mL of a COMBICLEA solution were added. In the cases were a CPO - CLEA was used, 660 μL of a CPO – CLEA were added. In the cases where a GOX – CLEA was used, 1 mL of a GOX – CLEA was added.

CLEA Preparations

CPO-CLEA: 50 μL of CPO (1118 Units), buffer (50 μL) and 200 μL of PEHA (100 mM, 244 μL PEHA in 10 mL of 0.1 M phosphate buffer pH = 6.4) were stirred for 20 min. To this an albumin solution (50 μL, 46 nmoles) was added to the reaction and after being stirred for 20 min, the mixture was added to 1,2-dimethoxyethane (900 μL) and stirred for another 30 min. Then 52 μL of glutaraldehyde was added to the reaction and the solution stirred for 2 h. Finally, $NaBH_3CN$ (126 μL, 100 mM) was added and the mixture stirred for 1 h. The suspended CLEA was centrifuged (13000 rpm, 15 min) and washed 3 times with 1 mL buffer to remove all remaining glutaraldehyde and non cross-linked protein.

GOX CLEA

Same procedure as above with 16,5 mg of GOX (3050 Units).

COMBICLEAs

50 μL of CPO were added to 50 μL of buffer (pH=6.4), 200 μL of PEHA (100 mM, 244 μL PEHA in 10 mL of 0.1 M phosphate buffer pH = 6.4) and an albumin

solution (50 μL, 46 nmoles). The mixture was left stirring for 30 minutes. The same procedure was repeated with 5 mg of GOX in the 1:1 COMBICLEA (25 mg of GOX on the 1:5 COMBICLEA) and a second mixture was obtained. The two mixtures were combined and 1,8 mL of 1,2-dimethoxyethane were added. This was stirred for 30 minutes. Then 62 μL of glutaraldehyde were added and stirred for 3 hours. Finally $NaBH_3CN$ (252 μL, 100 mM) was added. The suspended COMBI-CLEA was centrifuged (13000 rpm, 15 min) and washed 3 times with 2 mL buffer.

Sampling

100 μL of reaction mixture were removed, added to 1 mL of a 5 mM solution of anisole (external standard) in ethyl acetate and shaken. To this Na_2SO_3 and $MgSO_4$ were added. The mixture was shaken, centrifuged and analyzed.

Analysis

(*R*) and (*S*)-methyl-phenyl-sulfoxide were detected in a Shimadzu GC 17A with AOC-201 automatic injector using a Cyclodex Beta (50m*0.25mm*0.25 um). Anisole, thioanisole and methyl-phenyl-sulfoxide were detected in a Varian 3400 CX with Varian 6577 autosampler using a Cpwax-52B (50m x 0.53mm x 2um).

Acknowledgment

We thank Daniel I. Perez for the preparation and supplying of the CPO and GOX CLEAs as well as COMBICLEAs.

References

---

1 Hatti-Kaul, R.; Törnvall, U.; Gustafsson, L.; Börjesson, P.; *Trends in Biotechnology* **2007**; 25, 3, 119 - 124

2 de Hollander, J.A.; *Applied Microbiology and Biotechnology* **1994**; 42, 508 – 515.

3 van Maris, A.J.A.; Konings, W.N.; van Dijken, J.P.; Pronk, J.T.; *Metabolic Engineering* **2004**; 6, 245 – 255

4 Elander, R.P.; *Applied Microbiology and Biotechnology* **2003**; 61, 385 - 392

5 Kallenberg, A.I.; van Rantwijk, F.; Sheldon, R.A.; *Advanced Synthesis and Catalysis* **2005**; 347, 905 - 926

6 Bruggink, A.; Roos, E.C.; de Vroom, E.; *Organic Process Research & Development* **1998**; 2, 128 - 133

7 Chandel, A.K.; Rao, L. V.; Narasu, M.L.; Singh, O.V.; *Enzyme and Microbial Technology* **2008**; 42, 199 - 207

8 Richardson, T.H.; Tan, X.; Frey, G.; Callen, W.; Cabell, M.; Lam, D.; Macomber, J.; Short, J.M.; Robertson, D.E.; Miller, C.; *Journal of Biological Chemistry* **2002**; 277, (29), 26501 - 26507

9 Gavrilescu, M.; Chisti, Y.; *Biotechnology Advances* **2005**; 7 – 8, 471 - 499

10 Mayer, S.F.; Kroutil, W.; Faber, K.; *Chemical Society Reviews* **2001**; 30, 332 - 339

11 Schrittwieser, J.H.; Sattler, J.; Resch, V.; Mutti, F.G.; Kroutil, W.; *Current Opinion in Chemical Biology* **2011**; 15, 249 - 256

12 Ricca, E.; Brucher, B.; Schrittwieser, J.H.; *Advanced Synthesis and Catalysis* **2011**; 353, 2239 - 2262

13 Bruggink, A.; Schoevaart, R.; Kieboom, T.; *Organic Process Research and Development* **2003**; 7, 622 - 640

14 Fernández, I.; Khiar, N.; *Chemical Reviews* **2003**; 103, 3651 - 3705

15 Wong, C.M.; Wong, K.H.; Chen, X.D.; *Applied Microbiology and Biotechnology* **2008**; 78, 927 – 938

16 Meyer, A.S.; Isaksen, A.; *Trends in Food Science & Technology* **1995**; 61, 300 - 304

17 Wilkins E.; Atanasov, P.; *Medical Engineering Physics* **1996**; 18, 273 – 288

---

18 Ramachandran, S.; Fontanille, P.; Pandey, A.; Larroche, C.; *Food Technology and Biotechnology* **2006**; 44, 2, 185 – 195

19 van Rantwijk, F.; Sheldon, R.A.; *Current Opinion in Biotechnology* **2000**, 11, 6, 554 - 564

20 Perez, D.I.; Grau, M.M.; Arends, I.W.C.E.; Hollmann, F.; *Chemical Communications* **2009**; 6848 – 6850

21 Shevelkova, A.N.; Ryabov, A.D.; *Biochemistry and Molecular Biology International* **1996**; 39, 4, 665 - 670

22 van Deurzen, M.P.J.; van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron* **1997**; 53, 39, 13183 - 13220

23 Seelbach, K.; van Deurzen, M.P.J.; van Rantwijk, F.; Sheldon, R.A.; Kragl, U.; *Biotechnology and Bioengineering* **1997**; 55, 2, 283 - 288

24 Borole, A.; Dai, S.; Cheng, C.L.; Rodriguez Jr, M.; Davison, B.H.; *Applied Biochemistry and Biotechnology* **2004**; 113 – 116, 273 - 285

25 Kohlmann, C.; Lütz, S.; *Engineering and Life Sciences* **2006**; 6, 2, 170 - 174

26 Karmee, S.K.; Roosen, C.; Kohlmann, C.; Lütz, S.; Grainer, L.; Leitner, W.; *Green Chemistry* **2009**, DOI: 10.1039/b820606f

27 Narayanan, R.; Zhu G.; Wang, P.; *Journal of Biotechnology* **2007**; 128, 86- 92

28 Zhang L-H; Bai, C.H.; Wang, Y.S.; Jiang, Y.C.; Hu, M.C.; Li, S.N.; Zhai, Q.G.; *Biotechnology Letters* **2009**, 31, 1269 - 1272

29 Katchalski-Katzir, E.; Kraemer, D.M.; *Journal of Molecular Catalysis B: Enzymatic* **2000**; 10, 157 - 176

30 Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R.; *Enzyme and Microbial Technology* **2007**; 40, 1451 - 1463

31 Sheldon, R.A.; *Advanced Synthesis and Catalysis* **2007**; 349, 1289 – 1307

32 Brady, D.; Jordaan, J.; *Biotechnology Letters* **2009**; 31: 1639 - 1650

33 Mateo, C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J.M.; *Biotechnology and Bioengeneering* **2000**; 68, 1, 98 - 105

34 Bosley, J.A.; Peilow, A.D.; *Journal of the American Oil Chemical´s Society* **1997**; 74, 2, 107 - 111

35 Marquez, L.D.S.; Cabral, B.V.; Freitas, F.F.; Cardoso, V.L.; Ribeiro, E.J.; *Journal of Molecular Catalysis B: Enzymatic* **2008**; 51, 86 - 98

36 van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron* **2004**; 60, 501 - 519

37 De Vroom, E.; **1997**; WO 9704086

38 Rosenborg, M.; Rebroš, M.; Krištofíková, L.; Malátová, K.; *Biotechnology Letters* **2005**; 27, 1943 - 1947

39 Hanefeld, U.; Gardossi, L.; Magner, E.; *Chemical Society Reviews* **2009**; 38, 453 - 468

40 Coiffier, A.; Coradin, T.; Roux, C.; Bouvet, O.M.M.; Livage, J.; *Journal of Materials Chemistry* **2001**; 11, 2039 - 2044

41 Cao, L; van Langen, L.; Sheldon, R.A.; *Current Opinion in Biotechnology* **2003**; 14, 387 - 394

42 Habeeb, A.F.S.A.; *Archives of Biochemistry and Biophysics* **1967**; 119, 264 - 268

43 Quiocho, F.A.; Richards, F.M.; *Proceedings of the American National Academy of Sciences of the USA* **1964**; 52, 833 - 839

44 Cao, L.; van Rantwijk, F.; Sheldon, R.A.; *Organic Letters* **2000**; 2, 10, 1361 - 1364

45 Brady, D.; Steenkamp, L.; Skein, E.; Chaplin, J.A.; Reddy, S.; *Enzyme and Microbial Technology* **2004**; 34, 283 - 291

46 Cao; L. ; van Rantwijk, F.; Sheldon, R.A.; *Organic Letters* **2000**; 2, 1361-1364

47 Sheldon, R.A.; *Biochemical Society Transactions* **2007**; 1583 - 1587

48 Sheldon, R.A.; *Applied Microbiology and Biotechnology* **2011**; 92, 467 - 477

49 Sheldon, R. A.; Schoevaart, R.; van Langen, L.M.; *Biocatalysis and Biotransformations* **2005**; 23, 141 - 147

50 Mateo, C.; Chmura, A.; Rustler, S.; van Rantwijk, F.; Stoltz, A.; Sheldon, R.A.; *Tetrahedron: Asymmetry* **2006**; 17, 3, 320 - 323

51 Taboada-Puig, R.; Junghanns, C.; Demarche, P.; Moreira, M.T.; Feijoo, G.; Lema, J. M.; Agathos, S.N.; *Bioresource Technology* **2011**; 102, 6593 - 6599

52 van Deurzen, M.P.J.; Remkes, I.J.; van Rantwijk, F.; Sheldon, R.A.; *Journal of Molecular Catalysis A: Chemical* **1997**; 117, 329 - 327

53 van de Velde, F.; Lourenço, N.D.; Bakker, M.; van Rantwijk, F.; Sheldon, R.A.; *Biotechnology and Bioengineering* **2000**; 69, 286 - 291

Optimizing the Chloroperoxidase - Glucose Oxidase System: The Effect of Glucose Oxidase on Activity and Enantioselectivity

5

The contents of this chapter have been published in Pereira, P.C.; Arends, I.W.C.E.; Sheldon, R.A.; *Proc Biochem* **2015**; 50, 746 – 751

Chapter 5

Introduction

Biocatalytic reactions involving incorporation of oxygen are potentially very useful in synthetic chemistry [1,2]. Monooxygenases (E.C. 1.14.X.X.), for example, catalyze the incorporation of one oxygen atom of dioxygen into organic substrates [3], with concomitant formation of a molecule of water according to reaction (1). The reducing equivalents are supplied by a NADH or NADPH cofactor and, hence, require regeneration in a separate step.

Peroxidases (E.C 1.11.1.X), in contrast, catalyze mainly oxidative dehydrogenation of organic substrates according to reaction (2) using hydrogen peroxide or alkyl hydroperoxides as the primary oxidant [4,5,6,7,8,9].

Reaction 1 $RH + O_2 + 2\,[H] \rightarrow ROH + H_2O$

Reaction 2 $2\,RH + H_2O_2 \rightarrow R\text{-}R + 2\,H_2O$

Reaction 3 $Cl^- + H_2O_2 \rightarrow ClO^- + H_2O$

Chloroperoxidase (CPO E.C. 1.11.1.10) secreted by the fungus *Caldariomyces fumago* is a heavily glycosylated heme dependent haloperoxidase, with a molecular mass of 42 kDa. *In vivo* it catalyzes the oxidative chlorination of 1,3-cyclopentadione by generating hypochlorite *in situ* by oxidation of chloride ion (reaction 3). In the absence of halide ions it is able to catalyze a variety of synthetically useful oxygen insertions (see Figure 1), such as: olefin epoxidation [10,11,12,13], sulfoxidation, [14,15,16], hydroxylations [17,18,19], oxidation of indoles [20,21] and oxidation of primary alcohols to aldehydes [22,23,24,25,26,27].

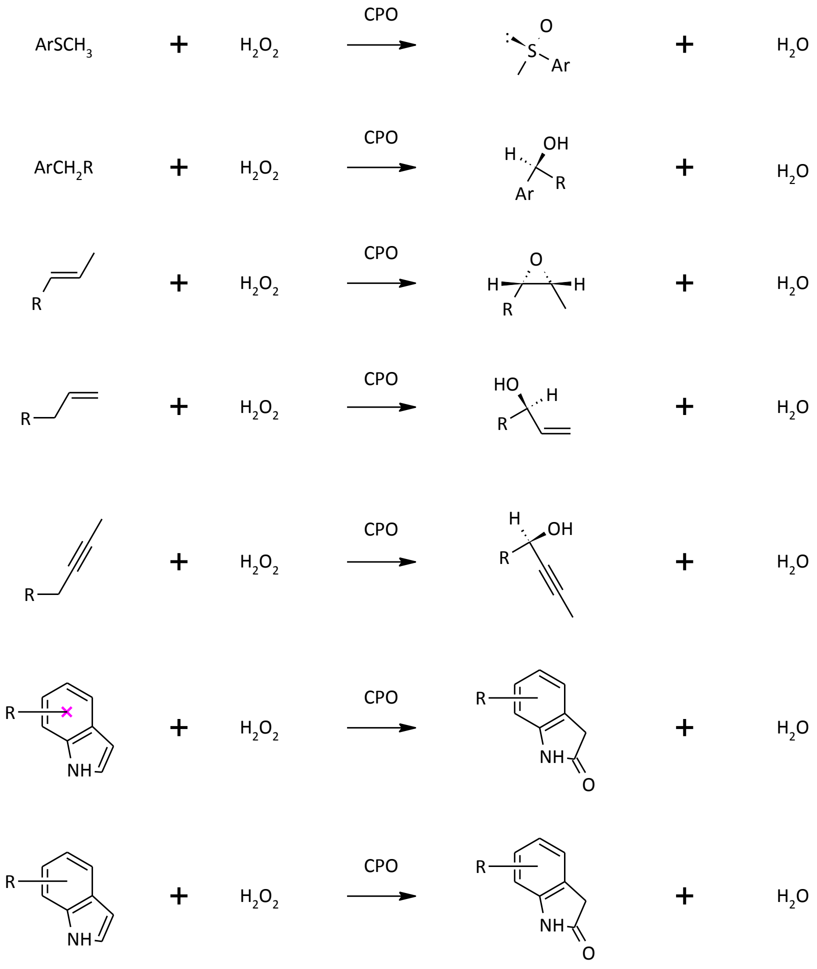


Figure 1: Oxidations catalyzed by CPO

This makes CPO a catalyst with an extremely high commercial potential. There is, however, no example of its use as catalysts in industrial organic synthesis, due to limited commercial availability, high price and low operational stability [28]. The latter is a result of competing oxidative degradation of the enzyme (suicide inactivation) by hydrogen peroxide [2,29]. Because of the high price of the enzyme, it is necessary that very high turnovers are obtained and the activity and, hence, the turnover, is strongly dependent on the type of reaction.

The suicide inactivation is caused by oxidation of the heme group and involves different activated oxygen species such as hydrogen peroxide or hydroxyl radicals, which are common to heme peroxidases [9, 30]. One strategy to circumvent CPO inactivation is to ensure that the hydrogen peroxide steady-state concentration is kept as low as possible by controlling the addition to the reaction medium. This is crucial as a concentration of just 30 µM causes complete inactivation of CPO [31].

Different modes of adding the oxidant to the reaction medium have been described [2,31,32] and it is noteworthy that the total turnover number (moles of product / moles of enzyme) strongly depends on the type of hydrogen peroxide addition (Table 1).

External addition generates locally high concentrations of hydrogen peroxide, which can lead to a rapid inactivation of CPO [8]. On the other hand, the “feed-on-demand” system requires the use of diluted solutions, which translates into volume increase. In terms of total turnover number, the best result in the asymmetric oxidation of thioanisole was obtained when using *in situ* generation of hydrogen peroxide with glucose oxidase (GOX; EC 1.1.3.4) from *Aspergillus niger*, glucose and oxygen [33].

Optimizing the Chloroperoxidase – Glucose Oxidase System: The Effect of Glucose Oxidase on Activity and Enantioselectivity

Table 1: Effect of H_2O_2 addition mode in CPO catalyzed batch oxidation of thioanisole in water / *t*-BuOH mixtures

Type of H_2O_2 Addition	**TTN (moles of product / moles of enzyme)**	**Reference**
Stepwise	41,000	[2]
Continuous	108,000	[2]
Sensor controlled “Feed-on-demand”	148,000	[2,36]
In situ generation (with co-immobilized glucose oxidase)	250,000	[2,33]

In situ generation of H_2O_2 seems therefore a logical strategy to overcome these challenges and different approaches have been reported. The use of electrochemical generation of hydrogen peroxide is an atom efficient methodology, as it uses no sacrificial reductant, but requires the use of specialized equipment [34]. Another reported approach is the use of visible light and flavins, but the reductants used afford unwanted side-products [32,35].

The method of *in situ* hydrogen peroxide generation which afforded the highest total turnover number in the oxidation of thioanisole was the co-immobilized chloroperoxidase and glucose oxidase system, under an oxygen atmosphere (Table 2).

Table 2: Asymmetric oxidation of thioanisole with *in situ* H_2O_2 generated. Total turnover numbers for CPO.

Method of H_2O_2 *in situ* Generation	TTN	$[S]_0$ (mM)	Yield (%)	e.e. (%)	Reference
Photochemical (Flavin/EDTA/*hv*)	22,400	8	100	> 99	[32]
Electrochemical (Cathode/O2)	95,000	20	66	> 98	[34]
Enzymatic (co-immobilized glucose oxidase/glucose/O2)	250,000	15	100	> 99	[33]

[S] is thioanisole concentration. Yield refers to % methyl phenyl sulfoxide formation relative to substrate added

Notwithstanding a reported decrease in CPO activity when using buffered aqueous *t*-BuOH, selectivities were improved in the oxidation of aryl alkyl sulfides by using this co-solvent [16]. Moreover, the use of *t*-BuOH improved the stability in the oxidation of indole [31], possibly due to its reported hydroxyl radical scavenging properties [29,36]. In parallel this increased the solubility of hydrophobic substrates, overcoming the problems associated with substrate solubility and diffusion limitations.

The chloroperoxidase - glucose oxidase combination in water / *t*-butyl alcohol mixtures is a promising and very attractive system, as it requires readily available glucose and an inexpensive auxiliary enzyme. Moreover it affords water and gluconolactone as side products, which are both non-toxic compounds. It furthermore circumvents both the use of specialized equipment, as well as handling of hydrogen peroxide as this is generated *in situ*.

Optimizing the Chloroperoxidase – Glucose Oxidase System: The Effect of Glucose Oxidase on Activity and Enantioselectivity

This system is, however, not fully characterized. For example, previous reports do not include a study over a broad range of enzyme concentrations or a description of how the system is influenced by the ratio of both enzymes [37,38,39]. More insight into these variables could form the basis for further improvement of its practical utility, for example in the development of more efficient co-immobilized preparations. In this work we have studied the influence of different enzyme concentrations, in their native formulations, in the oxidation of thioanisole with the purpose of determining an optimum operational ratio between the two biocatalysts.

Results and Discussion

In order to determine an optimum operational ratio, we tested several combinations of chloroperoxidase (CPO) and glucose oxidase (GOX) concentrations (see table 3). As previously shown, the CPO catalyzed oxidation of thioanisole with H_2O_2 occurs selectively, with methyl phenyl sulfoxide as the only product with a high stereoselectivity towards the (*R*) enantiomer [16].

The rate of hydrogen peroxide supply was previously reported to be crucial in CPO catalyzed oxidations and it was concluded that the H_2O_2 formation rate should be limiting, as accumulation resulted in complete inactivation of this enzyme [18,40].

With an activity unit-based ratio of 1:2 (CPO:GOX) the initial formation of hydrogen peroxide appeared to be rate-limiting for the activity of CPO. Thus, under these conditions, a two-fold increase in the amount of CPO to ratio 1:1 did not result in any increase of the initial rate of thioanisole oxidation (Figure 2).

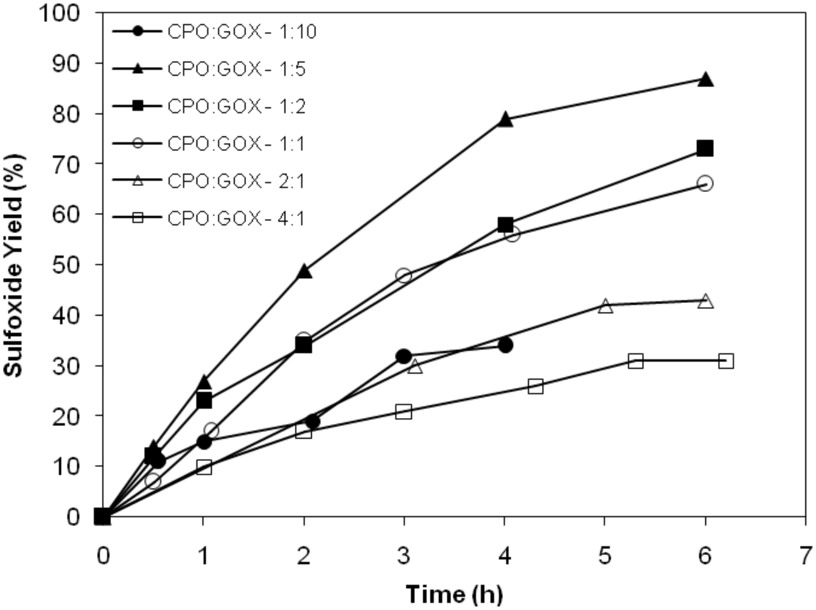


Figure 2: Sulfoxide formation in the CPO:GOX catalyzed thioanisole oxidation (50 mM) in 25 mL of a 50% (v/v) H_2O / *t*-BuOH mixture with 1.22 kU of CPO (full marker) or 0.61 kU of CPO (empty marker) and variable GOX amounts

As expected, reactions with the same amount of CPO (1.22 kU) and decreasing amounts of GOX lead to decreasing thioanisole oxidation rates. However, in these cases also the final yields decreased (Table 3).

Inactivation of CPO by hydrogen peroxide seemed unlikely, as accumulation should not have taken place in any of these cases (see above). Furthermore, the lowest yields were obtained with the lowest hydrogen peroxide formation rates rather than with the highest rates.

Although CPO is relatively stable in aqueous mixtures up to 30% aqueous *t*-BuOH [41], a possible explanation for these results is that an increase to 50% might be deleterious for the life time of CPO, or even both biocatalysts, at prolonged reaction times.

Table 3: CPO:GOX catalyzed thioanisole (50 mM) oxidation in 25 mL of H_2O / *t*-BuOH mixtures, using different enzyme ratios.

CPO:GOX Ratio	*t*-BuOH (%)	Yield $_{4\text{ h}}$ (%)	e.e. $_{4\text{ h}}$ (%)	Yield $_{22\text{ h}}$ (%)	e.e. $_{22\text{ h}}$ (%)
$1:10^*$	50	34	93	93	66
$1:5^*$	50	79	83	91	75
$1:2^*$	50	58	87	100	64
$1:1^+$	50	56	84	73	76
$2:1^+$	50	35	> 85	52	81
$4:1^+$	50	26	87	41 $_{\text{23 hrs}}$	81 $_{\text{23 hrs}}$
$1:5^\#$	50	82	74	100 $_{\text{6 hrs}}$	66 $_{\text{6 hrs}}$
$1:5^\#$	20	68	94	100 $_{\text{6 hrs}}$	91 $_{\text{6 hrs}}$

$^+$: experiments performed with 1,22 kU of CPO

*: experiments performed with 0,61 kU of CPO

$^\#$: experiments performed with 3 kU of CPO and 15 kU of GOX and 250 mM of glucose

The optimum result was obtained at a CPO:GOX ratio of 1:5 (Figure 2). Increasing the GOX concentration to 1:10 resulted in a decrease in rate. This was possibly due to accumulation of H_2O_2 in the early reaction phase, with consequent CPO inactivation.

The maximum yields after 22 hours, as a function of CPO and GOX loading, increased with the loading of GOX (Figure 3).

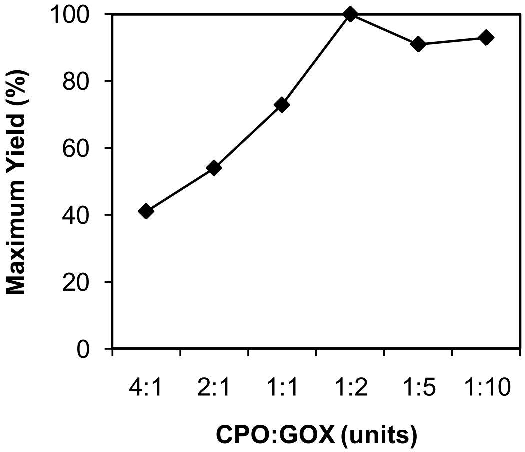


Figure 3: Maximum yields for sulfoxide formation after 22 hrs in the CPO:GOX catalyzed thioanisole oxidation with 1,22 kU or 0,61 kU of CPO and variable amounts of GOX

As a further evaluation of this CPO:GOX system, the enantiopurity of the reaction product was measured in the course of the reactions (Table 3). Previous work has shown that oxidation of sulfides with H_2O_2 catalyzed by CPO yields products in high enantiopurity and formation of optically impure products has been attributed to a chemical background oxidation [16].

In this system, the enantiomeric excesses obtained tended to decrease in the course of the reaction (Table 3), suggesting the presence of a blank reaction, with growing contribution towards the end of the process. This can be rationalized as being due to a decrease of CPO activity by inactivation, or rate decrease due to substrate depletion, in parallel with an unselective oxidation, due to the presence of hydrogen peroxide.

Optimizing the Chloroperoxidase – Glucose Oxidase System: The Effect of Glucose Oxidase on Activity and Enantioselectivity

In this respect, the reactions performed with ratios containing low GOX content deserve attention, as the immediate consumption of peroxide should eliminate, or at least minimize, the contribution of a chemical hydrogen peroxide oxidation reaction in the early phase of the reaction. Remarkably, however, we observed the opposite: at higher CPO:GOX ratios, the enantiopurity of the products, towards the (*R*) enantiomer, tended to be lower, already from the start of the process.

The ratio which combines highest product enantiopurity with highest product formation is the ratio 1:5, which is simultaneously the best ratio tested for reaction rate (see above). We therefore concluded that in this system, higher enantiomeric excesses are obtained when the initial rate of CPO is higher.

In order to gain further understanding on the unselective reaction in our system, we have tested the chemical oxidation of thioanisole with hydrogen peroxide. Blank reactions were performed in the presence of excess H_2O_2, without enzymes, both in the presence of D-glucose and in the presence of D-gluconolactone.

The results are presented in Figure 4 and a rate of formation of sulfoxide of approximately 0.25 mM / hr was observed, at an initial concentration of 250 mM (5 equivalents) of hydrogen peroxide. The presence of gluconolactone did not have a significant influence on this blank reaction compared with glucose. It has to be noted that in these experiments an excess of H_2O_2 was present. In our catalytic experiments the real concentration of H_2O_2 (1 equiv. maximum) will be much lower, and thereby also the background reaction slower than in Figure 4.

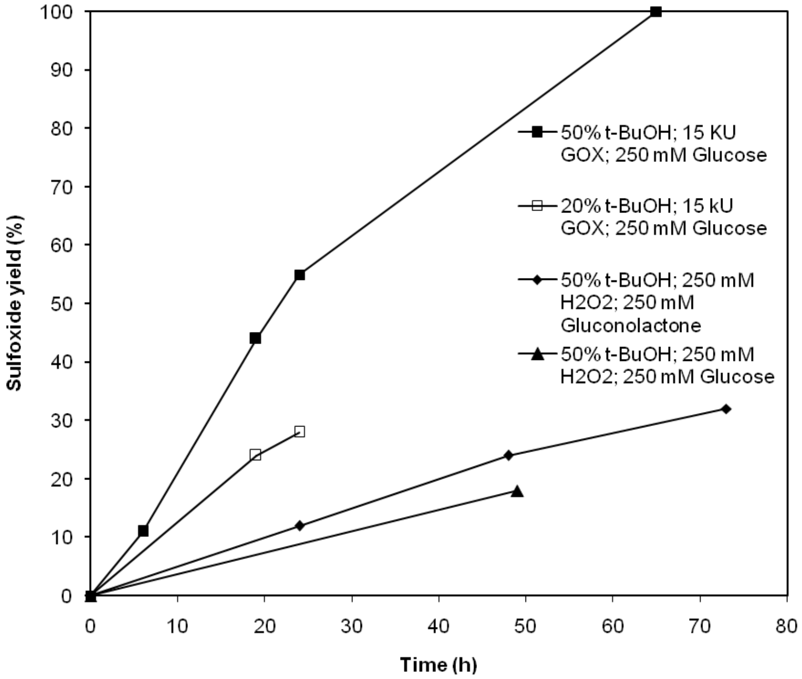


Figure 4: Comparison of thioanisole (50 mM) oxidations as a function of *t*-BuOH concentration with GOX/glucose, H_2O_2/glucose and H_2O_2/gluconolactone

Reactions performed in the presence of 15 kU of GOX and 250 mM of glucose surprisingly increased the rate of the background reaction to approximately 1 mM / hr. In this reaction the obtained sulfoxide was racemic.

A possible explanation for the observed novel GOX catalyzed sulfoxidation can be found in the fact that GOX is a flavin-dependent oxidase. It is known that flavin-dependent oxidases, such as Baeyer-Villiger monooxygenases (BVMOs), can catalyze aerobic sulfoxidations [42].

Optimizing the Chloroperoxidase – Glucose Oxidase System: The Effect of Glucose Oxidase on Activity and Enantioselectivity

The active oxidant is a flavin hydroperoxide, formed by the reaction of the reduced flavin with oxygen. The flavin hydroperoxide oxidizes the sulfide to the corresponding sulfoxide with concomitant formation of the oxidized flavin and a molecule of water. In the BVMO system the oxidized flavin is then reduced by the NADPH cofactor but in the case of an alcohol oxidase, such as GOX, the oxidized flavin can be reduced by the alcohol, in this case glucose, to complete the catalytic cycle. In the natural reaction the flavin hydroperoxide is hydrolyzed to give hydrogen peroxide and the oxidized flavin.

However, in our case promiscuous activity of glucose oxidase seems unlikely as no enantioselectivity was observed. We suggest that a plausible and more likely explanation is that the free flavin, leached from the GOX, is catalyzing the non-enantioselective sulfoxidation. It is known that flavins are able to catalyze sulfoxidations using either dioxygen [42,43] (in the presence of a reducing agent which could be the alcohol) or hydrogen peroxide [44,45,46,47] as the terminal oxidant.

Moreover, it is consistent with our observation that this background reaction was dependent on the presence of *t*-BuOH. Reducing the amount of *t*-BuOH from 50% to 20% (v / v) resulted in a significant decrease in the GOX-catalyzed background reaction (Figure 4), strongly suggesting that *t*-BuOH is responsible for leaching of the flavin from the GOX.

It is known that polar solvents, such as glycerol, can cause leaching of the flavin cofactor from GOX [48]. Furthermore, the observed higher enantioselectivity under these conditions is consistent with the suppression of the background sulfoxidation at the lower *t*-BuOH concentration (Table 3): 100% yield was obtained after 6 h, with an e.e. of 91%.

Another possibility, which we consider to be less likely, is that additives in the glucose oxidase preparation catalyze sulfoxide formation. It is commonly known that thioanisole oxidation with hydrogen peroxide in organic solvents is greatly accelerated by acidic, nitrilic as well as electrophilic agents [49]. A related study appeared on the effect of organic solvents on activity and enantioselectivity in asymmetric sulfoxidation, using heme-peroxidases as the catalyst [50].

Conclusions

The chloroperoxidase catalyzed asymmetric oxidation of thioanisole in *t*-BuOH / water mixtures with *in situ* generated hydrogen peroxide using glucose oxidase, shows an optimum rate and selectivity at a ratio of 1:5 CPO:GOX, on the basis of activity Units.

Final yields decreased with lower glucose oxidase concentrations, showing that under these conditions moderating the hydrogen peroxide formation rate does not lead to improvement in CPO stability.

Our results suggest that the stability of both enzymes deserves attention. Maximum yields depended on the amount of GOX and quantitative yields could be obtained by increasing the overall amount of both enzymes.

Interestingly, we observed a novel, hitherto unknown non-selective GOX-catalyzed aerobic sulfoxidation of thioanisole, affording racemic sulfoxide which we attribute to competing sulfoxidation catalyzed by the flavin cofactor which is leached from the GOX. This novel side reaction is worthy of further study. The side reaction was dependent on the co-solvent concentration and, hence, product enantiopurity could be improved by decreasing the co-solvent concentration.

Optimizing the Chloroperoxidase – Glucose Oxidase System: The Effect of Glucose Oxidase on Activity and Enantioselectivity

Experimental Part

Materials

Chloroperoxidase from *Caldariomyces fumago* (EC 1.11.1.10) was purchased from Sigma (18500 U / mL) and glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) was purchased from Sigma (185 U / mg). Both enzymes were used as received. Thioanisole, anisole, sulfoxide and *tert*-butyl alcohol were purchased from Aldrich in analytical grade and used without further purification.

Oxidation Reactions

Typical conditions: In a 50 mL round bottom flask containing 12.5 mL of *t*-BuOH and 12.5 mL of phosphate buffer pH 5, 100 mM, 1.25 mmol of thioanisole (1.47 µL) and 227 mg of D-glucose (1 equivalent) were added. To this 33 µL (610U) or 66 µL of chloroperoxidase were added and the mixture was stirred for 5 minutes. The reaction was started with various amounts of glucose oxidase (see tables and Figures for conditions).

Sampling

100 µL of reaction mixture were removed and added to 1 mL of a 5 mM solution of anisole (external standard) in ethyl acetate. To this Na_2SO_3 and $MgSO_4$ were added. The mixture was shaken, centrifuged and analyzed.

Analysis

(*R*) and (*S*)-methyl phenyl sulfoxide were analyzed with gas chromatography using a Shimadzu GC 17A with AOC-201 automatic injector using a Cyclodex Beta column (50m*0.25mm*0.25 um). This was run isothermally at 160 °C for 20 minutes with a column flow of 0,9 mL/min and the retention times were 3,4 minutes for thioanisole; 9,85 minutes for *R*-methyl phenyl sulfoxide and 13,0 minutes for *S*-methyl phenyl sulfoxide. Anisole, thioanisole and methyl phenyl sulfoxide were analyzed with gas chromatography using a Varian 3400 CX gas chromatograph with a Varian 6577 auto sampler using a Cpwax-52B (50m x 0.53mm x 2um).

References

---

1 Duetz, W.A.; van Beilen, J. B.; Witholt, B.; *Current Opinion in Biotechnology* **2001**; 12, 419 - 425

2 Leak, D.J.; Sheldon, R.A.; Woodley, J.M.; Adlercreutz, P.; *Biocatalysis and Biotransformations* **2009**; 27, 1, 1 - 26

3 Torres, P.D.E., Winklerb M, Glieder A, Fraaije M.W.; *Journal of Biotechnology* **2010**; 146, 9–24

4 Monti, D.; Ottolina, G.; Carrea, G.; Riva, S.; *Chemical Reviews* **2011**; 111, 4111 - 4140

5 Hollmann, F.; Arends, I.W.C.E.; Buehler, K.; Schallmey, A; Bühler, B.; *Green Chemistry* **2011**; 13, 226 - 265

6 Regalado, C.; García-Aldemdaréz, B.E.; Duarte-Vázquez, M.A.; *Phytochemistry Reviews* **2004**; 3, 243 - 256

7 Colonna, S; Gaggero, N.; Richelni, C.; Pasta, P.; *Trends in Biotechnology* **1999**; 17, 163 - 168

8 van de Velde, F.; van Rantwijk, F.; Sheldon, R.A.; *Trends in Biotechnology* **2001**; 19, 73 - 80

9 van Deurzen, M.P.J.; van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron* **1997**; 53, 13183 - 13220

10 Allain, E.J.; Hager, L.P.; Deng, L.; Jacobsen, E.N.; *Journal of the American Chemical Society* **1993**; 115, 4415 - 4416

11 Dexter, A.F.; Lakner, F.J.; Campbell. R.A.; Hager, L.P.; *Journal of the American Chemical Society* **1995**; 117, 6412 - 6413

12 Lakner, F.J.; Hager, L.P.; *Journal of Organic Chemistry* **1996**; 61, 3923 - 3925

---

13 Lakner, F.J.; Cain, K.P.; Hager, L.P.; *Journal of Organic Chemistry* **1997**; 119, 443 - 444

14 Colonna, S.; Gaggero, N.; Casella, L.; Carrea, G.; Pasta, P.; *Tetrahedron: Asymmetry* **1992**; 3, 95 - 106

15 Colonna, S.; Gaggero, N; Carrea, G; Pasta, P.; *Chemical Communications* **1997**; 5, 439 - 440

16 van Deurzen, M.P.J.; Remkes, I.J.; van Rantwijk, F.; Sheldon, R.A.; *Journal of Molecular Catalysis A: Chemical* **1997**; 117, 329 - 337

17 Miller, V.P.; Tschirret-Guth, R.A.; de Montellano, P.R.O.; *Archives of Biochemistry and Biophysiscs* **1995**; 319, 333 - 340

18 Zaks, A.; Dodds, D.R.; *Journal of the American Chemical Society* **1995**; 117, 10419 - 10424

19 Hu, S.; Hager, L.P.; *Journal of the American Chemical Society* **1999**; 121, 872 - 873

20 Corbett, M.D.; Chipko, B.R.; *Biochemical Journal* **1979**; 269 - 276

21 van Deurzen, M.P.J.; van Rantwijk, F.; Sheldon, R.A.; *Journal of Molecular Catalysis B: Enzymatic* **1996**; 2, 33 - 42

22 Geigert, J.; Dalietos, D.J.; Neidleman, S.L.; Lee, T.D.; Wadsworth, J.; *Biochemical and Biophysical Research Communications* **1983**; 114, 1104 - 1108

23 van Deurzen, M.P.J.; van Rantwijk, F.; Sheldon, R.A.; *Journal of carbohydrate Chemistry* **97**, 16, 299 - 309

24 Kiljunen, E.; Kanerva, L.T.; *Tetrahedron: Asymmetry* **1999**; 10, 3529 - 3535

25 Kiljunen, E.; Kanerva, L.T.; *Journal of Molecular Catalysis B: Enzymatic* **2000**; 9, 163 - 172

26 Samra, BK; Andersson, M; Adlercreutz, P.; *Biocatalysis and Biotransformation* **1999**; 17, 381 - 391

27 Hu, S.; Dordick, J.S.; *Journal of Organic Chemistry* **2002**; 67, 314 - 317

28 Okrasa, K.; Falcimaigne, A.; Guibé-Jampel, E. ; Therisod, M.; *Tetrahedron: Asymmetry* **2002**; 13, 519 - 522

29 Park, J.B.; Clark, D.S.; *Biotechnology and Bioengineering* **2006**; 93, 1190 - 1195

30 Ayala, M; Batista, C.V.; Vazquez-Duhalt R.; *Journal of Biological Inorganic Chemistry* **2011**; 16; 63–68

31 Seelbach, K.; van Deurzen, M.P.J.; van Rantwijk, F.; Sheldon, R.A.; Kragl, U.; *Biotechnology and Bioengineering* **1997**; 55, 283 - 288

32 Perez, D.I.; Grau, M.M.; Arends, I.W.C.E.; Hollmann,F.; *Chemical Communications* **2009**; 6848 - 6850

33 van de Velde, F.; Lourenço, N.D.; Bakker, M.; van Rantwijk, F.; Sheldon, R.A.; *Biotechnology and Bioengineering* **2000**; 69, 286 – 291

34 Lutz, S.; Steckhan, E.; Liese, A.; *Electrochemistry Communications* **2004**; 6, 583 - 587

35 Churakova, E.; Arends I.W.C.E.; Hollmann F.; *ChemCatChem* **2013**; 5, 565-568

36 van Rantwijk, F.; Sheldon, R.A.; *Current Opinion in Biotechnology* **2000**; 11, 554 - 564

37 Borole, A.; Dai, S.; Cheng, C.L.; Rodriguez Jr, M.; Davison, B.H.; *Applied Biochemistry and Biotechnology* **2004**; 113 – 116, 273 – 285

38 Jung, D.; Streb, C.; Hartmann, M.; *Microporous and Mesoporous Materials* **2008**; 113, 523 – 529

39 Jung, D.; Streb, C.; Hartmann, M.; *International Journal of Molecular Sciences* **2010**; 11, 762 - 778

40 van Deurzen, M.P.J.; Seelbach, K; van Rantwijk, F.; Kragl, U.; Sheldon, R.A.; *Biocatalysis and Biotransformation* **1997**; 15, 1 - 16

41 Sanfilippo, C.; D´Antona, N.; Nicolosi, G.; *Biotechnology Letters* **2004**; 26, 1815-1819

42 Rioz-Martinez, A.; de Gonzalo, G.; Pazmiño, D.E.T; Fraaije, M.W.; Gotor, V.; *European Journal of Organic Chemistry* **2010**; 6409 - 6416

43 Imada, Y.; Kitagawa, T.; Wang, H-K.; Komiya, N.; Naota, T.; *Tetrahedron: Letters* **2013**; 54, 621–624

44 Imada, Y.; Iida, H.; Ono, S.; Masui, Y.; Murahashi, S-I.; *Chemistry - An Asian Journal* **2006**; 1–2, 136–147

45 Lindén, A.A.; Johansson, M.; Hermanns, N.; Bäckvall, J-E.; *Journal of Organic Chemistry* **2006**; 71, 3849–3853

46 Lindén, A.A.; Krüger, L.; Bäckvall, J-E.; *Journal of Organic Chemistry* **2003**; 68, 5890–5896

47 Minidis, A.B.E.; Bäckvall, J-E.; *Chemistry - A European Journal* **2001**; 7, 297-302

48 Ellis, P.B.; Morris, D.L.; *US 4268631* **1981**

49 Młochowski, J.; Peczyńska-Czoch, W.; Piętka-Ottlik, M.; Wójtowicz-Młochowska, H.; *Open Catalysis Journal* **2011**; 4, 54–82

50 Dai, L.; Klibanov, A.M.; *Biotechnology and Bioengineering* **2000**; 70, 353-357

Chapter 5

Screening of the Biocatalytic Potential of Limonene Epoxide Hydrolase

6

Introduction

Enantiopure epoxides are attractive intermediates for producing chiral compounds with high added-value, like pharmaceuticals. They are versatile compounds due to their high reactivity towards different nucleophiles, affording bifunctional compounds [1,2] (Figure 1).

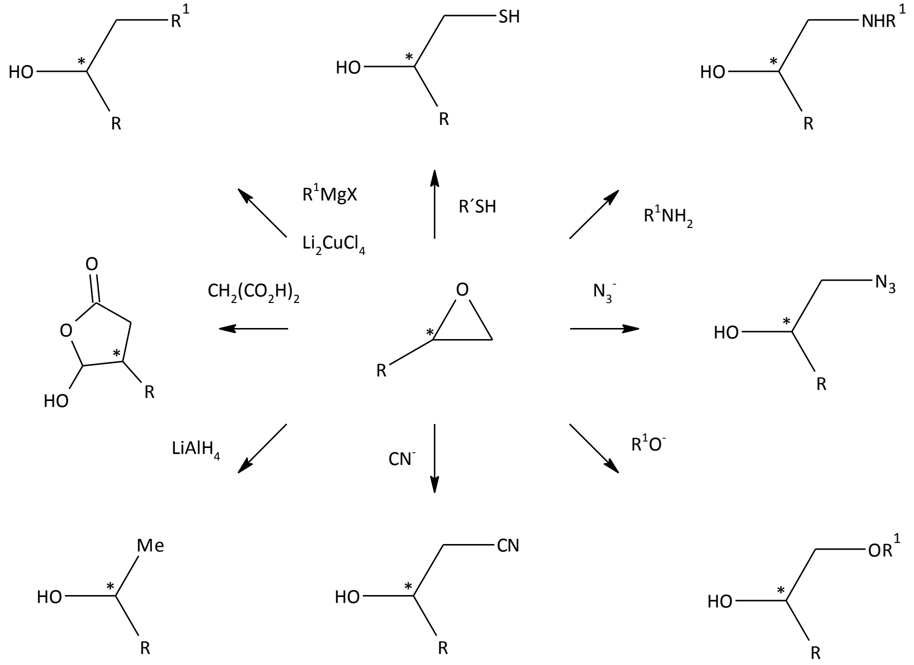


Figure 1: Examples of reactions of epoxides with a chiral centre

A representative example is the case of *cis*-aminoindanol, a key intermediate in the synthesis of protease inhibitor Indanavir sulfate (CRIXIVAN), which is used in the treatment of AIDS. This chiral aminoalcohol is derived from chiral indeneoxide [3] (Figure 2).

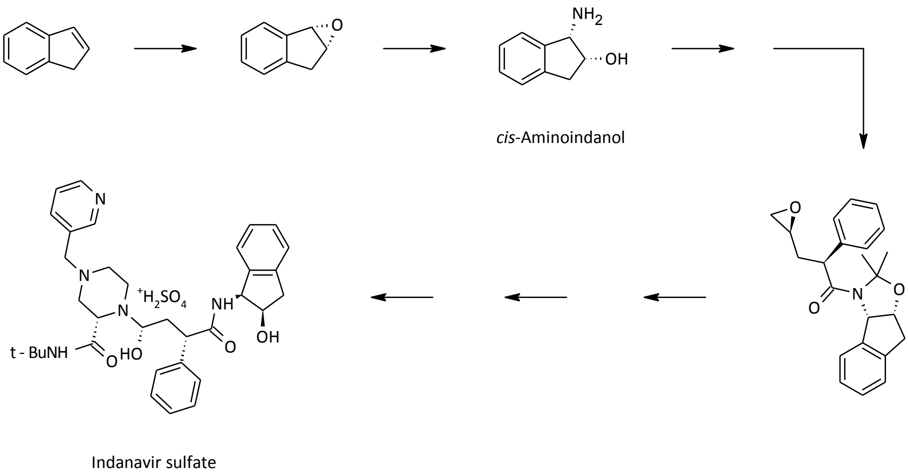


Figure 2: Synthesis of Indanavir sulfate (CRIXIVAN) via chiral Indene oxide

The preparation of enantio enriched epoxides is therefore a significant target for asymmetric synthesis and, particularly, the identification of catalytic asymmetric olefin oxidation methods has been an area of research for several decades [4]. The Sharpless asymmetric epoxidation reaction and the use of chiral (Salen) Mn^{III} complexes are known examples of methods that have provided general access to enantiomerically enriched epoxides. Methods involving organocatalysts have also been reported [5,6,7].

The oxidative synthesis of chiral epoxides can also be achieved using enzymes [1]. Monoxygenases such as cytochromes P-450, and peroxidases such as chloroperoxidase, are representative examples.The former can use molecular oxygen and incorporate one oxygen atom into the substrate while reducing the other to water, converting alkenes into epoxides [8]. The latter was shown to be highly effective in the enantioselective catalytic epoxidation of various simple olefins using hydrogen peroxide [9].

Another approach to prepare enantiopure epoxides is by kinetic resolution of racemic mixtures, for which several chemical [10] and biocatalytic [2] procedures are available. The major disadvantage of this strategy is that the theoretical maximum yield of the chiral compound, either the unconverted substrate or the product of the reaction, is only 50% (but see later).

Epoxide hydrolases can catalyze the hydrolytic kinetic resolution of epoxides, leading to the formation of enantiopure diols and remaining enantiopure epoxides [11]. Halohydrin dehalogenases are enzymes that catalyze the conversion of vicinal halohydrins into their corresponding epoxides. Alternatively they can catalyze the kinetic resolution of racemic epoxides by enantioselective non-hydrolytic ring-opening using anionic nucleophiles, affording bifunctionalized compounds and the unconverted enantiopure epoxides (Figure 3) [12,13].

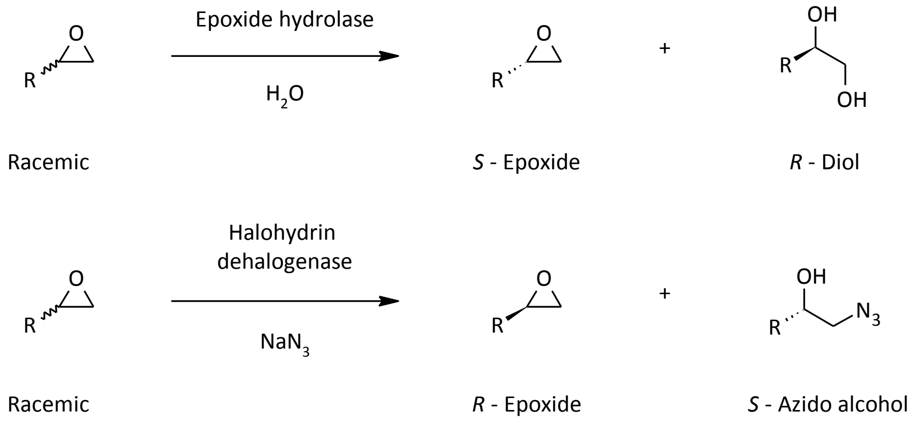


Figure 3: Reaction schemes of a kinetic resolution using an epoxide hydrolase and a kinetic resolution using a halohydrin dehalogenase

The ability of halohydrin dehalogenases to insert an unnatural nucleophile has been used to synthesize enantiopure cyanoalcohols from racemic chlorohydrins in one pot (Figure 4).In the first step the racemic halohydrin is resolved affording an enantiopure epoxide and, in the presence of cyanide, the enzyme will convert this epoxide into its corresponding cyanoalcohol [14,15].

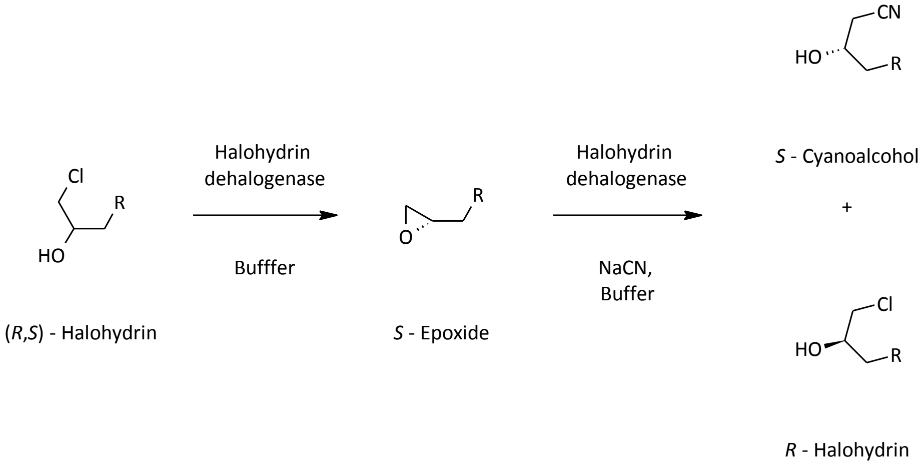


Figure 4: Reaction scheme of a sequential kinetic resolution affording an enantiopure cyanoalcohol and an enantiopure halohydrin

Another example of the variety of processes involving epoxides is the enantioconvergent synthesis of enantiopure diols with two different epoxide hydrolases. It is noteworthy mentioning this process because enantiopure diols are interesting and valuable compounds.

This procedure overcomes the intrinsic limitation of 50% theoretical yield characterizing kinetic resolutions [16]. The two selected enzymes accept the opposite enantiomers and insert a water molecule in a different position, affording only one enantiopure product (Figure 5).

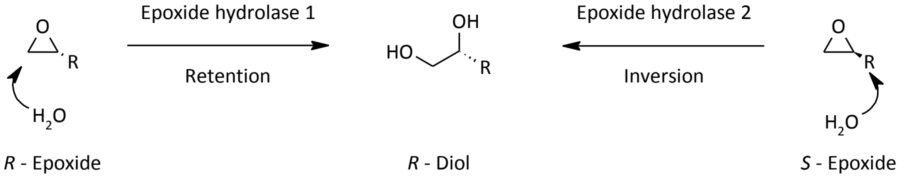


Figure 5: Reaction scheme of an enantioconvergent synthesis of an enantiopure diol using two different epoxide hydrolases

Most epoxide hydrolases belong to the α/ß-hydrolase fold epoxide hydrolases. In this case, epoxide ring opening proceeds via a catalytic mechanism involving a covalent intermediate with an aspartate residue, followed by hydrolysis in the second step [17] (Figure 6B). Other epoxide hydrolases display ring opening mechanisms which do not go via a covalent intermediate, for example leukotriene A4 epoxide hydrolases in which the ring opening is facilitated by a zinc atom. Epoxide ring opening with halohydrin dehalogenases occurs via non-covalent catalysis, assisted by tyrosine and arginine residues [18].

One example of an epoxide hydrolase that does not belong to the α/ß-hydrolase fold family limonene epoxide hydrolase from *Rhodococcuserythropolis*. LEH is a cytoplasmic enzyme with a molecular mass of 17 kDa.

This separates it from the class of α/ß-hydrolase fold epoxide hydrolases, which have an estimated minimum molecular mass of 25 kDato allow the reaction mechanism used by this class [19]. The putative LEH mechanism, as depicted in Figure 6A, is substantially different from that of the α/ß-hydrolase fold epoxide hydrolases, as it seems to occur without the formation of a covalent enzyme-substrate intermediate [20].

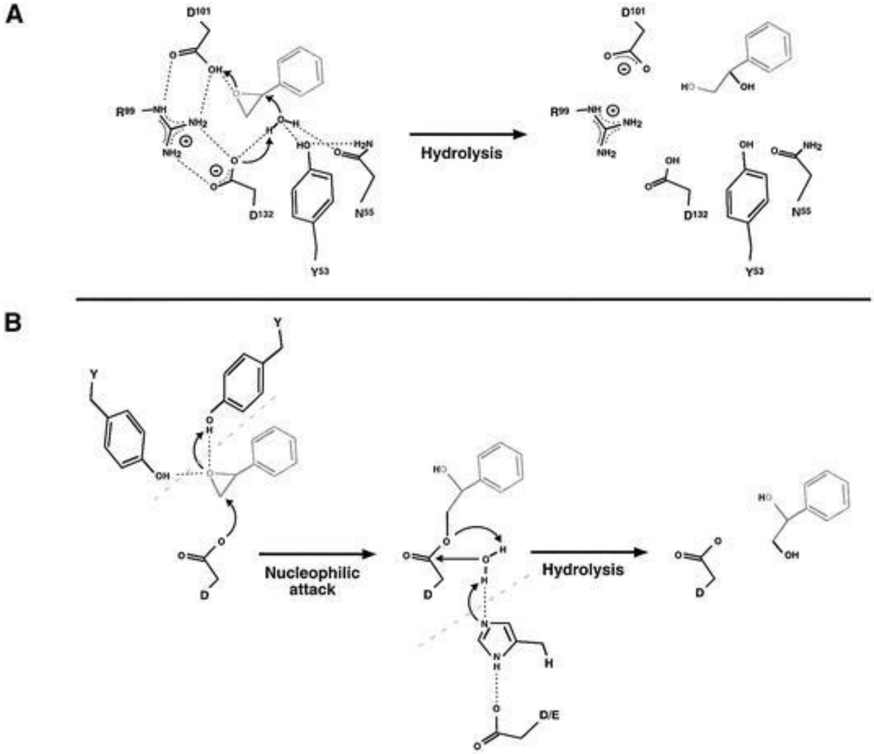


Figure 6: Mechanisms of epoxide hydrolases. Proposed mechanism for LEH (A) and α/β-hydrolase fold epoxide hydrolases mechanism (B) with styrene oxide as example (adapted from [20]).

The selectivity of LEH differs from that observed in α/β-hydrolase fold epoxide hydrolases [21]. LEH mediated hydrolysis of 1-methylcyclohexeneoxide occurs with attack on the more substituted carbon, contrary to the α/β-hydrolase fold epoxide hydrolases, suggesting an acid-catalyzed mechanism (Figure 7).

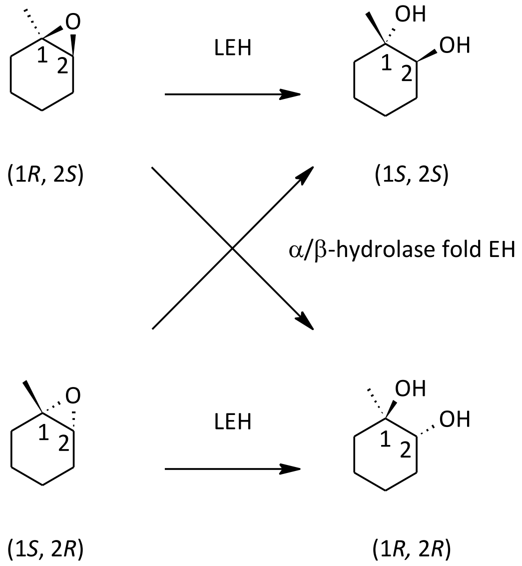


Figure 7: Selectivity difference between LEH and α/ß-hydrolase fold epoxide hydrolases, in the hydrolysis of 1-methylcyclohexe oxide.

On the other hand, hydrolysis of the four stereoisomers of limonene-1,2-epoxide only happens with attack on the most substituted carbon with two of the four possible isomers: the (-)-*cis*(1*S*,2*R*,4*S*) and the (+)-*cis*(1*R*,2*S*,4*R*). In the other two cases, the (-)-*trans*(1*R*,2*S*,4*S*) and the (+)-*trans*(1*S*,2*R*,4*S*), the attack occurs at the least substituted carbon.

A comparison between1-methylcyclohexene oxide and limonene-1,2-oxide shows that when the substituent at C_4 is *cis*, or has no substituent, then the nucleophilic attack occurs at C_1. If the substrate is *trans* then the attack occurs at C_2.

In both cases there is inversion of configuration of the attacked carbon. This means the orientation of the substituent group in $C_4$is what determines the final product configuration, although it is far from the epoxide ring (Figure 8).

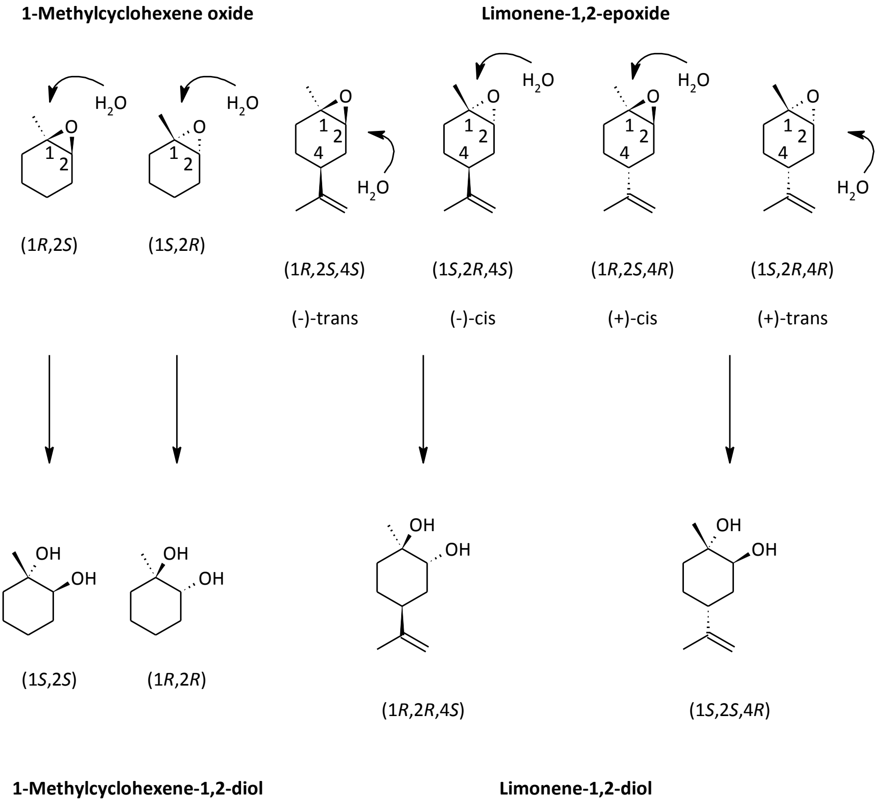


Figure 8: LEH different selectivity in the hydrolysis of 1-methylcyclohexene oxide and in the enantioconvergent hydrolysis of limonene-1,2-epoxide, with inversion of configuration of the attacked carbon atom.

A theoretical examination of the catalytic mechanism of LEH concluded it is a generic acid catalyzed mechanism involving protonation of the epoxide by an aspartate residue, followed by nucleophilic attack of a water molecule.

Simultaneously, it was also concluded that the factors that control regioselectivity in the hydrolysis of limonene-1,2-epoxide are conformational and not electronic [22].

LEH catalyzes the hydrolysis of some alicyclic and 2-methyl-1,2-epoxides but has a narrow substrate specificity [19,23]. The best substrate for the enzyme is the (1*R*,2S,4*R*), or (+)-*cis*, isomer of limonene-1,2-oxide.

The difference in activity between 1-methylcyclohexe oxide and cyclohexene oxide suggests a methyl group at the oxirane ring is required for enzymatic activity (Table 1).The influence in reactivity of a substituent group in the C_4 position is not known.

Table 1: LEH substrate specificity [19,23]

Substrate	***Relative Activity (%)***
(+)-*cis*Limonene-1,2-epoxide (1*R*,2*S*,4*R*)	100
(-)-*trans*Limonene-1,2-epoxide(1*R*,2*S*,4*S*)	65
(-)-*cis* Limonene-1,2-epoxide (1*S*,2*R*,4*S*)	58
(+)-*trans* Limonene-1,2-epoxide (1*S*,2*R*,4*R*)	7,8
1-Methylcyclohexene oxide	47
Cyclohexene oxide	4,0
Indene oxide	57
1,2-Epoxy-2-methylheptane	10
1,2-Epoxy-2-methyl-6-heptene	4,0
1,2-Epoxy-2,6.dimethyl-5-heptene	4,0

In the following work we explore the scope of limonene epoxide hydrolase from *Rhodococcuserythropolis* using different approaches such as nucleophile screening, solvent engineering and immobilization. The use of 4-methylcyclohexene oxide as substrate was also tested.

The viability of immobilizing LEH, for example as a CLEA, opens the door for the possibility of a COMBI-CLEA with one or more enzymes (see chapter 4). An example of an attractive prospect is a COMBI-CLEA comprising LEH, GOX and CPO (see chapter 5). This would allow for a cascade procedure towards an enantiopure vicinal diol, simply starting from an olefin and glucose, without necessity to isolate the intermeadiates (Figure 9).

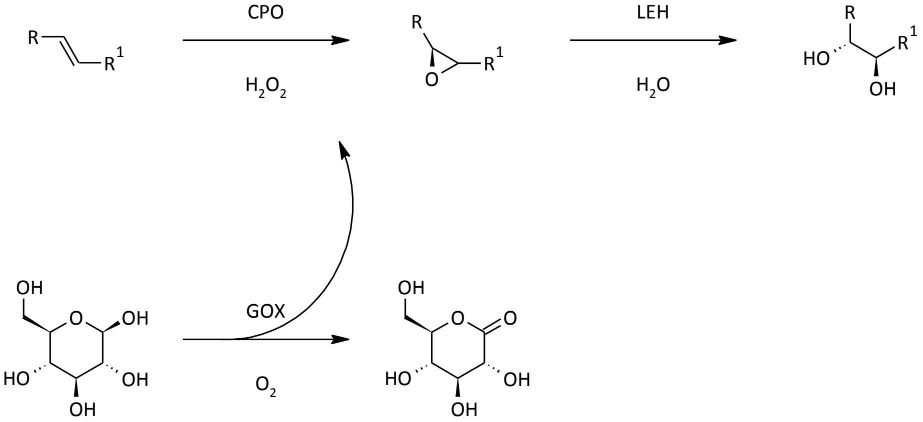


Figure 9: Cascade synthesis of an enantiopure vicinal diol in one pot, using a triple COMBI-CLEA with glucose oxidase, chloroperoxidase and limonene epoxide hydrolase.

Similarly, enantiopure bifunctionalized compounds could be synthesized using a putative capacity of LEH to accept other nucleophiles than water (Figure 10).

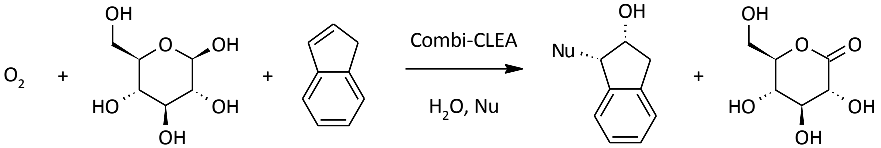


Figure 10: Cascade synthesis of an enantiopure bifunctionalized compound in one pot, using a triple COMBI-CLEA with glucose oxidase, chloroperoxidase and limonene epoxide hydrolase.

Results and Discussion

Characterization of native LEH

Limonene epoxide hydrolase was obtained by rupture of cells of *Rhodococcus erythropolis*. The cell-free extract was characterized in the natural reaction, (Figure 11) under different substrate and enzyme concentrations (Figure 12), in order to define a valid activity test for comparing various LEH formulations in this work.

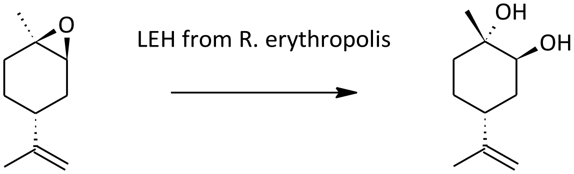


Figure 11: (+)-*cis*-limonene-1,2-epoxide (1*R*,2*S*,4*R*) hydrolysis with limonene epoxide hydrolase from *Rhodococcus erythropolis*

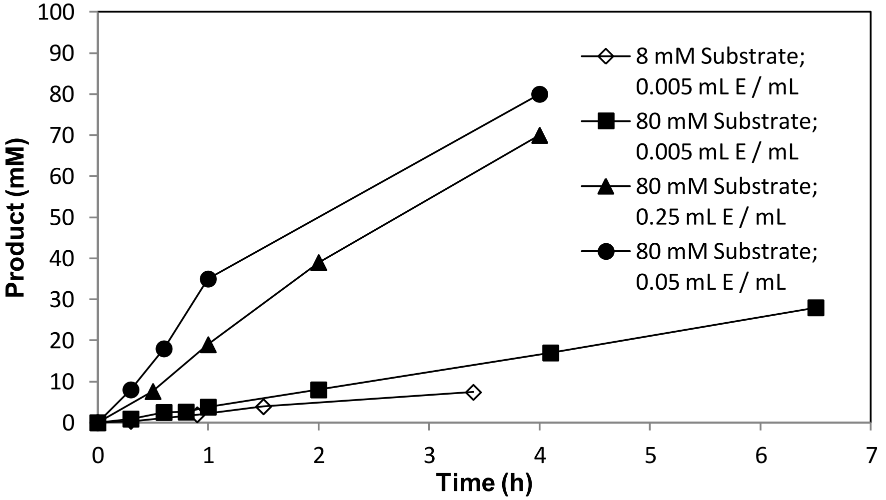


Figure 12: LEH catalyzed (+)-*cis*-limonene-1,2-epoxide hydrolysis with different substrate and enzyme concentrations (mL cell free extract / mL), at 40 °C in phosphate buffer (50 mM; pH 7).

Table 2: Initial rates found in the reaction conditions tested (Figure 11)

[S] (*mM*)	*V* (*mL*)	*LEH* (*µL*)	*V_i* (*mM/hr*)	*V_i* (*µmol/min.mL*)
8	20	100	2,24	7,5
80	2	100	4,23	14
80	2	50	19,2	13
80	2	10	38,8	13

At 80 mM (+)-*cis*-limonene-1,2-epoxide we have found proportionality between enzyme amount and initial reaction rate, validating the activity test (Figure 13).

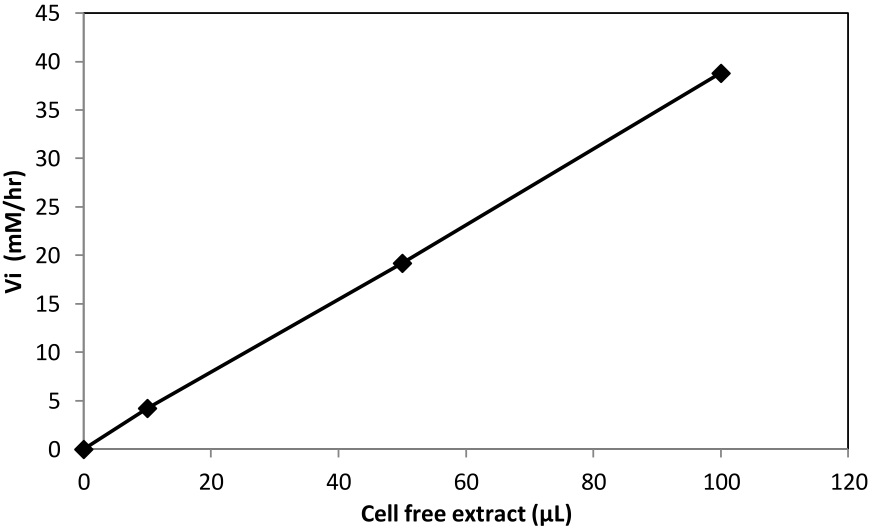


Figure 13: Initial rate in the LEH catalyzed hydrolysis of (+)-*cis*-limonene-1,2-epoxide with different enzyme concentrations (µL cell free extract / mL) at 40 °C in phosphate buffer (50 mM; pH 7).

The specific activities measured at different substrate concentrations (Table 2) allowed us to estimate the values of V_{max} and K_M (Table 3). These confirm that at 80 mM we are close enough to V_{max}, ensuring the limiting factor is solely the activity of the enzyme and not the amount of substrate available.

Table 3: Estimated K_M and V_{max} for limonene epoxide hydrolase

LEH formulation	*V_{max}(µmol/min.mL)*	*K_M (mM)*
LEH cell free extract	14,1	7,1

Nucleophile Screening

The synthetic potential of hydrolases can be increased by the possibility of using other nucleophiles than water. Known examples are the cases of lipases where amines were used to prepare amides [24], penicillin acylase in the resolution of amines through acylation [25] and of glycosidases in the coupling of sugars to an alcohol [26].

In epoxide ring-opening chemistry this would permit obtaining enantiopure ß-substitued alcohols from racemic epoxides via an enzymatic kinetic resolution. Epoxide ring-opening by nucleophiles such as halides, cyanide, azide and nitrite, was reported using the enzyme halohydrin dehalogenase [12].

The choice of nucleophile proved to be crucial due to the thermodynamic equilibrium of the reaction. For example, ring opening by halides yielded little or no halohydrin whereas azide resulted in almost full conversion into the corresponding azido-alcohol [13].

In the case of a hydrolytic epoxide ring-opening such as ours, we have reasoned that the screening of different nucleophiles in an aqueous mixture would be severely hampered by the competing hydrolysis. We therefore opted to look for inhibition of the hydrolytic process in the presence of several nucleophiles.

We started by testing the effect of chloride up to a concentration of 4 M (Table 4). The enzyme was found to lose activity at high concentrations of sodium chloride. Because this loss occurs suddenly at very high saline concentrations, we attributed this effect to the high ionic strength rather than competitive inhibition from chloride.

Table 4: LEH catalyzed hydrolysis of (+)-*cis*-limonene oxide in the presence of sodium chloride at pH 7

[NaCl] (M)	*Yield* $diol_{1\ hr}$ *(%)*
0,0	55
1,0	55
2,0	42
4,0	2,0

We proceeded by testing cyanide, thiocyanate, hydroxylamine, ethanolamine and azide up to 0,5 M concentrations(Table 5). The diol formation was found to decrease to some extend in the presence of these nucleophiles.

Table 5: LEH catalyzed hydrolysis of a mixture of (+)-*cis/trans*-limonene oxide in the presence of several nucleophiles

Second Nucleophile	*Concentration (mM)*	*pH*	Yield $diol_{4\ hrs}$ (%)
-	-	7	52
-	-	8	42
Cyanide	250	7	51
Thiocyanate	250	7	44
Hydroxylamine	250	8	41
Ethanolamine	250	7	35
Azide	250	7	32
Cyanide	500	7	42
Thiocyanate	500	7	36
Hydroxylamine	500	8	42
Ethanolamine	500	7	36

To further investigate the effects of the presence of unnatural nucleophiles in LEH catalyzed openings, we have chosen to study the reactivity of azide in more detail. Azide is an interesting nucleophile because organic azides are useful in organic synthesis. Moreover, the formation of the corresponding azidohydrin of limone oxide should be thermodynamically favorable.

For this purpose we have synthesized the corresponding azidohydrins of both (+)-*cis*-limonene oxide and (+)-*trans*-limonene oxide, with sodium azide (10%) in a mixture of water and acetonitrile. In each separate reaction only one product was obtained (Figure 14).

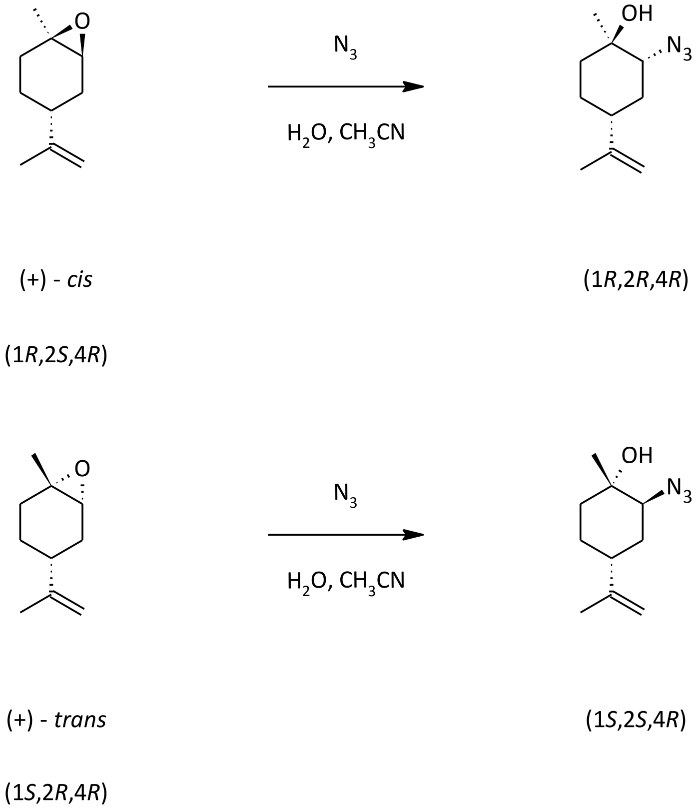


Figure 14: Chemical azidolysis of (+)-*cis* and (+)-*trans* limonene-1,2-oxide

This suggests the mechanism of chemical azidolysis was SN_2 with nucleophilic attack at the least substituted carbon atom and not SN_1 via an intermediary carbocation, in the most substituted carbon, which would have led to the formation of two diastereomers.

The SN_2 mechanism is consistent with the use of a strong nucleophile such as azide and mild basic conditions. The two synthesized C_2 azidohydrins could be discriminated by GC and were identified with GC MS.

Limonene epoxide hydrolase is enantioconvergent, which means that when starting from the (+)-*cis*/*trans* limonene-oxide mixture there is only one hydrolysis product and the same happens when starting from the (-)-*cis*/*trans* mixture. These two products are enantiomers. The preferred substrate isomers are always the (1*R*,2*S*) and the nucleophilic attack occurs at C_1 when the substrate is *cis* and at C_2 when the substrate is *trans* (Figure 15).

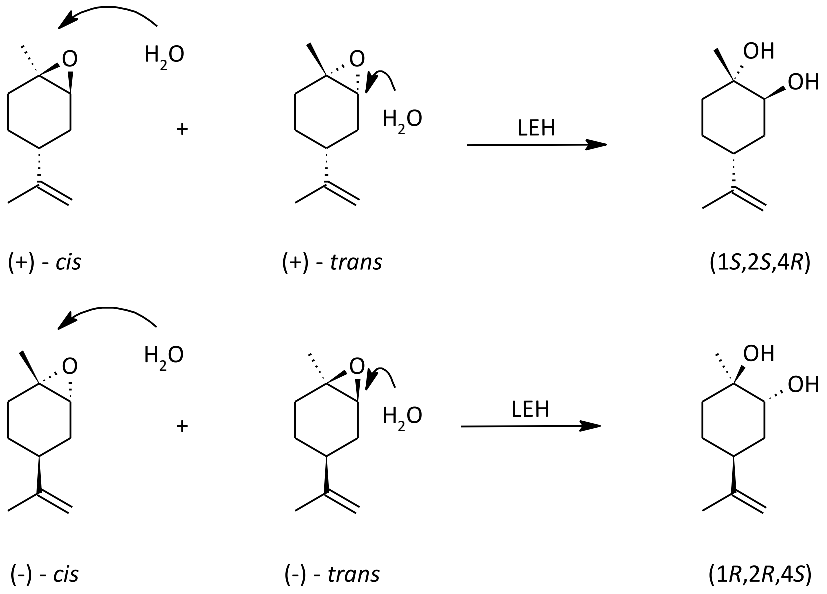


Figure 15: Enantioconvergency of LEH in the hydrolysis of limonene-1,2-oxide

Because the position where water is inserted depends on the substrate molecule configuration, we reasoned that a study of LEH mediation in azide insertion could only be complete by testing the four limonene-1,2-epoxide isomers.

According to the known selectivity (Figure 8 and Figure 15) an enzymatic azide insertion using the (+)-*trans* isomer of limonene-1,2-epoxide would be expected to occur at the same position as the chemical background reaction, because with this substrate the enzyme mediated water attack occurs at C_2 (Figure 16). It should be noted that this isomer is the enzyme´s less preferred isomer and consequently a high competition from the chemical azidolysis reaction is to be expected.

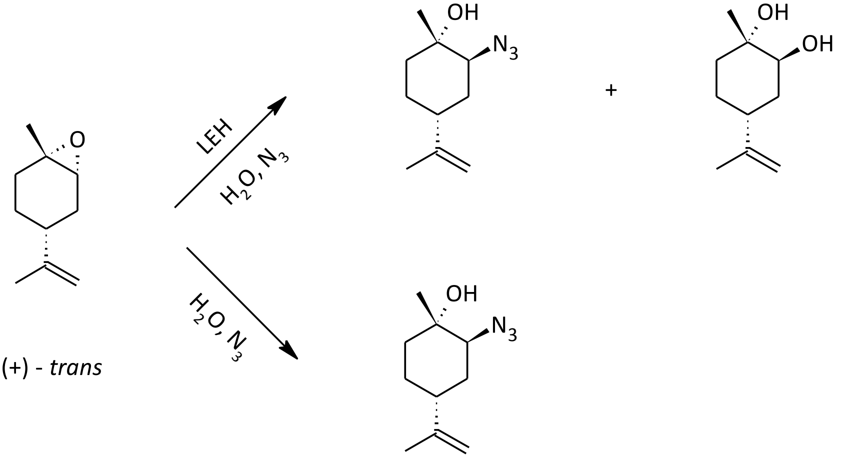


Figure 16: Chemical azidolysis product of (+)-*trans* limonene-1,2-oxide, LEH hydrolysis product and LEH catalyzed azidolysis expected product in aqueous media

The reactions performed with LEH afforded higher amounts of azidoalcohol then the background reaction. However, this difference remained approximately the same in the course of time (Table 6).

Table 6: Azidolysis of (+)-*trans* limonene-1,2-oxide in the presence of LEH

Substrate	*Time (hrs)*	*[E] (U)*	*[N_3] (mM)*	*Yield* *diol*(%)	Yield azidohyrin(%)
(+)-*trans*	1	0,6	250	0,2	1,5
(+)-*trans*	1	-	250	0,0	0,0
(+)-*trans*	4	0,6	250	3,0	6,6
(+)-*trans*	4	-	250	0,0	3,9
(+)-*trans*	6	0,6	250	4,2	10
(+)-*trans*	6	-	250	0,0	7,5

In the following experiment we used the (+)-*cis* isomer, which is the preferred substrate isomer. An enzymatic insertion would afford an azidoalcohol with the azide group at C_1, a compound we have not synthesized (Figure 17).

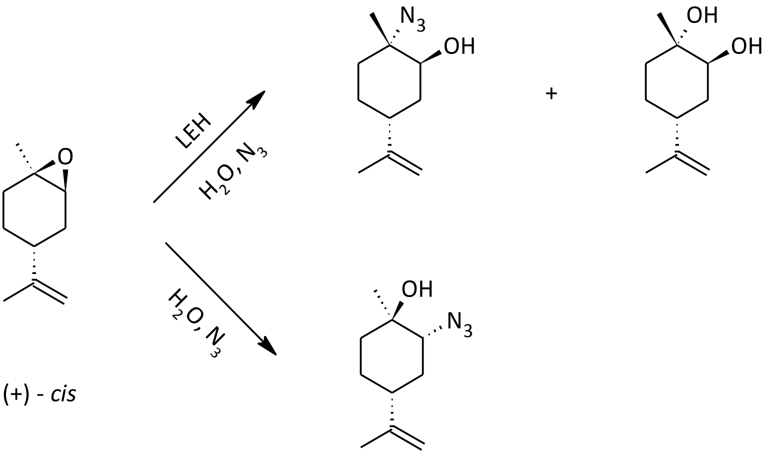


Figure 17: Chemical azidolysis product (+)-*cis* limonene-1,2-oxide, LEH hydrolysis product and LEH catalyzed azidolysis expected product in aqueous media

We have however not detected the formation of a new product with the (+)-*cis* isomer. The azidohydrin yield was comparable both in presence and in absence of the enzyme (Table 7).

Table 7: Azidolysis of (+)-*trans* limonene-1,2-oxide in the presence of LEH

Substrate	*Time* *(hrs)*	*[E]* *(U)*	*[N_3]* *(mM)*	*Yield*$_{diol}$ *(%)*	Yield$_{azidohyrin}$ (%)
(+)-*cis*	3	1,2	250	8,6	4,4
(+)-*cis*	3	-	250	0,0	2,5
(+)-*cis*	6	1,2	250	17	4,1
(+)-*cis*	6	-	250	0,0	6,2

When starting with the (+)-*cis*/*trans* limonene-1,2-oxide isomeric mixture, the possible products from an aqueous enzyme mediated azidolysis are one diol and three azidoalcohols (Figure 18). These are a compound with azide at C_1 derived from the (+)-*cis* isomer together with two azidohydrins at C_2 derived from the from both *cis* and *trans*. Enzyme activity would generate a diastereomeric excess favoring the formation of the (1*S*,2*S*,4*R*) azidoalcohol.

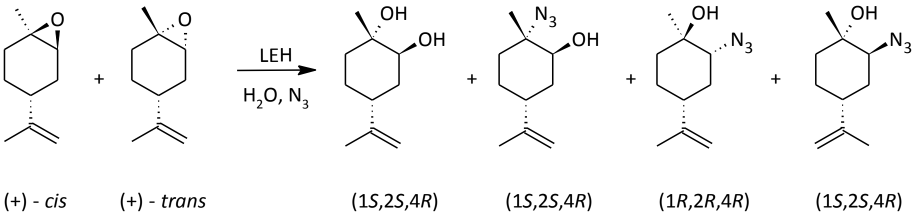


Figure 18: Possible products in the LEH catalyzed azidolysis of (+)-*cis/trans* limonene-1,2-oxide mixture in aqueous media

The reactions performed with LEH afforded an enriched mixture with the (1*S*,2*S*,4*R*) azidoalcohol when compared with the background reactions and the absolute amount formed was higher than in the background reaction (Table 8). However, we have not observed a third azidoalcohol formation which would correspond to the enzymatic product of the reaction with the (+)-*cis* isomer.

Table 8: Azidolysis of (+)-*cis/trans* limonene-1,2-oxide in the presence of LEH

Time (hrs)	*[E] (U)*	*[N$_3$] (mM)*	*Yield (%)* $_{diol}$	*Yield (%) C$_{2\ azidohyrin}$ (1R,2R,4R)*	*Yield (%) C$_{2\ azidohyrin}$ (1S,2S,4R)*	**d.e. (%)**
1	0,6	250	14	0,5	2,0	57
1	-	250	0	0,3	0,0	100
4	0,6	250	32	0,1	4,4	95
4	-	250	0	2,0	2,0	0
6	0,6	250	38	0,1	6,4	96
6	-	250	0	2,9	3,1	4,1

The diastereomeric excess obtained in the reactions carried out with enzyme can be explained by a faster consumption of the (+)-*cis* isomer in the hydrolysis reaction, leaving higher amounts of the (+)-*trans* available for the chemical azidolysis. On the other hand, we could not explain the higher formation of the (1*S*,2*S*,4*R*) azidohydrin than in the background reaction.

This effect had already been previously observed with the experiments using solely the (+)-*trans* isomer. The observed yields of the chemical background azidolysis (Table 9) must be the total chemical azidolysis because a C_1 azidoalcohol could not be formed chemically, as shown above. This experiment was repeated with two other enzyme concentrations and these results could be reproduced (Table 9).

Table 9: Azidolysis of (+)-*cis/trans* limonene-1,2-oxide in the presence of LEH

Time (hrs)	*[E] (U)*	*[N_3] (mM)*	*Yield (%) diol*	*Yield (%) C_2 azidohyrin (1R,2R,4R)*	*Yield (%) C_2 azidohyrin (1S, 2S,4R)*	d.e. (%)
6	7,2	250	45	0	5,2	100
6	-	250	0	2,4	3,5	18
6	4,3	250	21	0,2	7,4	94
6	-	250	0	2,6	3,6	8,0

The relative activity of the enzyme towards the two isomers tested thus far, which correspond to the (+)-*cis/trans* mixture, is different than the one towards the isomers of the (-)-*cis/trans* mixture (Table 10). In the first case, the preference for the *cis* isomer is more than ten-fold higher than for the *trans* (entries 1 and 4). On the other hand, with the (-)-*cis/trans* mixture the preference for each isomer is comparable and the preferred isomer is the *trans* (entries 2 and 3).

In addition to this, we have further measured the activity for the (-)-*cis/trans* mixture. In Table 10 the hydrolysis activity for the different is listed and we have obtained a lower value than the one published (entry 6).

Table 10: LEH activity with isolated limonene-1,2-oxide isomers and with the (-)-*cis/trans* mixture

Entry	*Limonene-1,2-oxide isomer*	*Activity (%)*
1	(+)-*cis* limonene-1,2-oxide (1*R*,2*S*,4*R*) [23]	100
2	(-)-*trans* limonene-1,2-oxide (1*R*,2*S*,4*S*) [23]	65
3	(-)-*cis* limonene-1,2-oxide (1*S*,2*R*,4*S*) [23]	58
4	(+)-*trans* limonene-1,2-oxide (1*S*,2*R*,4*R*) [23]	7,8
5	(-)-*cis/trans* limonene-1,2-oxide [19]	27
6	(-)-*cis/trans* limonene-1,2-oxide	18

Then we proceeded with the testing of azide in the presence of LEH. The possible products in the case of the (-)-*cis/trans* mixture are the corresponding enantiomers of the possible products in the case of the (+)-*cis/trans* mixture (Figure 19). In the case of enzyme mediation in the (-)-*cis/trans* azidolysis, one azidohydrin with the azide in C_1 is expected together with an enriched mixture of C_2 azidohydrins favouring the (1*R*,2*R*,4*S*) isomer.

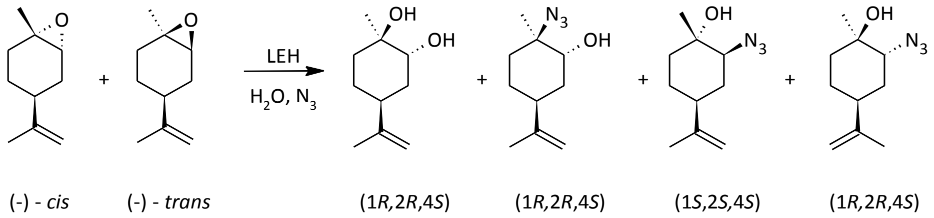


Figure 19: Possible products in the LEH catalyzed azidolysis of (-)-*cis/trans* limonene-1,2-oxide mixture in aqueous media

Table 11: Azidolysis of (-)-*cis/trans* limonene-1,2-oxide in the presence of LEH

Time (hrs)	*[E] (U)*	*[N$_3$] (mM)*	*Yield (%) $_{diol}$*	*Yield (%) C$_{2\ azidohyrin}$ (1S,2S,4S)*	*Yield (%) C$_{2\ azidohyrin}$ (1R,2R,4S)*	d.e. (%)
4	0	500	0	3,7	4,1	4,5
4	1,9	500	42	5,3	2,2	41
4	3,8	500	73	6,3	0,8	76
4	0	250	0	2,3	2,2	1,9
4	1,9	250	41	2,1	1,3	23
4	3,8	250	64	2,5	0,6	61
4	0	125	0	1,4	1,1	12
4	1,9	125	41	1,1	0,7	26
4	3,8	125	63	1,7	0,8	34

We have again observed an increasing diastereomeric excess with increasing amounts of enzyme (Table 11). Similarly to the case of the (+)-*cis/trans* mixture this can be explained by higher availability of one of the substrate isomers, due to a faster consumption of the other isomer in the hydrolysis reaction.

Another similarity to the previous case is the higher absolute formation of one azidohydrin when the reaction was performed with enzyme. However, the differences between background and enzymatic reaction are smaller than those observed previously (Table 11). Moreover, the overall azidohydrin formation was clearly dependent on the azide concentration and the (1*S*,2*S*,4*S*) azidoalcohol isomer, which was formed in growing amounts when in presence of LEH, corresponds to the product which could have only been formed chemically.

Taking the known selectivity of the enzyme as a starting hypothesis, we could not find evidence for an enzyme mediated azidolysis. However, it is noteworthy that the d.e.´s we found are quite high, already at low conversion values. In the case of a substrate depletion induced d.e., we would expect lower azidohydrin formation rates and as a result much lower diastereomeric excesses.

In addition to this, the hypothesis of an enzyme mediated formation of a C_1azidohydrin was not verified, as we have only synthesized two reference C_2azydohyrins. In that case, the possibility that these compounds would overlap with our references cannot be excluded.

In order to pursue unnatural nucleophilic insertion studies development towards the use of this enzyme in anhydrous media must be performed, in order to reduce the competition from the enzymatic hydrolysis and the chemical background reaction.

Solvent Engineering

The use of organic solvents, or aqueous mixtures of organic solvents, is a useful tool to improve the applicability of enzymes. An often used system is a two phase system, consisting of a water phase and a water-immiscible solvent phase.

The solvent phase acts here as a reservoir for the hydrophobic reactants, while catalysis takes place in the water layer. To stabilize the resulting emulsions, surfactants may be added [27].

Homogeneous water-solvent mixtures are another possible system, mainly with the purpose of increasing substrate solubility. A well-known example is *t*-butanol, which is tolerated in concentrations as high as 50% (v/ v) by some enzymes (see chapter 5). However, the presence of miscible co-solvents can result in loss of activity.

In the previously mentioned cases there is sufficient water to ensure retention of enzymatic activity. In some cases enzymes retain activity in the absence of water or in the presence of very small amounts [28]. Immobilized lipases are used in complete anhydrous systems, allowing for reactions with unnatural nucleophiles [29,30].

The choice of solvent can affect selectivity, rate and stability of the biocatalyst, regardless of the system. Solvent engineering is, therefore, a relevant part of the development of a biocatalytic process. In a first attempt to evaluate the tolerance of LEH for organic solvents, we screened a series of solvents at high concentrations, measuring the formation of diol (Table 12).

Table 12: LEH hydrolysis of *cis*-(+)-Limonene-1,2-epoxide in solvent:buffer (v / v) mixtures

Solvent	*Yield$_{2\ hrs}$ (%)*
Dioxane (95%)	0
Dimethoxyethane (95%)	0
t-Butanol (25%)	0
t-Amylalcohol (95%)	0
Diethylether (95%)	0
Ehtylacetate (95%)	0
Tetrahydrofuran (95%)	0
Dimethylformamide (95%)	0
Dimethylsulfoxide (95%)	0
Acetone (95%)	0

All solvents tested resulted in complete inactivation of the enzyme, so we studied the effect of increasing amounts of one solvent (acetonitrile) in more detail, at relatively low concentrations (Figure 20). A concentration of 5% had no hindering effect but already with 10% both activity and stability suffer severe losses and with 15 % no activity is observed. This low tolerance of LEH for organic media is corroborated by its complete inactivation in 25% *t*-butanol (Table 12).

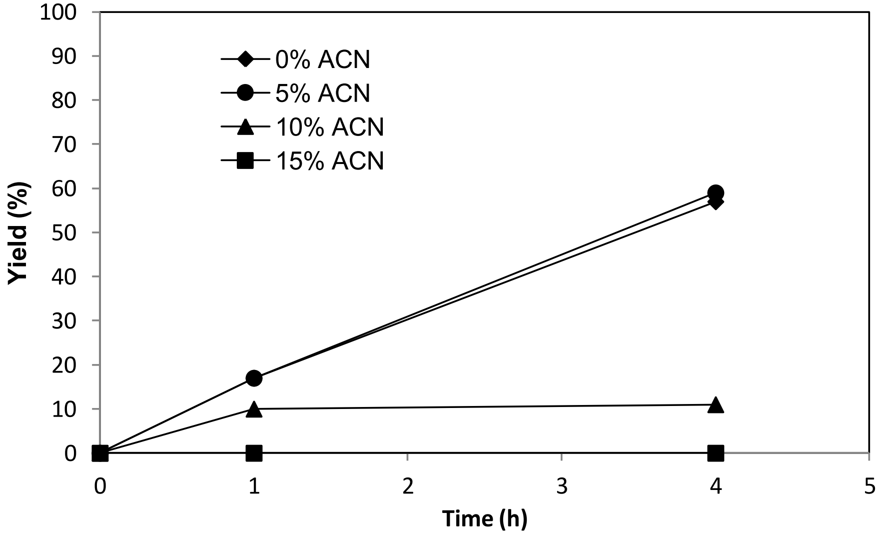


Figure 20: LEH catalyzed (+)-*cis*-limonene-1,2-epoxide hydrolysis in phosphate buffer (50 mM; pH 7) with different acetonitrile concentrations (v / v) at 40° C.

Out of curiosity we have tested the activity of LEH using one ionic liquid as co-solvent. Ionic liquids are simultaneously organic molecules and ionic salts, which are liquid at room temperature. Their use as design solvents for biotransformations has received great attention in the last decade and due to their properties they are a relevant alternative to both aqueous and organic media in solvent engineering [31].

We have chosen the ionic liquid butyl-methyl-imidazoliumdicyanamide ([C4mim][dca]), that was shown to allow for enzymatic activity in the case of lipases and has a low viscosity [32]. In the performed experiments at two different ionic liquid concentrations, we have not found hydrolysis product following the standard analytical procedure. We have, however, observed substrate consumption (Figure21).

This could be explained by poor extraction of the reactants, which is a known difficulty when using ionic liquids [33]. Although the enzyme loses activity after one hour for both concentrations, it is noteworthy that a higher concentration of ionic liquid afforded a higher conversion.

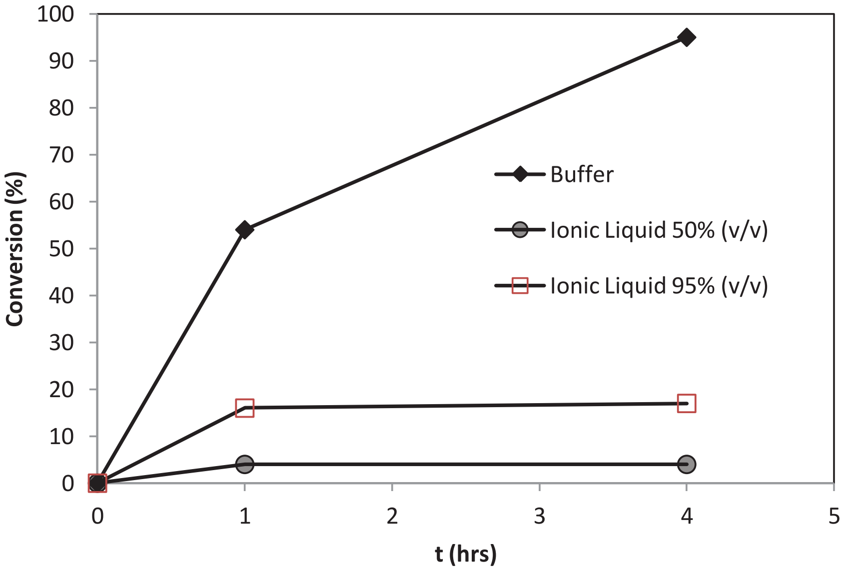


Figure21: LEH catalyzed (+)-*cis*-limonene-1,2-epoxide hydrolysis in phosphate buffer (50 mM pH 7) : 1-butyl-3-methylimidazolium dicyanamide [C4 mim][dca] mixtures; with different concentrations (v / v) at 40° C.

Our overall conclusion is that LEH has a low tolerance for organic solvents and stabilization techniques are required to enable its use in non-aqueous media.

Immobilization as Cross-Linked Enzyme Aggregates - CLEA

Immobilization is an important tool to improve the operational stability of enzymes, including the use in organic solvents [34]. We have chosen a cross-linking strategy to investigate the enzyme´s potential with immobilization techniques. This is a technique that does not require a solid carrier and usually affords a biocatalyst with high volumetric activities (see Chapter 4).

The native LEH was subjected to a standard CLEA procedure [35]. The cell-free extract was treated with ammonium sulfate to precipitate the protein, which was followed by cross-linking by addition of glutaraldehyde in different concentrations, resulting in three different LEH-CLEA preparations (Table 6).

Table 6: Description of the prepared LEH-CLEAs

LEH Formulation	*Precipitant*	*Glutaraldehyde (mM)*
CLEA 1	NH_4SO_4	5
CLEA 2	NH_4SO_4	20
CLEA 3	NH_4SO_4	50

After overnight incubation the immobilized particles were washed and the resuspended CLEA mixture was assayed in the hydrolysis of limonene oxide (Table). We have found slightly higher activity retention with higher amounts of glutaraldehyde.

Table 14: LEH-CLEA preparations activity retention in standard activity test conditions (see above), measured after 4 hours.

LEH Formulation	*Activity (%)*
Cell Extract	100
CLEA 1	18
CLEA 2	24
CLEA 3	22

Our preliminary studies with the cross-linking technique show it is a viable immobilization strategy for LEH. The immobilization procedure was not optimized suggesting higher activity retentions can be obtained.

Lyophilized LEH-CLEAs

Enzymatic activity in anhydrous organic solvents requires drying. Lyophilization is a known drying procedure [36] and generally considered a mild method. We have made the corresponding lyophilizates of the previously made CLEAs through freeze-drying, the obtained solids were resuspended and their activity measured (Table 7).

Table 7: Lyophilized LEH-CLEA preparations activity retention measured after 4 hours and with [S]=40 mM, other conditions as standard (see above).

LEH Formulation	*Activity (%)*
Cell extract	100
Lyo CLEA 1	8
Lyo CLEA 2	27
Lyo CLEA 3	16

When comparing the activity retention of the prepared LEH-CLEAs and the lyophilized LEH-CLEAs, we conclude the lyophilization procedure did not translate in a significant loss of activity with exception of the CLEA prepared with the lowest concentration of glutaraldehyde (Table 8).

Table 8: LEH Lyophilized CLEA and LEH CLEA suspension activity retention.

LEH - CLEA	*Lyophilized (%)*	*Suspension (%)*
CLEA 1	8	18
CLEA 2	27	24
CLEA 3	16	22

The majority of the observed loss in the prepared immobilized formulations occurred in the cross-linking step, which shows there is room for further optimization. This could be achieved by screening a wider concentration range of cross-linking agent, enzyme concentration, cross-linking time and eventually other precipitating agents.

Substrate Specificity

The difference in selectivity observed between 1-methylcyclohexene oxide and limonene-1,2-epoxide is possibly induced by the substituent at the C4 position. These hypotheses could be further elucidated by studying the LEH catalyzed hydrolysis of similar substrates with substituents in the mentioned position, for example 4-methylcyclohexene oxide.

We have not found any report of a substituent without a methyl group at the oxirane ring and with a substituent at the C4 position. So it is not known how, for example a methyl group at that position, can influence enzymatic activity. In order to validate this hypothesis, we have tested 4-methylcycloehexene oxide and used (-)-limonene-1,2-epoxide as control experiment.

The relative activity found for the (-)-*cis/trans* limonene epoxide was slightly lower than the one reported in [19] and we have found LEH accepts 4-methylcyclohexene oxide. The relative activity found for this substrate is lower than the one reported for 1-methylcyclohexene oxide (47%) [19] but five-fold higher than the one reported for cyclohexene oxide, showing the presence of a methyl group also at the C_4 position greatly contributes to the reactivity of the substrate.

Table 9: Relative activity of LEH found with4-methylcyclohexene epoxide

Substrate	*Relative Activity (%)*
(+)-*cis*-Limonene-1,2-epoxide	100
(-)-Limonene-1,2-epoxide	18
4-methylcyclohexene epoxide	22
(-)-Limonene-1,2-epoxide [19]	27
1-methylcyclohexene epoxide [19]	47
Cyclohexene oxide [19]	4,0

Conclusions

The use of nucleophiles other than water by LEH could not be observed in aqueous media. Further studies in anhydrous media that circumvent the native reaction could possibly lead to better results. However the enzyme´s instability in the presence of organic solvents needs to be overcome in this case.

Immobilization of LEH as a cross-linked aggregate showed this to be a viable method. Lyophilization of the LEH-CLEAs did not result in loss of activity. Further testing of these LEH-CLEA´s is required in order to establish if these preparations stabilize the enzyme in organic solvents.

In addition to solvent and nucleophiles screening, the present substrate scope of the enzyme was extended to 4-methylcyclohexene oxide. The activity was five-fold higher than the one reported for cyclohexene oxide, showing that the introduction of a methyl group at the C_4 position of the substrate influences the reactivity.

Experimental Part

Materials

Cell Extraction

The cells were allowed to defreeze at room temperature and centrifuged at 20 krpm for 20 minutes and the supernatant discarded. They were further washed with phosphate buffer (pH 7; 100 mM) and the procedure repeated twice. The cells were resuspended in 4 mL of the same buffer and 250 μLof DTT were added. The cells were sonicated with glass beads for 15 minutes at 7,5 microns, centrifuged at 20 krpm for 20 minutes, the extract recovered and the cell debris discarded.

The different cell extracts obtained as described above had specific activities that varied between 0,5 UmL^{-1} and 19 UmL^{-1}.The activity of LEH was assayed in the hydrolysis of a 80 mM solution of (+)-*cis* limonene-1,2-oxide in 50 mM phosphate buffer at pH 7. One unit (U) of LEH is defined as 1 μmol of substrate converted in 1 minute at 40 ºC.

Generic LEH Catalyzed Limonene Epoxide Hydrolysis Reactions

The appropriate volume of a LEH cell free extract is added to an 80 mM limonene epoxide solution in phosphate buffer 50 mM, pH 7, making a total volume of 2 mL. The reaction mixture is shaken at 40 ºC. The reaction is stopped by addition of 2 mL of ethyl acetate followed by direct extraction of the organic layer. This was dried with sodium sulfate and analyzed by GC.

Synthesis of (1*R*,2*R*,4*R*)-2-azido-1-methyl-4-(prop-1-en-2-yl)cyclohexanol

0,98 g of (+)-*cis*-limonene-1,2-oxide was dissolved in a mixture of 10 mL of water and 20 mL of acetonitrile. 1 g of sodium azide was added to this solution and the mixture was allowed to react at room temperature overnight. The acetonitrile was evaporated, the aqueous residue was extracted three times with ethyl acetate, the organic layer was dried and the solvent was evaporated, affording 630 mg of a yellowish liquid. This was distilled at reduced pressure for analytical purposes.

GC-MS (m/z): 196 (M_w+1); 168; 152; 148; 134; 123; 108, 94; 81; 67; 57; 43

Synthesis of (1*S*,2*S*,4*R*)-2-azido-1-methyl-4-(prop-1-en-2-yl)cyclohexanol

Same procedure as above, starting from 1,015 g of (+)-*trans*-limonene-1,2-oxide.

GC-MS (m/z): 196 (M_w+1); 166; 158; 135; 123; 109; 94; 81; 67; 53; 43

Synthesis of 4-Methylcyclohexene oxide

10,32 g of 4-Methylcyclohexene were added to a mixture of 500 mL and of dichloromethane and 500 mL of phosphate buffer (pH8, 500 mM) under stirring and the mixture was cooled to 2ºC. At this temperature 21 g of 3-

chloroperbenzoic acid were added. The mixture was kept at this temperature for 30 minutes, after which it was allowed to reach room temperature and react overnight. The phases were separated and the aqueous phase was extracted twice with dichloromethane. The organic phases were gathered and washed twice with thiosulfate, twice with saturated bicarbonate and twice with water. The organic layer was dried under sodium sulfate, the solvent was evaporated and 8,8 g of a yellowish liquid was obtained, which corresponds to a yield of 75%. The product was GC pure.

Preparation of LEH-CLEAs

Three different preparations were made containing 1,8 U of LEH and 2,7 mL of a saturated solution of ammonium sulfate (pH 7). To which 15 µL, 60 µL and 150 µL of glutaraldehyde (1M) were added correspondingly. The solutions were kept overnight at room temperature under stirring. 7 mL of water were added to each solution and stirred for 3 hours. The solutions were centrifuged (3000 rpm; 10 minutes) and the supernatant collected. This procedure was repeated twice and the pellets were finally ressuspened in 3 mL of phosphate buffer (50 mM; pH 7).

Preparation of LEH-Lyophilized CLEAs

The CLEA suspensions (1,5 mL) were frozen with liquid nitrogen. They were freeze dried at 0,4 mbar for 5 hours. The obtained solids were resuspended in 1,0 mL of phosphate buffer (50 mM; pH 7).

Analysis

Limonene epoxide, Limonene1,2-diol, 1-Methylcyclohexene oxide, 1-Methylcyclohexene diol, 4-Methylcyclohexene oxide, 4-Methylcyclohexene diol, Cyclohexene oxide, Cyclohexene diol and decane (internal standard) were analyzed with gas chromatography using a Varian 3400 CX gas chromatograph with a Varian 6577 auto sampler using a Cpwax-52B (50m x 0.53mm x 2um).

References

---

1 Besse, P.; Veschambre, H.; *Tetrahedron***1994**; 50, 30, 8885 - 8927

2 Archelas, A.; Furstoss, R.; *Annual Review of Microbiology***1997**; 51, 491 - 525

3 Buckland, B.C.; Drew, S.W.; Connors, N.C.; Chartrain, M.M.; Lee, C.; Salmon, P.M.; Gbewonyo, K.; Zhou, W.; Gailliot, P.; Singhvi, R.; OlewinskiJr, R.R.C.; Sun, W.J.; Reddy, J.; Zhang, J.; Jackley, B.A.; Taylor, C.; Goklen, K.E.; Junker, B.; Greasham, R.L.; *Metabolic Engineering***1999**; 1, 63 - 74

4 Schaus, S.E.; Brandes, B.D.; Larrow, J.F.; Tokunaga, M.; Hansen, K.B.; Gould, A.E.; Furrow, M.E.; Jacobsen, E.N.; *Journal of the American Chemical Society***2001**; 124, 7, 1307 - 1315

5 Rossiter, B.E.; Katsuki, T.; Sharpless, K.B.; *Journal of the American Chemical Society***1981**; 103, 464 - 465

6 Katsuki, T.; *Coordination Chemistry Reviews***1995**; 140, 189 - 214

7 Shi, Y.; *Accounts of Chemical Research***2004**; 37, 488 - 496

8 Ortiz de Montellano, P.R.; Fruetel, J.A.; *Journal of the American Chemical Society***1991**; 113, 3195 - 3196

9 Allain, E.J.; Hager, L.P.; Deng, L.; Jacobsen, E.N.; *Journal of the American Chemical Society***1993**; 115, 4415 - 4416

10 Jacobsen, E.N.; *Accounts of Chemical Research***2000**; 33, 421 - 431

11 Archelas, A; Furstoss, R.; *Current Opinion in Chemical Biology***2001**; 5, 112 - 119

12 Spelberg, J.H.L.; Tang, L; van Gelder, M.; Kellog, R.M.; Janssen, D.B.; *Tetrahedron: Asymmetry***2002**; 13, 1083 - 1089

13 Spelberg, J.H.L.; van Hylckama Vlieg, J.E.T.; Tang, L.; Janssen, D.B.; Kellog, R.M.; *Organic Letters***2001**; 3, 1, 41 - 43

14 Elenkov, M.M.; Tang, L.; Hauer, B.; Janssen, D.B.; *Organic Letters***2006**; 8, 19, 4227 - 4229

15 Davis, S.C.; Grate, J.H.; Gray, D.R.; Gruber, J.M.; Huisman, G.W.; Ma, S.K.; Newman, L.M.; Sheldon, R.A.; Wang, L.A.; *US7,807,423 B2***2010**

16 Kim, H.S.; Lee, O.K.; Hwang, S.; Kim, B.J.; Lee, E.Y; *Biotechnology Letters***2008**; 30, 127 - 133

17 Borhan, B.; Jones, A.D.; Pinot, F.; Grant, D.F.; Kurth, M.J.; Hammock, B.D.; *Journal of Biological Chemistry***1995**; 270, 45, 26293 - 26930

18 de Vries, E.J.; Janssen, D.; *Current Opinion in Biotechnology***2003**; 14, 414 - 420

19 van der Werf, M.; Overkamp, K.M.; de Bont, J.A.M.; *Journal of Bacteriology***1998**; 180, 19, 5052 - 5057

20 Arand, M.; Halberg, B.M.; Zou, Jinyu; Bergfors, T.; Oesch, F.; van der Werf, M.J.; de Bont, J.A.M.; Jones, T.A.; Mowbray, S.L.; *The European MolecularBiologyOrganization Journal***2003**; 22, 11, 2583 – 2592

21 van der Werf, M.; de Bont, J.A.M.; Swarts, H.J.; *Tetrahedron: Asymmetry***1999**; 10, 4225 - 4230

22 Hopmann, K.H.; Hallberg, M.; Himo, F.; *Journal of the American Chemical Society***2005**; 127, 41, 14339 - 14347

23 van der Werf, M.J.; Orru, R.V.A.; Overkamp, K.M.; Swarts, H.J.; Osprian, I.; Steinreiber, A.; de Bont, J.A.M.; Faber, K.; *Applied Microbiology and Biotechnology***1999**; 52, 380 - 385

24 Gotor, V.; *Journal of Biotechnology***2002**; 96, 35 - 42

25 vanLangen, L.M.; Oosthoek, N.H.P.; Guranda, D.T.; van Rantwijk, F.; Švedas, V.K.; Sheldon, R.A.; *Tetrahedron: Asymmetry***2000**; 4593 - 4600

26 Rémond, C; Ferchichi,M; Aubry, N.; Plantier-Royon, R.; Portella, C.; O´Donohue, M.J.; *Tetrahedron Letters***2002**; 9653 - 9655

27 Martinek, K.; Levashov, A.V.; Klyachko, N.; Khmelnitski, Y.L.; Berezin, I.V.; *European Journal of Biochemistry***1986** ; 155, 453 - 468

28 Klibanov, A.M.; *Trends in Biochemical Science***1989**; 14, 4, 141 - 144

29 Wegman, M.A.; Hacking, M.A.P.J.; Rops, J.; Pereira, P.; van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron: Asymmetry***1999**; 10, 1739 - 1750

30 van Rantwijk, F.; Sheldon, R.A.; *Tetrahedron***2004**; 60, 501 - 519

31 Sheldon, R.A.; Lau, R.M.; Sorgedrager, M.J.; van Rantwijk, F.; Seddon, K.R.; *Green Chemistry***2002**; 4, 147 - 151

32 Galonde, N.; Nott, K.; Debuigne, A.; Deleu, M.; Jerôme, C.; Paquot, M. ;Wathelet, J.-P.; *Journal of Chemical Technology and Biotechnology***2012**; 87, 4, 451 - 471

33 Park, S.; Kazlauskas, R.J.; *Current Opinion in Biotechnology***2003**; 14, 432 - 437

34 Sheldon, R.A.; Schoevaart, R.; van Langen, L.M.; *Biocatalysisand Biotransformations***2005**; 23, 141 - 147

35 Sheldon, R.A.; *Biochemical Society Transactions***2007**; 35, 1583 - 1587

36 Klibanov, A.M.; *Nature***2001**; 409, 241 - 246

Summary

Biocatalysis is an established and widely used method for the production of chemicals and specially enantiopure compounds. Moreover, its applications are not limited to organic synthesis and can be found in a wide scope of the chemical industry, ranging from the food to the pharma industry.

The main obstacles to large scale implementation are often associated with enzyme stability, low activity or substrate toxicity. The different components of an enzymatic system comprise the enzyme, the reaction medium, the substrate and the post translational biocatalyst. These can be engineered in order to make a system viable, as well as to design new synthetic routes. Biocatalysis engineering is the integrated development of an enzymatic system by engineering the different variables of that system.

The first part of ***Chapter 1*** gives an overview of biocatalysis, ranging from an historical perspective to the current growth opportunities, while covering a wide description of its applications in the chemical industry.

The second part of ***Chapter 1*** describes in more detail the concrete engineering aspects of the different variables of a biocatalytic system, with the exception of reactor engineering. These comprise engineering of the system by manipulation of the substrates, the reaction media or even the amount of enzymes used simultaneously. In addition the engineering of the enzyme is dealt with either by altering the protein sequence to obtain an enzyme with modified properties, or the engineering of the post translation enzyme, which is to alter the final formulation of an enzyme without changing the protein structure.

In this thesis we describe a variety of industrially relevant examples illustrating the approaches described in ***Chapter 1***, with the exception of protein

engineering. We have addressed the development of new routes using (i) an already developed biocatalyst, (ii) a new co-immobilized biocatalyst and a study of this system, and (iii) the screening of the potential of applying the different engineering tools in a new enzyme. In short, we have addressed three different phases of biocatalysis engineering.

In ***Chapters 2*** and ***3*** we describe a new chemo-enzymatic synthetic route towards enantiopure diketopiperazines, which are an important class of biologically active compounds. In both chapters the focus was on taking advantage of the enzyme´s substrate scope for achieving viable and robust syntheses, and not on the development or study of the biocatalyst itself.

In ***Chapter 2*** we have tested this route while targeting untested natural amino acids as substrates. To achieve this we have screened a broad scope of enantiopure compounds, for which the route proved largely successful. In two of the cases tested, the final chemical step remarkably afforded the same compound, which was in each case not the expected product.

In ***Chapter 3*** the strategy was to start from racemic mixtures of unnatural amino acids, while targeting specific final products. For this we have used racemic mixtures different to the ones corresponding to the substrates used in ***Chapter 2***. The route was successful in all cases, thus broadening the scope of application.

Chapters 4 and ***5*** address the research towards a viable biocatalyst comprised of two enzymes, namely chloroperoxidase (from *Caldariomyces fumago*) and glucose oxidase. Chloroperoxidase (CPO) is an interesting haloperoxidase which uses hydrogen peroxide as oxidant, and is capable of a wide array of transformations. However, chloroperoxidase is hindered by a poor operational stability. Although hydrogen peroxide is an accepted substrate, it easily leads to the enzyme´s inactivation.

One of the different approaches to overcome this obstacle involves the use of a bi-enzymatic system, using glucose oxidase (GOX) to reduce molecular oxygen and generate hydrogen peroxide *in situ*. One co-immobilized formulation of these enzymes was previously proven to be an efficient way to circumvent chloroperoxidase inactivation.

We have started by investigating the viability of a new co-immobilized formulation of CPO and GOX in ***Chapter 4***, together with systems where only one of the enzymes was immobilized. Although results were disappointing for the co-immobilized formulation, we found an unexpected improvement when GOX was immobilized but not CPO.

In ***Chapter 5*** we address the study of the system with more depth, in order to gain more information which could be useful for any type of future immobilization. For this purpose both enzymes were used in their natural formulations and a screen over a broad range of enzyme ratios was tested. Remarkably we found a glucose oxidase unselective sulfoxidation hitherto unknown, and that this is influenced by the amount of co-solvent.

Finally, in ***Chapter 6*** we screened several methods for engineering a system with a new enzyme. The enzyme used was limonene epoxide hydrolase (LEH), which is an atypical epoxide hydrolase. This family of enzymes is interesting because optically pure epoxides and derivatives are important compounds in the pharmaceutical industry. In addition to this, the selectivity of LEH seems to be influenced by the substrate.

Both unnatural and natural substrates were tested, as well as different reaction media and immobilization as cross-linked aggregates (CLEAs). We have ascertained that the substrate scope of this enzyme includes a new compound (4-methylcyclohexene oxide), which could be used in further studies regarding the enzyme´s selectivity. Furthermore, we concluded that the position of the methyl group in the cyclohexene oxide ring has an influence on the reactivity.

Experiments carried out in aqueous media did not show precedence for acceptance of unnatural substrates by LEH. Azidolysis was studied, and these studies were hampered by significant background activities. On the other hand we were able to create a carrier free active immobilized formulation of this enzyme, which could allow for further substrate research under water-free conditions.

Samenvatting

Voor de productie van chemische verbindingen zoals enantiozuivere stoffen wordt veel gebruik gemaakt van biokatalyse. Daarnaast kent biokatalyse ook toepassingen in de voedingsmiddelen- en farmaceutische industrie.

De grootschalige implementatie van biokatalyse wordt vaak bemoeilijkt door factoren die samenhangen met een ontoereikende enzymstabiliteit, lage activiteit of beperkte bestendigheid t.a.v. de gebruikte reactanten, producten en reagentia. Elk van deze factoren kan d.m.v. techniek worden verbeterd om een proces, bestaand of nieuw te ontwerpen, techno-economisch haalbaar te maken. De Engelstalige term voor deze geïntegreerde technologie is *biocatalysis engineering*. Bij gebrek aan een goede vertaling wordt dat Engelse begrip in dit proefschrift ook in het Nederlands gebruikt.

Het eerste deel van ***Hoofdstuk 1*** geeft een overzicht van biokatalyse vanuit historisch perspectief tot en met de huidige kansen. Ook worden verschillende toepassingen in de chemische industrie beschreven.

Het tweede deel van ***Hoofdstuk 1*** gaat meer in detail in op concrete ontwerpaspecten van een biokatalyseproces: keuze van de uitgangsstoffen, het reactiemedium, het gebruik van meerdere enzymen tegelijkertijd en het verbeteren van het enzym zelf middels protein engineering of formulering.

Vervolgens worden in dit proefschrift een aantal industrieel relevante voorbeelden aangehaald ter illustratie van de in ***Hoofdstuk 1*** besproken ontwerpaspecten. Aan bod komt de ontwikkeling van nieuwe routes waarbij gebruik wordt gemaakt van (i) een bestaande biokatalysator; (ii) een nieuwe multicomponenten biokatalysator met bijbehorende studie; en (iii) een screening van de toepassingsmogelijkheden van verschillende engineeringstechnieken in een nieuw enzym. Kortom, er is aan drie verschillende fasen van *biocatalysis engineering* gewerkt.

In de ***Hoofdstukken 2 en 3*** wordt een nieuwe chemo-enzymatische route voor de synthese van enantiozuivere diketopiperazines beschreven. De focus in beide hoofdstukken lag op het benutten van de substraatacceptatie van het enzym voor het ontwikkelen van robuste en succesvolle syntheses en niet op het ontwikkelen of bestuderen van het enzym.

In ***Hoofdstuk 2*** is de nieuwe route getest met niet eerder gebruikte natuurlijke L-aminozuren als substraat. In de meeste gevallen was de reactie succesvol maar in twee gevallen was het verkregen product hetzelfde en anders dan verwacht.

In ***Hoofdstuk 3*** hebben we vergelijkbare reacties bestudeerd met racemische mengsels van aminozuren welke in alle gevallen succesvol waren en daarmee de toepasbaarheid van de route vergroot.

Hoofdstukken 4 en ***5*** gaan over een multi-enzymsysteem bestaande uit chloroperoxidase (van *Caldaryomyces fumago*) en glucose oxidase. Chloroperoxidase is een interessant enzym dat gebruikmaakt van waterstofperoxide als oxidant en een scala aan reacties kan katalyseren. Hoewel waterstofperoxide onmisbaar is als substraat, leidt het ook tot inactivering van het enzym waardoor de toepasbaarheid ernstig wordt beperkt. Een bekende oplossing om de toepasbaarheid van chloroperoxidase te vergroten is het gebruik van glucose oxidase om *in-situ* waterstofperoxide te maken uit zuurstof en glucose.

In ***Hoofdstuk 4*** werd een nieuwe gecombineerde immobilisatie van de twee enzymen vergeleken met de apart geïmmobiliseerde enzymen. Tegen de verwachting in werd een verbetering gevonden wanneer aleen glucose oxidase was geïmmobiliseerd maar niet chloroperoxidase.

In ***Hoofstuk 5*** is het systeem meer in detail bestudeerd m.b.t. de enzymen in oplossing en een reeks verschillende ratio’s daarvan. Verrassend was de ontdekking dat glucose oxidase een tot op heden onbekende niet-selective sulfoxidatie katalyseerde, die werd beïnvloed door de hoeveelheid cosolvent.

Tot slot zijn in ***Hoofdstuk 6*** verschillende methodes gescreend voor het ontwikkelen van een reactiesysteem met het enzym limoneen-epoxidehydrolase (LEH), een atypisch epoxidehydrolase. Deze enzymen zijn interessant want optisch zuivere epoxiden en derivaten daarvan zijn belangrijke stoffen voor de farmaceutische industrie en de selectiviteit van LEH is blijkbaar afhankelijk van het substraat.

Na studie van natuurlijke en niet-natuurlijke substraten, verschillende reactiemedia en immobilisatie als CLEA, werd een nieuw substraat (4-methylcyclohexeenoxide) geïdentificeerd dat gebruikt kan worden in vervolgstudies om de slectiviteit van het enzym te bestuderen. Tevens bleek de methylgroep van dit substraat van invloed te zijn op de reactiviteit.

Zowel natuurlijk en onnatuurlijke substraten werden getest, als verschillende reactie media en immobilizatie door cross-linked enzyme aggregates (CLEA). We hebben vastgesteld dat de scope van substraten van dit enzym bevat een nieuwe stof (4-methylcyclohexene oxide), welke gebruikt kan worden in verdere studies naar de selectiviteit van het enzym.

Bovendien hebben we geconcludeerd dat de positie van de methyl groep in de cyclohexene oxide ring van invloed is op de reactiviteit. In waterig milieu bleek het enzym geen voorkeur te hebben voor niet-natuurlijke substraten. Azidolyse werd bestudeerd maar werd bemoeilijkt door een niet-enzymatische blanco-reactie. De immobilisatie als CLEA was wel succesvol en opent de weg naar verder onderzoek onder watervrije condities.

Dankwoord

Dear Roger (Prof. Dr. Roger A. Sheldon), I would like to thank you the opportunity you gave me for doing a PhD in your group. When I met you first as an Erasmus student, I became deeply impressed with your way of thinking and identified with it strongly. The work topics of your group were ones I already had an interest at the time: organic chemistry and enzyme technology. I then thought if I would do a PhD I would do it with you and in your group. When the opportunity appeared I could not refuse and took it with great enthusiasm.
I would especially like to thank you for the support you gave me in very crucial stages of my thesis, particularly the last, without which I could not have finished.

Dear Isabel (Prof. Dr. Isabel W.C.E. Arends), I would like to thank you for having accepted to be my Promotor when Roger could not and I would further like to extend this to the support you offered in the final stages. I am equally thankful for you not having micromanaged me when you were my supervisor, while never letting me forget the practical aspects. Thank you for everything I have learned from you.

Dear Luuk (Luuk van Langen), GS, KHV, James, Victor, Lucas, LeClerc, my former colleague and the Mr Spock of my imaginary spaceship, de kleine balletjes singer and the king of the 10k. I could write another thesis just about you, but maybe it would take even longer. Thank you for all the discussions, fights, and for everything I learned with you. It was a pleasure, Sir, en vrees niet want Pedro is er bij.

Dear Mike (Michiel Janssen), my Broertje. Thank you for always being available when I had questions, for the interest you showed in them, for speaking your mind straight and for not trying to convince me you were right when I wasn´t wrong.

Dankwoord

Dear Hilda (Hilda Ismail), my Sista. Thank you for being the best office mate one could ask for. Thank you for your patience and for teaching me so many things. Terima kasih.

Dear Kristina (Kristina Djanashvili), Kris. More than an excellent professional you were a great colleague. And more than a great colleague you are a good friend. I have not enough words to thank you for always having time to discuss NMR and for always having time when I was generating several samples per hour. And, naturally, thank you for our philosophical discussions. Madloba, girl.

Dear Mieke (Mieke van der Kooij), thank you for making everything happen, for your support and for supporting everything, thank you for not leaving anything to chance, thank you for being excellent.

Dear Joop (Joop Peters), thank you for always being available for my questions and doubts, and for all our discussions.

Dear Adrie (Adrie Knol-Kalkman) en Maarten (Maarten Gorseling), thank you so much for the excellent support and for more often than not going the extra mile to help me.

Dear Menno-Pá (Menno Sorgedrager), Sander-Pá (Sander van Pelt) and Bruno-Pá (Bruno Fernandes), you were either my former bench mates, or colleagues in the Practicum classes, or office mates, or several of them. In one way or the other, the times I spent with you, the experiences, the conversations and moments we shared will remain forever a huge part of my PhD experience and therefore of my life. Thank you, guys.

Dear van Vliet (Michiel van Vliet), RL. Thank you for your advices whenever I needed them, for being a walking encyclopedia and for the soundtrack when you worked on the office next door. You are not just a great chemist, scientist and cocktail maker, you can also think like an engineer.

Dear Hacking (Michiel Hacking), Hackelaar “the catastrophic scenario”. Thank you for the uncomplicated and comprehensive views on all things chemical, all your support and the great times, the fun and easy as well as the less fun and more difficult.

Dear Rute (Rute Lau), my former colleague in the TU and from the Lisbon days, thank you for the advices about ionic liquids.

Dear Dani (Daniela Sordi) and Pereirinha (Sílvia Pereira), the mixture of you two in my office for a brief period of time was not just a great experience, it was great fun. Thank you, Ladies.

Dear Seda (Seda Aksu Kanbak), Inga (Inga Mattijosite), Alekssandra (Alekssandra Kotlewska) and John (John van der Toorn), my former group colleagues, I´m sure none of us will ever forget our “very catalytic” work-meetings, just as I´m sure none of you will ever forget my “short & sweet” 30 minute presentations.

Dear Serena (Serena Gargiulo) and Katya (Ekateryna Shurakova), my last office mates, I would like to thank you the not so long but good times we shared in the office and outside.

Dear Christophe (Christophe Bouvier), thank you for being one of the clearest voices that kept me going in difficult times.

Dankwoord

Dear Sweetie (Sarah Campbell), if at first you felt my wing over your shoulder, in the course of the years it was always yours over mine. Thanks.

Dear Irmandade (João, Leila and Cátia), much of the path I made was done in your company, with your support and ever present friendship. Thank you. Let us pray together and yet again offer sacrifice.

Dear Aretha (Vasco), without you there could not have been the unforgettable "Aretha & Cher" duets or the "Pepper-Cherando Motto", for which the world is not yet ready.

Dear Getugatz, Buitenlanders and Coeca crowd & Cia, thank you for all the great moments, and even more the discussions about the all and the nothing and everything in between, which somehow brought me back to normality.

Dear Annelies, Dinneke and Peter, with you I´ve learned to twist, bend, sit, stand on my hands, elbows, shoulders, head, on one, and sometimes even on both feet. With you I could make another discovery journey, this time within myself, which gave me the essential balance. Dank je wel.

Dear Poilu (Tristan) and Sander, thank you both for giving me the essential peace at home, without which home easily becomes something else. Et la marmotte elle met le chocolat dans le papier d´alu.

Mãe, sem o teu apoio, amor e exemplo, eu nunca teria tido a força.

Pai, já não consegui acabar a tempo nem tu pudeste esperar mais. Mas tenho a certeza que ainda me vês daí desse lado. Sem ti nada disto teria sido possível.

Dear Gerjanne, AH. Zonder jou was dit boekje niet tot stand gekomen. Thank you for Everything and not just everything. Obrigado por tudo o que me deste e por tudo o que fizeste por mim HMNvS.

Het is onmogelijk om iedereen persoonelijk te noemen die me heeft geholpen of ondersteund, maar het waren er ontzettend veel. Hartelijk dank, danke, tak, thank you, merci, graciès, gracias, grazzie, köszönöm, dziękuję, ačiū, spasibo, madloba, teşekkürler, dhaniavaad, xièxiè, arigatō, terima kasih, a todos muito obrigado!

Dankwoord

Publications

Pedro C. Pereira; Isabel W.C.E. Arends; Roger A. Sheldon; Optimizing the chloroperoxidase-glucose oxidase system: The effect of glucose oxidase on activity and enantioselectivity; *Process Biochemistry* **2015**, 50, 746 - 751

Pedro C. Pereira; Isabel W.C.E. Arends; Roger A. Sheldon; A green & expedient synthesis of enantiopure diketopiperazines via unnatural amino acid kinetic resolution; *Tetrahedron Letters* **2014**, 55, 4991-4993

Pedro C. Pereira; Isabel W.C.E. Arends; Roger A Sheldon, Robust and straightforward chemo-enzymatic enantiopure dipeptide syntheses and diketopiperazines thereof; Tetrahedron: *Asymmetry* **2014**, 25, 825-832

Gonçalo Gamboa da Costa; Pedro C. Pereira; Mona I. Churchwell; Frederick A. Beland; M. Matilde Marques; DNA Adduct Formation in the Livers of Female Sprague-Dawley Rats Treated with Toremifene or α-Hydroxytoremifene; *Chemical Research Toxicology* **2007**, 20 (2), 300-310

M.A. Wegman; M.A.P.J. Hacking; J. Rops; P. Pereira; F van Rantwijk; R.A. Sheldon; Dynamic kinetic resolution of Phenylglycine esters via lipase-catalyzed ammonolysis*; Tetrahedron: Asymmetry* **1999**, 10, 1739-1750