UNIVERSITÀ DEGLI STUDI DI MILANO

DIPARTIMENTO DI
SCIENZE FARMACEUTICHE

SCUOLA DI DOTTORATO IN SCIENZE E TECNOLOGIE CHIMICHE

CORSO DI DOTTORATO IN CHIMICA DEL FARMACO

XXVIII CICLO

Biocatalytic synthesis of chiral pharmaceutical intermediates

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Anno accademico 2014/2015

Summary

The Doctoral Thesis has been structured into two parts preceded by a general introduction that covers basic concepts on biocatalysis with special focus on application of whole cells of unconventional yeasts and a new ketoreducatese from *Pichia glucozyma*. All these thematic issues give a background to the following two parts, where the experimental results have been deeply discussed.

Part 1 The biocatalytic potential of whole cells of *P. glucozyma* have been studied through biotransformations using substrates of different chemical nature having interesting pharmaceutical and synthetic aspects. After optimization of the reaction conditions through the transformation of acetophenone in (*S*)-1-phenylethanol, the attention was focused on the reduction of different aromatic ketones. All the reactions occurred with high yields and high enantiomeric excess for the corresponding (*S*)-alcohols.

Part 2 Bioreduction catalysed by NADPH-dependent benzil-reductase (KRED1-Pglu) from *Pichia glucozyma* provides an attractive approach for selectively reducing a broad range of aromatic ketones. After protein identification, characterization and over-expression in *E. coli*, we have established a procedure for the purification and storage of KRED1-Pglu. After optimization of the reaction parameters, a thorough study of the substrate range of KRED1-Pglu was conducted. KRED1-Pglu prefers space demanding substrates which are often converted with high stereoselectivity. The observed activities and enantioselectivities were explained with a molecular modelling study for understanding the structural determinants involved in the stereorecognition experimentally observed and unpredictable on the basis of steric properties of the substrate.

In conclusion, different efficient and sustainable methods for the stereoselective reduction of prochiral ketones using both whole cells of unconventional yeasts such as *P. glucozyma* CBS 5766 and the isolated enzyme KRED1-Pglu have been proposed in this PhD work.

Riassunto

Questa Tesi di Dottorato è stata strutturata in due parti differenti precedute da un'introduzione riguardante i principi fondamentali della biocatalisi con particolare attenzione sull'applicazione di cellule intere di lieviti non convenzionali e una nuova chetoreduttasi proveniente dal lievito *Pichia glucozyma*. Tutte queste tematiche rappresentano il "background" per poter meglio comprendere le parti sperimentali descritte nei successivi paragrafi.

Parte 1 Il potenziale biocatalitico rappresentato da cellule intere di *P. glucozyma* è stato studiato usando substrati di differente natura chimica interessanti sia dal punto di vista farmaceutico che sintetico. Dopo l'ottimizzazione delle condizioni di reazione, effettuata sulla trasformazione dell'acetofenone in (*S*)-1-feniletanolo, l'attenzione è stata focalizzata sulla riduzione di differenti chetoni aromatici. Tutte le reazioni generalmente avvengono con elevate rese ed elevata selettività per il corrispondente (*S*)-alcol.

Parte 2 Riduzioni catalizzate da una benzil-reduttasi (KRED1-Pglu) NADPH-dipendente proveniente da *P. glucozyma*, rappresentano un' interessante strategia sintetica per la riduzione stereoselettiva di chetoni aromatici. Dopo identificazione, la caratterizzazione e l'over-espressione della proteina in *E. coli*, un metodo di purificazione e conservazione dell'enzima è stato messo a punto. Dopo ottimizzazione dei parametri di reazione, è stato effettuato un accurato studio riguardante i substrati accettati da KRED1-Pglu. Questa proteina preferisce substrati stericamente ingombrati, spesso convertiti con elevata stereoelettività. La differente enantioselettività sperimentalmente osservata, è stata spiegata mediante studi di docking molecolare.

In conclusione, due differenti metodi efficienti ed eco-sostenibili per la riduzione stereoselettiva di diversi chetoni aromatici, utilizzando sia cellule intere di lieviti non convenzionali tra cui *P. glucozyma* CBS 5766 e l'enzima isolato KRED1-Pglu sono stati presentati in questo lavoro di tesi.

A very special thank you to:

for funding this PhD project.

To my parents and my sister,
thank you for believing in me...

“It takes courage to be happy.”

Karen Blixen

Introduction

Aim of the project

Investigation of *Pichia glucozyma* as whole cells biocatalyst

Importance of Prostaglandins and their analogues

Employment of a new ketoreductase from *P.glucozyma* for stereoselective reductions

Enzymatic reduction of acetophenone derivatives: electronic and steric effects on activity and enantioselectivity

Stereoselective enzymatic reduction of ethyl secodione: preparation of key-intermediates for the total synthesis of steroids

Conclusions

Acknowledgments

Introduction

Introduction

1.1 Efficiency in organic chemistry

In the recent history of organic chemistry the concept of reaction efficiency has undergone substantial changes. For long time the success of a chemical process was entirely related to the yield of the desired product. Aspects such as waste regeneration or toxic properties of the chemicals and solvents were neglected. For example till 1980 the production of 1 Kg of phloroglucinol (1,3,5-benzentriol), an antispasmodic agent with a lot of applications also as precursor of pharmaceuticals and explosives, could generate up to 40 Kg of solid waste in a industrial process[1].

From a modern perspective, this limited viewpoint must be enlarged. In 90's Trost redefined the reaction efficiency in terms of atom economy, as the percentage of reactants molecular mass incorporated in the final products.[2] In this context synthetic method should be designed to maximize the incorporation of all materials used during the process, in the final product.

Additionally, increasing the environmental consciousness, in 1998 Anastas and Warner published the twelve principles of environmentally driven chemical processes[3] (Fig. 1). The main topics were the utilization of raw materials with minimization of waste production through the implementation of catalytic process

[1] R.A. Sheldon, *Green Chem*. **2007**, *9*, 1273-1283
[2] B.M. Trost, *Science* **1991**, *254*, 1471-1477
[3] P.T Anastas, J.C Warner, *Green Chemistry: Theory and Practise*, Oxford University Press, Oxford, 1998

and safety issues about the use of chemicals with particular emphasis on the research for alternative reaction media to replace the most health hazardous reaction conditions. The concept of *Green Chemistry* was coined and its principles have became relevant in chemical process design.

As a result, an efficient chemical process started to be perceived as a procedure where minimal amount of waste was produced. The most widely accepted measure of the environmental impact of a chemical processes is the *E factor*, defined as the weight of waste generated in producing one Kg of product, including auxiliary reagents such as catalysts, solvents and others.[4] Water is generally excluded as waste in this approximation. Other metrics have been proposed for measuring the environmental acceptability of processes such as mass intensity (MI) or carbon efficiency (CE) especially used in fine and pharmaceutical industry.[5]

P Prevent wastes
R Renewable materials
O Omit derivatization steps
D Degradable chemical products
U Use of safe synthetic methods
C Catalytic reagents
T Temperature, Pressure ambient
I In-Process monitoring
V Very few auxiliary substrates
E E-factor, maximise feed in product
L Low toxicity of chemical products
Y Yes, it is safe

Fig. 1 The twelve principles of *Green Chemistry*

[4] R.A. Sheldon, *CHEMTECH* **1994**, *24*, 38-47
[5] W.J.W. Watson, *Green Chem*. **2012**, *14* 251-259

$$E = \frac{\text{Total mass of waste}}{\text{Mass of final product}} \qquad MI = \frac{\text{Total mass in process}}{\text{Mass of final product}} \qquad CE\ \% = \frac{\text{Carbon in product x 100}}{\text{Total carbon in reactants}}$$

Fig. 2 Most commonly used metrics for process efficiency

The stoichiometric ratio and the use of solvents and catalysts are the practical problems associated with the realization of a chemical process dealing with the consumption of raw material and the production of waste as well as the yield. However, we are still far from an accurate assessment of environmental impact of a chemical process. The analyzed parameters, in fact, take into account the amount of waste but they do not consider their characteristics.

Furthermore, the responsible management of natural resources through development of sustainable processes has motivated the research for the utilization of renewable resources such as biomass to produce biofuels, chemicals, pharmaceuticals and bioplastics.[6] Sustainable development is defined as the environmental, economic and social well-being that allows "meeting the needs of the present generation without compromising the ability of the future generations to meet their own".[7]

[6] K. Alfonsi, *Green Chem*. **2008**, *10*, 31-36

[7] World Commission on Enviromental and development (WCED). *Our common future.* Oxford University Press, Oxford, 1987

1.2 Reaction media in a sustainable process

Particular attention has been dedicated to the reaction media in synthetic chemistry because the major source of waste (around 50%) is represented by the solvent losses[8]. Furthermore, health and/or safety matters associated with many traditional organic solvents have led to their severely reduced use. On the other hand solvent utilization is necessary to facilitate mass and heat transfer during the reaction and affects reaction rate and selectivity. The American Chemical Society Green Chemistry Institute® Pharmaceutical Roundtable (ACSGCIPR), taking into account the major issues of the use of organic media, such as carcinogenicity of halogenated solvents and flammability of highly volatile ones, has delivered guidelines for solvent use in pharmaceutical industry in order to improve the green chemistry worldwide (Fig 3).

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

Fig. 3 Guide for organic solvents regarding safety principles

[8] C. Jiminez-Gonzales, *Int. J. Life Cycle Assess.*, **2004**, *9*, 115–121

1.3 Water in organic chemistry

Recently, many efforts have been devoted to the finding of sustainable reaction media. In this contest, water has been regarded as naturally green solvent for conducting reaction processes. Indeed, water offers many advantages from both economic and environmental points of view because it is a cheap, readily available, non-toxic and non-inflammable solvent. On the other hand, water is considered a poor solvent for organic transformations because of the low solubility of organic compounds so, for a long time, it was considered a reaction contaminant.[9] However, it is now well established that the unique structure and physicochemical properties of water lead to particular interactions improving reactivity and selectivity of many reactions, simplifying the work-up procedure and recycling catalyst.

The most important example of enhanced reactivity and selectivity is represented by Diels-Alder reactions conducted in water, recently redefined by *Sharpless et al*. "on water reactions" [10] using reactants hardly soluble in this solvent at room temperature. The cycloaddition of quadricyclane and dimethyl azodicarboxilate was performed in water giving an excellent yield in a very short time as shown in Fig. 4a. Another example is the Passerini[11] condensation, a multicomponent reaction transforming three or more starting materials in a single product. In this case, using 3-methylbut-2-enoic acid, 3-methylbutanal and 2-isocyano-2-methylpropane in water, the desired product was obtained quantitatively in 3.5 hours (Fig. 4b).

[9] M.O Simon *et al., Chem. Soc. Rev.* **2012**, *41*, 1415-1427
[10] S. Narayan *et al., Angew. Chem. Int. Ed.* **2005**, *44*, 3275
[11] M.C. Pirrung *et al., J. Am. Chem. Soc.* **2003**, *126,* 444-445

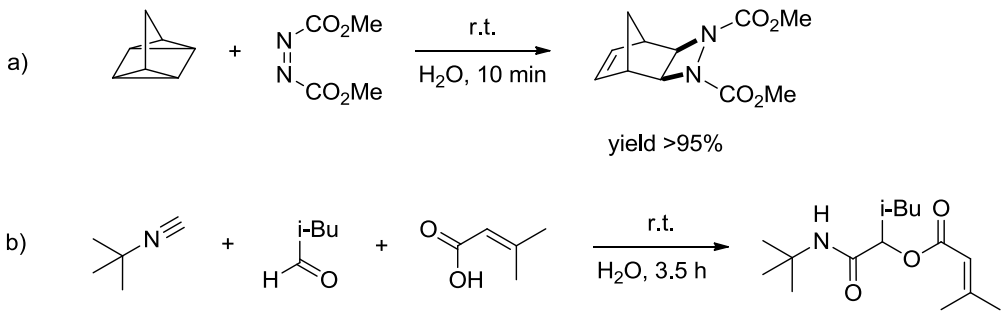


Fig. 4 Dies-Alder cycloaddition reaction and multicomponent Passerini condensation in water

The increasing of reactivity can be explained by the repulsive interactions between hydrophobic molecules and water, which leads to the formation of hydrophobic aggregates which reduces the contact surface between them. In order to maintain the network of hydrogen bonds, water wraps itself around these aggregates, thus accelerating reactions such as Dies-Alder cycloadditions or Passerini multicomponent condensations. Moreover water can improve the yield of products because it reduces side reactions. An interesting example is the industrial synthesis by Novartis of 1-substituted-4cyano-1,2,3-triazoles from 2-chloroacrylonitrile and organic azides (Fig. 5a)[12].

[12] R.Portman, *World Patent*, WO 9802423

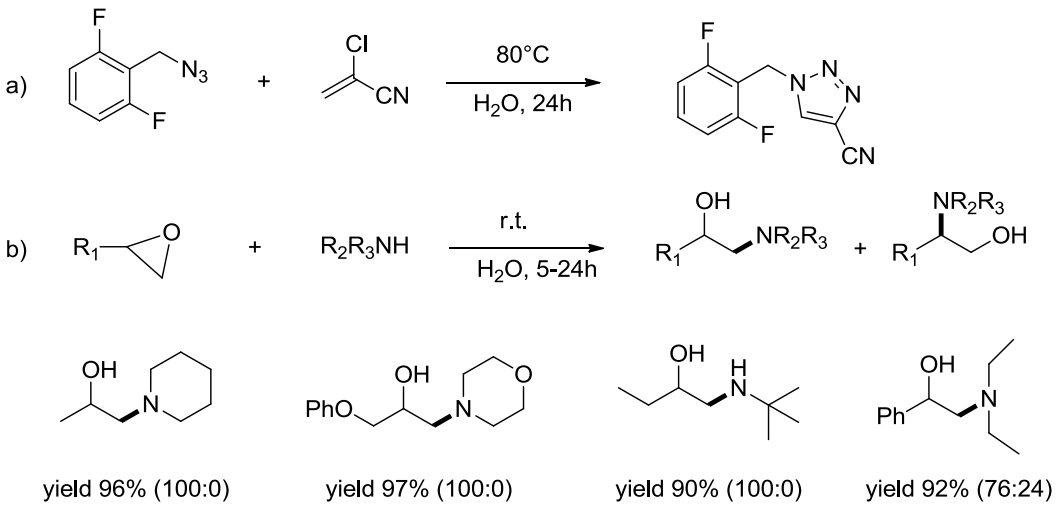


Fig. 5 (a) Minimization of side products formation and (b) enhancement of chemo- and regioselectivity in water as reaction medium.

In this reaction, the 1,3-dipolar cycloaddition is followed by an aromatization, which generates hydrogen chloride as a side product. 2-Chloroacrylonitrile is known to polymerize under acid conditions resulting in a low yield of desired product. The use of water as medium reaction leads to solubilize hydrogen chloride allowing minor polymerization of the alkene.

Selectivity is also a very important parameter for sustainable chemistry because a non-selective reaction increases the environmental impact both increasing waste, and determining through tedious and complicated purification steps. A good and well-known example is the nucleophilic opening of epoxides in the synthesis of a lot of natural products. As shown in Fig. 5b, β-aminoalcohols could be synthesized in high

yields by a reaction between epoxides and amines in water at room temperature[13]. In most cases, a total region- and stereoselectivity was obtained. In the case of styrene oxide however, both regioisomers were obtained but their yields were maximized using water as a reaction medium.

1.4 Biocatalysis

Biocatalysis is the use of biologically-derived catalysts (i.e. enzymes or whole cells) to perform chemical transformations of organic compounds. Biocatalysis offers an increasing potential for the production of goods to meet various human needs. New biocatalytic processes, have been developed to produce compounds of food, chemical and pharmaceutical interest. Enzymes are also important molecules both in terms of environmental, analytical and diagnostic aims. The driving forces in the development of the industrial enzyme technology are

- the obtainment of new products or processes to meet these needs;
- the improvement of the quality of processes for the preparation of existing products starting from raw materials.

Both these approaches may lead to an innovative product or process that is not only competitive, but also meet sustainability criteria.[14]

[13] N.Azizi *et al., Org. Lett.* **2005**, *7,* 3649-3651

[14] K. Buchhols, V. Kasche, U. T. Bornscheuer *Biocatalysts and Enzyme Technology*, Wiley-VCH Verlag GmbH & Co. **2005**

1.5 Advantages and disadvantages of biocatalysis

Biocatalysis combines many appealing features that could overcome its certain limitations. The employment of biologic systems, such as whole cells or isolated enzymes, offers important advantages:

- Chemo- regio- and stereoelectivity. These intrinsic properties indicate, respectively, the enzyme capability of selectively interacting with a functional group in presence of other similar or more reactive groups, of distinguishing between chemical groups located in different positions and of producing molecules with a specific stereochemistry. Triple-level of substrate recognition by a biocatalyst consists of chemo- regio- and stereodiscrimination which in practice leads to shorter time of synthesis due to elimination of protection and deprotection steps as well as to cleaner work-up due to prevailed formation of only one product.[15]
- Catalytic efficiency. While chemical catalysts are generally used in concentrations of 0.1-1%, enzymatic reactions can be conducted with biocatalyst concentration of 10^{-3}-10^{-4}%.
- Mild reaction conditions. Most of the enzymes works in water around physiological pH and temperature between 20 and 40 °C. These reaction conditions let the reduced utilization of solvents, toxic reagents and heavy metals.

[15] K. Faber, *Biotransformation in organic chemistry. A textbook*, 5th Ed., Springer, Heidelberg, **2004**.

- Sustainability. Biocatalysts are completely biodegradable substances.
- One-pot multienzymatic reactions. It is possible to set up multienzymatic cascade systems because many proteins are stable and active under similar reaction conditions.[16] In this way, reaction time, costs and product recovery steps can be reduced. Moreover, the isolation of instable intermediates can be avoided.
- Protein ability to accept a broad range of organic molecules. Enzymes and whole cells can transform very different substrates from those involved in the conventional cellular metabolisms. Finally, enzymatic reactions can usually proceed in both directions since enzymes do not modify the thermodynamic equilibrium of the reaction, but act on the activation energy. Moreover, recent advances in metabolic and protein engineering make possible to adapt biocatalysts to very different and unnatural substrates.

On the other hand, some of these same advantageous traits of enzymes may also constitute their limitations.

- Narrow operational window. The temperature, pH and salts concentration conditions may be unfavourable for organic compounds as well as the aqueous environment.
- Low productivity. Proteins are subjected to substrate and/or product inhibition phenomena. To overcome this problem, continuous processes may be advantageously applied.

[16] P.A. Santacoloma *et al.,* *Org. Process Res. Dev*. **2011**, *15*, 203-212.

- Low number of commercially available biocatalyst.
- High time required for the development of an industrial process.
- Cofactor-depending enzymes. Many proteins require specific and very expensive cofactors, such as NAD(P)H or $FADH_2$ for the redox reactions. It is possible to overcome this limitation by using recycling systems such as a second protein able to re-establish the initial oxidized/reduced cofactor form or whole cells bearing recombinant enzymes.
- Enzyme enantiospecificity. In nature often enzymes show only one defined enantiospecifity and a natural enantiocomplementary counterpart is not common. However it is common to find enzymes showing stereocomplementary behaviour, as they possess "mirror-image" active site.[17] Therefore, a desired stereospecific enzyme has to be found in tedious screenings, or by means of molecular engineering techniques,[18] altering the enzyme structure to produce the opposite enantiomer.
- Catalyst recovery and reuse. A requisite to cut down the costs of a process is the possibility to recycle the (bio)catalysts. In the case of enzymes or whole cells, they are often used as immobilized biocatalysts to improve their recovery and reuse.

To overcome these issues various strategies are necessary. It is possible to stabilize biocatalysts by protein engineering and/or metabolic interventions in order to

[17] P.F. Mugford *et al., Amgew. Chem. Int. Ed*. **2008**, *47*, 8782-8793
[18] G.A. Behrens *et al., Adv. Synth. Catal*. **2011**, *353*, 2191-2215

produce industrial, selective and robust catalysts; or to immobilize them for their use in continuous (flow) reactors.

1.6 Isolated enzymes versus whole cells systems

These two systems show different features and it is necessary to consider the type of reaction, cofactor recycling and biotransformation scale in order to decide to employ microorganisms or purified proteins as catalysts. In Table 1 possible advantages and disadvantages of the two kind of types of biocatalysts are summarized.[19]

Biocatalyst	Form	Advantages	Disadvanatges
Isolated enzymes	Dissolved in water	✓ Simple apparatus ✓ Simple workup ✓ Better productivity ✓ High substrate concentration tolerance ✓ High catalytic activity	✓ Cofactor recycling necessary ✓ Side reaction possible ✓ Lipophilic substrates insoluble ✓ Workup requires extraction
	Suspended in organic solvents	✓ Easy to perform ✓ Simple workup ✓ Lipophilic substrates soluble	✓ Reduced catalytic activity

[19] K. Faber *Biotranformations in Organic Chemistry. A Textbook*. 6th Ed. Springer- Verlag Berlin Heidelberg **2011**

		✓ Easy enzyme recovery	
	Immobilized	✓ Easy enzyme recovery ✓ Enzymatic recycling ✓ Possibility to use continuous flow reactors	✓ Loss of activity during immobilization
Whole cells	Growing cultures or resting cells	✓ No cofactor recycling necessary ✓ High activity ✓ Simple workup ✓ Fewer byproducts	✓ Expensive equipment ✓ Tedious workup ✓ Low productivity ✓ Low substrate concentration tolerance ✓ Low organic solvents tolerance ✓ Uncontrolled side reactions
	Immobilized cells	✓ Possible cell re-use ✓ Possibility to use continuous flow reactors	✓ Loss of activity during immobilization

Table 1 Advantages and disadvantages in using isolated enzymes or whole cells

1.7 Thermodynamic aspects of enzyme catalysis

By analogy to other catalytic processes, enzymes act on the activation energy of the reaction by lowering the energy barrier between substrate and product. An enzyme reaction consists of a first binding step and a subsequent reaction step. In a non-catalyzed reaction, one or more substrates are, at the beginning, in their respective *ground states*. During the reaction, the reactants pass the point of maximum free energy, called *transition state*, and continue to the ground state of the products. (Fig. 6).

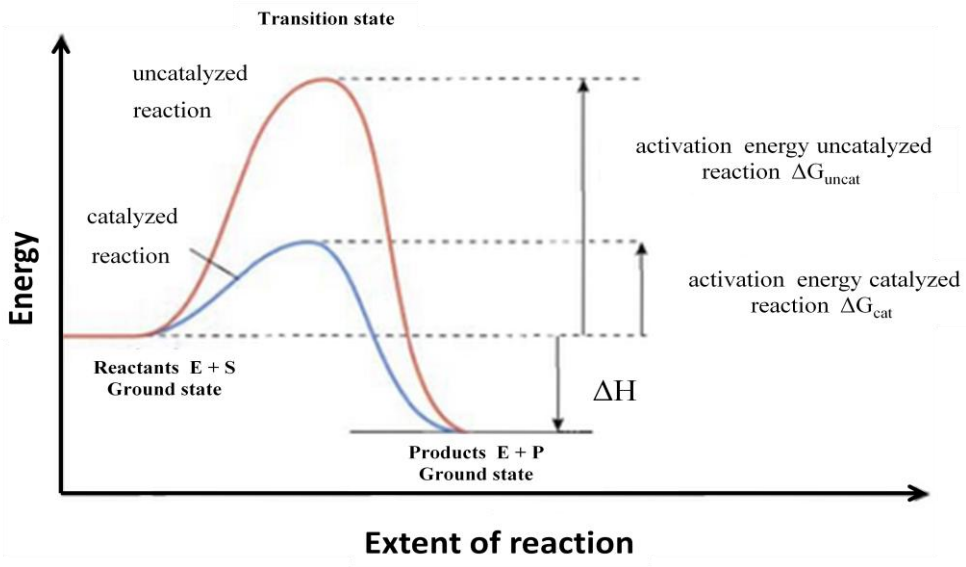


Fig. 6 Thermodynamic aspects of enzymatic catalysis

In an enzyme-catalyzed reaction, initially, free enzyme **E** and free substrate **S** likewise are in their respective *grounds state*. From the *ground state*, enzyme and substrate become a reversible enzyme-substrate complex [**ES**]. The [**ES**] complex passes through

another lower *transition state*, and finally the enzyme-product complex [**EP**] on the *ground state* of free enzyme **E** and free product **P**. The maximum of the Gibbs free energy between the *ground state* of substrate and product forms the Gibbs free energy of activation ΔG, which determines the rate of the reaction. Like every catalyst, an enzyme decreases the value of ΔG accelerating the reaction.[20]

1.8 Enzyme classification and nomenclature

The widely accepted international enzyme classification was introduced by the Enzyme Commission. For identification purpose, every enzyme has a four-digit number in the general form EC A.B.C.D., where EC stands for 'Enzyme Commission', **A** denotes the type of reaction catalyzed as shown in Table 2 , **B** indicates the substrate class or type of transferred molecule, **C** indicates co-substrate class and **D** is the individual enzyme number.

[20] A. S. Bommarius, B.R. Riebel *Biocatalysis*, Wiley-VCH Verlag GmbH & Co. **2004**

EC number	Enzyme class	Reaction Type
1	Oxidoreductases	Oxidation-reduction of double bonds, alcohols/ carbonyl groups
2	Transferases	Transfer of methyl, acyl, amino, carbonyl, glycosyl, or phosphoryl groups
3	Hydrolases	Hydrolysis-formation of esters, lactones, amides, lactams, epoxides, anhydrides, nitriles, glycosides.
4	Lyases	Addition-elimination of small molecules to multiple bounds like C=C, C=O, C=N
5	Isomerases	Racemization, epimerization, rearrangement
6	Ligases	Formation-cleavage of C-C, C-O, C-N, C-S bonds with concomitant triphosphate cleavage

Table 2 Enzyme classification

Information about catalytic activity is expressed by *katal* (1 kat = 1 mol s^{-1} of substrate transformed), but it has not widely accepted because of its too big magnitude to practical application. As consequence a more used measure has been *International Unit* (1 I.U.= 1 µmol min^{-1} of substrate transformed). The biocatalyst catalytic power can be described by *turn over frequency* (TOF). It indicates the number of substrate molecules converted by a single catalyst molecule during a specific time. For the majority of enzymes

TOF values are between 10 and 1000 s^{-1}, whereas for chemical catalyst about one order of magnitude lower. The productivity of a biocatalyst is indicated by the *turn over number* (TON). It represents the number of substrate molecules converted by a single catalyst during its lifetime and indicates the durability of the catalytic system.[21]

1.9 Development of a biocatalytic process in the pharmaceutical industry

The use of biocatalyst has acquired importance as a complement to conventional chemical techniques in various application fields. In particular, biocatalyst selectivity is important for obtaining pharmaceuticals, whose stereochemistry affect their pharmacological characteristics. The isomer with the highest activity is defined 'eutomer' and its enantiomer with less or undesired activities 'distomer'.[22]

For a convenient employment of a biocatalyst in an industrial process, it is necessary to consider also development time of a large-scale process and final productivity.[23] Within an industrial process a biocatalyst has to be active on elevate substrate concentrations (around 50-100 g/L) and the ratio between product grams and catalyst grams should be about 1000.[24] The scale-up from laboratory conditions to industrial ones could be a problematic step. In fact, the extension of reaction time, the increasing of substrate concentration, the improvement of energy and instrumentations for large tanks could cause catalyst degradation or loss of activity and productivity. In order to

[21] P. Atkins, J.De Paula *Physical Chemistry*, 8th Ed. Chapter 23, Oxford University Press, New York, **2006**

[22] K. Faber *Biotranformations in Organic Chemistry. A Textbook*. 6th Ed. Springer- Verlag Berlin Heidelberg **2011**

[23] J.D. Pollard *et al., Trend Biotechnol.* **2006**, *25*, 66-73

[24] J. D. Rozzel *et al.*, *Bioorg. Med. Chem.,* **1999**, *7*, 2253-2261.

obtain high products yield, different strategies are possible such as product removal from reaction environment or controlled substrate supplying using hydrophobic resins, ionic liquids or crystallization/precipitation techniques.[25]

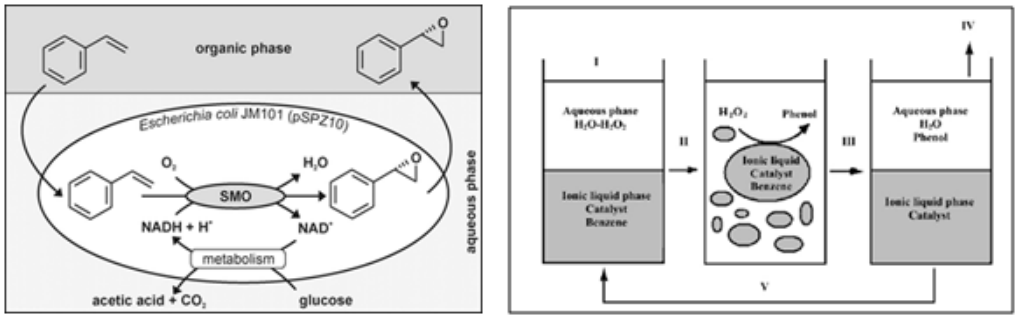


Fig. 7 Example of biphasic system and liquid ionic solvent biotransformation

Most likely, the most important reason for the biocatalysts employing in medicinal chemistry is their stereoselectivity. The production of optically pure compounds by pharmaceutical industries has become a very important issue overall after the publication of guidelines provided by the Food and Drug Administration (FDA) in 1992. In this document is underlined the importance to characterize and verify the pharmacological and toxicological effects of both the enantiomers of a racemic mixture.[26] In this contest, the chiral synthesis for obtaining a single isomer has become a key point for synthetic medicinal chemistry. In Fig. 8 is shown the increasing of the optical pure active molecules on sale from 1980 to 2000. In the 1990 the pure optical pharmaceutical preparations were only the 25% while in 2000 they were improved to 60%.

[25] G. J. Lye *et al., Trend Biotechn.* **1999**, *17*, 395-402
[26] www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122833.htm

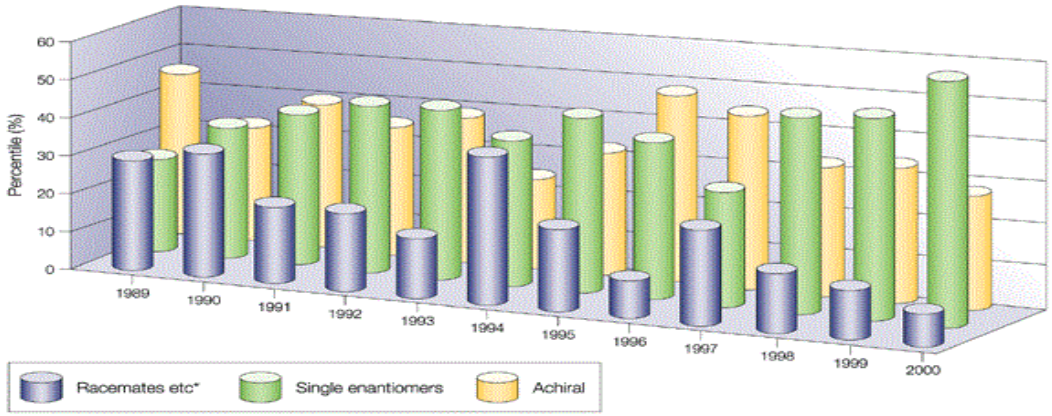


Fig. 8 The increasing of the production of pure optical pharmaceuticals

The possibility of using a biocatalytic process is particularly meaningful if it is adopted during the first phase of a drug development, because the late substitution of an optimized chemical step is very difficult. Firstly, the industrial employment of isolated enzymes is more convenient because of the possibility to quickly produce pilot drug amounts for clinical tests (phase I and II). Subsequently, for the production on large scale, the utilization of whole recombinant cells with over-expressed desired enzymes is economically favourable.[27] For an industrial process development various disciplines such as chemistry, molecular biology, enzymology, microbiology and engineering, are needed. Moreover, in order to reduce developing time, to utilize low substrate amounts and to obtain many process data, preliminary studies are made in miniaturized conditions using for example ninety-six wells microplates containing lyophilized isolated enzymes or using microscale analytic techniques. For the same aims, mathematic models furnishing

[27] M.D. Truppo *et al.*, *Org. Proc. Res. Dev.* **2006**, *10*, 592-598; A.J.J. Straathof *et al., Curr. Opin. Biothecnology* **2002**, *13*, 548-556; D. Pollard *Tet. Asym.* **2006**, *17*, 554-559

simulating reaction conditions are applied. These models are less widespread for biocatalytic processes than chemical ones.

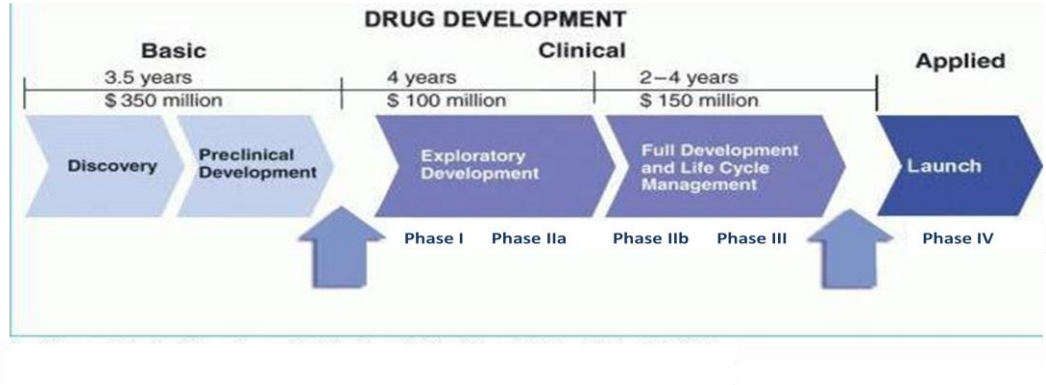


Fig. 9 Drug discovery timeline and cost

1.10 Examples of chiral drugs

Biocatalytic methods for the preparation of some popular drugs, such as profens, statins and ACE inhibitors, have been thoroughly studied.

The profens family belongs to the most important non-steroidal anti-inflammatory drugs (NSAIDs); 2-arylpropionic acids are the key intermediates in the preparation of NSAIDs used for the inhibition of cyclooxygenase-1 and 2 (COX-1 and COX-2), responsible for the synthesis of prostaglandins involved in inflammatory

process.[28] It is known that the pharmacological effect is determined by (*S*)-enantiomer, so the production of this optically pure compound is of great interest.[29]

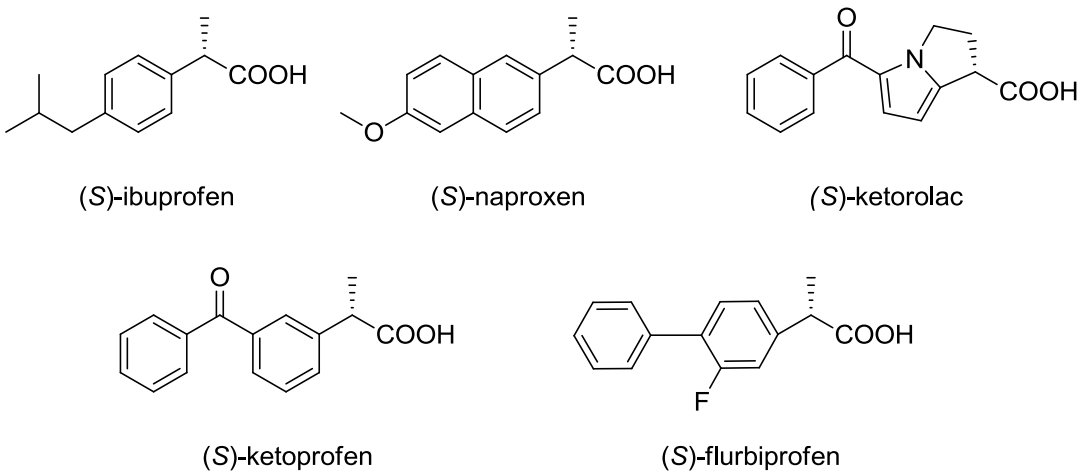


Fig. 10 Non-steroidal anti-inflammatory drugs

Ibuprofen, well-known anti-inflammatory drug, can be produced as (*S*)-enantiomer by various biocatalytic processes involving lipases. One method comprises the use of an immobilized lipase from *Candida rugosa* (CRL) for catalysing the kinetic resolution (KR) of racemic ibuprofen methylester. The lipase catalyzes the hydrolysis of the racemic mixture of ibuprofen methylester, with selectivity for the (*S)*-enantiomer (Fig. 11). In order to obtain good yields, it has been developed a two-phase system where the esterified substrate better dissolves in the hydrophobic phase whereas (*S*)-ibuprofen partitions in aqueous phase. The CRL is immobilized on a membrane placed to the interface.

[28] J.L. Masferrer *et al. Am. J. Ther.* **1995**, *2*, 607-610
[29] C. Reichel *et al. Mol.Pharm.* **1997**, *51*, 576-582.

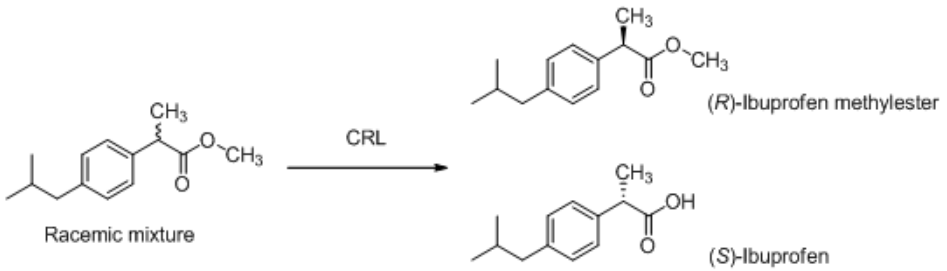


Fig. 11 Enantioselective hydrolysis of Ibuprofen methylester

KR has been applied also to obtain the (*S*)- enantiomer of flurbiprofen. (*S*)-flurbiprofen has anti-inflammatory properties, while the (*R*)-enantiomer shows anticancer effects *in vivo* and *in vitro* studies. KR of (*R*,*S*)-flurbiprofen may be achieved *via* esterification carried out in organic media (Fig. 12); a particular innovative one, using an immobilised lipase B from *Candida antarctica* (Novozym 435®) in a continuous flow reactor, has been reported to exhibit a relatively high enantiopreference toward (*R*)-flurbiprofen.

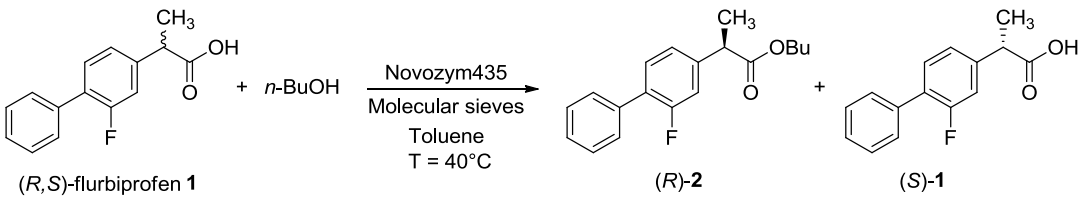


Fig. 12 Enantioselective esterification of (*R*,*S*)-flurbiprofen **1** with *n*-BuOH catalysed by Novozym 435 with the addition of molecular sieves, in toluene.

One general limitation of KR is the maximum conversion obtainable (50%) for recovering an enantiopure product; dynamic kinetic resolution (DKR), where the slow-reacting enantiomer racemizes with a rate faster than the asymmetric transformation, offers an impactful alternative, since 100% of the racemic substrate can be converted to the enantiopure product. Continuous flow reactors could help to overcome some of the limitations of traditional enzymatic KR in batch, such as long reaction times, product inhibition of the enzymes scalability. Moreover, in continuous flow reactors recovery of the products and set-up of DKR processes are relatively easy. In the case of the esterification of racemic flurbiprofen, the use of a continuous flow reactor allowed for higher reaction rates, higher biocatalyst stability, and recovery of the unreacted (*S*)-carboxylic acid using an ionic exchange resin. The process was further implemented by racemization of the unreacted flurbiprofen directly into the polymer-supported resin and recycle of the racemic substrate. [30]

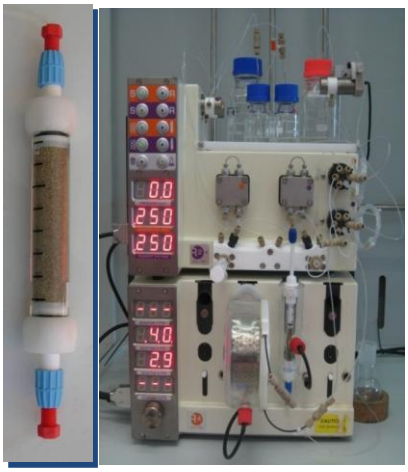


Fig. 13 Flow chemistry reactor and immobilized Novozyme 435 column

[30] L. Tamborini *et al J. Mol.Catal B,* **2012**, *84*, 78-82

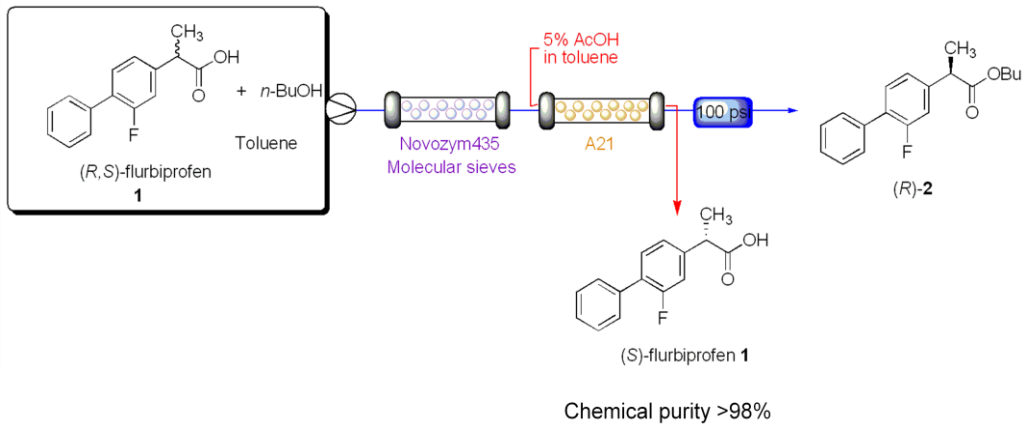


Fig. 14 Kinetic resolution of Flurbiprofen and in-line purification

Statins are cholesterol lowering drugs, which inhibit the enzyme HMG-CoA reductase, an enzyme playing a central role in the production of endogenous cholesterol. Statins are administered to prevent cardiovascular disease and mortality in those who are at high risk.[31]

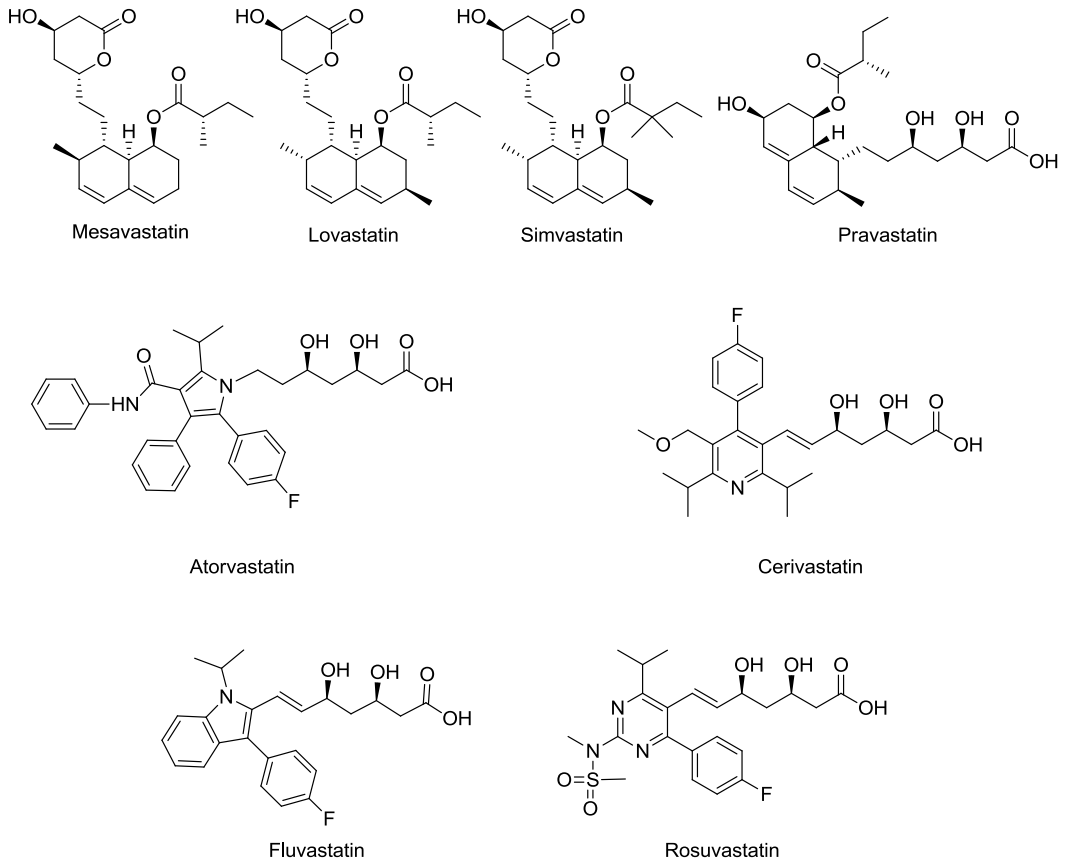


Fig. 15 Statins

The essential structural components of all statins are a dihydroxyheptanoic acid unit and a ring system with different substituents. The statin pharmacophore is a modified

[31] E. A. Stein *et al. Clin.Cardiol.* **2003**, *26*, 25-31

hydroxyglutaric acid component, which is structurally similar to the endogenous substrate HMG-CoA. It binds the same active site as the substrate of HMG-CoA and inhibits the HMGR enzyme. It has also been shown that HMGR is a stereoselective enzyme and, as a result, all statins need to have the required 3*R*,5*R* stereochemistry.[32] The chemical synthesis could be very complex, in particular for obtaining of the correct stereochemistry. The conventional production of atorvastatin requires numerous steps and drastic reaction conditions such as temperature -90°C or use high quantities of solvents. To overcome these problems several biocatalytic alternatives for the synthesis of the different building blocks, in particular ethyl (*R*)-4-cyano-3-hydroxybutyrate, also called hydroxynitrile and its 5-hydroxy-3-keto derivative have been developed.

[32] D.E. Butler et al. US patent 5155251, **1993**.

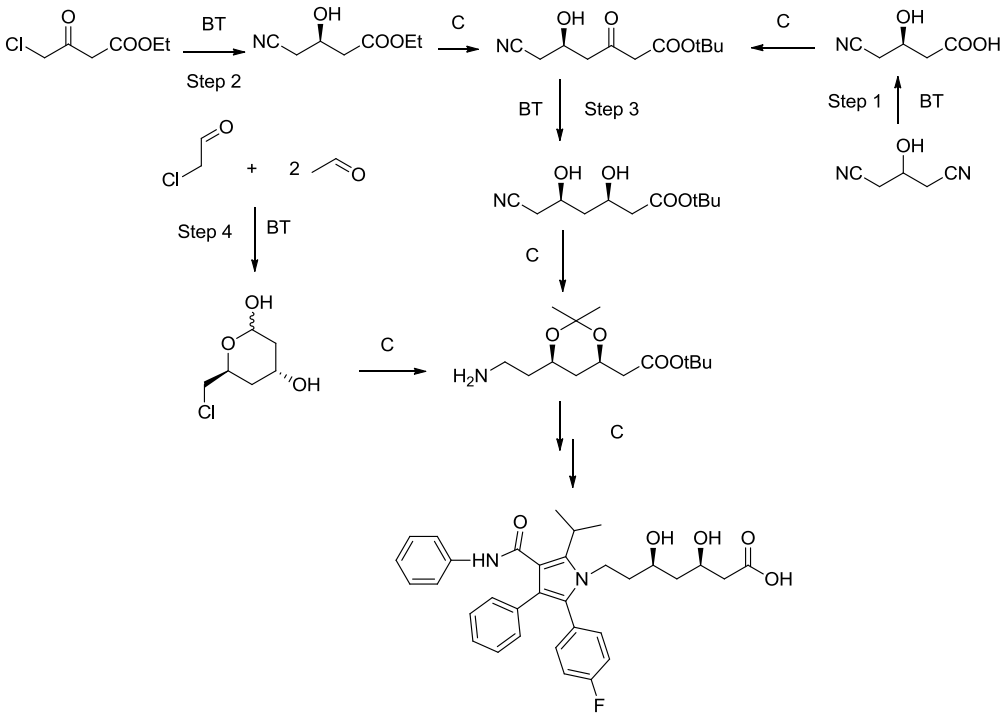


Fig. 16 Synthesis of atorvastatin with biocatalityc (BT) and chemical steps (C)

As shown in Fig. 16 (*R*)-4-cyano-3-hydroxybutyrate can be obtained by a mutant nitrilase from 3-hydroxy glutaronitrile (Step 1).[33] The acid was esterified with *tert*-butyl acetate and oxidized to give the 5-hydroxy ketoderivate. Two alternative steps can be used for synthesizing this intermediate: an asymmetric reduction of 4-chloroacetoacetate (Step 2) and a subsequent oxidation to obtain the 5-hydroxy ketoderivate. For the synthesis of this key intermediate, Codexis has developed a biocatalytic method using three different enzymes: a ketoreductase (KRED) to obtain (*S*)-CHBE with an excellent e.e. (> 99.0%), a glucose dehydrogenase (GDH) for the recycle of NADPH used in the first step by KRED, and a halohydrin dehalogenase (HHDH) which, by removing hydrogen chloride, generates

[33] G. Desantis *et al., J. Am. Soc*, **2002**, *124*, 9024-9025; G. Desantis *et al., J. Am. Soc*, **2003**, *125*, 11476-11477.

an epoxy ring; the stoichiometric HCl produced in this step activates sodium cyanide allowing for the opening of epoxy ring.[34]

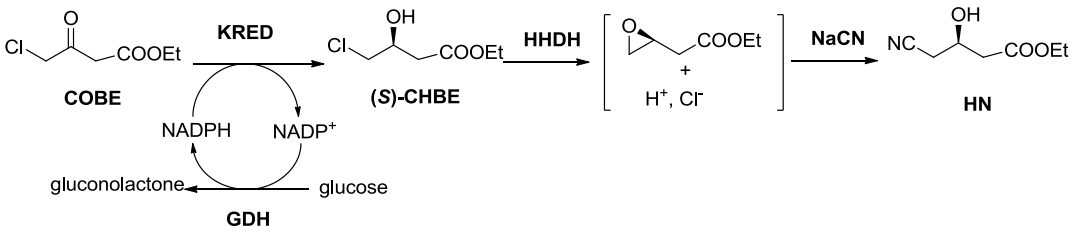
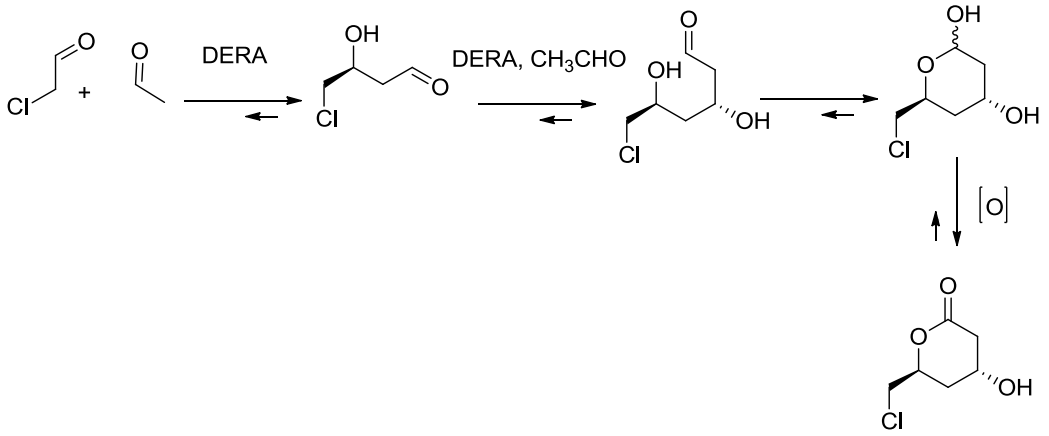


Fig. 17 Syntesis of **HN** by Codexis

For the reduction of ketone of the 3-hydroxy-5-cyano derivate, an alcohol dehydrogenase NADPH-dependent from *Lactobacillus brevis* over-expressed in *E. Coli* has been employed. The same enzyme is able to regenerate the cofactor because of the oxidation of 2-propanol into acetone.[35] The last biotransformation step forms the lactone from chloro acetaldehyde by employing a mutant aldolase (DERA) as shown in Fig. 18.



.

Fig. 18 Biocatalytic synthesis of lactone

[34] M. Schallmey *et al. Chem.Bio. Chem*. **2013**, *14*, 870-881
[35] M. Wolberg *et al. Bioprocess Biosyn*. *Eng.* **2008**, *31*, 183-191

Angiotensine Converting Enzyme (ACE) inhibitors are pharmaceuticals used for the treatment of hypertension and congestive heart failure. They inhibit the enzyme responsible for the conversion of Angiotensin I into Angiotensin II, important component of the rennin-angiotensin-aldosterone system. The most important drug is Captopril (Fig. 19)

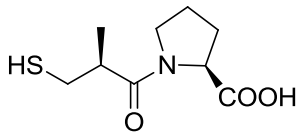


Fig. 19 Captopril

The pharmaceutical effect depends on side chain containing the –SH group. Noteworthy is the *S* configuration of the chiral centre in 2 position, which confers a hundred times major activity than the opposite enantiomer.[36] There for, a stereoselective synthesis of the side chain is very important. Lipases from *Rhizopus orizae* and *Pseudomonas cepacia* hydrolyze stereoselectively the thioester of racemic mixture of 3-acetylthio-2-methylpropanoic acid, in order to obtain the (*S*)-enantiomer (yield 28%, e.e. 95%). The best yield and enantiomeric excess were obtained using an esterase from *Pseudomonas sp.* MRC, which catalyzes the hydrolysis of the methyl ester of 3-acetylthio-2-methylpropanoic acid (yield 49%, e.e. >99.0%) as shown in Fig.20.[37]

[36] M.A. Ondetti et *al,. J. Med. Chem.,* **1981**, *24*, 355-361
[37] R.N. Patel, *Coord. Chem. Rev.* **2008**, 252, 659-701

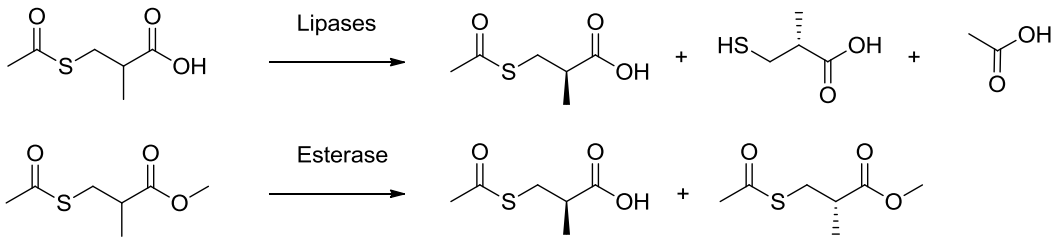


Fig. 20 Enantiselective reactions to obtain the key intermediate for Captopril synthesis

Another alternative for the preparation of (*S*)-acid of the side chain of Captopril could be an enantioselective esterification of the racemic mixture of the 3-benzothio-2-methylpropanoic acid. Lipases of *Rhizopus orizae* and *Pseudomonas cepacia* in toluene are able to esterify only the (*R*)-enantiomer, leaving the unreacted acid in the *S*-form.[38]

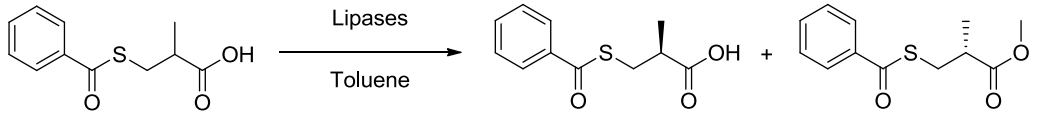


Fig. 21 Enantioselective esterification of the 3-benzothio-2-methylpropanoic acid

An early chirogenic step has been introduced by oxidation of different meso compunds, as shown in Figure 22. (*R*)-3-hydroxy-2-methylpropanoic acid has been obtained by hydroxylation of isobutyric acid or by oxidation of 2-methyl-1,3-propanediol. Both the microbial oxidation proceeded with high yields and enantioselectivity.

[38] R.N. Patel *et al., Appl. Microbiol. Biotechnol*. **1991**, *36*, 29-34

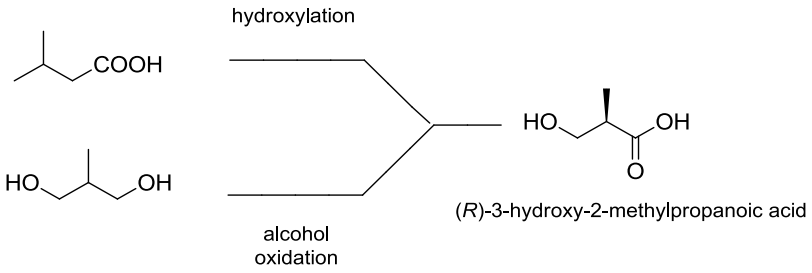


Fig. 22 Oxidation of different molecules for obtaining the key intermediate for the synthesis of Captopril

Also for the synthesis of the Monopril, biocatalitic strategies have been studied in order to obtain the (*S*)-monoacetate ester, key intermediate for the synthesis of this ACE-inhibitor. Lipases such as porcine pancreatic lipase (PPL), or that from *Chronobacterium viscosum*, stereoselectively hydrolyze diacetate esters as shown in Fig. 23.[39]

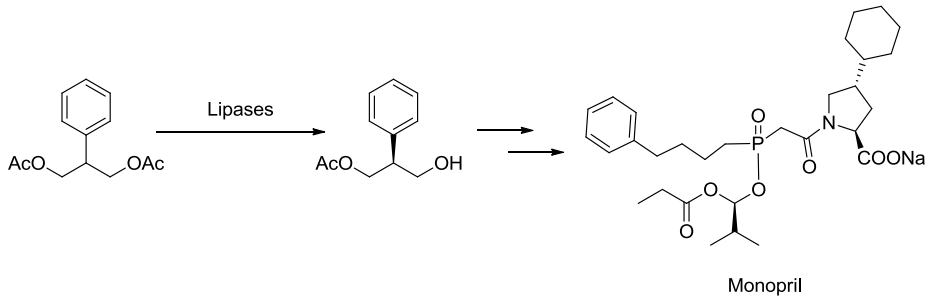


Fig. 23 Monopril and its key intermediate

[39] O.A. Lednev *et al., Klin.Med (Mosk.),* **2009**, *87*, 48-49

Aim of the project

2.1 Aim of the PhD project

This PhD project is divided in two parts. The first one aims at exploring the biocatalytic capabilities of the whole cells of the unconventional yeast *Pichia glucozyma* CBS 5766, evaluating its reducing activity towards different substrates of pharmaceutical interest. The second one aims at isolation, biochemical characterization, cloning and over-expression of one of the enzyme responsible for the stereoselective reduction of aromatic ketones.

Pichia glucozyma whole cells

The investigation of *Pichia glucozyma* as whole cells biocatalyst

3.1 Why yeasts?

In recent times, because of their peculiar features and history, yeasts have become important experimental models in both microbiological and genetic research as well as the main characters in many pharmaceutical industrial fermentative production processes. For these reasons yeasts have a great importance both in science and in every days human life. The modern exploitation of yeast in biotechnology has found place in a wide range of different fields such as food/chemical industries, health-care industries, fundamental biological research, biomedical research, environmental technologies and fermentation industries.[40] Such uses are also possible because of genetic modifications, especially for the strains of *Saccharomyces cerevisiae*, which is by now the best characterized yeast specie.[41] The wide utilization of yeast in industrial processes goes together with the fact that since they are widely cultivated and the same time genetically well accessible, they have represented as an ideal model system. Multiple features make this simple eukaryote a model organism of choice. First of all, an increasing number of species are well genetically and genomically defined. Moreover, highly annotated online database have become available, which provide multitudes of detailed information on yeast genes and proteins. Yeast is easy to grow, with a doubling time relatively short and survives for long time in frozen glycerol stock. Other versatile characteristics of this

[40] G.M Walker Yeast phisiology and biotechnology. **1998**, John Wiley and Sons (ed.), Chichester UK
[41] J. Hansen *et al, J.Biotechnol*. **1996**, *49*, 1-12

organism are the ease transformation and the ability to integrate genes by homologous recombination. Although not ubiquitous as bacteria, yeasts are widespread in the natural environment. Yeast cells lack chlorophyll (are not photosynthetic) and strictly chemoorganotrophic, meaning that they require fixed, organic forms of carbon for growth. Sources of carbon for yeast metabolism can be various such as sugars, polyols, organic fatty acids, aliphatic alcohols, hydrocarbons and different heterocyclic compounds. Furthermore, yeasts are usually growing well also at lower pH value than those that are optimal for bacteria, and they are insensitive to antibiotics that inhibit bacteria growth: that means the possibility to keep free of contamination by fast-growing bacteria yeast culture. Because of their large size yeast are more easily and cheaply harvested than bacteria. Regarding yeast taxonomy they are classified as plants and trees: strains are grouped into species, species are arranged into genera and genera in families. Families are assembled in orders and orders in classes and these ones in divisions. While some yeasts use exclusively aerobic respiration, others, in oxygen absence, can use an anaerobic respiration process, called fermentation. These yeast types produce energy by converting sugars in carbon dioxide and ethanol. In the alcoholic beverages fermentation the production of ethanol is useful, while in the leavened bread and derivates, the carbon dioxide raises the dough and ethanol evaporates.[42] Respiration processes are more favourable from metabolic point of view and are the more used conditions for biocatalytic reactions.

[42] P.F. Stanbury and A. Whitaker *Principles of Fermentation Technology*, **1993** Pergamon press, Oxford

3.2 *Pichia glucozyma*

Pichia glucozyma (*Hansenula glucozyma* or *Ogatea glucozyma*) CBS 5766 is an unconventional yeast belonging to ascomycota *phylum.* It was isolated from parasites of *Picea engelmanii*, a typical North American fir.[43] Macroscopically it appears between white and cream colour. Budding is its reproduction mode. In our laboratory this strain grows up in M5YE medium (5 g/L yeast extract dissolved in malt infusion) at 28°C in 48 hours and it was conserved in frozen glycerol stocks.

The increasing attention for this microorganism derives from its stereoselective reducing activity. In the last few years it was widely studied and employed as whole cell biocatalyst for obtaining optically pure products interesting from pharmaceutical point of view.

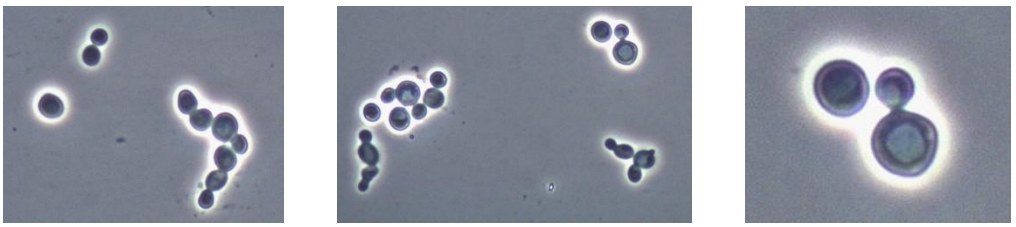


Fig. 24 Whole cells of *P. glucozyma* (magnification 400X)

3.3 Aromatic ketones and ketoesters as substrates

Enantiomerically pure alcohols including α- β- and γ-hydroxyesters are important and valuable intermediates in the synthesis fine chemicals and pharmaceuticals[44] such as penem and carbapenem antibiotics, L-carnitine and inhibitors of HMG-CoA

[43] L. J. Wickerham, *Mycopathol. Mycol. Appl.* **1969**, *37*, 15-32
[44] D. Zhu *et al., J. Org. Chem*. **2006**, *71*, 4202-4205

reductase.[45] They also can be considered synthons for the preparation of a large group of anti-depressants and α- or β-adrenergic drugs.[46] Various synthetic methods have been investigated to obtain optically pure alcohols, α- β- and γ- hydroxyl acids and their derivates. Among these methods, a straightforward approach is the reduction of prochiral ketones to chiral alcohols. In this contest a different chemical catalysts such as metal complexes in particular Rhutenium, Rhodium and Iridium ones[47], or organometallic reagents (DIBAL-H, BINAL-H)[48] have been developed for the asymmetric ketones reduction. In comparison to metal hydride reducing agents ($NABH_4$, $LiAlH_4$) these reagents offer conveniences in handling and stoichiometric addition as well as major or completely stereoselectivity. However, in many cases, some difficulties remain in the process operations and for obtaining the optically pure desired product. Moreover, residual metal in the final products originated by using metal catalysts presents another challenge because of ever more stringent regulatory restrictions on the level of metal allowed in pharmaceutical products.[49] An alternative to chemical asymmetric reduction processes is the biocatalytic transformation with whole cells of yeasts, as said before, offers advantages such as mild and environmentally conditions, high chemo- regio- and stereoselectively, and being void a residual metal in the alcohols.

[45] A. Matsuyama *et al., Org. Process Res. Dev.* **2002**, *6*, 558-561
[46] D. Zhu *et al., Tet. Asymm.* **2005**, 1*6*, 3275-3278
[47] A. Zanotti-Gerosa *et al., Platinum Metals Rev.* **2005**, *49*, 158-165
[48] P. Daverio *et al., Tet. Asymm*. **2001**, *12*, 2225-2259
[49] A. Thayer, *Chem. Eng. News* **2005**, *83*, 55-58

Tetrahedron Letters 55 (2014) 7051–7053

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Biotransformation of aromatic ketones and ketoesters with the non-conventional yeast *Pichia glucozyma*

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ARTICLE INFO

Article history:
Received 22 August 2014
Revised 21 October 2014
Accepted 27 October 2014
Available online 31 October 2014

Keywords:
Aromatic ketones
Aromatic ketoesters
Reduction
Enantioselective
Ketoreductase
Esterase

ABSTRACT

The non-conventional yeast *Pichia glucozyma* CBS 5766 has been used for the biotransformation of different aromatic ketones and ketoesters. The growth and biotransformation conditions were optimised for the reduction of acetophenone and under optimised conditions, propiophenone, butyrophenone and valerophenone were reduced to the corresponding (*S*)-alcohols with high yields and enantioselectivity. Ketoreductase(s) of *Pichia glucozyma* showed high catalytic activities also towards aromatic β- and γ-ketoesters, being often competitive with esterase(s). These concurrent activities allowed for the preparation of hydroxyesters, hydroxyacids and lactones often in a very selective manner.

The asymmetric reduction of prochiral ketones using isolated or cell-bound ketoreductases is a well-recognised method for the preparation of chiral alcohols.[1] The major drawback encountered with enzymatic reduction using isolated enzymes is the necessity for cofactor recycling, which can be circumvented by two-enzyme or one-enzyme recycling methodologies.[2] Microbial whole cells exhibit as a major advantage that cofactors are already present and can be recycled via the oxidation of a second substrate (i.e., glucose or glycerol).[3] Beside the easily available *Saccharomyces cerevisiae*,[4] other microbial species have been widely employed for asymmetric reductions or more generally as sources for new selective ketoreductases.[5] However, there is still a need for new biocatalysts able to perform stereoselective reductions with different or ameliorated chemo-, regio- and stereoselectivity, especially towards bulky substrates.[6] Non-conventional yeasts are plentiful sources of different carbonyl reductases and during our past works on enantioselective carbonyl reduction we have found that whole cells of the yeast *Pichia glucozyma* CBS 5766 (now reclassified as *Ogataea glucozyma*) catalysed the stereoselective reduction of different ketones, often showing remarkable results in terms of stereoselectivity.[7] In this work, we have studied in detail the potential of whole cells of *Pichia glucozyma* CBS 5766 for the biotransformation of various aromatic ketones and ketoesters under optimised conditions.

Reduction of acetophenone (**1a**) was firstly studied with whole cells of *Pichia glucozyma* grown using different culture media. Experiments were performed with cells harvested after 24 and 48 h and suspended in phosphate buffer (0.1 M, pH 7) in the presence of 5% glucose. The best results were observed when *P. glucozyma* was grown in a medium composed with malt extract and 0.5% yeast extract for 24 h: reduction of **1a** with *Pichia glucozyma* grown under these conditions, furnished (*S*)-1-phenylethanol (**2a**) with good enantioselectivity (92%) and yield (70%). The conditions of the sequential experimental trials aimed at the optimisation of the biotransformation conditions were selected employing the Multisimplex® 2.0 software;[8] control variables were biocatalyst and substrate concentrations, pH and temperature. The best results in terms of yields and enantioselectivity were observed employing 25 mg of dry cells and 20 mM substrate concentration, in the presence of glucose (277 mM, 50 g/L) at pH 7.0 and 28 °C; whole cell based specific activity towards acetophenone under these conditions was 1.33 mmol/$g_{dry\ cells}$ min (see Supplementary data for definition). The influence of different co-substrates (ethanol, glucose, glycerol, sucrose xylose at 50 g/L) was independently assayed. The results obtained using glucose, glycerol and xylose were very similar, while a slight decrease of enantioselectivity was detected with ethanol.

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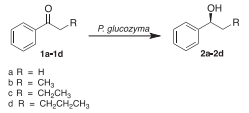
http://dx.doi.org/10.1016/j.tetlet.2014.10.133
0040-4039/

Table 1
Reduction of aromatic ketones (20 mM) with *Pichia glucozyma* CBS 5766



Entry	Substrate	Yield (%)	ee **2** (%)	Time (h)
1	**1a**	39	97 (*S*)	4
2	**1a**	90	97 (*S*)	18
3	**1b**	28	97 (*S*)	1
4	**1b**	97	97 (*S*)	6
5	**1c**	41	>98 (*S*)	1
6	**1c**	96	>98 (*S*)	4
7	**1d**	46	>98 (*S*)	1
8	**1d**	97	>98 (*S*)	4

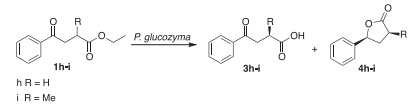
Biotransformations were carried out with freshly prepared cells (50 $mg_{dry\ weight}$/ml) suspended in phosphate buffer (0.1 M, pH 7) in the presence of 5% glucose. Enantiomeric excesses were determined by chiral HPLC analysis (see Supplementary data for details).

Reduction of acetophenone (**1a**), propiophenone (**1b**), butyrophenone (**1c**) and valerophenone (**1d**) under optimised conditions (resting cells 20 $mg_{dry\ weight}$/ml suspended in phosphate buffer 0.1 M, pH 7 in the presence of 50 g/L of glucose) is reported in Table 1.[9] Reductions occurred with moderate to high conversion and with reaction rates increasing when hydrophobicity of the substrates was increased; high enantioselectivity was always observed, giving the corresponding (*S*)-alcohol (Prelog rule). The absolute configuration was determined by comparison with reported chiral HPLC data.[6a,7a]

Aromatic β-ketoesters were also assayed (Table 2). It is known that biotransformations of ketoesters with yeasts occur with competition between ester hydrolysis and carbonyl reduction, due to the occurrence of cell-bound esterase activities;[10] this competitive enzymatic activity can be modulated by using appropriate substrate/co-substrate concentrations: with high co-substrate concentrations (e.g., glucose >50 g/L) the activity of ketoreductases is generally predominant, as a consequence of redox cofactor regeneration, whilst esterase activity is mostly observed in the absence of cosubstrates.[7g] The biotransformations were performed in optimal conditions for favouring ketone reduction (50 g/L glucose; Table 2).

The biotransformation of ethyl benzoylacetate **1e** showed that ketoreductase activity was predominant in the first part of the biotransformation leading to (*S*)-ethyl-3-hydroxy-3-phenylpropanoate (**2e**, yield 87%, ee 86% after 4 h; Table 2, entry 1), which was further enzymatically hydrolysed at a prolonged time (24 h), giving optically pure (*S*)-3-hydroxy-3-phenylpropionic acid (**3e**) and leaving low amounts of (*R*)-**2e** (ee 95%; Table 2, entry 2). The absolute configuration of **2e** and **3e** was determined by comparison with reported chiral chromatographic data.[11,12] These results can be explained by suggesting the occurrence of an enantioselective esterase able to preferentially hydrolyse (*S*)-**2e**, therefore leaving unreacted (*R*)-**2e** with high ee. Ethyl 2-methyl-3-oxo-3-phenylpropanoate **1f** was reduced to the corresponding alcohol **2f** with limited enantiomeric and diastereomeric excesses (Table 2, entry 3); absolute configuration of **2f** was determined by comparison with reported chiral chromatographic data.[6b], No traces of ester hydrolysis were found even at prolonged times, most likely due to the steric hindrance caused by the methyl in 2-position. Ethyl-2-(benzamidomethyl)-3-oxo-phenylpropanoate (**1g**) was selectively reduced to ethyl-2-(benzamidomethyl)-3-hydroxy-phenylpropanoate (**2g**); the biotransformation occurred with higher conversion rate and yield than what was reported before with cells used under non-optimised conditions of growth and biotransformation (Table 2, entry 4).[7g]

Table 3
Biotransformation of ethyl γ-ketoesters with *P. glucozyma*



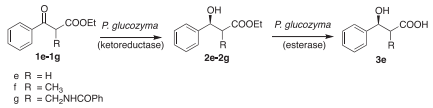
	Substrate	**3**		**4**			
		Yield (%)	ee (%)	Yield (%)	ee (%)	de (%)	Time (h)
1	**1h**	55	—	40	14(*S*)		12
2	**1i**	44	82(*R*)	40	70	92(3*S*,5*S*)	24

Biotransformations were carried out with freshly prepared cells (50 $mg_{dry\ weight}$/ml) suspended in phosphate buffer (0.1 M, pH 7) in the presence of 50 g/L glucose. Stereochemical compositions were determined by chiral HPLC analysis (see Supplementary data for details).

Biotransformation of ethyl γ-ketoesters **1h–i** gave a mixture of lactones and ketoacids (conversion and stereochemical composition after 12 and 24 h, Table 3).

Biotransformation of ethyl 4-oxo-4-phenylbutanoate **1h** gave a prevalence of the corresponding ketoacid **3h** (55%), whereas lactone (*S*)-**4h** was obtained in 40% yield and low enantioselectivity (14%; Table 3, entry 1). Racemic ethyl 2-methyl-4-oxo-4-phenylbutanoate **1i** was resolved as a result of the action of enantioselective esterase(s) with formation of the ketoacid (*R*)-**3i** (ee 82% at 44% conversion), while reduction of the carbonyl afforded lactone (3*S*,5*S*)-3-methyl-5-phenyldihydrofuran-2(3H)-one (**4i**) with high diastereoselectivity and good enantioselectivity (Table 3, entry 2). Stereochemical compositions were determined by combining chiral HPLC analysis and ^{1}H NMR.[13a–c]

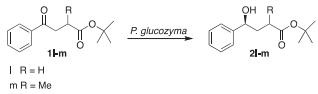
Table 2
Reduction of aromatic ethyl β-ketoesters (20 mM) with *Pichia glucozyma* CBS 5766



Entry	Substrate	**2**			**3**		
		Yield (%)	ee (%)	de (%)	Yield (%)	ee (%)	Time (h)
1	**1e**	87	86(*S*)	—	<5	—	4
2	**1e**	14	95(*R*)	—	75	>97(*S*)	24
3	**1f**	96	80(*S*)	34(2*S*,3*S*)	0	—	24
4	**1g**	95	>98(*S*)	70(2*R*,3*S*)	0	—	16

Biotransformations were carried out with freshly prepared cells (50 $mg_{dry\ weight}$/ml) suspended in phosphate buffer (0.1 M, pH 7) in the presence of 50 g/L glucose. Stereochemical compositions were determined by chiral HPLC analysis (see Supplementary data for details).

Table 4
Biotransformation of *tert*-butyl γ-ketoesters with *P. glucozyma*



Entry	Substrate	2			
		Yield (%)	ee (%)	de (%)	Time (h)
1	**1l**	60	10(*S*)		18
2	**1m**	70	75(*S*)	<5	24

Biotransformations were carried out with freshly prepared cells (50 $mg_{dry\ weight}$/ml) suspended in phosphate buffer (0.1 M, pH 7) in the presence of 50 g/L glucose. Stereochemical compositions were determined by chiral HPLC analysis (see Supplementary data for details).

For the preparation of γ-hydroxyester by microbial reduction, we decided to test the activity of *Pichia glucozyma* using (enzymatically) difficult to hydrolyse *tert*-butyl γ-ketoesters (Table 4).

Reduction of *tert*-butyl 4-oxo-4-phenylbutanoate (**1l**) gave (*S*)-*tert*-butyl 4-hydroxy-4-phenylbutanoate (**2l**) with sluggish enantioselectivity (Table 4, entry 1), whereas the formation of the corresponding γ-lactone was not observed; *tert*-butyl 2-methyl-4-oxo-4-phenylbutanoate (**1m**) was also converted into *tert*-butyl 4-hydroxy-2-methyl-4-phenylbutanoate **2m**, as the only detectable product (70% yield after 24 h) affording the *syn*- and *anti*-diastereomers (2*R*,4*S*)-**2m** and (2*S*,4*S*)-**2m** with 75% ee (Table 4, entry 2). The stereochemical composition of **2m** was determined after purification of the four stereoisomers by preparative chiral HPLC. Relative configuration of the *syn*- and *anti*-diastereomers was assigned by ^{1}H NMR,[13c] whereas comparison of the retention times in chiral HPLC with literature data[13d] allowed for assignment of the absolute configuration.

In conclusion, we have shown that whole cells of *Pichia glucozyma* CBS 5766 can be used for the enantioselective reduction of different aromatic ketones; enantioselectivity towards simple aromatic ketones under optimised conditions is sometimes remarkable. Enantioselectivity was generally lower with γ-ketoesters respect to β-ketoesters; the occurrence of competitive esterase activities can be modulated by choosing the best biotransformation conditions and/or the suited substrate, turning whole cells of *P. glucozyma* into a useful system for multistep reactions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014.10.133.

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9. *Typical procedure for the preparation of the biocatalysts and for biotransformations:* *Pichia glucozyma* CBS 5766 was cultured using malt extract +0.5% yeast extract medium (malt broth, yeast extract 5 g/L, pH 6.0) in a 3.0 L fermenter with 1.0 L of liquid medium for 24 h, at 28 °C and agitation speed 100 rpm. Cells from submerged cultures were harvested by centrifugation and washed with 0.1 M phosphate buffer, pH 7.0. Reductions were carried out in 100 mL screw-capped test tubes with a reaction volume of 50 mL with cells (2.5 g, dry weight) suspended in 0.1 M phosphate buffer, pH 7.0 containing 50 g/L of glucose. After 30 min of incubation, substrates (20 mM) were added and the incubation continued for 24 h under magnetic stirring. When the reaction was over, pH was brought to pH 1 by the addition of 1 M HCl and 35 mL of EtOAc was added and the resulting mixture was shaken and centrifuged; the aqueous phase was extracted twice more with 35 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were purified by flash chromatography.
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Supplementary Data

Biotransformation of aromatic ketones and ketoesters with the non-conventional yeast *Pichia glucozyma*

Martina Letizia Contente, Francesco Molinari, Paolo Zambelli, Valerio De Vitis, Raffaella Gandolfi, Andrea Pinto, Diego Romano*

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1. General

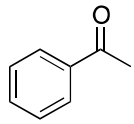
^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) are expressed in Hz.

Gas chromatography (GC) analyses were performed with a Dani 6500 gas cromatograph: gas carrier H_2 (0.6 bar, T=100°C), Detector FID (Flame Ionization Detector), T_{max} = 300°C. The following chiral capillary GC columns were used: DMePeBeta-CDX-PS086 (diameter 0.25 mm, length 25 m, thickness 0.25 μ, MEGA), Mega-Dex DET beta (diameter 0.25 mm, length 25 m, thickness 0.25 lm, MEGA).

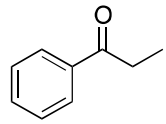
HPLC analyses were performed with a Jasco Pu-980 equipped with a UV-vis detector Jasco UV-975. The following chiral HPLC columns were used: Lux Amylose-2 column (150 x 4.6 mm, Phenomenex), Chiralcel OD (250 x 4.6 mm, Daicel), Chiralcel OD-H (250 x 4.6 mm, Daicel), Chiralpack AD-H (250 x 4.6 mm, Daicel), Chiralpack AD (250 x 4.6 mm mm, Daicel). All the columns were equipped with a 80 x 4 mm precolumn.

Preparative HPLC was performed with a 1525 Extended Flow Binary HPLC pump, equipped with a Waters 2489 UV-vis detector and Phenomenex Lux-Amylose-2 column (21.2 x 250 mm) at flow rate of 15 mL/min.

1. Substrates



1a



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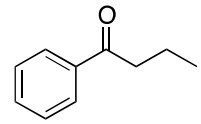
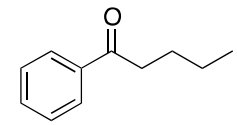
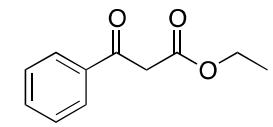


1c

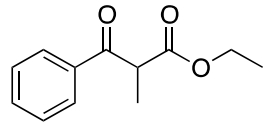


1d

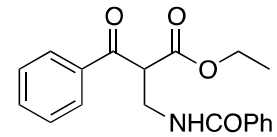


1e

Substrates **1a-e** were from Aldrich.

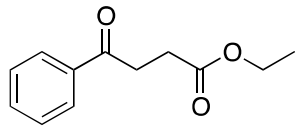


1f

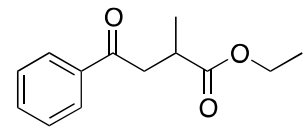


1g

Substrates **1f**[1] and **1g**[2] was prepared according to the literature methods.



1h



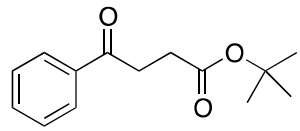
1i

Substrates **1h** and **1i** were prepared from commercially available **4-oxo-4-phenylbutanoic acid** and **2-methyl-4-oxo-4-phenylbutanoic acid** (Aldrich), respectively, using conventional Steglich esterification. Typical procedure: 37.5 mg of 4-dimethylaminopyridine (4-DMAP) and 610 mg of dicyclohexylcarbodiimide (DCC) were added to a stirred solution of **2-methyl-4-oxo-4-phenylbutanoic acid** (500 mg) in dry CH_2Cl_2 (20 mL) at 4°C. The reaction mixture was stirred for 2 h at room temperature and then paper-filtered and the filtrate evaporated at 35 °C under vacuum. The residue was purified by flash chromatography (n-hexane/EtOAc = 98:2) to give 478 mg of pure **1i** (84%) as a colourless liquid.

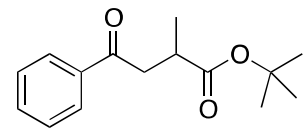
Ethyl 4-oxo-4-phenylbutanoate 1h: ^{1}H-NMR ($CDCl_3$, 300 MHz) (A): δ 1.26 (t, *J*= 7.2 Hz, 3H), 2.75 (t, *J*= 6.4 Hz, 2H), 3.31 (t, *J*= 6.4 Hz, 2H), 4.15 (q, *J*= 7.2 Hz,

2H), 7.46 (dd, J^1= 7.8 Hz, J^2= 7.2 Hz, 2H), 7.56 (t, J= 7.2 Hz, 1H), 7.98 (d, J= 7.8 Hz, 2H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (A): δ 14.1, 28.2, 33.3, 60.6, 128.0, 128.5, 133.1, 136.6, 172.8, 198.1 ppm.

Ethyl 2-methyl-4-oxo-4-phenylbutanoate 1i: ^{1}H-NMR ($CDCl_3$, 300 MHz) (A): δ 1.26 (t, J= 7.0 Hz, 3H), 1.33 (d, J= 7.0 Hz, 3H), 3.02 (dd, J^1=17.3, J^2= 5.6, 1H), 3.08-3.18 (m, 1H), 3.48 (dd, J^1= 17.3, J^2= 7.6, 1H), 4.15 (q, J= 7.0, 2H), 7.39 (dd, J^1= 8.0 Hz, J^2= 7.4 Hz, 2H), 7.50 (t, J= 7.4, 1H), 7.90 (d, J= 8.0, 2H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (A): δ 14.4, 17.5, 35.3, 42.2, 60.8, 128.3, 128.8, 133.3, 137.7, 176.1, 198.3 ppm.



1l



1m

Substrates **1l** and **1m** were prepared from commercially available **4-oxo-4-phenylbutanoic acid** and **2-methyl-4-oxo-4-phenylbutanoic acid** (Aldrich) using *tert*-butyl trichloroacetamidate as esterification reagent.[3] Typical procedure: *tert*-butyl trichloroacetamidate (1.364 g) and BF_3-Et_2O (62 mg) were added to a stirred solution of **4-oxo-4-phenylbutanoic acid** (600 mg) in dry CH_2Cl_2 (18 mL) and dry THF (10 mL) at room temperature. The reaction mixture was stirred for 21 hours at room temperature and then evaporated under reduced pressure; 10 mL of CH_2Cl_2 were added to the crude residue and the solution washed with 10 mL of an aqueous solution (5%) of $NaHCO_3$. The aqueous phase was re-extracted twice with 10 mL of CH_2Cl_2 and the collected organic extracts were dried over Na_2SO_4 and the solvent evaporated. The crude residue was purified by flash chromatography (9:1 hexane:EtOAc) giving 700 mg (78%).

***tert*-Butyl 4-oxo-4-phenylbutanoate (1l)** Colourless oil; ^{1}H-NMR ($CDCl_3$, 300 MHz) (A): δ 1.45 (s, 9H), 2.68 (t, J= 7.0 Hz, 2H), 3.26 (t, J= 7.0, 2H), 7.45 (dd, J^1=

8.0 Hz, J^2= 7.2 Hz,, 2H), 7.57 (t, J= 7.2 Hz, 1H), 7.98 (d, J= 8.0 Hz, 2H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (A): δ 28.2, 29.4, 33.3, 80.6, 128.0, 128.5, 133.1, 136.6, 172.8, 198.1 ppm.

***tert*-Butyl 2-methyl-4-oxo-4-phenylbutanoate (1m)** White solid, m.p. 59-60 °C; ^{1}H-NMR ($CDCl_3$, 300 MHz) (A): δ 1.25 (d, J= 7.0 Hz, 3H), 1.44 (s, 9H), 2.90-3.10 (m, 2H), 3.42 (dd, J^1= 17.2 Hz, J^2= 7.6 Hz, 1H), 7.45 (dd, J^1= 7.8 Hz, J^2= 7.4 Hz, 2H), 7.55 (t, J= 7.4, 1H), 7.98 (d, J= 7.8, 2H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (A): δ 17.6, 28.2, 35.3, 42.2, 80.2, 128.3, 128.8, 133.3, 137.7, 176.1, 198.3 ppm.

2. Preparation of the biocatalyst and set-up of biotransformations

Preparation of the biocatalyst

Pichia glucozyma CBS 5766 was cultured using different media in 3.0 L fermenters with 1.0 L of liquid medium for 24 and 48 h, at 28°C and agitation speed 100 rpm The liquid media used were:

1) malt extract medium (malt broth pH 6.0);
2) malt extract + 0.5% yeast extract medium (malt broth, yeast extract 5 g/L, pH 6.0);
3) malt extract + 5% yeast extract medium (malt broth, yeast extract 50 g/L, pH 6.0);
4) YPD medium (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, pH 5.6).

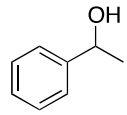
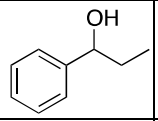
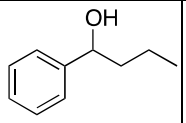
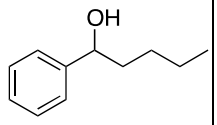
Fresh cells from submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0 prior the use for biotransformations.

Biotransformations

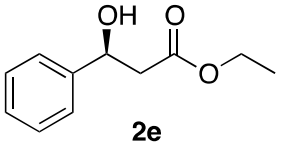
Reductions were carried out in 100 mL screw-capped test tubes with a reaction volume of 50 mL with cells (25-75 g/L, dry weight) suspended in 0.1 M phosphate buffers with pH ranging from 5.8 to 8.0 with or without the addition of different co-substrates (ethanol, glucose, glycerol, sucrose, xylose) in concentration of 50 g/L. After 30 min of incubation, neat substrates were added and the incubation continued for 24 h under magnetic stirring. When the reaction was over, pH was brought to pH 1 by the addition of 1 M HCl and 35 mL of EtOAc were added and the resulting mixture was shaken and centrifuged; the aqueous phase was extracted twice more with 35 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were purified by flash chromatography.

1. Analytical data of the products

Chiral HPLC analyses for determination of e.e. of **2a-2d**

Compound	**Column**	**Conditions** (λ 254 nm)	**Retention times** (min)
2a 	Chiralcel OD	*n*-hexane/2-propanol 97/3 flow 0.7 ml/min	14.8 (*R*), 18.0 (*S*)
2b 	Chiralcel OD-H	*n*-hexane/2-propanol 96/4 flow 0.5 ml/min	17.6 (*R*), 21.4 (*S*)
 **2c**	Chiralcel OD-H	*n*-hexane/2-propanol 96/4 flow 0.5 ml/min	16.4 (*R*), 17.5 (*S*)
 **2d**	Chiralcel OD-H	*n*-hexane/2-propanol 96/4 flow 0.5 ml/min	15.9 (*R*), 16.8 (*S*)

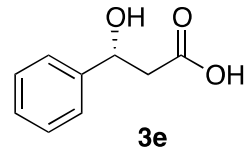
2.



2e

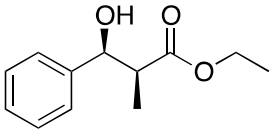
Ethyl-3-hydroxy-3-phenylpropanoate (2e) was purified by flash chromatography (9:1 hexane:EtOAc). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.26 (t, *J*= 7.2 Hz, 3H), 2.80-2.90 (m, 2H), 3.30 (d, *J*= 3.3 Hz, 1H), 4.20 (q, *J*= 7.2 Hz, 2H), 5.10-5.20 (m, 1H), 7.22-7.42 (m, 5H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 14.3, 43.4, 60.9, 70.3, 125.5, 127.6, 128.3, 142.2, 172.1 ppm. $[\alpha]_D^{25}$ = -47.2 (*c*=1.0 $CHCl_3$); lit. = -52

(c=1.0 $CHCl_3$).[4] Chiral GC analysis using a Mega-Dex DET beta column using the following temperature program: 3 min at 70 °C, increased to 170 °C over 25 min and then held at 170 °C for 10 min; t_r (*S*)-**2e** = 22.1 min; t_r (*R*)-**2e** = 22.4 min. Anal. calcd for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27; found: C, 67.83; H, 7.64.



3e

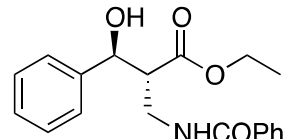
3-Hydroxy-3-phenylpropionic acid (**3e**) was purified by flash chromatography (99:1 EtOAc:AcOH). ^{1}H-NMR (C_3D_6O, 300 MHz): δ 2.67 (dd, J^1= 15.3 Hz, J^2= 4.8 Hz, 1H), 2.71 (dd, J^1= 15.3 Hz, J^2= 8.5 Hz, 1H), 5.19 (dd, J^1= 8.5 Hz, J^2= 4.8 Hz, 1H), 7.25 (dd, J^1= 8.0 Hz, J^2= 7.2 Hz, 2H), 7.33 (t, J= 7.2 Hz, 1H), 7.39 (d, J= 8.0 Hz, 2H) ppm. ^{13}C-NMR (C_3D_6O, 75.5 MHz): δ 42.5, 70.2, 124.7, 127.5, 128.2, 145.0, 174.3 ppm. $[\alpha]_D^{25}$ = -20.0 (c=0.5, CH_3OH); lit. $[\alpha]_D^{23}$ –21.9 (c 4, MeOH).[5] The stereochemical outcome of the reaction was monitored by GC after treatment of the organic extracts with CH_2N_2. Chiral GC was performed using a Mega-Dex DET beta column using the following temperature program: 3 min at 70 °C, increased to 170 °C over 25 min and then held at 170 °C for 10 min; t_r (*S*)-**3e** = 10.9 min; t_r (*R*)-**3e** = 11.3 min. Anal. calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07; found: C, 64.80; H, 6.43.



2f

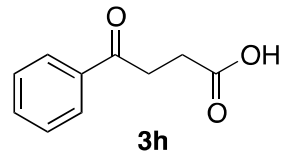
Ethyl 3-hydroxy-2-methyl-3-phenylpropanoate (**2f**) was purified by flash chromatography (8:2 hexane:EtOAc). ^{1}H-NMR *syn*-product ($CDCl_3$, 300 MHz): δ

1.15 (d, *J*= 7.1 Hz, 3H), 1.21 (t, *J*= 7.1 Hz, 3H), 2.35 (m, 1H, OH), 2.75-2.80 (m, 1H), 4.12 (m, 2H), 5.10 (d, *J*= 4.4 Hz, 1H), 7.30-7.40 (m, 5H) ppm. ^{13}C-NMR *syn*-product ($CDCl_3$, 75.5 MHz): δ 10.8, 14.0, 46.4, 60.7, 73.7, 125.5, 128.0, 128.3, 141.4, 175.9 ppm. ^{1}H-NMR *anti*-product ($CDCl_3$, 300 MHz): δ 1.00 (d, *J*= 7.1 Hz, 3H), 1.25 (t, *J*= 7.1 Hz, 3H), 2.35 (m, 1H, OH), 2.72–2.81 (m, 1H), 4.17 (q, *J*= 7.1 Hz, 2H), 4.73 (dd, J^1 = 8.4 Hz, J^2= 4.5 Hz, 1 H), 7.24–7.36 (m, 5H) ppm. ^{13}C-NMR *anti*-product ($CDCl_3$, 75.5 MHz): δ 14.1, 14.4, 47.1, 60.7, 76.3, 126.6, 128.0, 128.4, 141.6, 175.9 ppm. Chiral HPLC was performed with a Chiralcel OD-H column using *n*-hexane/2-propanol 97/3, flow 0.8 ml/min, λ= 216 nm: t_r (2*R*,3*R*)-**2f** = 13.4 min; t_r (2*S*,3*S*)-**2f** = 15.2 min; t_r (2*R*,3*S*)-**2f** = 17.8 min; t_r (2*S*,3*R*)-**2f** = 26.5 min. Anal. calcd for $C_{12}H_{16}O_3$: C, 69.21; H, 7.74; found: C, 68.96; H, 8.07.



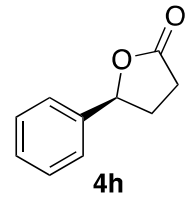
2g

Ethyl-2-(benzamidomethyl)-3-hydroxy-phenylpropanoate (2g) was purified by flash chromatography (3:7 cyclohexane:EtOAc). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.01 (t, *J*= 7.0 Hz, 3H,), 3.15–3.24 (m, 1H, anti diastereoisomers), 3.61–3.69 (m, 2H), 3.98 (q, *J*= 7.0, 2H), 4.12–4.18 (m, 2H), 4.95 (d, *J*= 7.3 Hz, 1H,), 6.72 (br s, 1H), 7.29–7.53 (m, 8H), 7.65-7.82 (m, 2H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 14.0 38.0 53.0 61.1 72.6 126.4–132.0 173.5. Chiral HPLC was performed with a Chiralpack AD column using *n*-hexane/2-propanol 90/10, flow 0.6 ml/min, λ= 230 nm: t_r (2*S*,3*R*)-**2g** = 49.3 min; t_r (2*R*,3*S*)-**2g** = 67.9 min. Anal. calcd for $C_{19}H_{21}NO_4$: C, 69.71; H, 6.47; N, 4.28; found: C, 69.53; H, 6.72; N, 4.12.



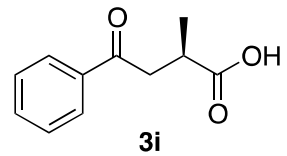
3h

4-Oxo-4-phenylbutanoic acid (3h) was purified by flash chromatography (99:1 EtOAc:AcOH). ^{1}H-NMR ($CDCl_3$, 300 MHz) (C): δ 2.81 (t, *J*= 6.6 Hz, 2H), 3.32 (t, *J*= 6.6 Hz, 2H), 7.42-7.58 (m , 3H), 7.96-8.00 (m, 2H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (C): δ 28.2, 33.4, 128.3, 128.9, 133.6, 136.65, 178.8, 198.0 ppm. Anal. calcd for $C_{10}H_{10}O_3$: C, 67.41; H, 5.66; found: C, 67.14; H, 5.94.



4h

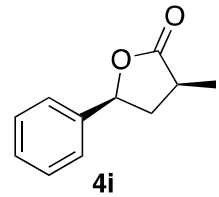
(*S*)-5-phenyldihydrofuran-2(3H)-one (4h) was purified by flash chromatography (85:15 hexane:EtOAc). ^{1}H-NMR ($CDCl_3$, 300 MHz) (B): δ 2.20 (m, 1 H), 2.67 (m, 3H), 5.50 (dd, J^1= 8.0 Hz, J^2= 6.3 Hz , 1H), 7.37 (m, 5H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (B): δ 29.0, 31.0, 81.3, 125.4, 128.5, 128.9, 139.5, 178.0 ppm. Chiral GC was performed using a Mega-Dex DET beta column using the following temperature program: 1 min at 110 °C, increased to 170 °C over 15 min and then held at 170 °C for 10 min; t_r (*S*)-**4h** = 16.4 min; t_r (*R*)-**4h** = 16.6 min. Anal. calcd for $C_{10}H_{10}O_2$: C, 74.06; H, 6.21; found: C, 73.72; H, 6.49.



3i

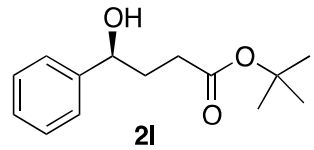
2-Methyl-4-oxo-4-phenylbutanoic acid (3i) was purified by flash chromatography (99:1 EtOAc:AcOH). ^{1}H-NMR ($CDCl_3$, 300 MHz) (D): δ 1.24 (d, *J*= 7.0, 3H),

3.00-3.21 (m, 2H), 3.50 (dd, J^1= 16.9, J^2=7.0, 1H), 7.50 (dd, J^1= 8.0 Hz, J^2= 7.4 Hz, 2H), 7.60 (t, J= 7.4, 1H), 7.90 (d, J= 8.0, 2H) ppm. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) (D): δ 17.3, 35.0, 42.0, 128.3, 128.8, 133.5, 136.8, 182.1, 198.0 ppm. $[\alpha]_D^{25}$ = +13.0 (*c*=0.3, $CHCl_3$); lit. $[\alpha]_D^{25}$ = +21.1 (*c*= 0.8, $CHCl_3$).[6] The stereochemical outcome of the reaction was monitored by HPLC after treatment of the organic extracts with CH_2N_2. Chiral HPLC was performed with a Chiralcel AD column using *n*-hexane/2-propanol 95/5, flow 1.0 ml/min, λ= 254 nm. Anal. calcd for $C_{11}H_{12}O_3$: C, 68.74; H, 6.29; found: C, 68.44; H, 6.53.



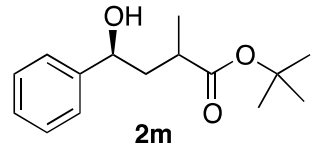
4i

3-Methyl-5-phenyldihydrofuran-2(3H)-one (**4i**) was purified by flash chromatography (9:1 hexane:EtOAc). ^{1}H-NMR *syn*-product ($CDCl_3$, 300 MHz): δ 1.33 (d, J= 7.2 Hz, 3H), 1.79-1.93 (m, 1H), 2.75-2.89 (m, 2H), 5.36 (dd, J^1= 10.7 Hz, J^2= 5.3 Hz, 1H), 7.30-7.44 (m, 5H) ppm. ^{13}C-NMR *syn*-product ($CDCl_3$, 75.5 MHz): δ 15.4, 36.8, 41.4, 79.6, 126.2, 128.9, 129.2, 139.9, 179.6 ppm. ^{1}H-NMR *anti*-product ($CDCl_3$, 300 MHz): δ 1.27 (d, J= 7.3 Hz, 3H), 2.24-2.45 (m, 2H), 2.67-2.75 (m, 1H), 5.51(dd, J^1= 7.6 Hz, J^2= 5.4 Hz, 1H), 7.25-7.37 (m, 5H) ppm. ^{13}C-NMR *anti*-product ($CDCl_3$, 75.5 MHz): δ 15.5, 33.7, 38.4, 77.8, 126.6, 128.7, 129.0, 138.4, 179.7 ppm. Chiral HPLC was performed with a Lux Amylose-2 column using *n*-hexane/2-propanol 90/10, flow 1.0 ml/min, λ= 220 nm. t_r (3*R*,5*S*)-**4i** = 10.5 min; t_r (3*R*,5*R*)-**4i** = 11.6 min; t_r (3*S*,5*R*)-**4i** = 12.4 min; t_r (3*S*,5*S*)-**4i** = 13.0 min. Anal. calcd for $C_{11}H_{12}O_2$: C, 74.98; H, 6.86; found: C, 74.60; H, 7.11 .



2l

***tert*-Butyl 4-hydroxy-4-phenylbutanoate (2l)** was purified by flash chromatography (9:1 hexane:EtOAc). ^{1}H-NMR (300 MHz, $CDCl_3$) (B): δ 1.44 (s, 9H), 2.00 (t, *J*= 7.0 Hz, 2H), 2.34 (t, *J*= 7.0 Hz, 2H), 4.7 (t, *J*= 6.5 Hz, 1H), 7.17-7.31(m, 5H) ppm. ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ 28.2, 32.0, 33.3, 72.3, 80.7, 126.0, 127.5, 128.5, 143.6, 172.8 ppm. Chiral HPLC was performed with a Chiralcel AD column using *n*-hexane/2-propanol 98/2, flow 1.0 ml/min, λ= 220 nm. t_r (*R*)-**2l**= 21.9 min; t_r (*S*)-**2l** = 23.0 min. Absolute configuration was assigned by comparison with previously reported chiral HPLC data.[7] Anal. calcd for $C_{11}H_{12}O_2$: C, 71.16; H, 8.53; found: C, 70.94; H, 8.70.



2m

***tert*-Butyl 4-hydroxy-2-methyl-4-phenylbutanoate (2m)** was purified by flash chromatography (9:1 hexane:EtOAc). ^{1}H-NMR *syn*-product ($CDCl_3$, 300 MHz): δ 1.15 (d, *J*= 7.2 Hz, 3H), 1.47 (s, 9H), 1.81 (ddd, J^1= 13.9 Hz, J^2= 9.1 Hz, J^3= 4.4 Hz, 1H), 2.04 (ddd, J^1= 13.6 Hz, J^2= 9.6 Hz, J^3= 3.7 Hz, 1H), 2.54-2.66 (m, 1H), 4.78 (dt, J^1= 9.1 Hz, J^2= 3.7 Hz, 1H), 7.27-7.37 (m, 5H) ppm. ^{13}C-NMR *syn*-product ($CDCl_3$, 75.5 MHz): δ 18.0, 28.3, 37.5, 43.2, 73.0, 80.3, 125.3, 127.2, 128.2, 144.1, 175.7 ppm. ^{1}H-NMR *anti*-product ($CDCl_3$, 300 MHz): δ 1.80 (d, *J*= 7.0 Hz, 3H), 1.46 (s, 9H), 1.69 (ddd, J^1= 14.0, J^2= 11.1, J^3= 5.2, 1H), 2.19 (ddd, J^1= 17.0, J^2= 14.0, J^3= 8.3, 1H), 2.50-2.57 (m, 1H) 4.73 (dt, J^1= 11.1 Hz, J^2= 8.3 Hz, 1H), 7.25-7.37 (m, 5H) ppm. ^{13}C-NMR *anti*-product ($CDCl_3$, 75.5 MHz): 18.15, 28.3, 38.3, 43.1, 72.2, 80.3, 125.5, 127.3, 128.1, 144.2, 176.0 ppm. Chiral HPLC was performed with a Chiralpack AD-H column using *n*-hexane/2-propanol

97/3, flow 1.0 ml/min, λ= 220 nm. t_r (2*S*,4*R*)-**2m** = 12.0 min; t_r (2*R*,4*S*)-**2m** = 12.8 min; t_r (2*S*,4*S*)-**2m** = 13.8 min; t_r (2*R*,4*R*)-**2m** = 15.5min. The stereochemical composition of **2m** was determined after purification of the four stereoisomers by preparative chiral HPLC using a Phenomenex Lux-Amylose-2 column (21.2 x 250 mm) at flow rate of 15 mL/min. Relative configurations was determined by ^{1}H NMR,[8] while absolute configuration by comparison with previously reported chiral HPLC data.[7] Anal. calcd for $C_{11}H_{12}O_2$: C, 71.97; H, 8.86; found: C, 71.71; H, 9.03.

5. Supporting references

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Importance of Prostaglandins and their analogues

4.1 Prostaglandins and inflammation

Inflammation is the immune system's response to infection and damage and it is implicated in the generation of various pathologies such as arthritis, cancer, stroke as well as neurodegenerative and cardiovascular diseases. Inflammation is an intrinsically beneficial event because of the possibility to remove the offending factors and to restore tissues and physiological functions.[50] The cardinal signs of acute inflammation are:

- *rubor* (redness) caused by vasodilatation which produces the increasing of blood amount and the decreasing of blood rate flow in the injured area
- *calor* (heat) caused by temperature and increased metabolism
- *dolor* (pain) for biochemical local alterations
- *tumor* (swelling) produced by increasing of vascular permeability responsible for leakage of plasma proteins from vascular bed
- *functio laesa* (function inhibition) because of imbalances induced by inflammatory mechanisms.[51]

The usual outcome of acute inflammation is a good resolution with restoration of tissue damage and recovery of organ function.[52]

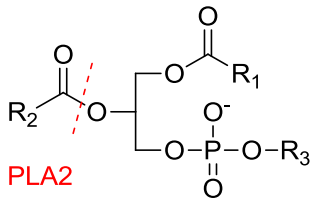
[50] E. Ricciotti *et al., Arterioscler. Thromb. Vasc. Biol.* **2011**, *31*, 986–1000
[51] C.D. Funk, *Science* **2001**, *294*, 1871-1875
[52] C. Nathan, *Nature* **2002**, *420,* 846-852

Prostaglandins, and more in general eicosanoids, whose biosynthesis is significantly increased in inflamed tissue, play a key role in the genesis of the inflammatory response because of their contribution to development of the cardinal signs of acute inflammation.

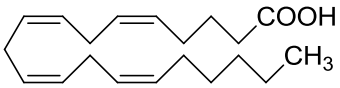
4.2 Eicosanoids biosynthesis

Prostaglandins and tromboxane A_2 (TXA_2), collectively termed eicosanoids, belong to the family of autacoids, substances released by cells into the extracellular environment where they induce biological responses by interacting with specific receptors on the cell that generated them or on cells in the immediate vicinity. They are synthesized from arachidonic acid, a 20-carbon unsatured fatty acid present in the cell membranes as an ester of phosphatidylinositol or other membrane phospholipids. It is released by phospholipase A2 able to attack the C2 position of the phospholipids generating monoglyceride phosphate and one molecule of arachidonic acid (Fig. 25).[53]

[53] P.W. Collins *et al., Chem. Rev.* **1999**, *93,* 1533-1504



PLA2 Activity



Arachidonic acid

Fig. 25 PLA2 activity and arachidonic acid

The term eicosanoids stands for several inflammatory mediators characterized by having a short plasma half-life as rapidly synthesized on demand and quickly inactivated by enzymes. The most important are leukotrienes and three types of prostanoids: prostaglandins, prostacyclin and thromboxanes. Prostanoids production (Fig. 26) depends on the activity of COXs, bifunctional enzymes that contain both cyclooxygenase and peroxidase activity and which exist as distinct isoforms referred to as COX-1 and COX-2.[54] COX-1, expressed constitutively in most cells, is the predominant source of prostanoids involved in homeostasis for example in gastric epithelial cytoprotection. COX-2, induced by inflammatory stimuli, is the most important source of prostanoid involved in inflammation and in pathogenesis of various diseases.[55] However, both enzymes contribute to the production of healthy prostanoids, and both can contribute to their release during inflammation.

The conversion of arachidonic acid takes place by means the uptake of two molecules of oxygen and cyclization of the hydrocarbon chain (cyclooxygenase activity),

[54] W.L. Smith *et al., Annu. Rev. Biochem*. **2000**, *69*, 145-182
[55] R.N. Dubois *et al., FASEB J.* **1998**, *12*, 1063-1073

followed by the reduction of hydroperoxide group into hydroxide one, crucial for biological activity and the generation of oxygen free radical (peroxidase activity).

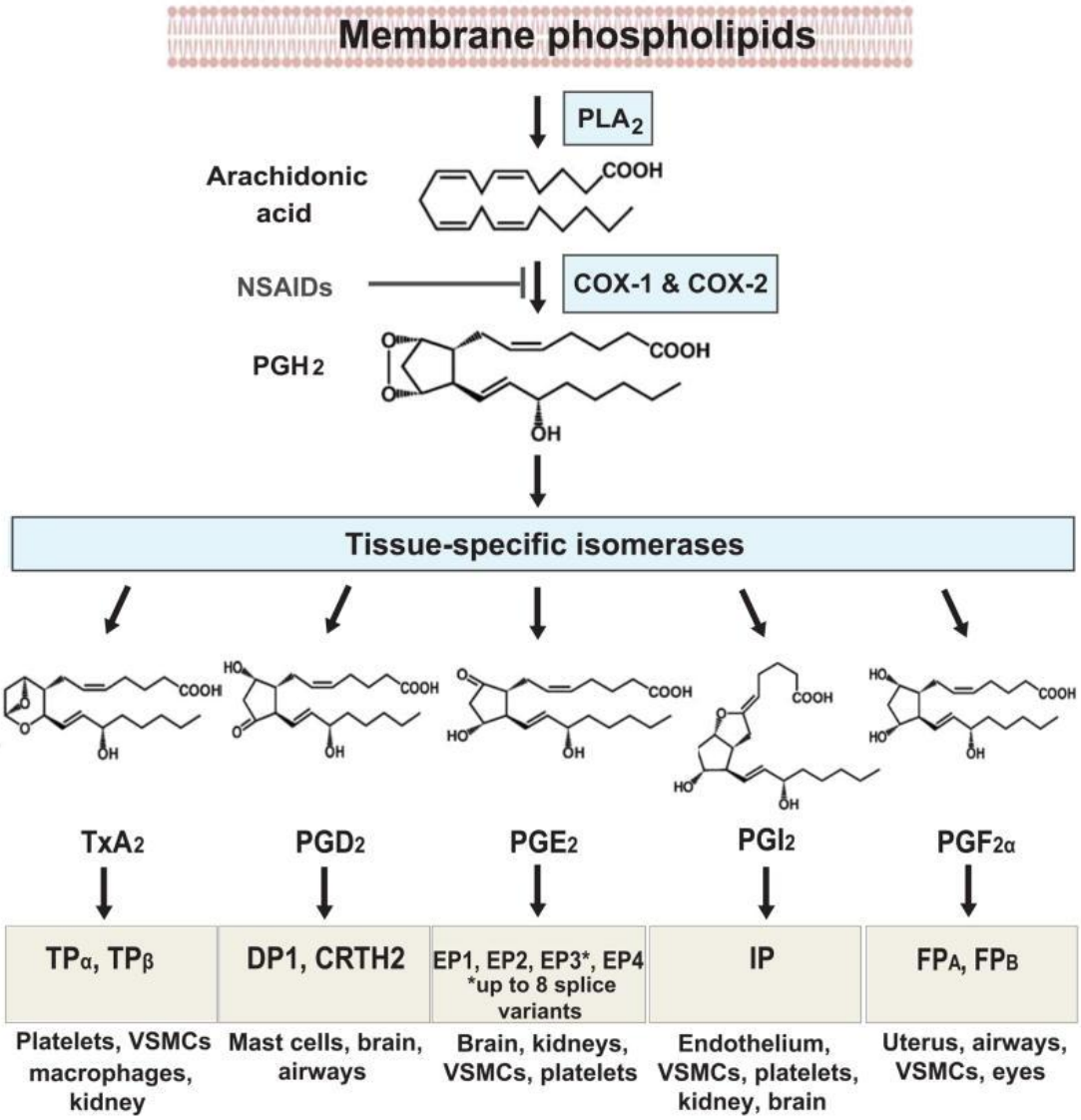


Fig. 26 Biosynthetic pathway of prostanoids

By further enzymatic modifications of PGH_2 the following prostanoids will be obtained:

TXA_2: it is predominantly produced by platelets and the renal cortex. It is responsible for platelets aggregation, vasoconstriction, decreased blood flow and glomerular filtration rate.

PGI_2: it is produced mainly by the vessel wall and from the renal cortex. It regulates tone of smooth muscles inducing vasodilation and increasing the renal blood flow and glomerular filtration.

PGD_2, PGE_2, PGF_2: they are produced mainly by monocyte - macrophages, hypothalamus, renal medulla, uterus and gastric cells. They regulate the decreasing of smooth muscle tone, gastric secretions, the increasing of body temperature, the release of neurotransmitters in the nervous system, the decreasing of the pain threshold.

4.4 Prostanoids receptors

Prostanoids exert their effect by activating seven transmembrane spanning G-protein coupled receptors (GPCRs). They can be grouped in different classes according to their trasduction signal Tab. 3.

Class	Subtype	G-protein coupled	Second Messenger
TxA_2	TP_α, TP_β	G_q, G_{13}, G_h, $G_s(TP_\alpha)$, $G_i(TP_\beta)$	$IP_3/DAG/Ca^{2+}$, RhoGEF cAMP
PGD_2	DP	G_s	cAMP
	CRTH2	G_i	cAMP, Ca^{2+}
PGE_2	EP1	G_q	$IP_3/DAG/Ca^{2+}$
	EP2	G_s	cAMP
	EP3	G_i, G_{12}	cAMP, Ca^{2+}, Rho
	EP4	G_s	cAMP
PGI_2	IP-IP	G_s	cAMP
	IP- TP_α	G_s	cAMP
$PGF_{2\alpha}$	FP_A, FP_B	G_q	$IP_3/DAG/Ca^{2+}$, Rho

Table 3 Signal transduction of prostanoid receptors

While EP2, EP4, IP and DP receptors activate adenylyl cyclase *via* Gs, increasing intracellular cAMP, EP1 and FP receptors activate phosphatidylinositol metabolism *via* Gq, increasing the formation of inositol triphosphate with mobilization of intracellular free calcium.[56] TP receptors stimulating different types of G-proteins, activate various transduction pathways mediated by phospholipase C and by adenylate cyclase. On the contrary, EP3 and CRTH2 being inhibitory receptors, mediate the reduction of intracellular cAMP and elevate the concentration of intracellular Ca^{2+}.

[56] K.L Pierce *et al., J. Biol. Chem.* **1999**, *274*, 35944-35949

However, the effects of prostanoids on these G protein–coupled signalling pathways may change depending on the ligand function, concentration or structure[57] (Fig. 27)[58].

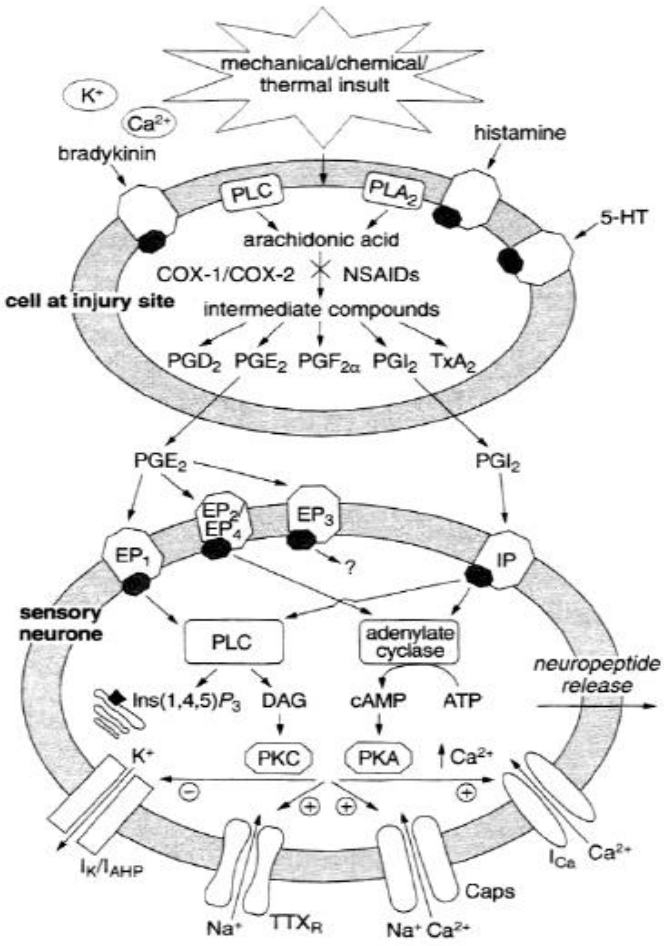


Fig. 27 Pathway of prostanoids

Because of the numerous biological effects, prostaglandins have been subject of study and research for the synthesis of analogues, which could be potential drugs. PGs may

[57] S. Narumiya *et al., J. Clin. Invest.* **2001**, *108*, 25-30
[58] Bley *et al.*, *Tips* **1998**, *19*, 141-147

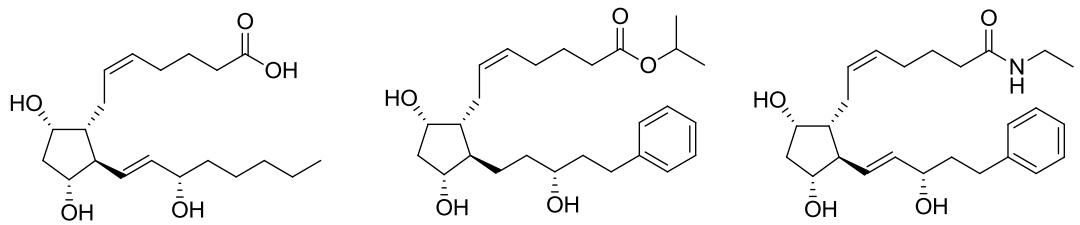
be useful in the therapy of several diseases. Open-angle glaucoma,[59] characterized by increased intraocular pressure which causes poor blood flow and progressive damage of the optic nerve, ischemia caused by corneal vasoconstriction and blood hypo-perfusion of the primary structures of the eye, are examples. Moreover, the PGs involved in therapy of peptic duodenal ulcer[60] provide an increased production of mucus and bicarbonate by the parietal cells of the stomach in order to decrease the acid level into the gastric lumen, characteristic of this disease. In addition, analogues of PGs are also used in obstetrics both to induce uterine contractions during labor because of the activity they have on smooth muscle and to induce abortion in combination with mifepristone.[61]

4.5 PGF2α prostaglandin analogues as a potent and selective anti-glaucoma agents

Glaucoma, is a progressive eye disorder, characterized by increasing of intraocular pressure (IOP), damage of the optic nerve, and gradual loss of vision. Initial therapy usually involves topical application of muscarinic agonists such as pilocarpine or adrenergic agonists and antagonists such as epinephrine and timolol, respectively. If treatment with such topically applied drugs is not effective, systemic administration of carbonic anhydrase inhibitors or surgical intervention may be employed.[60] For over forty years, attention has been focused on PGs, particularly on PGF2α as IOP-lowering substances.[62] Several studies indicate that PGF2α reduces IOP by increasing uveoscleral

[59] B. Resul *et al., J. Med. Chem*. **1993**, *36*, 243-248.
[60] D.Y Graham *et al., Ann. Inten. Med.* **1993**, *119*, 257-262
[61] H.K. Kallner *et al., Contraception* **2012**, *85*, 394–397
[62] LZ. Bito, *et al., Exp .Eye Res*. **1984**, *38*, 181-194; J.Villumsen *et al., Br. J. Ophta.* **1986**, *73*, 975-979;

outflow of aqueous humor.[63] Thus, there has been a great deal of interest in developing synthetic PGF2α analogues with IOP-lowering efficacy. PGs and their pro-drugs, such as PGF2α isopropyl ester, not only are able to reduce IOP, but also cause conjunctival hyperemia, foreign-body sensation and superficial irritation[64]. The development of pro-drugs of potent, selective synthetic prostaglandin FP receptors agonists as clinically effective IOP-lowering agents devoid of ocular side-effects has been an important advance in the glaucoma treatment.[65] Phenyl substituted PGF2α analogues such as Latanoprost and Bimatoprost (Fig. 28) are the active ingredients of topically medications that have revolutionized the glaucoma therapy.



PGF2α Latanoprost Bimatoprost

Fig. 28 Structures of PGF2α and analogues

In this context we have developed an alternative chemoenzymatic synthesis of analogues of PGF2α having potent and selective action in glaucoma treatment. Whole cells of *Pichia glucozyma* and of *Pichia anomala* have been proved excellent catalysts for the biosynthesis of key intermediates for the total synthesis of Latanoprost and Bimatoprost.

[63] A. F. Clark, *Emerg. Drugs* **1999**, *4*, 333-353
[64] A. Aim, *Curr. Opin. Ophththalmol.* **1993**, *4*, 44
[65] Z. Feng *et al., Biorg. Med. Chem*. **2009**, *17*, 576-584

Journal of Molecular Catalysis B: Enzymatic 114 (2015) 7–12

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

A new chemoenzymatic approach to the synthesis of Latanoprost and Bimatoprost

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ARTICLE INFO

Article history:
Available online 11 June 2014

Keywords:
Pichia anomala
Latanoprost
Bimatoprost
Biotransformation
Multi-enzyme processes

ABSTRACT

Bimatoprost (**1**) and Latanoprost (**2**) are prostaglandin analogues widely used for glaucoma treatment. We have developed a new chemoenzymatic synthesis for **1** and **2**, which utilizes a highly stereoselective sequence of biotransformations catalyzed by enzymes belonging to a single microorganism (the yeast *Pichia anomala*). The original synthesis, starting from (−)-Corey lactone benzoate (3a*R*,4*R*,5*R*,6a*S*)-**3**, was modified by replacing three synthetic steps (C=C reduction, stereoselective C=O reduction and hydrolysis/deprotection of the benzoate ester) with a one-pot, three-enzymes reaction. The overall biotransformation gave good yields and it was highly stereoselective; noteworthy, by engineering the reaction medium, C=C reduction could be modulated so that unsaturated (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**6** or saturated intermediate (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** could be preferentially obtained.

1. Introduction

Bimatoprost (**1**) and Latanoprost (**2**) (Scheme 1) are prostaglandin analogues used for controlling the progression of glaucoma by reducing intraocular pressure and have become billion-dollar drugs [1,2]. The industrial manufacture of Bimatoprost and Latanoprost is mostly based on variants of the original strategy developed by Corey [3–9], although different synthetic strategies have been proposed, including a shorter stereocontrolled organocatalytic synthetic procedure recently reported [10]. In the conventional route, the key ketoprostaglandin intermediate (3a*R*,4*R*,5*R*,6a*S*)-**4** is obtained by Horner-Wadsworth–Emmons (HWE) condensation of (−)-Corey lactone benzoate (3a*R*,4*R*,5*R*,6a*S*)-**3** with the suited ketophosphonate. Key intermediate (3a*R*,4*R*,5*R*,6a*S*)-**4** is then reduced by chemoselective hydrogenation (i.e. lithium selectride at low temperature) to give the unsaturated secondary alcohol (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**5** that can be used for the synthesis of Bimatoprost after hydrolysis to (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** (also known as **Lactondiol B**); alternatively, (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**5** can be reduced at the double bond with Pd/C catalytic hydrogenation and hydrolyzed at the ester moiety to furnish (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** (also known as **Lactondiol L**), the actual intermediate for Latanoprost synthesis (Scheme 1).

The transformations occurring in the overall conversion of (3a*R*,4*R*,5*R*,6a*S*)-**4** into (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** (C=O reduction and ester hydrolysis) or into (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** (C=O and C=C reduction, ester hydrolysis) can be also enzymatically catalyzed (Scheme 1, in blue colour); more specifically, enoate reductases catalyze the reduction of C=C conjugated to an electron withdrawing group (such as C=O groups) [11], dehydrogenases (carbonyl reductases) catalyze the reduction of C=O groups [12] and esterases catalyze the hydrolysis of esters [13]. Microbial reduction of (3a*R*,4*R*,5*R*,6a*S*)-**4** into the corresponding alcohol (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**5** has been already reported using different yeasts, with *Kloeckera jensenii* ATCC 20110 giving the best yields [14].

In this work, we have studied the possibility to set up a one-pot biocatalytic method for the stereocontrolled transformation of (3a*R*,4*R*,5*R*,6a*S*)-**4** directly into (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** (Lactondiol B) or (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** (Lactondiol L) by using different yeasts.

2. Experimental

2.1. General experimental methods

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Merck Silica Gel 60 F_{254} plates were used for analytical TLC; ^{1}H and ^{13}C

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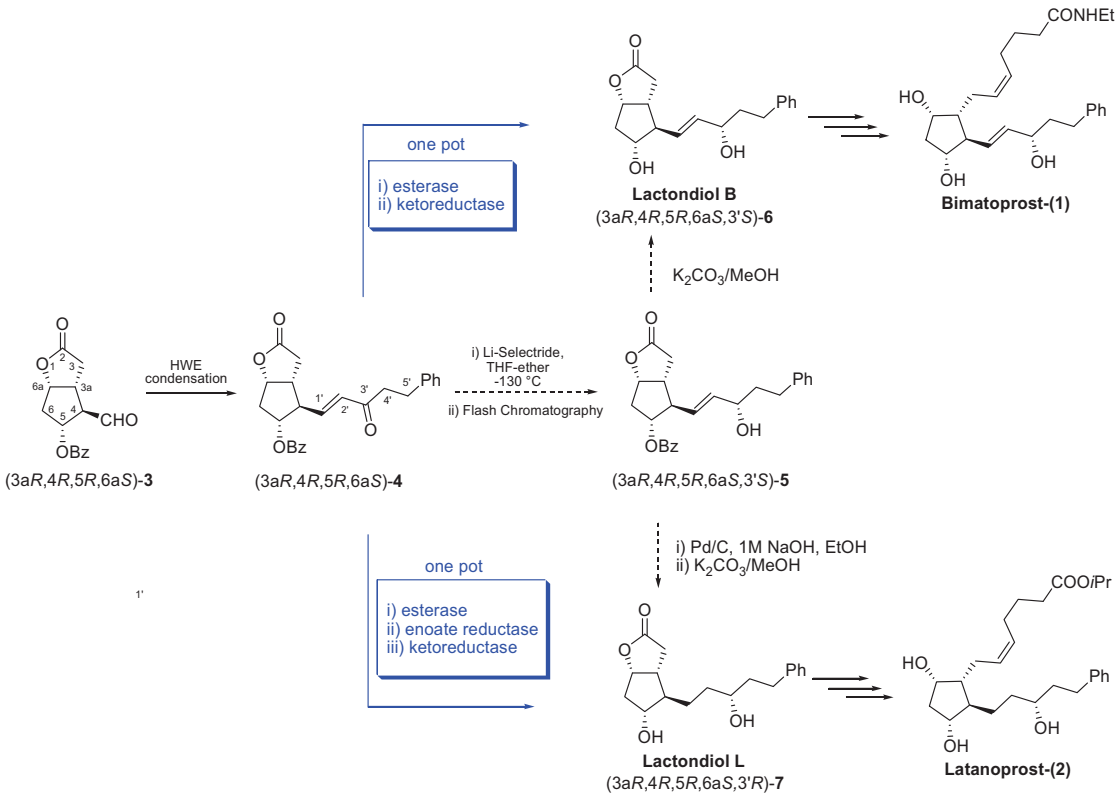
http://dx.doi.org/10.1016/j.molcatb.2014.05.022
1381-1177/



Scheme 1. Synthetic routes to Bimatoprost and Latanoprost.

NMR spectra were recorded on a Varian-Gemini 200 spectrometer. Flash column chromatography was performed on Merck Silica Gel (200–400 mesh). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm) and coupling constants (J) in Hertz (Hz). MS analyses were performed on a Thermo-Finnigan LCQ ADVANTAGE mass spectrometer equipped with an electron spray ionization (ESI) source. Microanalyses (C, H, N) of new compounds were within 0.4% of theoretical values.

2.2. General procedures for microbial screening of transformation of *4*

Eighteen yeasts were used in the primary screening for LB enone **4** modification; the strains were chosen among 12 yeasts known for esterase and/or ketoreductase activity (*Candida boidini* CBS6056, *Kluyveromyces lactis* CBS2359, *Kluyveromyces marxianus* CBS1553, *K. marxianus* var. *lactis* CL69 *Pichia anomala* CBS110, *Pichia etchellsii* MIM, *Pichia glucozyma* CBS 5766, *S. cerevisiae* CBS1782, *S. cerevisiae* CBS3093, *S. cerevisiae* CBS3081, *S. cerevisiae* NCYC 73, *S. cerevisiae* Zeus)[11] and 6 strains with ketoreductase and/or enoate reductases activity (*S. cerevisiae* BY4741, *S. cerevisiae* BY4741ΔOye1, *S. cerevisiae* BY4741ΔOye2, *S. cerevisiae* BY4741ΔOye2Cc, *S. cerevisiae* BY4741ΔOye2Ks, *S. cerevisiae* L12) [16,17,21].

Strains from official collections or from our collection (Microbiologia Industriale Milano) were routinely maintained on M5YE slants (Barley malt flour 100 g/L (Diagermal), 5 g/L yeast extract (Difco), agar 15 g/L, pH 5.6). To obtain cells for biotransformations, the microorganisms were cultured in 2 L Erlenmeyer flasks containing 300 mL of M5YE liquid medium (Barley malt flour 100 g/L (Diagermal), 5 g/L yeast extract (Difco), distilled water pH 5.6), incubated for 48 h at 28 °C on a reciprocal shaker (150 spm). Fresh cells from submerged cultures were centrifuged (5000 rpm, 20 min) and washed with 0.1 M phosphate buffer, pH 7.0, prior to use.

Biotransformations were carried out in 10 mL screw-capped test tubes with a reaction volume of 3 mL with cells (20 g/L, dry weight) suspended in 0.1 M phosphate buffer, pH 7, containing 5% of glucose and 1 g/L of **4**, at 28 °C under magnetic stirring (500 rpm).

2.3. Analyticals

Biotransformations were monitored by HPLC. Samples (0.5 mL) were taken at regular intervals, centrifuged and the aqueous phase was extracted with an equal volume of ethyl acetate; substrate and product concentrations were determined by HPLC using a Purospher Star RP18e250*4.6 mm (5 μm) (Merck, Darmstadt, Germany), UV detection at 220 nm with a Merck-Hitachi 655-22 detector and CH_3CN/H_2O/MeOH (18/72/10) as eluent with a flow rate of 0.8 mL/min.

The retention time of substrates and products was: **4** = 29.0 min; **5** = 37.8 min; **6** = 58.2 min; **7/11** = 61.6 min; **8** = 22.7 min; **9** = 69.8 min; **10** = 76.7 min.

The stereochemical composition of **6** was determined by HPLC using a Pinnacle II silica 250*4.6 mm (4 μm, Restek, Bellefonte, PA, US), UV detection at 220 nm with a Merck-Hitachi 655-22 detector and *n*-heptane/iPrOH (90/10) as eluent with a flow rate of 1.5 mL/min.

Separation of isomers **7** and **11** was performed by HPLC using a Phenomenex Lux Cellulose-1 column 250*4.6 mm (5 μm) (Phenomenex, Torrance, CA, US), UV detection at 220 nm with a Merck-Hitachi 655-22 detector and cyclohexane/*i*PrOH (85/15) as

eluent with a flow rate of 1.0 mL/min. Diastereoisomers were identified by comparison with authentic reference materials chemically synthesized.

2.4. Optimization studies

Optimization studies were carried out with *P. anomala* CBS 110. The desired amount of cells was suspended in different volumes of 0.1 M phosphate buffers containing co-substrates and neat substrate was added to reach the desired concentration; the suspensions obtained were shaken (150 rpm) at different temperatures. Samples of 100 μL were withdrawn from the reaction mixture, centrifuged, extracted with EtOAc (100 μL) and analyzed by HPLC.

2.5. Preparative scale synthesis

2.5.1. [(3aR,4R,5R,6aS)-5-hydroxy-4-((S,E)-3-hydroxy-5-phenylpent-1-en-1-yl)hexahydro-2H-cyclopenta[b]furan-2-one] (Lactondiol B, (3S)-6)

P. anomala CBS 110 was grown in a 3.0 L fermenter with 1.5 L of M5YE liquid medium for 48 h, 28 °C and agitation speed 150 rpm. Fresh cells from submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0, resuspended in 0.5 L of phosphate buffer to reach 25 $g_{dry\ cells}$/L concentration of cells and used for biotransformations. Reactions were carried out in 2 L Erlenmeyer flask by adding glycerol (25 g) and **4** (500 mg) dissolved in 1% of DMSO and keeping the reaction mixture under reciprocal shaking (150 spm) at 28 °C. After 7 days, the biotransformation mixture was centrifuged, and the aqueous supernatant was extracted twice with EtOAc (350 mL); the organic extract was dried over Na_2SO_4 and the solvent was evaporated to give a crude mixture and the single products were purified by flash chromatography (gradient 7:3; 5:5; 2:8 *n*-hexane/EtOAc), furnishing **6** (232 mg, 62%).

Colourless oil. R_f = 0.085 (*n*-hexane/EtOAc 4:6); $[\alpha]_D^{24} = -5.2$ (*c* = 1.00 CH_3CN); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.78–1.98 (m, 2H), 2.23–2.36 (m, 1H), 2.44–2.78 (m, 7H), 3.91 (q, *J* = 7.70, 1H, $CH_2CHOHCH_2$), 4.07 (q, *J* = 6.97, 1H, C*H*OH), 4.83–4.92 (m, 1H, C*H*OC=O), 5.44 (dd, J^1 = 8.06, J^2 = 15.40, 1H, C_{sp2}-H), 5.62 (dd, J^1 = 6.60, J^2 = 15.40, 1H, C_{sp2}-H), 7.16–7.29 (m, 5H, C_{sp2}-H).

^{13}C NMR ($CDCl_3$, 50.4 MHz): δ 177.06 (C=O), 141.79 (C_{sp2}quat.), 136.65, 130.51 (C_{sp2}-H), 128.68, 128.63, 126.20 (C_{sp2}-H), 82.69 (O*C*HCH$_2$), 76.74 (*C*HOH), 72.17 ($CH_2CHOHCH_2$), 56.39 ($CH_2CHCHCHOH$), 42.69 ($CH_2CHCHCHOH$), 40.05, 38.89, 34.38, 31.95 (CH_2).

MS (ESI + *m*/*z*%): 325.3 [M^++Na^+] (100).

2.5.2. [(3aR,4R,5R,6aS)-5-hydroxy-4-((R)-3-hydroxy-5-phenylpentyl)hexahydro-2H-cyclopenta[b]furan-2-one)] (Lactondiol L, (3R)-7)

P. anomala CBS 110 was grown in a 3.0 L fermenter with 1.5 L of M5YE liquid medium for 48 h, 28 °C and agitation speed 150 rpm. Fresh cells from submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0, resuspended in 0.5 L of phosphate buffer to reach 25 $g_{dry\ cells}$/L of cells and used for biotransformations. Reactions were carried out in 2 L Erlenmeyer flask by adding fumaric acid (719 mg) and **4** (500 mg) dissolved in 1% of DMSO and keeping the reaction mixture under reciprocal shaking (150 spm) at 28 °C. After 7 days, the biotransformation mixture was centrifuged, and the aqueous supernatant was extracted twice with EtOAc (350 mL); the organic extract was dried over Na_2SO_4 and the solvent evaporated to yield a crude mixture and the products were purified by flash chromatography (gradient 7:3; 5:5; 2:8 *n*-hexane/EtOAc), furnishing **7** (308 mg, 82%).

Colourless oil. R_f = 0.087 (*n*-hexane/EtOAc 4:6); $[\alpha]_D^{24} = -25.0$ (*c* = 2.00 CH_3CN); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.22–1.28 (m, 1H), 1.46–1.59 (m, 3H), 1.73–1.81 (m, 3H), 2.21–2.28 (m, 2H), 2.43–2.50 (m, 2H), 2.64–2.80 (m, 3H), 3.60–3.66 (m, 1H, $CH_2CHOHCH_2$), 3.97 (q, *J* = 5.1 1H, C*H*OH), 4.88–4.96 (m, 1H, C*H*OC=O), 7.16–7.27 (m, 5H, C_{sp2}-H).

^{13}C NMR ($CDCl_3$, 50.4 MHz): δ 177.95 (C=O), 142.13 (C_{sp2}quat.), 128.68, 128.62, 126.15 (CH_{sp2}), 84.20 (O*C*HCH$_2$), 77.65 (*C*HOH), 71.52 ($CH_2CHOHCH_2$), 54.16 ($CH_2CHCHCHOH$), 43.43 ($CH_2CHCHCHOH$), 40.69, 39.31, 36.22, 35.43, 32.26, 29.18 (CH_2).

MS (ESI + *m*/*z*%): 327.3 [M^++Na^+] (100).

2.5.3. [(3aR,4R,5R,6aS)-5-hydroxy-4-((E)-3-oxo-5-phenylpent-1-en-1-yl)hexahydro-2H-cyclopenta[b]furan-2-one] (**9**)

Intermediate **9** was obtained following the same procedure described above, stopping the reaction after 4 days. The reaction mixture was purified by flash chromatography (gradient 7:3; 1:1; 2:8 *n*-hexane/EtOAc), furnishing **9** (67 mg, 18%).

Brown oil. R_f = 0.28 (*n*-hexane/EtOAc 4:6); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.97–2.10 (m, 1H), 2.39–2.56 (m, 3H), 2.67–2.97 (m, 6H), 4.10 (q, *J* = 6.90, 1H, C*H*OH), 4.90–4.98 (m, 1H, C*H*OC=O), 6.15 (d, *J* = 15.30, 1H,C_{sp2}-H), 6.50 (dd, J^1 = 6.23, J^2 = 15.33, 1H, C_{sp2}-H), 7.20–7.32 (m, 5H, C_{sp2}-H).

^{13}C NMR ($CDCl_3$, 50.4 MHz): δ 198.94 (C=O), 176.34 (OC=O), 144.47 (CH_{sp2}) 141.12 (C_{sp2}quat.), 131.79, 128.74, 128.63, 126.43 (CH_{sp2}), 82.62 (O*C*HCH$_2$), 76.78 (*C*HOH), 56.32 ($CH_2CHCHCHOH$), 42.60 ($CH_2CHCHCHOH$), 42.82, 40.64, 34.62, 30.26 (CH_2).

Anal. calcd for $C_{18}H_{20}O_4$: C 71.98, H 6.71; found: C 71.59, H 6.93.

MS (ESI + *m*/*z*%): 301.2 [M^+] (70).

2.5.4. [(3aR,4R,5R,6aS)-2-oxo-4-(3-oxo-5-phenylpentyl)hexahydro-2H-cyclopenta[b]furan-5-yl benzoate] (8) and [(3aR,4R,5R,6aS)-5-hydroxy-4-(3-oxo-5-phenylpentyl)hexahydro-2H-cyclopenta[b]furan-2-one] (***10***)

S. cerevisiae BY4741 ΔOye2Ks was grown in a 3.0 L fermenter with 1.5 L of M5YE liquid medium for 48 h, 28 °C and agitation speed 150 rpm. Fresh cells from submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0, resuspended in 0.5 L of phosphate buffer to reach 20 $g_{dry\ cells}$/L concentration of cells and used for biotransformations. Reactions were carried out in 2 L Erlenmeyer flask by adding glucose (50 g) and **4** (500 mg) dissolved in 1% of DMSO and keeping the reaction mixture under reciprocal shaking (150 spm) at 28 °C. After 7 days, the biotransformation mixture was centrifuged, and the aqueous supernatant was extracted twice with EtOAc (350 mL); the organic extract was dried over Na_2SO_4 and the solvent was evaporated to yield a crude mixture and the single products were purified by flash chromatography (gradient 7:3; 1:1; 2:8 *n*-hexane/EtOAc), furnishing 392 mg of **8** (392 mg, 78%) and **10** (30 mg, 8%).

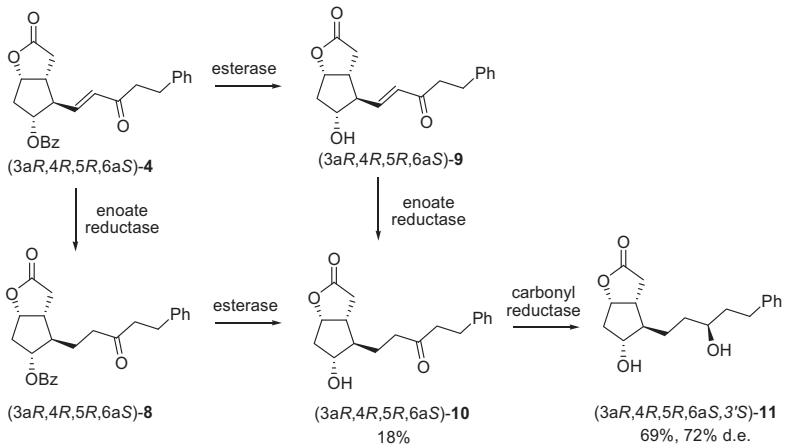
8: White solid. R_f = 0.55 (*n*-hexane/EtOAc 4:6); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.56–1.72 (m, 3H), 2.00–2.20 (m, 1H), 2.35–2.42 (m, 2H), 2.49–2.66 (m, 3H), 2.70–2.95 (m, 5H), 5.08–5.15 (m, 1H, C*H*OC=O), 5.16–5.20 (m, 1H, PhC=OOC*H*), 7.10–7.29 (m, 5H, C_{sp2}-H), 7.40–7.60 (m, 3H, C_{sp2}-H), 7.95–7.99 (m, 2H, C_{sp2}-H).

^{13}C NMR ($CDCl_3$, 50.4 MHz): δ 209.10 (C=O), 176.78 (OC=O), 166.23 (PhC=O), 141.06 (C_{sp2}quat.), 133.55, 129.85 (CH_{sp2}), 129.81 (OC=OC_{sp2}quat.), 128.76, 128.72, 128.52, 126.39 (CH_{sp2}), 84.50 (O*C*HCH$_2$), 79.96 (PhC=OO*C*H), 52.54 (CHCHCHOC=OPh), 44.56 (CH_2), 44.00 ($CH_2CHCHCHOC$=OPh), 40.79, 37.87, 36.37, 29.97, 26.94 (CH_2).

Anal. calcd for $C_{25}H_{26}O_5$: C 73.87, H 6.45; found: C 73.50, H 6.65.

MS (ESI + *m*/*z*%): 429.4 [M^++Na^+] (100).

10: Colourless oil. R_f = 0.25 (*n*-hexane/EtOAc 4:6); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.50–1.80 (m, 3H), 1.90–2.10 (m, 2H),



Scheme 2. Biotransformation of **4** with *Pichia glucozyma*.

2.25–2.52 (m, 4H), 2.60–2.94 (m, 5H), 3.85 (q, J = 5.6, 1H, C*H*OH), 4.84–4.93 (m, 1H, C*H*OC=O), 7.15–7.32 (m, 5H, C_{sp2}-H).

^{13}C NMR ($CDCl_3$, 50.4 MHz): δ 209.89 (C=O), 177.24 (OC=O), 141.10 (C_{sp2}quat.), 128.74, 128.59, 126.43 (CH_{sp2}), 83.45 (O*C*HCH$_2$), 77.43 (*C*HOH), 53.2 (CH_2*C*HCHCHOH), 44.50 (CH_2), 43.19 (CH_2*C*HCHCHOH), 40.86, 35.69, 35.69, 30.05, 26.08 (CH_2).

Anal. calcd for $C_{18}H_{22}O_4$: C 71.50, H 7.33; found: C 71.13, H 7.53.

MS (ESI + m/z%): 325.4 [M^++Na^+] (100).

*2.5.5. [(3aR,4R,5R,6aS)-5-hydroxy-4-((S)-3-hydroxy-5-phenylpentyl)hexahydro-2H-cyclopenta[b]furan-2-one] (**11**)*

P. glucozyma CBS 5766 was grown in a 3.0 L fermenter with 1.5 L of M5YE liquid medium for 48 h, 28 °C and agitation speed 150 rpm. Fresh cells from submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0, resuspended in 0.5 L of phosphate buffer to reach 25 $g_{dry\ cells}$/L concentration of cells and used for biotransformations. Reactions were carried out in 2 L Erlenmeyer flask by adding glucose (25 g) and **4** (500 mg) dissolved in 1% of DMSO and keeping the reaction mixture under reciprocal shaking (150 spm) at 28 °C. After 7 days, the biotransformation mixture was centrifuged, and the aqueous supernatant was extracted twice with EtOAc (350 mL); the organic extract was dried over Na_2SO_4 and the solvent was evaporated to give a crude mixture and the single products were purified by flash chromatography (gradient 7:3; 1:1; 2:8 *n*-hexane/EtOAc), furnishing **11** (260 mg, 69%). Absolute configuration was assigned by comparison with authentic reference materials chemically synthesized.

3. Results and discussion

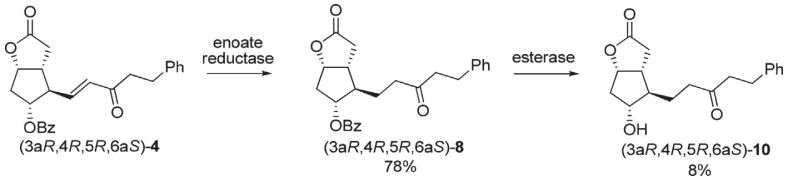
A screening for the identification of yeasts (see experimental section for the complete list) able to transform (3a*R*,4*R*,5*R*,6a*S*)-**4** into (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** and/or (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** was carried out using resting cells suspended in phosphate buffer in the presence of 5% glucose and neat (3a*R*,4*R*,5*R*,6a*S*)-**4** (1 g/L). Two wild-type strains (*P. anomala* CBS110 [15,16] and *P. glucozyma* CBS 5766 [17–20]) and the recombinant strain *S. cerevisiae* BY4741ΔOye2Ks (which has the original OYE2 deleted and bears the highly expressed enoate reductase gene from *Kazachstania spencerorum*) [21] gave molar conversion of (3a*R*,4*R*,5*R*,6a*S*)-**4** above 30%.

P. glucozyma CBS 5766 gave ketone (3a*R*,4*R*,5*R*,6a*S*)-**10** (18% yield) and (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**11** (69% yield, 72% d.e.) as final products. During the reaction, traces of the transient intermediates (3a*R*,4*R*,5*R*,6a*S*)-**8** and (3a*R*,4*R*,5*R*,6a*S*)-**9** were observed, indicating that the overall transformation likely occurred as reported in Scheme 2. The reduction of the carbonyl was partially enantioselective, furnishing (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**11** with 72% d.e. Absolute configuration was assigned by comparison with authentic reference materials chemically synthesized.

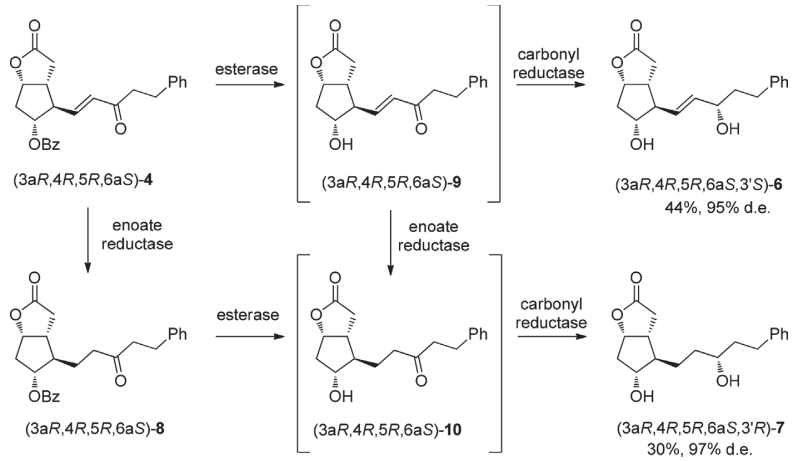
S. cerevisiae BY4741ΔOye2Ks showed, as expected, high enoate reductase activity giving the saturated ketone (3a*R*,4*R*,5*R*,6a*S*)-**8** as the main product (78%) and minor amounts of **10**, deriving from subsequent ester hydrolysis (Scheme 3).

P. anomala CBS 110 gave a mixture of Lactondiol B (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** (44% yield, 95% d.e.) and (*R*)-Lactondiol L (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** (30% yield, 97% d.e.). Interestingly, the opposite epimer (with respect to the one obtained with *P. glucozyma*) was obtained by C=O reduction, with high stereoselectivity (d.e. 97%). Scheme 4 shows the biotransformations involved in the conversion of (3a*R*,4*R*,5*R*,6a*S*)-**4** with *P. anomala*, based on the different intermediates observed during the overall transformation.

It is known that the use of whole cells of yeasts generally furnishes a mixture of saturated and unsaturated alcohols in the reduction of α,β-unsaturated carbonyls, depending on the strain and conditions employed [22]; this is due to the relative rates of enoate reductase and carbonyl reductase activities, which



Scheme 3. Biotransformation of **4** with *Saccharomyces cerevisiae* BY4741ΔOye2Ks.



Scheme 4. Biotransformation of **4** with *Pichia anomala*.

Table 1
Biotransformation of (3a*R*,4*R*,5*R*,6a*S*)-**4** with *P. anomala* in presence of different cosubstrates.

Entry	Co-substrate	Concentration of co-substrate (g/L)	**6**		**7**	
			Yield (%)	d.e. (%)[a]	Yield (%)	d.e. (%)[a]
1	Ethanol	5	5	n.d.	12	92
2	Ethanol	50	10	97	20	92
3	Fumaric acid	5	8	n.d.	75	97
4	Fumaric acid	50	13	97	70	97
5	Glucose	5	38	95	25	97
6	Glucose	50	44	95	30	97
7	Glycerol	5	57	97	29	97
8	Glycerol	50	58	97	30	97
9	Xylose	5	–	n.d.	21	96
10	Xylose	50	–	n.d.	33	95

[a] Determined by HPLC analysis.

determine the final ratio of the products. The type of co-substrates added for favouring co-factors regeneration may play a crucial role in the activity of the different reductases. Thus, the biotransformation with *P. anomala* was carried out using various conventional co-substrates (glucose, ethanol, glycerol, xylose) showing the predominant formation of Lactondiol B (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** with d.e. in the range of 95–97%; in particular the use of glucose gave (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** with 44% yield and 95 d.e., while in presence of glycerol 58% yield and 97% d.e. were observed. Interestingly the use of fumaric acid as cosubstrate in the same reaction system gave higher amounts of Lactondiol L (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** (75% yield, 97% d.e.); fumaric acid is an unconventional co-substrate for cofactor regeneration and it is assumed that the yeast like *P. anomala* may oxidize fumaric acid in Krebs cycle, therefore allowing for regeneration of NAD(P)H. Table 1 summarizes the results obtained using different co-substrates.

The biotransformation of (3a*R*,4*R*,5*R*,6a*S*)-**4** catalyzed by *P. anomala* was optimized using the Multimplex® experimental design, which simultaneously evaluates different parameters of the biotransformation and had previously shown to be suited for the fast optimization of different biotransformations [23,24]. The response parameters were product yields and d.e., while the control variables were temperature, pH, concentration of cells, DMSO and co-substrate. The best performance for the preparation of (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** (82% yield, 97% d.e.) was obtained using 0.1 mM phosphate buffer at pH 7.0, 28 °C, 1 g/L substrate concentration in the presence of 1.5 g/L fumaric acid (corresponding to a 5/1 molar ratio of fumaric acid/substrate), while the best conditions for the preparation of (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** (62% yield, 97% d.e.) were found using 0.1 mM phosphate buffer at pH 7.2, 28 °C, 1 g/L substrate concentration in the presence of 50 g/L glycerol.

4. Conclusions

In conclusion, here we propose an alternative chemoenzymatic approach for the synthesis of Bimatoprost and Latanoprost, based on the biotransformation of 15-ketoprostaglandin (3a*R*,4*R*,5*R*,6a*S*)-**4** with whole cells of non-conventional yeast *P. anomala*. This appears like a useful method for preparing optically pure Lactondiol B (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** and Lactondiol L (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** in high yields depending on the co-substrate employed: Lactondiol B (3a*R*,4*R*,5*R*,6a*S*,3′*S*)-**6** was obtained with 62% yield using glycerol, whereas the use of fumaric acid gave 82% yield of Lactondiol L (3a*R*,4*R*,5*R*,6a*S*,3′*R*)-**7** in a simple one-pot, three-step modification of substrate (3a*R*,4*R*,5*R*,6a*S*)-**4**.

Supporting information

Copies of ^{1}H NMR, ^{13}C NMR and mass spectra of all characterized compounds **6**–**10** are provided.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2014.05.022.

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KRED1-Pglu

Employment of a new ketoreductase from *P. glucozyma* for stereoselective reductions

5.1 Productions of recombinant proteins

The demand of recombinant proteins has increased, as more applications in several fields become a commercial reality. Recombinant proteins have been utilized as tools for cellular and molecular biology. Various applications areas have experienced substantial advances thanks to the possibility of producing large amounts of recombinant proteins by an increasing availability of genetically manipulated microorganisms. For instance, a lot of diseases have been treated because of the accessibility of therapeutic and prophylactic proteins that in the past could be obtained in a very small amount and using unsafe sources. To date, more than 75 recombinant proteins are used as pharmaceuticals and more than 360 new therapeutic proteins are under development.[66] The use of recombinant DNA for cloning and gene expression in a host microorganism was introduced in 1973. In particular the *Salmonella thyphimurium* gene coding for the streptomycin resistance was cloned in *Escherichia coli*, verifying the antibiotic tolerance.[67] Among the many systems available for heterologous proteins production, the Gram-negative bacterium *Escherichia coli* still remains one of the most attractive because of genetically and physiologically well characterized. Among its strengths the ability to rapidly grow up on inexpensive substrates, easily to genetically manipulate, the high level

[66] L.A. Palomares *et al., Methods in Molecular Biology* **2004**, *267*, 15-51
[67] S.N Cohen *et al., PNAS* **1973**, *70*, 3240-3244

of protein expression, the availability of an increasingly large number of cloning vectors and mutant host strains.[68] On the other hand the recombinant proteins expressed in *E. coli*, sometimes do not present the post-trasductional correct modifications such as phosphorilations and glycosylations, and the overproduction of heterologous proteins in *E. coli* cytoplasm could be accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies. To overcome this limit it is necessary the reduction of the transcription and traduction efficiency for example lowering the expression temperature at 15-20 °C. In this way the proteins have the time to fold and became active. [69] For the heterologous protein expression in eukaryotes the yeasts *Pichia pastoris* and *Saccharomyces cerevisiae*, microorganisms rapidly to grow and easily to genetically manipulate are the most used. Moreover, they have the ability to carry out many post-trasductional modifications such as glycosylation. However they lack some post-traductional pathways which could be important when recombinant proteins have animal origin, such as hydroxylation, amidation and many types of phosphorilation and glycosylation.

In order to maximize the production of heterologous protein is necessary take into account these aspects:

- Selection of the host cell
- Selection and design of the promoter for the interested gene. Promoters of *E. coli* consist of a hexanucleotide sequence located approximately 35 bp upstream of the transcription initiation base separated by a short spacer from another hexanucleotide sequence. A useful promoter

[68] F. Baynex, *Curr. Opin. Biotechnol.* **1999**, *10*, 411-421
[69] X.Dai *et al., Biochem. Biophys. Res. Commun*. **2005**, *332*, 593-601; E.D. Clark, *Curr. Opin. Biotechnol.* **2001**, *12*,202-207

exhibits several desirable features: it is strong, it has a low basal expression level (it is tightly regulated), it is easily transferable to other *E. coli* strains to facilitate testing of a large number of strains for protein yields, and its induction is simple and cost effective .

- Increase of mRNA half-life
- Adaptation of codons to codon-usage of host microorganism. Genes in both prokaryotes and eukaryotes show a non-random usage of synonymous codons. The systematic analysis of codon usage patterns in *E. coli* led to the following observations: (i) Certain codons are most frequently used by all different genes irrespective of the abundance of the protein; for example, CCG is the preferred triplet encoding proline. (ii) Highly expressed genes exhibit a greater degree of codon bias than do poorly expressed ones. (iii) The frequency of use of synonymous codons usually reflects the abundance of their cognate tRNAs. These observations imply that heterologous genes enriched with codons that are rarely used by *E. coli* may not be expressed efficiently in *E. coli*.
- Modifications of gene sequence in order to send the protein to a specific cellular compartment such as cytoplasm, periplasm or extracellular secretion.
- Optimization of fermentation conditions (composition of cell growth medium, temperature, pH, oxygen, agitation) [70]

[70] S.C. Markrides, *Microbiol. Rev.* **1996**, *60*, 512-538

5.2 Expression systems

The *E. coli* expression systems are based on the utilization of expression vectors which both simplify the cloning operations and require high stability obtained with antibiotics resistance markers. The success of the production of heterologous protein often depends on the accurate choice of expression system suitable for both the protein and for the coding gene. The expression system most used for *E.coli* is the "pET Expression System" (Novagen). A pET vector is a bacterial plasmid designed to enable the quick production of large quantity of any desired protein when activated. The plasmid contains several important elements such as a *lacI* gene which codes for lac repressor protein, a T7 phage promoter which is specific only for T7 RNA-polymerase (not bacterial ones) and also does not occur anywhere in the prokaryotic genome, a *lac* operator which can block transcription, a polylinker, an f1 origin the replication, an ampicillin resistance gene and ColE1 origin of replication (Fig. 29).

To start the process the gene (YFG) is cloned into pET vector at the polylinker site. Both the T7 promoter and the *lac* operator are located 5' to YFG. When the T7 RNA polymerase is present and the *lac* operator is not repressed, the transcription of YFG proceeds rapidly. Because T7 is a viral promoter, it transcribes rapidly and profusely for as long as the T7 RNA polymerase is present. The expression of the protein (YFP) increases rapidly as the amount of mRNA transcribed from YFG increases. Within a few hours, YFP is one of the most prevalent components of the cell.

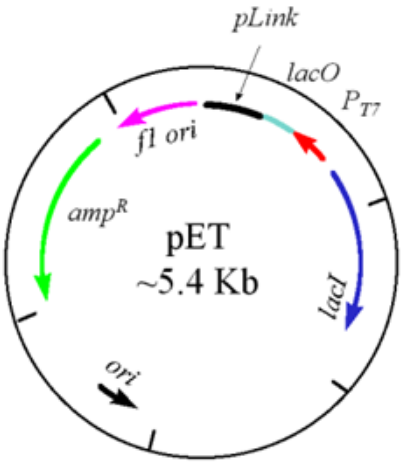

Fig. 29 The pET vector

One of the most important parts of the pET Expression System involves the fact that YFG is not transcribed unless the T7 RNA polymerase is present. Prokaryotic cells do not produce this type of RNA, and therefore the T7 RNA polymerase must be added. Usually, the host cell for this expression system is a bacteria which has been genetically engineered to incorporate the gene for T7 RNA polymerase, the *lac* promoter and the *lac* operator in its genome. When lactose or a molecule similar to lactose is present inside the cell, transcription of the T7 RNA polymerase is activated. Typically, the host cell used is *E. coli* strain *BL(DE3)*.

Control of the pET expression system is accomplished through the *lac* promoter and operator. Before YFG can be transcribed, T7 polymerase must be present. The gene on the host cell chromosome usually has an inducible promoter which is activated by IPTG. IPTG displaces the repressor from the *lac* operator. Since there are *lac* operators on both

the gene encoding T7 polymerase and YFG, IPTG activates both genes. Therefore, when IPTG is added to the cell, the T7 polymerase is expressed, and quickly begins to transcribe YFG which is then translated as YFP. IPTG works to displace a *lac* repressor since IPTG is an analogue of lactose. The *lac* genes express enzymes which are involved in the breaking down of lactose, and therefore, the presence of lactose (or it's analogue) would trigger the initiation of transcription of *lac* genes (Fig. 30)

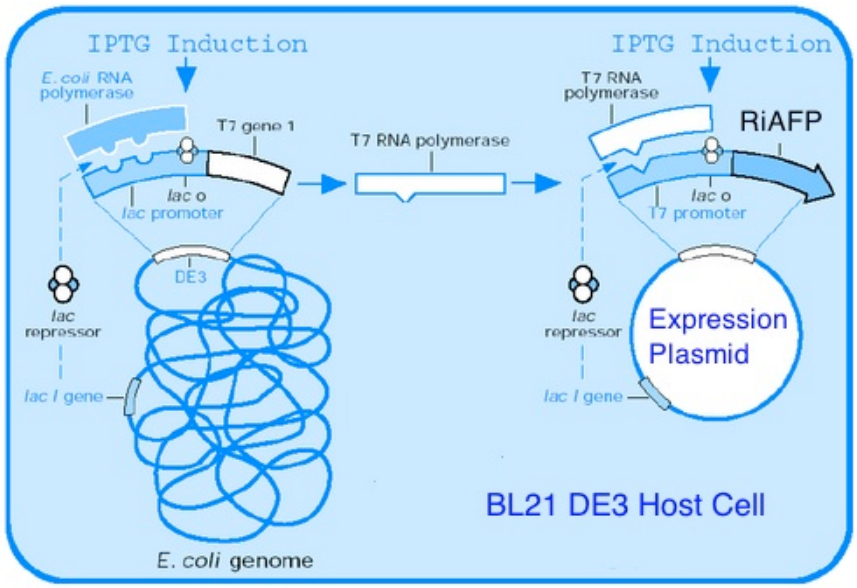

Fig. 30 IPTG mechanism of action

In order to facilitate the recombinant protein expression and purification many fusion partners have been developed. [71] In *vivo* the fusion partners can be advantageous

[71] R.C. Stevens, *Struct. Fold. Des.* **2000**, *8*, 177-185

because they could protect the new produced protein from intracellular proteolysis[72] and could increase their solubility.[73]

5.3 Choice of host microorganism

The genetic base of the host strain is an important characteristic for the production of recombinant proteins. The expression strains could be mutated for the natural host proteases, maintaining stable the new formed recombinant protein and lending the strain many relevant genetic elements in the expression system. *E. coli* BL21 is the most utilized host, it is a robust strain able to vigorously grow up on a minimal media. BL21 strain lacks OmpT and Lon, two different proteases that could interfere with the production of recombinant protein degrading it. Moreover, the mutated strain utilized in this PhD thesis, BL21 DE3Star, presents a mutation on rne gene, coding for truncated RNasi E unable to degrade mRNA.[74] This endonuclease consists of 1061 amino acids and it is involved in the mRNA degradation. The truncated enzyme lacks the C-terminal tail (about 477 a. a.) appointed for this function, so the increased stability of mRNA. Because of the choice of pET Expression System, in which the interesting gene is under the control of the T7 promotor, it is required that the chosen host has the phage fragment DE3 coding for the polymerase T7.

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[72] A. Jaquet *et al., Protein Expr. Purif.* **1999**, *17*, 392-400; A. Martinez *et al., Biochem J*. **1995**, *306*, 589-597.

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5.4 Oxidoreductases

This class of enzymes, responsible for redox transformations in living organisms is composed by three different families according to the funtion: (i) dehydrogenases, catalyze the oxidations of alcohols to ketones and formation of double bonds, as well as the reversive reactions (ii) oxigenases, catalyze the hydroxylation of non-activated C-H bonds, epoxidation or dihydroxylation of C=C bonds, and (iii) oxidases, which catalize electron transfer processes.[75] Common feature shared by the members of oxidoreductases is their dependence on a specific cofactor molecule whose presence in the active site ensures the enzymes performance. For example, alcohol dehydrogenases (ADH) also called ketoreductases or carbonyl reducatases are able of reducing carbonyl groups into alcohols as well as the opposite oxidation reaction, because of the presence of nicotinamide cofactors NAD(P)H or $NAD(P)^+$ which provides the hydride atom to the carbonyl substrate or abstract the hydride from the alcohol. In more details, as showed in Fig. 31, the hydride could be transferred in four different stereochemical ways to the prochiral substrate. It can attack either *si* or *re* face of the carbonyl depending on the orientation of the substrate in the active site. On the other hand, enzyme facilitates the transfer of either *pro-R* or *pro-S* hydride of the cofactor depending on the type of ADH. Moreover, stereospecificity of ADH-catalyzed reactions is classified following Prelog's rule based on the size of the substituents bonded to the carbonyl group. It is based on the stereochemisry of microbial reduction using *Curvularia falcata* cells and it states that the ADH delivers

[75] D. Gamenara, G.A. Seoane, P.Saenz-Méndez, P. Dominguez de Maria, *Redox Biocatalysis: Fundamentals and Applications.* Wiley & Sons, New Jersey, **2013**, 12-18

the hydride from *re*-face of a pro-chiral ketone affording the (*S*)-alcohol. On the contrary, in case of (*R*)-enantiomer is produced, the ADH is describe as an anti-Prelog enzyme.

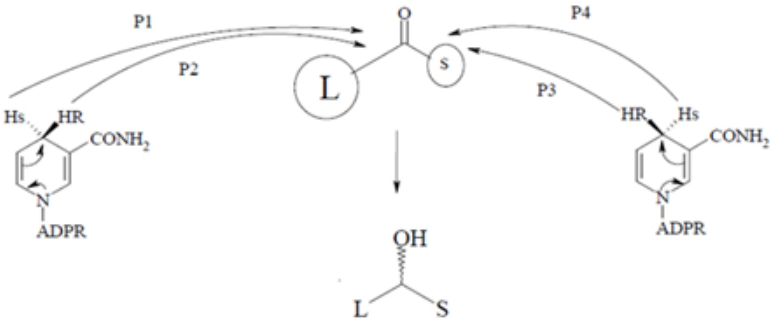

Fig. 31 Stereospecificity of ADHs is determined by the mutual position of the nicotinamide cofactor and carbonylic substrate in the active site.

The majority of the commercially available ADHs used for the stereospecific reduction of ketones and the majority of microorganisms (for instance, *S. cerevisiae*) follow the Prelog's rule.[76]

[76] K. Faber *Biotranformations in Organic Chemistry. A Textbook*. 6th Ed. Springer- Verlag Berlin Heidelberg **2011**

5.5 Cofactors recycling

In the redox processes catalyzed by dehydrogenases, the cofactors like nicotinamide NAD(P), flavin FNM or FAD derivatives play a key role by providing the necessary redox equivalents, acting as electron donor or acceptor.

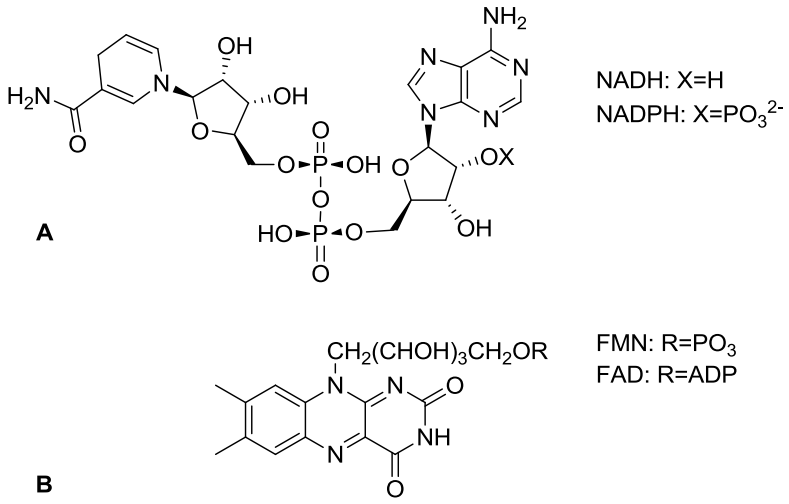

Fig. 32 Chemical structure of oxidoreductase cofactors: (A) nicotinamide; (B) flavin.

The cofactors are molecules very expensive and unstable that are consumed in a stoichiometric amount. As shown in Fig. 33, the oxidation of a generic alcohol to ketone determines the consumption of $NAD(P)^+$, while the reduction of a generic alcohol leads the consumption of $NAD(P)H^+$. For the development of an industrial application of oxidoreductases, this problem has been overcome by the *in situ* recycling techniques allowing the use of catalytic amounts of these compounds.

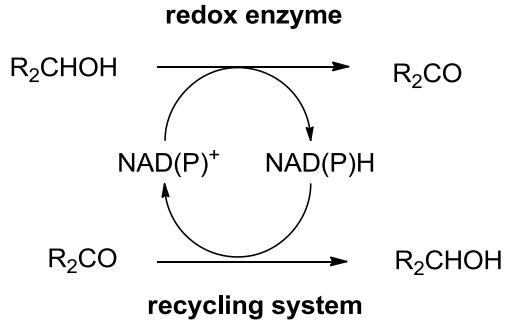

Fig. 33 Redox system using a co-substrate for the recycle of cofactors

The main strategies for the regeneration of cofactors concern the use of enzymatic reactions, and electrochemical or photochemical systems.

- Electrochemical regeneration: it usually employs metals to obtain the electron transfer between an electrode and reduced/oxidized cofactors. Fig. 34a
- Photochemical regeneration: it employs photosensitizers in homogeneous phase derived from complexes such as zinc complexes or colloids such as derivatives of cadmium or titanium dioxide. Fig. 34b
- Enzimatic regeneration: it is divided in two different categories. The "enzyme coupled system" based on the use of another enzyme such as GDH (glucose dehydrogenase) with glucose, or FDH (formate dehydrogenase) with a formate salt, consists in two parallel different reactions catalyzed by different enzymes. The first one is the interesting reaction that is the transformation of the reagent in the desired product, while the second one is the regeneration of the cofactor. The most important features of this system are both the substrate specificity of the different employed biocatalysts and

the high affinity for the same cofactor. On the other hand, "the coupled substrate system" based on the constant regeneration of cofactor by the same enzyme because of the addition of the co-substrate. The auxiliary substrate let the biocatalyst performs a secondary reaction in order to have the desired cofactor form. While in the first case the "enzyme coupled system" is thermodynamically favoured because of the shifted equilibrium towards the formation of the product, the "coupled substrate system" is less thermodynamically favoured. It is a common good practise the adding of a huge molar excess of co-substrate in order to force the equilibrium of the interesting reaction to have the desired product. Fig. 35.

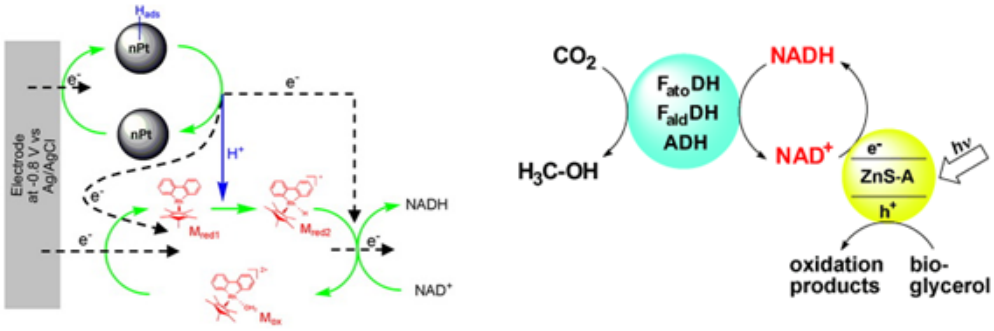

Fig. 34 A: platinum nanoparticles (n-Pt) in electrolyte enhance electron transferfrom electrode to NAD^+ in electrolyte during an indirectelectrochemical regenerationof NADH. The intermediate nPt-Hads, formed a negative potential, helps the primary mediator turnover by donating proton and electron in a kinetically favourable way.[77] B: CO^2 conversion into CH_3OHusing light to regenerate the enzyme cofactor (NADH) by ZnS-A photocatalysis, with bioglicerol as H^- and e^- donor[78]

[77] C. Hwang *et al.*, *Scientific report* **2015**, *5*, 1-10.
[78] A. Dibenedetto *et al. Chem. Sus. Chem.* **2012**, *5*, 373-378.

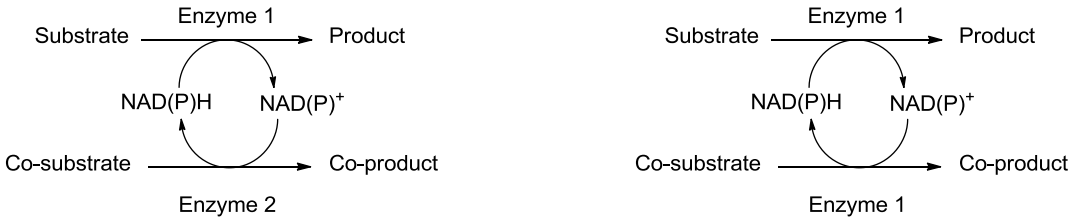

Fig. 35 Enzymatic regeneration of cofactors

5.6 Benzilreductases and SDR (short chain dehydrogenases)

The benzilreductase is an oxidoreductase NAD(P)H-dependent belonging to the family of SDR (short chain dehydrgenases), proteins with a simpler molecular architecture, with largely one-domain subunit without metal derived from coenzyme-binding fold and with more or less extension. Members of the SDR superfamily are non-metalloenzymes with molecular masses between 25 and 35 kDa. Substrates for the SDR include prostaglandins, steroids, aliphatic and aromatic compounds and xenobiotcs, too. The benzyl-reductase, catalyzing the reduction of aromatic ketones in particular the stereoselective reduction of benzil into (*R*)- or (*S*)-benzoin, has at least two binding domains, one for the substrate bond and important to determine the substrate specificity, it contains the amino acids involved in the catalysis, and the so called Rossman fold, a N–terminal domain binding the nucleotidic co-factor and showing a very conserved sequence formed by 70 residues with a super secondary structure type βαβαβ. The conserved sequence is made up of GXGXXG in which the first and the second glycines are involved

in the binding with cofactor while the third facilitates the approach between the α-helix and the β-sheet.[79]

The typical catalytic triad of the active site in the SDR family is composed by a Tyr, Lys and a Ser, which form a proton relay system. An additional Asn also play an important role by maintaining the active site configuration during the proton transfer.[80] In particular it is involved both by maintaining the Lys position during the proton relay and by stabilizing the side chain interaction. The Tyr residue plays an important role in the catalytic system because it is the responsible for the abstraction or donation (in oxidation or reduction respectively) of the proton from/to the substrate. Moreover, the Lys residue participates with its amino group in binding the ribose of the co-factor, while Ser residue stabilizes the oxygen atom of the product. [81] In the end, the carbonyl group of Asn residue binds with an H-bond a water molecule to the Lys chain stabilizing the proton relay system.

[79] S. Rao *et al*., *J. Mol. Biol.* **1973,** *76*, 241-256
[80] C.Filling *et al., J. Biol. Chem.* **2002**, *277*, 2567-2584
[81] A. Lerchner *et al., Biothecnol. Bioeng.* **2013**, *110*, 2803-2814

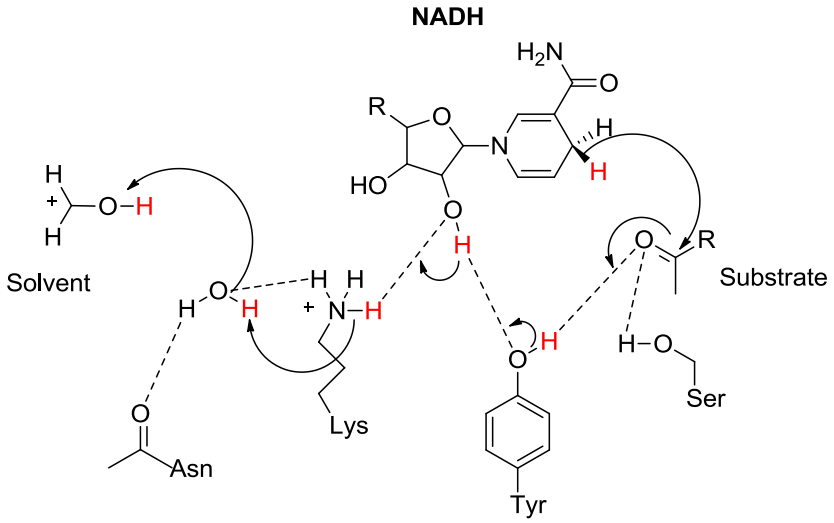

Fig. 36 SDR (3β/17β-HSD) mechanism. Tyr 151 is the catalytic base, Ser 138 fixes the substrate while Lys 155 forms the H-bon with NADH.

5.7 The synthetic importance of the α-hydroxyketones

Enantiomerically pure α-hydroxyketones can be used as precursors for the synthesis of pharmaceuticals, such as antitumor antibiotics *e.g.* olivompicina A and chromomycin A3, farnesyl transferase inhibitors *e.g.* kurasoin A and B2, inhibitors of the production of β-amyloid proteins and antidepressants such as bupropion. They also can be exploited for the preparation of important structures such as amino alcohols and diols.[82]

[82] T. Tanaka *et al. Bioorg. Med. Chem.* **2004**, *12*, 501-505. O.B. Wallace *et al. Bioorg. Med. Chem. Lett.* **2003**,*13*, 1203-1206. Q. Fang et *al. Tet. Asym*. **2000**, *11*, 3659-3663.

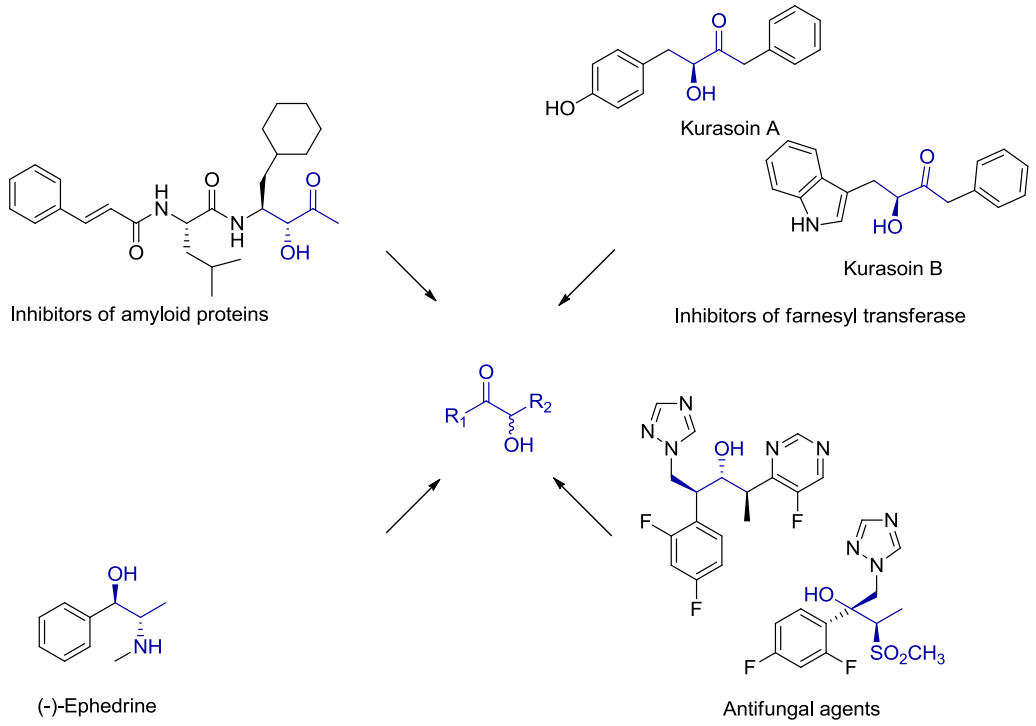

Fig. 37 Derivatives of hydroxyketones

In literature numerous methods for the synthesis of hydroxyketones such as benzoin and derivatives have been reported. They include both chemical methods and enzymatic ones. Between these we include the ketohydroxylation of olefins,[83] the asymmetric mono-oxidation of the corresponding 1,2- diol,[84]as well as the kinetic resolution *via* oxidation of racemic α-hydroxyketone. Moreover, a lot of organocatalytic techniques consisting into α–oxygenation of ketones in the presence of proline or alanine [85] have been proposed. Finally, benzoin condensation by the use of chiral salts such as

[83] B. Plietker, *Eur. J. Org. Chem.* **2005**, *9*, 1919-1929.
[84]O.Onomura *et al., Tet. Lett*. **2007**, 48, 8668-8672.
[85] P. Muthupandi *et al., Chem. Commun*. **2009**, 3288-3290. S. Alamsetti *et al., Chem. Eur. J.* **2009**, *15*, 5424-5427.

thiazole, imidazole and triazole, which increase both the yield of the product and the selectivity has been reported.

These salts exploit the same mechanism of the thiazolic ring of thiamine (vitamin B1), coenzyme universally present in all living systems; after the formation of the carbanion because of the removal of hydrogen of the thiazolic group, it attacks the electrophile carbon of a molecule of benzaldehyde, which adds another molecule of this aldehyde. The use of thiamine or molecules with analogous thiazole ring as catalysts allows dimerization in a basic environment (*e.g.*triethylamina) of many either aliphatic or aromatic aldehydes.

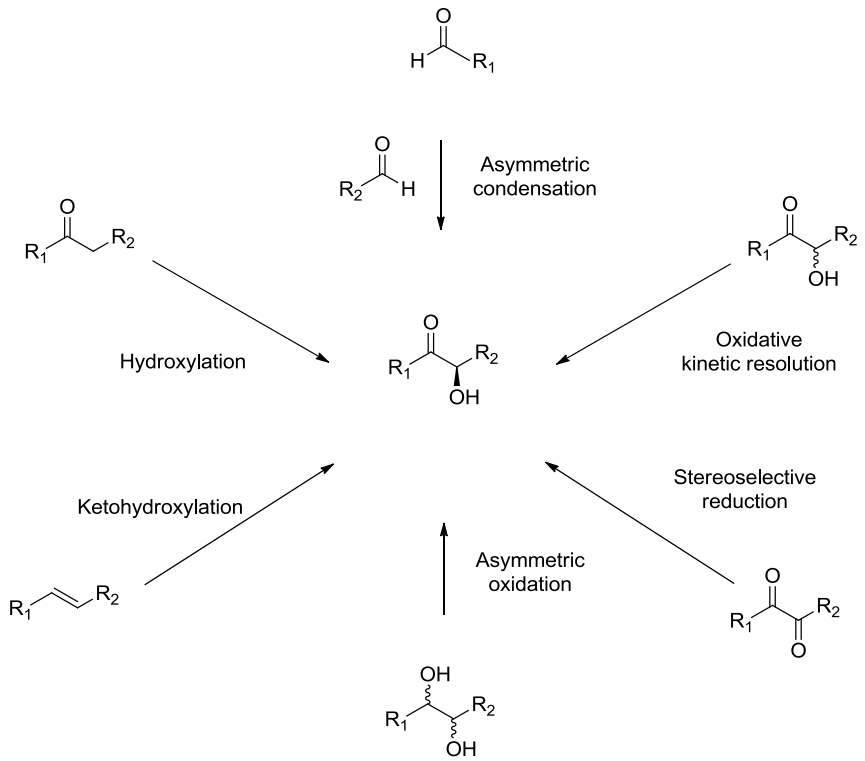

Fig. 38 Chemical strategies for the asymmetric synthesis of α-hydroxyketones

To overcome the problems of chemical approaches such as significant number of steps, reduced yields and difficulty to obtaining enantiopure products several biocatalytic methods were proposed. Among these the use of lyase thiamine diphosphate-dependent for aldehydes condensation, lipases for stereoselective hydrolysis and oxidoreductases for stereoselective reduction/oxidation are noteworthy.

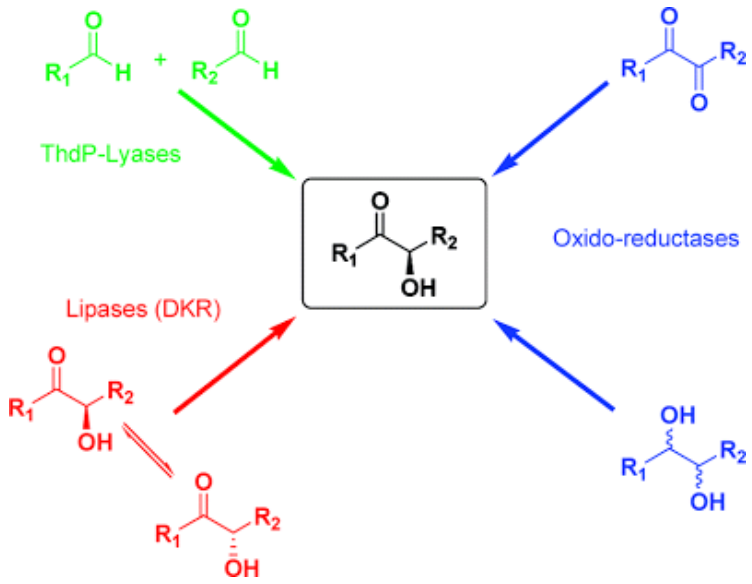

Fig. 39 Biocatalytic strategies for the asymmetric synthesis of α-hydroxyketones

The ThdDP-lyase catalyzes condensation of aldehydes to give α-hydroxyketones. The more studied enzymes are benzaldehyde lyase (BAL) isolated from *Pseudomonans fluorescens* [86]and pyruvate decarboxylase (PDC). The first one catalyzes the carboligation both of aromatic aldehydes and of aliphatic ones, the second is a good example of a robust catalyst used in industrial scale processes for the production of L-

[86] M. Muller *et al., FEBS J.* **2009**, *276*, 2894-2904.

phenylacethylcarbinol (CAP), the precursor of ephedrine, from benzaldehyde and pyruvic acid.[87]

Hydrolases, such as lipases and esterases, convert both aliphatic and aromatic compounds, giving different α-hydroxyketones;[88] *Jeon et al* . reported the enantioselective transesterification of racemic ketones by an immobilized lipase B from *Candida Antarctica* (CAL-B) in organic solvents.[89]

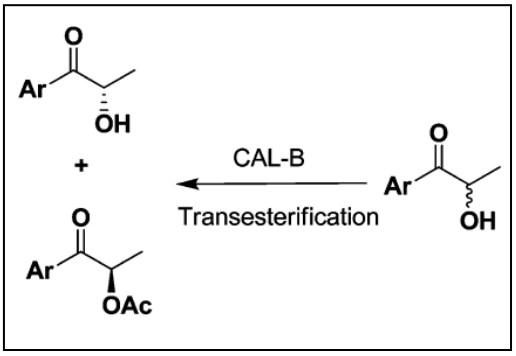

Fig. 40 Example of esterification of (*R)*-2-hydroxy-1-phenylpropan-1-one mediated by CAL-B

Finally, an important role is played by the oxidoreductases, which act both as isolated enzymes and as whole cells. In literature, there are many examples of microorganisms employed as biocatalysts for the reduction of α-diketones.[90]

[87] M. Pohl *et al. Curr. Opin. Biothecnol*. **2004**, *15*, 335-342

[88] Y. Aoyagi *et al.Tet. Lett.* **2000**, *41*, 10159-10162. Y. Aoyagi *et al. J. Org. Chem.* **2001**, *66*, 8010-8014.

[89] H . Ohta *et al. Chem Lett.* **1986**, 1169-1172.

[90] P.Hoyos *et al. Tet.* **2008,** 64, 7929-7936. A. S. Demir *et al. J. Mol. Cat. B: Enzym.* **2008**, *55,* 164-168. T.Saito *et al. Appl. Biochem. Biotechnol.* **2003**, *111*, 185-190. T. Matsuda *et al. Tet. Lett.* **2000**, *41*, 4135-4138. T. Matsuda *et al. J. Org. Chem*. **2000**, *65*, 157-163.

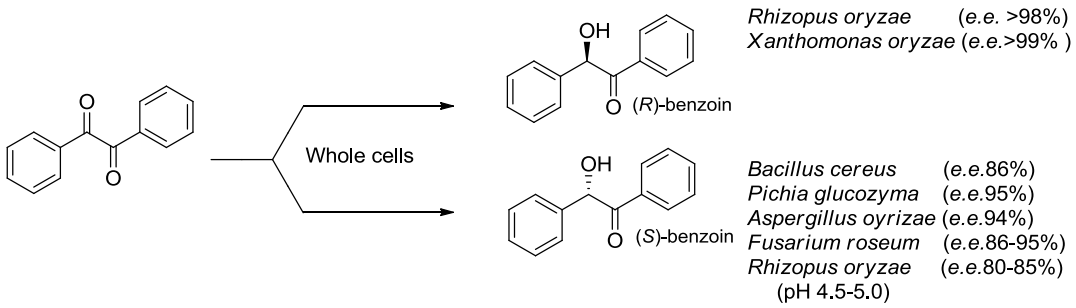

Fig. 41 Microorganisms employed for the reduction of benzoin

Another strategy involves the application of dehydrogenases or oxidases capable of performing reaction of (de)racemization. For example *Rhizopus oryzae* is able to reverse the chirality of benzoin depending on the pH of the reaction medium,[91] while the whole cells of *Pichia glucozyma* are able to deracemize a racemic mixture of (*R*)- and (*S*)-benzoin because of the activity of different dehydrogenases: the first one responsible for the oxidation of (*R*)-benzoin to benzil and the second one responsible for the reduction of benzil in the opposite enantiomer.[92]

[91] A.S. Demir *et al., Tet. Asym.* **2004**, *15*, 2579-2582. A.S. Demir *et al., Tet. Let.* **2002**, *43*, 6447-6449.
[92] M.C. Fragnelli *et al., Tet*. **2012**, *68*, 523-528.

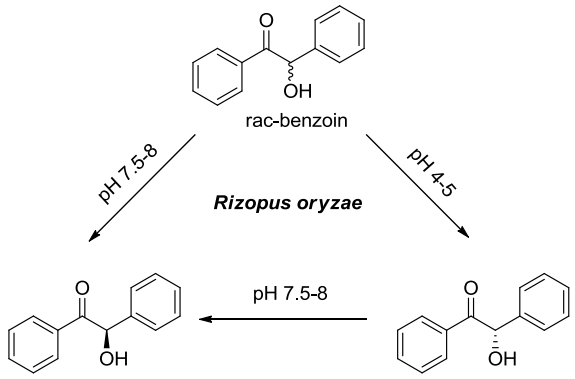

Fig. 42 Deracemization of benzoin catalyzed by *Rhizopus oryzae*

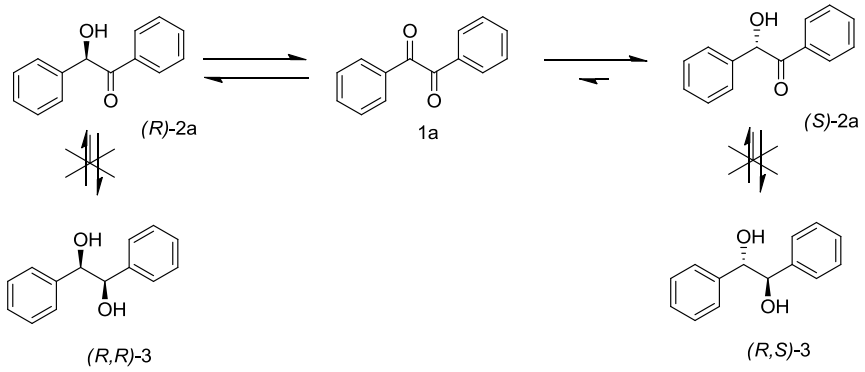

Fig. 43 Deracemization of (*R*)-benzoin catalyzed by *Pichia glucozyma*

Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6961-y

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Stereoselective reduction of aromatic ketones by a new ketoreductase from *Pichia glucozyma*

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Received: 25 May 2015 / Revised: 20 July 2015 / Accepted: 23 August 2015

Abstract A new NADPH-dependent benzil reductase (KRED1-Pglu) was identified from the genome of the non-conventional yeast *Pichia glucozyma* CBS 5766 and overexpressed in *E. coli*. The new protein was characterised and reaction parameters were optimised for the enantioselective reduction of benzil to (*S*)-benzoin. A thorough study of the substrate range of KRED1-Pglu was conducted; in contrast to most other known ketoreductases, KRED1-Pglu prefers space-demanding substrates, which are often converted with high stereoselectivity. A molecular modelling study was carried out for understanding the structural determinants involved in the stereorecognition experimentally observed and unpredictable on the basis of steric properties of the substrates. As a result, a new useful catalyst was identified, enabling the enantioselective preparation of different aromatic alcohols and hydroxyketones.

Keywords Ketoreductase · Carbonyl reductase · *Pichia glucozyma* · Enantioselective reduction · Biocatalysis · Stereoselective

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Introduction

The use of ketoreductases (KREDs, often also described as carbonyl reductases (CR) or alcohol dehydrogenases (ADH)) is a well-recognised method for the enantioselective reduction of prochiral ketones into stereodefined chiral alcohols (Hall and Bommarius 2011; Hollmann et al. 2011). Reduction of aromatic ketones has been studied with different reductases, with particular focus on the reduction of sterically hindered (bulky) substrates, not easily recognised by most of the available KREDs (Lavandera et al. 2008). Commercially available or engineered KREDs can reduce structurally different (including bulky) ketones and their use has been also scaled-up to industrial processes (Huisman et al. 2010; Liang et al. 2010). Other alcohol dehydrogenases, such as the ones from *Rhodococcus ruber* (ADH-A; Stampfer et al. 2004), *Ralstonia* sp. (RasADH; Lavandera et al. 2008), and *Lactobacillus brevis* (LBADH; Haberland et al. 2002) have been identified as suitable biocatalysts for the reduction of different aromatic bulky substrates. Steric effects of the substrates on the activity of LBADH towards aromatic ketones (acetophenone derivatives) were accurately studied. In most cases, a tendency for higher activity with smaller substrates could be detected, but noticeable deviations from this general trend were observed due to stereoelectronic effects (Rodríguez et al. 2014). Understanding how substrate-binding residues of the protein affect substrate binding orientation and thus stereorecognition is necessary not only for predicting enantioselectivity, but also for designing rational or semirational mutations, aimed at increasing or changing the stereobias of a given reduction (Baerga-Ortiz et al. 2006; O'Hare et al. 2006). This approach was employed for mutating a carbonyl reductase from *Sporobolomyces salmonicolor* AKU4429 (SSCR) and switching its enantiopreference in the reduction of *para*-substituted acetophenones (Zhu et al. 2008). This SSCR

Published online: 16 September 2015

variant has been recently used for the enantioselective reduction of asymmetric benzophenone derivatives (Li et al. 2009).

Whole cells of the non-conventional yeast *Pichia glucozyma* CBS 5766 (now reclassified as *Ogataea glucozyma*) have been used for the reductive biotransformation of various aromatic ketones and ketoesters (including bulky ones), often showing interesting stereoselectivity (Forzato et al. 2001; Gandolfi et al. 2009; Rimoldi et al. 2011a; Husain et al. 2011; Rimoldi et al. 2011b; Contente et al. 2015). The reduction of these substrates frequently occurred with activity and enantioselectivity strongly dependent on the conditions of growth and biotransformation, suggesting the possible occurrence of different ketoreductases acting on aromatic ketones. Notably, whole cells of *P. glucozyma* carried out the selective monoreduction of different 1,2-diarylethanediones (benzils) (Hoyos et al. 2008; Fragnelli et al. 2012). Enzymes with benzil reductase activity, belonging to the short-chain dehydrogenase/reductase (SDR) family, have been found in bacteria (Maruyama et al. 2002; Pennacchio et al. 2013) and yeasts (Johanson et al. 2005). Here, we report the identification of a benzil reductase from *P. glucozyma* and a study of its activity/enantioselectivity towards different aromatic ketones using the recombinant protein (KRED1-Pglu) overexpressed in *Escherichia coli*.

Materials and methods

General

Phusion DNA polymerase was from New England Biolabs (EuroClone S.p.A., Pero (MI), Italy). pET26b(+) vector was from Novagen (Merck Millipore, Vimodrone (MI), Italy).

HIS-Select® Nickel Affinity Gel used for protein purification was purchased from Sigma Aldrich (Milano, Italy).

Pichia glucozyma CBS 5766 was from CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands). Stocks of the strain were stored at −20 °C in 15 % *v/v* glycerol and revitalized liquid YPD (yeast extract 5 g/L, peptone 5 g/L, glucose 20 g/L, pH 6.0) medium prior use. The bacterial strain *E. coli* XL1 blue was used for the propagation of the recombinant plasmid. *E. coli* BL21(DE3)Star (Merck Millipore, Vimodrone, Italy) was used for expression of KRED1-Pglu. Both were used according to the instruction of the suppliers.

All the DNA manipulations and bacterial transformations were carried out according to standard protocols (Sambrook and Russell 2001) or manufacturers' instructions, unless otherwise stated.

Protein concentration was determined with the Bradford method on a Pharmacia Biotech Ultrospec 1000 using bovine serum albumin as standard (Bradford 1976).

Production of the recombinant protein

Identification of KRED1-pglu gene

Genome of *P. glucozyma* CBS 5766 was sequenced by Baseclear (BaseClear B.V., Leiden, The Netherlands) using Illumina Casava pipeline version 1.8.0; assembly has been performed using the "de novo assembly" option of the CLC Genomics Workbench version 5.1. Annotation has been made on the assembled contig or scaffold sequences using the GAPS Annotation Pipeline from Progenus (Gembloux, Belgium). Upon analysis, 311 oxidoreductases, 517 transferases, 512 hydrolases, 71 lyases, 53 isomerases and 109 ligases were annotated and, among the oxidoreductases, a unique benzil reductase gene (named KRED1-Pglu) was found after BLAST analysis.

Cloning

KRED1-Pglu gene, coding for a putative benzil reductase, was amplified from the genomic DNA of *P. glucozyma* by PCR using the following primers, carrying *Nde*I and *Hin*dIII restriction sites: forward 5′-ATAC**CATATG**ACGAAGGTGA CTGTTGTGAC-3′; reverse 5′-AGAG**AAGCTT**GG CGTACTCCTTCAACTCTG-3′.

The amplified gene was then cloned into a pET26b(+) vector using the *Nde*I and *Hin*dIII restriction sites. With this cloning strategy, the resulting protein is expressed with an N-terminal His_6 tag. The correct construction of the expression plasmid was confirmed by direct sequencing.

Protein expression

Cultures of *E. coli* BL21(DE3)Star transformed with the resulting plasmid were grown overnight at 37 °C in LB medium supplemented with 25 μg/mL kanamycin. The seed culture was then diluted into a stirred bioreactor (Biolafitte & Moritz bioreactor, Pierre Guerin Technologies) containing 4 L of cultivation medium (Terrific Broth, 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 g/L glycerol, 2.3 g/L KH_2PO_4, 9.4 g/L K_2HPO_4, pH 7.2) to an initial OD_{600nm} of 0.05. Cultivation was carried out in batch-mode at 37 °C, 250 rpm stirring and 250 L/h aeration rate. Cells were grown until OD_{600nm} reached the value of 0.8. The cultures were induced for 20 h with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cells were then harvested by centrifugation at 4500 rpm for 30 min, washed once with 20 mM phosphate buffer at pH 7.0 and stored at −20 °C.

Protein purification

Cells were suspended in 50 mM Tris–HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 buffer. Proteins were extracted by sonication (5 cycles of 30 s each, in ice, with 1 min interval) and cell debris were harvested by centrifugation at 15,000 rpm for 30 min at 4 °C. The enzyme was purified by affinity chromatography with HIS-Select® Nickel Affinity Gel. Briefly, the column was equilibrated with 50 mM Tris–HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 and the crude extract loaded; column was then washed with 50 mM Tris–HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0; finally, the adsorbed enzyme was eluted with 50 mM Tris–HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0.

Pellets, crude extracts and collected fractions were analysed by SDS-PAGE. The fractions showing the presence of a band of the expected size (27 kDa) were pooled, dialysed against 50 mM Tris–HCl buffer pH 8.0 and stored at −20 °C. Typically, starting from 4 g of wet cell paste, it was possible to obtain 70 mg of pure protein.

Benzil-reductase activity test

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

Temperature and pH activity and stability studies

Temperature and pH optima were studied conducting the benzil-reductase test at temperatures between 15 and 50 °C and at pH values between 4 and 12 (using suitable buffers). To investigate its temperature-dependent stability, the enzyme was incubated at different temperatures (15, 28, 37, 50 and 60 °C) in 50 mM Tris–HCl buffer pH 8.0; residual activity was assessed conducting the benzil reductase test under reference conditions at different time points between 0 and 24 h of incubation. pH-dependent stability was investigated preincubating the enzyme in different buffers at pH between 5 and 11 at 25 °C. Residual activity was measured at pH 8.0, 25 °C, at different time points between 0 and 24 h, and compared to the value before incubation.

Substrate range

KRED1-Pglu substrate range was studied carrying out the reduction assay under reference conditions (0.25 mM NADPH, 50 mM Tris–HCl buffer pH 8.0, 25 °C), using 0.5 mM of each compound (in final 0.1 % DMSO); relative activities were expressed as per cent values versus benzil.

Determination of kinetic parameters

Kinetic parameters for the reduction of benzil were determined using the reduction assay (0.07 g/L of KRED1-Pglu, 0.25 mM NAD(P)H, 50 mM Tris–HCl buffer pH 8.0, 30 °C). Data were fitted to suitable kinetic models with KaleidaGraph software (Synergy Software Inc., USA); kinetic parameters were calculated with the same program.

Preparative reductions

Molar conversion and enantioselectivity towards different aromatic ketones and ketoesters were determined by performing biotransformations at 50-mg scale, using an enzyme-coupled system (glucose-glucose dehydrogenase (GDH) from *Bacillus megaterium*; Bechtold et al. 2012) for cofactor recycling. Reductions were carried out in 10-mL screw-capped test tubes with a reaction volume of 5 mL with KRED1-Pglu (20 mU/mL), GDH (1 U/mL), $NADP^+$ (0.1 mM), substrate (1 g/L), glucose (4× mmol of substrate) suspended in 50 mM Tris/HCl buffer pH 8.0. GDH activity and stability in our reaction system were checked before its use (data not shown).

Analyses ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are expressed in Hz.

HPLC analyses were performed with a Jasco Pu-980 equipped with a UV–vis detector Jasco UV-975. The following HPLC columns were used: LichroCART (250×4.6 mm), Chiralcel OD (250×4.6 mm, Daicel), Chiralcel OD-H (250×4.6 mm, Daicel), Chiralcel OJ (250×4.6 mm, Daicel), Lux cellulose 2 (250×4.6 Phenomenex) Lux cellulose 3 (250×4.6 Phenomenex). (*R*) and (*S*) isomers were identified by comparison of their retention times with those of synthetic standards.

Analytical data of product of biotransformations are as follows:

Benzoin (**2a**)

$[\alpha]_D^{25}$=+37.1° (*c* 0.8 Acetone) lit.[1] $[\alpha]_D^{25}$=+44.2° (*c* 1.0 acetone)

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.88–7.93 (m, 2H), 7.48–7.53 (m, 1H), 7.23–7.41 (m, 7H), 5.95 (d, J=6 Hz, 1H), 4.55 (d, J=6 Hz, 1H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 199.1, 139.2, 134.0, 133.7, 129.3, 129.2, 128.8, 128.7, 127.9, 76.4 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 90:10, 0.7 mL/

min, 254 nm): t_r (*S*)-benzoin 14.7 min, t_r (*R*)-benzoin 21.6 min).

Diphenylmethanol (**2b**)

^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.19 (d, J=4.0 Hz, 1H), 5.86 (d, J=4.0 Hz, 1H), 7.25–7.28, (m, 2H), 7.32–7.35 (m, 4H), 7.38–7.40 (m, 4H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 143.8 128.5, 127.6, 126.5, 76.2 ppm.

The molar conversion was determined by HPLC using LichroCART 250–4,6 MeOH/H_2O 65:35, 0.7 mL/min, 220 nm: t_r diphenylmethanol 12.5 min, t_r benzophenone 20.6 min.

Ethyl mandelate (**2d**)

$[\alpha]_D^{25}$=+88.7° (*c* 0.7 EtOH) lit.[2] $[\alpha]_D^{22}$=+97.33° (*c* 1.1 EtOH).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.35–7.42 (m, 5H), 5.15 (s, 1H), 4.15–4.25 (m, 2H), 1.20 (m, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 173.7, 138.5, 128.7, 128.5, 126.6, 73.0, 62.3, 14.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD-H column (n-hexane/*i*-PrOH 90:10, 1.0 mL/min, 210 nm): t_r (*S*)-ethyl mandelate 6.0 min, t_r (*R*)-ethyl mandelate 10.0 min).

1-Phenylethanol (**2e**)

$[\alpha]_D^{25}$=+40.0° (*c* 0.5 chloroform) lit.[3] $[\alpha]_D^{25}$=+36.0° (*c* 1.0 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.20–7.35 (m, 5H), 4.85 (q, J=6.4 Hz, 1H), 1.46 (d, J=6.4 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ 145.9, 128.5, 127.5, 125.4, 70.4, 25.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 95:5, 0.7 mL/min, 254 nm): t_r (*R*)-1-phenylethanol 14.2 min, t_r (*S*)-1-phenylethanol 18.0 min.

1-Phenylpropanol (**2f**)

$[\alpha]_D^{25}$=+20.5° (*c* 0.2 chloroform) lit.[4] $[\alpha]_D^{20}$=+22.2° (*c* 1.0 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.35–7.22 (m, 5H), 4.54 (t, J=6.5 Hz, 3H), 2.20 (br s, 1H, OH), 1.83–1.68 (m, 2H), 0.89 (t, J=7.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 144.6, 128.4, 127.5, 126.0, 76.0, 31.9, 10.1 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD-H column (n-hexane/*i*-PrOH 98:2, 1.0 mL/min, 254 nm): t_r (*R*)-1-phenylpropanol 16.5 min, t_r (*S*)-1-phenylpropanol 19.5 min.

1-Phenylbutanol (**2g**)

$[\alpha]_D^{25}$=−40.5° (*c* 0.2 chloroform) lit.[7] $[\alpha]_D^{25}$=−43.5° (*c* 1.0 chloroform).

^{1}H-NMR (300 MHz, CDCl3): δ 7.34–7.32 (m, 4H), 7.27–7.24 (m, 1H), 4.66 (t, J=6.5 Hz, 1H), 1.93 (br s, 1H, OH), 1.80–1.63 (m, 2H), 1.46–1.27 (m, 2H), 0.92 (t, J=7.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, CDCl3): δ 145.0, 128.4, 127.5, 125.9, 74.4, 41.2, 19.0, 13.9 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 99:1, 0.5 mL/min, 254 nm): t_r (*R*)-1-phenylbutanol 19.4 min, t_r (*S*)-1-phenylpbutanol 23.2 min.

1-Pyridin-3yl-ethanol (**2i**)

$[\alpha]_D^{25}$=−38.04° (*c* 0.4 ethanol) lit.[4] $[\alpha]_D^{20}$=−39.0° (*c* 0.93 ethanol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.46–8.33 (m, 2H), 7.75–7.70 (m, 1H), 7.24 (dd, J^1=8.0 Hz, J^2=5.0 Hz, 1H), 4.90 (q, J=6.5 Hz, 1H), 4.5 (br s, 1H, OH), 1.49 (d, J=6.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 148.0, 147.0, 141.8, 133.5, 123.5, 67.5 25.1 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 2 column (n-hexane/*i*-PrOH 90:10, 0.7 mL/min, 254 nm): t_r (*R*)-(1-pyridin-3yl)ethanol 20.46 min, t_r (*S*)-(1-pyridin-3yl)ethanol 25.30 min.

1-Pyridin-3yl-propanol (**2j**)

$[\alpha]_D^{25}$=−40.0° (*c* 0.2 methanol) lit.[6] $[\alpha]_D^{20}$=−41.4° (*c* 2.1 methanol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.31 (m, 1H), 8.25 (m, 1 H), 7.63 (m, 1 H), 7.15 (m, 1 H), 4.90 (s, 1 H), 4.51 (t, J=6.6 Hz, 1 H), 1.68 (m, 2 H), 0.82 (t, J=7.41 Hz, 3 H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$,): δ 147.9, 147.4, 140.6, 133.9, 123.3, 72.8, 31.9, 9.8 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 2 column (n-hexane/*i*-PrOH 90:10, 0.7 mL/min, 254 nm): t_r (*R*)-(1-pyridin-3yl)propanol 18.2 min, t_r (*S*)-(1-pyridin-3yl)propanol 21.1 min.

(1-Thiophen-2-yl)ethanol (**2k**)

$[\alpha]_D^{25}$=−20.80° (*c* 0.2 chloroform) lit.[4] $[\alpha]_D^{20}$=−23.2° (*c* 0.79 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25–7.18 (m, 1H), 6.95–6.92 (m, 2H), 5.07 (q, J=6.5 Hz, 1H), 2.54 (br s, 1H, OH), 1.56 (d, J=6.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 150.0, 126.6, 124.4, 123.2, 66.2, 25.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 3 column (n-hexane/*i*-PrOH 90:10, 0.5 mL/min, 254 nm): t_r (*S*)-(1-thiophen-2-yl)ethanol 13.5 min, t_r (*R*)-(1-thiophen-2-yl)ethanol 15.90 min.

(1-Furan-2-yl)ethanol (**2l**)

$[\alpha]_D^{25}$=−20.5° (*c* 0.2 chloroform) lit.[4] $[\alpha]_D^{20}$=−13.6° (*c* 0.55 chloroform).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.36 (dd, J^1=2.0 Hz,J^2=1.0 Hz, 1H,), 6.32 (dd, J^1=3.0 Hz,J^2=2.0 Hz, 1H,), 6.22 (d, J=3.0 Hz, 1H,), 4.86 (q, J=6.5 Hz, 1H,), 2.35 (br s, 1H, OH), 1.52 (d, J=6.5 Hz, 3H,) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ 157.7, 141.8, 110.1, 105.1, 63.6, 21.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 3 column (n-hexane/*i*-PrOH 98:2, 0.5 mL/min, 220 nm): t_r (*S*)-(1-furan-2-yl)ethanol 19.3 min, t_r (*R*)-(1-furan-2-yl)ethanol 21.3 min.

3-Hydroxy-3-phenylpropanenitrile (**2m**)

$[\alpha]_D^{25}$=−40.5° (*c* 0.2 ethanol) lit.[8] $[\alpha]_D^{20}$=−57.7° (*c* 2.6 ethanol).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.30–7.45(m, 5H), 5.1 (t, *J*=6.0,1H), 2.79 (d, *J*=6 Hz, 2H), 2.36 (br s, 1H, OH) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ 140.1, 128.9, 128.8, 125.5, 117.6, 70.2, 28.1.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OJ column (n-hexane/*i*-PrOH 90:10, 1 mL/min, 216 nm): t_r (*S*)-3-hydroxy-3-phenylpropanenitrile 21.4 min, t_r (*R*)-3-hydroxy-3-phenylpropanenitrile 25.0 min.

2-Hydroxy-1-phenylpropan-1-one (**2v**)

$[\alpha]_D^{25}$=−100° (*c* 0.2 chloroform) lit.[8] $[\alpha]_D^{27}$=−91.1° (*c* 0.3 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.87 (d, *J*=8.2 Hz, 2H), 7.57 (t, *J*=7.42 Hz, 1H), 7.46 (t, *J*=7.7 Hz, 2H), 5.17 (m, 1H), 3.5 (br s, 1H, OH), 1.45 (d, *J*=7.0 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ 202.3, 133.9, 133.4, 128.7, 128.6, 69.3, 22.2.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 95:5, 0.4 mL/min, 254 nm): t_r (*R*)-2-hydroxy-1-phenylpropan-1-one 17.2 min, t_r (*S*)-2-hydroxy-1-phenylpropan-1-one 21.9 min.

3-Hydroxy-1-phenyl-butan-1-one (**2w**)

$[\alpha]_D^{25}$=+54.2° (*c* 0.1 chloroform) lit.[10] $[\alpha]_D^{27}$=+67.5° (*c* 1.2 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.97 (dd, J^1=8.5, J^2= 2.0 Hz, 2H), 7.50 (m, 3H), 4.42 (m, 1H), 3.24 (br s, 1H, OH), 3.10 (d, *J*=6.0 Hz, 2H) 1.30 (d, *J*=6.4 Hz 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ 200.95, 136.8, 133.6, 128.8, 128.1, 64.1, 46.5, 22.5 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD-H column (n-hexane/*i*-PrOH 95:5, 0.8 mL/min, 254 nm): t_r (*R*)-3-hydroxy-1-phenyl-butan-1-one 12.1 min, t_r (*S*)-3-hydroxy-1-phenyl-butan-1-one 13.6 min.

Accession numbers

GenBank accession number of *KRED1-Pglu* gene is KR080472.

Results

Analysis of the genome of *Pichia glucozyma* CBS 5766 revealed the occurrence of a sequence (called KRED1-Pglu) with high homology with known benzil reductases. The corresponding His-tagged protein was successfully expressed in *E. coli*, yielding an active enzyme accounting for about 40 % of the total protein content of the cell extract.

Activity and enantioselectivity

The activity of KRED1-Pglu was first investigated for reduction of benzil, observing that the enzyme is NADPH-dependent. The enzyme exhibited the highest activity at pH between 7.0 and 8.0, being stable at pH between 6.0 and 8.0 (residual activity of 90 % and 80 %, respectively). KRED1-Pglu showed the highest activity between 30 °C and 35 °C and was quite stable up to a temperature of 30 °C, above which its activity started to decrease. As a compromise between activity and stability, the following studies have been carried out at 30 °C in 50 mM Tris/HCl buffer pH 8.0. Kinetic parameters for monoreduction of benzil by KRED1-Pglu were determined using 0.07 mg/ml of protein, NADPH as cofactor, at 30 °C and pH 8.0 (Table 1).

The initial rates of the biotransformation of different aromatic ketones and ketoesters were determined using a substrate-independent assay (Table 2, column 5); relative activities were expressed as per cent values versus benzil. Molar conversion and enantioselectivity were determined by performing the reduction of the substrates on a 50 mg scale, using an “enzyme-coupled” system (glucose/glucose dehydrogenase, GDH) (Table 2).

Preparative reduction of benzil (**1a**) resulted in total conversion into (*S*)-benzoin (ee >98 %) in 2 h; bulky benzophenone (**1b**) (which is transformed at low rates) was reduced to diphenylmethanol in 24 h with good yields (66 %), showing that a sluggish initial activity does not necessarily imply low conversion, as already observed for the reduction of aromatic ketones using LBADH (Rodríguez et al. 2014). Ethyl 2-oxo-2-phenylacetate (**1d**) was totally reduced within 30 min, albeit with mediocre *S*-enantioselectivity (ee 40 %). No consistent trend could be detected between bulkiness of the substrates and initial rates; for instance, the series **1e-h** showed an erratic tendency of activities with respect to the increasing bulkiness of the substrate, similar to what observed with LBADH (Rodríguez et al. 2014). KRED1-Pglu gave pronounced anti-Prelog stereorecognition in the reduction of acetophenone (**1e**) and propiophenone (**1f**) to yield the corresponding (*R*)-1-aryl-2-ethanol, whereas other aromatic ketones (**1g** and **1i-1m**) provided the corresponding (*S*)-alcohol (Prelog rule) with high enantioselectivity. This remarkable switch of enantioselectivity between similar substrates is not common among ketoreductases able to reduce aromatic ketones (Lavandera et al. 2008; Zhu et al. 2006). Finally, KRED1-Pglu showed no activity towards much hindered substrates like β-γ-ketoesters (**1n-1u**).

Table 1 Kinetic parameters for monoreduction of benzil by KRED1-Pglu

k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
0.14±0.02	0.64±0.10	0.22

Table 2 Investigation of the substrate range of KRED-Pglu concerning the reduction of different aromatic ketones and ketoesters

Entry	Substrate	Ar	R	Activity (%)[a]	Yield (%)	ee (%)	Time (h)[b]
1	**1a**	Phenyl	COPh	100	>95	>98 (*S*)	2
2	**1b**	Phenyl	Phenyl	15	66	–	24
3	**1c**	Phenyl	CH_2Ph	<5	<5	<5	24
4	**1d**	Phenyl	COOEt	115	>95	40 (*S*)	0.5
5	**1e**	Phenyl	CH_3	21	40	95 (*R*)	24
6	**1f**	Phenyl	CH_2CH_3	25	60	97 (*R*)	24
7	**1g**	Phenyl	$CH_2CH_2CH_3$	18	56	97 (*S*)	24
8	**1h**	Phenyl	$CH_2CH_2CH_2CH_3$	<5	<5	<5	24
9	**1i**	3-Pyridyl	CH_3	58	>95	>98 (*S*)	24
10	**1j**	3-Pyridyl	CH_2CH_3	60	>95	>98 (*S*)	24
11	**1k**	2-Thienyl	CH_3	51	>95	>98 (*S*)	24
12	**1l**	2-Furanyl	CH_3	48	>95	>98 (*S*)	24
13	**1m**	Phenyl	CH_2CN	55	>95	95 (*S*)	24
14	**1n**	Phenyl	CH_2COOEt	<5	<5	<5	24
15	**1o**	Phenyl	$CH_2COOtBu$	<5	<5	<5	24
16	**1p**	Phenyl	$CH(CH_3)COOEt$	<5	<5	<5	24
17	**1q**	Phenyl	$CH(CH_3)COOtBu$	<5	<5	<5	24
18	**1r**	Phenyl	CH_2CH_2COOEt	<5	<5	<5	24
19	**1s**	Phenyl	$CH_2CH_2COOtBu$	<5	<5	<5	24
20	**1t**	Phenyl	$CH_2CH(CH_3)COOEt$	<5	<5	<5	24
21	**1u**	Phenyl	$CH_2CH(CH_3)COOtBu$	<5	<5	<5	24

[a] Relative activities measured using the benzil-reductase activity test and expressed as percent values referred to benzil

[b] Referred to the maximum yield

1-Phenylpropane-1,2-dione (**1v**) and 1-phenylbutane-1,3-dione (**1w**) were also tested; both diketones were exclusively reduced at the less hindered carbonyl group with formation of (*S*)-2-hydroxy-1-phenylpropan-1-one (**2v**) and (*S*)-3-hydroxy-1-phenylbutan-1-one (**2w**) as the only reaction products, with complete enantioselectivity (Fig. 1).

The observed activities and enantioselectivity cannot be explained by simply assuming a model of the active site with two hydrophobic pockets differing in size, where the large pocket binds the phenyl moiety, while the small one defines whether the other substituent can be properly accommodated and thus controls reaction rates and enantioselectivity. Since the experimental results were unpredictable on the basis of just the steric properties of the substrates, a molecular modelling study was carried out for understanding the structural determinants for the observed stereorecognition.

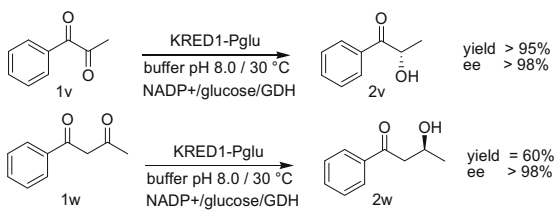

Fig. 1 Reduction of 1-phenylpropane-1,2-dione (**1v**) and 1-phenylbutane-1,3-dione (**1w**) with KRED1-Pglu

As the starting point for homology modelling, the PDB database (http://www.rcsb.org/pdb/) was scanned with BLAST against the target sequence of the KRED1-Pglu while setting the maximal E-threshold=100; the search retrieved several items. The top-scoring identified template, an uncharacterized oxidoreductase YIR035C from *Saccharomyces cerevisiae* (UniProt entry P40579), and the target share 46 % identity; YIR035C has been crystallized in its *apo* form (X-ray structure as PDB entry 3KZV). The two sequences were automatically aligned and manually adjusted, and a model for the target protein was built by comparative modelling. The structure so obtained did not allow, however, for any effective docking of NAD(P)H to it. The second best match, *cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase from *Comamonas testosteroni* (UniProt entry Q46381), shares 31 % identity with the target and has been crystallized in a complex with NAD (PDB entry 2Y99). After alignment of the sequences with *T-coffee* and superposition of the cognate structures, NAD was imported from the template to the model and the complex was energy-minimized, which resulted in minor RMSD overall and a negligible displacement of the amino acids predicted to be involved in the catalytic process (annotations to UniProt entry P40579). However, once more,

no effective docking of the test substrates could be obtained to the model•NAD complex.

Various PDB entries of *holo* enzymes, crystallized as to contain both NAD and a substrate/substrate analog/inhibitor (2JAP, 1NAS, 4NBU), were thus inspected from the previous BLAST output; clavulanic acid dehydrogenase from *Streptomyces clavuligerus* (UniProt entry Q9LCV7; PDB entry 2JAP; 30 % identity with the target) was selected as further template because of the comparable size of its substrate to our test substrates. After superposition of the structures, substrate clavulanic acid was imported from 2JAP to our model•NAD. Effective docking was possible to the ternary complex model•NAD•clavulanic acid after energy minimization; we will refer to the outcome of this procedure as KRED1-Pglu model.

Docking to KRED1-Pglu model was carried out using different *protocols* (rigid receptor, induced fit), various *forcefields* (MMF94x, Amber12:EHT) and different *electrostatics setups* (distance-dependent dielectric, reaction field); comparing the position of the redox centre in the top scoring poses for the test substrates in each procedure with that of the redox centre of clavulanic acid taken as reference suggested that the combination: *rigid receptor*, *Amber12:EHT*, and *distance-dependent dielectric* performs best. This protocol was then used to dock to the KRED1-Pglu model all selected substrates.

The procedures of molecular modelling provided insight into the ways of enzyme-substrate interaction and allowed computing the affinities (dissociation constant values, K_ds) for different substrates, with an accuracy within one order of magnitude, as discussed by Eberini et al. (2006). Table 3 lists the in silico affinities of the enzyme for selected substrates alongside the in vitro activities (same as in Table 2). Binding of a substrate to the enzyme active site is necessary, but not sufficient for catalysis to take place: once this prerequisite is met, the rate of the process is controlled by the stereoelectronic property of the substrate, a feature not directly addressed by the classical docking procedure. Within these boundaries, the relative order of the computational data is in agreement with the experimental results and the enzyme is most active on benzil (entry 1), the substrate with the highest affinity for the catalytic site.

When the series of aryl aliphatic ketones is docked into the enzyme active site, the orientation of the top-scoring placements changes depending on the length of the aliphatic chain, which defines the stereochemistry of the reductive reaction. Figure 2 shows the relative placement of acetophenone (substrate **1e**; colour = green) and butyrophenone (substrate **1h**; colour = yellow), and their position with respect to the reductive hydride (arrow) of NAD(P)H (CPK colours). Lengthening the aliphatic chain produces steric clashes with amino acids in the enzyme catalytic site (specifically, W147 and L208) and results in an inversion of the predominant orientation during the interaction. As a result, the stereochemistry predicted for the stereocenter of the alcohol products is different in either case and identical to what is obtained in vitro.

Table 3 In silico affinities of KRED-Pglu for selected substrates

Entry	Substrate	Ar	R	Activity (%)	K_i
1	**1a**	Phenyl	COPh	100	7.94×10^{-6}
2	**1b**	Phenyl	Phenyl	5	1.21×10^{-5}
5	**1e**	Phenyl	CH_3	11	6.15×10^{-5}
6	**1f**	Phenyl	CH_2CH_3	35	3.36×10^{-5}
7	**1g**	Phenyl	$CH_2CH_2CH_3$	10	2.32×10^{-5}
8	**1h**	Phenyl	$CH_2CH_2CH_2CH_3$	<5	1.38×10^{-5}

Discussion

Ketoreductases (KREDs) stereoselectively reduce prochiral ketones giving access to optically pure secondary alcohols (Hall and Bommarius 2011; Hollmann et al. 2011). Bulky aromatic ketones can be reduced by specialised KREDs (Haberland et al. 2002; Stampfer et al. 2004; Lavandera et al. 2008; Huisman et al. 2009). Benzil reductases (Maruyama et al. 2002; Johanson et al. 2005; Pennacchio et al. 2013) are specific KREDs with high affinity towards very hindered 1,2-diphenyl-ethanediones. Sequence comparison among the benzil reductases from bacteria, yeasts and mammals showed that conserved amino acid residues are essential for benzil reductase activity and can be useful for fishing out their genes from genomes. Whole cells of wild-type *Pichia glucozyma* CBS 5766 have been used for a number of reductions of aromatic ketones, including symmetric and asymmetric 1,2-diaryl-ethanediones (benzils); the genome of *P. glucozyma* CBS 5766 was annotated and a putative gene for benzil reductase was identified based on sequence homology with known benzil reductases and cloned in *E. coli*. The His-tagged protein (named as KRED1-Pglu) was purified and used for the reduction of different aromatic ketones; the enzyme showed a marked preference for NADPH and was used on

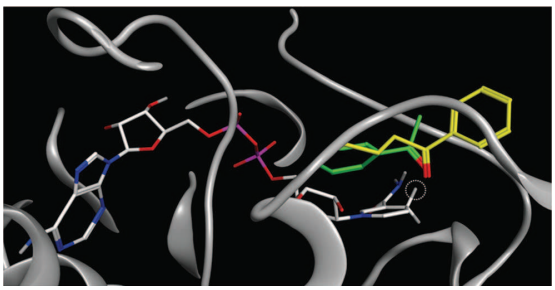

Fig. 2 Close-up to the catalytic site in the model of KRED1-Pglu. NADH is rendered with CPK colors; the hydride atom involved in the reduction process is marked with a dotted circle. Two substrates, acetophenone (**1e**) and butyrophenone (**1 h**) are rendered in green and yellow. Their orientation in the enzyme pocket is opposite, leading to inverted configuration in the alcohol products

preparative scale coupled with a glucose dehydrogenase (GDH) for cofactor recycling. Benzil was reduced giving (*S*)-benzoin as the sole observable product, whereas the use of whole cells yielded lower enantiomeric excesses and further reduction to hydrobenzoin (Fragnelli et al. 2012), indicating the occurrence in *P. glucozyma* of other KR(s) active not only on benzil but also on benzoin. Very hindered benzophenone was also reduced, as previously observed with selected KRs (Truppo et al. 2007). Aromatic ketoesters were poorly reduced by KRED1-Pglu, whereas whole cells of *P. glucozyma* CBS 5766 were very effective and sometimes highly enantioselective on these substrates, indicating the occurrence of other enantioselective KRs in this unconventional yeast (Contente et al. 2014). Acetophenone and propiophenone were reduced to the corresponding (*R*)-alcohols (anti-Prelog's rule), whereas butyrophenone was converted into the corresponding alcohol with different stereobias, giving (*S*)-phenylbutanol with high enantioselectivity. This shift of stereopreference was explained by modelling the interaction between substrate and protein and observing that the length of the aliphatic chain governs the stereochemistry of the biotransformation, determining which prostereogenic face of the ketone can be favourably attacked by the hydride. The key role of the aliphatic chain can also be seen in the case of valerophenone, where the longer chain determines steric clashes with amino acids in the enzyme catalytic site and the ketone is not reduced. KRED1-Pglu was also employed for the reduction of 1-phenylpropane-1,2-dione and 1-phenylbutane-1,3-dione leading to the enantioselective formation of the α-methyl alcohols as the only product, suggesting that KRED1-Pglu has potential as catalyst for the preparation of hydroxyketones with high chemical and optical purity.

In conclusion, a new (bio)catalyst for the preparation of bulky aromatic chiral alcohols and hydroxyketones with high enantioselectivity by reduction of the corresponding ketones has been identified and produced from an unconventional yeast. The different and erratic stereopreferences towards simple aromatic ketones were explained on the basis of modelling studies. Further studies could be useful/help to identify, generate and analyse KRED1-Pglu mutants, in which the stereoselectivity of the reductive reaction can be carefully tuned in order to customize and scale-up specific reactions with industrial relevance.

Acknowledgments Recombinant GDH from *Bacillus megaterium* was kindly provided by Prof. Daniela Monti (Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco 9, 20131 Milano, Italy).

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All the authors declare they have no conflict of interest.

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Enzymatic reduction of acetophenone derivatives: electronic and steric effects on activity and enantioselectivity

6.1 Correlation between structure and activity

The ketoreductase KRED1-Pglu from *P. glucozyma* was used for the enantioselective reduction of different mono-substituted acetophenones in order to study the correlation between reaction rates and electronic effects described by σ-Hammett coefficients. In fact, it is known that some ketoreductases show a quite broad substrate acceptance which is often limited by the electronic structure and polarization as well as by size and chirality of the substrates.[93] In particular, the biocatalytic reduction of acetophenones is influenced by the different substituents of the aromatic ring.[94]

The effects of ring substituents on electrophilic aromatic substitution (S_NAr) have been studied and quantified for the first time in the 1930 by Hammett[95] which correlated the electronic effects of the substituents and the consequent ionization constant of substituted benzoic acids with reaction rate in S_NAr.[96] This model was subsequently expanded in order to predict different chemical and biochemical reactions, moreover the new equation provides σ Hammett coefficients for various substrate structures.[97]

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6.2 Hammet equation

The Hammett equation relates the electronic and steric effects of functional groups present on a molecule with the reactivity of the same molecule. It allows to estimate the effects of various substituents able to change the electronic distribution both with inductive and mesomeric effects on the reactivity of a molecule.

$$\text{Log } K = \rho\sigma$$

Fig. 44 Hammet equation

K is the equilibrium constant, while ρ and σ indicate respectively the reaction constant (depending on the type of reaction but not on the substituent) and the substituent constant, a measure of the total electronic effects exerted by the substituents on the reaction centre, σ is a positive value for electron withdrawing groups and negative for electron donor groups.

Although it has been calculated for the dissociation reaction of different benzoic acids, the σ value is not dependent on the type of reaction and therefore can be used to estimate the influence of the introduction of a substituent on any type of reactions.

The reaction constant ρ is the slope of the line correlating Log *K* with the σ values of the substituents. The sign of the slope tells if a reaction rate is enhanced or suppressed by electron donating versus electron withdrawing groups. Whereas negative ρ is diagnostic for the presence of positive charge at the reactive centre in the transition state,

the meaning of positive ρ is the development of a negative charge during the transition state. In the first case the reaction rate is suppressed by electron withdrawing substituents, while in the second case the reaction rate is enhanced by electron withdrawing substituents. The magnitude of ρ describes the susceptibility of the reaction to the electronic features of the substituents.

In our study the σ Hammett parameters were used to assess the influence of the nature of *para*- and *meta*-substituents on the kinetic of the hydride transfer to acetophenone. σ Hammett coefficients were in excellent correlation with reaction rates of *para*-acetophenones (R^2= 0.934), showing that the activity of KRED1-Pglu was predominantly dependent on electronic properties, while substrate size and/or hydrophobicity had negligible effects (ρ > 0) as a consequence of the negative charge in the transition state caused by the attack of the hydride on the carbonyl group.

The collected data about *meta*-derivatives showed a co-occurrence of steric and electronic effects in fact a low correlation between reaction rates and σ Hammett coefficients (R^2= 0.721) was observed. This was confirmed by the reduction of *ortho*-derivatives: only the substrates bearing substituents with low steric impact (F and CN) were reduced by KRED1-Pglu. *(Data not published)*.

Enzymatic reduction of acetophenone derivatives with a benzil reductase from *Pichia glucozyma* (KRED1-Pglu): electronic and steric effects on activity and enantioselectivity

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A ketoreductase from *Pichia glucozyma* (KRED1-Pglu) was employed for the enantioselective reduction of various mono-substituted acetophenones. Reaction rates of the reduction of *meta*- and *para*-derivatives were mostly consistent with the electronic effects described by σ-Hammett coefficients; Only o*rtho*-substituted acetophenones with low steric impact (i.e., F and CN substituted) showed activity.

Introduction

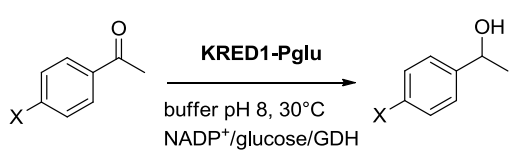
Substituted 1-phenylethanols comprise an important structural class of compounds for synthetic purposes, and their obtainment by enantioselective reduction of the corresponding acetophenones has been thoroughly studied using chemocatalysis [1] and biocatalysis [2, 3]. Various microbial alcohol dehydrogenases (ADHs), carbonyl reductases (CRs), and ketoreductases (KRs) are able to reduce acetophenone derivatives giving access to both the enantiomers of the corresponding 1-phenylethanols [4]. An ADH from *Rhodococcus erythropolis* proved efficient in the reduction of a number of mono-, di- and tri-substituted

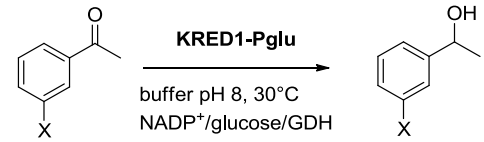
acetophenones, giving always the corresponding (*S*)-1-phenylethanols (Prelog rule) [5]; the same stereopreference was observed with others ADHs, i.e., from *Ralstonia* sp. [6] and from *Thermoanaerobacter* sp. [7]. A general preference for Prelog rule was also detected in a screening with 24 recombinant KRs tested on thirteen mono-susbstituted acetophenones [8]. Conversely, optically pure (*R*)-1-phenylethanol can be prepared by reduction of (unsubstituted) acetophenone using specific enzymes, such as LK-ADH from *Lactobacillus kefir* [9], PF-ADH from *Pseudomonas fluorescens* [10] and carbonyl reductase from *Candida magnoliae* (CMCR) [11]. The effect of ring-substituents on catalytic efficiency of a number of mono-substituted acetophenone derivatives was studied using *Candida tenuis* xylose reductase (CtXR), which showed, in all the cases, preference for the formation of (*S*)-1-phenylethanols [12]. For this enzyme, σ-Hammett coefficients of *para*- and *meta*-substituents were in good correlation with the reaction velocities, indicating again that enzyme-substrate binding has a subordinated influence on the CtXR catalytic efficiency on these substrates; interestingly, *ortho*-derivatives were sometimes reduced with higher rates, indicating a beneficial mode of binding, in spite of steric effects. An interesting effect of the nature of the *para* -substituents was observed with ADH from *Lactobacillus kefir* and *Thermoanaerobacter* sp., where acetophenones with neutral *para* -substituents were efficiently and selectively reduced, whereas those with ionizable substituents could not be reduced [13]. Whole cells of the non-conventional yeast *Pichia glucozyma* enantioselectively reduced different aromatic ketones [14-16], including acetophenone, with a marked preference for the formation of (*S*)-1-phenylethanol [17, 18]; interestingly, a benzil ketoreductase (KRED1-Pglu) isolated from the same yeast reduced acetophenone as recombinant protein with opposite enantioselectivity [19]. In this study we have investigated the reduction of different mono-substituted acetophenones using KRED1-Pglu for evaluating the potential of this new KR as enantioselective biocatalyst and understanding the correlation between enzymatic activity and the stereoelectronic features of the substrates.

Results and discussion

The recombinant benzil ketoreductase KRED1-Pglu [19] was initially investigated using different *para*-substituted acetophenone derivatives (**1a**-**1c** and **1e**-**1h**); acetophenone (**1d**) was used for comparative purposes (Table 1). The substrates were transformed with very different rates, being 4'-aminoacetophenone **1a** (entry 1, Table 1) the only substrate not accepted. Reduction followed Prelog rule, giving the corresponding (*S*)-1-phenylethanols; most of the biotransformations occurred with high enantioselectivity, with the exception of 4'-methoxyacetophenone **1b** (entry 2, Table 1), which was reduced with only 60% ee. The observed stereopreference is opposed to the one obtained with (unsubstituted) acetophenone **1d**, which furnished (*R*)-1-phenylethanol **2d** with 95% ee.

KRED1-Pglu was also used for the reduction of a set of *meta*-substituted acetophenones (Table 2), having the same substituents of the *para*-acetophenones previously tested (**1i-1o**). In addition, in this case, the substituent affected the reaction rate and only poor reaction could be observed with the amino derivative **1i**. All the *meta*-derivatives were reduced to the corresponding (*S*)-1-phenylethanols with high-to-excellent enantioselectivity. Notably, 3'-methoxyacetophenone **1j** was reduced to optically pure (*S*)-1-(3-methoxyphenyl)ethanol **2j**, whereas the corresponding *para*-derivative **1b** gave moderate enantioselectivity.





Entry	Substrate	X	Rel. Rate (%)[a]	Yield (%)[b]	ee (%)
1	**1a**	NH_2	0	0	-
2	**1b**	OCH_3	16	20	60 *S*
3	**1c**	CH_3	23	30	> 98 *S*
4	**1d**	H	22	40	95 *R*
5	**1e**	F	35	40	93 *S*
6	**1f**	CF_3	51	42	95 *S*
7	**1g**	CN	68	70	97 *S*
8	**1h**	NO_2	100	100	> 98 *S*

Table 1

Entry	Substrate	X	Rel. rate (%)[a]	Yield (%)[b]	ee (%)
1	**1i**	NH_2	7	10	-
2	**1j**	OCH_3	30	67	> 98 *S*
3	**1k**	CH_3	15	18	> 98 *S*
4	**1d**	H	22	40	95 *R*
5	**1l**	F	61	85	> 98 *S*
6	**1m**	CF_3	31	25	94 *S*
7	**1n**	CN	46	49	95 *S*
8	**1o**	NO_2	53	78	> 98 *S*

Table 2

[a]Inizial rates were calculated from initial progress curves 100% corresponds to the activity of the highest reacting substrate (**1h**).

[b]Molar conversion after 24h

The σ-Hammett coefficients were used to evaluate the influence of the nature of the *para* and *meta* - substituents on the kinetics of the hydride transfer to acetophenone. The initial rates were plotted against the σ-values of the substituents; σ_{para} and σ_{meta} were independently correlated (Figure 1).

σ_{para}-Hammett coefficients were in excellent correlation with the reaction rates, showing that for *para*-substituents, the activity of KRED1-Pglu was mostly dependant on electronic properties, and substrate size and/or hydrophobicity had

negligible effects; as expected, since the attack of the hydride on the carbonyl cause a negative charge in the transition state, the reaction rate is consequently accelerated by electron withdrawing groups ($\rho > 0$). Electronic charge density distribution and C=O length have been previously used as parameters for correlating the activity of ADHs on different mono-substituted acetophenones [12, 13]. Hence, we correlated the activity of KRED1-Pglu with the electronic charge distribution on the carbonyl (Figure 2) and with C=O length (Figure 3); electronic charge on the C=O carbon and C=O lengths were calculated in the gas phase through the MOE SCF Calculation interface to the GAMESS software *[20]*. The DFT calculations were based on the B3LYP hybrid functional with the 6-31G* basis *[21]*. A high correlation between activity and electronic features of the substituents was found with *para*-derivatives, whereas a much lower correlation could be detected with *meta*-derivatives as detected with other enzymes *[12, 31*]. This behaviour is different from the one observed with ADHs from *Lactobacillus brevis* and *Thermoanaerobacter* sp., where the electron withdrawing or donating properties of the substituents did not exert a significant effect [13]. All these collected data indicate that *para* derivatives are reduced with reaction rates not dependent on steric interactions of the substrates with the enzyme, whereas steric effects have a (minor) impact on the reactivity of *meta* derivatives. The co-occurrence of steric effects for *meta*-derivatives is confirmed by the observation that 3'-fluoroacetophenone **1l** (bearing the less sterically hindered substituent still being an electron withdrawing group) was the preferred substrate.

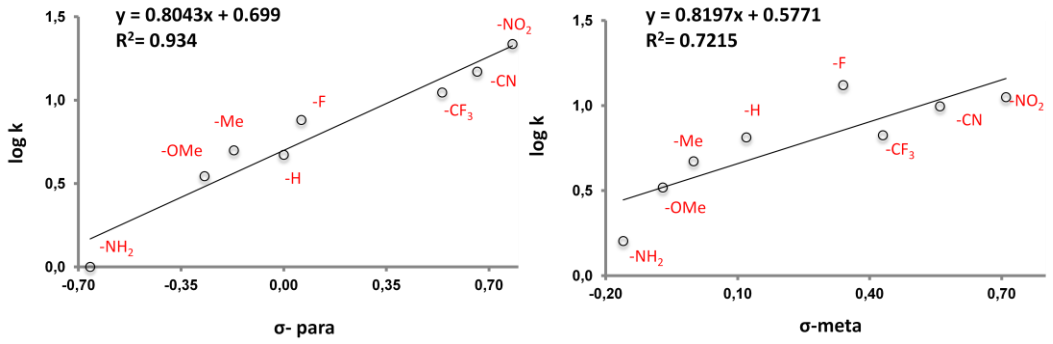


Figure 1 Correlation between activity and the Hammett σ-values.

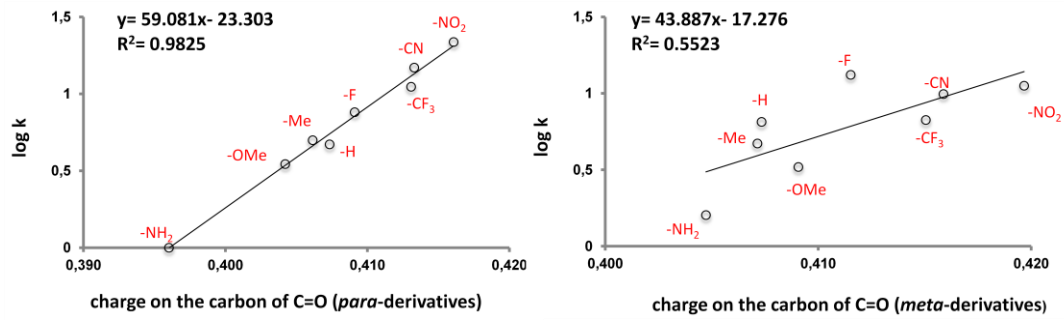


Figure 2 Correlation between activity and charge distribution on the C=O Carbon.

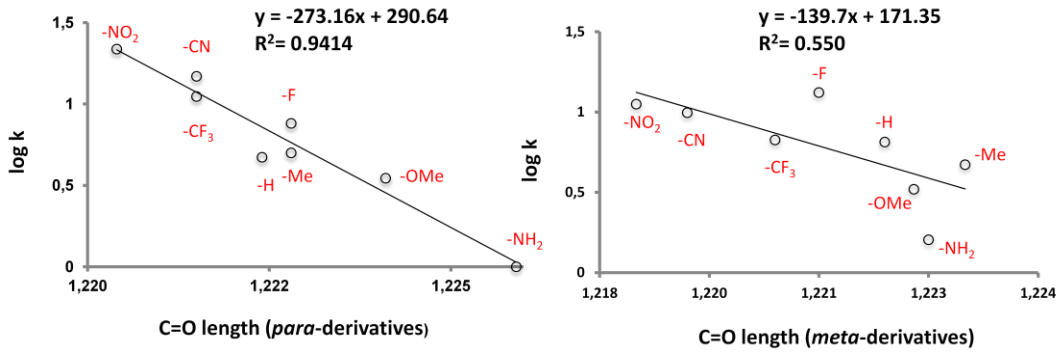
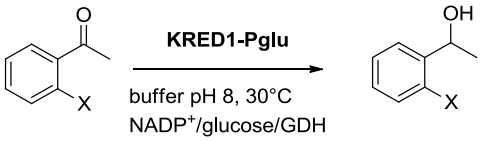


Figure 3 Correlation between activity and C=O length.

Ortho-derivatives were finally assayed (Table 3). KRED1-Pglu showed activity only towards 2'-fluoroacetophenone **1s** and 2'-cyanoacetophenone **1u**, the substrates bearing electron withdrawing with the lower steric impact. *Ortho*-substituents introduce significant steric effects in the reactivity of acetophenone derivatives, and therefore a simple Hammett's approach to quantify the substituent effects on a reaction center cannot be applied. Other enzymes, such as *Candida tenuis* xylose reductase [12], reduce *ortho*-substituted acetophenones better than *para*- and *meta*-derivatives, pointing out the crucial role of the binding mode between enzyme and substrate which is still in progress.



Entry	Substrate	X	Rel. rate (%)[a]	Yield (%)[b]	ee (%)
1	**1p**	NH_2	0	-	-
2	**1q**	OCH_3	0	-	-
3	**1r**	CH_3	0	-	-
4	**1d**	H	22	40	95 *R*
5	**1s**	F	76	97	95 *S*
6	**1t**	CF_3	0	-	-
7	**1u**	CN	82	98	> 98 *S*
8	**1v**	NO_2	0	-	-

Table 3

[a]Inizial rates were calculated from initial progress curves 100% corresponds to the activity of the highest reacting substrate (1h). [b]Molar conversion after 24h

In conclusion, we showed that benzil reductase from Pichia glucozyma (KRED1-Pglu) is an efficient biocatalyst for the enantioselective reduction of various mono-sobstituted acetophenones. Reaction rates of *meta*- and *para*-derivatives were consistent with the electronic effects described by σ-Hammett coefficients; on the contrary, molecular modelling studies to understand the enantioselectivity determined by binding mode between enzyme and substrate, are still in progress. Reduction of *ortho*-derivatives occurred only with the substrates bearing substituent with low steric impact (i.e. F, CN).

Supporting Information for

Enzymatic reduction of acetophenone derivatives with a benzil reductase from *Pichia glucozyma* (KRED1-Pglu): electronic and steric effects on activity and enantioselectivity

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Contents:

Materials

All reagents and solvents were obtained from Sigma–Aldrich–Fluka and used without further purification or drying. TLC was performed with Merck silica gel 60 F_{254} pre-coated plates. Silica gel column chromatography was carried out on silica gel 60 M (40–63 mm). Recombinant KRED1-Pglu was produced in *Escherichia coli* and purified to apparent

homogeneity as described earlier [19]. Recombinant glucose dehydrogenase (GDH) was a kind gift from Dr. Daniela Monti. For each compound the alcohol racemic mixture was obtained by $NaBH_4$ reduction (0.25 x mMol of substrate).

Analyticals

^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Mercury 300 (300 MHz) or 500 (500 mHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) are expressed in Hz. GC analyses were performed with a Dani 6500 gascromatograph: gas carrier H_2 (0.6 bar, T=100°C), Detector FID (Flame Ionization Detector), T_{max} = 300°C. Chiral capillary GC columns used: DMePeBeta-CDX-PS086 (diameter 0.25 mm, length 25 m, thickness 0.25 μ, MEGA) and Mega-Dex DET beta (diameter 0.25 mm, length 25 m, thickness 0.25 μ, MEGA). HPLC analyses were performed with a Jasco Pu-980 equipped with a UV-vis detector Jasco UV-975. Chiral HPLC columns used: Chiralcel OD-H (250 x 4 mm, Daicel), Chiralcel OD (250 x 4 mm, Daicel), Lux cellulose-2 column (4.6 x 150 mm, Phenomenex) and Lux cellulose-3 column (4.6 x 150 mm, Phenomenex). Rotary power determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat.

Enzymatic activity

Enzymatic activity was measured spectrophotometrically at 340 nm by determining the consumption of NADPH during the reduction of acetophenones at 22°C in a half-micro cuvette (total volume 1 mL) for 5 min. The cuvette contained the ketone (0.47 mM) dissolved in DMSO (final concentration 0.1%) and NADPH (0.25 mM) in Tris-HCl 50 mM pH 8.0 buffer (up to 1 mL). The reaction was initiated by the addition of KRED1-Pglu (0.07mg). The reaction rates were calculated from measurements determined in triplicate. One unit (U) of activity is defined as the amount of enzyme which catalyses the consumption of 1 μmol of NADPH per minute. Relative rates were calculated taking into account the maximal activity registered (towards 4'-nitroacetophenone).

Preparative reduction of acetophenones

General procedure for the ketone reduction using a NADPH recycle system was as follows: reductions were carried out in 10 mL screw-capped test tubes with a reaction volume of 5 mL with KRED1-Pglu (20 mU/mL), GDH (1 U/mL), $NADP^+$ (0.1 mM), substrate (1 g/L), glucose (4 x mMol of substrate) suspended in 50 mM Tris/HCl buffer pH 8.0. The samples were extracted with ethyl acetate (3 x 5 mL). The organic extracts were dried over anhydrous sodium sulfate and subjected to chiral GC or HPLC analysis.

Characterization of the products

(*S*)-**2b** (1-(4'-methoxyphenyl)ethanol): $[\alpha]_D^{25}$ = - 25.0° (*c* 0.2 chloroform) lit.[22] $[\alpha]_D^{25}$ = - 51.9° (*c* 0.718 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.25-7.30 (m, 2H), 6.83-6.87 (m, 2H), 4.82 (q, *J* = 6.5 Hz, 1H), 3.78 (s, 3H), 1.44 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 158.5, 138.0, 126.4, 114.0, 69.7, 55.1, 25.0 ppm. The enantiomeric excess (% *ee*) was determined by HPLC using Chiralcel OD-H column (n-hexanes/*i*-PrOH 99:1, 1 mL/min, 210 nm): t_r (*R*)-1-(4'-methoxyphenyl)ethanol min, t_r (*S*)-1-(4'-methoxyphenyl)ethanol 22.6 min.

(*S*)-**2c** (1-(4'-tolyl)ethanol): $[\alpha]_D^{20}$ = - 51.4.0° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 53.4° (*c* 0.85 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.20-7.24 (m, 2H), 7.10-7.15 (m, 2H) 4.8 (q, *J* = 6.5 Hz, 1H), 2.32 (s, 3H), 1.45 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 143.0, 136.5, 129.1, 128.3, 124.7, 68.6, 25.1, 21.0 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(4-tolyl)ethanol 14.1 min, t_r (*R*)-1-(4-tolyl)ethanol 14.6 min.

(*R*)-**2d** (1-phenylethanol): $[\alpha]_D^{25}$ = + 40.0° (*c* 0.5 chloroform) lit.[24] $[\alpha]_D^{25}$ = + 36.0° (*c* 1.0 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.20–7.35 (m, 5H), 4.85 (q, *J* = 6.4 Hz, 1H), 1.46 (d, *J* = 6.4 Hz, 3H) ppm.^{13}C-NMR (75 MHz, $CDCl_3$): δ = 145.9, 128.5, 127.5, 125.4, 70.4,

25.2 ppm. The enantiomeric excess (% *ee*) was determined by HPLC using Chiralcel OD column (n-hexanes/*i*-PrOH 95:5, 0.7 mL/min, 254 nm): t_r (*R*)-1-phenylethanol 14.2 min, t_r (*S*)- 1-phenylethanol 18.0 min.

(*S*)-**2e** (1-(4'-fluorophenyl)ethanol): $[\alpha]_D^{20}$ = - 78.6.0° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 81.5° (*c* 1.58 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.30-7.35 (m, 2H), 7.00-7.05 (m, 2H) 4.85 (q, *J* = 6.5 Hz, 1H), 1.45 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 162.0 (d, *J* = 242.5 Hz), 140.8 (d, *J* = 2.5Hz), 126.8 (d, *J* = 8.8 Hz), 114.4 (d, *J* = 20.0 Hz, Ar-C), 70.0 (CH), 25.2 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(4-Fluorophenyl)ethanol 12.9 min, t_r (*R*)-1-(4-Fluorophenyl)ethanol 13.5 min.

(*S*)-**2f** (1-(4'-trifluoromethylphenyl)ethanol): $[\alpha]_D^{20}$ = - 40.50° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 32.0° (*c* 0.86 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.58 (d, *J* = 8.0 Hz, 2H,), 7.45 (d, *J* = 8.0 Hz, 2H,), 4.95 (q, *J* = 6.5 Hz, 1H,), 1.46 (d, *J* = 6.5 Hz, 3H,) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 148.9, 129.5 (q, *J* = 32.0 Hz), 124.3, 125.4 (q, *J* = 3.9 Hz), 123.1, 70.0 (CH), 25.2 ppm. The enantiomeric excess (% *ee*) was determined by HPLC using Chiralcel OD-H column (*n*-hexane/EtOH 98:2, 0.8 mL/min, 220 nm): t_r (*S*)-1-(4-trifluoromethylphenyl)ethanol 16.5 min, t_r (*R*)-1-(4-trifluoromethylphenyl)ethanol 17.25 min.

(*S*)-**2g** (1-(4'-cyanophenyl)ethanol): $[\alpha]_D^{20}$ = - 45.2° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 46.4° (*c* 2.40 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.61 (d, *J* = 8.5 Hz, 2H,),7.50 (d, *J* = 8.0 Hz, 2H,), 4.98 (q, *J* = 6.5 Hz, 1H,), 1.48 (d, *J* = 6.5 Hz, 3H,) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 151.5, 133.4, 126.1, 119.0, 111.0, 69.5, 25.2 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(4-cyanophenyl)ethanol 23.7 min, t_r (*R*)-1-(4-cyanophenyl)ethanol 24.0 min.

(*S*)-**2h** (1-(4'nitrophenyl)ethanol): $[\alpha]_D^{25}$ = - 25.0° (*c* 0.2 chloroform) lit.[25] $[\alpha]_D^{20}$ = - 22.6° (*c* 1.0 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 8.18 (d, *J* = 8.5 Hz, 2H,), 7.51 (d, *J* = 8.0 Hz, 2H,), 4.95 (q, *J* = 6.5 Hz, 1H,), 1.50 (d, *J* = 6.5 Hz, 3H,) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 153.1, 147.1, 126.1, 123.7, 69.4, 25.4 ppm. The enantiomeric excess (% *ee*) was determined by HPLC using Phenomenex LUX Cellulose-3 column (*n*-hexane/ *i*-PrOH 90:10, 0.5 mL/min, 254 nm): t_r (*S*)-1-(4-nitrophenyl) ethanol 23.4 min, t_r (*R*)-1-(4-nitrophenyl) ethanol 24.9 min.

(*S*)-**2j** (1-(3'-methoxyphenyl)ethanol): $[\alpha]_D^{20}$ = - 40.2° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 38.9° (*c* 1.27 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.20-7.25 (m, 1H,), 6.90-6.95 (m, 2H,), 6.80 (ddd, *J* = 8.5 Hz, 1.5 Hz and 1.0 Hz, 1H,), 4.84 (q, *J* = 6.5 Hz, 1H,), 3.80 (s, 3H), 1.46 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 159.8, 147.6, 130.0, 116.5, 113.0, 111.0 , 70.3, 56.2, 25.1 ppm. The enantiomeric excess (% *ee*) was determined by HPLC using Chiralcel OD column (*n*-hexane/*i*-PrOH 95:5, 0.5 mL/min, 216 nm): t_r (*R*)-1-(3-methoxyphenyl)ethanol 23.1 min, t_r (*S*)-1-(3-methoxyphenyl)ethanol 26.4 min.

(*S)*-**2k** (1-(3'-tolyl)ethanol): $[\alpha]_D^{20}$ = - 45.4.0° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 47.2° (*c* 1.09 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.00-7.25 (m, 4), 4.84 (dq, , *J* = 6.5 Hz and 3.5 Hz, 1H), 2.35 (s, 3H), 1.47 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 144.0, 137.1, 128.4, 128.2, 126.1, 122.4 , 68.4, 25.1, 21.4 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(3'-tolyl)ethanol 14.8 min, t_r (*R*)-1-(3'-tolyl)ethanol 15.1 min.

(*S)*-**2l** (1-(3'-fluorophenyl)ethanol): $[\alpha]_D^{20}$ = - 25.60° (*c* 0.2 chloroform) lit.[26] $[\alpha]_D^{20}$ = - 24.4° (*c* 1.24 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.25-7.32 (m, 1H), 7.10-7.15 (m, 1H), 6.88–6.98 (m, 2H), 4.85 (q, *J* = 6.5 Hz, 1H), 1.45 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75

MHz, $CDCl_3$): δ = 163.0 (d, *J* = 246.1 Hz), 148.5 (d, *J* = 6.4 Hz), 129.9 (d, *J* = 7.7 Hz), 120.9 (d, *J* = 2.6 Hz), 114.2 (d, *J* = 21.2 Hz), 112.3 (d, *J* = 21.2 Hz), 69.7 (d, *J* = 2.1 Hz), 25.2 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(3'-fluorophenyl)ethanol 17.0 min, t_r (*R*)-1-(3'-fluorophenyl)ethanol 17.5 min.

(*S*)-**2m** (1-(3'-trifluoromethylphenyl)ethanol): $[\alpha]_D^{20}$ = - 30.50° (*c* 0.2 chloroform) lit.[23] $[\alpha]_D^{20}$ = - 27.6° (*c* 1.05 chloroform).^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.63-7.25 (m, 4H), 4.91 (q, *J* = 6.5 Hz, 1H,), 1.48 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 146.7, 130.8 (q, *J* = 32.5 Hz), 128.9, 128.8, 124.2 (q, *J* = 4.6 Hz), 124.1 (q, *J* = 270.8 Hz), 122.2 (q, *J* = 4.4 Hz), 69.8, 25.2 ppm
The enantiomeric excess (% *ee*) was determined by HPLC using Chiralcel OD-H column (*n*-hexane/EtOH 98:2, 0.8 mL/min, 220 nm): t_r (*S*)-1-(3'-trifluoromethylphenyl)ethanol 18.5 min, t_r (*R*)-1-(3'-trifluoromethylphenyl)ethanol 19.3 min.

***(S)*-2n** (-1-(3'-cyanophenyl)ethanol): $[\alpha]_D^{20}$ = - 25.2° (*c* 0.2 chloroform) lit.[27] $[\alpha]_D^{20}$ = - 26.6° (*c* 0.3 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.66 (s, 1H,), 7.60 (d, *J* = 7.3 1H,), 7.52-7.55 (m, 1H,), 7.40-7.45 (m, 1H), 4.90 (q, *J* = 6.1 Hz, 1H,), 1.48 (d, *J* = 6.1 Hz, 3H,) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 146.5, 130.5, 129.9, 129.0, 128.5, 119.0, 111.0, 69.5, 25.2 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(3'-cyanophenyl)ethanol 23.7 min, t_r (*R*)-1-(3'-cyanophenyl)ethanol 24.0 min.

(*S*)-**2o** 1-(3'-nitrophenyl)ethanol**:** : $[\alpha]_D^{25}$ = - 22.0° (*c* 0.2 chloroform) lit.[28] $[\alpha]_D^{25}$ = - 14.5° (*c* 1.0 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 8.18 (t, *J* = 1.8. Hz, 1H,), 8.05 (ddd, *J* = 8.3, 2.1 and 0.9 Hz, 1H,), 7.65 (d, *J* = 7.8, 1H), 7.50 (t, *J* = 7.8. Hz, 1H,), 4.95 (q, *J* = 6.5 Hz, 1H,), 1.50 (d, *J* = 6.5 Hz, 3H,) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 149.1, 147.1, 131.5,

129.1, 123.7, 120.2, 69.4, 25.4 ppm. The enantiomeric excess (% *ee*) was determined by HPLC using Phenomenex LUX Cellulose-2 column (*n*-hexane/*i*-PrOH 98:2, 0.5 mL/min, 254 nm): t_r (*R*)-1-(3'-nitrophenyl)ethanol 60.0 min, t_r (*S*)-1-(3'-nitrophenyl)ethanol 67.5 min.

(*S*)-**2s** (1-(2'-fluorophenyl)ethanol)**:** $[\alpha]_D^{20}$ = - 43.1° (*c* 0.2 chloroform) lit.[29] $[\alpha]_D^{25}$ = - 47.8° (*c* 1.46 chloroform). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.45-7.50 (m, 1H), 7.27-7.20 (m, 1H), 7.15–7.05 (m, 1H), 7.04-6.98 (m, 1H), 4.85 (q, *J* = 6.5 Hz, 1H), 1.43 (d, *J* = 6.5 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 159.9 (d, *J* = 246.1 Hz), 132.5 (d, *J* = 13.4 Hz), 128.9 (d, *J* = 8.2 Hz), 126.9 (d, *J* = 4.6 Hz), 124.2 (d, *J* = 3.4 Hz), 115.3 (d, *J* = 21.2 Hz), 65.7, 24.2 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(2'-fluorophenyl)ethanol 12.09 min, t_r (*R*)-1-(2'-fluorophenyl)ethanol 13.9 min.

(*S*)-**2u** (1-(2'-cyanophenyl)ethanol)**:** $[\alpha]_D^{20}$ = - 45.6° (*c* 0.2 dichloromethane) lit.[30] $[\alpha]_D^{20}$ = - 47.6° (*c* 1.0 dichloromethane). ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.60-7.70 (m, 1H,), 7.55 (d, *J* = 7.3 2H,), 7.20-7.36 (m, 1H,), 5.20 (q, *J* = 6.4 Hz, 1H,), 1.48 (d, *J* = 6.4 Hz, 3H,) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 148.9, 133.1, 132.5, 127.8, 125.4, 116.5, 109.0, 66.7, 24.2 ppm. The enantiomeric excess (% *ee*) was determined by GC using Mega-dex DET Beta column, gas carrier: H_2 (0.6 bar, T = 100°C), detector FID (flame ionization detector), temperature: 70-170°C 4°/min: t_r (*S*)-1-(2'-cyanophenyl)ethanol 26.4 min, t_r (*R*)-1-(2'-cyanophenyl)ethanol 27.2 min.

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Stereoselective enzymatic reduction of ethyl secodione: preparation of key-intermediates for the total synthesis of steroids

7.1 Nature and action of hormones

In the superior organisms the functional integration of the various organs is possible by the "information" transmitted *via* nervous system or through bloodstream. Although functionally distinct, these two information and regulation systems are closely coordinated by a common control center, the hypothalamus. The hormones are chemical messengers secreted in the blood and distributed throughout the body to explicate their action on target cells having receptor able to recognize them. Target cells also have elements for translating the hormonal stimulation in appropriate functional and metabolic changes in order to adapt the organism to the internal or external environment conditions maintaining the homeostasis.

Chemically heterogeneous, the hormones can be classified in i) peptide hormones such as insulin, glucagon, pituitary hormones, ii) steroidal hormones, sexual and adrenocortical ones, iii) derivatives of amino acids such as adrenalin, noradrenalin, thyroxin, triiodothyronine. The hydrophilic hormones circulate in the free form in the bloodstream whereas the hydrophobic ones are carried out in the blood by specific binding proteins.

The hormones are not secreted with regular rhythm, but following cycles or after stimulation. For example gonadotropins are released following menstrual cycle or after occasional events such as pregnancy or breastfeeding, the insulin and glucagon secretion is

regulated by the glycaemia level, the calcitonin and parathormone release by level of serum calcium.

The hormone's average life is short: once explicated their action, molecules are quickly inactivated for example using proteolysis, in this way the need of a controlled and transitory response is satisfied. The continuity of hormone response, when necessary, is assured by pulsing secretions.

Another characteristic of hormones is to exert their action at very low concentrations (10^{-9}-10^{-12} M).[98]

7.2 Progesterone

Progesterone, that in the adrenal cortex is the key intermediate for the synthesis of adrenocortical hormones, is synthesized in high amount in the ovarian corpus luteum after the stimulation of pituitary LH (luteinizing hormone). It is the predominant hormone in the second part of menstrual cycle and, essentially, it acts in the uterus for the endometrium preparation to fecundated ovum implantation. In case of pregnancy the secretion of progesterone, supported by placenta, is maintained until the labor. The administration of progesterone during specific periods of menstrual cycle inhibits the ovulation. On this action is based its use as contraceptive.

All the steroidal hormones derive from cholesterol through pregnenolone. The transformation of pregnenolone in progesterone consists of in two different steps: in the first the hydroxyl in 3 position is oxidized into the corresponding ketone by the 3-β-olo-

[98] Suliprandi and Tettamanti. *Biochimica Medica.* 3th Ed. Piccin, Padova, **2005**.

dehydrogenase. In the second step the carbon-carbon double bond is transferred in delta[4] position by delta 4,5 isomerase. Both the reactions occur in the cytosol. [99]

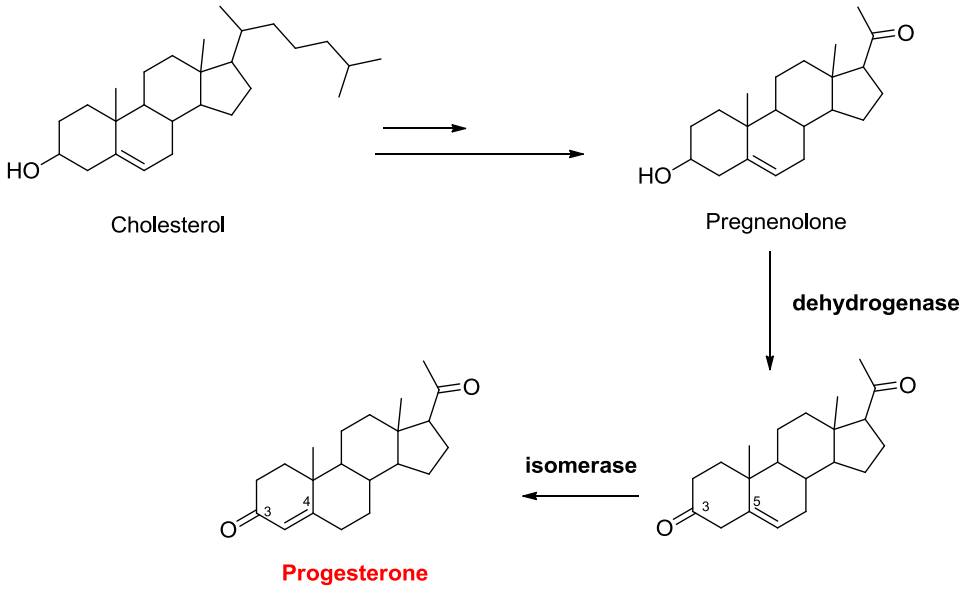

Fig. 45 Synthesis of progesterone in the human cells

7.3 Levonorgestrel

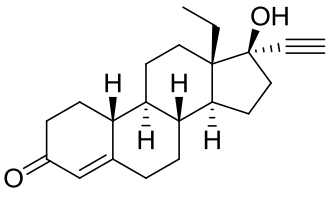

Fig. 46 Structure of Levonorgestrel

[99] W.L. Miller *Endocr. Rev.* **1988**, *9*, 295-318

Norgestrel was synthesized in 1950s as racemic mixture. Many studies about pharmacokinetics and pharmacodynamic parameters showed that norgestrel was an extremely potent progestogen with progestational and anti-ovulatory activities higher than those existing progestogens. The total activity of norgestrel resides in the left-handed isomer levonorgestrel, so it quickly become the progestogen used in therapy as a contraceptive both in association with ethinylestradiol as birth control pill, and in monotherapy as an emergency contraceptive.[100] Its activity on the prevention of ovulation and fecundation,[101] in particular the transformation of the endometrium in unsuitable environment for the implantation of the embryo makes it one of the most used and effective nonsurgical method for spacing and fertility control.[102] Levonorgestrel is a second-generation progestin agonist having high affinity for progesterone receptors. It binds the intracellular cytosolic receptors formed by three different regions: the amino terminal responsible for the dimerization of the receptor, the central portion that presents affinity for specific DNA sequences and the carboxy-terminal region responsible for the binding with ligand and for the migration of the complex ligand-receptor in the cell nucleus. The carboxy-terminal domain when occupied by the hormone or by an agonist confers the active conformation upon the receptor. In this domain are present two subdomains, one participant in the modulation of transcription process and the other binding particular proteins called HSP (heat shock proteins). As long as HSPs are bonded, the central interaction domain with DNA is locked and the receptor is inactive. The hormone entry, inducing the receptor dimerization, removes the HSPs transforming the receptor in its active form. After dimerization the receptor enters the cell nucleus and

[100] K. Fotherby, *Clin. Pharmacokinet*. **1995**, *28*, 203-215
[101] K. Gemzell-Danielsson, *Contraception* **2010**, *82*, 404-409
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binding specific DNA sequences, facilitates or inhibits transcription. The difference in the central receptor regions is the reason of the binding specificity with DNA sequences called HRE (Hormone Responsive Elements) responsible for the transcription triggering and for the activation of signal transduction pathway with the consequent release of second messengers. These, intervening on the state of protein phosphorylation produce effects of metabolic regulation. [103]

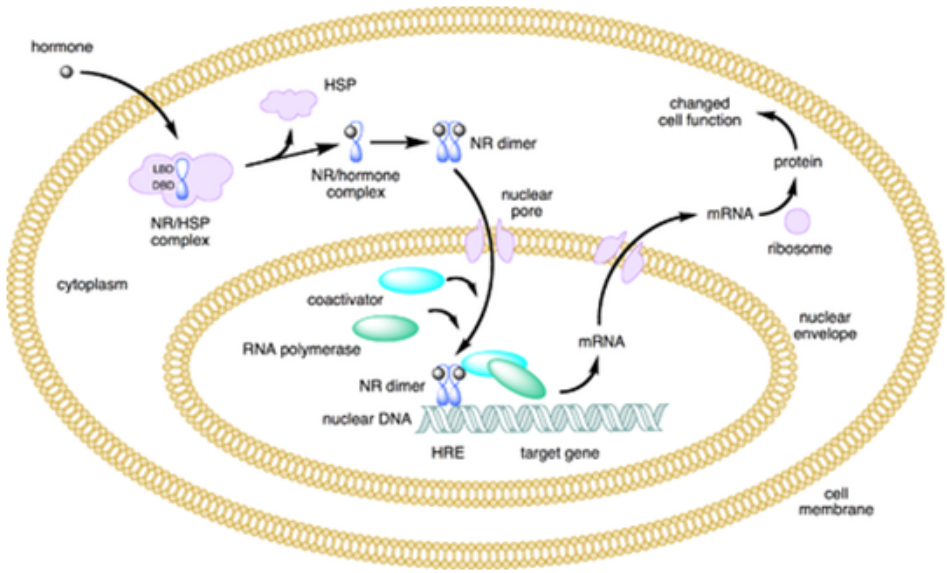

Fig. 47 Action mechanism of progesterone and its receptor

[103] Suliprandi and Tettamanti. *Biochimica Medica.* 3th Ed. Piccin, Padova, **2005**.

Based on this key role in metabolism, progesterone derivatives are compounds of great interest for pharmaceutical industry. Their complex and multi-functional structure requires highly selective synthetic strategies. Our chemoenzymatic synthesis, exposed above, combines the flexibility of chemical synthesis and the high selectivity encontered with enzymatic transformations both using whole cells of various yeasts and the recombinant benzil-reductase from *P. glucozyma*, being an effective approach for the preparation of Levonorgestrel.

Stereoselective enzymatic reduction of ethyl secodione: preparation of a key-intermediate for the total synthesis of steroids

Martina Letizia Contente,[a] Francesco Molinari,[a] Immacolata Serra,[a] Andrea Pinto,*[b] Diego Romano*[a]

Abstract: Ethyl secodione (**1**) has been enantioselectively reduced using different biocatalysts for the preparation of (13*R*, 17*S*)-**2a**. The recombinant ketoreductases KRED1-Pglu converted the substrate with the highest reaction rate and stereoselectivity (ee > 98%), whereas whole cells of *Pichia minuta* CBS 1708 showed the highest productivity. Stereoselective reduction of **1** provides the key chiral precursor for the synthesis of a number of hormonal contraceptives (i.e., desogestrel, norgestrel, gestodene).

Chemoenzymatic synthesis combines the flexibility of chemical synthesis and the high (stereo)selectivity often encountered with enzymatic transformations, being an effective approach for the synthesis of many drugs, including steroids.[1] Total synthesis of steroids has become widely applicable mostly due to the discovery by Ananchenko and Torgov of a straightforward process making possible the assembly of a steroidal A/B bicyclic core.[2, 3] This strategy can be used for the synthesis of second generation oral contraceptives, after reduction of the easily available ethyl secodione derivative **1**. Only 13-(*R*)-ethyl secols are useful for the synthesis of contraceptive hormones (i.e., desogestrel, levonorgestrel, gestodene (Figure 1).[3]

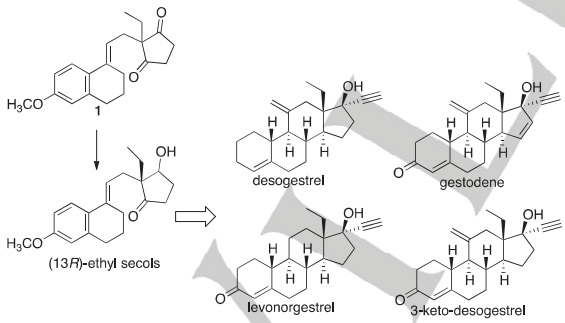

Figure 1. Second generation oral contraceptives from ethyl secodione **1**.

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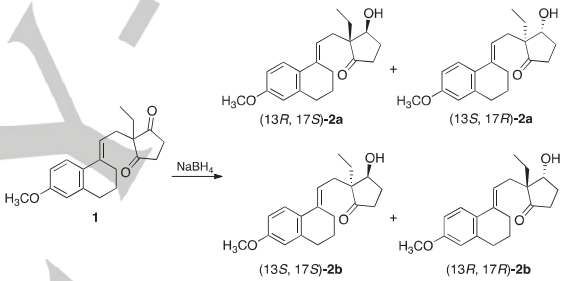
Although biocatalytic reduction of compound **1**, has been studied by industrial groups since the mid-sixties,[4] no detailed (stereo)chemical characterization of the reduction products of **1** has been reported in literature. To this aim, compound **1** was reduced with $NaBH_4$ and the four possible stereoisomers of **2** were isolated and fully characterized (Scheme 1).



Scheme 1. Monoreduction of ethyl secodione **1** using $NaBH_4$.

Firstly, *syn* racemate (±)-**2a** was separated from the *anti* racemate (±)-**2b** by semi-preparative silica HPLC. The relative stereochemistry of compounds (±)-**2a** and (±)-**2b** was initially deduced from the NOESY spectra.

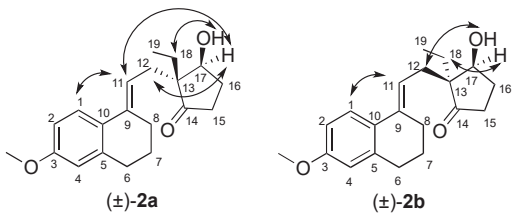


Figure 2. NOESY correlations in *syn* racemate (±)-**2a** and *anti* racemate (±)-**2b**.

Concerning derivative (±)-**2a**, the observation of NOESY correlations from H-17 to H-12 and H-11, and from H-18 to 17-OH, indicated that 17-OH and ethyl group are in a *syn* configuration (Figure 2). On the other hand, for derivative (±)-**2b**, the observation of NOESY correlations from H-12 to 17-OH, and from H-18 to H-17, indicated that 17-OH and ethyl group are in an *anti* configuration. The racemates (±)-**2a** and (±)-**2b** were, in turn, resolved into their pure enantiomers by semi-preparative

chiral HPLC using, in both cases, tris-(3,5-dimethylphenyl)carbamoyl amylose as chiral stationary phase (see Supplementary information).

We then succeeded in growing diffraction-quality crystals of one of the two enantiomers of **2b** The relative stereochemistry was unequivocally determined as *syn* by accurate single-crystal X-ray experiments (Figure 3), confirming the results obtained by the NOESY technique (see Supplementary information for further details).

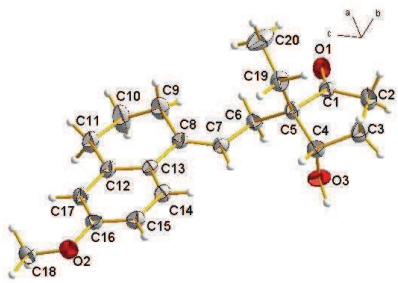


Figure 3. X-ray structure of the asymmetric unit of (–)-**2b** at room temperature, with the atom numbering scheme. Thermal ellipsoids are drawn at the 30% probability level.

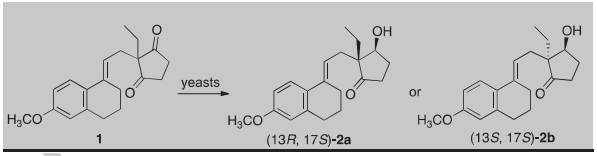
To assign the absolute configuration of the four stereoisomers, we decided to follow the Mosher's methodology. To this aim starting from enantiomerically pure (–)-**2a** and (–)-**2b**, (*R*)- and (*S*)-MTPA esters of the alcohol at C-17 were prepared and the absolute configuration at C-17 was unambiguously assigned as 17*S* in both cases (see Supplementary information). Based upon the already assigned relative configuration, the absolute configuration of (–)-**2a** was established as 13*R*, 17*S* and the absolute configuration of (–)-**2b** was established as 13*S*, 17*S*.

Corey and coworkers developed a method for stereoselective reduction of methyl secodione (Torgov's diketone) using oxazaborolidine catalysis, which furnishes enantiopure (13*R*, 17*R*)-methyl secol in 86% yield with 99% ee after recrystallization,[5] whose preparation opened the route to a stereoselective version of Torgov's synthesis of estrone. Our attempts to apply Corey's method to the reduction of **1** gave poor results, with formation of (13*R*, 17*R*)-**2b** in low yields (25-30%) and poor enantioselectivity (75% ee).

Biocatalytic reduction (either using isolated ketoreductases or ketoreductases bound to whole microbial cells) is an effective alternative for obtaining enantiopure compounds.[6] A recombinant ketoreductase (KRED1-Pglu), recently reported as an efficient stereoselective biocatalyst for the reduction of bulky diketones,[7] was employed for the reduction of **1**. KRED1-Pglu was used in the presence of a catalytic amount of $NADP^+$ and an enzyme-coupled system (glucose/glucose dehydrogenase, GDH) for the regeneration of the cofactor. Different parameters of the biotransformation (substrate and enzyme concentrations, pH, type and co-substrate concentrations) were optimized using a Multisimplex, approach, previously used for biotransformations;[8] KRED1-Pglu under optimized conditions (0.05M Tris-HCl at pH 8.0, 30°C, 6.5 mM substrate concentration in the presence of 3.2% EtOH and 2.5% glucose) gave the desired stereoisomer (13*R*, 17*S*)-**2a** with outstanding stereoselectivity (> 98% ee) and good reaction rates, but with moderate yields (65% after 6 hours). Notably, no further reduction to diol was observed even at prolonged times, indicating the total selectivity of KRED1-Pglu.

The relative low yield observed with the recombinant ketoreductases, led us screening various yeasts for finding more productive biocatalysts. The screening was carried out with yeasts previously known for their ability to reduce structurally different ketones.[9-12] Table 1 gives the results of the biotransformation at the time of maximum production (only reactions with yields above 50% are reported).

Table 1. Reduction of ethyl secodione **1** using different yeasts



1 (13*R*, 17*S*)-**2a** (13*S*, 17*S*)-**2b**

Yeasts[a]	Product	ee[b] (%)	Yield (%)	Time (h)
Pichia anomala DBVPG 2873	(13*R*,17*S*)-**2a**	20	65	24
P. capsulata CBS 1993	(13*R*,17*S*)-**2a**	51	62	96
P. etchellsii CBS 2011	(13*R*,17*S*)-**2a**	50	75	24
P. glucozyma CBS 5766	(13*S*,17*S*)-**2b**	94	85	2
P. minuta CBS 1708	(13*R*,17*S*)-**2a**	90	80	24
Rhodotorula glutinis NRRL 1587	(13*S*,17*S*)-**2b**	90	78	24
R. rubra MIM146	(13*S*,17*S*)-**2b**	76	92	24
S. cerevisiae CEN.PK113-7D	(13*R*,17*S*)-**2a**	96	81	48

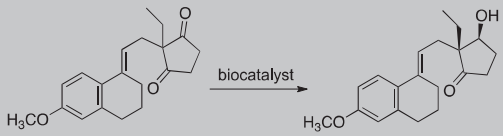
[a] To compare yields, all biotransformations were performed with the same optical density of yeast culture (OD_{600}/ml = 60). [b] Determined by analytical chiral HPLC (details in the Supporting Information).

Six strains (*Pichia etchellsii* CBS 2011, *Pichia glucozyma* CBS 5766, *Pichia minuta* CBS 1708, *Rhodotorula glutinis* NRRL-Y1587, *Rhodotorula rubra* MIM 146, *Saccharomyces cerevisiae* CEN.PK113-7D) gave monoreduction of **1** with yields higher than 70%. *P. minuta* CBS 1708 and *S. cerevisiae* CEN.PK113-7D afforded the desired stereoisomer (13*R*, 17*S*)-**2a** with ee ≥ 90%; different stereoselectivity was detected with *P. glucozyma* CBS 5766 and *R. glutinis* NRRL-Y1587, giving (13*S*, 17*S*)-**2b** in high ee. The biotransformation with *P. minuta* CBS 1708 and *S. cerevisiae* CEN.PK113-7D were further optimized by evaluating different parameters (temperature, pH, substrate and cells concentration, and co-substrate type and concentration).

Table 2 reports the comparison between the results obtained in the biotransformation of **1** accomplished at 1 litre scale with the best-performing biocatalysts (recombinant KR or yeasts)

used under optimized conditions. Although *S. cerevisiae* and the recombinant ketoreductase furnished the highest stereoselectivity, *P. minuta* allowed for the obtainment of the highest amounts of enantiomerically pure (13*R*, 17*S*)-**2a** after a single crystallization from diisopropyl ether (DIPE). Actually, *P. minuta* allowed us to perform the biotransformation at substrate concentration up to 4.7 g/L (15 mM) with > 95% yield, whereas *S. cerevisiae* and KRED1-Pglu provided high yields only at much lower concentrations.

Table 2. Optimized reduction of **2** into (13*R*, 17*S*)-**4a**.



1 (13*R*, 17*S*)-**2a**

Biocatalyst	ee (%)	Yield[a] (%)	Product recovered[b] (g)	Time (h)
P. minuta[c]	92	> 95	3.42	24
S. cerevisiae[d]	> 98	> 95	2.75	24
KRED1-Pglu[e]	> 98	65	1.15	8

[a] Analytical yield determined by HPLC [b] After crystallization from DIPE [c] Conditions: 0.1 M phosphate buffer at pH 7.2, 30°C, 15 mM substrate concentration in the presence of 3.2% EtOH and 25 $g_{dry\ weight}$/L of yeast cells; [d] Conditions: 0.1 M phosphate buffer at pH 6.5, 30°C, 10 mM substrate concentration in the presence of of 3.5% EtOH and 30 $g_{dry\ weight}$/L of yeast cells; [e] Conditions: 0.05M Tris-HCl buffer at pH 8.0, 28°C, 6,5 mM substrate concentration in the presence of 3.2% EtOH, 2.5% glucose, KRED1-Pglu and GDH.

In summary, we used different biocatalysts (yeasts or recombinant ketoreductase) for chemo- and stereoselective carbonyl reduction of secodione derivative **1**. This approach is a simple and complementary strategy for the enzymatic generation of optically pure intermediates to be used for the synthesis of commercially relevant steroids.

Experimental Section

General procedure for the preparative asymmetric reduction with recombinant enzyme

Biotransformation using KRED1-Pglu was carried out by addition of substrate (6.5 mM) dissolved in ethanol (32 mL) to a solution containing KRED1-Pglu (20 mU/mL), GDH (1 U/mL), $NADP^+$ (0.1 mM), and glucose (25 mM) in 50 mM Tris/HCl buffer pH 8.0 (1 L). The reaction mixture was stirred at 28°C for 6 hours and then extracted with 600 mL of EtOAc; the aqueous phase was extracted twice more with 500 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent evaporated. The crude residues were crystallised with DIPE.

General procedure for the preparative asymmetric reduction with whole cells of yeasts

Biotransformation using *Pichia minuta* and *Saccharomyces cerevisiae* was carried out by addition of substrate as ethanolic solution (32 mL of ethanol in the case of *P. minuta,* 35 mL of ethanol in the case of *S. cerevisiae*). The biotransformation was kept under reciprocal shaking (150 rpm) at 30°C. When the reaction was over, 600 mL of EtOAc were added and the resulting mixture was shaken and centrifuged; the aqueous phase was extracted twice more with 500 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were crystallised with DIPE.

See Supporting Data for all the details about preparation of the biocatalysts, stereochemical analysis and analytical data of the products.

Acknowledgements

We would like to thank Dr. Roberto Lenna for providing us the substrates and for his precious help about the analytical section.

Keywords: enzymatic reduction • yeast • steroids • desogestrel • secodione • levonorgestrel• gestodene

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Supplementary Data

Stereoselective enzymatic reduction of ethyl secodione: preparation of a key-intermediate for the total synthesis of steroids

Martina Letizia Contente, Francesco Molinari, Immacolata Serra, Andrea Pinto, Diego Romano

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General

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Substrate **1** was kindly furnished by Dr. Roberto Lenna (Industriale Chimica). Elemental analyses were carried out on a Carlo Erba Model 1106 (Elemental Analyzer for C, H, and N), and the obtained results are within 0.4% of theoretical values. Merck Silica gel 60 F254 plates were used for analytical TLC; flash column chromatography was performed on Merck Silica gel (200–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^{1}H and ^{13}C chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) in hertz (Hz). IR spectra were recorded on a FT-IR PerkinElmer 16 PC. Rotary power determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. MS analyses were performed on a Thermo-Finnigan LCQ ADVANTAGE mass spectrometer

equipped with an electrospray ionization (ESI) source. HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV/Vis detector Jasco UV-975 (l 220 nm) and a Lux 5µ Cellulose-2 column (4.6 X 250 mm, Phenomenex) at a flow rate of 0.5 $mLmin^{-1}$. Preparative HPLC was performed with a 1525 Extended Flow Binary HPLC pump equipped with a Waters 2489 UV/Vis detector and Luna 5 µm C18 (2) 100 A, (21.2 X 250 mm; Phenomenex) or a Kromasil 5-AmyCoat column (21.2 X 250 mm, AkzoNobel) at a flow rate of 15 $mLmin^{-1}$.

Preparation of (13*R, 17*S**)-E-13-ethyl-3-methoxy-8,14-secogona-1,3,5(10),9(11)-tetraene-17-ol-14-one [(±)-2a] and (13*R**, 17*R**)-E-13-ethyl-3-methoxy-8,14-secogona-1,3,5(10),9(11)-tetraene-17-ol-14-one [(±)-2b]**

To a solution of ethyl secodione **1** (200 mg, 0.64 mmol) in EtOH (4 mL) was added $NaBH_4$ (6.1 mg, 0.16 mmol) and the reaction mixture was kept under agitation at room temperature. After 15 minutes, the reaction was evaporated under vacuum and the crude residue was purified by flash chromatography (*n*-hexane/EtOAc 6/4) to give a mixture of (±)-**2a** and (±)-**2b** (102 mg, 0.32 mmol). (±)-**2a** and (±)-**2b** were separated by preparative HPLC under the following conditions: Luna 5 µm C18 (2) 100 A, (21.2 X 250 mm; Phenomenex); hexane/isopropanol 95:5; 15 $mLmin^{-1}$; Retention times of (±)-**2a** and (±)-**2b** were 21.6 and 22.9 minutes, respectively.

(±)-**2a**: R_f= 0.61 (n-hexane/EtOAc 1:1); ^{1}H NMR (300 MHz,C_3D_6O): δ= 0.91 (t, J = 7.5 Hz, 3H; CH_3[19]), 1.63-1.73 (m, 2H; CH_2 [18]), 1.78 (qui, J = 6.3 Hz, 2H; CH_2 [8]), 1.88-1.97 (m, 1H; CH [16]), 2.13-2.40 (m, 5H; CH_2 [12], [15]; CH [16]), 2.49 (t, J = 5.6 Hz, 2H; CH_2 [9]), 2.73 (t, J = 6.2 Hz, 2H; CH_2 [7]), 3.77 (s, 3H; -OCH_3), 4.05 (d, J = 4.4 Hz, 1H; OH), 4.32 (q, J = 5.5 Hz, 1H; CH [17]), 5.86 (t, J = 7.5 Hz, 1H; CH [11]), 6.65 (d, J = 2.8 Hz, 1H; CH_{ar} [4]), 6.72 (dd, J= 2.8, 8.7 Hz, 1H; CH_{ar} [2]), 7.46 (d, J = 8.7 Hz, 1H; CH_{ar} [1]) ppm; ^{13}C NMR (300 MHz,C_3D_6O): δ= 8.0 (CH_3), 21.6 (CH_2), 23.4 (CH_2), 26.7 (CH_2), 27.9 (CH_2), 30.5

(CH_2), 30.7 (CH_2), 35.0 (CH_2), 54.8 (CH_3), 56.6 (C_q), 75.2 (CH), 112.7 (CH), 113.2 (CH), 117.1 (CH), 125.2 (CH), 129.3 (C_q), 135.9 (C_q), 138.7 (C_q), 159.0 (C_q), 218.8 (C=O) ppm; MS (ESI+ *m*/*z* %): 337.4 (100) $[M+Na]^+$; 315.4 (36) $[M+H]^+$; IR (KBr pellet): 3500, 2943, 2835, 1723, 1602, 1570, 1496, 1454, 1442, 1304, 1232, 1166, 1150, 1118, 1040, 957, 826, 801, 789, 735, 723, 675, 563, 530 cm^{-1}; elemental analysis: calcd (%) for $C_{20}H_{26}O_3$ (314.2): C 76.40, H 8.33; found: C 76.74, H 8.56.

(±)-**2b**: R_f= 0.58 (n-hexane/EtOAc 1:1); ^{1}H NMR (300 MHz,C_3D_6O): δ= 0.82 (t, *J* = 7.5 Hz, 3H; CH_3 [19]), 1.53 (non, *J* = 7.5 Hz, 2H; CH_2 [18]), 1.78 (qui, *J* = 6.2 Hz, 2H; CH_2 [8]), 1.95-2.07 (m, 1H; CH [16]), 2.23-2.31 (m, 3H; CH_2 [15]; CH [16]), 2.43-2.46 (m, 2H; CH_2 [12]), 2.51 (t, *J* = 6.2 Hz, 2H; CH_2 [9]), 2.74 (t, *J* = 6.2 Hz, 2H; CH_2 [7]), 3.77 (s, 3H; CH_3 [20]), 4.13 (d, *J* = 4.2 Hz, 1H; OH), 4.32 (q, *J* = 5.3 Hz, 1H; CH [17]), 6.0 (t, *J* = 7.5 Hz, 1H; CH [11]), 6.64 (d, *J* = 2.8 Hz, 1H; CH_{ar} [4]), 6.72 (dd, *J*= 2.8, 8.7 Hz, 1H; CH_{ar} [2]), 7.50 (d, *J* = 8.7 Hz, 1H; CH_{ar} [1]) ppm; ^{13}C NMR (300 MHz,C_3D_6O): δ= 8.2 (CH_3), 23.6 (CH_2), 25.3 (CH_2), 26.6 (CH_2), 26.8 (CH_2), 28.4 (CH_2), 30.9 (CH_2), 34.8 (CH_2), 54.9 (CH_3), 57.2(C_q), 75.1 (CH), 112.8 (CH), 113.2 (CH), 118.1 (CH), 125.3 (CH), 129.6 (C_q), 135.4 (C_q), 138.7 (C_q), 159.0 (C_q), 219.1 (C=O) ppm; MS (ESI+ *m*/*z* %): 315.3 (100) $[M+H]^+$; 337.3 (52) $[M+Na]^+$; IR (KBr pellet): 3405, 2968, 2935, 2916, 2835, 1716, 1606, 1573, 1497, 1456, 1440, 1316, 1234, 1164, 1088, 1037, 902, 824, 792, 741, 703, 675, 629, 545 cm^{-1}; elemental analysis: calcd (%) for $C_{20}H_{26}O_3$ (314.2): C 76.40, H 8.33; found: C 76.65, H 8.66

Preparative chiral HPLC resolution of (±)-**2a**

Preparative chiral HPLC conditions: Kromasil 5-AmyCoat column (21.2 X 250 mm, AkzoNobel); hexane/isopropanol 9:1; 15 $mLmin^{-1}$; Retention times of (–)-**2a** and (+)-**2a** were 14.0 and 19.4 minutes, respectively.

(–)-**2a**: $[\alpha]_D^{20}$ = −14.5 (*c*= 1.0 in dioxane);

(+)-**2a**: $[\alpha]_D^{20}$ = +14.5 (*c*= 1.0 in dioxane).

Preparative chiral HPLC resolution of (±)-**2b**

Preparative chiral HPLC conditions: Kromasil 5-AmyCoat column (21.2 X 250 mm, AkzoNobel); hexane/isopropanol 9:1; 15 mL/min; Retention times of (+)-**2b** and (–)-**2b** were 12.8 and 14.0 minutes, respectively.

(+)-**2b**: $[\alpha]_D^{20}$ = +11.0 (*c*= 1.0 in dioxane);

(–)-**2b**: $[\alpha]_D^{20}$ = −11.0 (*c*= 1.0 in dioxane).

Single-crystal X-ray diffraction.

Colourless prismatic crystals of (–)-**2b** were grown by slow evaporation from diisopropyl ether at room temperature. Two diffraction-quality specimen were selected for the subsequent X-ray analysis. Diffraction data were measured at room temperature by a Bruker three-circle SMART APEX diffractometer equipped with an APEX II CCD area detector. Graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) was employed to perform a full data collection up to a maximum resolution of $\sin\theta/\lambda$ = 0.65 $Å^{-1}$ for both the specimens. As independent data processing led to identical structural results, the two sets of diffraction intensities were scaled and merged together, giving an overall 99.8 % complete dataset consisting of 45511 reflections (4028 unique) with R_{int} = 0.0334. The crystal system is orthorhombic, space group $P2_12_12_1$ (nº 19), with lattice constants *a* =7.324(2) Å, *b* = 10.495(8) Å, *c* =22.664(2) Å and cell volume *V* = 1742(1) $Å^3$. The molecular structure was solved by direct methods and refined through the shelx software package,[1] giving final agreement factors as low as R1(F) = 0.0462 (3388 $F_o > 4\sigma(F_o)$), R1(F) = 0.0569 and wR(F^2) = 0.1355 (all data), goodness-of-fit = 1.057. The maximum and minimum Fourier residuals were as low as +0.21 / –0.28 $e\cdot Å^{-3}$. Due to the lacking of anomalous scatterers in the unit cell, it was not possible to unequivocally determine the absolute structure of (–)-**2b**. Anyhow, the

relative configurations of the two stereogenic centres were determined as *syn*. CCDC1437502 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.

(–)-2b crystal structure

Figure S1 shows the X-ray diffraction-derived structure of the compound (–)-**2b**.

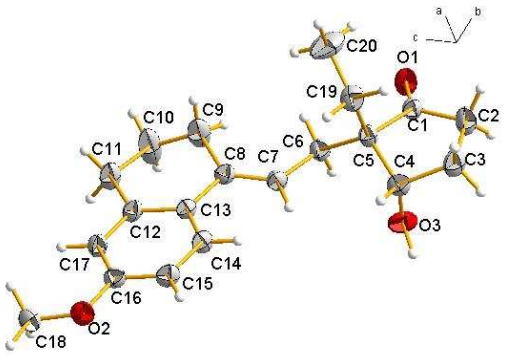
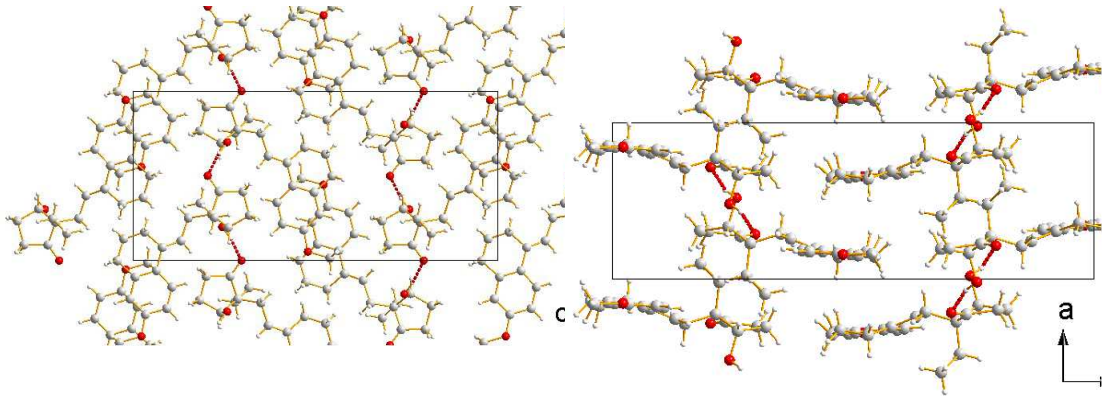


Figure S1. X-ray structure of the asymmetric unit of (–)-**2b** at room temperature, with the atom numbering scheme. Thermal ellipsoids are drawn at the 30% probability level.

Figure S2 displays the main packing features of (–)-**2b**.



(a) (b)

Figure S2. Ball-and-stick representation of the crystal packing of (–)-**2b**, as seen along the **a** (a) and **b** (b) cell edges. Carbon atoms are drawn in grey, Oxygen atoms in red and Hydrogen atoms as small white spheres. Short O–H···O hydrogen-bonded contacts are highlighted as dashed red lines.

The molecule bears a unique hydrogen donor group (–OH), which is involved in a short hydrogen-bonded contact with the facing keto Oxygen atom O1 at –x, –1/2+y, 3/2–z (Table S1). Antiparallel chains of hydrogen-bonded molecules determine the formation of zig-zag motifs along the **b** cell edge (Figure S2 (a)). The $–C19H_2–C20H_3$ ethyl group is accommodated in the free space between adjacent layers of molecules along the **c** axis (Figure S2 (b)).

Table S1. Hydrogen-bonded contact geometry for the symmetry-independent O3–H3···O1 interaction. Least-squares estimated standard deviations from the structural refinement are given in parentheses.

Donor-H···Acceptor	D–H / Å	H···A / Å	D···A / Å	D-H·A / deg
O3–H3···O1	0.93(4)	1.88(4)	2.796(3)	166(4)

Synthesis of Mosher's esters of (–)-2a

To a solution of (–)-**2a** (20 mg, 0.064 mmol) in anhydrous CH_2Cl_2 at 4°C was added (*R*)-MTPA (16.8 mg, 0.072 mmol). The reaction mixture was kept at 4°C under stirring and DCC (15.6 mg, 0.075 mmol) DMAP (2.5 mg, 0.020 mmol) were added; the reaction was left at room temperature for 2 hours, then filtered and the filtrate was concentrated under vacuum. The crude residue was purified by flash

chromatography (*n*-hexane/EtOAc 9/1) to give 30 mg of (*R*)-MTPA ester of (–)-**2a**.

***(R)*-MTPA ester of (–)-2a:**

^{1}H NMR (300 MHz,C_3D_6O): δ= 0.73 (t, J = 7.5 Hz, 3H; CH_3), 1.32-1.60 (m, 2H; CH_2), 1.77 (qui, J = 6.2 Hz, 2H; CH_2), 2.11-2.18 (m, 1H; CH), 2.23-2.35 (m, 2H; CH_2) 2.39-2.45 (m, 3H; CH_2; CH), 2.49-2.60 (m, 2H; CH_2), 2.73 (t, J = 6.2 Hz, 2H; CH_2), 3.57 (s, 3H; CH_3), 3.76 (s, 3H; CH_3), 5.59 (t, J = 5.7 Hz, 1H; CH_{17}), 5.75 (t, J = 7.5 Hz, 1H; CH_{11}), 6.64 (d, J = 2.8 Hz, 1H; CH_{ar}), 6.70 (dd, J = 2.8, 8.7 Hz, 1H; CH_{ar})7.42-7.49 (m, 4H; CH_{ar}) 7.55-7.57 (m, 2H; CH_{ar}) ppm.

The remaining Mosher's esters were prepared using the above general procedure:

***(S)*-MTPA ester of (–)-2a:**

^{1}H NMR (300 MHz,C_3D_6O): δ= 0.87 (t, J = 7.5 Hz, 3H; CH_3), 1.50-1.70 (m, 2H; CH_2), 1.77 (qui, J = 6.2 Hz, 2H; CH_2), 1.90-2.07 (m, 1H; CH), 2.21-2.36 (m, 5H; CH_2; CH), 2.40-2.56 (m, 2H; CH_2), 2.73 (t, J = 6.2 Hz, 2H; CH_2), 3.52 (s, 3H; CH_3), 3.75 (s, 3H; CH_3), 5.59 (t, J = 5.7 Hz, 1H; CH_{17}), 5.80 (t, J = 7.5 Hz, 1H; CH_{11}), 6.65 (d, J = 2.8 Hz, 1H; CH_{ar}), 6.72 (dd, J = 2.8, 8.7 Hz, 1H; CH_{ar})7.45-7.47 (m, 4H; CH_{ar}) 7.52-7.56 (m, 2H; CH_{ar}) ppm.

***(R)*-MTPA ester of (–)-2b:**

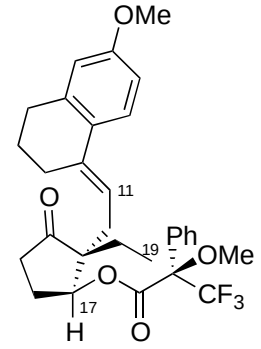
^{1}H NMR (300 MHz,C_3D_6O): δ= 0.77 (t, J = 7.5 Hz, 3H; CH_3), 1.35-1.60 (m, 2H; CH_2), 1.66-1.75 (m, 2H; CH_2), 2.16-2.38 (m, 4H; CH_2) 2.42 (t, J = 7.9 Hz, 2H; CH_2), 2.53-2.64 (m, 2H; CH_2), 2.70 (t, J =6.2 Hz, 2H; CH_2), 3.57 (s, 3H; CH_3), 3.77 (s, 3H; CH_3), 5.68-5.76 (m, 2H; CH_{17}; CH_{11}), 6.64 (d, J = 2.8 Hz, 1H; CH_{ar}), 6.74 (dd, J = 2.8, 8.7 Hz, 1H; CH_{ar}), 7.38-7.49 (m, 4H; CH_{ar}) 7.55 (d, J = 7.5 Hz, 2H; CH_{ar}) ppm.

***(S)*-MTPA ester of (–)-2b:**

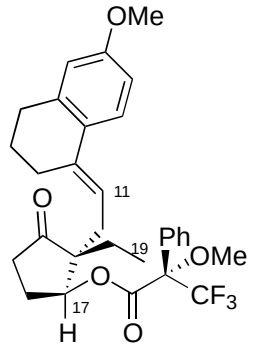
^{1}H NMR (300 MHz,C_3D_6O): δ= 0.84 (t, J = 7.5 Hz, 3H; CH_3), 1.54-1.64 (m, 2H; CH_2), 1.67-1.78 (m, 2H; CH_2), 2.08-2.19 (m, 1H; CH), 2.22-2.43 (m, 5H; CH_2; CH), 2.46-2.58 (m, 2H; CH_2), 2.71 (t, J =6.2 Hz, 2H; CH_2), 3.52 (s, 3H; CH_3), 3.75 (s, 3H; CH_3), 5.67 (t, J =5.7 Hz, 1H; CH_{17}), 5.86 (t, J =7.5 Hz, 1H; CH_{11}), 6.65 (d, J = 2.8 Hz, 1H; CH_{ar}), 6.71 (dd, J = 2.8, 8.7 Hz, 1H; CH_{ar}) 7.36-7.48 (m, 4H; CH_{ar}) 7.54 (d, J = 7.5 Hz, 2H; CH_{ar}) ppm.

Determination of the absolute configuration of 2a

Based upon the already assigned relative configurations of (–)-**2a** and (–)-**2b** and assuming also that the Mosher's esters adopt the conformations shown in Figure 1 (CF_3, carbonyl and methine hydrogen in the same plane), the hydrogens that are on the same side of the phenyl ring would be shielded by the phenyl ring and thus further upfield in the ^{1}H NMR spectrum.



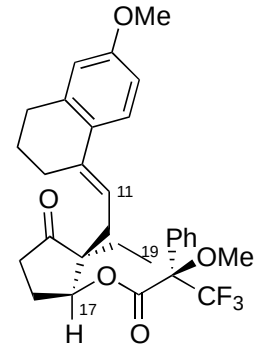
(*R*)-Mosher ester of (–)-**2a**



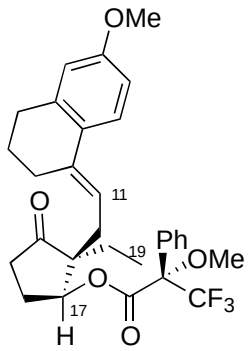
(*S*)-Mosher ester of (–)-**2a**

This is indeed the case for the (*R*)- and the (*S*)-Mosher's esters of (–)-**2a** shown above. So for instance, H-11 and H-19 in the (*R*)-Mosher's ester of (–)-**2a** are on the same side of the molecule as the phenyl ring, and they are further upfield (5.75 and 0.73 ppm respectively) than the H-11 and H-19 in the (*S*)-Mosher's ester in which are opposite to the phenyl ring (5.80 and 0.87 ppm respectively). These

observations are in agreement with an absolute configuration of (13*R*, 17*S*) for (–)-**2a**.



(*R*)-Mosher ester of (–)-**2b**



(*S*)-Mosher ester of (–)-**2b**

The same holds true for compound (–)-**2b**. H-11 and H-19 in the (*R*)-Mosher's ester of (–)-**2b** are on the same side of the molecule as the phenyl ring, and they are further upfield (5.75 and 0.77 ppm respectively) than the H-11 and H-19 in the (*S*)-Mosher's ester, in which these hydrogens are opposite to the phenyl ring (5.86 and 0.84 ppm respectively). These observations are in agreement with an absolute configuration of (13*S*, 17*S*) for (–)-**2b**.

Preparation of the biocatalysts

Yeasts were cultured in liquid medium (malt extract + 0.5% yeast extract medium, pH 5.8) in 3.0 L fermenters with 1.0 L of liquid medium for 48 h, at 28°C and agitation speed 150 rpm. Fresh cells from submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0 prior the use for biotransformation.

KRED1-Pglu was prepared as recombinant protein in *Escherichia coli*.[2] KRED1-Pglu gene was amplified from the genomic DNA of *P. glucozyma* by PCR using the following primers, carrying *Nde*I and *Hind*III restriction sites:

Forward: 5'-ATAC**CATATG**ACGAAGGTGACTGTTGTGAC-3'

Reverse: 5'-AGAG**AAGCTT**GGCGTACTCCTTCAACTCTG-3'

The amplified gene was then cloned into a pET26b(+) vector using the *Nde*I and *Hind*III restriction sites. With this cloning strategy, the resulting protein is expressed with an N-terminal His_6 tag. The correct construction of the expression plasmid was confirmed by direct sequencing.

Cultures of *E. coli* BL21(DE3)Star transformed with the resulting plasmid were grown overnight at 37 °C in LB medium supplemented with 25 µg/mL kanamycin. The seed culture was then diluted into a stirred fermenter containing 4 L of cultivation medium (Terrific Broth, 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 g/L glycerol, 2.3 g/L KH_2PO_4, 9.4 g/L K_2HPO_4, pH 7.2) to an initial OD_{600nm} of 0.05. Cultivation was carried out in batch-mode at 37 °C, 250 rpm stirring and 250 L/h aeration rate. Cells were grown until OD_{600nm} reached the value of 0.8. The cultures were induced for 20 h with IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.5 mM. Cells were then harvested by centrifugation at 4500 rpm for 30 min, washed once with 20 mM phosphate buffer at pH 7.0 and stored at -20 °C.

Protein purification was carried out with cells suspended in 50 mM Tris-HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 buffer. Proteins were extracted by sonication (5 cycles of 30 s each, in ice, with 1 min interval) and cell debris were harvested by centrifugation at 15000 rpm for 30 min at 4 °C. The enzyme was purified by

affinity chromatography with HIS-Select® Nickel Affinity Gel. Briefly, the column was equilibrated with 50 mM Tris-HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 and the crude extract loaded; column was then washed with 50 mM Tris-HCl, 100 mM NaCl, 6 mM imidazole; finally, the adsorbed enzyme was eluted with 50 mM Tris-HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0.

Biotransformations: screening with yeasts

Screening of the biotransformation of **1** with yeasts was carried out in 100 mL screw-capped test tubes with a reaction volume of 50 mL with cells (25 g/L, dry weight) suspended in 0.1 M phosphate buffer, pH 7.0. Ethyl secodione **1** (80 mg) was added as hot ethanolic solution (1.25 mL of ethanol). The biotransformation was kept under alternative stirring at 28°C. At different times, 2 ml of the biotransformation medium were withdrawn, extracted with 2 mL of EtOAc and the resulting mixture was shaken and centrifuged. The organic phase was collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were analysed by TLC (*n*-hexane/EtOAc 1/1) and by chiral HPLC under the following conditions: Lux 5µ Cellulose-2 (4.6 X 250 mm, Phenomenex); hexane/isopropanol 85:15; 0.5 mL/min; Retention times of (13*S*, 17*S*)-**2b,** (13*S*, 17*R*)-**2a,** (13*R*, 17*S*)-**2b** and (13*R*, 17*S*)-**2a** were 18.9, 20.6, 23.3, 24.6 minutes, respectively.

Biotransformation of 1 with recombinant ketoreductase KRED1-Pglu

The enzymatic reaction was optimized by performing biotransformations at 2 ml-scale, using an enzyme-coupled system (glucose-glucose dehydrogenase – GDH – from *Bacillus megaterium*; for cofactor recycling. Reactions were carried out in 5 mL screw-capped test tubes with a reaction volume of 2 mL with KRED1-Pglu (20 mU/mL), GDH (1 U/mL), and $NADP^{+}$ (0.1 mM).

Preparative biotransformation using KRED1-Pglu was carried out by addition of 32 mL of ethanol containing 0.20 mol of substrate (6.5 mM) dissolved in ethanol (32 mL) to a solution containing KRED1-Pglu (20 mU/mL), GDH (1 U/mL), $NADP^{+}$

(0.1 mM), and glucose (25 mM) in Tris/HCl buffer pH 8.0 (0.05M, 1 L). The reaction mixture was stirred at 28°C for 6 hours and then extracted with 600 mL of EtOAc; the aqueous phase was extracted twice more with 500 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were crystallised with diisopropyl ether (DIPE), furnishing (13*R*, 17*S*)-**2a** as a pure single stereoisomer (1.15 g, 56% yield).

Preparative biotransformation of 1 with *Pichia minuta*

Centrifuged cells (25 $g_{dry\ weight}$) were suspended in 1 L of phosphate buffer (pH 7.0, 0.1M) and 32 mL of ethanol containing 0.47 mol of substrate were added. The biotransformation mixture was kept under reciprocal shaking (150 rpm) at 30 °C. After 24 hours, 600 mL of EtOAc were added and the resulting mixture was shaken and centrifuged; the aqueous phase was extracted twice more with 500 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were crystallised with diisopropyl ether (DIPE), furnishing (13*R*, 17*S*)-**2a** as a pure single stereoisomer (3.42 g, 73% yield).

Preparative biotransformation of 1 with *Saccharomyces cerevisiae*

Centrifuged cells (30 $g_{dry\ weight}$) were suspended in 1 L of phosphate buffer (pH 6.5, 0.1M) and 35 mL of ethanol containing 0.30 mol of substrate were added. The biotransformation mixture was kept under reciprocal shaking (150 rpm) at 30 °C. After 24 hours, 600 mL of EtOAc were added and the resulting mixture was shaken and centrifuged; the aqueous phase was extracted twice more with 500 ml of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residues were crystallised with diisopropyl ether (DIPE), furnishing (13*R*, 17*S*)-**2a** as a pure single stereoisomer (2.75 g, 87% yield).

References

[1] G. M. Sheldrick, *Acta Cryst.* **2008**, *A64*, 112-122.

[2] M. L. Contente, I. Serra, M. Brambilla, I. Eberini, E. Gianazza, V. De Vitis, F. Molinari, P. Zambelli, D. Romano, *App. Microbiol. Biotechnol.* **2015**. doi 10.1007/s00253-015-6961-y.

Conclusions

8.1 Conclusions

In summary, different efficient and sustainable methods for the stereoselective reduction of prochiral ketones using both whole cells of unconventional yeasts such as *P. glucozyma* CBS 5766 and the isolated enzyme KRED1-Pglu have been proposed in this PhD work.

The biocatalytic potential of whole cells of *P. glucozyma* have been studied through biotransformations using substrates of different chemical nature having interesting pharmaceutical and synthetic aspects. After optimization of the reaction conditions through the transformation of acetophenone in (*S*)-1-phenylethanol, the attention was focused on the reduction of different aromatic ketones. All the reactions occurred with high yields and high enantiomeric excess for the corresponding (*S*)-alcohols.

Aromatic ketoesters were also assayed; it is known that biotransformations of β-ketoesters with yeasts take place with competition between ester hydrolysis and carbonyl reduction, due to the occurrence of cell-bound esterase activities; this competitive enzymatic activity can be modulated by using appropriate co-substrate concentrations: with high co-substrate concentrations (e.g. glucose > 50 g/L) the activity of ketoreductases is generally predominant, as a consequence of redox cofactor regeneration, while esterase activity is predominant in absence of co-substrates. Biotransformations were performed in the optimal conditions for favouring ketone reduction, obtaining also in this case enantiomerically enriched alcohols.

The co-occurrence of different enzymatic activity in the whole cells of yeasts led us to investigate the possibility to carry out multi-steps enzymatic reactions using a single yeast. The target was to develop an alternative chemoenzymatic synthesis of Bimatoprost and Latanoprost utilized in the glaucoma treatment, which utilizes a stereoselective sequence of biotransformations catalyzed by enzymes belonging to a single microorganism. The best results were obtained using another yeast of the *Pichia* family: *P. anomala* CBS 110 that gave Lactondiol B or Lactondiol L, respectively key intermediate for the preparation of prostaglandins analogues Bimatoprost and Latanoprost, only changing the co-subtsrate for the regeneration of co-factors.

The good results obtained with whole cells of *P. glucozyma* led us to isolate the enzyme(s) responsible for the reduction of the previous substrates, in order to overexpress and characterize it (them).

The genome of *Pichia glucozyma* was sequenced and annotated by Baseclear company (Leiden, The Netherlands). Whole cells of *P. glucozyma* were able to efficiently reduce benzil, a substrate not easily reduced with high enantioselectivity by most of the known ketoreductase. Among all the dehydrogenases present in this yeast, only one was described as a "benzil-reductase"; the corresponding gene named *KRED1-PGLU* (GeneBank accession number KR080472) was amplified from the genomic DNA of *P. glucozyma* and cloned into BL21DE3STAR *E. coli* cells (pET 26 b+ expression vector) as a His-tagged construct. The pure protein was obtained (15.6 mg/g_{cells}) with one purification step through a Nickel Affinity Chromatography.

After biochemical characterization (pH, temperature profiles, k_m, k_{cat}) the pure protein was investigated for the reduction of different aromatic ketones using an enzyme-coupled system (glucose-glucose dehydrogenase – GDH – from *Bacillus megaterium*) for cofactor recycling. KRED1-Pglu showed high activity and selectivity for the production of (*S*)- alcohols, but lower activity towards acetophenone and derivates such as β- and γ-ketoesters compared to whole cells of *P. glucozyma*. Surprisingly (*R*)-phenylethanol, the opposite enantiomer obtained using whole cells was obtained; these results indicate the existence of at least another enzyme in *P. glucozyma* able to reduce these substrates with opposite enantioselectivity.

A deep study to understand the correlation between electronic and steric effects on the activity and enantioselectivity for the reduction of various mono-substituted acetophenones with KRED1-Pglu was carried out.

Reaction rates of the reduction of *meta*- and *para*-derivatives were mostly consistent with the electronic effects described by σ-Hammett coefficients; since unsubstituted acetophenone has been reduced to (*R*)-1-phenylethanol (95% ee), whereas all the other *meta*- and *para*-derivatives have been reduced to (*S*)-1-phenylethanols, generally with good enantioselectivity, molecular modelling studies in order to determine the substrate binding mode are still in progress. *Otrho*-substituted acetophenones were poorly reduced, with the remarkable exception of derivatives with low steric impact (F and CN).

Finally, the diastereoselective reduction of secodione for obtaining the key intermediate for the industrial relevant synthesis of Levonorgestrel was studied. Although

KRED1-Pglu was completely regio- and stereoselective, the best results in terms of productivity were obtained using whole cells of *P. minuta* CBS 1708 as a biocatalyst.

Acknowledgments

Mi sembra ieri di aver iniziato questa nuova e per me sconosciuta avventura ed invece sono già passati tre anni.. tre anni di sudore e fatica, ma di immense soddisfazioni che hanno arricchito le mie conoscenze, le mie passioni, i miei sogni.. Desidero scrivere questi ringraziamenti in un modo un po' particolare associando ad ognuno di voi un elemento chimico che più degli altri vi rispecchi:

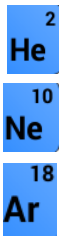
Non posso che partire dal primo elemento della tavola periodica, l'idrogeno, ed associarlo al Professor Molinari, che per primo ha creduto in me dandomi la possibilità di lavorare a questo progetto. Esplosivo nelle sue idee ha saputo trasmettermi l'amore per questo lavoro e più in generale la passione per la ricerca e la conoscenza..

Il titanio associato a colui che è in questi anni mi ha sempre sostenuto e dato forza, a Diego prezioso maestro ed aiuto.. Ricordo ancora la mia prima piastra Petri e tu che mi sgridavi perché mi tremavano le mani.. Grazie per avermi strappato un sorriso anche nelle giornate più grigie e grazie perché so che anche da lontano potrò sempre contare su di te..

Grazie ai miei colleghi di lab, che più che colleghi sono veri e propri amici, l'ossigeno che respiro ogni giorno.. Grazie a Valerio, Benedetta, Imma e Paolo per tutte le risate, gli sfoghi, i consigli, le intese anche solo con uno sguardo.. non potevo trovare compagni di viaggio migliori.. Grazie anche ai "piccolini" appena arrivati: Danila, Gabriele, Luca e Cesare.

Rutenio, rodio e palladio rappresentano per me la catalisi chimica, il sicuro, la certezza che una reazione avvenga, non posso che associarli alle tre certezze di questi anni, tre persone meravigliose che ho avuto la fortuna di incontrare lungo il mio cammino, Tiziana, Silvia e Stefania. Grazie per tutti i consigli, per avermi ascoltato, fatto riflettere e sempre spronato ad andare avanti anche innanzi alle difficoltà

che mi sembravano insormontabili.. Da me avrete sempre tanta ammirazione e stima sia dal punto di vista professionale che personale.



A Lucia, Andrea e alla Professoressa Paola Conti cui ho associato gli elementi nobili della tavola periodica come nobili e saggi sono stati tutti i vostri consigli..

Ai miei genitori, cui non potevo che associare l'elemento più importante dell'intera chimica organica, il carbonio.. Grazie per averci creduto anche questa volta, nonostante vi sembrasse l'ennesimo salto nel vuoto da parte mia. Come cita la mia frase iniziale "Per essere felici bisogna avere coraggio" ed io voglio essere felice tentando di diventare nella vita ciò che desidero.. Spero che questo traguardo vi renda orgogliosi di me..

All'oro della mia vita, la mia preziosa Diletta, l'altra metà di me senza la quale non sarei come sono, pregi e difetti.. Anche se saremo lontane rimarrai tra le persone più importanti della mia vita e sempre lo sarai..

Ai miei più cari amici, Christian, Valentina, Elisa, Mirko, Laura, Fabio, Emanuela, Simone e Simona che da sempre rappresentano l'omestasi della mia vita, il mio equilibrio.. Grazie perché mi fate sentire sempre importante e speciale anche con le mie mille insicurezze ed il mio basale sentirmi inadeguata.. Posso affermare con certezza che voi siete i fratelli che ho scelto!

Grazie ad Elisabetta, Laura, Marta e Vero, i miei elementi del buon umore che da anni condividono la mia stessa passione per la danza.. credo che il profumo del legno del palcoscenico, le luci che si accendono, lo spettacolo che comincia, la fatica,

l'emozione e la piacevole stanchezza saranno tra le sensazioni che più mi mancheranno quando sarò lontana..

A Michele: io, te e la promessa di rimanere insieme sempre per sempre come l'^{235}U e l'^{238}U, isotopi inserapabili..

Grazie alla Professoressa Rossi ed al Dott. Fulvio Benigni per aver contribuito a rendere possibile questo progetto di tesi, alla Professoressa Paradisi per aver accettato di ospitarmi presso i suoi laboratori durante il mio periodo post-doc, a chi seppur lassù, mi sta accanto e mi protegge, ed infine grazie a tutte le persone che durante questo periodo mi hanno dedicato il loro tempo, la loro pazienza ed i loro sorrisi..

Grazie a tutti voi.