Ph. D. thesis of Susanna Bertuletti

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Exploiting oxidoreductases, hydrolases, and transaminases as stereo- and regio-selective biocatalysts in organic synthesis.

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A Elena, Ottavio e Margherita,
perché sono la mia luce.

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Preface

The leitmotif of this doctoral thesis consists in the uses of biocatalysis in the organic synthesis of compounds of pharmaceutical interest. Before describing the main aspects of this work, therefore, two key questions must be addressed: what is biocatalysis? And how can it be a useful tool in organic synthesis?

Concerning the first point, biocatalysis can be defined as the use of entire living systems or of their parts (e.g., enzymes) to speed up chemical reactions. In a way, thus, all living systems are chemical machineries whose survival depends on the work of biocatalysts. And here the answer to the second question comes straightforward. The reactions carried out by biocatalysts in living systems are highly chemo-, regio- and stereo-selective, and they occur in conditions which are absolutely mild and eco-friendly: aqueous environment, low temperature, ambient pressure. For an organic chemist, such reactions can be looked at with a lot of interest, as they respond to the increasing needs of making chemistry more sustainable and eco-compatible, thus going from traditional chemistry to the so-called green chemistry. Particularly, for asymmetric synthesis, enzymes can become a useful and versatile tool for the synthetic organic chemist and indeed three of the enzyme classes pointed out by the International Union of Biochemistry[1,2] (namely oxidoreductases, hydrolases and lyases) represent to date the most applied examples.[3]

Following a brief introduction, the main focus of this thesis will follow, that is the use of oxidoreductases to perform selective synthesis or modification of compounds of pharmaceutical interest. In this framework, two main aspects have been addressed: enzymes have been used either to modify their natural substrates of medicinal interest or to investigate their ability to perform selective biotransformations on promiscuous substrates. Indeed, enzymes can work not only on their natural substrates, but also on exogenous molecules, thus revealing to possess a promiscuous catalytic action. The ability of enzymes to accept substrates differing from their natural ones, *alias* the substrate promiscuity of enzymes, is a matter of interest for the organic chemist. As a matter of fact, it implies that the relatively narrow panel of natural substrates, which could be object of biocatalytic transformations, can be widely expanded, at least as much as the enzymes' promiscuity permits. Moreover, exploiting the powerful tools made available by molecular biology, virtually all organic transformations could be carried out by biocatalysts, optimized by enzyme engineering.[4,5]

The first chapter of this thesis will deal with biocatalysis in the pharmaceutical industry. Specifically, the pros and cons of biocatalysis as a synthetic tool will be examined in the frame of green chemistry and of the synthesis of APIs (active pharmaceutical ingredients).

The second chapter will give an outline of the enzymes used in this work (namely, hydroxysteroid dehydrogenases, enoate reductases, amine transferases, and lipases), with focus on their 3D structure

and on their catalytic mechanism. Regarding hydroxysteroid dehydrogenases and amine transferases, the pharmaceutical relevance of the substrates they can work on will be pointed out.

Eventually, the experimental part is discussed, divided by topics and also by published papers.

Conclusive remarks are given in the end of the thesis, to sum up the main results and give perspectives about the future work that may herein be done.

Publications

Paper I: "Hydroxysteroid Dehydrogenases: An Ongoing Story."

Ferrandi, Erica Elisa; Bertuletti, Susanna; Monti, Daniela; Riva, Sergio.

European Journal of Organic Chemistry, **2020**, 2020(29), 4463-4473.

https://doi.org/10.1002/ejoc.202000192.

Paper II: "Insights into the Substrate Promiscuity of Novel Hydroxysteroid Dehydrogenases."

Bertuletti, Susanna; Ferrandi, Erica Elisa; Marzorati, Stefano; Vanoni, Marta; Riva, Sergio; Monti, Daniela.

Advanced Synthesis and Catalysis, **2020**, 362, 2474-2485.

https://doi.org/10.1002/adsc.202000120.

Paper III: "Biocatalytic Approaches to the Enantiomers of Wieland-Miescher Ketone and of Its Derivatives"

Bertuletti, Susanna; Bayout, Ikram; Bassanini, Ivan; Ferrandi, Erica Elisa; Bouzemi, Nassima; Monti, Daniela; Riva, Sergio.

European Journal of Organic Chemistry, **2021**, 2021(29), 3992-3998.

https://doi.org/10.1002/ejoc.202100174.

Paper IV: "Synthesis of ω-Muricholic Acid by One-Pot Enzymatic Mitsunobu Inversion using Hydroxysteroid Dehydrogenases."

Bertuletti, Susanna; Ferrandi, Erica Elisa; Monti, Daniela; Fronza, Giovanni; Bassanini, Ivan; Riva, Sergio.

ChemCatChem, **2021**, early view

https://doi.org/10.1002/cctc.202101307

The whole text of the published articles can be found in the end of the thesis in Appendix I.

Abbreviations

Ac:	Acyl ($CH_3C(=O)$-)
AcOEt:	Ethyl Acetate
ACN:	Acetonitrile
ACS:	American Chemical Society
ADH:	Alcohol Dehydrogenase
AE:	Atom Economy (or Atom Efficiency)
AKR:	Aldo-Keto Reductase
API:	Active Pharmaceutical Ingredient
ATA:	Amine Transferase
ATP:	Adenosine TriPhosphate
Bu:	n-butyl
CA:	Cholic Acid
CD:	Circular Dichroism
CDCA:	Chenodeoxycholic Acid
CPME:	CycloPentyl Methyl Ether
DCM:	Dichloromethane
DHCA:	Dehydrocholic Acid
DMSO:	Dimethyl Sulfoxide
EWG:	Electron Withrawing
FC:	Flash Chromatography
FDA:	Food and Drug Administration
FDH:	Formate Dehydrogenase
FMN:	Flavine Mononucleotide
FXR:	Farnesoid X (nuclear) Receptor
GDH:	Glucose Dehydrogenase
GlutDH:	Glutamate Dehydrogenase
GPCR:	G-Protein Coupled Receptor
GSK:	GlaxoSmithKline
HCA:	Hyocholic Acid
HDCA:	Hyodeoxycholic Acid
HSDH:	Hydroxysteroid Dehydrogenases

ISPR:	*In Situ* Product Removal
IUBMB:	International Union of Biochemistry and Molecular Biology
LCA:	Lithocholic Acid
LDH:	Lactate Dehydrogenase
MCA:	Muricholic Acid
MDR:	Medium Chain Dehydrogenases
NAD(P)(H):	Nicotinamide Adenine Dinucleotide (Phosphate) (reduced)
NBS:	N-Bromo Succinimide
NCBI:	National Center for Biotechnology Information
OPR:	12-OxoPhytodienoato Reductase
OYE:	Old Yellow Enzyme
ω-TA:	ω-Transaminase
PB:	(Potassium) Phosphate Buffer
PDB:	Protein Data Bank
PETNR:	PentaErythritol TetraNitrate Reductase
PPL:	Porcine Pancreatic Lipase
Py:	Pyridine
SDR:	Short Chain Dehydrogenases
T2DM:	Type 2 Diabetes Mellitus
TBME:	t-Butyl Methyl Ether
TGR5:	Takeda G-protein coupled Receptor 5
U:	Enzymatic Activity Unit
UCA:	Ursocholic Acid
UDCA:	Ursodeoxycholic Acid
WMK:	Wieland-Miescher Ketone

A: Introduction

Chapter 1

Biocatalysis in the pharmaceutical industry: a green alternative for sustainability

The first chapter of this thesis is conceived as an introduction for those who may wonder why one should discuss about biocatalysis when dealing with the synthesis of APIs (Active Pharmaceutical Ingredients). This overview is intended to be neither complete, nor exhaustive, but rather as a first glance of how biocatalysis can be an effective tool for the green organic synthesis of pharmaceuticals and other fine chemicals. As such, the following sections will build this chapter: at first, a definition of biocatalysis will be given, accompanied by prejudices that hamper its first-choice-use in organic synthesis; an analysis will then be made regarding the "greenness" of biocatalysis (is it just claimed or is it real?); some representative examples of the application of this tool in the industrial synthesis of drugs will be pointed out; a few words will be dedicated to other fields of industrial use of biocatalysts; in the end, a perspective will be given about what could be ameliorated to implement this tool in the large scale (up to tons) synthesis of fine chemicals.

1.1. Biocatalysis: a valuable tool for the organic chemist

1.1.1. What is biocatalysis?

The term biocatalysis refers to the use of enzymes or entire living organisms to perform chemical transformations.[6] Indeed, the use of enzymes as catalysts just constitutes an alternative to metals, organic molecules, photocatalysts or other kinds of catalysis, thus expanding the scope of the toolbox available for the organic chemist.[7]

Biocatalysis takes its inspiration by nature: indeed, living organisms can be observed by a scientist as very complex chemical machineries that perform a vast multitude of reactions, which would be rather more difficult if they were not aided by enzymes. The chemist can just look at them with admiration or, in addition, use them as a source of inspiration to perform exquisitely green and selective processes.

1.1.2. What is a(n) (enzymatic) biocatalyst?

We are exploring the framework of the biocatalytic world. To start doing that more consciously, it is important to have a basic idea of what our catalysts are. Enzymes are biological macromolecules that are composed of aminoacids, being able to catalyze biochemical reactions ranging from nutrient digestion to xenobiotic detoxification, comprising many biochemical reactions relevant to life. Even though they are constituted by just 20 kinds of aminoacids, their properties can vary enormously depending on their primary structure (i.e., the sequence of aminoacids constituting the given protein)

and the 3D-structure they possess (yielded by an ensemble of the so-called secondary, tertiary, and quaternary structures, see Figure 1.1 as an example).

The chiral 3D structure of enzymes is responsible for three major kinds of selectivities of these biocatalysts in their reactions: chemoselectivity, *alias* the ability to discriminate between many diverse reactive functional groups within the same molecule; regio- and diastero-selectivity, which imply that it is possible for them to distinguish chemically identical moieties in a given substrate and make them react selectively; enantioselectivity, given by the intrinsic chirality of enzymes (constituted only by L-aminoacids). [8]

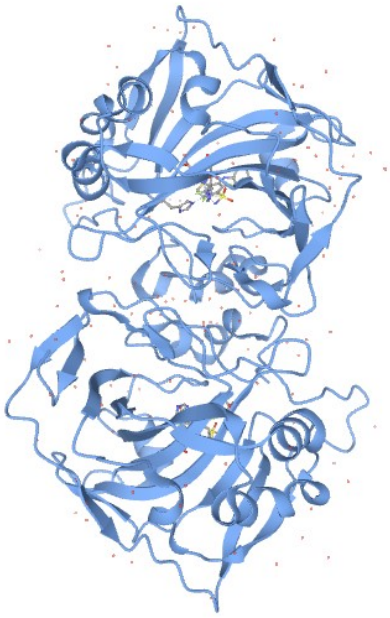


Figure 1.1: 3D structure of an enzyme (carbonic anhydrase IX) crystallized with an inhibitor (PDB code 4Q0L). [9]

It has been estimated that thousands of enzymes exist, only a fraction of these being known.[10] The enzymes that are known have been grouped by the IUBMB (International Union of Biochemistry and Molecular Biology) back in 1992 into six main classes depending on their reactivity, namely: oxidoreductases (performing redox reactions), transferases (transferring functional groups from a donor to an acceptor), hydrolases (hydrolyzing bonds), lyases (catalyzing electrophilic additions to unsaturated bonds of various kind), isomerases (racemizing, epimerizing or rearranging molecules) and ligases (forming or cleaving bonds).[11] Noteworthy, virtually all organic reactions could be catalyzed by enzymes,[12] even though nowadays the most industrially applied enzymes belong to the first three of the aforementioned six classes.[13]

A prejudice: what hampers the use of enzymes in organic synthesis?

At a first glance, enzymes look like fantastic catalysts: they are selective, they have already been optimized by nature in millions of years of evolutionary history and so on. Thus, what does it hamper their application in organic synthesis? It is here crucial to distinguish between real limitations and prejudices associated to the biocatalytic world.

A rather complete overview about the commercial use of biocatalysts was given 20 years ago by Rozzell,[14] and it will be briefly recapitulated here, starting with a short list of prejudices:

- First of all: enzymes are too expensive. Actually, this depends on the cost of the final product and on the purity required for the enzyme: crude extracts can indeed be rather inexpensive.
- Enzymes are too unstable. This is certainly true for some enzymes, but as the price, also the instability is something relative. Indeed, also reagents of common use (associated with fire danger) such as butyllithium or hydrogen are unstable. There are, however, ways of making them stable under given conditions, which, in the case of enzymes, can be pretty advantageous: atmospheric pressure, room temperature, aqueous buffer. Moreover, enzymes can be immobilized, as it will be pointed out later, making them much more stable and recyclable.
- The productivity is low. Actually, enzymes tend to accelerate the reactions they catalyze by 10^8-10^{10} times with respect to their non-catalyzed counterparts, this is certainly not a low productivity, and it is higher than that of most chemical catalysts.[8,15,16]
- Enzymes work only in their natural environment and on their natural substrates, thus they do not catalyze industrially meaningful reactions. This is false under all viewpoints: a wide number of literature examples reports of enzymes used in organic solvents on substrates also largely differing from their natural ones. This yielded the possibility of achieving industrially relevant enzymatic syntheses, as it will be pointed out later.

Leaving behind false beliefs about biocatalysis, we can start an analysis of its effective pros and cons. An excellent analysis in this respect was carried out by Prof. Kurt Faber,[8] and it will be shortly outlined in the following.

As such, the real drawbacks of biocatalysis are the following:

- Enzymes occur naturally in just a single enantiomeric form, thus, to reverse the stereochemical outcome of a given reaction it is compulsory to engineer the enzyme. It is not feasible to simply take the catalyst's mirror image as it can be done for a chemical catalyst.
- Enzymes have narrow operational parameters, therefore if a reaction does not perform well, it is not easy to make it work better by just changing solvent, pH or temperature without creating problems to the enzyme's stability.
- The cofactor promiscuity of enzymes is extremely rare, thus making it difficult to use more stable and less expensive chemical mimetics of natural cofactors, such as NAD(P)H, ATP, and others.

- Substrate and/or product inhibition are often encountered in biocatalysis, which require fine-tuned reaction engineering approaches such as timely addition of the substrate or *in situ* product removal (ISPR).
- Additionally, enzymes can cause allergies. However, this disadvantage can be severely limited by treating them with adequate precautions.

These limitations are well compensated by the mostly cited advantages of enzymes, namely their aforementioned selectivity, the mild operative reaction conditions which make them ideal for green applications in industry, the possibility to recycle the cofactors, their high catalytic efficiency and their intrinsic eco-sustainability and eco-compatibility.[6,13,17-19]

1.2. Biocatalysis is green: "is this the real life? Is it just fantasy?"

1.2.1. The 12 principles of Green Chemistry

It is commonly stated that biocatalysis is quintessentially green. However, how can this opinion be scientifically supported? Several years ago, Lord Kelvin stated: "To measure is to know". Then, let us measure how "green" biocatalysis is through some parameters and metrics. First of all, however, we must have clear in mind what "green" means, when it is referred to chemistry. Actually, the green chemistry "dogmas" were introduced in 1998, more than twenty years ago, by Anastas and Warner, with the so-called 12 principles of Green Chemistry (summarized in Figure 1.2).[20]

1. **Prevention**. It is better to prevent waste than to treat or clean up waste after it is formed.
2. **Atom Economy**. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. **Less Hazardous Chemical Synthesis**. Whenever practicable, synthetic methodologies should be designed to use and generate substances that pose little or no toxicity to human health and the environment.
4. **Designing Safer Chemicals**. Chemical products should be designed to preserve efficacy of the function while reducing toxicity.
5. **Safer Solvents and Auxiliaries**. The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary whenever possible and, when used, innocuous.
6. **Design for Energy Efficiency**. Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7. **Use of Renewable Feedstocks**. A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
8. **Reduce Derivatives**. Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9. **Catalysis**. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. **Design for Degradation**. Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. **Real-Time Analysis for Pollution Prevention**. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. **Inherently Safer Chemistry for Accident Prevention**. Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Figure 1.2: The 12 principles of Green Chemistry, picture created by Anastas and Eghbali.[21]

These principles can be seen as a guide to develop processes meant to prevent any kind of waste, with the aim of sustainability starting at the molecular level. Whether applied as a whole, they can truly lead to both economic and environmental advantages, as they strive both to reduce the number of steps and reagents/byproducts involved in a synthesis and to prevent waste generation, leading to reduced costs also in the waste disposal phase, which by itself is not a trivial matter.[7] In few words, green chemistry aims at re-designing (or *de novo* designing) chemical products and processes in order to minimize the presence and the generation of hazardous substances and of waste, so as to avoid the subsequent need to use decontamination processes.[22]

1.2.2. Green Chemistry through parameters: how green is a process?

As it is possible to see, these principles are a guideline, but they only allow a qualitative approach to green chemistry, rather than a quantitative one. In order to apply these principles and to compare the greenness of a process with respect to another one, e.g., in the attempt of process optimization, it became clear that there was the need of some parameters that might allow to define the greenness. Many efforts have been done in this respect, mainly by Roger Sheldon, who introduced the E-factor to quantify waste in 1992,[23] and by Barry Trost, who invented the concept of Atom Economy in 1990.[24]

The E-factor is a number that states how many kg of waste material are generated by a given process to produce 1 kg of the desired product. For example, if the E factor of a given process is 50, it means that the process under analysis produces 50 kg of waste for 1 kg of target substance. Usually, the major contribution to this number is given by the solvents, which can be partly recycled. In the past, water was not included in these calculations. However, today the growing trend is that of taking in consideration also water, as it is often contaminated, and it has to be treated. The E-factor helps in underlining the critical aspects of a process, thus allowing for punctual ameliorations.[25]

The Atom Economy (or Atom Efficiency, AE) is another key parameter, expressing how many atoms of the reagents end up building the product's skeleton. Mathematically speaking, it is given by the ratio between the molecular weight of the product and the sum of the molecular weights of the reagents. The closer this number is to 1 (or 100%), the more efficient the process is in terms of atom economy. For example, multicomponent reactions or cycloadditions in general feature a very high AE.[21]

Taken together, these (and other) parameters can lead to highlight which parts of the process could be improved, e.g., in terms of solvent use, number of synthetic steps, analytical methods, and catalysts.

However, it is important to be realistic and keep in mind that no chemical process can ever be really green: everything has a cost in terms of energy and materials and waste is always generated. So, when discussing about green chemistry, quantification is fundamental and a self-critical comparison with other methods at disposal is always opportune.[26]

1.2.3. Is biocatalysis green?

The aforementioned parameters allow to evaluate more consciously whether biocatalysis is green or not. Indeed, many papers, including those of the fathers of Green Chemistry, point out the conclusion that biocatalysis is a green tool in chemical synthesis.[3,25,27–29]

Enzymes work under mild reaction conditions (that is, ambient temperature and pressure, water, physiological pH), they are biodegradable and thus eco-sustainable and eco-compatible, they come from renewable feedstocks (mostly microorganisms) and, last but not least, they are designed to be selective by Nature. So, they can catalyze even extremely difficult reactions in a very selective fashion and in a low number of steps, avoiding the use of tedious (albeit useful) protecting group chemistry.[25] All of these features should be enough to make them look attractive from a green perspective but let us analyze enzymes according to the 12 principles of green chemistry.

The aforementioned principles of Green Chemistry have been recapitulated in an efficacious acronym, PRODUCTIVELY, which states for: Preventing waste; Renewable materials; Omit derivatization steps; Degradable chemical products; Use safe synthetic methods; Catalytic reagents; Temperature, pressure ambient; In-process monitoring; Very few auxiliary substances; E-factor, maximize feed in product; Low toxicity of chemical products; Yes, it is safe.[30]

Sure enough, enzymes allow to prevent waste by reducing the number of synthetic steps and auxiliaries required, they are catalytic, and they come from renewable sources (mostly bacteria, yeasts, and fungi), they are degradable and eco-compatible, and they work in mild and safe conditions. The critical points about the acronym, i.e., in-process monitoring and low toxicity of the chemicals, do not directly depend on the enzyme used, but rather on the synthetic process that is considered. As such, apparently enzymes seem to agree with the statements of the acronym PRODUCTIVELY, which has led them to be actually used in the industry of food, cosmetics, bulk chemicals and, relevant to us, pharmaceuticals.

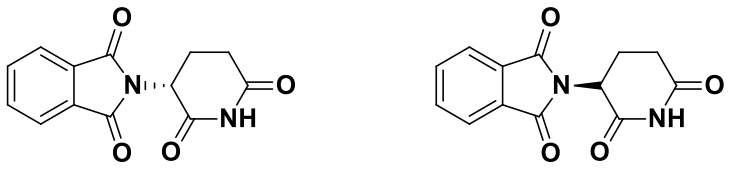
1.3. Biocatalysis in the pharmaceutical industry

Up to now, we have seen that enzymes are selective and green catalysts (paragraphs 1.1 and 1.2). The question we need to address at this point is: how might these properties be desirable in the pharmaceutical industry? We have already addressed the second point, that is, green chemistry is needed to move towards a sustainable and circular economy. But why is selectivity a crucial factor as well?

1.3.1. Thalidomide and the need for asymmetric synthesis

The selectivity of enzymes is not a novelty. Indeed, it was already known at the end of the XIX century by the notorious chemist Emil Fischer, which discussed about the lock-and-key model in 1894 and the ability of these molecules to perform asymmetric synthesis in 1898.[31,32]

It is also well known that living organisms are chiral environments, thus within a living being two enantiomers of the same molecule must be regarded as two distinct species. This may not be so evident in all cases: for the sake of clarity, the two enantiomers of ibuprofen are both active, even though one is more effective than the other. However, in some cases the effect of one enantiomer can be positive, whereas the other one can trigger detrimental processes in our body. This is the basis of the story of Thalidomide, a well-known drug that was sold half a century ago. The (*R*)-enantiomer can cure nausea and has calming and hypnotic effects, while the (*S*)-counterpart became infamous due to its high teratogenicity, which caused the birth of about 10 thousand children with severe health issues (the structures of the two mirror images are shown in Figure 1.3).[33,34]



(*R*)-Thalidomide (*S*)-Thalidomide

Figure 1.3: The two enantiomers of Thalidomide. This drug readily racemizes within our body, thus the administration of the (R)-enantiomer alone would not prove efficacious to prevent toxic effects.

This chapter of the drug market's history led the regulatory agencies to be significantly stricter about the sale of racemic drugs, with new FDA regulations being introduced 30 years ago (in 1992).[35] This does not mean that racemic drugs are prohibited in their entirety, but they are much more regulated and must pass a more troublesome pre-clinical development.[36] This discourages their use, leading to the need of effective means to perform asymmetric synthesis, rather than racemate resolution (which would imply the discard of 50% of the desired product).

1.3.2. Biocatalysis implementation in the pharmaceutical industry: what is wrong?

Asymmetric synthesis can be excellently performed by biocatalytic actors, but we have previously mentioned that enzyme usage poses some drawbacks.[37] Solving the issues arising from the use of such macromolecules is becoming easier and easier with the advances made in biotechnology, both in terms of more facile access to biocatalysts and of protein engineering, which led to the Nobel Prize in Chemistry assigned to Frances Arnold in 2018 for the development of efficient techniques for the directed evolution of enzymes. These techniques allow the optimization of enzymes, making them act as wished, so that they can be made much more stable or performant on molecules that they would not normally transform. Thus, virtually all disadvantages of these biocatalysts could in principle be solved, making them suitable for industrial applications without any sort of problems. This would be the ideal case.

The development of the "perfect" biocatalyst is still a challenging and rather long process, although it is getting faster thanks to the previously described biotechnological research advances. However, a

long and costly process development is of severe concern in the pharmaceutical industry. Changing the synthetic process of a drug leads to facing more of the aforementioned regulatory requirements, which becomes increasingly problematic after Phase 2, as there is a risk of impacting on the Phase 3 supplies. This could, in the worst case, invalidate the clinical trial of a drug.[29] As such, the ideal biocatalyst should be known and operative in the early phases of the drug discovery process before it is known if the drug has any hope to be marketed. This additional uncertainty in a process that already has a high attrition rate actually pushes the pharmaceutical companies to use well-known classic organic synthesis.[38] In addition to this, after the FDA approval, the residual patent life usually is of about 12 years, adding more barriers in the pharmaceutical industry to the use of alternative approaches.[39] Roschangar and others resumed this in a recent perspective article by means of the following meaningful picture (Figure 1.4).[29]

Figure 1.4: The main barriers to the implementation of green chemistry in the pharmaceutical industry, according to Roschangar, Sheldon and Senanayake.[29]

The use of biocatalysis in the pharmaceutical industry is hampered by problematics that are peculiar to this productive sector, and they must therefore be specifically addressed. As it was stated by Woodley and Pollard, "the ability of biocatalysis to reach its full potential in pharmaceutical synthesis will require cost-reduction techniques and complete integration with chemistry".[38]

1.3.3. Biocatalysis implementation in the pharmaceutical industry: what is right?

Albeit some barriers exist for the use of biocatalysis in the production of drugs, many marketed drugs can be produced or are already made exploiting enzyme catalysis. The highly complex structure of many drugs and their frequent similarity with natural products' skeletons make biocatalysis a very attractive tool for the green synthesis of such compounds. The stories mentioned in the following subsections regard very efficient process optimization, but, as it was outlined in the previous paragraph, in the future the first-choice implementation of enzymes as green catalysts could be desirable in the pharmaceutical industry. Indeed, many reactions which were considered challenging

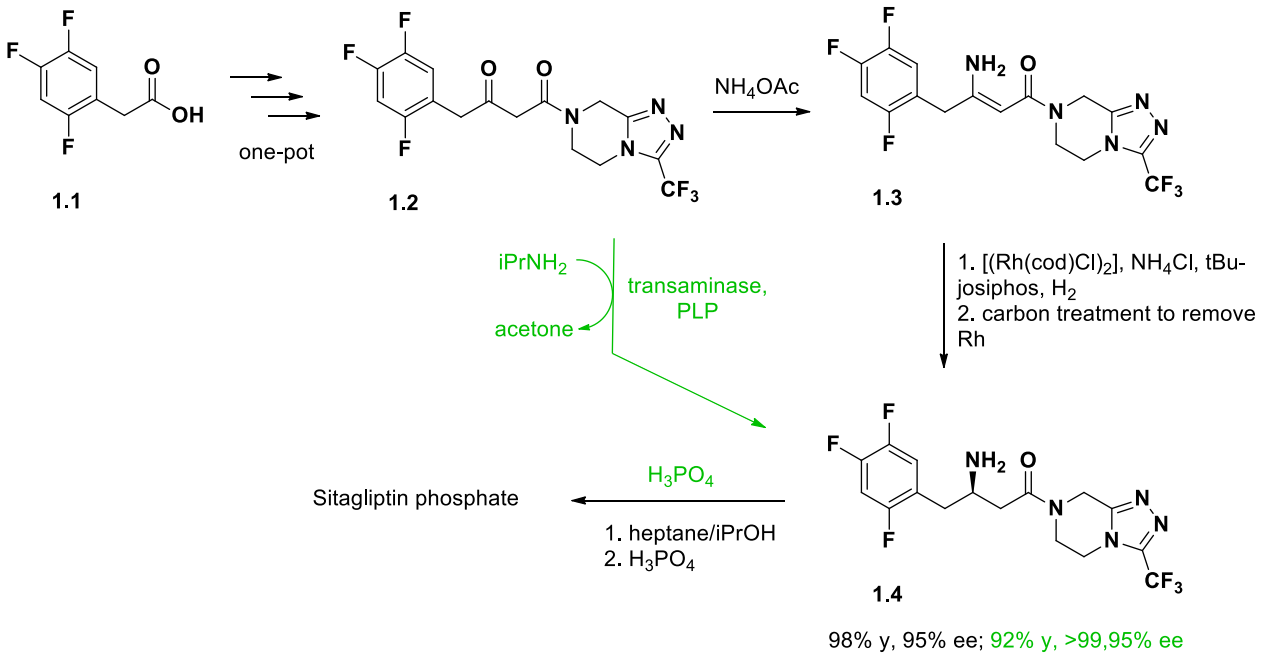
to this sector by the ACS Green Chemistry Institute Pharmaceutical Roundtable in 2007 can be rather easily performed by different enzymes (*inter alia*, chiral amine synthesis can be catalyzed by transaminases, asymmetric hydrocyanation by nitrilases, asymmetric hydrogenation of olefins by enoate reductases, C-H activations of alkyl and aromatic groups by monooxygenases and laccases, ester hydrolysis and amidation by lipases, chiral alcohol synthesis by alcohol dehydrogenases).[40,41] A recent GSK perspective (published on *Advanced Synthesis and Catalysis* in 2019) points out clearly how enzymes can be well exploited in a "cost effective and sustainable pharmaceutical manufacturing" or how they hold the potential to tackle synthetic challenges in a green way.[42]

The subsequent sections will therefore be dedicated to examples of problematic reactions performed industrially in a green way thanks to the aid of biocatalysts.

1.3.3.1. Chiral amine synthesis: Sitagliptin

Sitagliptin is the active ingredient in a drug used to treat type-2 diabetes and it is produced yearly in ton-scale.[43] The synthetic process to obtain this API has gone along a path of amelioration that has led to one of the most well-known examples of biocatalysis' and Green Chemistry's success in the drug manufacturing. Chiral amine synthesis has been regarded for long as a challenge for Green Chemistry by the ACS Green Chemistry Pharmaceutical Roundtable, but now it is amenable with biocatalysis.[40]

Indeed, as it is described in Scheme 1.1, the so-called 3rd generation process for Sitagliptin manufacturing (green arrows) exploits an engineered transaminase that allows to obtain a chiral amine directly from a carbonyl precursor, using a cheap amine donor (iso-propylamine) which is transformed into an easily removable by-product, acetone. Compound **1.4** is obtained in high yield and excellent enantiomeric excess (92% and 99,95%, respectively). The previous 2nd generation process (black arrows) had two disadvantages when compared to the new one: a) an additional synthetic step was needed, even though the synthesis was conducted in a one-pot fashion from compound **1.1** to intermediate **1.3**; b) a heavy metal catalyst was used in the final synthetic step, and as a consequence a very efficacious (and expensive) treatment was required to remove traces of it in the final product. Overall, the 3rd generation process proved to be greener in terms of reagents, solvents, and step economy. Moreover, higher yield (additional 13%) and productivity (increase of 53%) were obtained, accompanied by a reduction of waste by 19%.[44]



Scheme 1.1: Sitagliptin manufacturing and comparison between 2nd generation process (black arrows) and 3rd generation biocatalytic process (green arrows). The pathway from compound 1.1 to intermediate 1.2 is common to both processes and it is not shown for the sake of clarity.[44]

1.3.3.2. Stereo-controlled C-C bond formation and chiral alcohol synthesis: Atorvastatin

Statins are drugs that can be used to lower the level of cholesterol, constituting a class of inhibitors of HMG-CoA reductase. Their structural peculiarity resides in the side chain, which features two chiral alcohol moieties, as shown by the skeleton of the API atorvastatin.

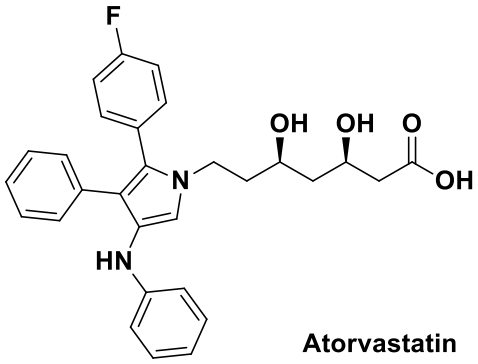
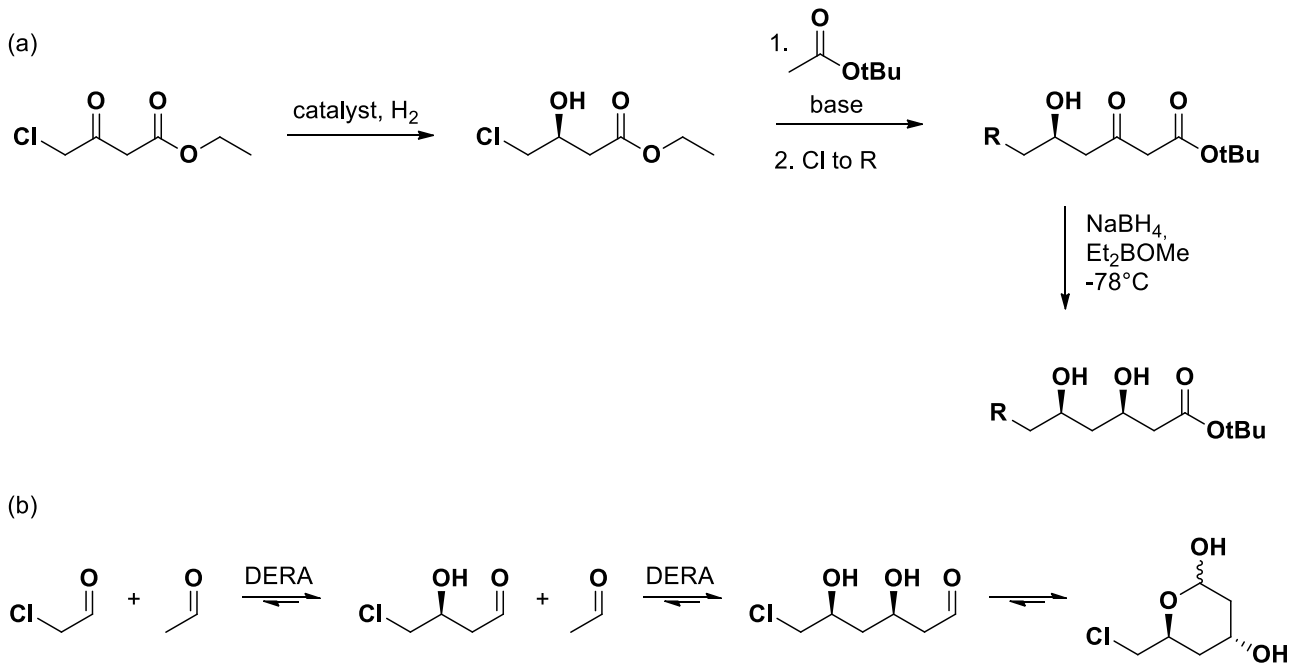


Figure 1.5: Structure of Atorvastatin (marketed by Pfizer under the name of Lipitor).

A significant effort has been posed on the synthesis of the side chain. This process was chemically performed according to the procedure shown in Scheme 1.2(a) and it featured several criticalities: low atom economy, use of metal catalysts, cryogenic conditions in the Claisen condensation and in the following reductive step to form the second chiral center (performed at -78°C).[45]



Scheme 1.2: Statin side chain by (a) chemical synthesis; (b) DERA-mediated one-pot synthesis. The schemes were adapted from "*Green Chemistry in the Pharmaceutical industry*", Chapter 6.[45]

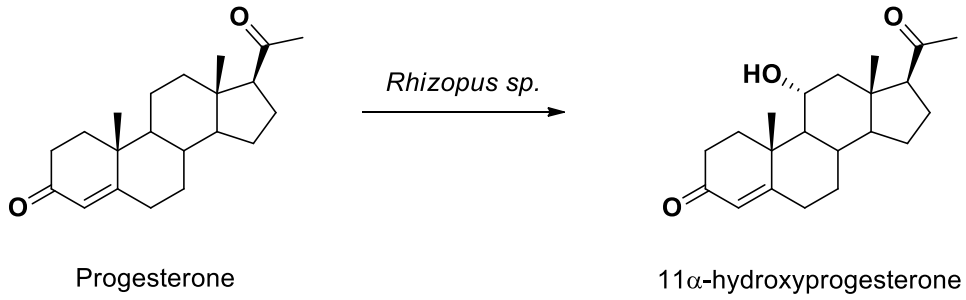
The enzymatic synthesis (Scheme 1.2(b)), on the other hand, allowed to obtain the side chain in one step starting from the cheap and readily available acetaldehyde and chloroacetaldehyde, thanks to the catalytic aid of the deoxyribose-phosphate aldolase (DERA) from *E. coli*. The exquisite selectivity of this enzyme allowed to obtain in a one-pot mode the C-C bond formations and two stereogenic centers.

The enzymatic pathway was optimized. An early reaction engineering (pH, temperature, enzyme loading, substrate concentration and ratio of aldehydes) allowed a 10-fold improvement in reaction efficiency, while enzyme engineering via directed evolution led to industrial applicability.[45]

It must be noted that other biocatalytic approaches to the side chain of Atorvastatin were investigated and are cited elsewhere.[46]

1.3.3.3. Aliphatic C-H activation: Cortisone

Cortisone is an anti-inflammatory steroid that is produced on the ton-scale every year. The industrial synthesis of cortisone was based on a 1st generation 30-step chemical process, which could be replaced thanks to biocatalytic methods by a 2nd generation 15-step chemo-microbial synthesis, the key step being the conversion of progesterone to 11α-hydroxy-progesterone (see Scheme 1.3). The much shorter and obviously also greener process led to a reduction of hydrocortisone's price by a factor of 50.[46,47]



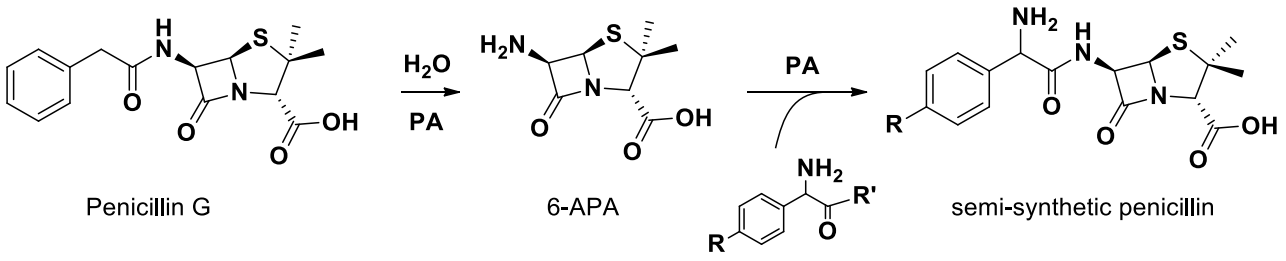
Scheme 1.3: Microbial transformation of progesterone to its monohydroxylated derivative functionalized on C11 with α configuration.[46]

Noteworthy, the microbial transformation is carried out with very high regio- and stereo-selectivity on a non-activated and aliphatic C-H bond, chemically identical to many others in the molecule.

1.3.3.4. Amide bond formation and hydrolysis: antibiotics (penicillins and cephalosporins)

7-ACA (7-aminocephalosporic acid) is a key intermediate for the synthesis of cephalosporins and it has been widely used to obtain semi-synthetic antibiotics. This precursor is industrially obtained via a two-step enzymatic process from the deacylation of cephalosporin-C, using a D-aminoacid oxidase and a 7β-(4-butoxybutanamido)-cephalosporanic acid acylase.[48]

Similarly, the synthesis of 6-APA (6-aminopenicillanic acid) is industrially applied thanks to the aid of penicillin acylase, which can be remarkably exploited both for the amide hydrolysis and for the new amide bond formation in a two-step sequence shown in Scheme 1.4.[49]



Scheme 1.4: Hydrolysis of penicillin exocyclic amide bond by penicillin acylase and subsequent reamidation. Penicillin G is produced via fermentation.[49]

Indeed, many other drugs and fine chemicals are made biocatalytically. A few mention-worthy ones are vitamin C, pregabalin, L-DOPA, vitamin B3, diltiazem, ephedrine, ibuprofen, ketoprofen and L-aspartate. Many more examples, together with these ones, can be found in extensive reviews in the literature.[18,48,50-54]

1.3.4. Future challenges for biocatalysis in the drug market

The previous sections have shown the huge potential that biocatalysis, with its "pros and cons", can offer to the drug manufacturing process. Just a quick glance at the economic and environmental advantages brought by this tool in industrial synthesis could encourage a lot its use.

Not only biocatalysis can help in the synthesis of already known or "soon-to-be-marketed" drugs, but it can also be a very effective tool for the generation of combinatorial libraries of, e.g., natural compounds with potential biological activity. Furthermore, it can be used indirectly for semisynthetic processes such as the one that affords a key precursor for paclitaxel synthesis. Moreover, it is applied in the study of drug metabolites.[46]

The crucial aspect that should be underlined at this point is that the advances in biotechnology were impressive in the last decades, both in terms of accessing to enzymes and of making them more suitable to organic synthesis through enzyme engineering and immobilization. Previously, the process had to be adapted to the available enzyme. Nowadays the enzymes can be adapted to the process. This makes the real difference, as we can exploit nature's tools, i.e., enzymes, and modify them according to our needs.[51] Moreover, with more and more studies being made on the substrate promiscuity of enzymes, many more molecules result to be suitable for biocatalytic modifications.

1.4. Biocatalysis in other fields: an overview

The use of resting cells for biotransformations dates back to 1897, when Buchner made the pioneering discovery that cell-free extracts from yeast could perform alcoholic fermentations. Since then, many advancements have been made, permitting to use enzymes in industrial transformations. The relevance of this topic was high also back in 2000, when a whole book entitled "*Industrial Biotransformations*" was published.[55]

Just one year later, in 2001, a review article was published on *Nature*, giving a perspective of industrial biocatalysis in the production of bulk or fine chemicals, including *inter alia* acrylamide, aspartame, t-Leucine, phenolic resins. Moreover, this review highlighted how commercial enzymatic processes share the advantages of being selective (no undesirable by-products), having high productivity, working with high substrate loadings, and not needing expensive cofactors (or exploiting efficient cofactor regeneration systems).[56]

More recent examples in the literature include a whole issue in *Current Opinion in Chemical Biology* of 2006[57] and a recent review article of 2014.[19] The former is dedicated to the roles of biocatalysis and biotransformations in modern research, giving an idea of how much potential biocatalysis holds. The latter is instead an overlook of how biocatalysis can be used in biofuel, detergent, textile, pulp and paper industries, food processing (mainly cheese manufacturing and production of high fructose corn syrup), pharmaceutical and cosmetics production.

As it was mentioned in the beginning of our digression (paragraph 1.1), it has been estimated that 25000 enzymes exist and only a few of them are currently known (about 6500 in 2018).[8] The world of biocatalysis remains therefore largely unexplored, with novel enzymes awaiting to be discovered, studied for their substrate promiscuity, and engineered to expand the already broad scope of industrial applications of biocatalysis and making it ever more economic and applicable.

1.5. Biocatalysis in the industrial world: what is next?

Enzymes have been used for centuries in some productive processes, e.g. in fermentations, but the potential they hold in modern chemical synthesis is being really understood in these years, since newer and better empirical models and modes for biocatalysts' stabilization have been discovered in the latest decades.[58] Indeed, the global enzyme market is in continuous expansion: according to the BCC (Business Communication Company), it is expected to increase from 5.5 billion dollars in 2018 to 7.0 billion dollars in 2023.[59]

A major contribution to the use of enzymes in the industries is given by the possibility of using them in a recombinant form, which is readily accessible, and by enzyme immobilization and directed evolution for meaningful stabilization to process conditions (organic solvents, higher temperatures, and wide pH ranges). In particular, directed evolution has paved the way to opening new routes in biosynthetic chemistry, as it was well demonstrated by the research team of Frances Arnold, who achieved C-Si and C-B bond formation, cyclopropanation, nitrene and carbene transfer and anti-Markovnikov alkene oxidations.[60]

As it was previously stated, enzyme engineering has taught us that now it is the biocatalyst that can be adapted to a process and not *vice versa*. Moreover, the development of a biocatalytic process still requires a significant optimization effort, mainly regarding reaction media and biocatalysis engineering (in terms of enzyme, substrate, reactor, substrate addition and product removal, and cascade implementations).[27]

Even though there have been several advancements in protein and bioprocess engineering, further improvements are still being waited for a wide-scale application of biocatalysis in industries. The main research areas that could prove to be helpful in this respect encompass both practical and computational efforts. The former research should focus on process standardization, downstream processing, new reactor, and continuous-flow technologies, whereas the latter could apply mathematical modelling and machine learning to improve/predict enzyme performances, to make protein engineering more predictable and to implement enzyme cascades in an efficient way.[61,62]

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Chapter 2

An overview of the enzymes used in this work

This second introductive Chapter will be devoted to the enzymes used in the framework of this thesis. As it was mentioned in the previous section, enzymes have been classified by the IUBMB into six main classes, outlined below (Table 2.1)

Enzyme Class Number	Enzyme Class Name	Catalytic function
E.C. 1	Oxidoreductases	Redox reactions
E.C. 2	Transferases	Transfer of functional groups
E.C. 3	Hydrolases	Bond cleavage via water addition
E.C. 4	Lyases	Bond cleavage without the use of water
E.C. 5	Isomerases	Rearrange atoms within a molecule
E.C. 6	Ligases	Join two molecules with energy expense

Table 2.1: enzyme classes as defined by IUPAC and IUB in 1972.[1]

The enzymes used in the present work belong to the first three enzyme classes. As such, the following sections will discuss oxidoreductases (Section 2.1, with focus on hydroxysteroid dehydrogenases and old yellow enzymes), transferases (Section 2.2, dealing with transaminases), and hydrolases (Section 2.3, where lipases will be described).

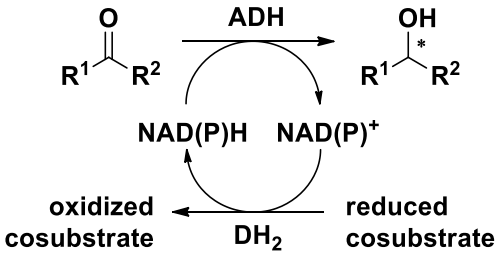
2.1. Oxidoreductases: what are they and what can they do?

This class is constituted by enzymes that are able to perform redox reactions. Alcohol Dehydrogenases (ADHs) are a relevant example of this category, being able to oxidize/reduce hydroxyl/oxo moieties of a wide variety of natural and non-natural substrates (e.g., glucose and other sugars, formate, acetone, steroids). These catalysts find wide application in organic synthesis and in industries.[2-4] In general, the reactions catalyzed by ADHs can be resumed as it is shown in Scheme 2.1.

Scheme 2.1: reactions catalyzed by alcohol dehydrogenases (left: oxidation; right: reduction).

Other relevant oxidoreductases are Baeyer-Villiger monooxygenases, amine dehydrogenases, enoate reductases, oxidases, and peroxidases. These enzymes are able to perform radical coupling, lactonization of ketones, reduction of biologically relevant moieties and of enone C=C double bonds, epoxidations, and many other interesting reactions.[5] The ability of enzymes to perform so many kinds of redox reactions is very attractive from a green chemistry perspective.[6]

These processes have a main drawback, *i.e.*, in order to work they need a stoichiometric amount of cofactor, which is often very expensive. This limitation can be partially overcome by coupling the reaction of interest with an apparatus to recycle *in situ* the cofactor. The regeneration system can be chemical, photocatalytic, electrochemical, but to date the best solution is given by an enzymatic approach. Indeed, another dehydrogenase that depends on the same cofactor and works on a cost-effective sacrificial substrate (such as formate or glucose) can be used as it is shown in Scheme 2.2.[7]



Scheme 2.2: *in situ* cofactor regeneration enzymatic apparatus. DH_2 can be, for instance, glucose or formate dehydrogenase. The reduced cosubstrate changes accordingly with the specificity of DH_2.

Another example of widely used oxidoreductases is given by laccases, which use oxygen as electron acceptor and find wide industrial application.[8] In any case, bio-oxidations and reductions of relevant intermediates are being studied with increasing interest.

Relevant to us, this subsection will deal mostly with hydroxysteroid dehydrogenases (for short and from now on, HSDHs), after a brief outlook at the subclass of redox enzymes they belong to, that is, short chain dehydrogenases (SDRs), and secondly with old yellow enzymes (for short, OYE).

2.1.1. SDRs: biological characteristics and catalytic functions

2.1.1.2. SDR structure: from primary to quaternary

Short Chain Dehydrogenases (SDRs) are a large superfamily of enzymes that mostly are NAD(P)(H)-dependent ADHs of microbial origin.[9,10,11,12]

As their name may suggest, these enzymes are characterized by a short primary aminoacid sequence of approximately 250 residues. The level of sequence identity among the SDR class is about 10-30%, which is barely enough to define enzymes as homologs. However, there are two consensus sequences that allow to identify an enzyme as an SDR, which have already been pointed out by early studies on this class almost 30 years ago by Persson et al:[13] a N-terminal Gly-X-X-X-Gly-X-Gly motif that is within the cofactor binding region, with the spacing among glycine residues corresponding to the one expected at the cofactor binding site in many DHs;[14] the catalytic triad Ser, Tyr, and Lys (among which Tyr is the most conserved), with special focus to the active site pattern Tyr-X-X-X-Lys.[9]

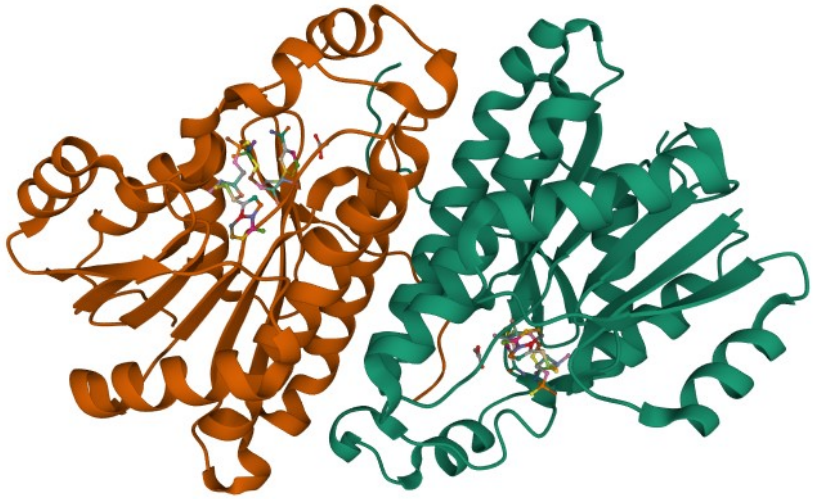


Figure 2.1: 3D structure of the SDR ADH from *Drosophila melanogaster* (PDB: 1MG5).[15]

While SDRs share a low primary sequence identity, they display in some way a highly conserved 3D structure, characterized by the presence of the so-called Rossmann-fold motif in the nucleotide binding region. This structural feature consists in an α/ß folding pattern, as it is shown in Figure 2.1 and in Figure 2.2, which is made by a central twisted ß-sheet motif (with six-seven strands) surrounded by a variable number of α-helices (three-four at each side of the sheet).[16] This motif provides the possibility to bind the cofactor, and also the specificity for either NAD(H) or NADP(H), depending on the presence

of specific charged residues (Asp for NAD(H)-dependent SDRs, and Lys and Arg for NADP(H)-dependent SDRs).[10,11,17] On the other hand, the substrate recognition is due to the highly variable C-terminal part of the proteins, that displays the catalytic triad as well.

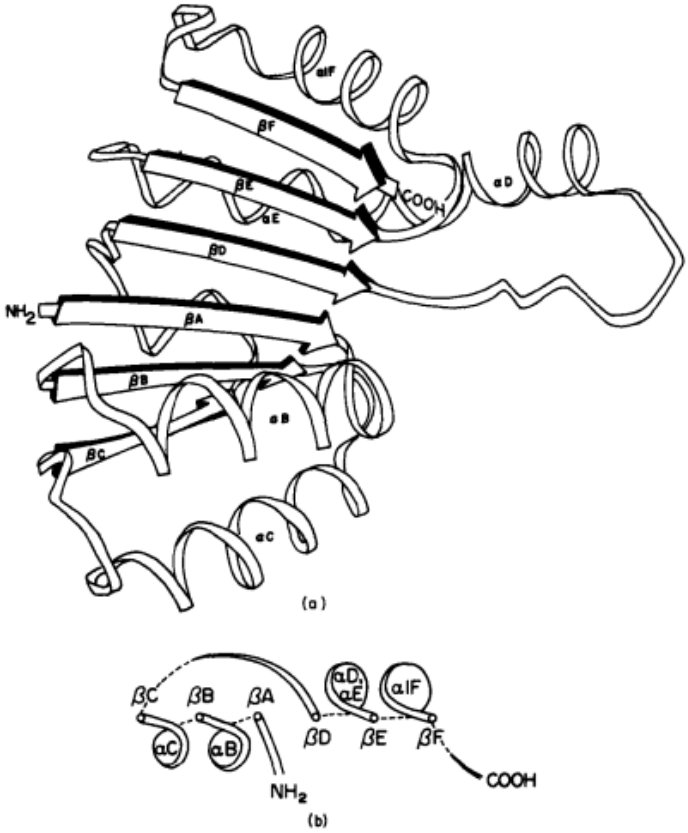


Figure 2.2: schematic representations of the Rossman-fold structural pattern as it was depicted by Rossman et al.[15]

The vast majority of SDRs tend to form oligomers, with a quaternary structure that is usually homodimeric or homotetrameric.

2.1.1.2. SDR catalytic action: a first glance

The mechanistic features of the SDR superfamily are preeminently linked to the catalytic consensus sequence Tyr-X-X-X-Lys that was mentioned above. The catalytic action of these residues is illustrated in Figure 2.3: Tyr acts as a catalytic acid in the reductive sense (or as a base in the oxidative sense), giving a proton to the alcoholate intermediate that results from the carbonyl reduction, while Lys has the dual roles of orienting the coenzyme and of lowering the pKa of Tyr via electrostatic effects.

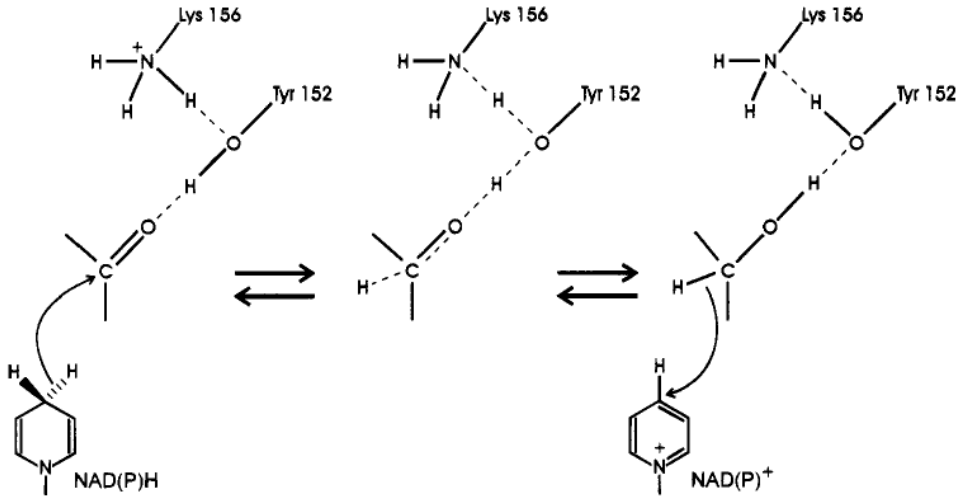


Figure 2.3: SDR catalytic action on carbonyl substrates (here, the acetone/2-propanol redox couple, Jörnvall et al).[12]

In many enzymes of the SDR superfamily, a widely conserved Ser residue has both a substrate positioning and an intermediate stabilizing role, but this is not always the case.[18] It has been demonstrated via site-directed mutagenesis that this Ser residue can be substituted by a Thr with no observable loss of activity.[19]

The mechanism has been proposed to be a *bi-bi* one. The cofactor enters before the substrate, then the substrate induces conformational changes (mostly in the C-terminal segment) prior to binding. The catalytic action happens and the oxidoreduction takes place. The product leaves the catalytic site prior to the cofactor.[10] However, a more detailed inspection of the reaction mechanism will be given in the following subsection 2.1.2.2, dealing with the catalytic mechanism of the 7α-HSDH from *E. coli*.

2.1.2. HSDHs: biology and catalysis

HSDHs will be analyzed in the following in terms of general outlook (2.1.2.1), 3D structures and mechanism of action (2.1.2.2), and medicinal relevance of their natural substrates (2.1.2.3).

2.1.2.1. What are they and what do they do?

HSDHs are NAD(P)(H)-dependent oxidoreductases that preeminently belong to the previously analyzed superfamily of SDRs, although many of them, which will not be considered in our analysis, belong to the superfamilies of aldo-keto reductases (AKRs) and medium-chain dehydrogenases (MDRs).[20]
Hydroxysteroid dehydrogenases are alcohol dehydrogenases that accept and transform steroidal compounds as natural substrates (Figure 2.4, gonane, *alias* cyclopentanoperhydrophenanthrene nucleus, accompanied by side chains).

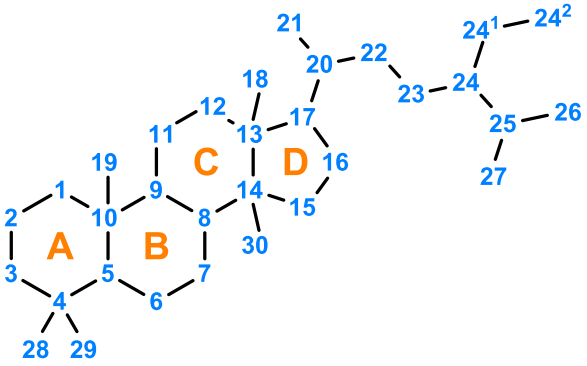
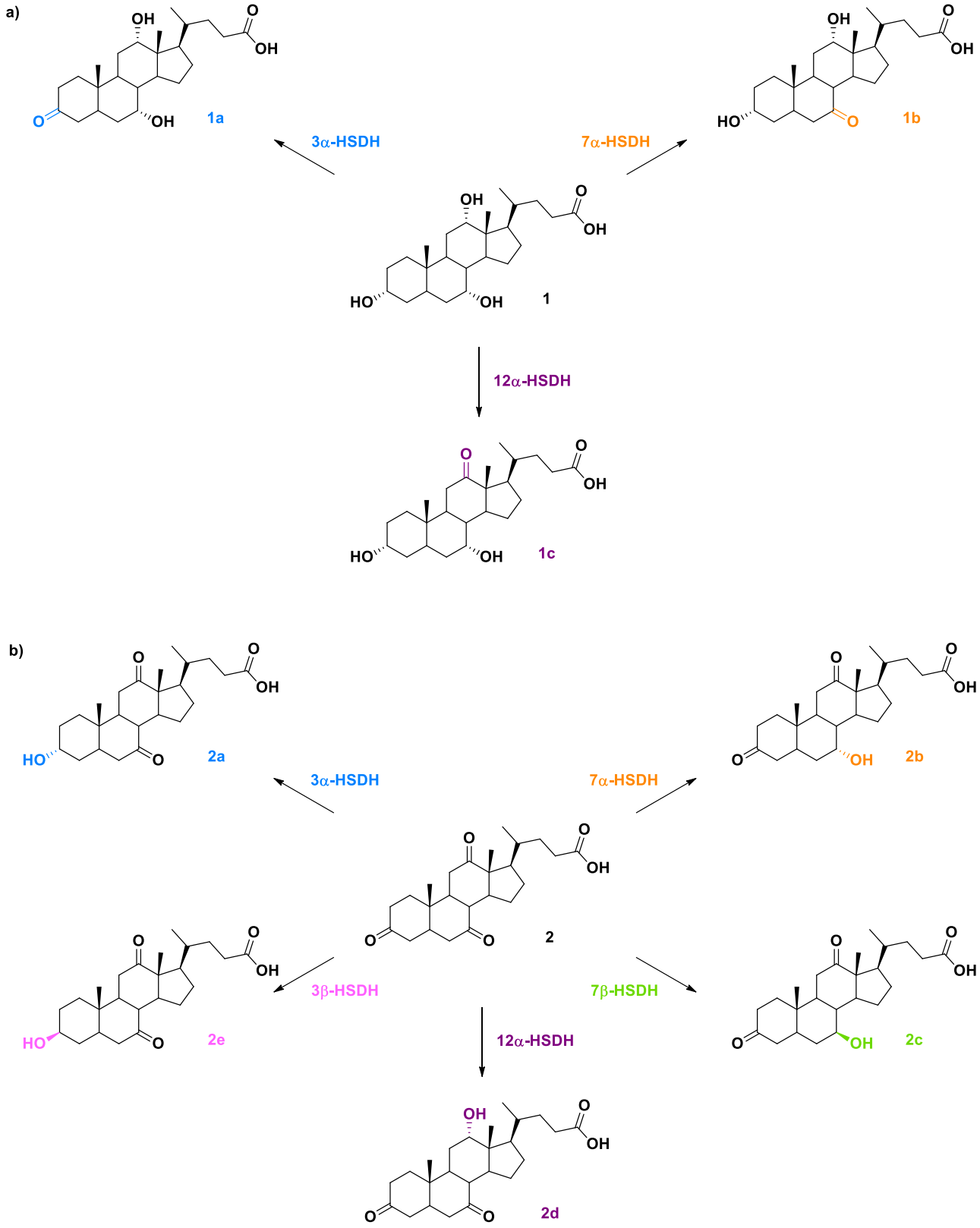


Figure 2.4: Enumeration of the general structure of a steroid core.[21]

These enzymes are able to regio- and stereo-specifically transform hydroxyl/oxo moieties at given positions of the steroid core (e.g., C3, C7, C11, C12, C17). Indeed, not only can HSDHs discriminate hydroxyl/oxo moieties that are chemically almost identical, but they can also distinguish as substrates (or form as products) hydroxyl moieties that are either below or above the plane of the steroid molecule (in the so-called α- and ß-configurations, respectively). So, *exempli gratia*, a 7α-HSDH will only catalyze the oxidation of α-hydroxyls at C7 to carbonyls and the reversed reduction (Scheme 2.3 a), from compound **1** to **1b**). [22] An illustrative example of the selectivity of these enzymes is given in Scheme 2.3 on the model substrates cholic acid (compound **1**) and dehydrocholic acid (compound **2**).

Aside from the high synthetic interest of these enzymes, which will be thoroughly pointed out in the following, HSDHs are relevant in the in vivo metabolism of steroidal compounds, thus they hold many functional roles. Both microbial and host HSDHs exist,[20] even though the attention of this thesis will be focused on those of microbial origin.



Scheme 2.3: Regio- and stereospecific action of different HSDHs on a) cholic acid (oxidation of compound **1**) and b) on dehydrocholic acid (reduction of compound **2**).[23]

2.1.2.2. 3D structure and catalytic mechanism

As it was previously mentioned, most HSDHs of microbial origin belong to the SDR superclass. As such, these enzymes are characterized by the typical SDR structural motifs (Rossmann fold, high 3D structure similarity, and the catalytic triad Ser-Tyr-Lys). Differences in the active site of HSDHs confer them the typical regio- and stereo-selectivities they are endowed with.

The ability of intestinal microbial strains of oxidizing cholic acid was already published in 1942.[24] Nevertheless, HSDHs started to be isolated and characterized in the 1960s, the first individuals being purified from *Comamonas testosteronii* (3α-HSDH and 3(17)ß-HSDH).[24,25] Since then, many HSDHs have been isolated, purified, characterized, and in recent years cloned and produced in recombinant form.[26-37]

2.1.2.2.1. 7α-HSDH from *Escherichia coli* (*Ec*7α-HSDH)

One of the most studied individuals from this group of enzymes is the 7α-HSDH from Escherichia coli.[18,26,38-41] *Ec*7α-HSDH was isolated and characterized in 1973 by Macdonald et al,[26] and since then it has become not only a structural model for HSDHs, but also for the SDR superfamily.[40,41]

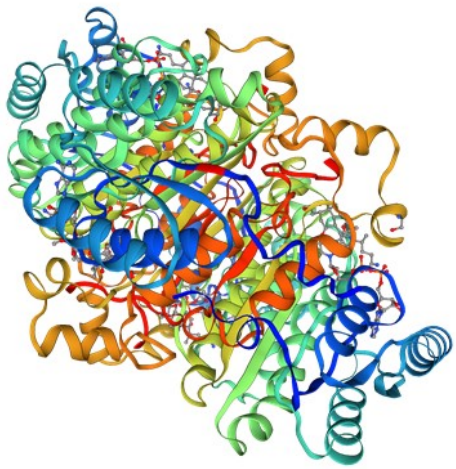


Figure 2.5: 3D ribbon representation of *Ec*7α-HSDH (PDB: 7ENY).[42]

The crystal structure of *Ec*7α-HSDH shows a homotetrameric form, where the four subunits are related by the 222-point group symmetry (Figure 2.5, Figure 2.6). A single subunit is characterized by the presence of the Rossmann fold (in this case, seven parallel ß-strands organized in a ß-sheet and surrounded by three α-helices on each side).

Figure 2.6: 3D ribbon representation of tetrameric *Ec*7α-HSDH (binary complex with NAD^+, as it was represented by Tanaka et al in 1996).[41]

As a classic SDR, *Ec*7α-HSDH is characterized by the presence of the catalytic triad Ser-Tyr-Lys (Ser146, Tyr159, and Lys163, in the numbering of this enzyme), as well as by the Gly-X-X-X-Gly-X-Gly motif (highlighted in green and orange, respectively, in the sequence shown in Figure 2.7).

MFNSDNLRLDGKCAIITGAGAGIGKEIAITFATAGASVVVSDINADAANHVVDEIQQLGG
QAFACRCDITSEQELSALADFAISKLGKVDILVNNAGGGGPKPFDMPMADFRRAYELNVF
SFFHLSQLVAPEMEKNGGGVILTITSMAAENKNINMTSYASSKAAASHLVRNMAFDLGEK
NIRVNGIAPGAILTDALKSVITPEIEQKMLQHTPIRRLGQPQDIANAALFLCSPAASWVS
GQILTVSGGGVQELN

Figure 2.7: primary sequence of *Ec*7α-HSDH (Uniprot code P0AET8), composed of 255 aminoacids. The SDR characteristic motifs are highlighted in orange (N terminus) and in green (C terminus).

A few years ago, a wonderful report on the structural features of the 7α-HSDH from *Clostridium absonum*, for short, *Ca*7α-HSDH, confirmed the similarity of this enzyme to its homologue *Ec*7α-HSDH.[35]

2.1.2.2.2. 7α-HSDH from *Escherichia coli* (*Ec*7α-HSDH): a mechanistic overview

The roles of each of the aminoacids involved in the catalytic action of *Ec*7α-HSDH have been thoroughly characterized by Tanabe et al in 1998.[18] A closer look at the active site (Figure 2.8) and at the proposed catalytic mechanism (Figure 2.9) can clarify the way this enzyme works.

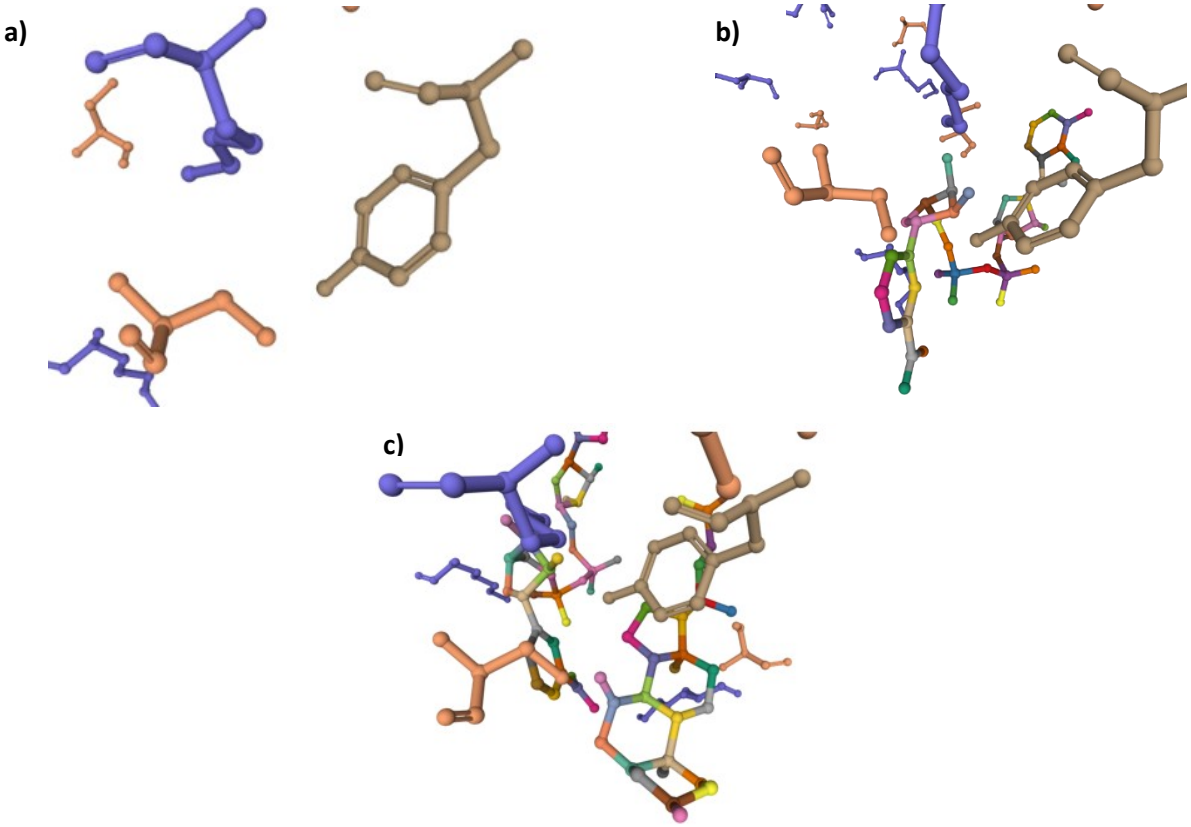


Figure 2.8: 3D ball and stick representation of a) the active residues Ser146 (bottom-left, salmon pink), Tyr159 (top-right, beige), and Lys163 (top-left, blue) [PDB: 7ENY];[43] b) the triad and the bound NAD^+ cofactor [PDB: 1AHH];[41] c) the triad, the bound cofactor, and the co-crystallized substrate Chenodeoxycholic acid [PDB 1AHI].[41] The pictures were generated on the PDB interface.

The availability of the crystal structures of the holoenzyme (Figure 2.8a),[43] of the binary co-crystal with the cofactor (Figure 2.8b), and of the ternary co-crystal with the cofactor and a natural substrate (chenodeoxycholic acid, Figure 2.8c)[41] makes it possible to compare the different forms of the active site. Noteworthy, both in the holoenzyme and in the binary complex, Tyr159 and Ser146 interact via a hydrogen bond, while in the ternary complex both of the residues interact with the C7 hydroxyl of the bound natural substrate. This suggests that hydrogen cascades and hydrogen bonds could have a relevant role in the catalytic mechanism of *Ec*7α-HSDH. On the other hand, the Lys residue is both close to the Tyr-OH and to the 2'- and 3'-hydroxyl groups of the cofactor's ribose, thus suggesting an acid-base role in catalysis and a structural role in cofactor positioning.

An insight on the mechanism of this enzyme has shown that Tyr159 acts as a base, whose pKa value is lowered by the nearby Lys163, while Ser146 has a relevant role in the stabilization of the negatively charged intermediate (Figure 2.9).

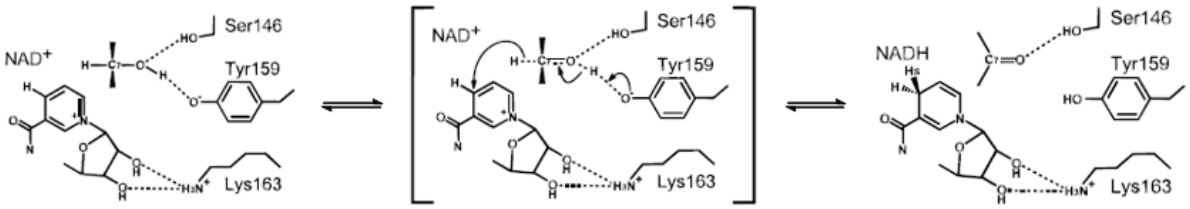
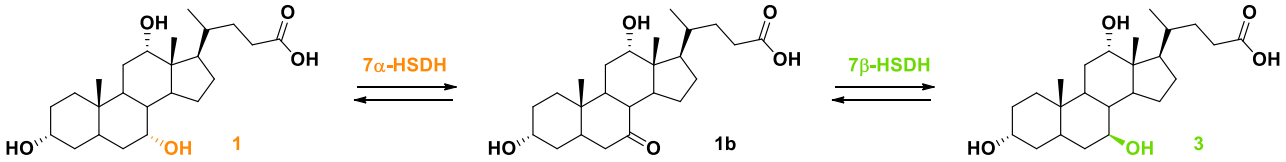


Figure 2.9: Schematic representations of the roles of Ser146, Tyr158, and Lys163 by Tanaka et al.[41]

This catalytic mechanism is shared among the SDR-HSDHs, although some differences in the active sites can confer different regio- and stereo-selectivity to these highly selective enzymes.

2.1.2.2.3. 7α- vs 7β-HSDHs: insights into the stereoselectivity of HSDHs

7α- and 7β-HSDHs catalyze a reaction that has the same regiochemistry (i.e., selectivity for C7 hydroxyl/oxo moieties of the steroid core), but an opposite stereochemistry (Scheme 2.4).



Scheme 2.4: Catalytic activity of 7α-HSDHs (orange) and of 7β-HSDHs (green).

Extensive efforts have been reported in the literature to rationalize this opposite stereoselectivity. For instance, it was demonstrated that the substrate binding site of the 7β-HSDH from *Collinsella aerofaciens* (*Cae*7β-HSDH) is substantially different from that of *Ec*7α-HSDH, mainly for the presence of an additional segment of aminoacids that fold into additional α-helices (Figures 2.10 and 2.11).

MNLREKYGEW GLILGATEGV GKAFCEKIAA GGMNVVMVGR REEKLNVLAG EIRETYGVET
KVVRADFSQP GAAETVFAAT EGLDMGFMSY VACLHSFGKI QDTPWEKHEA MINVNVVTFL
KCFHHYMRIF AAQDRGAVIN VSSMTGISSS PWNGQYGAGK AFILKMTEAV ACECEGTGVD
VEVITLGTTL TPSLLSNLPG GPQGEAVMKI ALTPEECVDE AFEKLGKELS VIAGQRNKDS
VHDWKANHTE DEYIRYMGSF YRD

Figure 2.10: primary sequence of *Cae*7β-HSDH (Uniprot code A4ECA9),[44] composed of 263 residues. The SDR characteristic motifs are highlighted in orange (N terminus) and in green (C terminus).

Aside from that, the cofactor binding mode and the catalytic triad are apparently well conserved (Figure 2.11). The difference in the C-terminus was however proven via deletion experiments not to be the determinant feature for the opposite stereoselectivity, as the deleted mutant did not display a 7α-activity.[44]

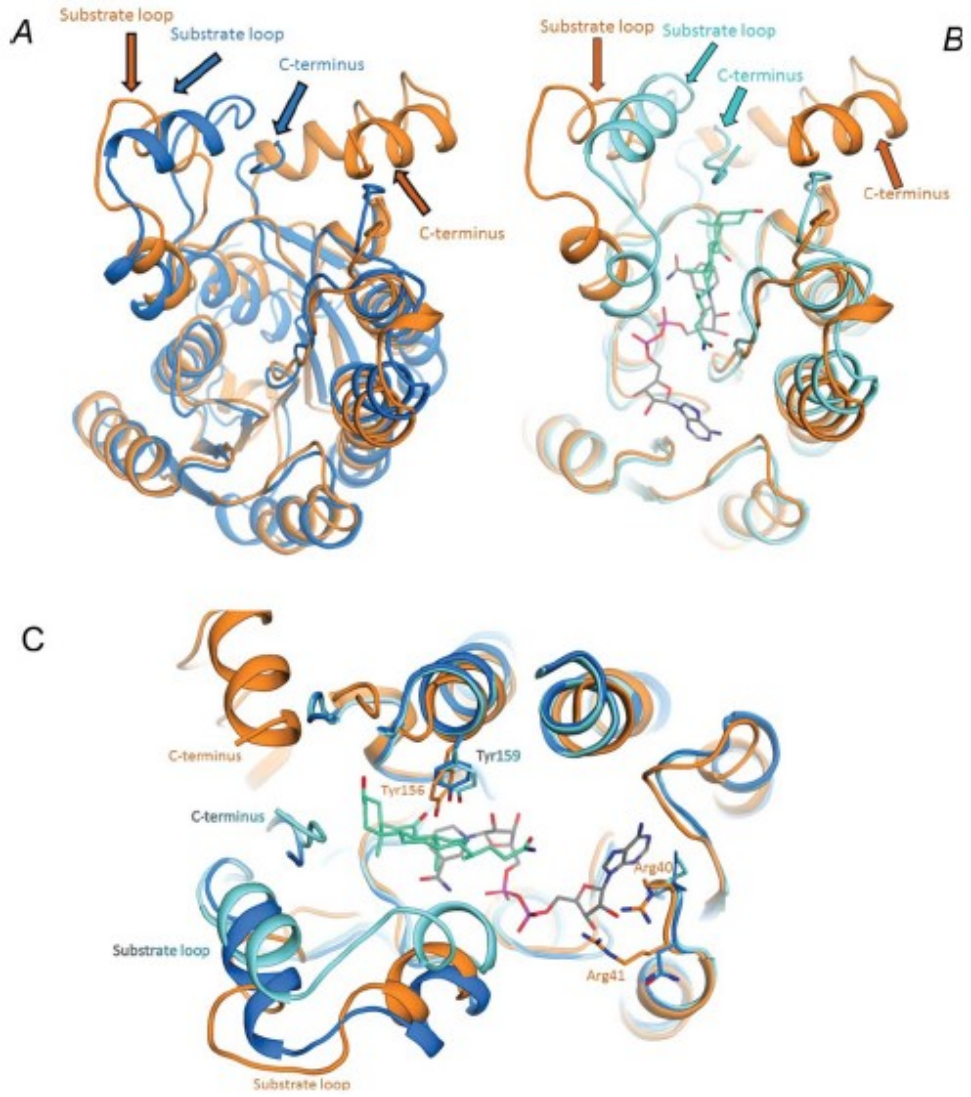


Figure 2.11: Superimposition of *Ec*7α-HSDH (blue) and apo-*Cae*7β-HSDH (orange) made by Savino et al [PDB: 5FYD].[44] This picture shows well the 3D structural similarity of the two enzymes and the additional α-helices at the C-terminus of *Cae*7β-HSDH(A), the differences in the cofactor binding modes (B), and the conserved active site (C).

A more detailed insight in the cofactor binding mode was given by Wang et al.,[45] who were able to crystallize the binary complex of *Cae*7β-HSDH with bound $NADP^+$ [PDB: 5GT9]. This crystal not only confirmed the previous findings, but it revealed that the cofactor features two binding modes (one in each of the protein monomers): the first is essentially identical to that of NAD^+ in *Ec*7α-HSDH, as predicted by Savino et al.; the other is twisted approximately 90° in the nicotinamide part (Figure 2.12).

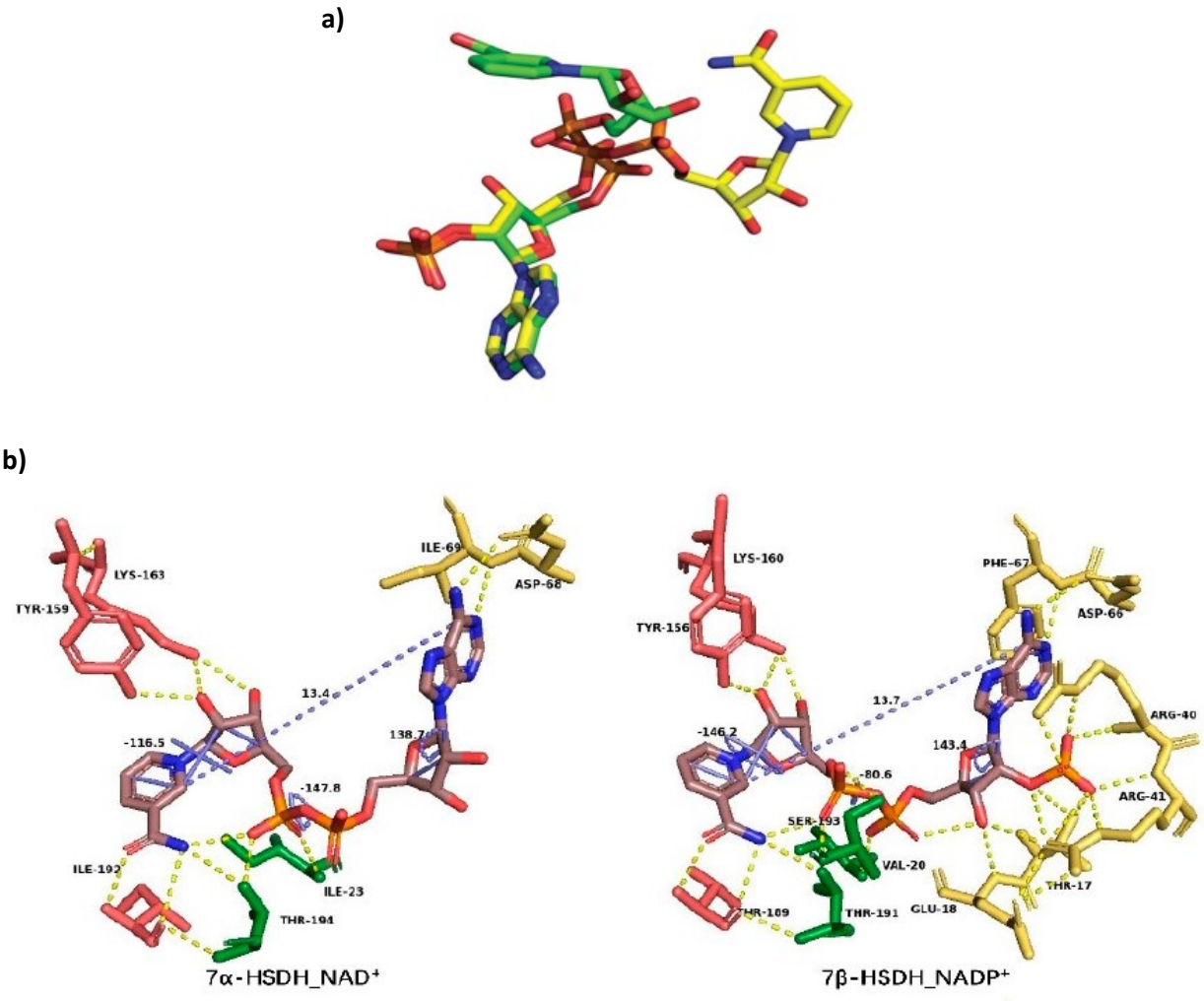
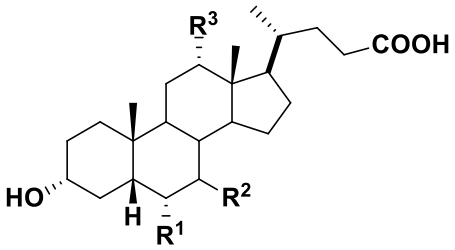


Figure 2.12: a) alignment of the two different binding modes of $NADP^+$ as shown by Wang et al.[45] b) comparison of the cofactor binding sites of *Ec*7α-HSDH (on the left, [PDB: 1FMC])[41] and *Cae*7β-HSDH (on the right, [PDB: 5GT9])[45] as it was shown by Miaomiao et al.[46]

The differences in the cofactor binding mode have been recently proposed to be involved in the different stereoselectivity of 7α- and 7β-HSDHs.[46] Nevertheless, it had also been suggested in the past that the opposite stereochemistry may arise from different substrate orientations in the substrate pocket, implying a substantial difference in the structures of the active sites in the two enzymes.[44] Apparently, both of the statements are true. Indeed, a Ser193 residue in 7β-HSDHs (*Cae*7β-HSDH sequence numbering) would cause the conformational change in the cofactor, thus forming a barrier that would prevent the substrate to enter the active site with the same orientation it does in 7α-HSDHs.[46]

2.1.2.2.1. Natural substrates: bile acids

Bile acids are amphiphilic molecules built by a C24 skeleton (Figure 2.13). These steroidal compounds are characterized by a carboxylic acid moiety at C24 and a variable hydroxylic substitution pattern, characteristics that endow them with a hydrophilic "face" and a hydrophobic one.[47]



1: R^1 = H, R^2 = R^3 = α-OH (cholic acid, CA)
3: R^1 = H, R^2 = β-OH, R^3 = α-OH (ursocholic acid, UCA)
4: R^1 = R^3 = H, R^2 = α-OH (chenodeoxycholic acid, CDCA)
5: R^1 = R^3 = H, R^2 = β-OH (ursodeoxycholic acid, UDCA)
6: R^1 = R^2 = α-OH, R^3 = H (hyocholic acid, HCA)
7: R^1 = R^2 = R^3 = H (lithocholic acid, LCA)

Figure 2.13: Structures and nomenclature of some relevant bile acids.

Bile acids are endogenous compounds which exist in many vertebrate species mainly as glycine and taurine conjugates, and they are involved in many physiological functions. According to the considered species, the functionalization pattern of the steroid core varies (e.g., C12-OH are typical of some mammals,[48] C6-OH are widespread in mice,[49,50] while C16 and C15-OH are representative in birds and reptiles[51,52]), alongside with the physicochemical properties of these compounds. It has been hypothesized that the common ancestor of bile acids could be chenodeoxycholic acid (CDCA, Figure 2.13, compound **4**), with additional hydroxylations happening in different species.[53]

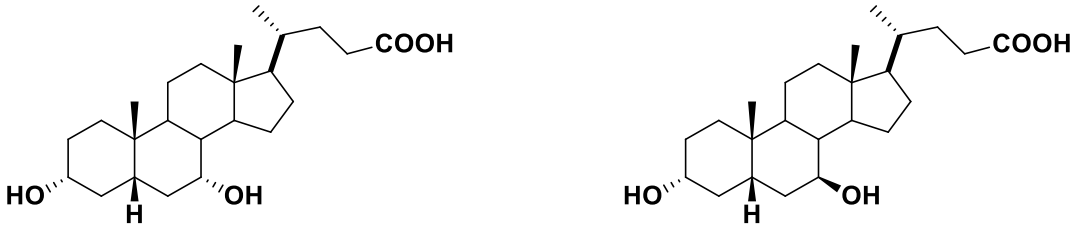
The interest for these compounds is rising a lot in the last decades, as it has been shown that not only they have many regulatory functions (e.g., they can self-regulate their own biosynthesis), but they can also be biomarkers for the early-stage detection of various diseases and some of them are already sold as drugs or hold the potential to be used as medicines (UDCA is the case study of this topic).[54-66]

2.1.3. HSDHs and UDCA synthesis: an ongoing story

2.1.3.1. UDCA as a drug

The chemical structure of bile acids was first elucidated in 1932,[67] but the scientific interest on these molecules remained low until the sixties. Indeed, before the first clinical trials on cholic acid and on some of its derivatives it was thought that bile acids held no useful therapeutic properties.[68] However,

in 1965, cholic acid was shown to suppress bile acid and cholesterol biosynthesis,[68] and few years later the other primary bile acid chenodeoxycholic acid was shown to decrease biliary cholesterol saturation and to be able to dissolve cholesterol gallstones.[69–71] Meanwhile, the ability of gallstone dissolution was also confirmed in Japan for ursodeoxycholic acid (for short and from now on, UDCA).[72,73] UDCA was firstly isolated from bear bile in 1927,[74] and it was administered in suboptimal doses as a liver tonic, only because at the time it was thought that bear bile had therapeutic effects with no real scientific basis.



Chenodeoxycholic acid
(**4**, CDCA)

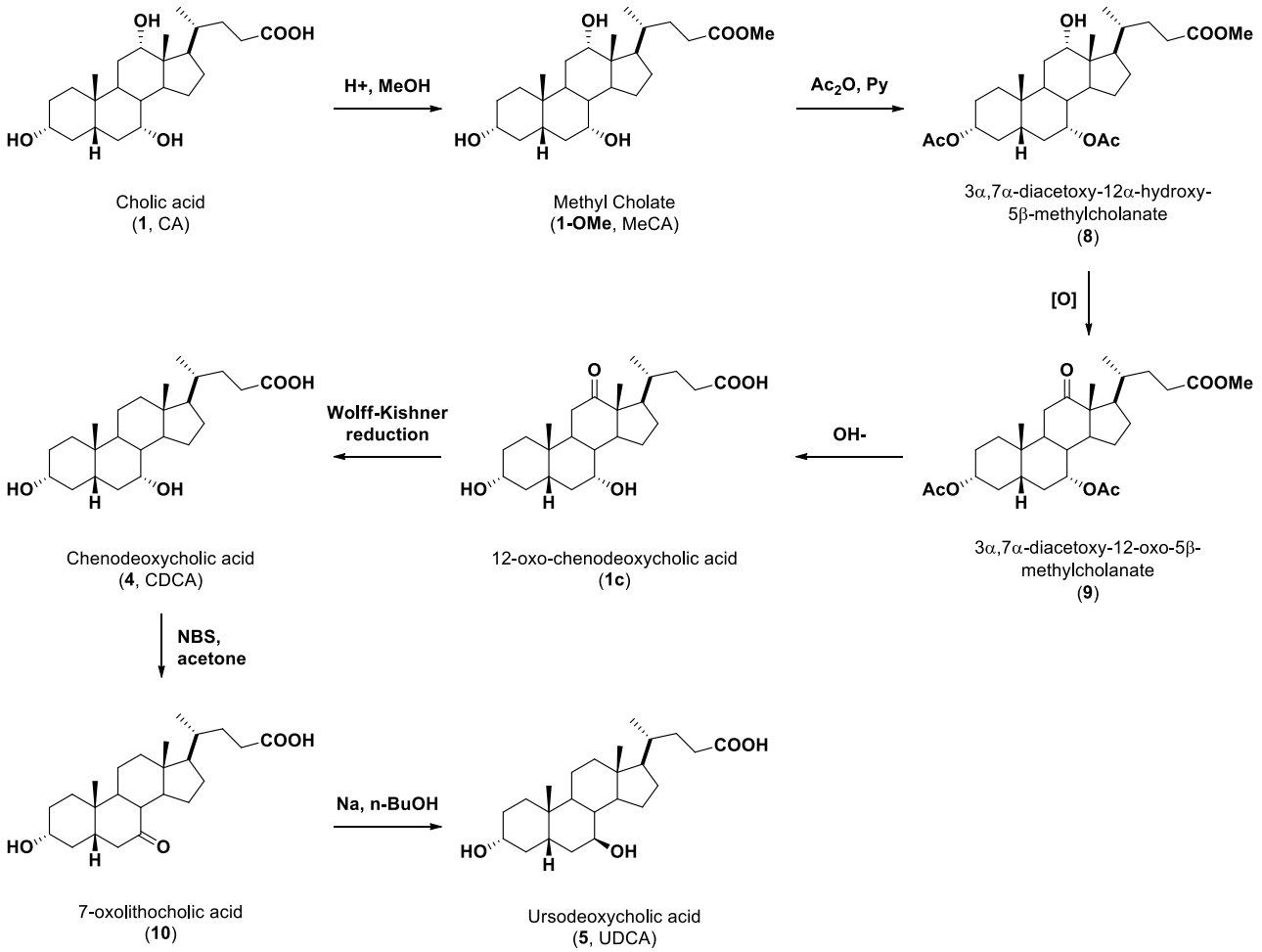
Ursodeoxycholic acid
(**5**, UDCA)

Figure 2.17: UDCA (compound **4**) and CDCA (compound **5**, see also Figure 2.13).

The interest in bile acids thereafter increased a lot, most of all towards UDCA. Indeed, in the following years, UDCA started to be produced on a ton-scale on a yearly basis, as it is currently the only alternative to surgical intervention for the treatment of cholesterol gallstones.[75] Moreover, this compound has been shown to possess other clinically relevant properties: it is used as a first-choice treatment for patients with chronic cholestatic diseases (such as primary biliary cirrhosis, primary sclerosing cholangitis, and several less common disorders in adults and infants);[76,77] it possesses cytoprotective and anti-apoptotic properties, *e.g.*, preventing the bioaccumulation of the most hydrophobic bile acids in hepatocytes;[78] it has been proposed as an anticancer agent or as a chemoprotective drug against colon cancer.[79,80]

2.1.3.2. UDCA synthesis

The great synthetic interest of obtaining this compound is evident by its many effective and potential applications in the pharmaceutical field. Nowadays, the synthesis of UDCA is performed via a 7-step chemical process (Scheme 2.4), which is considered under many aspects optimizable both in terms of overall yield, hazardous reactants, step economy, waste reduction, and environmental impact.[81,82]



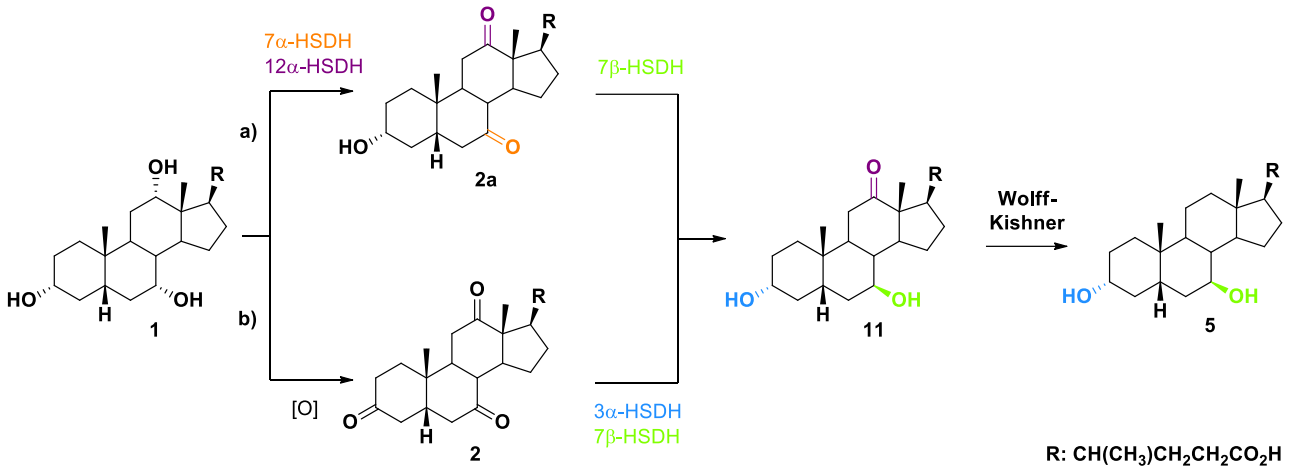
Scheme 2.4: 7-step chemical synthesis of UDCA (*ndr*, [O] states for oxidation, as Hofmann described the use of CrO_3 in 1963, but nowadays this method is not applied anymore in industries).

The synthesis of UDCA starts industrially by the readily available and cheap cholic acid, which is less similar to UDCA than CDCA is, but for supply and economic reasons it is the only viable option as starting material nowadays.

2.1.3.3. UDCA "biosynthesis"

The limitations of UDCA's chemical synthesis pushed the scientific community to work on a chemoenzymatic approach since the time when HSDHs started to be isolated.[83,84]

In the literature, two approaches that yield UDCA starting from CA are adopted: the first approach (Scheme 2.5a) epimerizes C7 hydroxyl from an α- to a ß-configuration, and oxidizes C12 α-OH to an oxo moiety, subsequently reduced to a methylene via a Wolff-Kishner reduction; the second pathway (Scheme 2.5b) fully oxidizes CA (compound **1**) to dehydrocholic acid (DHCA, compound **2**), and then it reduces each position of DHCA to yield the desired UDCA (i.e., C3 to an α-hydroxyl, C7 to a ß-hydroxyl, and C12 to a methylene).[22,85,86]



Scheme 2.5: chemoenzymatic processes from CA to UDCA.

Concerning the first approach, the enzymatic oxidation at C12[87-91] of CA and the sequential and one-pot enzymatic epimerization at C7 (of both CA and CDCA)[84,92-103] have been extensively reported in the literature. Moreover, a one-pot strategy for the synthesis of 12-oxoursodeoxycholic acid (compound **11**) starting from cholic acid was proposed about 12 years ago, where three HSDHs and two cofactor regeneration systems worked in a row.[104]

Regarding the second approach, green chemical methods have been developed for bile acid oxidation.[105-107] The subsequent enzymatic reduction can be performed with 3α- and 7β-HSDHs, to yield 12-oxoursodeoxycholic,[108-113] which will be reduced via the Wolff-Kishner reduction or analogous protocols.[114]

The synthetic limitations of applying HSDHs in industry are related to both enzyme stability and to substrate/product inhibition. However, some studies have partially overcome these limitations in search of thermostable or engineered enzymes.[22]

2.1.4. Ene Reductases: Old Yellow Enzymes

2.1.4.1. An overview on OYEs

As it was mentioned in the beginning of Section 2.1, Oxidoreductases (E.C.1) are extremely versatile enzymes, which are able not only to perform redox reactions on hydroxyls/carbonyls, but also on other synthetically relevant functional groups. A very peculiar case is that of ene reductases, which are flavoproteins able to stereoselectively reduce activated double C=C bonds, such as those bearing an electron withdrawing (EWG) substituent (e.g., α,β-unsaturated aldehydes, ketones, carboxylic acids, esters, nitriles, and nitroalkenes).

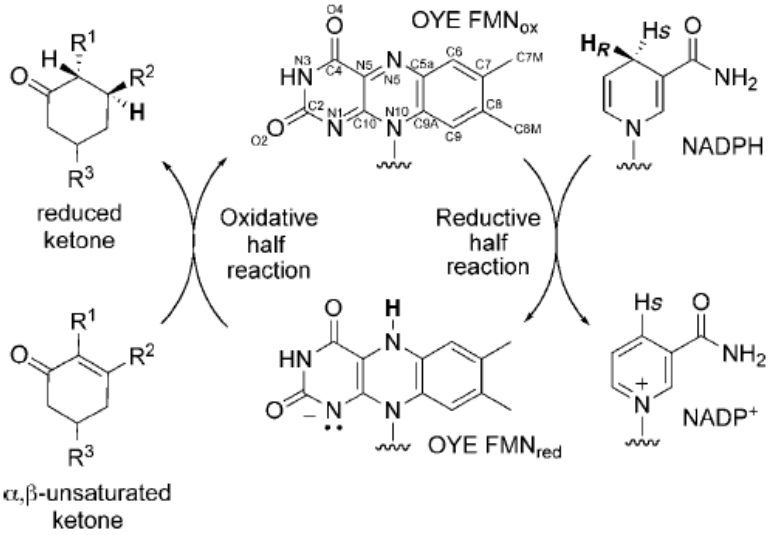
A large family of flavoproteins is constituted by Old Yellow Enzymes (for short and from now on, OYEs, E.C.1.6).[115] The curious name of these biocatalysts comes from their history and from their biological

characteristics. Indeed, the discovery of OYEs dates back to 1932, with the isolation of the first individual from *Saccharomyces pastorianus* (previously, *Saccharomyces carlsbergensis*), which was named "*das gelbe Ferment*" (the yellow enzyme) after its colour.[116] Two years later, another similar enzyme was isolated and it was (not much creatively) named "*das neue gelbe Ferment*" (the new yellow enzyme), leading the first enzyme to be identified thereafter as the Old Yellow Enzyme.[117] The discovery of other similar individuals from other yeasts led to the whole class to be named as Old Yellow Enzymes, a name which is still used today, as notably neither the natural substrates nor the physiological function of OYEs are known. Moreover, the yellow color is an intrinsic characteristic of the majority of OYEs, due to the presence of the yellow flavin cofactor.

2.1.4.2. The mechanism of OYEs

OYEs are flavoproteins that contain a non-covalently bound flavin cofactor (flavin mononucleotide, FMN), the oxidation state of which is regenerated by a nicotinamide cofactor accessing the active site (NADPH is preferred to NADH). Even though the natural substrates of OYEs are not known, the extensive structural characterization of these enzymes has permitted the identification of both the nicotinamide and the substrate binding site, which have been shown to be the same.[117-120]

The kinetic studies on the mechanism of OYEs have allowed to define a *bi-bi* ping-pong mechanism, where: at first, NADPH would enter the binding site, reduce the flavin cofactor, and leave the binding pocket (this process is referred to as the reductive half reaction, since the FMN is reduced by external NADPH); secondly, the substrate can access the flavin proximities and be reduced (oxidative half reaction). These studies are consistent with the hypothesis that the binding site for the cofactor and the substrates is shared, and thus they cannot occupy it simultaneously.[117]



Scheme 2.6: Depiction of the ping-pong OYE mechanism.[117]

2.1.4.3. Structural insights and OYE subfamilies

In contrast with other enzyme classes, such as the previously analyzed one of SDRs, OYEs tend to feature a high conservation of the primary sequence of selected regions of the protein, mainly involved in catalysis, substrate, and cofactor recognition, and FMN binding.[121]

OYEs tend to aggregate in multimeric quaternary structures of variable dimensions (from dimers to dodecamers).[122] Despite that, the structure of their monomers is overall conserved, being characterized by the presence of a TIM barrel fold (a secondary structure composed of 8 α-helices and 8 ß-strands that alternate and fold in a cylindrical fashion, the $(\alpha\beta)_8$-barrel), with additional secondary structural elements that vary among OYE subclasses and individuals. The FMN binding domain is within the barrel, at the C-terminal end of the ß-strands, non-covalently bound via a net of hydrogen bonds at the *re*-face.[117] The additional secondary structure elements help in forming the substrate binding pocket (Figure 2.18).

The structural analyses made on the YqjM OYE individual, isolated from from *Bacillus subtilis*, revealed novel properties, allowing thus to divide the OYE family into two major subfamilies, namely: the classical OYEs; the thermostable OYEs.[118]

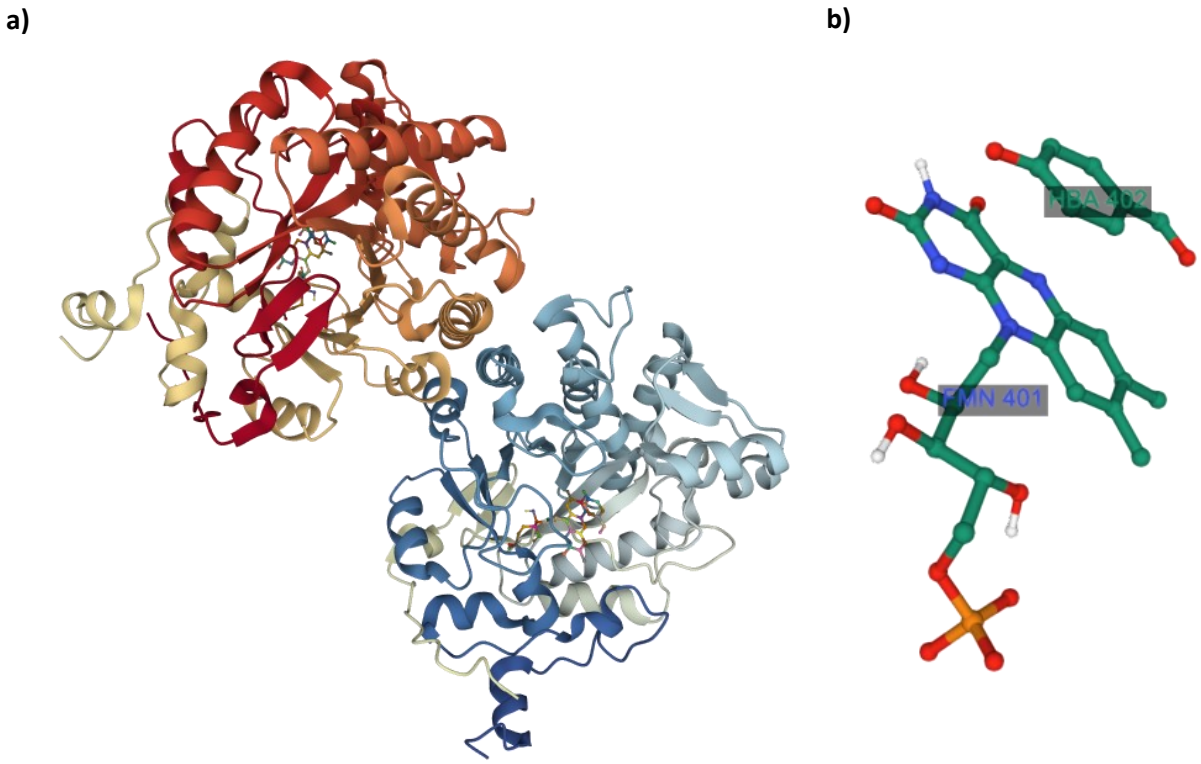


Figure 2.18: a) the 3D-structure of OYE1 [PDB: 1OYB]; b) the 4-hydroxybenzaldehyde ligand and FMN [PDB: 1OYB].[119]

More recent structural and bioinformatic insights have confirmed this classification and have further divided the classical family into three more subclasses: OYE1-like; OPR-like (12-oxophytodienoato reductase-like); PETNR-like (pentaerythritol tetranitrate reductase-like). A phylogenetic tree of all the classes is shown below (Figure 2.19), alongside with a structural comparison of four representative individuals (Figure 2.20, one for each class, so YqjM for thermostable OYEs, OYE1 for OYE1-like, LEOPR3 for OPR1-like, and PETNR for PETNR-like individuals).[123]

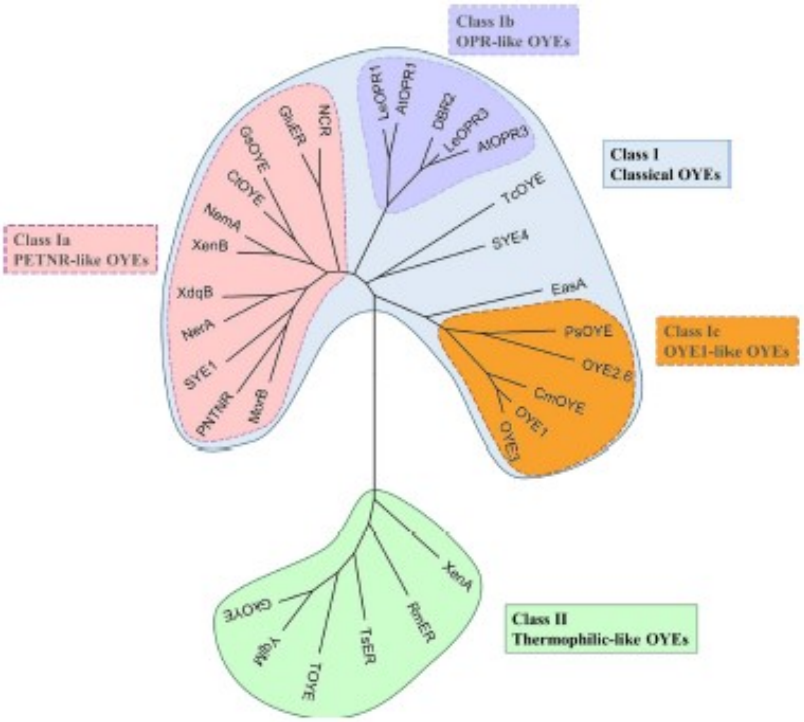


Figure 2.19: Phylogenetic tree of some representative OYE individuals by Shi et al.[123]

The overall 3D-structures of the enzymes belonging to the different subclasses of the OYE family are similar, and they all conserve the TIM barrel motif, even though some additional secondary structure elements are present in some individuals.

On the other hand, a look in the active sites gives an idea of why thermostable OYEs are able to reduce bulkier substrates: the binding pocket of such enzymes is much less hindered, leaving the possibility to host larger molecules (Figure 2.20).

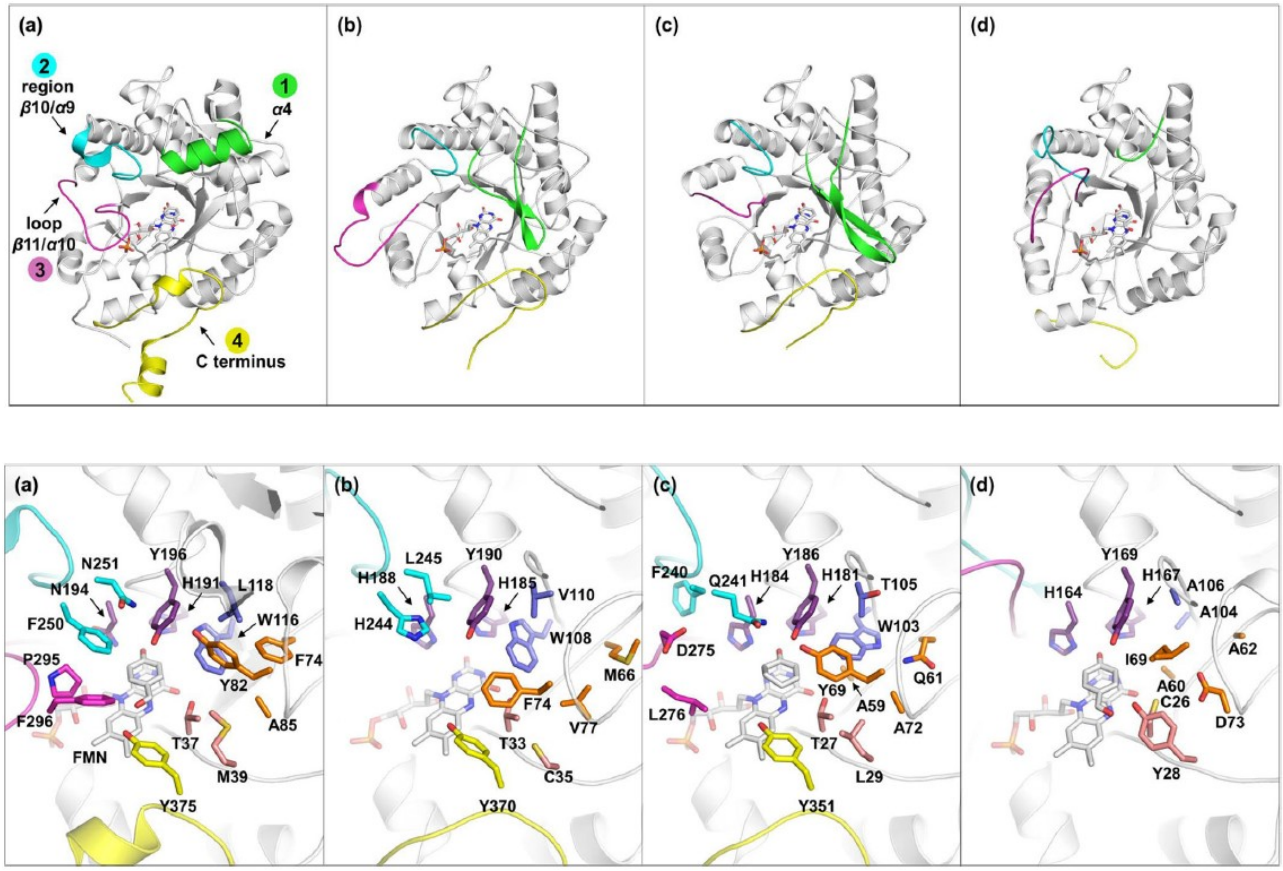


Figure 2.20: Above: Overall structural comparison of a) OYE1 [PDB: 1OYB];[119] b) LEOPR3 [PDB: 2HSA];[124] c) PETNR [PDB: 1GVQ];[125] YqjM [PDB: 1Z42].[118] Below: Active site comparison of a) OYE1 [PDB: 1OYB];[119] b) LEOPR3 [PDB: 2HSA];[124] c) PETNR [PDB: 1GVQ];[125] YqjM [PDB: 1Z42].[118] Depicted by Shi et al.[123]

2.1.4.4. Mutagenesis as a tool to reverse OYE stereoselectivity

One of the most notable features of the OYE enzymes is that their stereoselectivity in the reduction of enones can be modulated from many viewpoints.[126]

Secondly, point mutations of selected residues in these enzymes allow to obtain stereocomplementary biocatalysts. This was the case, among others, of OYE1 and its single mutant OYE1 W116L, which reduced carvone forming a *R*- and an *S*- new stereocenter, respectively (Figure 2.21).[123,127,128]

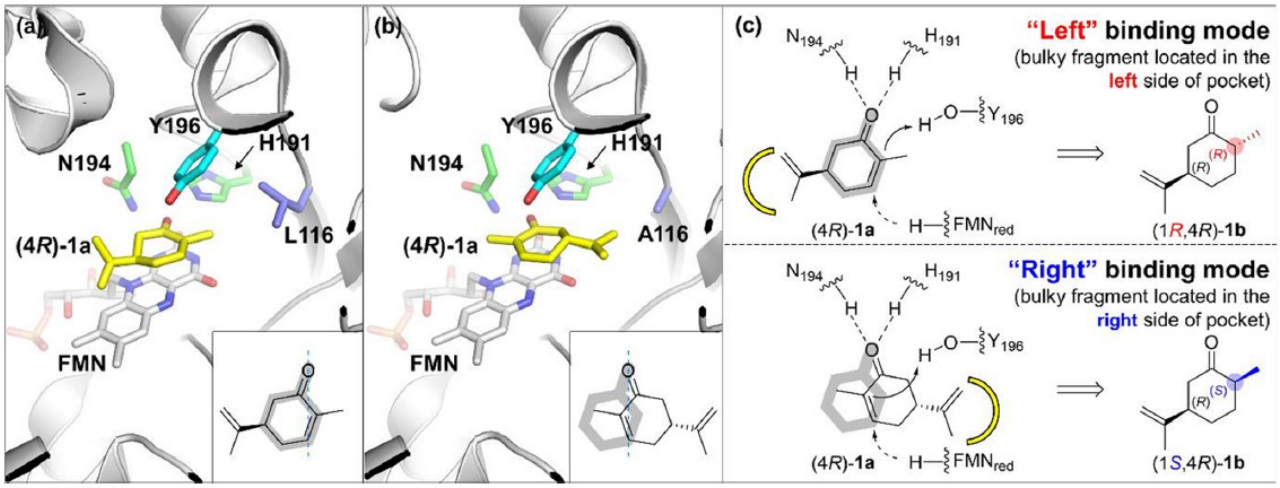


Figure 2.21: Carvone stereocomplementary reduction by OYE1 and its single mutant OYE1 W116L, resulting by two opposite flipped-binding modes of the substrate, as depicted by Shi et al.[123,128]

Many studies of this kind have been made on other OYEs, such as those on YqjM, where punctual mutations at C26 and I69 have been made with interesting outcomes.[129,130]

The engineering of OYEs holds a great potential to further integrate the use of these redox enzymes in the industrial protocols for the reduction of activated double bonds, as a valuable alternative to toxic metal catalysts.[131]

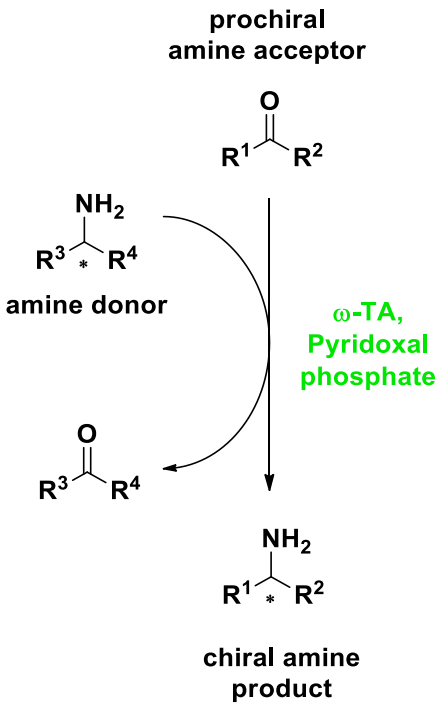
2.2. Transferases

Transferases (EC 2) are enzymes which catalyze the transfer of a functional group from a molecule to another. Many examples of different transferases exist, for instance catalyzing the transfer of acyl groups (acyltransferases), phosphates (kinases, phosphotransferases), methyl group (methyl transferases), glycosides (glycosyltransferases) or amines (transaminases). The latter group of enzymes will be discussed in further details in the following way: an outline on the nature and properties of these enzymes will be given (section 2.2.1.1); their relevance in industries and fine chemical synthesis will be briefly pointed out (section 2.2.1.2).

2.2.1. Transaminases: biology and catalysis

2.2.1.1. What are they and what do they do?

Transaminases (or aminotransferases) are a group of transferases. They are pyridoxal-5'-phosphate (PLP) dependent enzymes catalyzing the stereoselective transfer of amino groups to α-ketoacids or ketones. The donor amino group is transformed into a ketonic moiety, while the ketoacid or the ketone acting as acceptor becomes an α-aminoacid or an amine, respectively (see Scheme 2.7). A novel stereogenic centre is formed during the reaction and both (*S*)-selective and (*R*)-selective aminotransferases exist, although the former ones are naturally much more represented than the latter.[132,133]



Scheme 2.7: general reaction scheme of a transaminase.

Amino transferases are normally classified into two categories: α-TAs, converting only α-aminoacids and α-ketoacids; ω-TAs, accepting amino acids that have a distal carboxylic group as substrates.[134] The latter enzymes have been also denominated amine transaminases (ATAs).

The action of this group of enzymes depends on the cofactor PLP, as it was previously mentioned (see Figure 2.22 for the catalytic action).

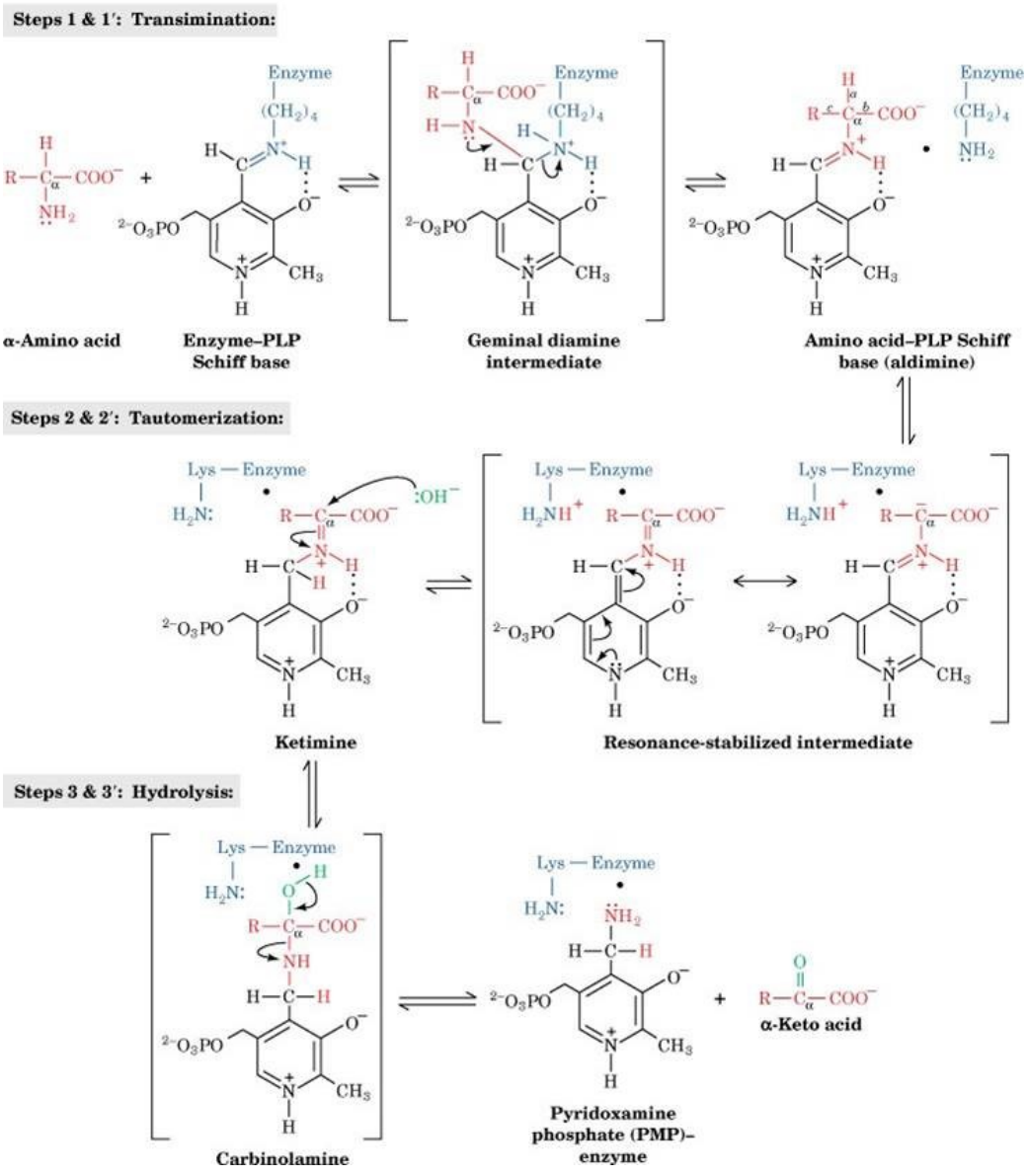


Figure 2.22: first half of the catalytic mechanism of transaminases.[134]

Pyridoxal phosphate has a reactive aldehyde moiety, which is activated by a nearby lysine residue in the active site. This transforms the carbonyl of PLP into an iminium ion, which is very susceptible to the attack of nucleophiles due to the high electrophilicity impaired by the positive charge on the electron withdrawing nitrogen atom. This allows the amine donor (*e.g.*, alanine) to attack the iminium ion, generating a tetrahedral intermediate which undergoes hydrolysis, leading to the formation of the

ketonic sacrificial product (*e.g.*, pyruvate), and to pyridoxamine phosphate (PMP), the active cofactor form which can then transform the prochiral ketonic acceptor in the chiral amine of interest, regenerating in turn PMP, ready for the following catalytic cycle.

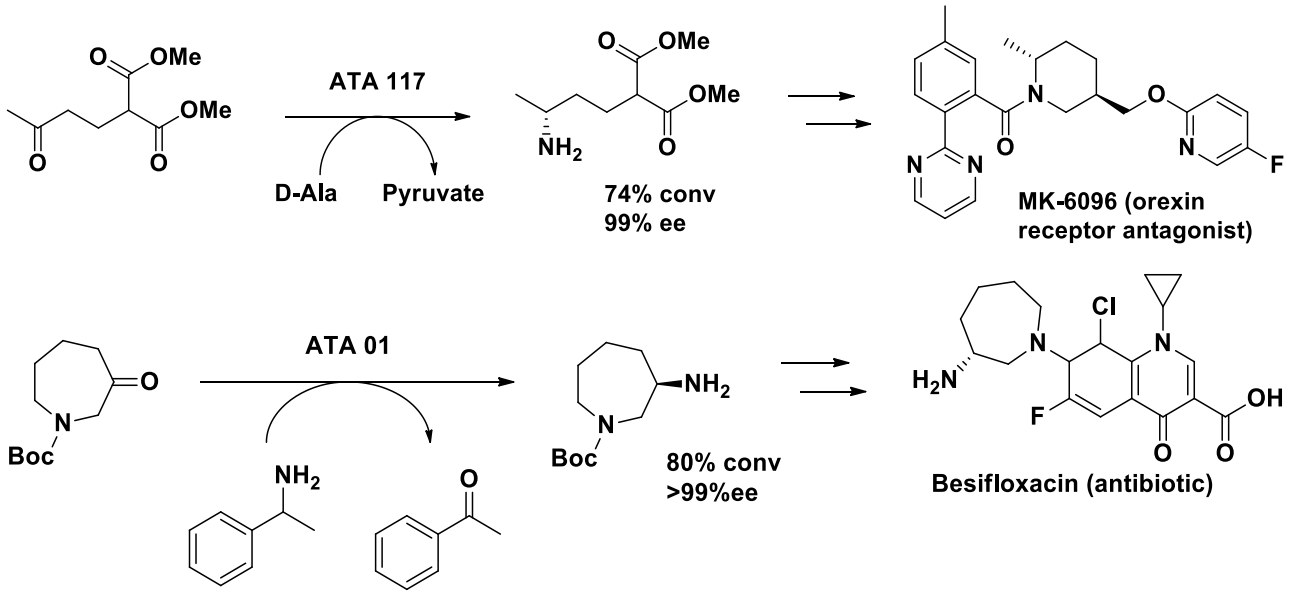
Transaminases have fundamental roles *in vivo*, as they are involved in the biosynthesis and degradation of aminoacids. Additionally, some ω-TAs can also accept simple amines as substrates and this peculiarity makes them feasible for synthetic purposes.[135]

2.2.1.2. Transaminases in chiral amine synthesis

The enantiomerically pure amines are of interest in the framework of drug design and development since they are potent pharmacophores, therefore they can be used as building blocks for new active pharmaceutical ingredients (APIs).[136–138]

The development of new sustainable methods alternative to the currently applied metal-based reductive amination is of primary importance for pharmaceutical industries, as the use of biocatalysts allows to circumvent the limitations associated with traditional transition metal catalysts used for asymmetric synthesis of amines, which are toxic and quite often require high pressure conditions.[135]

TAs seem to be valid candidates for this purpose, due to their inherent high chemo-, regio- and stereo-selectivity and to their catalytic activity. As it was previously outlined, both (*S*)- and (*R*)-selective ATAs exist in nature and they have been used either for kinetic resolution of racemic amines or for asymmetric synthesis of chiral amines starting from the corresponding ketones.[135] For instance, two applications of ATAs in large-scale synthesis of bioactive molecules are shown below.[134]



Scheme 2.8: chiral amine API synthesis via amino transaminases.

Currently, the number of natural ATAs that are used is quite restricted (nearly 20 (S)-selective from bacteria and approximately the same number of (R)-selective from fungi)[134,139] This might be due to the inherent instability of such macromolecules to organic solvents, conditions which are often unavoidable for preparative scale applications.

There are two possible ways to overcome the previously mentioned difficulties: protein engineering of existing ATAs or the exploitation of newly discovered thermostable ATAs. The former solution has already led to industrial applications of such enzymes, whereas the latter has recently started to be tackled.[140]

Focusing on the latter solution, the use of metagenomics (*i.e.*, the study of genetic materials coming from environmental samples) at SCITEC-CNR has recently led to the discovery of three novel thermostable (*S*)-selective ATAs from thermophilic microorganisms. These enzymes also feature a good compatibility with organic solvents. Both their thermostability and their organic solvent compatibility make them attractive for preparative scale applications. One of these enzymes, named B3-TA, appears to be particularly promising, due to its hyperthermophilic behavior. Indeed, its activity increases with temperature up to nearly 90°C. B3-TA has also proven to be the most stable to organic solvents among the three newly discovered enzymes. Moreover, all of them (namely B3-TA, It6-TA, Is3-TA) have been shown to tolerate a wide range of donors and acceptors, thus they seem to hold a great potential as biocatalysts for synthetic applications.[140]

2.3. Hydrolases

Hydrolases constitute the third enzyme class (E.C. 3), and they break chemical bonds using a water molecule. Many kinds of bonds can be broken by these enzymes, for instance esters, ethers, amides, glycosides, carbon-halogen/sulfur/phosphorus bonds, and sulfur-sulfur bonds. The generic reaction scheme of a hydrolase-catalyzed reaction is the following one, where the A-B bond is cleaved by the insertion of a water molecule.

$$A\text{–}B + H_2O \rightarrow A\text{–}OH + B\text{–}H$$

In the body, these enzymes hold a digestive role, and the building blocks obtained via hydrolysis (*e.g.*, of fatty acid triglycerides by lipases) can be re-used for biosynthetic purposes or as energy sources. Moreover, these enzymes can have a physiological function, such as the hydrolase secreted by the intestinal microbe *Lactobacillus jensenii*, which stimulates the bile salt release from the liver for food digestion.[141]

2.3.1. Lipases: hydrolases for industrial applications

2.3.1.1 An overview on lipases

Lipases are a subclass of hydrolases (in particular, esterases) which catalyze the hydrolysis of lipids.[142]

These esterases are the enzymes that find the widest use in organic synthesis, exploiting their significant regio- and stereo-selectivity. This is mainly due to their commercial availability, broad specificity and remarkable stability in media containing organic solvents.[143]

Actually, reactions that are thermodynamically unfavorable in water (*e.g.*, esterification and transesterification) can be catalyzed by lipases in organic solvents. Furthermore, thanks to the broad specificity of lipases, many substrates and acylating agents can be used, despite the fact that the natural substrates of lipases are fatty acid triglycerides. Past works performed at SCITEC-CNR have shown that lipases can catalyze the regioselective esterification of polyhydroxylated compounds using activated esters of di-carboxylic acids.[144,145]

2.3.1.2. The structure and the mechanism of lipases

Lipases in nature are highly diversified, with many kinds of folding patterns. However, most of them share an α/ß hydrolase pattern of 8 ß-sheets connected by 6 α-helices (see Figure 2.23).[146]

Lipases' catalytic action is performed through a catalytic triad Ser-His-Asp, consisting of a nucleophilic serine residue, a basic histidine, and an aspartic acid residue. The mechanism of a lipase-mediated hydrolysis is depicted in Figure 2.24, where the roles of the catalytic triad members are outlined.

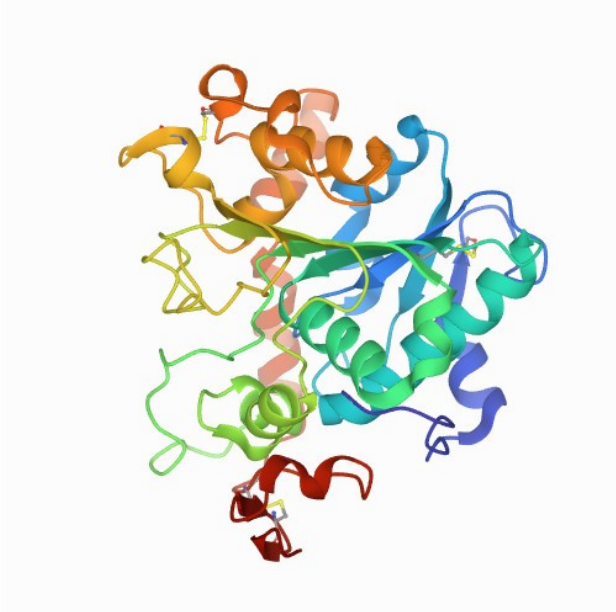


Figure 2.23: 3D-structure of the CAL-B lipase from Candida antarctica [PDB: 4K5Q].[147,148]

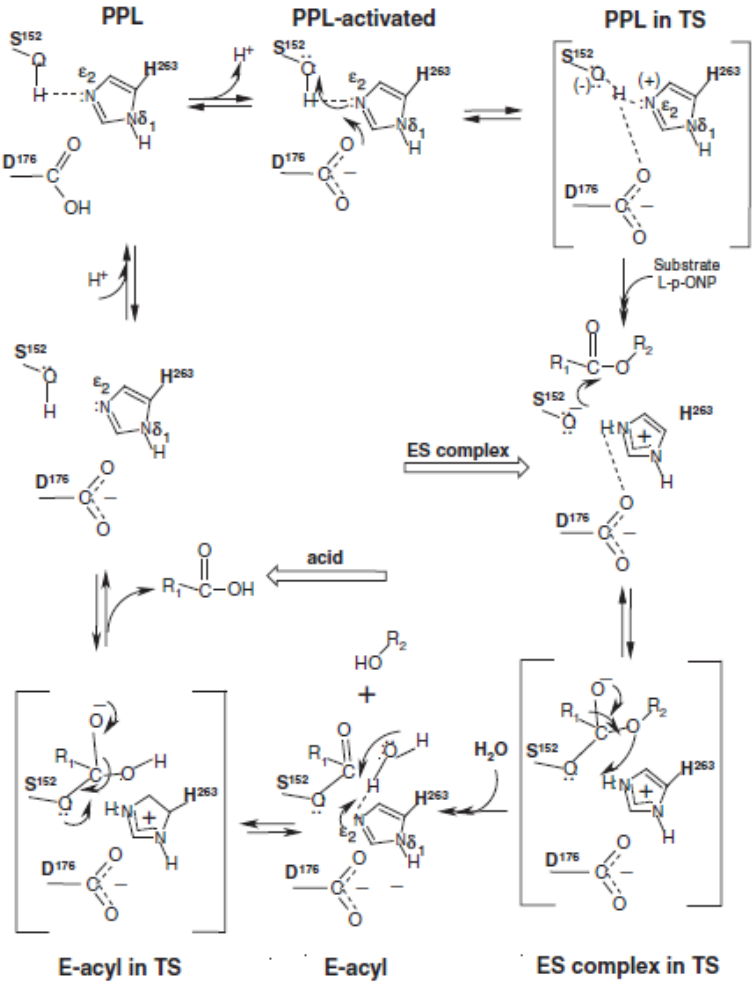


Figure 2.24: catalytic mechanism of lipase-mediated hydrolyses pictured by Stergiou et al.[149]

2.3.1.3. Lipases in organic solvents

Lipases can work both in their natural hydrolytic sense or in the opposite direction, *i.e.*, they can make ester bonds (see Figure 2.24 and 2.25 for the catalytic mechanism of direct and reverse reactions, respectively). A typical use of lipases is that of performing acylation reactions.

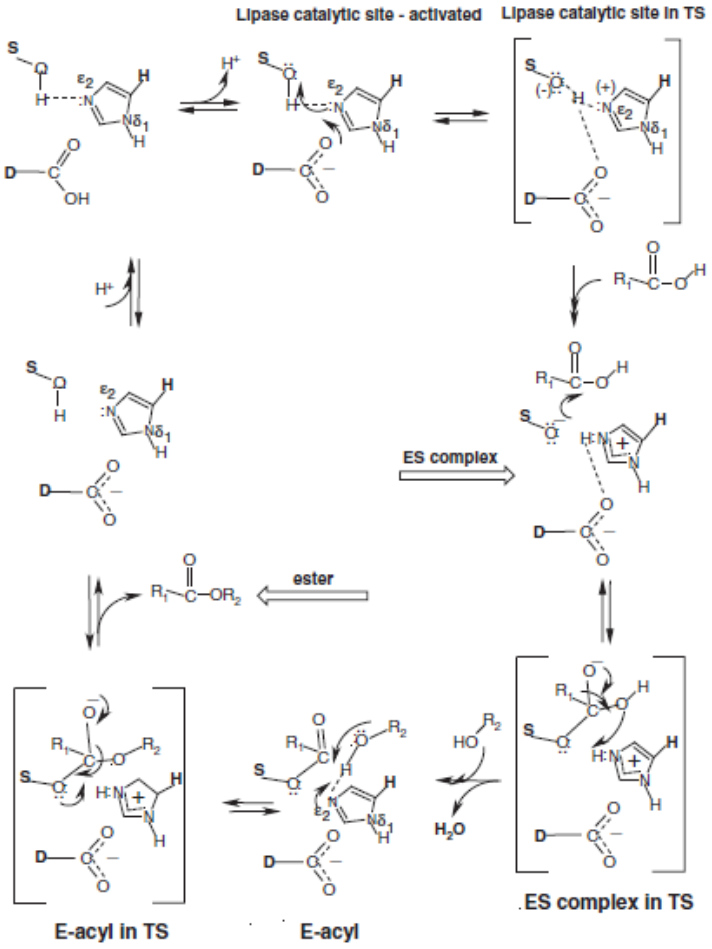


Figure 2.25: catalytic mechanism of lipase-mediated acylation.[149]

These reactions need a strictly dry environment, to prevent water molecules from the re-hydrolysis of the newly formed ester bond. Indeed, lipase-mediated esterifications in dry organic solvents are widely applied as a green method to form ester bonds.[150]

The low cost of these enzymes, together with the fact that they often come from animal waste, and that they are particularly suitable for working in organic media, makes their use industrially viable. Indeed, the lipase can make regioselective esterifications, thus avoiding the tedious use of protecting group chemistry, but also of condensing agents (such as carbodiimides) that are typically employed in the synthesis of esters, as the enzyme by itself acts as a sort of condensing agent.[151] A further advantage of lipases is that they are able to accept a wide variety of substrates both for hydrolytic cleavages and for esterifications, making them suitable for generalizable protocols.

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B. Experimental Section

General information

Reagents and solvents

All reagents, cofactors, commercial enzymes, and solvents were of the highest purity grade (95-99%) and were used with no further purification. They were purchased by the following suppliers: Merck (Darmstadt, Germany); Fluka Chemika (Buchs, Switzerland); Fluorochem (Hadfield, UK); Alfa-Aesar (Ward Hill, Massachusetts, USA); Steraloids Inc. (Newport, Rhode Island, USA).

Hyocholic acid was a generous donation from Dipharma Francis S.r.L. (Baranzate, Italy). The NADP(H) dependent FDH from methylotrophic bacterium *Pseudomonas* sp. 101 was a kind gift from Prof. Tishkov (M.V. Lomonosov Moscow State University).[1]

Analytics and instruments

The **NMR** spectra were acquired in $CDCl_3$, DMSO-d6, or in CD_3OD at room temperature on a Bruker AV 400 MHz spectrometer with a z gradient at 400 MHz for ^{1}H-NMR analysis and 101 MHz for ^{13}C-NMR.[2]

ESI-MS spectra were recorded on a Bruker Esquire 3000 PLUS instrument (ESI Ion Trap LC/MSn System), equipped with an ESI source and a quadrupole ion trap detector (QIT). The samples were dissolved in methanol to 1-2 g L^{-1} and then directly syringed in the ESI-MS at 4 µL min^{-1} rate. The analyses were performed in positive mode. The acquisition parameters were optimized as such: 4.5 kV needle voltage, 10 L h^{-1} N_2 flow rate, 40 V cone voltage, trap drive set to 46, 115.8 V capillary exit, 13000 (m/z) s^{-1} scan resolution over the 35-900 m/z mass/charge range, source temperature 250°C.

Biotransformations were performed with a G24 Environmental Incubator New Brunswick Scientific **Shaker** (Edison, USA) or a **Thermomixer** Comfort (Eppendorf, DE).

Reactions were monitored by **TLC** (thin-layer chromatography) on precoated glass plates silica gel 60 with fluorescent indicator UV_{254} and developed either with UV light (254 nm), or treated with A) Komarowski's reagent, *i.e.*, an oxidizing solution [4-hydroxybenzaldehyde (6.3 g), H2SO4 (50% v/v in H2O, 40 mL), MeOH (400 mL)] or B) Pancaldi's reagent, a molybdate oxidizing solution [$(NH4)_6Mo_7O_{24}$·4 H_2O (4.2 g); $Ce(SO4)_2$ (0.2 g); H_2SO_4 98%, (6.2 mL); H_2O (100 mL)].

Products were purified via **FC** (flash chromatography) on silica gel 60 (70–230 mesh), with eluents based on CH_2Cl_2/AcOEt mixtures.

Dehydrogenase **activities** of HSDHs and *Bm*GDH were determined spectrophotometrically by measuring the reduction of $NAD(P)^+$ at 340 nm (ε: 6.22 $mM^{-1}cm^{-1}$), while the activities of Is2-SDR and RmLDH were

measured by following the oxidation of NAD(P)H at the same wavelength. Assays were carried out in polyethylene cuvettes at room temperature by adding the opportune amount of purified dehydrogenase to the following assay mixtures (1 mL final volume in cuvette):

- HSDH assay: 2.5 mM substrate (cholic acid for 7α- and 12α-HSDHs, ursodeoxycholic acid for 7β-HSDHs); 50 mM potassium phosphate buffer (PB), pH 9.0; 0.20 mM $NAD(P)^+$.
- Is2-SDR assay: 10 mM ethyl 3-methyl-2-oxobutyrate; 50 mM PB, pH 8.0; 0.20 mM NADPH.
- *Bm*GDH assay: 50 mM glucose; 50 mM PB, pH 7.0; 0.20 mM $NAD(P)^+$.
- FDH assay: 200 mM ammonium formate; 50 mM PB, pH 7.0; 0.20 mM $NAD(P)^+$.
- RmLDH: 10 mM sodium pyruvate; 100 mM PB, pH 7.0; 0.20 mM NADH.

One unit (U) is defined as the enzyme activity that reduces/oxidizes 1 µmol of NAD(P)(H) per min under the assay conditions described above. The activities were determined either with a Jasco V-600 or with a Jasco V-730 **spectrophotometer**.

Chiral phase **HPLC** analyses were performed on a Shimadzu LC-20AD high performance liquid chromatography system endowed with a Shimadzu SPD-20A UV detector and equipped either with a Phenomenex Lux 3µ Cellulose-2 chiral column (250 mm x 4.6 mm) used in direct phase mode (mobile phase 2-propanol/petroleum ether in variable proportions) or with a Phenomenex Lux Cellulose-1 5µ chiral column (150 mm x 4.6 mm) used in reversed phase mode (mobile phase acetonitrile/water with 0.05% trifluoroacetic acid in variable amounts).

Chiral phase **GC** analyses were carried out on an Agilent Technologies 6850 Network GC system gas chromatograph equipped with split/splitless injector, FID detector and MEGA-DEX DAC Beta chiral capillary column (25 m × 0.25 mm × 0.25 µm).

GC-MS analyses were performed using an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 µm) on a Finnigan TRACE DSQ GC/MS instrument (ThermoQuest, San Jose, CA).

Optical rotations were measured on a Jasco P-2000 polarimeter. The specific rotation was calculated as the $[\alpha]\lambda T=\alpha 100/cd$, where α represents the recorded optical rotation, c the analyte concentration (mg mL^{-1}), d the cuvette length (dm). As such, the specific rotation is expressed as (10^{-1}deg $cm^{-2}g^{-1}$). λ is reported in nm and T in °C. λ corresponds to sodium D line (589 nm), thus the optical rotation is referred to as $[\alpha]_D$. T, c and the solvent were chosen according to references reported in literature.

Circular dichroism analyses were conducted with a Jasco J-1100 CD spectrometer for the determination of enzymes' T melting or secondary structure overall content.

Organization of the experimental section

The experimental section will discuss in detail the work that was made in the framework of this doctoral thesis. Specifically, the work will be subdivided in sections corresponding either to the published papers or to investigations that still have to be communicated to the scientific community. The discussion will be organized as follows: firstly, the use of HSDHs on their natural substrates (Chapter 3 on Hyocholic acid and its derivatives); secondly, a perspective on the substrate promiscuity of HSDHs (Chapter 4, divided in 4.1 on α-ketoesters and ketones, 4.2 on the Wieland-Miescher ketone, and 4.3 on HSDH-mediated modification of α-diketones, accompanied by the side use of other enzymes on these substrates); in the end, the conclusion will be devoted to a potentially synthetically relevant SDR (namely, Is2-SDR, in Chapter 5).

Each of the aforementioned Chapters will be subdivided in a brief discussion on the state of the art, followed by a presentation of the results and in the end by experimental details for those interested to have a deeper insight in the characterization of compounds.

Abbreviations and additional information can be found in Appendix I (abbreviations), II (compounds' reference numbers and names), and III (libraries of enzymes).

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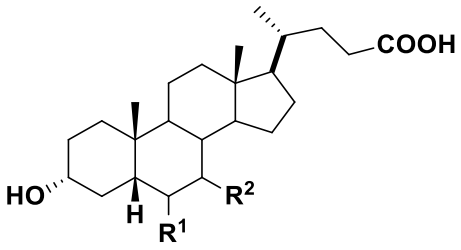
Chapter 3

Hyocholic acid's derivatization by means of HSDHs

This discussion is outlined in the published paper on *ChemCatChem* "Synthesis of ω-muricholic acid by one-pot enzymatic Mitsunobu inversion using hydroxysteroid dehydrogenases" (see Appendix I, paper IV).

3.1. Hyocholic acid and its derivatives: state of the art

Hyocholic acid (HCA, Compound **6**, Figure 2.13 and Figure 3.1) was isolated for the first time in 1923 by Windhaus.[1,2] Its structure was however fully elucidated only after thirty three years, in 1956, by Ziegler.[3] This compound can be found as a primary bile acid in hog bile, accompanied by the more abundant hyodeoxycholic acid (HDCA, Compound **12**, Figure 3.1). HCA, HDCA, and their conjugated derivatives account for about 80% of the bile acid content in hog bile.[4] Other C6-hydroxylated bile acids, such as muricholic acids, can be found in rodent bile as well, and they are depicted in Figure 3.1 as a reference for the subsequent discussion.



6: $R^1 = R^2 = \alpha$-OH (hyocholic acid, HCA)
12: $R^1 = \alpha$-OH, R^2 = H (hyodeoxycholic acid, HDCA)
13: $R^1 = \alpha$-OH, $R^2 = \beta$-OH (ϖ-muricholic acid, ϖ-MCA)
14: $R^1 = \beta$-OH, $R^2 = \alpha$-OH (α-muricholic acid, α-MCA)
15: $R^1 = \beta$-OH, $R^2 = \beta$-OH (β-muricholic acid, β-MCA)

Figure 3.1: Hyocholic acid and its metabolically relevant derivatives.

Nowadays, there is a strong interest in bile acid research, not only for their importance in the synthesis of drugs of relevance such as UDCA (Chapter 2.1.3.1), but the need of understanding their signaling properties is growing exponentially, after the discovery that they can modulate the G-Protein Coupled Receptor (GPCR) TGR5 and the nuclear farnesoid X receptor (FXR).[5-7] Moreover, it has been recently pointed out that bile acids hold the potential not only to be used as biomarkers for early-stage diagnosis of metabolic disorders or diseases, but they could be used as putative drugs to treat obesity or type 2 diabetes mellitus (T2DM), as well.[8-11]

3.1.1. Bioactivity of Hyocholic acid and Muricholic acids

As it was the case for bear bile and UDCA, also HCA has been administered for a long time in Chinese traditional medicine as a component of pig bile. Indeed, back in 1593 the *Compendium of Materia Medica* reported about the first known use of pig bile to treat *xiao-ke*, a disorder that nowadays is called diabetes.

The case of HCA and C6-hydroxylated bile acids is of growing interest, as it is in the very last years that signaling profiling of these molecules highlighted the high potential they hold as drugs to target metabolic disorders.[10,11] Clinical studies have been made both *in vitro* and *in vivo* on model animals (rodents and pigs), demonstrating that HCA and its conjugates can control glucose homeostasis through a double action on two receptors: the transmembrane TGR5 and the nuclear FXR.

It is noteworthy that HCA turned out to be more effective than tauroursodeoxycholic acid (UDCA taurine conjugate) in improving glucose tolerance in diabetic mouse models. Furthermore, despite their obesogenic diet, pigs showed an outstanding resistance to the development of diabetes, which was related to the high content of HCA bile acids in its bile by Zheng and coworkers.[11]

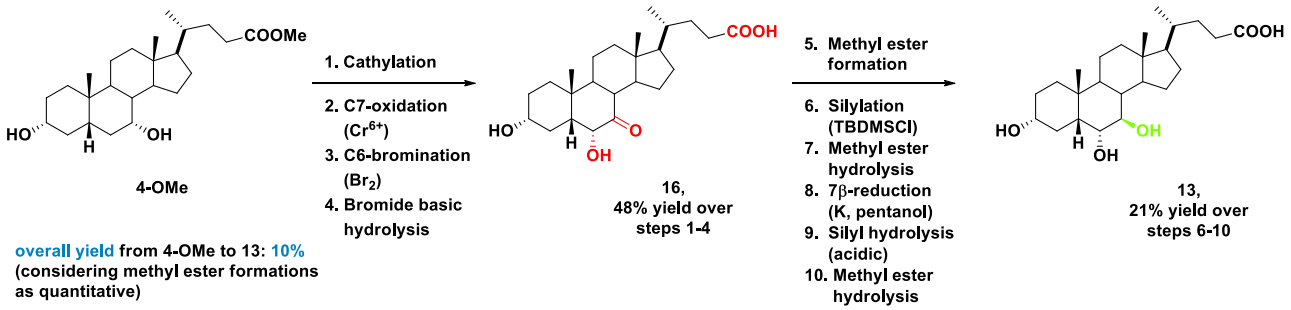
Further clinical studies and toxicology profiling will allow the scientific community to understand whether C6-hydroxylated bile acids may be good drug candidates.

3.1.2. The synthesis of muricholic acids in the past

Due to the metabolic and biological relevance of C6-hydroxylated bile acids, it is of growing interest to have the possibility to handle sufficient amounts of these species for studies on their signaling abilities. While this is possible for hyocholic acid, muricholic acids are in mixture in rodent bile, and even though they account for about 35% of the bile acid content of mice bile, they cannot be easily isolated in a pure form and in appreciable amounts, as it is shown by their high prices (*e.g.*, more than 250 USD for 1 mg of ω-MCA, reported in MolPort).[10]

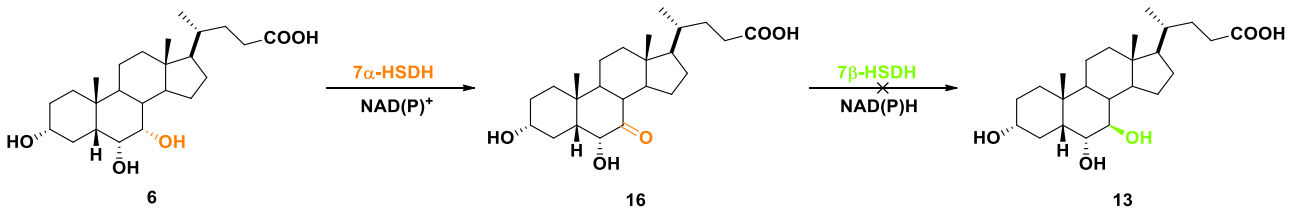
The synthesis of MCAs could start from HCA, as it was extensively described in 1989 by Iida et al.[12] This report described the synthesis of all the isomers of 3,6,7-trihydroxycholan-24-oic acids, among which, relevant to us, ω-muricholic acid.

ω-muricholic acid could be synthesized in the past via the low yield multistep process shown below in Scheme 3.1 (ten steps from CDCA methyl ester, with extensive use of protecting groups, use of harmful reagents, and overall yield of about 10%).



Scheme 3.1: chemical synthesis of ω-muricholic acid by Iida in 1989.[12]

Some preliminary studies on the enzymatic modification of HCA (among other bile acids) to reverse its C7-stereochemistry and afford ω-muricholic acid in a two-step procedure were made a few years later, in 1993, by Bovara et al. However, once the oxidation of HCA to its 7-oxo derivative (Compound **16**, 7-oxodeoxychyocholic acid, for short 7-oxoDHCA) was made, the reaction could not go any further to ω-MCA (Scheme 3.2) with the enzyme available at that time.[13]



Scheme 3.2: enzymatic modification of HCA by Bovara et al in 1993.[13]

It was therefore hypothesized that the steric hindrance provided by C6-α-OH would hamper the reduction, which was indeed effective on the 6β-stereoisomer α-muricholic acid (Figure 3.2).

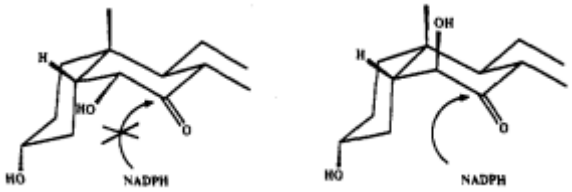


Figure 3.2: steric hindrance of C6-α-OH vs C6-β-OH on the C7-carbonyl for a 7β reduction.[13]

Another *in vitro* enzymatic biotransformation was carried out from β-MCA to ω-MCA with intestinal microorganisms (*Eubacterium lentum* and *Fusobacterium* sp. strains), but the transformation stopped between 50 and 90% conversion and the reaction scale was not specified.[14]

3.2. Results and discussion on ω-muricholic acid synthesis

All the novel 7β-HSDHs discovered and isolated at SCITEC-CNR (or previously described, in one case)[15] were able to perform the reduction of 7-oxoDHCA (compound **16**). As such, the work described herein managed to epimerize HCA at C7, forming the rare and expensive ω-MCA.

Hyocholic acid was at first successfully oxidized by the panel of 7α-HSDHs from the library at disposal in our lab (enlisted in Table 3.1). The reactions were scaled up to a 500 mg scale and coupled with a suitable cofactor regeneration system for the *in situ* regeneration of the oxidized NAD(P)$^+$ cofactor.[16,17] Compound **16** was isolated quantitatively and the structural characterization confirmed both the presence of the C7-oxo moiety and the unchanged stereochemistry at C5 and C6 (the $J_{H5\text{-}H6}$ was similar to that of HCA).

Enzyme	Activity	Cofactor	Source
*Ca*7α-HSDH	7α-HSDH	NADP(H)	*Clostridium absonum*
*Dm*7α-HSDH	7α-HSDH	NAD(H)	*Deinococcus marmoris*
*Ec*7α-HSDH	7α-HSDH	NAD(H)	*Escherichia coli*
*Hh*7α-HSDH	7α-HSDH	NAD(H)	*Halomonas halodenitrificans*
Ng1-7α-HSDH	7α-HSDH	NAD(H)	Metagenome

Table 3.1: 7α-HSDHs at disposal at SCITEC-CNR.

Then, compound **16** was subjected to 7β-HSDH-mediated reduction and all the screened HSDHs (shown in Table 3.2) were able to quantitatively reduce it to a more polar compound. The fastest reactions, catalyzed by the 7β-HSDHs from *Collinsella aerofaciens* and from *Staniera cyanosphaera*, were scaled up to a 100-200 mg scale and coupled with the suitable cofactor regeneration system.[18-20]

Enzyme	Activity	Cofactor	Source
*Bsp*7β-HSDH	7β-HSDH	NAD(H)	*Bacillus* sp.
*Cae*7β-HSDH	7β-HSDH	NADP(H)	*Collinsella aerofaciens*
*Hh*7β-HSDH	7β-HSDH	NAD(H)	*Halomonas halodenitrificans*
*Rs*7β-HSDH	7β-HSDH	NAD(H)	*Rhodococcus sphaeroides*
*Sc*7β-HSDH	7β-HSDH	NAD(H)	*Stanieria cyanosphaera*

Table 3.2: 7β-HSDHs at disposal at SCITEC-CNR.

This allowed the isolation and characterization of the desired ω-MCA, the stereochemistry at C7 confirmed by NMR analysis (both the J_{H6-H7} and J_{H7-H8} constants rose up to 9.4 Hz, indicating all the hydrogens to be in an axial position, see Figure 3.3).

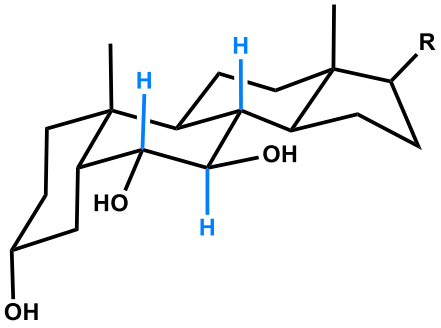
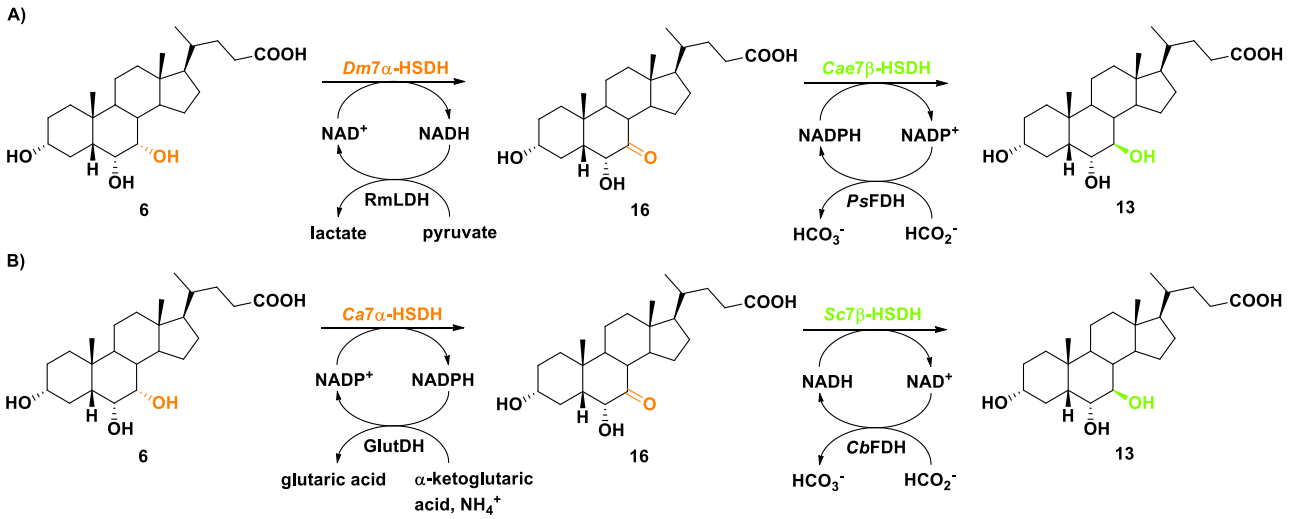


Figure 3.3: ω-MCA, with axial protons at C6, C7, and C8 highlighted in light blue.

Having confirmed the viability of both of the steps of HCA epimerization at C7 from an α to a ß configuration, the process was optimized in order to be run in one-pot, to mimic a Mitsunobu-like stereoinversion, but with an ameliorated atom efficiency and a more desirable green chemistry profile in general.[21] This result could be achieved by choosing enzymes with strict and different cofactor specificities for the two reactions (C-7 regioselective oxidation and C-7 stereoselective reduction), as it is shown in Scheme 3.3. The biotransformation in Scheme 3.3A was scaled to 100 mg of HCA and a one-pot road to the quantitative isolation of ω-MCA was set. This result could be achieved by choosing enzymes with strict different cofactor specificities for the two reactions (C-7 regioselective oxidation, C-7 stereoselective reduction), as it is shown in Scheme 3.3.



Scheme 3.3: one-pot Mitsunobu-like stereoinversion of HCA at C7 to obtain ω-MCA. RmLDH: Lactate Dehydrogenase from rabbit muscle; *Ps*FDH: Formate Dehydrogenase from *Pseudomonas* sp.; GlutDH: commercial Glutamate Dehydrogenase from Merck; *Cb*FDH: Formate Dehydrogenase from *Candida boidinii*.

Encouraged by these preliminary results on the C7 epimerization of HCA to ω-MCA, we envisaged the possibility to use HCA as an alternative starting material for UDCA instead of cholic acid (Figure 3.4).

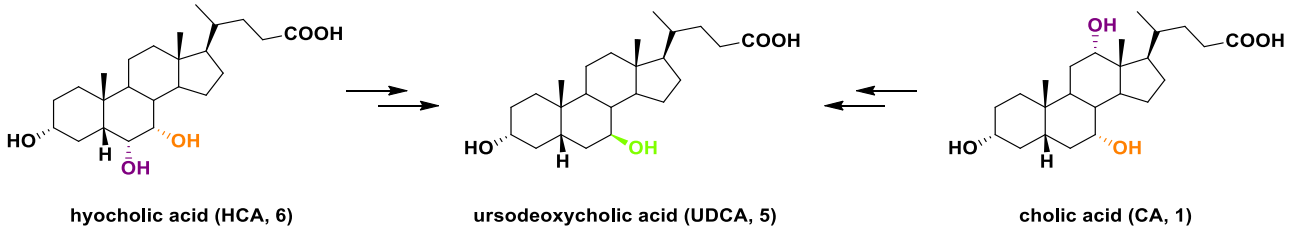
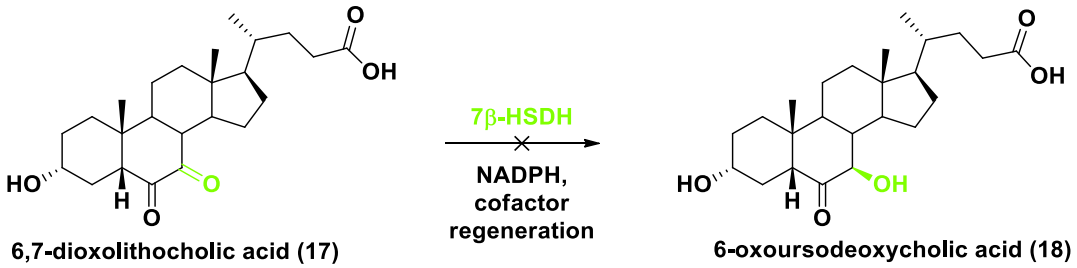


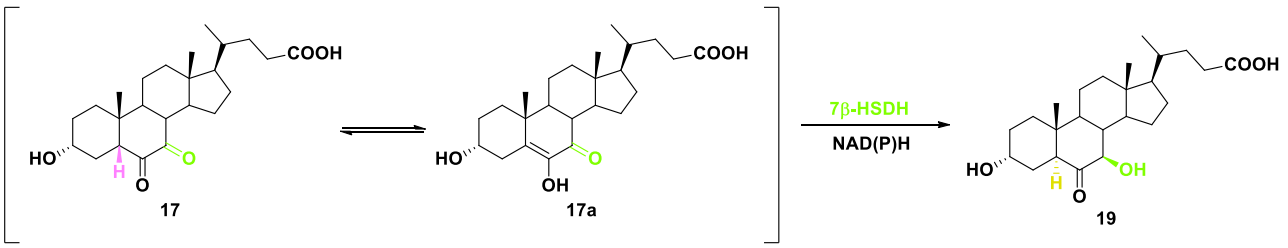
Figure 3.4: different possible starting materials for UDCA synthesis.

To do so, we investigated the 7β-HSDH-mediated reduction (Scheme 3.4) of the putative synthetic intermediate 6,7-dioxolithocholic acid (Compound **17**, for short 6,7-dioxoLCA), available by a commercial supplier. All the investigated enzymes were able to perform this reduction to the supposedly compound **18** (Scheme 3.4, 6-oxoursodeoxycholic acid, for short 6-oxoUDCA).



Scheme 3.4: HSDH-mediated reduction of 6,7-dioxoLCA (compound **17**).

Nevertheless, a scale-up of the best performing reaction, catalyzed by the 7β-HSDH from *Collinsella aerofaciens*, allowed to determine that an isomerization of the A-B ring junctions of the steroid core from a *cis*- to a *trans*-configuration had happened, thus leading to the isolation of *allo*-6-oxoUDCA (compound **19**, Scheme 3.5; the prefix *allo*- is used to refer to the 5α-epimers of cholanoic acids, with a *trans*-junction between rings A and B).



Scheme 3.5: keto-enol tautomerism of 6,7-dioxoLCA led to the isolation of compound **19**.

This was indeed consistent with previous studies reported in the literature, that mentioned the lability of C5 configuration in the presence of a 6-oxo moiety, and in some cases this fact was exploited to

synthesize brassinosteroids (plant hormones of agricultural interest).[12,22,23] Further confirmation of the enolization mechanism came from both ^{1}H- and ^{13}C-NMR studies of the starting diketone **17**, showing that it exists as its enol tautomer **17a** in DMSO-d6.

For this reason, the idea of synthesizing UDCA from HCA was temporarily abandoned.

The experimental details are outlined in the Paper IV in Appendix I.

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Chapter 4

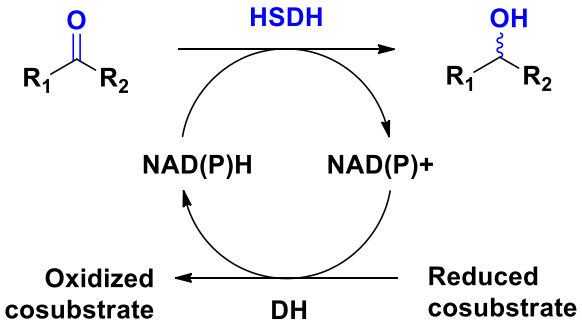
Insights into the substrate promiscuity of HSDHs

As it was outlined in the Introduction, bacterial HSDHs are NAD(P)H-dependent enzymes that mainly belong to the superfamily of SDRs. These enzymes display peculiar features, mainly connected to their high chemo-, regio- and stereoselectivity, that make them attractive for industrial applications on steroidal compounds (*e.g.*, in the synthesis of UDCA).[1]

Although these enzymes have been thoroughly investigated during the last years, little is currently known regarding their possible uses on alcoholic or ketonic substrates that differ from steroids.[2]

In order to fill this gap, the substrate promiscuity of a library of fifteen recombinant enzymes was investigated. Specifically, we dealt with thirteen different 7α-, 7β-, or 12α-HSDHs and two SDRs, which originated both from known and well-characterized microbial sources as well as from newly identified (meta)genomic sequences.

Accordingly, these enzymes were tested for the stereoselective reduction of a panel of putative carbonylic substrates, as shown in Scheme 4.1. The screened compounds included α-ketoesters of pharmaceutical interest and selected ketones that partially resemble the structural features of steroids.



Scheme 4.1: stereoselective reduction of ketonic moieties catalyzed by HSDHs.

Section 4.1 will deal with three bicyclic ketones (namely, *trans*-1-decalone, α- and β-tetralone) and three α-ketoesters of interest in different API syntheses (methyl benzoylformate, ethyl 3-methyl-2-oxobutanoate, and ethyl 4-phenyl-2-oxobutanoate).[3] Section 4.2. will analyze enzymatic modifications on the pharmaceutically relevant Wieland-Miescher ketone (with HSDHs as a main focus and lipases and OYEs for further modifications).[4] In the end, Section 4.3 will deal with α-diketones (the use of other enzymes on these molecules, namely, transaminases, will be dealt with as well).

4.1. HSDHs and their substrate promiscuity: α-ketoesters and bicyclic ketones

4.1.1. State of the art

The investigation on HSDHs' substrate promiscuity started with almost an absence of literature information. Indeed, only few reports were found which mentioned the substrate promiscuity of HSDHs.[2,5-9] One of those regarded a close analogue of the enzymes in our library (*i.e.*, the 7α-HSDH from *Bacteroides fragilis*), which was reported to be able to reduce a panel of bulky α-ketoesters with high selectivity for the *R*-enantiomer of the α-hydroxyketonic product.

Moreover, in search of further substrates to expand or panel of investigation, we found a recent paper in the literature that described via *in silico* modeling the potential ability of a ketoreductase to stereoselectively reduce *trans*-1-decalone. Therefore, this compound and two closely related analogs, namely α- and ß-tetralones, were also chosen for our studies.

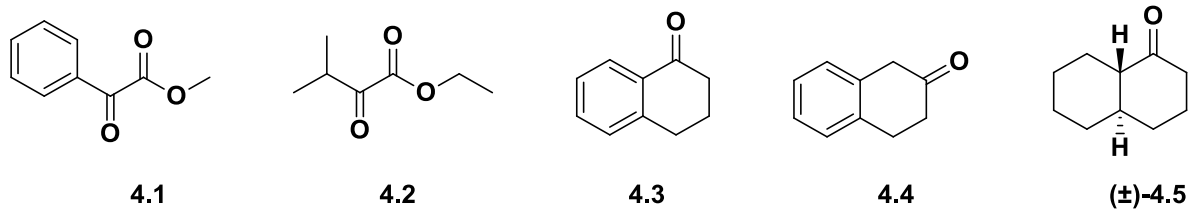


Figure 4.1: panel of α-ketoesters and bicyclic ketones chosen for preliminary investigations on HSDHs' promiscuity.

Figure 4.1 shows the panel of substrates we have chosen. Specifically, the α-ketoesters methyl benzoylformate (compound **4.1**) and ethyl 3-methyl-2-oxobutanoate (compound **4.2**) were chosen for their metabolic and pharmaceutical relevance (e.g., in the synthesis of potential Caspase 3 inhibitors to treat degenerative diseases or of other pharmaceuticals as pemoline).[10-16]

Regarding the bicyclic ketones, *trans*-1-decalone (compound **4.5**) is a known substrate for the ketoreductase module in fatty acid synthase and it turned out to be a substrate both in modeling and in experimental studies on different ketoreductases (e.g., the ketoreductase modules of both diketide synthase and erythromycin polyketide synthase).[17-21] Some of these reports also studied the reduction of α-tetralone (compound **4.3**). As expected, no study was found in the literature on the HSDH-mediated reduction of bicyclic ketones.

4.1.2. Results and discussion

In order to get further insights into the substrate promiscuity of HSDHs, this result was approached first of all by increasing the diversity of the individuals available in SCITEC-CNR's HSDH library. Indeed, most of the known microbial HSDHs are produced either in gut or in soil bacteria, which makes these enzymes not much diversified.

4.1.2.1. Discovery and characterization of ten novel SDRs

The diversity of the library of HSDHs at our disposal was increased via bioinformatic screening of (meta)genomes that were either public or assembled in-house. With these analyses, ten novel putative HSDH sequences were selected, cloned, and characterized after recombinant expression in *E. coli*. Their origins and characteristics are resumed in Table 4.1. Notably, two metagenomic individuals (Is2-SDR and Ngi7-SDR) turned out to have no HSDH activity, but they featured a substrate profile typical of ketoreductases (see Chapter 5 for further details on Is2-SDR). The other individuals were confirmed to be HSDHs and their regio- and stereo-selectivity was established via activity assays.

Entry	Enzyme	Source	Identity with known HSDHs (%)	HSDH activity	Cofactor	Yield (U)	Yield (mg)	Specific activity ($U mg^{-1}$)
1	Dm7α-HSDH	*Deinococcus marmoris*	75.40 (Sm7α-HSDH)	7α-HSDH	NAD(H)	788	14	56
2	Hh7α-HSDH	*Halomonas halodenitrificans*	74.51 (Sh7α-HSDH)	7α-HSDH	NAD(H)	1986	64	31
3	Hh7β-HSDH	*Halomonas halodenitrificans*	38.89 (Bs7α-HSDH)	7β-HSDH	NAD(H)	955	95	10
4	Sc7β-HSDH	*Stanieria cyanosphaera*	38.40 (Gri7α-HSDH)	7β-HSDH	NAD(H)	261	7	38
5	Bsp7β-HSDH	*Brucella* sp.	36.00 (Pa7α-HSDH)	7β-HSDH	NAD(H)	8400	91	92
6	Rs7β-HSDH	*Rhodobacter sphaeroides*	39.68 (Bs7α-HSDH)	7β-HSDH	NAD(H)	2348	80	29
7	Ngi1_7α-HSDH	metagenome	69.05 (Sm7α-HSDH)	7α-HSDH	NAD(H)	24780	174	142
8	Is2-SDR	metagenome	38.49 (Bsp7α-HSDH)	n.a.	NADP(H)	–	107	–
9	Ngi7-SDR	metagenome	35.50 (Csp12α-HSDH)	n.a.	NAD(H)	–	326	–
10	Ls12α-HSDH	*Lysinibacillus sphaericus*	47.39 (El12α-HSDH)	12α-HSDH	NAD(H)	348	135	2.6

Table 4.1: Characteristics of novel SDRs and HSDHs found at SCITEC-CNR.[3]

The screening of public (meta)genomes led to the identification of seven out of the ten novel individuals. *Dm*7α-HSDH and *Hh*7α-HSDH were found in the genome of extremophilic bacteria, and they had never been characterized before our study. *Hh*7β-HSDH was found by checking for SDR-encoding sequences in the proximity of the *Hh*7α-HSDH gene. This check was made since it was reported in the literature that genes encoding for HSDHs tend to be clustered in bacteria.[22] *Hh*7β-HSDH sequence was used as a model for new searches in the National Center for Biotechnology Information (NCBI), leading to the identification of three uncharacterized SDRs sharing up to 79% identity with the sequence of *Hh*7β-HSDH and coming from *Stanieria cyanosphaera*, *Brucella* sp., and *Rhodobacter sphaeroides*. The last individual from public genomic databases was *Ls*12α-HSDH from *Lysinibacillus sphaericus*, which was found to show a sequence identity of about 50% with a known 12α-HSDH.

On the other hand, the other three individuals were identified by screening of in-house metagenome samples collected in extreme environments. Ngi7_7α-HSDH was shown to share a high sequence identity with known 7α-HSDHs, while the other two individuals (Is2-SDR and Ngi7-SDR) showed no activity on the panel of tested bile acids.

A phylogenetic tree of the novel SDRs was made, showing some peculiarities (Figure 4.2, with new SDR individuals in bold). Indeed, *Dm*7α-HSDH and Ngi7_7α-HSDH showed a moderate thermophilicity and a 75% similarity to the recently isolated thermophilic *Sm*7α-HSDH,[23] constituting a different clade (highlighted in red) from the other known HSDHs (including the novel *Hh*7α-HSDH), that show a mesophilic behavior. *Ls*12α-HSDH was phylogenetically related to the other 12α-HSDHs. Noteworthy, the most interesting finding was the isolation of four NAD(H)-dependent 7β-HSDHs, that were in a distant clade (highlighted in blue) from the other known 7β-HSDHs, which are almost exclusively NADP(H)-dependent.

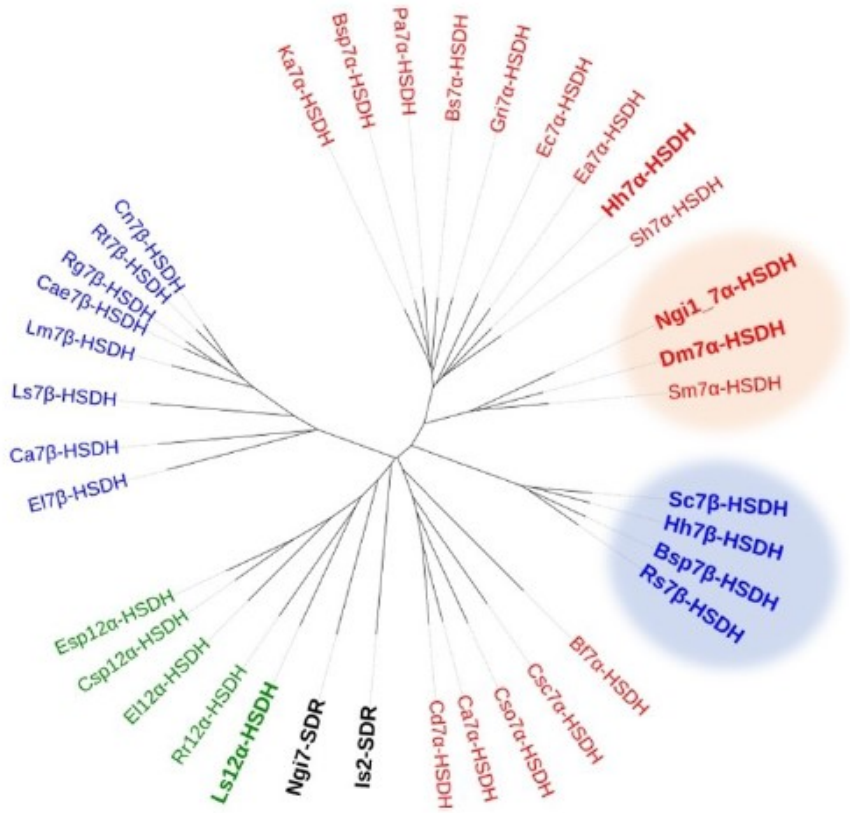


Figure 4.2: phylogenetic tree encompassing known and novel SDR/HSDH sequences.[3]

4.1.2.2. Substrate scope determination of the novel HSDHs

Due to the high regio- and stereoselectivity of this class of enzymes, a deeper insight into their substrate scope was evaluated to be of potential synthetic interest, maybe as a fertile ground for enzyme engineering as well.

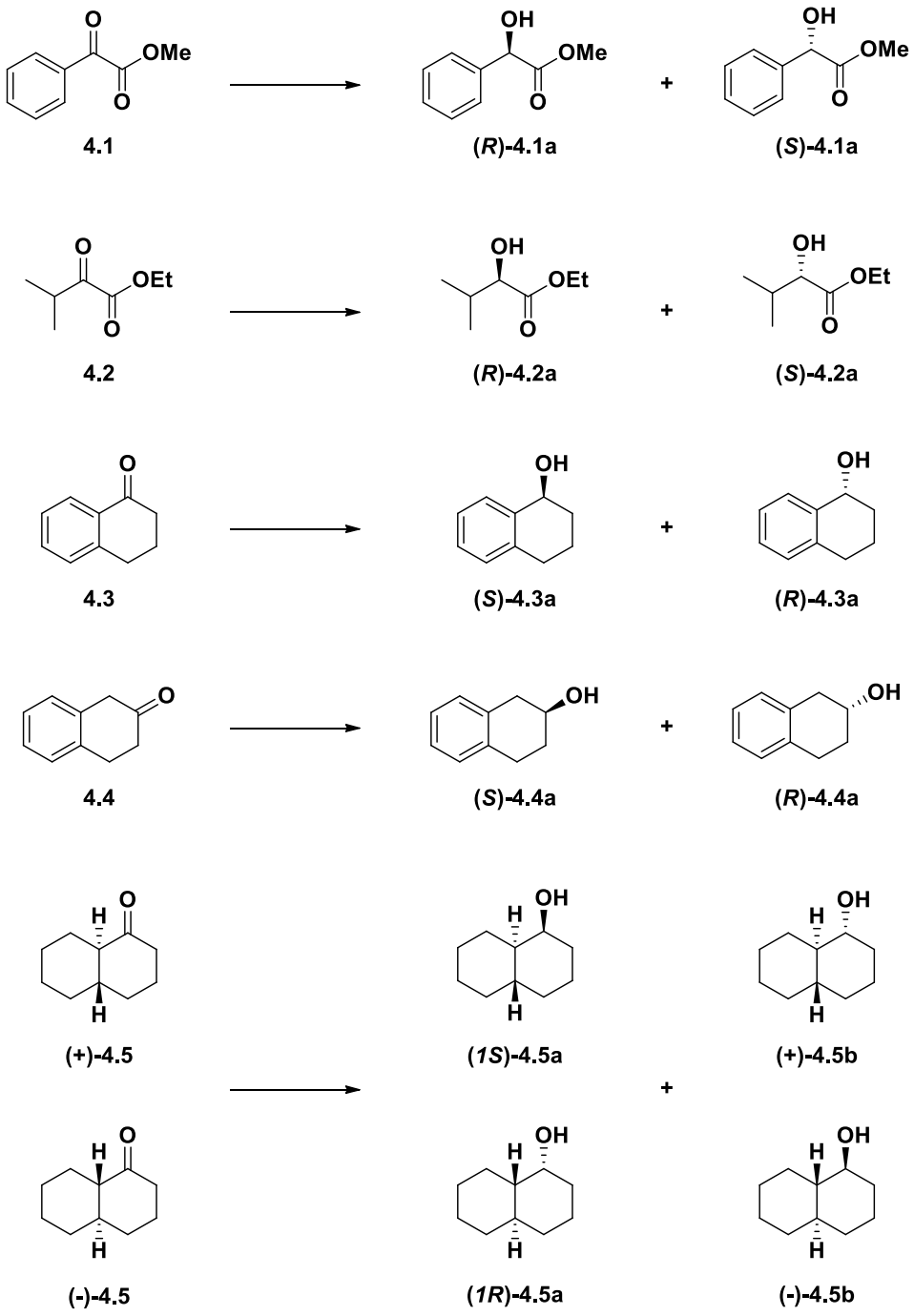
The new HSDHs and SDRs were screened with the compounds **4.1**-**4.5**, alongside with five previously described HSDH individuals (*Ca*7α-HSDH, *Ec*7α-HSDH, *Ca*7β-HSDH, *Cae*7β-HSDH, and *C*sp12α-HSDH). The whole library is enlisted in Table 4.2.

Enzyme	Activity	Cofactor	Source
*Ca*7α-HSDH	7α-HSDH	NADP(H)	*Clostridium absonum*
*Dm*7α-HSDH	7α-HSDH	NAD(H)	*Deinococcus marmoris*
*Ec*7α-HSDH	7α-HSDH	NAD(H)	*Escherichia coli*
*Hh*7α-HSDH	7α-HSDH	NAD(H)	*Halomonas halodenitrificans*
Ngi1_7α-HSDH	7α-HSDH	NAD(H)	Norwegian metagenome
*B*sp7β-HSDH	7β-HSDH	NAD(H)	*Brucella* sp.
*Ca*7β-HSDH	7α-HSDH	NADP(H)	*Clostridium absonum*
*Cae*7β-HSDH	7β-HSDH	NADP(H)	*Collinsella aerofaciens*
*Hh*7β-HSDH	7β-HSDH	NAD(H)	*Halomonas halodenitrificans*
*Rs*7β-HSDH	7β-HSDH	NAD(H)	*Rhodococcus sphaeroides*
*Sc*7β-HSDH	7β-HSDH	NAD(H)	*Stanieria cyanosphaera*
*C*sp*12* α-HSDH	12α-HSDH	NADP(H)	*Clostridium* sp.
Ls12 α-HSDH	12α-HSDH	NAD(H)	*Lysinibacillus sphaericus*
Is2-SDR	n.a.	NADP(H)	*Icelandic metagenome*
Ngi7-SDR	n.a.	NAD(H)	Norwegian metagenome

Table 4.2: library of HSDHs and SDRs investigated in this study.

Scheme 4.2 shows all the possible products that can be originated from the reduction of compounds **4.1**-**4.5**. In the case of methyl benzoylformate (**4.1**), the reduction yields the two synthetically relevant enantiomers of methyl mandelate (compounds **4.1a**), which were at disposal as enantiomerically pure standards in our laboratory. On the other hand, the analytical standards of the other reduced compounds were prepared by $NaBH_4$ reduction by us. Chiral phase GC or HPLC allowed us to assess the enantiomeric excesses of the products, while the comparison with the standards or

optical rotation measurements (compounds **4.2a**, and **4.5b**) permitted to assess the absolute configurations.



Scheme 4.2: chemical reduction of compounds **4.1**-**4.5** yields all the stereoisomeric products.

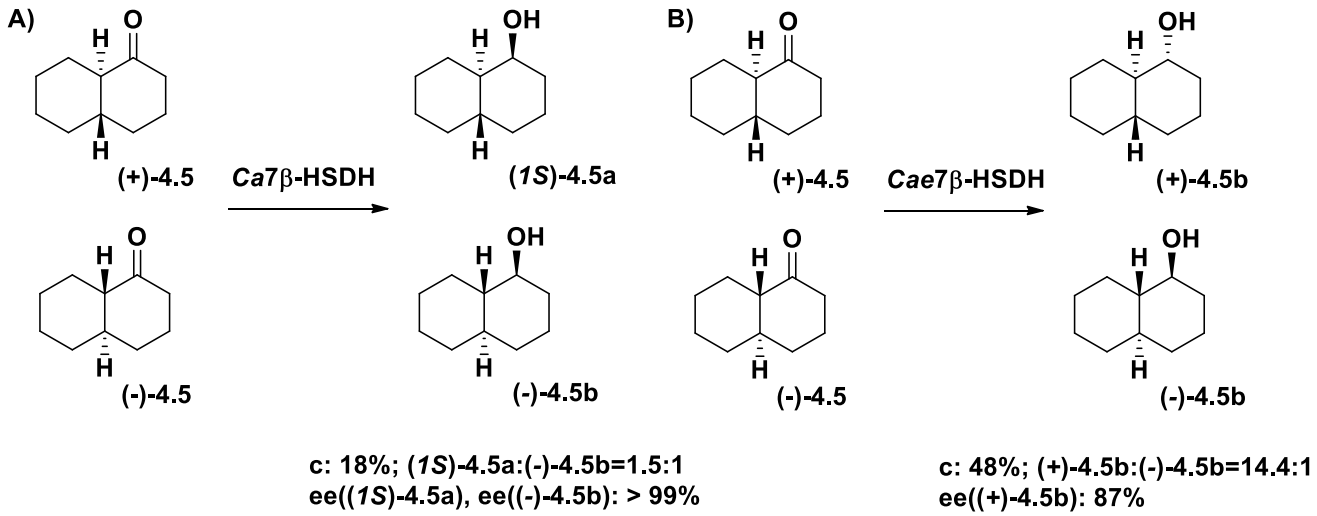
The α-ketoesters were widely accepted within our HSDH library with good to quantitative conversions and good to excellent enantiomeric excesses for the corresponding *R*-α-hydroxyesters, in accordance with the study on the panel of α-ketoesters reduced by *Bf*7α-HSDH. The conversions and ees of the HSDH-mediated reductions are resumed in Table 4.3.

Selectivity	Enzyme	Compound 4.1		Compound 4.2	
		C (%)	ee_R (%)	C (%)	ee_R (%)
12α	*Csp*12α-HSDH	94.5	> 99	93.9	85.7
12α	*Ls*12α-HSDH	94.1	64.7	> 99	93.6
7α	*Ca*7α-HSDH	> 99	88.3	42.3	91.1
7α	*Dm*7α-HSDH	> 99	82.9	> 99	93.9
7α	*Ec*7α-HSDH	89.7	90.2	81.6	> 99
7α	*Hh*7α-HSDH	95.9	97.9	81.0	> 99
7α	Ngi1-7α-HSDH	26.1	> 99	64.9	92.4
7β	*Bsp*7β-HSDH	> 99	89.6	> 99	96.0
7β	*Ca*7β-HSDH	> 99	45.2	> 99	89.3
7β	*Cae*7β-HSDH	> 99	23.3	> 99	97.2
7β	*Hh*7β-HSDH	97.4	95.2	65.8	64.9
7β	*Rs*7β-HSDH	75.5	> 99	> 99	95.5
7β	*Sc*7β-HSDH	61.7	92.5	65.8	64.9

Table 4.3: selectivity of HSDHs on α-ketoesters.

Notably, in some cases the stereoselectivity in the reduction of compound **4.1** was much lower than that observed for compound **4.2** (*Ca*7β-HSDH, *Cae*7β-HSDH, and, to a minor extent, *Ls*12α-HSDH). This may suggest that the aromatic ring in methyl benzoyl formate may promote more than one substrate binding mode (either pro-*R* or pro-*S*), while compound **4.2** would be bound only in a pro-*R* fashion. The assessment of the absolute *R*-stereochemistry of the α-hydroxyesters was made by comparison with analytical standards for compound **4.1**. Regarding compound **4.2**, the reaction catalyzed by *Cae*7β-HSDH, which combined the best stereoselectivity and the highest conversion, was scaled up to isolate **4.2a,** with an $[\alpha]_D^{25}$ value of -2.70°, consistent with the value reported in literature for the *R*-enantiomer.[13]

Regarding the bicyclic ketones, HSDHs did not accept the tetralones **4.3** and **4.4** as substrates, while only few HSDH individuals in the library reduced racemic *trans*-1-decalone **4.5** (*Ca*7β-HSDH and *Cae*7β-HSDH), but with different selectivity, as it is summed up in Scheme 4.3.

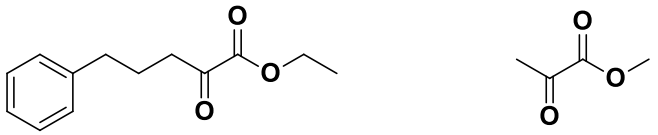


Scheme 4.3: A) stereoselective reduction of *trans*-1-decalone by *Ca*7β-HSDH produces the diastereomers of (*1S*)-*trans*-1-decalol; B) kinetic resolution over racemic *trans*-1-decalone by *Cae*7β-HSDH produces the enantiomers of *cis-trans*-1-decalol enriched with compound **4.5b1**, namely (*1R,4aR,8aS*)-1-decalol, in 87% ee.

The reductions on *trans*-1-decalone gave only moderate conversions under unoptimized reactions conditions. Notably, both the enzymes were 7Β-HSDHs, so close homologues. However, the two enzymes featured a different selectivity. Indeed, *Ca*7Β-HSDH performed a highly stereospecific reduction, producing the two diastereomers of (*1S*)-*trans*-1-decalol with high enantiomeric excesses (> 99%). On the other hand, *Cae*7Β-HSDH catalyzed a kinetic resolution on racemic **4.5,** reducing preferentially **(+)-4.5** to yield the unreacted **(-)-4.5** in 72% ee ($[\alpha]_D^{25}$: +2.75°) and the enantiomers of *cis-trans*-1-decalol (**(+)-** and **(-)-4.5b)** enriched with (*1R,4aR,8aS*)-1-decalol **(+)-4.5b** in 87% ee ($[\alpha]_D^{25}$: +44.45°). The absolute stereochemistry of the remaining substrate and of the products were assigned by scale up of the reduction catalyzed by *Cae*7Β-HSDH, NMR analysis and optical rotation measurements compared with those reported in the literature.[24,25]

4.1.3. Unpublished complementation to the work: ethyl 4-phenyl-2-oxobutanoate reduction with HSDHs

Based on the encouraging results obtained in the reduction of aromatic α-ketoesters and on our work on methyl benzoylformate and ethyl 3-methyl-2-oxobutanoate,[2,3] the reduction of a further α-ketoester of pharmaceutical interest, namely ethyl 4-phenyl-2-oxobutanoate (compound **4.6**), was investigated with HSDHs.



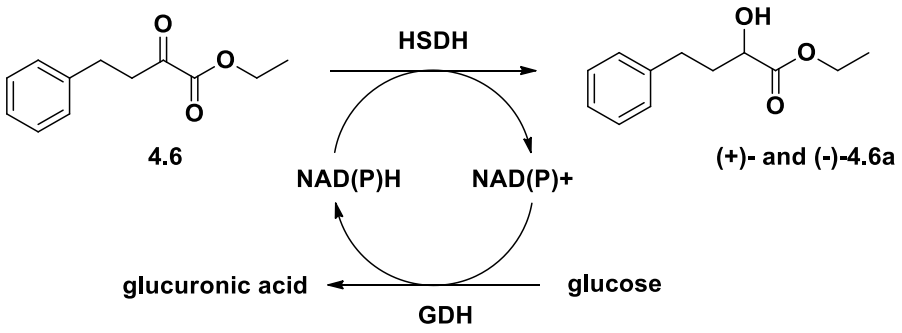
4.6 4.7

Figure 4.3: additional α-ketoesters as putative substrates for HSDH-mediated reduction.

Enzyme	C (%)	ee (4.6a/b)
*Csp12*α-HSDH	5.4	28.6
*Ls12*α-HSDH	3.5	61.6 (e1)
*Dm7*α-HSDH	14.4	82.7 (e2)
Ec7a-HSDH	32.6	14.3 (e2)
*Hh7*α-HSDH	14.3	9.0 (e2)
Ngi1-7α-HSDH	2.3	47.6 (e1)
*Ca7*α-HSDH	6.7	7.4 (e2)
*Bsp7*β-HSDH	28.6	80.2 (e1)
*Hh7*β-HSDH	3.0	53.3 (e1)
*Rs7*β-HSDH	14.9	71.0 (e1)
*Sc7*β-HSDH	29.1	75.3 (e1)
*Ca7*β-HSDH	1.9	n.d.
*Cae7*β-HSDH	41.9	64.6 (e1)

Table 4.4: conversions and ees of HSDH-mediated ethyl 4-phenyl-2-oxobutanoate reductions.

The screening was conducted on analytical scale (1 mL) with the aforementioned general protocol used for substrates 4.1-4.5. Chiral phase HPLC analyses were carried out on a Phenomenex Lux cellulose-2 column, T_{oven}: 33°C; $T_{detector}$: 40°C; $\lambda_{detection}$: 254 nm; elution: PE:iPrOH=9:1, flow 0.5 mL min^{-1}. retention times: 4.6: 10.98 min; (+)- and (-)-4.6a: 14.02 min, 15.14 min. ε_{254}(4.6): 946.9 $M^{-1}cm^{-1}$; ε_{254}((+)- and (-)-4.6a): 1756 $M^{-1}cm^{-1}$. The results of this screening, reported in Table 4.4 and obtained via chiral phase HPLC analyses, were not published due to their scarce synthetic relevance.



Scheme 4.4: reduction of ethyl 4-phenyl-2-oxobutanoate by HSDHs with glucose/GDH to regenerate NAD(P)H.

However, some interesting observations can be made. Indeed, compound **4.6** was accepted by all individuals in the library, albeit with lower conversions and poorer selectivity with respect to compounds **4.1** and **4.2**. Moreover, HSDHs seemed to have an enantio-divergent behavior, with *7*α-HSDHs preferring to form one enantiomer of reduced **4.6**, and *7*β-HSDHs giving the opposite one (the absolute stereochemistry of the preferred products has not been determined yet). Maybe, this could be explained by the existence of both a pro-*R* and a pro-*S* alternative binding modes within the active site, as it was suggested for the reduction of methyl benzoyl formate by *Ca*7β-HSDH and *Cae*7β-HSDH. Alternatively, the differences recently found in the active sites of *7*α- and *7*β-HSDHs could explain this bizarre behavior.[26]

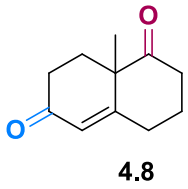
Regarding the steric demand of HSDHs, we tested the reduction of methyl pyruvate (compound **4.7**) and we saw that it was not reduced at all. It appears also that the carbon adjacent to the ketonic moiety could impair a higher stereoselectivity when it is functionalized with bulky substituents (*e.g.*, aromatic rings or *i*-Pr moiety), which could be an explanation of the reason why the stereoselectivity on compound **4.6** was poor.

A further hypothesis was made regarding the stereo-electronic demands of HSDHs, which will be considered in Chapter 4.3, and which will deal with the relevance of the α-dicarbonyl moiety for substrate acceptance by HSDHs.

4.2. HSDHs and their substrate promiscuity: the pharmaceutically relevant Wieland-Miescher ketone

4.2.1. State of the art

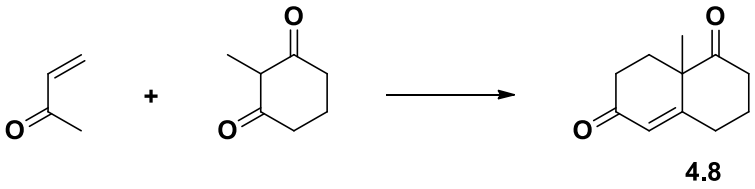
The Wieland-Miescher ketone (WMK, Compound **4.8**, Figure 4.4) is a synthetic intermediate that finds wide application in the total synthesis of natural and pharmaceutical products, mainly featuring a terpenoid-like, steroid-like or taxol-like structure.[27-29]



4.8

Figure 4.4: the structure of the Wieland-Miescher ketone (compound **4.8**).

The chemical synthesis of this compound can be easily achieved via the Robinson annulation, *i.e.*, a tandem reaction including a Michael addition and an intramolecular aldol condensation between 2-methylcyclohexane-1,3-dione and methyl vinyl ketone (Scheme 4.5).



4.8

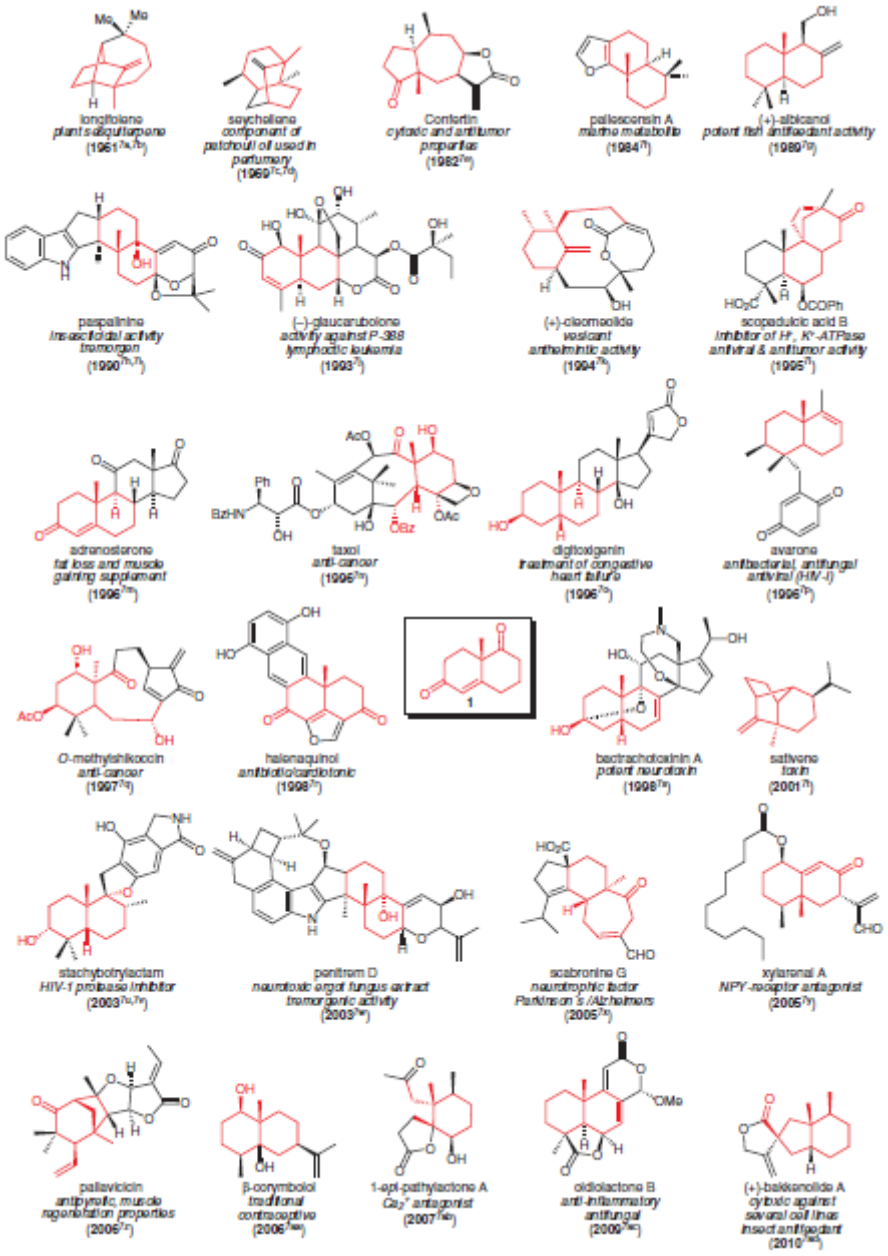
Scheme 4.5: the chemical synthesis of the Wieland-Miescher ketone via the Robinson annulation.

Due to its relevance as a synthetic intermediate, also asymmetric versions of the synthesis of WMK were developed, *e.g.*, by means of proline catalysts.[30,31]

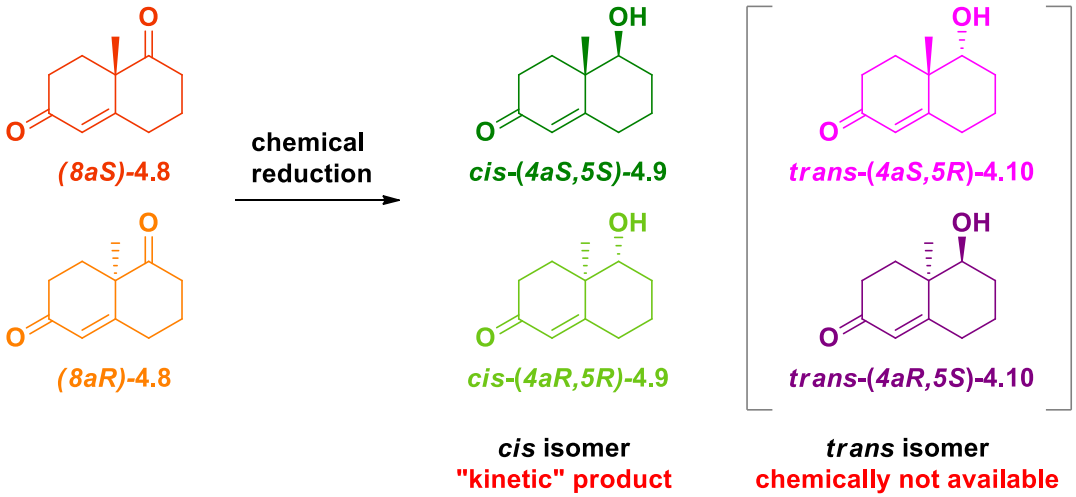
The enantiomerically enriched chiral derivatives **4.9** and **4.10** (Scheme 4.6) have been reported as important building blocks to synthesize either bioactive natural products or compounds of pharmaceutical interest.[32-36] Indeed, the molecular skeleton of these molecules can be easily further modified thanks to the presence of the enone moiety, which features a multifaceted reactivity profile. An illustrative example of the high synthetic versatility of the derivatives of 4.8 is shown in Picture 4.1, taken from the review article of Bradshaw and Bonjoch.[36]

The chemical reduction of this diketonic compound can in principle afford a plethora of products, encompassing all the diastereomers of the fully reduced diols, of the selectively reduced alcohol at C1 (carbonyl shown in purple in Figure 4.4) and of the allylic alcohol at C6 (carbonyl shown in light blue in Figure 4.4). The sole reduction of the C1 carbonyl moiety of compound **4.8** could yield a mixture of

four stereoisomeric alcohols (5-hydroxy-4a-methyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3H)-one), existing as two enantiomeric couples of *cis*- and *trans*-enantiomers (Scheme 4.6).



Picture 4.1: the skeleton of the Wieland-Miescher ketone derivatives is present in many pharmaceutical compounds.[36]



Scheme 4.6: representation of the selective chemical reduction of WMK's C1 carbonyl moiety.

However, it was demonstrated that the chemical reduction of compound **4.8** under mild conditions (0°C, $NaBH_4$) with common hydride-based reducing agents affords the racemic mixture of the enantiomeric *cis*-alcohols **4.9**, while the enantiomers of the *trans*-isomer **4.10** are chemically inaccessible. This is possibly due to the steric hindrance impaired by the C8a methyl group, adjacent to the carbonyl that has to be reduced (Scheme 4.6 and Figure 4.5).[35,37] The two *trans*-alcohols can be obtained in low yield, starting from the *cis*-isomer enantiomers, via a stereoinversion protocol that was invented *ad hoc* by Shimizu and coworkers, since the usual Mitsunobu protocol was ineffective on this substrate.[38]

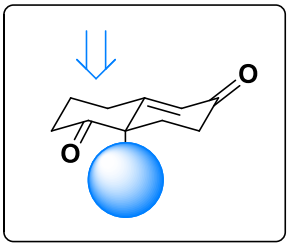


Figure 4.5: the steric hindrance of C8a-CH_3 hampers the formation of *trans*-alcohol **4.10**.

As it was widely discussed in the previous sections, when the asymmetric synthesis of compounds becomes of interest, biocatalysis starts to look as a much attractive tool to be used. For this reason, also in the case of the synthesis of the enantiomers of WMK or if its derivatives, "bio"-based strategies were envisaged to obtain these compounds as stereo-enriched species. In this respect, regarding the asymmetric synthesis of the Wieland-Miescher ketone, a noteworthy result was obtained by Danishefsky and coworkers, who developed a catalytic antibody to carry out the enantioselective Robinson annulation on 110 mg scale to yield *S*-**4.8** in 94% isolated yield and 96% optical purity.[39] Alternatively, a recent paper discusses the possibility to catalyze the Robinson annulation with the commercial porcine pancreatic lipase (PPL) with yield and ee that were higher to those of a L-proline

organocatalyzed reaction, even though it was thereafter demonstrated that a contaminant α-amylase was responsible for the catalytic action.[40,41]

Aside from asymmetric synthesis, enzymes also hold the potential to catalyze the kinetic resolution that leads to the separation of the enantiomers of compound **4.8**. This has been applied in quite a few reports in the literature, mostly focused on biotransformations mediated by microbial whole cells.[42–46] Apparently, before our work, no examples of the stereoselective reduction of WMK with isolated enzymes had been reported.[4] For this reason, we considered this substrate for further investigations of the substrate promiscuity of the enzymes belonging to our in-house collection of dehydrogenases, including new metagenomic and extremophilic enzymes.[3]

Furthermore, regarding the modification or the isolation of enantiomerically enriched compounds **4.9** and **4.10**, noteworthy, just a report by Shimizu et al. was found, mentioning an enzymatic asymmetric hydrolysis of the acetylated racemates of the considered *cis*- and *trans*-alcohols.[47] However, no report on the use of lipases in organic solvents was found regarding these hydrolyses. As such, our group considered the **4.9** and **4.10** as putative lipase substrates for acetylation with kinetic resolution.

4.2.2. Results and discussion

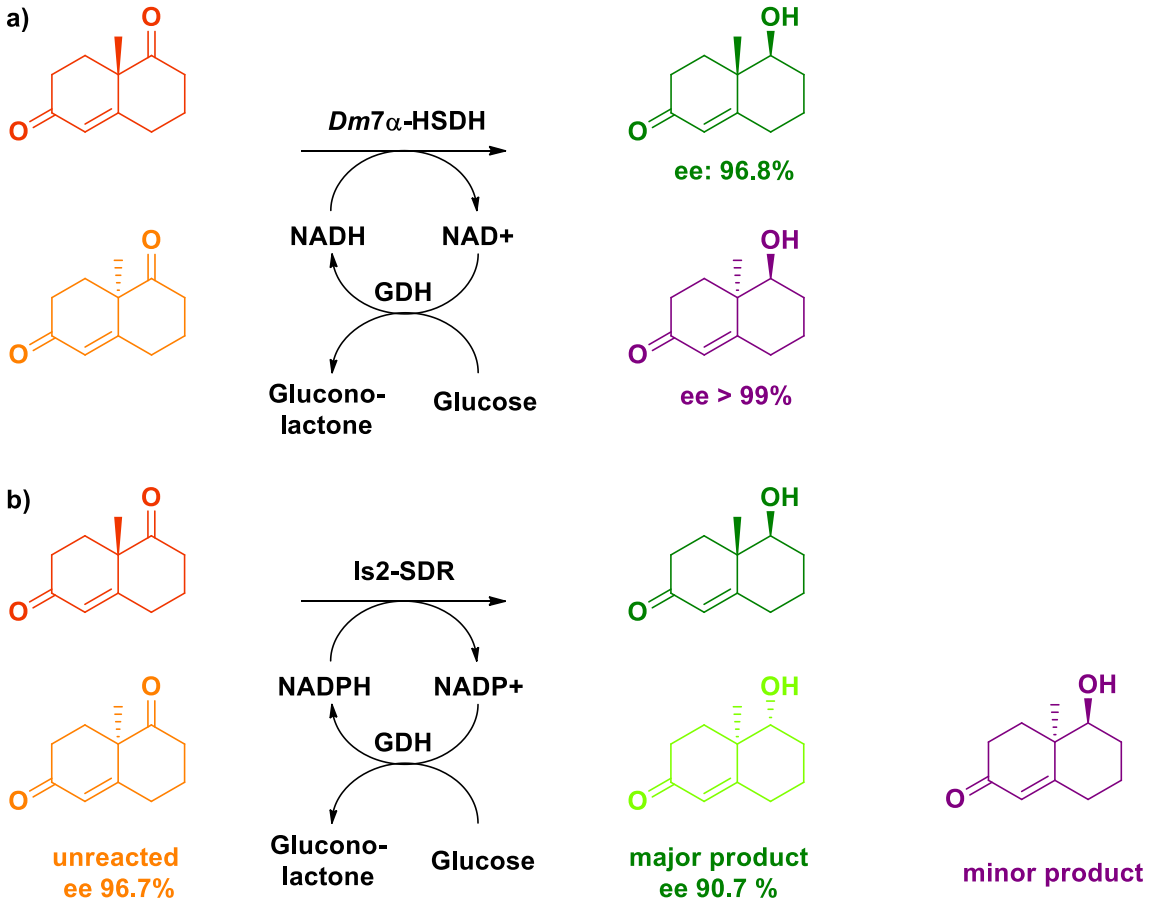
A preliminary screening of our panel of dehydrogenases for the asymmetric reduction of **4.8** was carried out and the results are summarized in Table 4.5.

Entry	Enzyme	Conversion
1	*Csp12*α-HSDH	< 1 %
2	*Ls12*α-HSDH	< 1 %
3	*Dm7*α-HSDH	< 99 %
4	*Ec7α-HSDH*	6 %
5	*Hh7*α-HSDH	< 1 %
6	Ngi1-7α-HSDH	< 1 %
7	*Ca7*α-HSDH	< 1 %
8	*Bsp7*β-HSDH	< 1 %
9	*Hh7*β-HSDH	< 1 %
10	*Rs7*β-HSDH	< 1 %
11	*Sc7*β-HSDH	< 1 %
12	*Ca7*β-HSDH	< 1 %
13	*Cae7*β-HSDH	< 1 %
14	Is2-SDR	66 %
15	Ngi7-SDR	12 %

Table 4.5: preliminary screening of HSDHs and SDRs for the reduction of racemic **4.8**.

Only four out of the fifteen individuals tested turned out to accept 4.8 as substrate, but only two of them gave up to complete conversions and for this reason they were further investigated (see Table 4.5, entries 3 and 14, respectively). The enzymes giving the most promising results were *Dm*7α-HSDH and Is2-SDR, and those individuals actually gave two different reaction outcomes.

Indeed, *Dm*7α-HSDH was able to fully convert WMK into two products, while Is2-sDR could catalyze a kinetic resolution over the enantiomers of WMK. The scale-up to a semi-preparative scale of the reactions catalyzed by these enzymes permitted to characterize their stereochemical outcome. Specifically, it was shown that *Dm*7α-HSDH stereospecifically converted WMK into the two enantiomerically pure diastereomers (*4aS,5S*)-**4.9** and (*4aR,5S*)-**4.10** (ee > 99 % in both cases) in a 1:1 ratio, forming the new stereocenter with a *S*-configuration, as it is shown in Scheme 4.7.

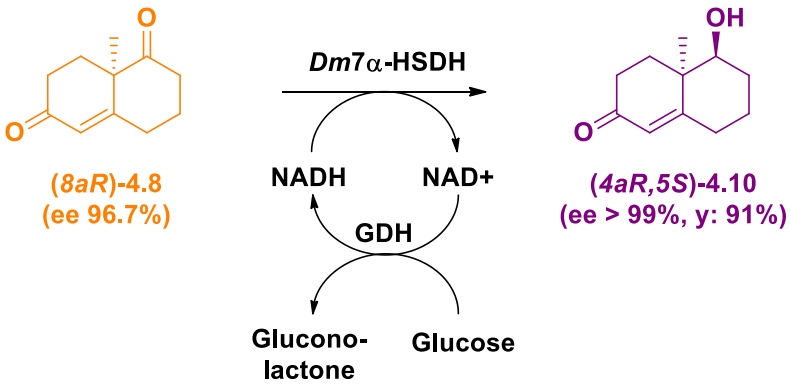


Scheme 4.7: reductions of the Wieland-Miescher ketone by a) *Dm*7α-HSDH and b) Is2-SDR.

The reaction catalyzed by *Dm*7α-HSDH (Scheme 4.7a) yielded the two diastereomers **(*4aS,5S*)-4.9** and **(*4aR,5S*)-4.10** in a 1:1 ratio. These compounds could eventually be separated via a Biotage® SP1 system for flash chromatography (FC), after many attempts (the complete separation was quite troublesome with classical FC).

For this reason, the synthesis of pure **(*4aR,5S*)-4.10** (> 99 % e.e., [α]D22: -111.6°, c 1.0 in CHCl3) was envisaged by *Dm*7α-HSDH-catalyzed reduction of the enantiomerically enriched **(*8aR*)-4.8**, obtained as

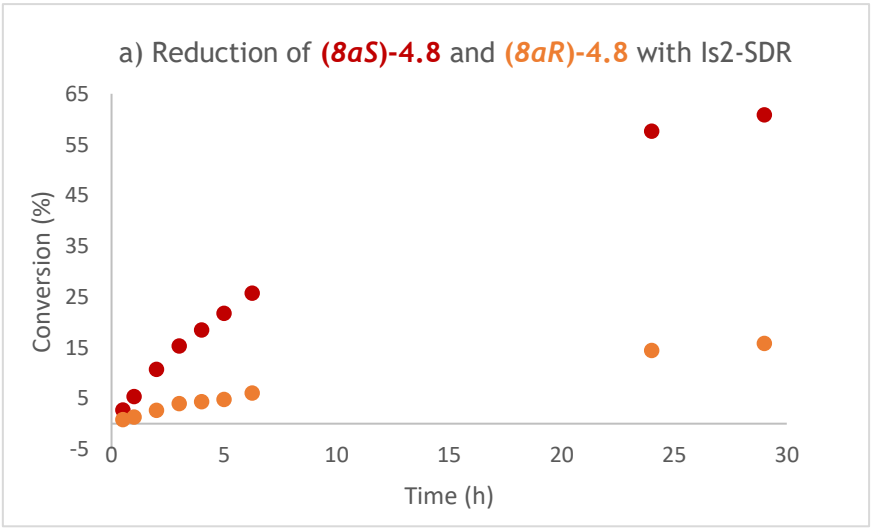
residual substrate from the reduction catalyzed by Is2-SDR, as it is shown in Scheme 4.8. Similar results were obtained in previous studies, albeit with lower enantiomeric excesses.[42,45,48]

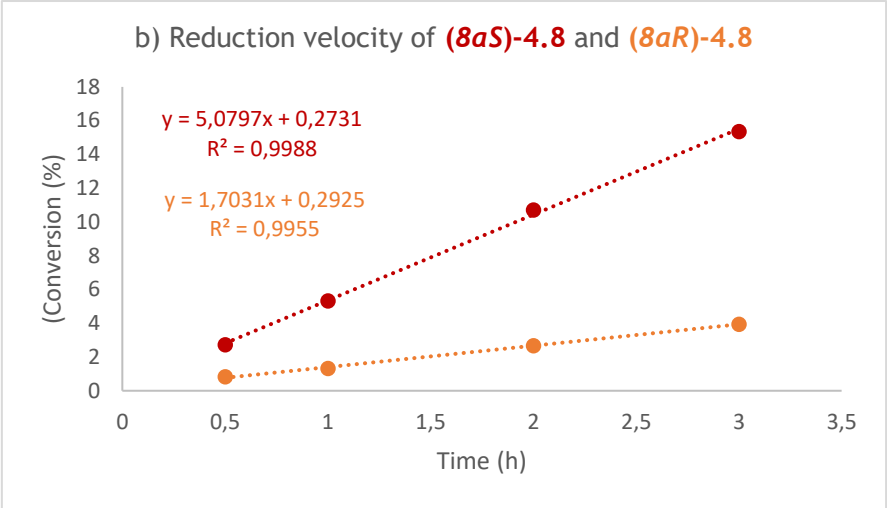


Scheme 4.8: stereoselective reduction of **(*8aR*)-4.8** to **(*4aR,5S*)-4.10** mediated by *Dm*7α-HSDH.

It is worthy to recall that **4.10** is chemically inaccessible in easy ways, while this enzyme provides a straightforward access to one of its enantiomers in highly pure form and in quantitative yield.[35,38]

The kinetic resolution over the enantiomers of **4.8** catalyzed by Is2-SDR allowed to isolate **(*8aR*)-4.8** with a high ee (96.7% ee, 32% isolated yield), consistently with other results obtained in the literature with whole cells and yeasts.[44,46,48] The kinetic resolution was studied more carefully, determining that **(*8aS*)-4.8** was preferentially reduced with respect to **(*8aR*)-4.8**. The relative ratio of the two reactions on the pure enantiomers were 3:1, as it is shown by Graphs 4.1a and 4.1b.





Graph 4.1: a) kinetics of reduction of **(*8aS*)-** vs **(*8aR*)-4.8** b) relative rates of the reduction of **(*8aS*)-** vs **(*8aR*)-4.8**.

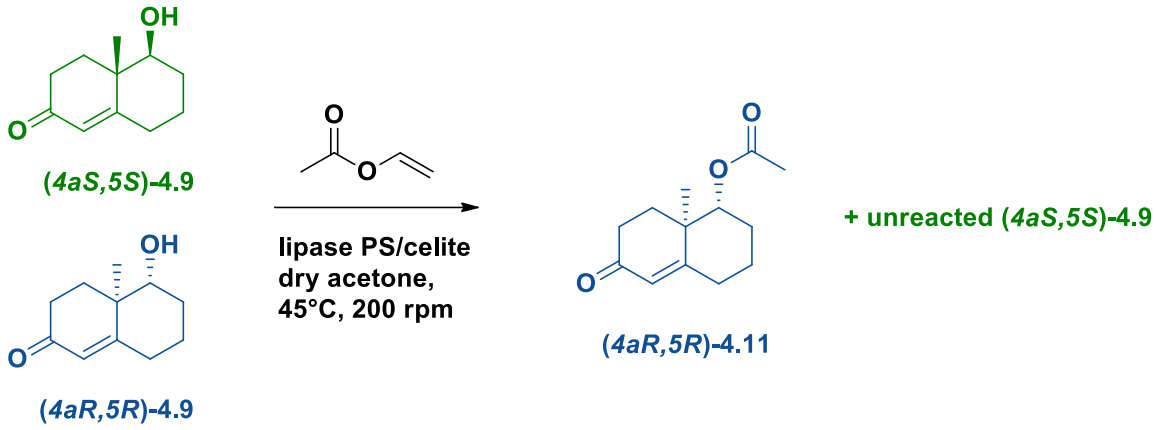
The products of this reaction were **(*4aS,5S*)-4.9** (90.1% ee) from the preferred **(*8aS*)-4.8** and **(*4aR,5S*)-4.10** and **(*4aR,5R*)-4.9** as minor products (in a relative ratio of 4:1) from **(*8aR*)-4.8** (see Scheme 4.7).

As a further investigation, due to the availability of racemic **4.9** by chemical reduction, our group decided to evaluate the possibility of separating the two enantiomers via a kinetic resolution carried out via a lipase-mediated acetylation in organic solvents.[49] This resolution had never been described before, so a diversified panel of lipases (see Table 4.6) were tested *ad hoc*.

Lipases
Porcine Pancreatic Lipase (PPL)
Lipase AK
Lipase CE
Candida rugosa lipase
Lipase PS on celite
Novozym435
Lipase N

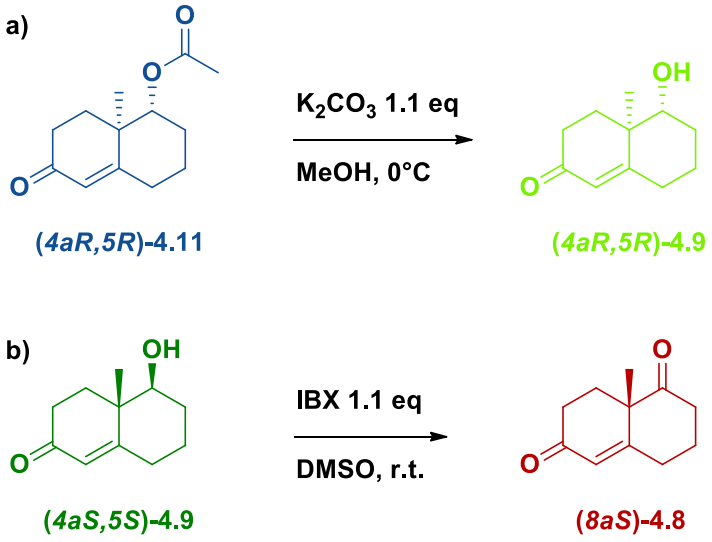
Table 4.6: Diversified lipase library composed of members among the individuals available at SCITEC-CNR.

Lipase PS supported on celite could perform an ideal kinetic resolution (the enantiomeric ratio "E" was as high as 2000) in acetone with 10% v/v vinyl acetate at 45°C. In this way, the two enantiomerically pure compounds **(*4aS,5S*)-4.9** and **(*4aR,5R*)-4.11** could be isolated (ee > 99% in each case, see Scheme 4.9). Notably, the reaction could be scaled up to a preparative scale of 1 g of racemic **4.9**.



Scheme 4.9: kinetic resolution in the acetylation of racemic **4.9** mediated by lipase PS on celite.

The subsequent mild hydrolysis of the ester **(*4aR,5R*)-4.11** furnished the pure missing enantiomer of the alcohol **(*4aR,5R*)-4.9**, while the 2-iodoxybenzoic acid(IBX)-mediated oxidation of the alcohol **(*4aS,5S*)-4.9** gave an entry to enantiomerically pure ketone **(*8aS*)-4.8** (see Scheme 4.10a and 4.10b, respectively). In this way, also the enantiomers of the ketone and of the cis-alcohol that were not directly formed by HSDHs/SDRs could be isolated in a highly pure form.

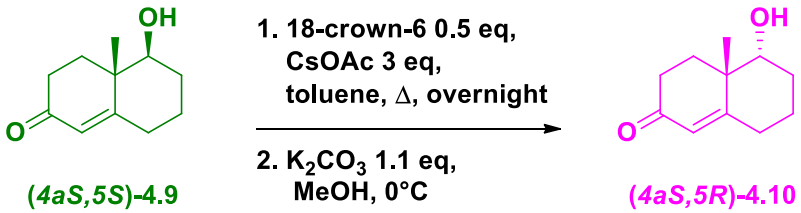


Scheme 4.10: chemical transformations to obtain a) **(*4aR,5R*)-4.9** and **b) (*8aS*)-4.8.**

The library of diversified lipases at our disposal (see Table 4.6) was also screened to approach an alternative separation of the mixture of **(*4aS,5S*)-4.9** and **(*4aR,5S*)-4.10**, obtained as products by *Dm*7α-HSDH, but none of the tested individuals was able to catalyze the acetylation on one of the diastereomers, so, to the best of our knowledge, the Biotage® SP1 system currently remains the best way to achieve the separation of these diastereomeric compounds.

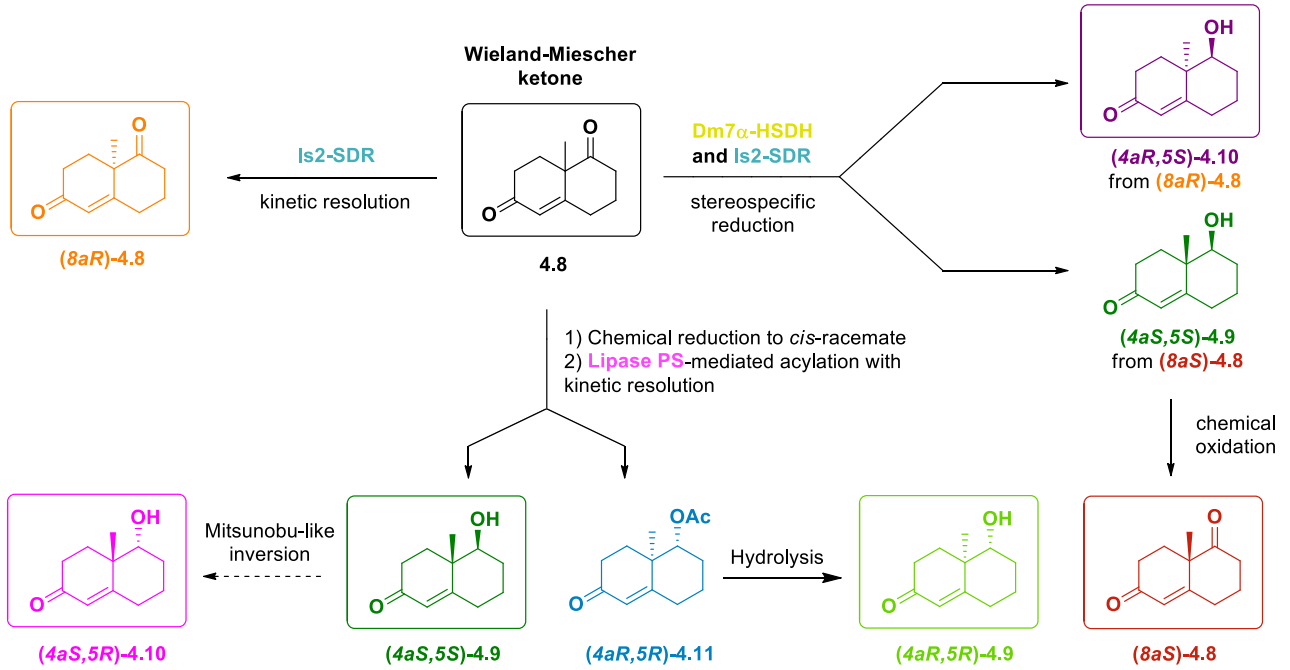
The last enantiomer of the trans-alcohol **4.10**, *i.e.*, **(*4aS,5R*)-4.10**, could be obtained as described by Shimizu et al in a two-step, low-overall yield procedure of Mitsunobu-like stereoinversion from the

enantiomerically pure **(*4aS,5S*)-4.9** (Scheme 4.11, about 30% isolated yield, m/z: 203.1 $[m+Na]^+$, HPLC rt 16.1 min vs 10.2 min of the opposite *trans*-enantiomer).[38]



Scheme 4.11: chemical Mitsunobu-like stereoinversion of ***cis*-(*4aS,5S*)-4.9** to ***cis*-(*4aS,5R*)-4.10**.

A summary of the chemoenzymatic transformations that were carried out on the Wieland-Miescher ketone within this work is outlined in Scheme 4.12.



Scheme 4.12: chemoenzymatic transformations on the Wieland-Miescher ketone.

Prior to the mild chemical oxidation of **(*4aS,5S*)-4.9** to **(*8aS*)-4.8**, also an enzymatic oxidation was attempted, but neither Is2-SDR nor *Dm*7α-HSDH were able to perform this reaction with acceptable conversion, even when racemic **4.9** was used as substrate instead of the pure **(*4aS,5S*)-4.9** to attempt a kinetic resolution. Since the reaction catalyzed by *Dm*7α-HSDH was expected to be the most selective, some kinetic studies were performed to assess whether **4.9** could act as an inhibitor of this enzyme. For this purpose, activity assays on the natural bile acid substrates (K_M in the μM range) were made with *Dm*7α-HSDH in the presence of increasing concentrations both of racemic **4.9**, and of the separate enantiomers **(*4aS,5S*)-4.9** and **(*4aR,5R*)-4.9**.

Indeed, it was seen that the *cis*-alcohol **4.9** would act as an inhibitor of *Dm*7α-HSDH, as it lowered the enzyme activity by 25% at a 10 mM concentration and by 60% at 40 mM. The effect was however stronger when the enantiomerically pure ***(4aS,5S)*-4.9** was used: the activity of the HSDH was reduced by 30% at 10 mM and halved at 40 mM. Since the K_M of WMK for *Dm*7α-HSDH was estimated to be of 98 mM by kinetic studies in our lab (thus, five orders of magnitude higher than that of the natural substrates), **4.9** could actually be an effective inhibitor for its own oxidation, which would explain why *Dm*7α-HSDH could not reach oxidation conversions higher than 20% even after 10 days of reaction.

Further experimental details are presented in Appendix I, article 3.

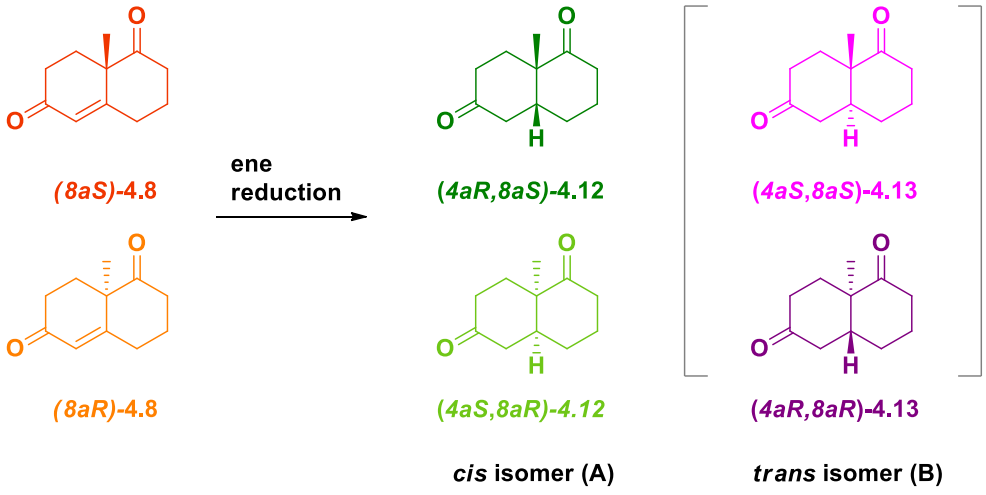
4.2.3. Unpublished complementation to the work: OYEs and the Wieland-Miescher ketone - some preliminary modifications

To further expand the possible enzymatic modifications on the pharmaceutically relevant core of the Wieland-Miescher ketone, a group of ene reductases (specifically, of old yellow enzymes, *alias* OYEs) was tested, as well, for the reduction of the C=C double bond within the enone moiety.

It has been recently demonstrated that some engineered ene reductases were able to reduce this compound and other sterically challenging ketones, but no comparison was made with other classical wild-type enzymes.[50] For this reason and for other purposes, our group screened a library of OYEs (see Tables 4.7 and 4.8) for the stereoselective reduction of compound **4.8**.

The ultimate aim was that of investigating if it would be possible to obtain both the *cis*- and *trans*-decal-1,6-diones (compounds **4.12** and **4.13**, respectively) for further enzymatic cascade modifications where OYEs would be coupled with HSDHs for stereo- and regio-selective carbonyl reductions. Future work will be done in this respect, and it will be compared with the results obtained on *trans*-1-decalone.[3]

The aforementioned reference in literature by Dobrijevic et al. let us suppose that *cis*-dione formula **4.12** would correspond to the major product (see Scheme 4.13), while the *trans*-dione formula **4.13** would be the minor product, but the structure of the OYE-mediated reduction products still has to be elucidated.[50]



Scheme 4.13: Biocatalyzed enone reduction catalyzed by OYEs.

Enzyme	Origin	Species
OYE1	Yeast	*Saccharomyces pastorianus*
OYE2	Yeast	*Saccharomyces cerevisiae*
OYE3	Yeast	*Saccharomyces cerevisiae*
PETNR	Bacterial	*Enterobacter cloacae*
YqjM	Bacterial	*Bacillus subtilis*
OYE2.6	Yeast	*Scheffersomyces stipitis*
KmOYE	Yeast	*Kluyveromyces marxianus*
OPR1	Plant	*Arabidopsis thaliana*
OPR3	Plant	*Arabidopsis thaliana*
LeOPR1	Plant	*Solanum lycopersicum*
NemA	Bacterial	*Escherichia coli*
PpNemA	Bacterial	*Pseudomonas putida*
SeOYE	Bacterial	*Synechococcus elogantus*
PpOYE	Bacterial	*Pseudomonas putida*
YNL134c	Yeast	*Saccharomyces cerevisiae*
LtB4DH	Animal	*Rattus norvegicus*

Table 4.7: Wild-type OYE enzymes available at SCITEC-CNR.

The conversions and the ratio of the major vs the minor product (products **A** and **B**, respectively) were obtained via GC-MS using an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm). Elution conditions: 60°C, 1 min; +10°C min-1 until 300°C; hold 1 min; flow rate: 1.0 mL min-1; inlet temperature: 250°C; ion source temperature: 250°C; MS transfer line temperature: 250°C. Retention times: (**4.8**): 13.7 min; (product **A**): 13.6 min; (product **B**): 13.3 min. The masses of both product 1 and product 2 (m/z: 180) correspond to a mono-reduced derivative of 4.8 (m/z: 178). The formation of alcoholic products by reduction of either of the two carbonyl moieties could be excluded because these compounds feature longer retention times in the column with respect to **4.8** (e.g., 14.6 min for **4.9** and 14.8 min for **4.10**), while both products **A** and **B** had a shorter retention time.

The reaction catalyzed by the most interesting individual (YqjM) was repeated with another cofactor regeneration system, namely the FDH from *Pseudomonas* sp. (*Ps*FDH), and higher conversions could be reached (about 70%).

Since a high conversion was reached, we thought it could be of interest to analyze the performances of single and double mutants of this YqjM, which were previously described in the literature and were at disposal in our lab (See Table 4.8).[51-55]

Enzyme	Mutant type
Yqjm WT	Wild-type
C26H	Single
C26N	Single
I69A	Single
I69Y	Single
H167A	Single
C26H-I69Y	Double
C26N-I69A	Double
I69Y-H167A	Double
I69A-H167A	Double

Table 4.8: YqjM ant its single and double mutants available at SCITEC-CNR.

To this purpose, we screened in the same conditions all the mutants enlisted in Table 4.8 for the bioreduction of compound **4.8**. The reaction conditions were comparable to those of the previous screening, but 0.2 U/mL of *Ps*FDH were used *au lieu de Bm*GDH and 70 mM ammonium formate was consequently used instead of 50 mM glucose.

GC-MS analyses of the crude reaction extracts in AcOEt gave the conversions enlisted in Table 4.9, alongside with the ratio between product **A** and product **B**.

Enzyme	C (%)	Product A (%)	Product B (%)	A:B ratio
Yqjm WT	68	73	27	3:1
C26H	70	88	12	9:1
C26N	90	66	34	7:3
I69A	17	82	18	4:1
I69Y	25	70	30	7:3
H167A	4.2	43	57	2:3
C26H-I69Y	1.6	51	49	1:1
C26N-I69A	51	91	9	9:1
I69Y-H167A	1.8	53	47	1:1
I69A-H167A	< 0.01	n.d.	n.d.	n.d.

Table 4.9: conversions and **A**:**B** ratio in the ene-reduction of WMK mediated by YqjM's selected mutants.

It is noteworthy that both the conversion and the **A**:**B** ratio changed significantly from one mutant to the other, consistently with the fact that the amino acids selected for mutation are hot spots in the active site of the enzyme.

The best results in terms of conversion were given in general by single mutants, especially by C26H and C26N, and by the double mutant C26N-I69A, giving the impression that the removal of the sulfur atom of C26 and its substitution with a basic residue (as histidine or asparagine) has a positive impact on the conversion of WMK. Regarding the **A**:**B** ratio, the best performances were given by C26H, C26N-I69A, and I69A, with **A** being the preferred product, while notably mutant H167A showed a slight preference for product **B** (the **A**:**B** ratio was 2:3 in this case).

The work carried out by Dobrijevic et al led us to think that product **A** could be the *cis*-dione **4.12**, while **B** could be the *trans*-dione **4.13**,[50] but further investigations need to be made both to clarify the structures of the products and their diastereomeric excess, and to rationalize the different selectivity of the YqjM mutants towards compound **4.8**.

To this purpose, the reaction catalyzed by C26H-YqjM, giving the best compromise between conversion and **A**:**B** ratio, will be scaled up and the product(s) will be isolated and structurally characterized.

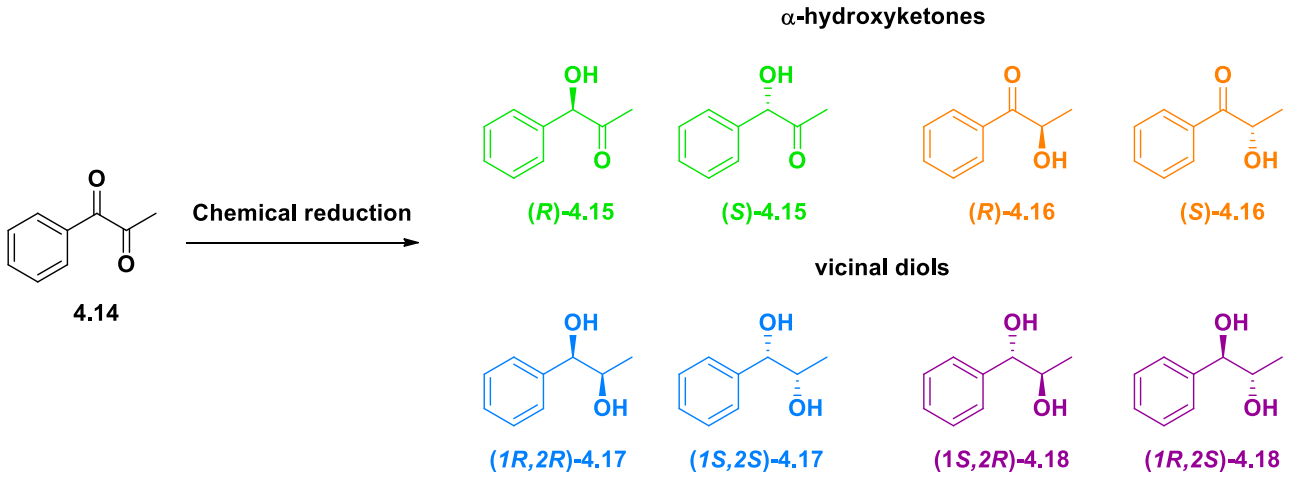
4.3. HSDHs and their substrate promiscuity: α-diketones

4.3.1. HSDH-mediated reduction of vicinal diketones

Additional investigations on the substrate scope of HSDHs were made in our laboratory for the reduction of vicinal diketones. This subsection will deal with a perspective on how to synthesize α-hydroxyketones from vicinal diketones, which will be followed by a discussion on the results we obtained by screening some test compounds for a bioreduction with HSDHs/SDRs. In the end, experimental details will be given.

4.3.1.1. Vicinal diketones: a first glance to a synthetic challenge

The reduction of these compounds indeed can feature selectivity problems that encompass both chemoselectivity (the α-hydroxyketone intermediate is usually further reduced to diol), regioselectivity (on asymmetric substrates, the diversely activated carbonyls may react differently), and stereoselectivity (usually, all the possible stereoisomers are formed from a chemical reduction, in proportions that may vary according to the reaction kinetics). An example of the plethora of products which can be formed in the reduction of a simple asymmetric vicinal diketone (namely, 1-phenyl-1,2-propanedione, compound **4.14**) is it shown in Scheme 4.14.



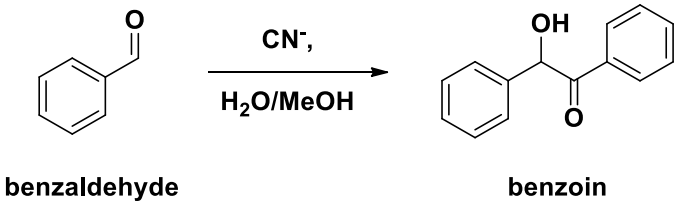
Scheme 4.14: spectrum of all the possible reduction products yielding from 1-phenyl-1,2-propanedione reduction.

From this simple example, it should be clear how challenging it can be to achieve a selective reduction of a vicinal diketone to the desired product (most of all, when the α-hydroxyketone intermediate is the desired product).

The chemical synthesis of α-hydroxyketones is *per se* quite a challenge from the classical chemistry viewpoint. Indeed, although some entries to these compounds exist, they are often poorly selective, or very specific for a narrow substrate scope, or limited by the presence of differently substituted

carbonyls that may help in dictating the regioselectivity in the reduction of the most activated one.[56,57] Moreover, these methods are usually not eco-friendly. Some bioreductions have also been attempted and optimized, *e.g.*, in the work on compound **4.14** by Nakamura et al., where pre-treated baker's yeast could reduce regio- and stereo-selectively **4.14** to **4.16** (80% yield under optimized conditions and high ees for the S-enantiomer).[58] However, the applicability of isolated alcohol dehydrogenases for the synthesis of α-hydroxyketone is still limited by the poor regioselectivity these enzymes usually feature (they tend to over-reduce these compounds to diols).

An alternative approach exists to obtain α-hydroxyketones, namely the so-called benzoin condensation. This process consists of the self-condensation of aldehydes catalyzed by nucleophiles such as cyanide (see Scheme 4.15).



Scheme 4.15: benzoin condensation reaction via cyanohydrin intermediate.

Enzymes exist that are able to catalyze benzoin condensation, namely carboligases, making it possible also to perform cross-coupling and to link together two different carbonyl compounds (one acting as nucleophile and the other as electrophile), and they have been applied to the synthesis of carbinols (an alternative name for α-hydroxyketones) for many decades.[59-63]

Even though these synthetic methodologies are at disposal to obtain chiral carbinols, their synthesis is still considered to be challenging.

4.3.1.2. Vicinal diketones as entries to the biocatalytic synthesis of α-hydroxyketones

The reports in the literature concerning the substrate promiscuity of HSDHs, together with the experimental information that were gathered at SCITEC-CNR on bicyclic ketones, α-ketoesters, and one ß-ketoester (methyl acetoacetate) were in accordance: the ß-ketoester was not accepted by these enzymes, whereas the α-ketoesters were stereo- and regio-selectively reduced to the corresponding *R*-α-hydroxyesters with high ees.[2,3]

These information gathered together raised a mechanistic hypothesis regarding the active site of HSDHs: what if these enzymes appreciate a vicinal di-carbonyl moiety to perform asymmetric catalysis?

For this reason, few model compounds (namely, the pharmaceutically relevant 1-phenyl-1,2-propanedione (**4.14**), 3,4-hexanedione (**4.19**), benzil (**4.20**), and the 9,10-dioxostearic acid (**4.21**)) were chosen, with the criterion of structural similarity to the two investigated α-ketoesters for

compounds **4.14** and **4.19** (see Figure 4.6). Moreover, benzil (**4.20**), and the 9,10-dioxostearic acid (**4.21**) were also considered as possible substrates.

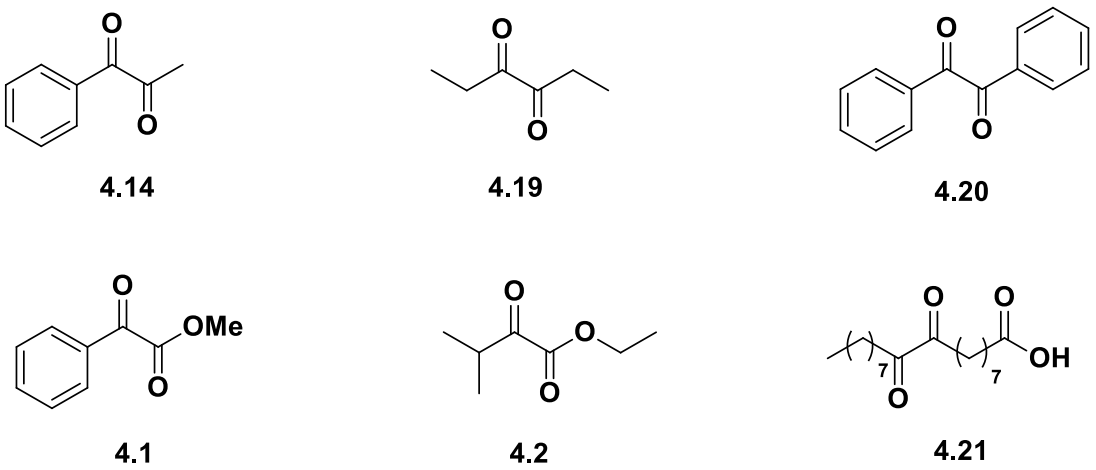


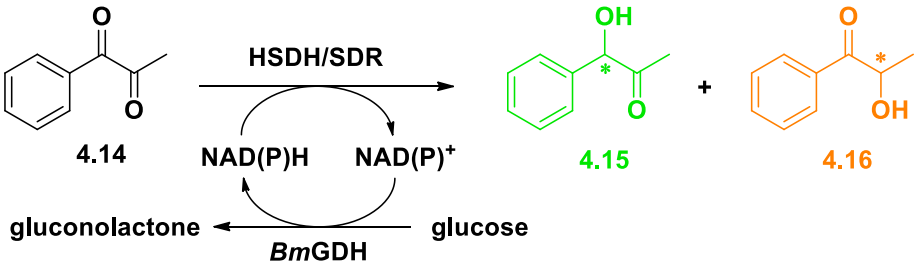
Figure 4.6: structural comparison between **4.1** and **4.14** (left) and between **4.2** and **4.19** (center); two additional putative substrates **4.20** and **4.21** (right).

The enzymatic synthesis of the α-hydroxyketonic products of compound **4.14** and **4.19**-**4.21** was thus attempted with our panel of HSDHs/SDRs. The regio- and stereo-selectivity in the reduction of these asymmetric and symmetric diketones was studies as well for compounds **4.14** and **4.14** and it will be determined for **4.20** and **4.21**.

4.3.1.2.1. 1-phenyl-1,2-propanedione as ADH substrate

A screening was performed to assess the ability of our HSDHs (see Table 4.9) to reduce 1-phenyl-1,2-propanedione to phenyl acetyl carbinol (PAC, compound **4.15**), an intermediate of interest in the synthesis of ephedrine derivatives.[64]

In this screening, none of the HSDHs catalyzed the reduction of the intermediate α-hydroxyketones to a diol, showing that indeed a vicinal di-carbonyl moiety is appreciated in the active site of the enzyme for its asymmetric catalysis to work. However, unluckily, the reactions biocatalyzed by these enzymes were poorly regio- and stereo-selective, yielding the desired compound **4.15** as major product accompanied by highly variable amounts (about 10-50%) of its regioisomer 2-hydroxypropiophenone **4.16** with unsatisfactory ees (see Scheme 4.16 and Table 4.10).



Scheme 4.16: SDR-mediated reduction of 1-phenyl-1,2-propanedione yields the corresponding α-hydroxyketones.

Despite that, in order to assign the absolute configuration to the product, the most regioselective reaction, *i.e.*, the one catalyzed by *Dm*7α-HSDH, was scaled up on 100 mg scale, yielding compound **4.15** in high conversion and about 40% ee determined by chiral phase GC analysis of the crude reaction extract. The obtained PAC product was thus purified and subjected to polarimetric analysis, allowing both to assign the *R* configuration to the major enantiomer, and to further understand the analytics of following enzymatic screenings on this compound.[60]

Enzyme	Conversion (%)	% 4.15	ee (4.15)	% 4.16
*Csp*12α-HSDH	42.0	62.8	42.6 (*R*)	37.2
*Ls*12α-HSDH	11.1	70.3	26.4 (*R*)	29.8
*Dm*7α-HSDH	93.8	> 99	38.9 (*R*)	n.d.
*Ec*7α-HSDH	96.8	86.4	65.7 (*R*)	13.6
*Hh*7α-HSDH	29.2	81.5	62.3 (*R*)	18.5
Ngi1-7α-HSDH	18.0	54.3	54.3 (*S*)	45.7
*Ca*7α-HSDH	15.8**	51.6	7.7 (*R*)	48.4
*Bsp*7β-HSDH	92.7	77.5	51.6 (*R*)	22.5
*Hh*7β-HSDH	< 1%	42.9	n.d.	57.1
*Rs*7β-HSDH	66.1	87.0	67.2 (*R*)	14.0
*Sc*7β-HSDH	42.3	64.0	50.6 (*R*)	36.0
*Ca*7β-HSDH	97.2	81.7	52.1 (*R*)	15.5
*Cae*7β-HSDH	82.5	89.6	57.9 (*R*)	10.4
Is2-SDR	94.1	80.5	64.2 (*R*)	19.5
Ngi7-SDR	96.4	78.2	32.9 (*R*)	12.0

Table 4.10: results of the HSDH/SDR screening for the biocatalytic reduction of compound **4.14**.

Evoxx ADH library screening

Due to the unsatisfactory result given by HSDHs, a screening for the reduction of compound **4.14** was performed with a panel of alcohol dehydrogenases (ADHs) as well, including 18 individuals from a commercial Evoxx kit, one from Merck and one from our in-house collection (see Table 4.11).

Enzyme number/name	Enzyme specificity	c %	% 4.15	ee 4.15 (%)	% 4.16	% (4.17+4.18)
1.1.010	*R*/*S*	98.7	58.2	60.3 (*S*)	14.8	30.1
1.1.020	*R*/*S*	96.6	46.3	81.2 (*S*)	53.7	-
1.1.030	*S*	>99.9	3.6	-	5.4	95.0
1.1.040	*R*/*S*	95.5	74.8	22.3 (*S*)	26.4	4.0
1.1.130	*R*	92.6	74.7	0.7 (*R*)	25.4	-
1.1.140	*S*	>99.9	74.7	32.6 (*S*)	25.4	-
1.1.190	*R*	80.6	75.8	75.8 (*S*)	23.1	41.1
1.1.200	*R*	>99.9	-	-	-	> 99
1.1.210	*S*	93.2	62.8	38.4 (*S*)	37.3	< 1
1.1.250	*S*	97.7	15.3	48.1 (*S*)	12.9	71.8
1.1.260	*R*/*S*	88.1	72.0	31.9 (*S*)	28.0	<1
1.1.270	*R*	99.0	14.1	92.5 (*S*)	3.0	82.9
1.1.380	*S*	97.9	1.3	-	51.8	48.2
1.1.420	*R*	95.3	46.9	91.3 (*S*)	30.7	22.4
1.1.430	*S*	96.6	32.0	43.1 (*S*)	45.7	22.3
1.1.440	*S*	86.5	19.9	80.1 (*R*)	0.7	71.3
1.1.441	*R*	>99.9	-	-	-	> 99
1.1.442	*R*/*S*	96.6	73.4	68.4 (*S*)	18.8	7.8
*Lk*ADH	*R*	95.4	51.2	75.9 (*R*)	8.2	40.6
R*Ec*ADH	*S*	96.4	64.6	35.7 (*S*)	35.5	-

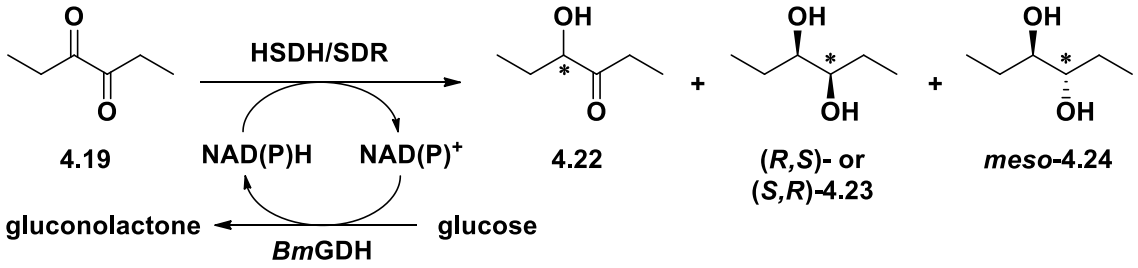
Table 4.11: results of the ADH library screening for the biocatalytic reduction of compound **4.14**.

The reactions were set according to the kit indications, but the substrate/enzyme ratio was increased by working with 10 mM substrate and 1 mg/mL enzyme (instead of 5 mM and 5 mg/mL, respectively). In these reactions, some ADHs were able to form chemoselectively the α-hydroxyketones, but most of them also formed the diols as mixtures enriched with either the *syn*- or the *anti*-diastereomer (compounds **4.17** and **4.18**, respectively). The conversions and the product spectra are outlined for

each reaction in Table 4.10.The tested ADHs gave better performances with respect to the tested HSDHs in terms of conversions (almost quantitative in nearly all the cases) and ees, even though these enzymes mostly preferred to form (*S*)-**4.15**, while HSDHs moderately preferred to form the (*R*)-**4.15**. Nevertheless, with this substrate most of these enzymes yielded product mixtures of scarce synthetic applicability.

4.3.1.2.2. 3,4-hexanedione as a synthetic and mechanistic curiosity

In line with the hypothesis made regarding the active site of HSDHs and their ability to accept α-dicarbonyl compounds, an additional screening was performed to assess the ability of HSDHs to reduce 3,4-hexanedione to the enantiomerically enriched 4-hydroxyhexan-3-one (**4.22**) or to the *syn*-/*anti*-3,4-hexanediol (**4.23** and **4.24**, respectively). The biocatalyzed reduction, which was as usual coupled with a cofactor regeneration system, is outlined in Scheme 4.17.



Scheme 4.17: Biocatalytic reduction of 3,4-hexanedione.

Again, as for the reduction of asymmetric 1-phenyl-1,2-propanedione, no reaction catalyzed by a HSDH formed the diols. The only enzyme that was able to reduce the α-hydroxy ketone intermediate was the non-HSDH enzyme named Is2-SDR. The conversions, ees and the product spectra were determined by chiral phase GC analyses, and they are outlined for each reaction in Table 4.12.

The reactions catalyzed by *Ls*12α-HSDH, by *Hh*7α-HSDH, and by *Hh*7β-HSDH gave the most promising results in terms of conversion (70-90%) and ee (95-98%) of the α-hydroxyketone product **4.22**. Thus, the reaction catalyzed by *Hh*7β-HSDH was scaled up so as to assess the absolute stereochemistry of the product, which turned out to be *R* based on the literature[68] and consistently with the fact that these enzymes seem to prefer to form *R*-hydroxyl compounds.[2,3]

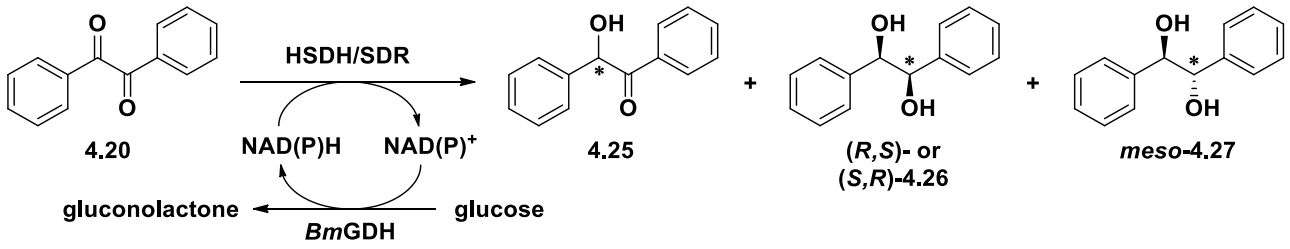
Enzyme	Conversion (%)	ee (4.22)	% diols	Diol ratio
*Csp*12α-HSDH	21.3	86.4	-	-
*Ls*12α-HSDH	70.0	97.7	-	-
*Dm*7α-HSDH	27.7	84.4	-	-
*Ec*7α-HSDH	31.1	67.8	-	-
*Hh*7α-HSDH	88.9	94.7	-	-
Ngi1-7α-HSDH	29.9	77.0	-	-
*Ca*7α-HSDH	73.2	85.8	-	-
*Bsp*7β-HSDH	26.3	85.4	-	-
*Hh*7β-HSDH	84.1	95.9	-	-
*Rs*7β-HSDH	16.8	89.8	-	-
*Sc*7β-HSDH	25.4	96.2	-	-
*Ca*7β-HSDH	69.2	95.1	-	-
*Cae*7β-HSDH	74.5	81.7	-	-
Is2-SDR	73.5	68.4	87.7	78 : 12
Ngi7-SDR	48.2	87.7	-	-

Table 4.12: results of the HSDH/SDR library screening for the biocatalytic reduction of compound **4.19**.

4.3.1.2.3. Benzil and 8,9-dioxostearic acid as further aromatic and aliphatic test substrates for HSDHs

Having seen the wide acceptance of α-diketones within the library members, two additional highly diverse compounds were tested as putative HSDH/SDR substrates, namely the bulky and symmetric benzyl (**4.20**, fully sp^2-hybridized) and the long-chained and asymmetric 9,10-dioxostearic acid (**4.21**, aliphatic and with a carboxylic acid moiety).

4.3.1.2.3.1. Benzil as an aromatic, bulky, and symmetric substrate



Scheme 4.18: Biocatalytic reduction of benzil.

To our surprise, in this case there was just one individual in our library that partially reduced this compound to the corresponding diol as well as to the α-hydroxyketone, namely *Dm*7α-HSDH. The other enzymes gave poor to moderate conversions, which were determined via chiral phase GC analyses, and which are outlined for each reaction in Table 4.13.

Enzyme	Conversion (%)	% 4.25	% (4.26+4.27)
*Csp*12α-HSDH	3.0	> 99	-
*Ls*12α-HSDH	3.6	97.7	-
*Dm*7α-HSDH	30.0	77.4	22.6
*Ec*7α-HSDH	9.7	> 99	-
*Hh*7α-HSDH	2.5	> 99	-
Ngi1-7α-HSDH	9.0	> 99	-
*Ca*7α-HSDH	17.4	> 99	-
*Bsp*7β-HSDH	6.7	> 99	-
*Hh*7β-HSDH	2.9	> 99	-
*Rs*7β-HSDH	4.2	> 99	-
*Sc*7β-HSDH	3.7	> 99	-
*Ca*7β-HSDH	38.6	> 99	-
*Cae*7β-HSDH	38.6	> 99	-
Is2-SDR	16.9	> 99	-
Ngi7-SDR	5.4	> 99	-

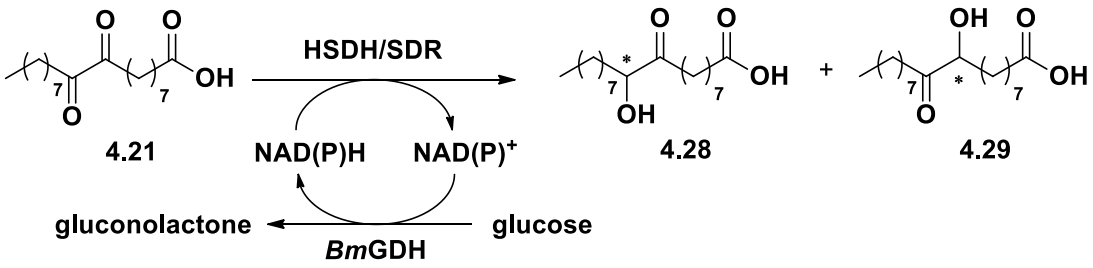
Table 4.13: results of the HSDH/SDR library preliminary screening for the biocatalytic reduction of compound **4.20;** the relative quantities of the 2-hydroxyketone and of the diols are represented as percentages of the total product amount.

These data are only preliminary, thus further investigations will be made to rationalize the diastero- and enantio-selectivity of these enzymes in the reduction on the aromatic and bulky benzyl. The most promising enzymes that may be investigated and whose reactions may be scaled to a semi-preparative scale are the ones catalyzed by *Ca*7β-HSDH, *Cae*7β-HSDH, and *Dm*7α-HSDH, as they give the highest conversions. *Ca*7β-HSDH and/or *Cae*7β-HSDH might allow to assign the absolute configuration to the α-hydroxyketone product, while the reaction catalyzed by *Dm*7α-HSDH may be pushed towards to isolate the

diol in appreciable amount and study the diastereoselectivity together with the enantioselectivity of this enzyme.

4.3.1.2.3.1. 9,10-dioxostearic acid as an aliphatic, bulky, and asymmetric substrate

The last screening of our enzyme library on vicinal diketones was led on the sterically demanding, albeit highly conformationally flexible, 9,10-dioxostearic acid (**4.21**). This compound was chosen due to the results obtained on 3,4-hexanedione (**4.19**), as we wanted to test the effects of a longer aliphatic chain. An additional curiosity was due to the high number of possible conformations this compound has, since this could lead it to fold up in the active site of the enzyme and find an optimal conformation that may resemble the polycyclic structure of steroids. Once again, the reduction of this compound can yield a plethora of products similar to that of **4.14**, being asymmetric.



Scheme 4.19: Biocatalytic reduction of 9,10-dioxostearic acid with HSDHs/SDRs.

Only few individuals in our library were able to reduce 9,10-dioxostearic acid, namely *Ca*7ß-HSDH, *Cae*7ß-HSDH, and Is2-SDR, but the regio- and stereoselectivity of these reductions were not quantified up to now and they will be object of future studies. The reactions were followed via TLC, and it was observed that no individual within our library apparently forms either the *syn*- or the *anti*-9,10-dihydroxystearic acids (**4.30** and **4.31**).

4.3.1.3. Experimental data

4.3.1.3.1. Analytics

The general analytics is reported in the beginning of Section B. Here, further details will be furnished.

Expressions and purifications of the HSDHs were carried out as it was described by us in the literature.[3]

Reactions were monitored *via* TLC as described in the beginning of Section B, using CH_2Cl_2 as eluent and Pancaldi's reagent to see the spots.

Reaction conversions and enantiomeric excesses were determined via chiral phase GC and/or achiral phase GC-MS and the standards of the mixtures of fully and partially reduced products were generated via $NaBH_4$-mediated reduction.

GC-MS elution conditions (bioreduction of **4.14**): 40°C, 1 min; +5°C min^{-1} until 150°C; hold 1 min. Flow rate 1.0 mL/min; inlet temperature: 250°C; ion source temperature: 270°C. Retention times: 1-phenyl-1,2-propanedione (**4.14**): 14.5 min; PAC (**4.15**): 16.0 min; regioisomer (**4.16**) 17.0 min; diols(*syn*+*anti*) (**4.17**+**4.18**): 18.7 min, 18.9 min.

Chiral phase GC elution conditions (bioreduction of **4.14**): column: MEGA-DEX DACBeta (25m x 0.25 mm x 0.25 µm); inlet temperature: 200°C; detector temperature: 250°C; flow: 1.4 mL min^{-1}; elution: 100°C, 1 min, +7°C min^{-1} until 200°C, hold 5 min. Retention times: 1-phenyl-1,2-propanedione (**4.14**): 6.20 min; R-PAC (**(*R*)-4.15**): 7.93 min; S-PAC (**(*S*)-4.15**): 8.25 min; regioisomer (**4.16**): 8.19 min (one enantiomer peak is missing); diols (*syn*+anti) (**4.17**+**4.18**): 10.53 min, 10.78 min, 10.97 min (one enantiomer peak is missing from one diastereomer).

Chiral phase GC elution conditions (bioreduction of **4.19**): column: MEGA-DEX DAC Beta; inlet temperature: 200°C; detector temperature: 250°C; flow: 1.4 mL min^{-1}; elution: 60°C, 1 min; +8°C min^{-1} until 200°C; hold 5 min. Retention times: 3,4-hexanedione (**4.19**): 5.2 min; α-hydroxyketone enantiomers (**4.22**): 8.5 min, 8.7 min; *syn*-3,4-hexanediol enantiomers (**4.23**): 10.85 min, 10.95 min; *anti*-3,4-hexanediol (**4.24**): 11.03 min.

Chiral phase GC elution conditions (bioreduction of **4.20**): column: MEGA-DEX DAC Beta; inlet temperature: 200°C; detector temperature: 250°C; flow: 1.4 mL min^{-1}; elution: 180°C, 1 min; +10°C min^{-1} until 200°C; hold 20 min. Retention times: benzil (**4.20**): 11.5 min; 2-hydroxy-2-phenylacetophenone (**4.25**): 12.0 min; diols(*syn*+*anti*) (**4.26**+**4.27**): 18.5 min, unsolved.

4.3.1.3.2. General synthetic procedures

4.3.1.3.2.1. Preparation of standard racemate mixtures

The reductions of substrates **4.14**, **4.19** and **4.20** were performed following a standard protocol of reduction with NaBH4.[25]

The substrate (100 mg scale) was dissolved 0.13 M in EtOH. At 0°C, $NaBH_4$ (1 eq) was added. When the reaction mixture became transparent, it was brought to room temperature and stirred for 2–4 h. The reaction was ended by addition of a saturated aqueous solution of NH_4Cl, then it was extracted with AcOEt. The organic phase was dried over Na_2SO_4, which was filtered away, and the solvent was evaporated under reduced pressure to yield the desired racemic products in quantitative yield. The products were characterized by ^{1}HNMR analysis and/or chiral GC or GC-MS analyses.

4.3.1.3.2.1. Chemical synthesis of 9,10-dioxostearic acid

2-iodoxybenzoic acid (IBX, 2.5 eq) was solubilized in DMSO (0.4 M). To the stirred colorless solution, s*yn*-9,10-dihydroxystearic acid (**4.30**, 1 eq) was added as a solid. The reaction was let at room temperature and

after 10 minutes it had developed an intense yellow color, typical of 1,2-dicarbonyls. A TLC (eluent AcOEt:petroleum ether=1:1) revealed the reaction was already terminated. The mixture was quenched by pouring it into saturated aqueous $NaHCO_3$ and it was extracted with AcOEt. The organic phase was dried over Na_2SO_4, which was filtered away, and the solvent was evaporated under reduced pressure to yield the desired product in 85% yield. The product was characterized by ^{1}HNMR analysis and GC-MS analyses.

*4.1.3.2.3. Enzymatic reductions of substrates **4.14** and **4.19***

A preliminary activity screening was performed with the enzyme library on the substrates of interest. The reactions were set on analytical scale (1 mL) and they contained: 10 mM substrate in 5% v/v DMSO + 95% v/v 50 mM PB pH 7.0; 50 mM glucose; 2 U HSDH/SDR; 1 U *Bm*GDH; 0.4 mM cofactor. 0.3-0.4 M NaCl was added to the reactions catalyzed by *Hh*7α-HSDH and *Hh*7β-HSDH. The mixtures were shaken at 25°C and 100 rpm for 24 to 48 h and monitored over time via TLC (eluent CH_2Cl_2) and chiral phase GC.

The reactions catalyzed by *Dm*7α-HSDH on substrate **4.14** and by *Hh*7β-HSDH on **4.19** were thereafter scaled up to a semi-preparative scale (100 mg, 0.7 mmol in 50 mL total volume) following the protocol described above. In both cases, the reaction mixture was extracted with AcOEt (3x). The collected organic phase was dried over Na_2SO_4, evaporated and the crude isolated products were purified by flash chromatography (eluent: gradient from CH_2Cl_2 to CH_2Cl_2:AcOEt=9:1) in 85% isolated yield for compound **4.14** and 60% for compound **4.19**. The purified products were characterized via ^{1}H-NMR, GC-MS, chiral phase GC and $[\alpha]_D^{25}$.

4.3.1.3.2. Structural characterization

1-phenil-1,2-propanedione (**4.14**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 – 7.99 (m, 2H), 7.70 – 7.60 (tt, 1H), 7.56 – 7.46 (tt, 2H), 2.53 (s, 3H).

3,4-hexanedione (**4.19**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.78 (q, *J* = 7.3 Hz, 1H), 1.09 (t, *J* = 7.3 Hz, 1H).

9,10-dioxostearic acid (**4.21**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.70 (t, *J* = 7.3 Hz, 2H), 2.29 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.49 (m, 5H), 1.39 – 1.19 (m, 19H), 0.87 (t, *J* = 6.9 Hz, 3H).

Racemic compounds

Racemic *syn*- and *anti*-1-phenyl-1,2-propanediol (**4.17** and **4.18**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 – 7.06 (m, 5H), 4.68 (d, *J* = 4.2 Hz, 1H), 4.00 (qd, 6.4, 4.3 Hz, 1H), 1.08 (d, *J* = 6.4 Hz, 3H); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 – 7.06 (m, 5H), 4.51 – 4.25 (m, 1H), 3.92 – 3.70 (m, 1H), 1.05 (d, *J* = 6.3 Hz, 3H).

Unsolved spectrum of racemic *syn*- and meso-*anti*-1-phenyl-3,4-hexanediol (**4.23** and **4.24**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.69 – 3.46 (m, 1H), 3.38 – 3.26 (m, 1H), 2.76 – 2.06 (bs, 4H), 1.69 – 1.33 (m, 6H), 1.22 – 0.66 (m, 12H).

Compounds isolated from scale-up reactions

(*R*)-4.15: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 – 7.13 (m, 1H), 5.07 (d, *J* = 9.6 Hz, 1H), 2.07 (s, 1H). Conversion: 97%, ee (chiral phase HPLC): 98%; $[\alpha]_D^{24}$: -84.16° (c: 1.10 in EtOH).

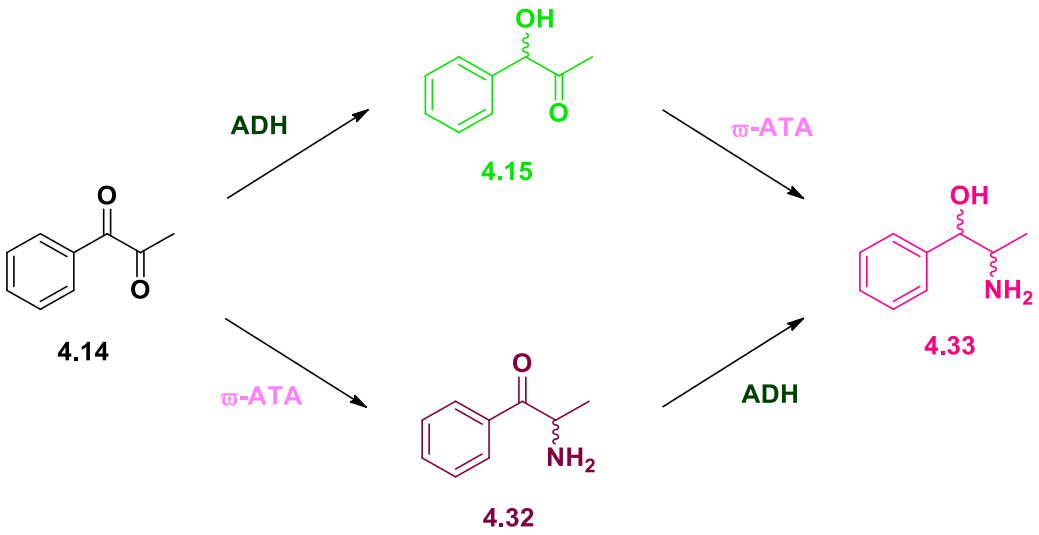
(*R*)-4.22: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.39 – 4.02 (m, 1H), 3.48 (d, *J* = 4.8 Hz, 1H), 2.61 – 2.38 (m, 1H), 2.01 – 1.79 (m, 2H), 1.69 – 1.50 (m, 2H), 1.22 – 1.05 (m, 3H), 1.02 – 0.84 (m, 3H). Conversion: 89%, ee (chiral phase GC): 40%; $[\alpha]_D^{24}$: -17.7° (c: 1.0 in EtOH).

4.3.2. ATA-mediated transamination of α-diketones and pyrazine synthesis

4.3.2.1. 1-phenyl-1,2-propanedione as a synthetic entry to ephedrine precursors and 1,4-diazine nucleus

As it was previously mentioned, the aim of reducing 1-phenyl-1,2-propanedione (compound **4.14**) is that of obtaining highly enantiomerically pure phenyl acetyl carbinol (PAC, compound **4.15**), an intermediate of interest in the synthesis of ephedrine derivatives.[64]

In order to do that, it is necessary to introduce an amino group in place of the phenethyl carbonyl moiety. This could in principle be done with enzymes called aminotransferases (or transaminases), and this specific reaction from **4.14** to cathinone (compound **4.32**) was demonstrated to be feasible in the literature in an exquisitely regio- and stereo-selective fashion.[66] Noteworthy, also cathinone is an intermediate of pharmaceutical relevance, which has effects on the central nervous system, acting as a serotonin re-uptake inhibitor (and thus, potentially, as an antidepressant agent) and stimulating the release of dopamine.



Scheme 4.20: putative biocatalytic ways to pseudoephedrines using 1-phenyl-1,2-propanedione as starting material.

As it is shown in Scheme 4.20, there are two possible fully enzymatic ways from **4.14** to the ephedrine precursor diastereomers **4.33**. The way shown in the upper part of the scheme consists in reducing 4.14 in a regio- and stereoselective fashion to enantiopure **4.15**, which undergoes a subsequent transamination. The preliminary studies we made on the reduction of **4.14** were not encouraging in terms of regio- and stereoselectivity, yielding product mixtures both with HSDHs and with commercial ADHs. To circumvent the regioselectivity problem in the dehydrogenase-mediated reduction, it could be envisaged to pursue the second synthetic pathway, thus, to previously perform the transamination of the aforementioned 1-phenyl-1,2-propanedione, as depicted in the scheme above. If the cathinone product **4.32** is then accepted by the chosen dehydrogenase, a two-step (in-flow) synthesis of ephedrine derivatives can in principle be obtained.

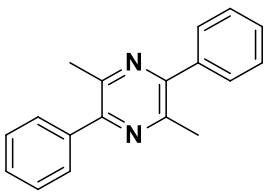
To this purpose, preliminary studies on the transamination of **4.14** were performed with a library of selected and diversified ω-ATAs among the individuals at disposal in the laboratories of SCITEC-CNR (see Table 4.13).

Enzyme	Selectivity
B3-like	*S*
B3-SUMO	*S*
B3-like SUMO	*S*
BV333	*S*
*Cv*ATA	*S*
Is3-TA	*S*
It6-TA	*S*
*Vf*ATA	*S*
*At*ATA	*R*
ATA117	*R*
*Nf*ATA	*R*

Table 4.13: diversified *R*- and *S*-transaminase library among the individuals available at SCITEC-CNR.

Among the *S*-selective ATAs, only the individual previously described in the literature,[67] namely *Cv*ATA, could perform this reaction. However, the *R*-selective enzymes were all able to complete the transamination within a few hours.

An attempt to isolate the cathinone intermediate with the scale-up of the reaction catalyzed by *At*ATA was made in the attempt of trying a subsequent reduction of the aminoketonic intermediate with ADHs. However, once it was isolated, this molecule was not *per se* stable, and it formed a pyrazine dimer as self-condensation product. The crude extract of the *At*ATA-catalyzed reaction showed indeed two stains visible in TLC at 254 nm. One of those was acetophenone resulting from the action of ATA on methylbenzylamine, the other one was the aforementioned 1,4-diazine derivative shown in Figure 4.8.



4.34

Figure 4.8: pyrazine (1,4-diazine) derivative obtained by cathinone intermediate self-condensation upon isolation.

A search in the literature led us to find out that this facile synthetic pathway for 1,4-diazines had already been described three years ago in an elegant report by Turner et al.[68]

However, if the reaction was not extracted, the cathinone intermediate remained apparently stable in the reaction medium, thus a one-pot or in-flow modification of the thus obtained cathinone intermediate could be envisaged, paying attention to the substrate specificity and to the enantioselectivities of the ADHs that would reduce cathinone to the ephedrine precursor **4.33**.

4.3.2.2. Experimental data

4.3.2.2.1. Analytics

The general analytics is reported in the beginning of Section B. Here, further details will be furnished.

Reactions were monitored *via* TLC as described in the beginning of Section B, using CH_2Cl_2:CH3OH=9:1 (+1% v/v of 30% aqueous ammonium hydroxide) as eluent and an ethanolic solution of ninhydrine (20 mg in 100 mL) to see the spots.

4.3.1.3.2. General synthetic procedures

*4.1.3.2.2. Enzymatic reductions of substrates **4.14** and **4.19***

A preliminary activity screening was performed with the transaminase library on substrate **4.14**. The reactions were set on analytical scale (0.500 mL), and they contained: 10 mM **4.14** in 1% v/v DMSO + 99% v/v 100 mM PB (pH 7.5 for *R*-ATAs and pH 9.0 for *S*-ATAs); 1 mg mL^{-1} ATA; 0.25 mM pyridoxal phosphate (PLP); 10 mM *R*- or *S*-methylbenzylamine. The mixtures were incubated at 30°C and shaken at 100 rpm for 24 h, and they were monitored over time via TLC.

The reaction catalyzed by the *R*-selective *At*ATA on substrate **4.14** was scaled up to a semi-preparative scale (100 mg of compound **4.14**) following the protocol described above. When the TLC showed that no more substrate and methylbenzylamine were present, the reaction mixture was extracted with AcOEt (3x). The collected organic phase was dried over Na_2SO_4, and the solvent was evaporated. The crude extract was purified via flash chromatography (eluent: gradient from CH_2Cl_2 to CH_2Cl_2:AcOEt=9:1) and the two isolated products were characterized via ^{1}H-NMR, ^{13}C-NMR, and ESI-MS.

4.3.1.3.2. Structural characterization

Compounds isolated from scale-up

acetophenone: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 – 7.87 (m, 2H), 7.57 (m, 1H), 7.54 – 7.40 (m, 2H), 2.61 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.12, 137.17, 133.09, 128.57, 128.30, 26.57.

4.27: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.62 (m, 2H), 7.55 – 7.48 (m, 2H), 7.48 – 7.42 (m, 1H), 2.66 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.04, 146.77, 137.82, 128.02, 127.50, 127.40, 21.63. ESI-MS: m/z $[m+H]^+$: 261.2; $[m+Na]^+$: 283.2.

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Chapter 5

Is2-SDR: a case-study of an enzyme with unusual properties

5.1 The discovery of an enzyme from hot spring metagenome

In search of novel thermostable HSDHs through bioinformatic and metagenomic tools, a new enzyme was found that turned out not to be a HSDH, even though it shared a 37% primary structure homology with the 7α-HSDH from *Bacteroides fragilis* and a 35% identity both with the 7α-HSDH from *Clostridium absonum* and with the 12α-HSDH from *Clostridium* sp. Indeed, during its preliminary characterization, this enzyme showed no activity against the panel of bile acids tested (functionalized with 3α-, 7 α-, 7β-, and 12α-hydroxyls and with 7- and 12-oxo functionalities).[1]

We have already named Is2-SDR many times in this thesis, mostly in chapter 4.2, when dealing with the derivatization of the Wieland-Miescher ketone, but further studies made in the laboratories of SCITEC-CNR regarding this enzyme made it appear very promising, and for this reason a whole chapter will be dedicated to what Is known up to now about this unusual SDR.

This enzyme is encoded by a DNA fragment extracted from an Icelandic ground soil sample in the next proximities of a geyser, and it was thereafter named Is2-SDR. The name indicates that this enzyme comes from Icelandic metagenome via the prefix Is, and that it apparently belongs to the same superfamily of HSDH, namely SDRs (short chain dehydrogenases). Indeed, despite featuring the primary structure motifs that are typical of this class of alcohol dehydrogenases, and despite its homology to HSDHs, this enzyme does not act like a common ADH: it is only able to reduce carbonyl moieties, but it is very slow in the oxidation of the corresponding alcohols.

As a classic SDR, Is2-SDR features the catalytic triad Ser-Tyr-Lys, as well as by the Gly-X-X-X-Gly-X-Gly motif (highlighted in green and in orange, respectively, in the sequence shown in Figure 5.1).

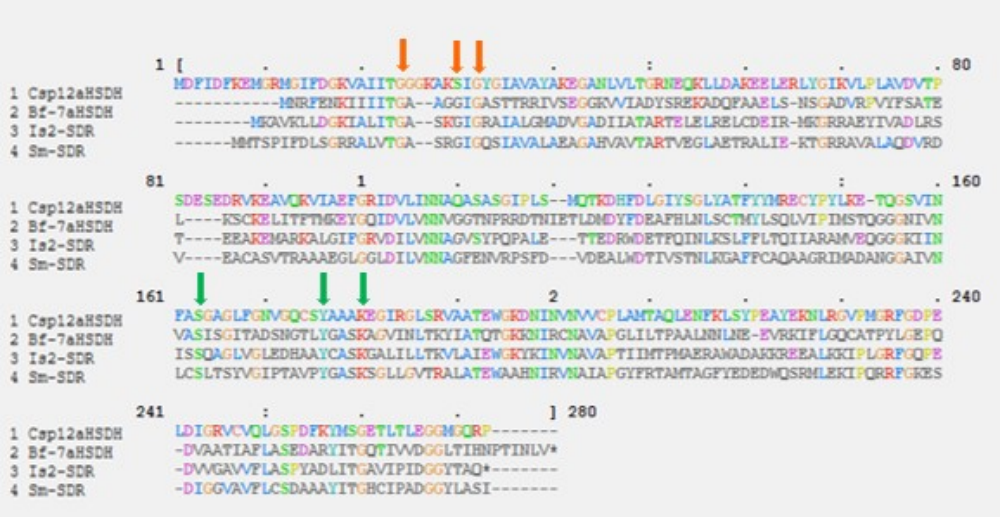


Figure 5.1: the primary sequence of Is2-SDR (sequence 3, Uniprot code A0A7G9U748) is shown, and it is composed of 253 aminoacids.[1] The SDR characteristic motifs are highlighted in orange (N terminus) and in green (C terminus). The sequence is aligned to those of the aforementioned *C*sp12α-HSDH, Bf7α-HSDH, and to the sequence of the SDR from *Shinorhizobium meliloti* (entry 1, 2, and 4, respectively).

5.2. The substrate scope of Is2-SDR, an extremely versatile ketoreductase

Is2-SDR has proven to accept an impressively wide variety of highly diversified ketones, which encompasses bicyclic ketones (both aromatic and aliphatic), α- and ß-ketoesters, aromatic and aliphatic α-diketones, benzylic aldehydes and ketones (only the unsubstituted benzaldehyde, acetophenone, and propiophenone were tested, even though different substitution patterns may be of interest for future studies), long chain natural substrates such as zingerone and 6-gingerol, and extremely bulky natural bile acids as dehydrocholic acid and 12-hydroxy-α-3,7-dioxo-5ß-cholan-24-oic acid.

Compared to the substrate scope of ADHs such as those sold in a kit of 18 individuals by Evoxx, composed of the members which were mentioned in chapter 4.3.1.2.1., Is2-SDR can work on much more diversified ketones. For example, individuals among the Evoxx library can work wither on benzylic ketones, or on long-chain ketones, or on less bulky ketones, or on α- and ß-ketoesters, but none of them is declared by Evoxx to be able to reduce all these substrates "at once".

Other ADHs were reported in the past being able to reduce diversified and bulky carbonyl compounds, but none of them showed such a wide substrate scope, to the best of my knowledge.[2-6]

All of the substrates represented in Table 5.1 were reduced with good to excellent ees and with moderate to quantitative conversions under unoptimized reaction conditions (10 mM substrate in 50 mM PB pH 7.0 + 5-10% v/v DMSO, 0.4 mM NADP+, 70 mM ammonium formate, 0.2 U/mL PsFDH, test volume of Is2-SDR). The analysis of the substrate scope of Is2-SDR was extended to all the substrates depicted in Figure 5.1.

For some of the substrates (namely, **4.4** and **5.1**-**5.4**), the conversion was determined via GC-MS, used as a first screening method, showing that benzaldehyde (**5.1**) was fully converted, while the other compounds gave conversions ranging from 25% (**5.2** and **5.4**) to 88% (**5.3**). In these cases, no information was gathered on the ee of the products, albeit the evaluation should be limited to compounds **4.4**, **5.2**, and **5.3**, since the other two compounds generate achiral alcohols.

On the other hand, the bioreductions of compounds **5.5**, **5.6**, **4.21**, **2**, **2d**, and **1a** were up to now evaluated just via TLC. The reaction on **2d** was complete, as it will be pointed out later, while the other reactions gave only partial conversions. Further evaluations will be made in this respect, also in terms of enantio- or diastereoselectivity in the formation of the chiral products.

The absolute configuration of the products still has to be determined for compounds **4.4**, **5.2**-**5.6**, and **4.19**-**4.21.**

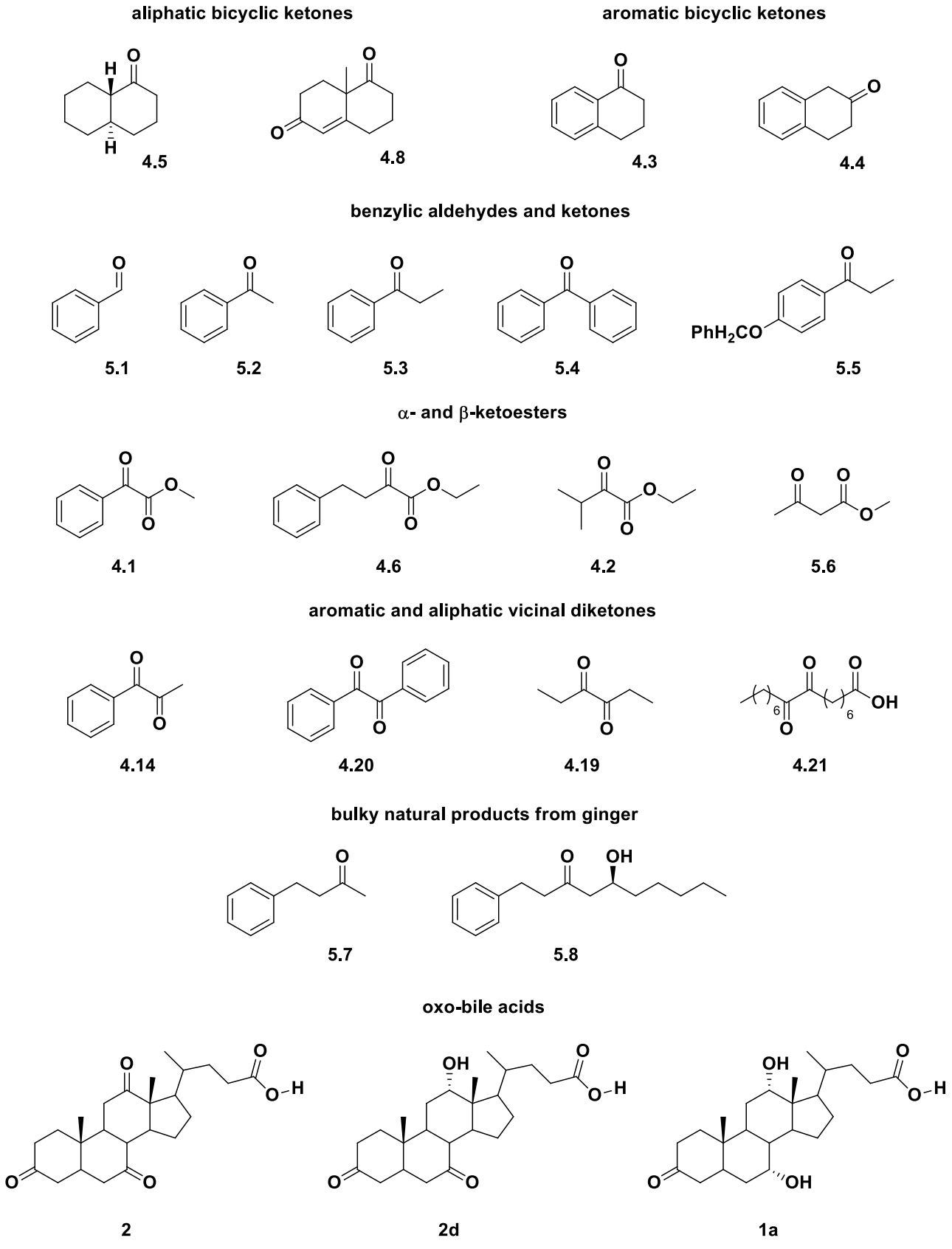
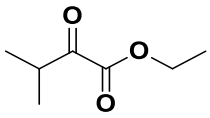
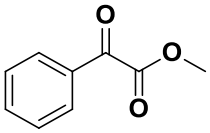
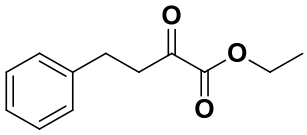
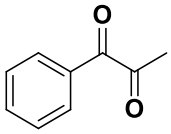
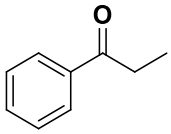
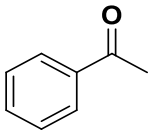
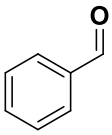
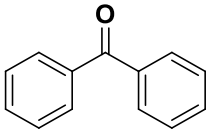
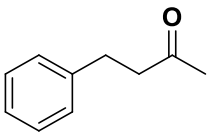
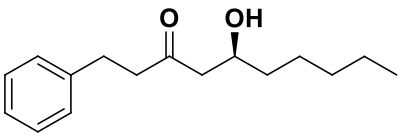


Figure 5.2: the highly diversified panel of substrates reduced by Is2-SDR.

Substrate	Compound number	C (%, 24 h)	e.e. (%)
	**4.2**	> 99	98.4 (*R*)
	**4.1**	> 99	82.0 (*R*)
	**4.6**	96	94.2 (n.d.)
	**4.14**	94	(***R***)**-4.15**: e.e. 64%
	**5.3**	88	n.d.
	**5.2**	25	n.d.
	**5.1**	> 99	-
	**5.4**	25%	-
	**5.7**	24	83 (*S*)
	**5.8**	50	> 99 (*R*)

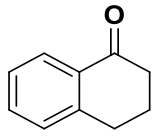
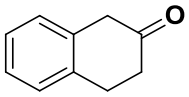
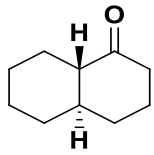
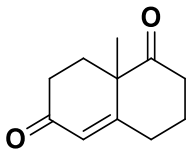
Substrate	Compound number	C (%) (24 h)	Ee (%)
	**4.3**	> 99	94.9 (n.d.)
	**4.4**	45	n.d.
	**4.5**	> 99	*cis*-OH: > 99 *trans*-OH: 61.2 (n.d.)
	**4.8**	58.0	See chapter 4.2

Table 5.1: the highly diversified panel of substrates reduced by Is2-SDR.

The screening on such a diversified panel of ketones demonstrated the high degree of substrate promiscuity of this enzyme, a property that is very promising for potential applications in the synthesis of highly valuable chiral alcohols. Among these, interestingly, there are zingerol and 4,6-gingerdiol (the reduced compounds corresponding to zingerone **5.7** and 6-gingerol **5.8**, respectively), which have been shown to possess many biological activities, including anti-tumor, anti-inflammatory and antioxidant effects.[7]

5.2.1. Is2-SDR and steroids: further investigations

As it was mentioned in the beginning of this chapter, Is2-SDR was demonstrated not to be a HSDH enzyme as none of the tested bile acids could be oxidized or reduced in spectrophotometric activity assays. However, having seen its astonishing ability to reduce bulky compounds, we recalled that we had not tested bile acids with a 3-oxo substitution. However, since Is2-SDR showed about 30-40% sequence homology with 7α- and 12α-HSDHs, but also with 3α-HSDH or 3β-HSDHs (the highest been 34% with *Ruminococcus gnavus* 3β-HSDH), some 3-oxo bile acids were tested alongside other oxidized bile acids. Indeed, as a further characterization of the substrate scope of Is2-SDR, and to understand whether this enzyme could to some extent work on steroids, the reduction of the fully oxidized dehydrocholic acid (compound **2**) and of partially oxidized derivatives of cholic acid (compound **1**) was tested. The tested compounds are shown, alongside with cholic acid, in Figure 5.3.

The reactions were carried out under standard screening conditions, *i.e.*: 10 mM substrate in 50 mM PB pH 8.3; 2 mg/mL Is2-SDR; 0.2 U/mL PsFDH; 0.4 mM $NADP^+$; 70 mM ammonium formate. The reactions were kept at 100 rpm and 25°C for 24 hours and monitored by TLC (eluent $CHCl_3$: MeOH: AcOH= 9: 1: 0.05, development: Komarovsky's reagent).

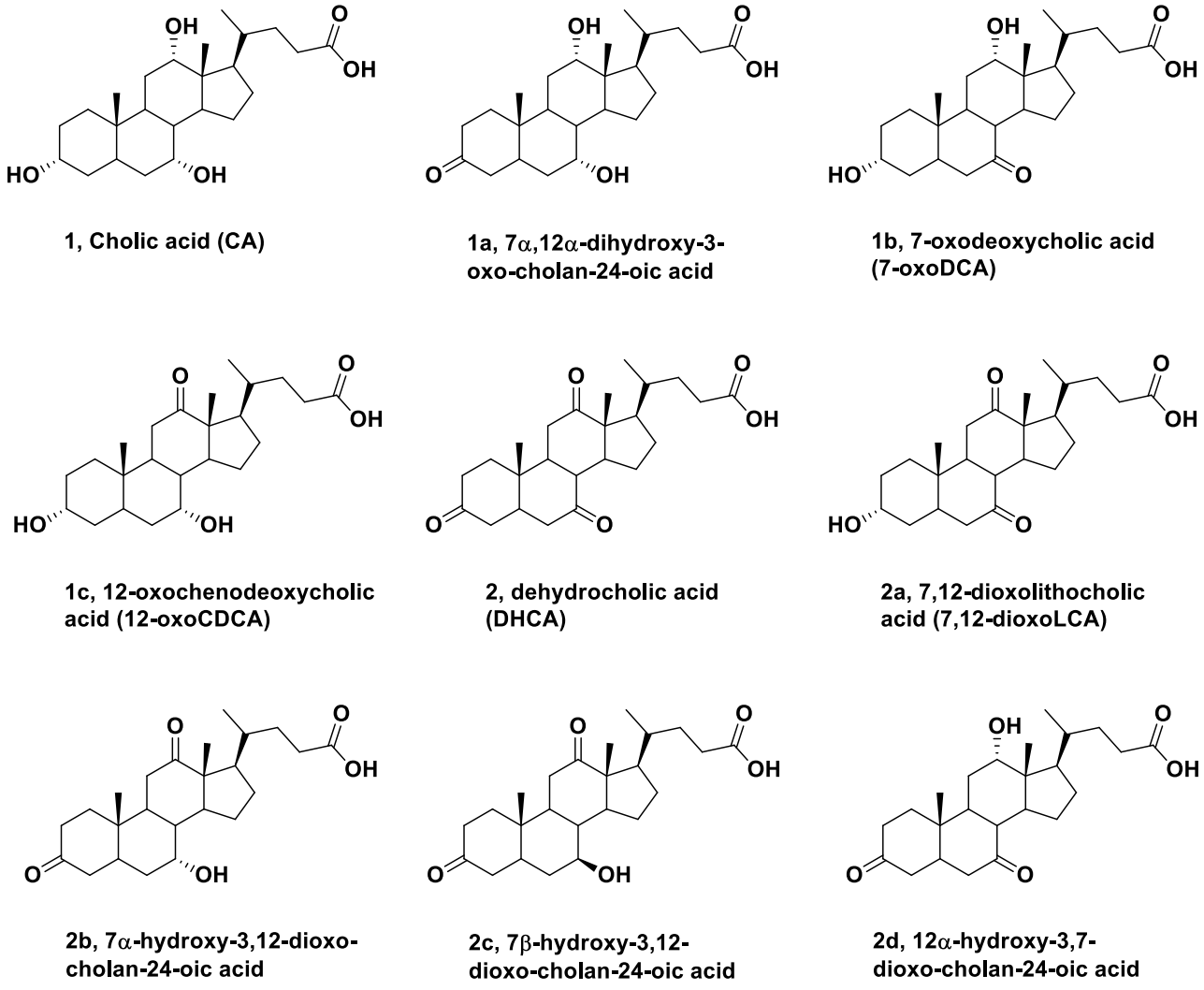


Figure 5.3: panel of bile acids tested as putative substrates for Is2-SDR-mediated reduction.

The TLCs showed that the substrates that did not contain a 3-oxo group had been left untouched, while compound **2** and **2d** had been partly (**2**) or fully (**2d**) converted to a more polar product. Even though the scale-up and isolation of the products still has to be carried out, a hypothesis can be made, based on these data and on the preliminary spectrophotometric activity assays: Is2-SDR may act as a 3β-HSDH. Indeed, during the preliminary activity screening, no 3β-hydroxy bile acid had been tested. This will be done in the next future to further understand and characterize the bizarre behaviour of this enzyme, together with the scale-up on the bioreductions of **2d**, which should at this point be converted to the corresponding 3β-hydroxy-7-oxo bile acid.

5.3. Insights into the structural stability of Is2-SDR

5.3.1. Homology modeling: putative structure of this enzyme

The structural and mechanistic overview on the SDR superfamily was given in Chapter 2.1. Here it will just be recalled that albeit SDRs share a low primary sequence identity, they display a highly conserved 3D structure, characterized by the presence of the so-called Rossmann-fold motif in the nucleotide binding region. The vast majority of SDRs tend to form oligomers, with a quaternary structure that is usually homodimeric or homotetrameric.

To have a first glance at the structure of Is2-SDR, homology models were built on SwissModel, using as templates five individuals that shared up to 50% identity with Is2-SDR. Specifically, I hereby report the structure built based on the Short Chain Dehydrogenase from *Shinorhizobium meliloti* 1021 [PDB: 4J2H],[8] that is classified as an SDR enzyme and shares 41.37% sequence identity with Is2-SDR. The structure was chosen because it had the highest QMEANDisCo scoring function value of 0.75±0.5 among the other models (which gives a quality estimate both on the global and on the local scale of the structural prediction based on a single template structure).

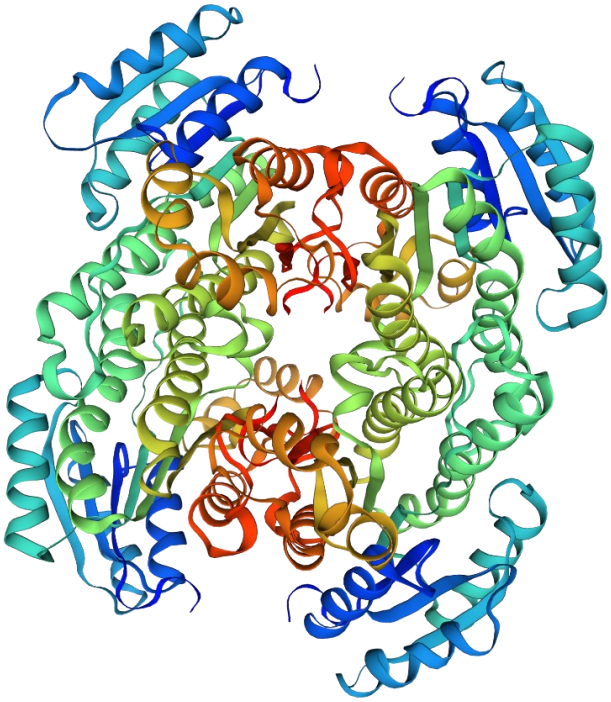


Figure 5.4: 3D-structure of Is2-SDR based on the SDR from *Shinorhizobium meliloti* 1021 [PDB: 4J2H].

Figure 5.4 shows the predicted 3D structure of Is2-SDR obtained by this homology modeling approach, while Figure 5.5 compares the template and model structures by superimposition. Both ribbon and rope views are given, as they highlight differently the structural diversities.

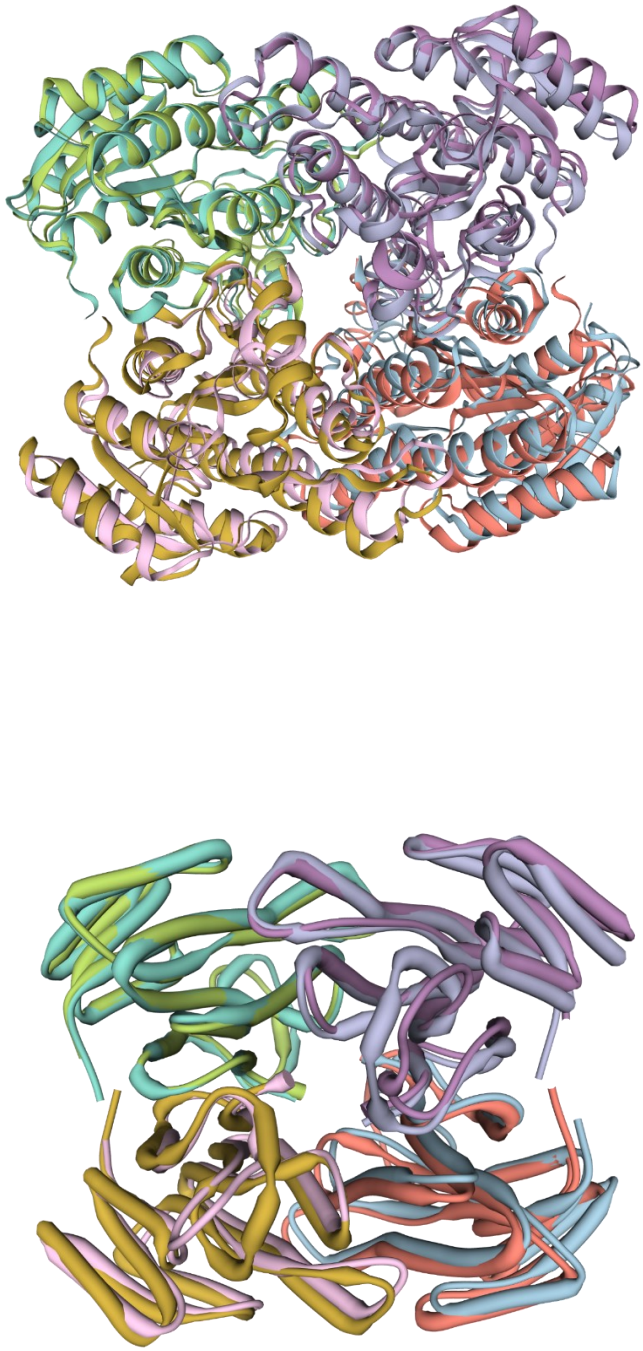


Figure 5.5: ribbon (above) and rope (below) superimpositions of the model 3D-structure of Is2-SDR and of the template SDR from *Shinorhizobium meliloti* 1021 show a high 3D-structure similarity, albeit local differences are present.

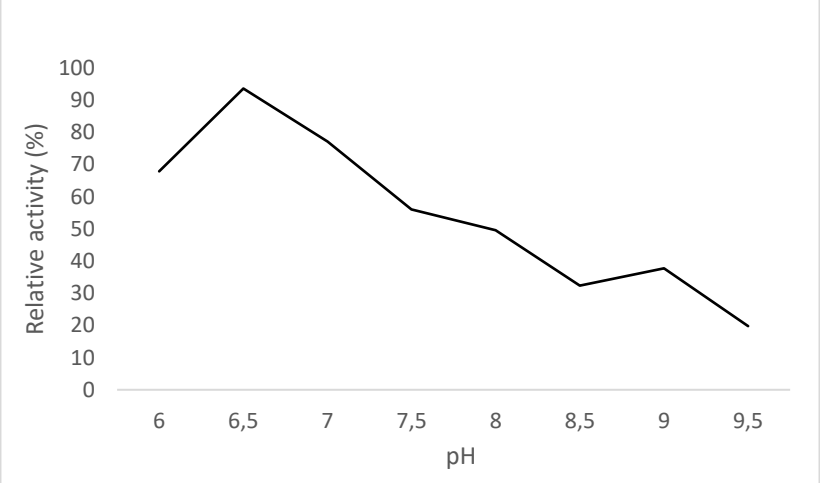
Up to now, little is known about the 3D-structure of this enzyme, although its biochemical properties and its substrate promiscuity make it a potentially interesting target for crystallization studies. Other models could be built as well, for a comparison with the homology obtained with SwissModel. YASARA could be used for another more precise homology modeling based on more than one template at a time, or *ab initio* calculations could be made with the revolutionary software Alphafold, able to predict with a high reliability the structure of proteins from their primary sequences.[9]

An information that we have on this enzyme is that it is totally inactive when the His-TAG is on the N-terminus, so we used a C-teminus His-Tagged Is2-SDR for our studies. It would be interesting to investigate which structural consequences the N-terminal His-TAG has on this enzyme that trigger its total inactivation.

5.3.2. Functional characterization of Is2-SDR

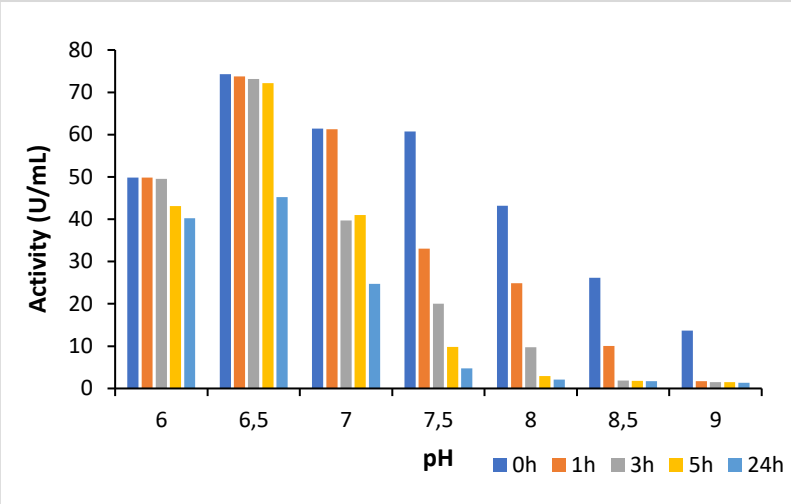
Due to its wide substrate scope and to the extremophilic origins of the metagenome that encodes for this enzyme, a functional characterization was started for Is2-SDR, to determine its optimal pH and temperature and its stability in time under storage conditions differing from -20°C or -80°C.

Activity assays using 10 mM ethyl 3-methyl-2-oxobutanoate **4.2** in 50 mM phosphate solutions at various pH values were carried out, and it was in this way determined that the optimum operational pH of this enzyme is around 6.5, as it is shown in Graph 5.1, in line with the fact that this enzyme performs reductions (best carried out around neutral pH, while oxidations work at more alkaline pH values). All the activity assays from now on are made with 10 mM ethyl 3-methyl-2-oxobutanoate and 0.2 mM NADPH in 50 mM phosphate buffer at pH 7.0.



Graph 5.1: determination of the pH optimum of Is2-SDR.

Similarly, the stability of this enzyme at room temperature at various pH values was tested, showing that it rapidly lost activity at pH 9 (in less than a h), while it retained nearly all of its activity at pH 6-6.5, as it is shown in Graph 5.2.



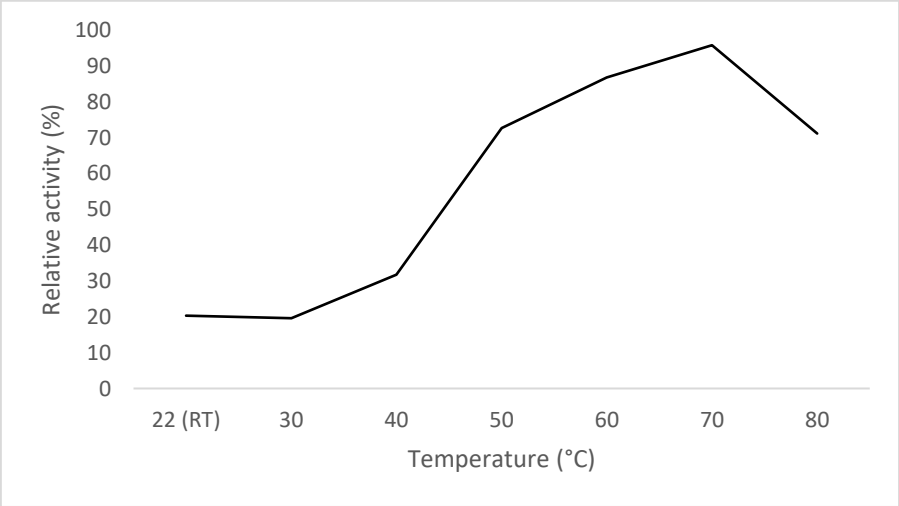
Graph 5.2: determination of the stability of Is2-SDR over time at different pH values.

Similar activity studies allowed to determine that this enzyme can be conserved in the fridge (4°C) for 12 days keeping more than 80% of its initial activity.

5.3.3. The thermostability of Is2-SDR

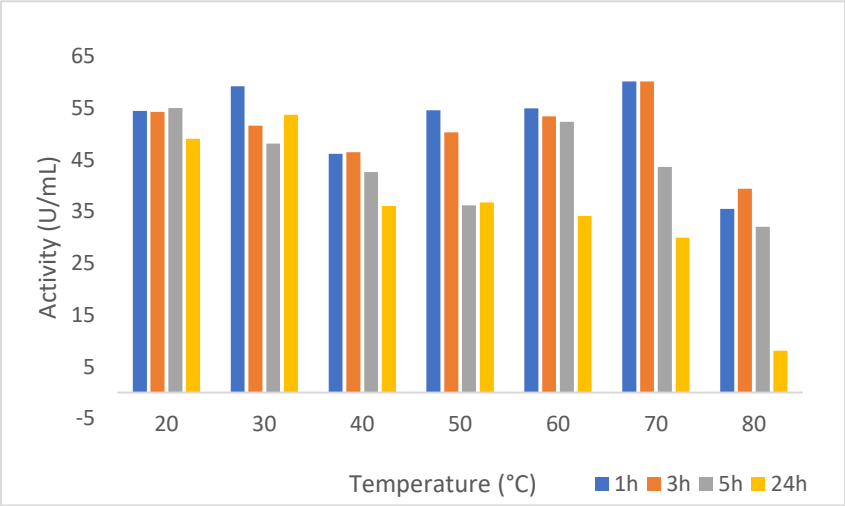
Aside from the high substrate versatility of this ketoreductase, that makes it *per se* an interesting enzyme, a deep biochemical characterization was thoroughly conducted in our laboratory. The native environment of the unknown microorganism that produces Is2-SDR is extreme, and as such it provides in principle the possibility for this enzyme to be thermostable.

Indeed, activity assays at different temperatures allowed to determine that the optimal temperature of this enzyme is of 70°C, where Is2-SDR has about 5 times the activity that it has at 20°C, as it is shown in Graph 5.3. This T optimum shows that Is2-SDR is highly thermophilic, *i.e.*, it works the best at high temperatures, which would be deleterious to common enzymes.



Graph 5.3: determination of the temperature optimum of Is2-SDR.

Having ascertained the thermophilicity of Is2-SDR, which is in line with its extremophilic origins, we wanted to test if this enzyme would be thermostable, as well. Indeed, an enzyme can be extremely thermophilic, but it can lose its activity very fast at high temperatures due to inherent structural liability. *Au contraire*, Is2-SDR showed to be not only thermophilic, but also thermostable. Indeed, incubation studies at various temperatures for up to 24 h showed no appreciable activity loss at up to 70°C, where the enzyme retains 50% of its activity after 24 h of incubation. The thermostability profile of Is2-SDR is outlined in Graph 5.4. Notably, even at 80°C no significant decrease in activity can be detected in the first 5 h of incubation.



Graph 5.4: determination of the thermostability of Is2-SDR.

The astonishing thermostability of Is2-SDR was furtherly confirmed via circular dichroism (CD) analyses, which have shown that this enzyme does not unfold even up to 90° (the limit temperature for analyses carried out in aqueous environments). Maybe, this enzyme could even be stable at higher temperatures in aqueous solutions containing cosolvents and salts that would rise the boiling point of the buffer due to ebullioscopic elevation.

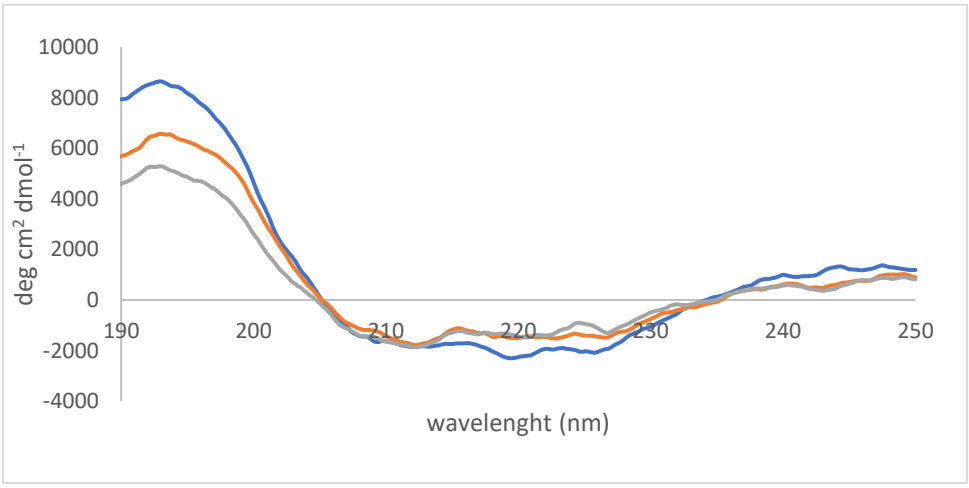
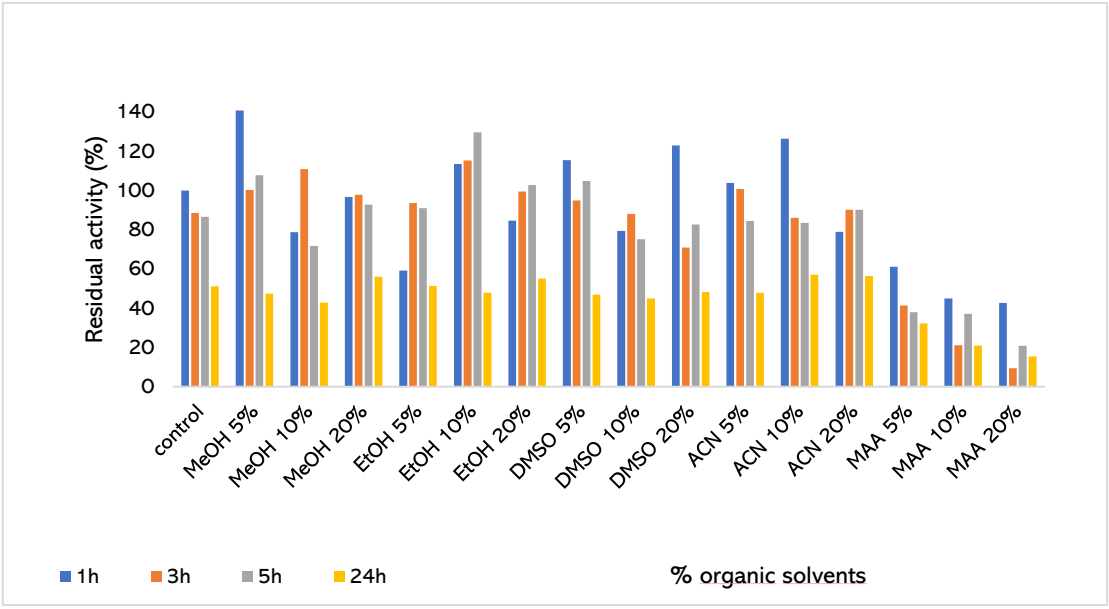


Figure 5.6: superimposition of the CD spectra of Is2-SDR at 25°C (blue), 80°C (orange) and 90°C (grey).

The CD spectra of this enzyme at different temperatures are almost superimposable, thereby confirming the structural stability of Is2-SDR at extremely high temperatures. No such thermostable enzyme has been reported to date, to the best of my knowledge.

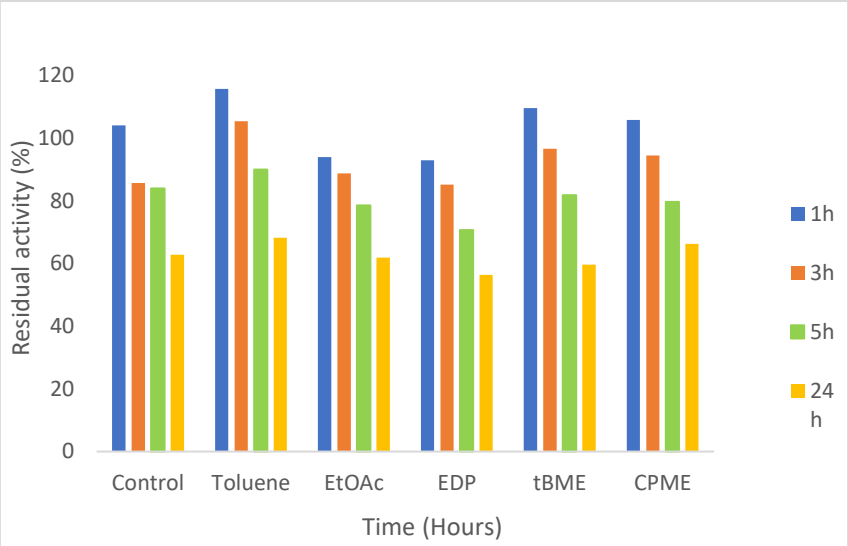
5.3.4. Compatibility with organic solvents

Since usually the thermostability of enzymes goes along with organic solvent compatibility, also this aspect was investigated for Is2-SDR with a variety of water miscible and immiscible organic solvents. The former encompassed MeOH, EtOH, acetonitrile (CAN), methyl acetoacetate (MAA), and DMSO and they are presented in Graph 5.5. The latter comprised toluene, t-butyl methyl ether (TBME), cyclopentyl methyl ether (CPME), petroleum ether (EDP), and ethyl acetate and they are detailed in Graph 5.6. In this case, too, Is2-SDR proved to be a very promising enzyme, as some solvents increased its activity even in high percentages (*e.g.*, 20% MeOH in aqueous buffer). Moreover, some solvents that are usually detrimental for enzymes did not affect Is2-SDR stability and activity.



Graph 5.5: determination of the stability of Is2-SDR in the presence of water-miscible organic solvents.

With the exception of MAA, which is deleterious to Is2-SDR, all the solvents appear to be not much influent on the activity of this enzyme, when compared to the control sample with no organic cosolvent. Noteworthy, some solvents, as MeOH in low percentages, lead to a significant increase in activity with respect to the control (about 40% more activity after 1h of incubation with 5% methanol was observed with respect to the control).



Graph 5.6: determination of the stability of Is2-SDR in biphasic systems with water-immiscible organic solvents.

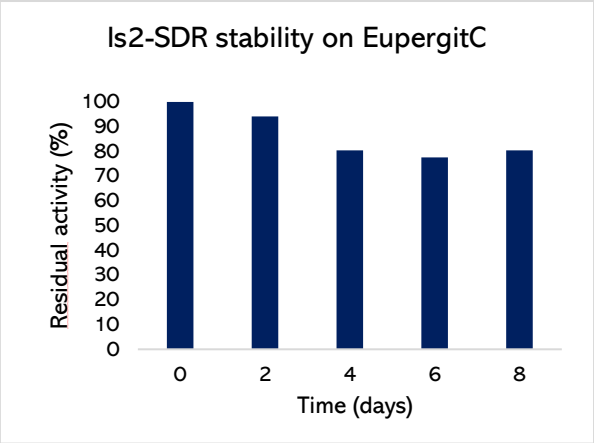
Additionally, Is2-SDR showed an excellent stability when exposed to water-immiscible organic solvents in biphasic systems. In particular, toluene, TBME, and CPME led to an increase in catalytic activity when compared to the control sample (see Graph 5.6).

5.4. Immobilization studies on Is2-SDR for synthetic applications

Considering the high synthetic interest of enantiomerically enriched chiral alcohols as synthons and intermediates, in the framework of reaction engineering via the exploitation of unconventional media, the immobilization of the remarkably stable SDR-Is2 on the commercially available EupergitC resin was carried out. In the preliminary study, the resin was loaded with a high amount of enzyme (1 mg of Is2-SDR every 20 mg of resin), with an activity recovery (meaning, the residual activity of the immobilized enzyme with respect to that of the free enzyme prior to immobilization) of 20%, which can be considered to be a good value due to the unoptimized immobilization conditions.

Is2@EupC (the short name of the immobilized enzyme) was tested both in term of shelf-life and for in-batch reductions, with performances that were slower, but comparable to those of the free enzyme.

Regarding the shelf-life, this study was carried to evaluate decrease in enzymatic activity for Is2@EupC in time. With this aim, every two days kinetic studies were performed on a given amount of wet resin (specifically, 0.5 mg) to reduce 2 mM ethyl 3-methyl-2-oxobutanoate with stoichiometric NADPH at pH 7.0 in 50 mM phosphate buffer. The conversions in time were monitored via GC-MS for each reaction. With this study, it was assessed that Is2@EupC kept about 80% of its initial activity after 8-10 days at 4°C (see Graph 5.7).



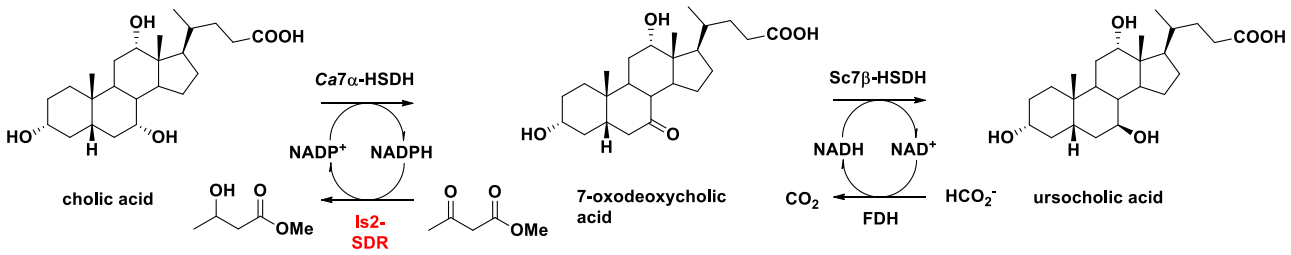
Graph 5.7: shelf-life of Is2@EupC.

After optimization of the immobilization conditions, Is2@EupC will also be used for the in-flow asymmetric synthesis of chiral alcohol synthons.

5.5. Is2-SDR as a cofactor regeneration system

Last but not least, this enzyme holds the potential to be used as cofactor regeneration for oxidized $NADP^+$. For instance, this enzyme has been coupled as a cofactor regeneration system for the oxidation of cholic and hyocholic acids by the NADP(H)-dependent *Ca*7α-HSDH. Since the cofactor regeneration systems for oxidized $NADP^+$ are not many, it should be evident that this extremely versatile enzyme holds a noteworthy synthetic potential.

The synthetic potential of this cofactor regeneration system was shown for example in the oxidation of cholic acid (compound 1) to its 7-oxo derivative was carried out on a 500 mg scale using Is2-SDR as a cofactor regeneration system, and the reaction came to completion in few hours, allowing the isolation of 7-oxodeoxycholic acid (compound 1b) in quantitative yield. Moreover, a one-pot stereoinversion of cholic acid to its 7β-epimer ursocholic acid (compound 4) could be carried out with the aid of Is2-SDR to regenerate NADP+ (see Scheme 5.1 and Figure 5.6).



Scheme 5.1: one-pot Mitsunobu-like C7 epimerization of cholic acid.

Figure 5.6: the TLC of the stereoinversion from cholic acid to ursocholic acid with the aid of Is2-SDR shows only ursocholic acid after less than one day (eluent: $CHCl_3$:MeOH:AcOH=9:1:0.05, development reagent: Komarowsky's oxidizing solution). The reaction does not result to be reversible.

The possibility to use this enzyme also in an immobilized form could render its recycling easier, thus making Is2-SDR very suitable for preparative scale biotransformations. The ability of immobilized Is2-SDR to catalyze reactions in organic solvents shall be considered as a further demonstration of the outstanding potential of this totally uncommon ketoreductase.

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C: Conclusive remarks

To sum up, this work is contextualized in the framework of biocatalysis, which was applied to the synthesis of chiral compounds, mainly having a pharmaceutical relevance. Enzymes belonging to different classes were used for this purpose, namely oxidoreductases, transferases, and hydrolases.

Specifically, the main focus of the present thesis was that of using hydroxysteroid dehydrogenases (HSDHs) to modify both their natural substrates (specifically, bile acids) and other carbonyl compounds.

The modification of bile acids is gaining ever increasing interest, as it has been pointed out in recent years that these molecules can act not only as biomarkers for the early diagnosis of metabolic disorders, but also as signaling compounds within the human organism. Specifically, attention has been given in the literature to hyocholic and muricholic acids, which were studied in this thesis to achieve a selective and efficient synthesis of the valuable derivative ω-muricholic acid. The use of HSDHs in the study of the biological relevance of bile acids may be desirable in the future, making the synthesis of these compounds easy, environmentally friendly, and with quantitative yields on appreciable amounts of the desired compounds, which may alternatively and in some cases be isolated only in trace amounts from biological samples.

Moreover, the substrate promiscuity of HSDHs was investigated. The synthesis of chiral alcohols and amino alcohols is relevant under a pharmaceutical viewpoint, and in this thesis the derivatization of some ketones to achieve the synthesis of compounds of biological relevance has been tackled, *i.e.*, α-hydroxyesters, derivatives of the Wieland-Miescher ketone and ephedrine precursors. The laboratory scale synthesis of such compounds was very efficient, with the possibility of further optimization that may lead these pathways from an academic study to an industrial application in the synthesis of drugs.

In the end, a novel enzyme has been thoroughly characterized, showing the great synthetic potential that it holds. The characteristics that this enzyme possess actually make it an excellent candidate for further studies as a generally applicable means to achieve the stereoselective reduction of diversified carbonyl compounds into chiral synthons. The high stability and versatility of Is2-SDR is of great relevance, as this enzyme is one of the many the “living” demonstrations that all the prejudices that hamper the use of biocatalysis in organic synthesis can be overcome not only by protein engineering, but also with the luck of finding excellent enzyme candidates in nature, that were already evolved to perform reactions in harsh conditions, while keeping a stability that certain classic and routinely used organic compounds can only dream of (it would be astonishing to use lithium aluminum hydride at room temperature and in aqueous environment, just to mention a non-stereoselective reducing agent).

The enzymes and the pathways that were investigated in this work could prove to be useful, after process optimization, for the green synthesis of pharmaceutical compounds, and my most sincere hope is that the work herein described may give the basis for some further studies and applications, maybe beyond those that I could see in this short final perspective.

Appendix I – Published papers

EurJOC
European Journal of Organic Chemistry

Minireview
doi.org/10.1002/ejoc.202000192

Hydroxysteroid Dehydrogenases

Hydroxysteroid Dehydrogenases: An Ongoing Story

Erica Elisa Ferrandi,[a] Susanna Bertuletti,[a,b] Daniela Monti,[a] and Sergio Riva*[a]

Abstract: Hydroxysteroid dehydrogenases (HSDHs) are NAD(P)(H)-dependent enzymes which belong to the so-called short-chain dehydrogenases/reductases (SDR) superfamily. These oxidoreductases catalyze the oxidation/reduction of hydroxyl/keto groups of steroids with high regio- and stereoselectivity. HSDHs were the first group of biocatalysts that was investigated by our group, as an original way to support the ongoing research activity in the area of steroid chemistry in the early seventies. Since then, the research activity on these enzymes has followed three main pathways: a) the isolation and production of new HSDHs; b) the development of new efficient enzymatic systems for the *in situ* regeneration of the cofactor NAD(P)(H); c) the synthetic exploitation of HSDHs. This minireview presents the results that have been published by various research groups in this area in the last 50 years, with a focus on the selective modification of bile acids.

1. Introduction

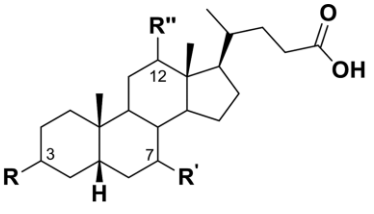
Biocatalysis, the exploitation of enzymes in preparative-scale organic synthesis, moved from its status of weird curiosity to become a topic that deserved to be acknowledged with the Nobel Prize in Chemistry, assigned to Prof. Frances Arnold in 2018.

The involvement of our group in biocatalysis started in the early seventies of the last century, as an original way to support the ongoing research activity in the area of steroid chemistry. The first group of biocatalysts that were investigated was a panel of NAD(P)(H)-dependent oxidoreductases known as hydroxysteroid dehydrogenases (HSDHs). These enzymes catalyze the oxidation/reduction of hydroxyl/keto groups of their natural substrates steroids and bile acids with high regio- and stereoselectivity. HSDHs belong to the short-chain dehydrogenases/reductases (SDR) superfamily, which constitutes one of the largest enzyme superfamilies.[1] Typically, HSDHs are homodimers or homotetramers, each monomer (about 250–300 residues) comprising a cofactor binding domain located at its N-terminus side and a catalytic domain located toward its C-terminus.[2]

These enzymes are widespread and have been identified in microorganisms, plants and animals. Among the various HSDHs described in the literature, this minireview is focused in particular on HSDHs that have been used for the oxidation/reduction of hydroxyl/keto groups of bile acids, i.e. 3α- and 3β-HSDHs, 7α- and 7β-HSDHs and 12α-HSDHs. It is worth noting that bile acids modifications catalyzed by HSDHs have been performed using mainly microbial enzymes. Good HSDHs producers may have evolved these peculiar enzymatic activities as detoxification mechanisms toward the bile acids present in mammalian intestines and released in soil.[3]

2. Early Studies

The first study related to intestinal microbial strains producing enzymes capable of oxidizing cholic acid (**1**, Figure 1) in mono-keto, diketo, and triketo derivatives dates back to 1942,[4] but it was only twenty years later that these enzymes gained significant attention. In 1964 Delin and co-workers purified two HSDHs to homogeneity for the first time, a 3α-HSDH and a 3β(17β)-HSDH from *Comamonas* (formerly *Pseudomonas*) *testosteroni*.[5] In 1970 Aries & Hill[6] prepared cell extracts from Clostridia, *Bacteroides*, *Bifidobacterium* and Enterobacteria strains containing NAD(P)(H)-dependent HSDHs catalyzing the oxidoreduction of the 3α-, 7α- and 12α-hydroxyl groups in cholic



1	R = R' = R'': α-OH	**10**	R = R' = R'': =O
2	R = R'': α-OH; R': β-OH	**11**	R: α-OH; R' = R'': =O
3	R = R': α-OH; R'':H	**12**	R: β-OH; R' = R'': =O
4	R: α-OH; R': β-OH; R'': H	**13**	R': α-OH; R = R'': =O
5	R: =O; R' = R'': α-OH	**14**	R': β-OH; R = R'': =O
6	R': =O; R = R'': α-OH	**15**	R'': α-OH; R = R': =O
7	R'': =O; R = R': α-OH		
8	R: α-OH; R': =O; R'': H		
9	R: α-OH; R': β-OH; R'': =O		

Figure 1. Mono-keto derivatives (**5–7**) of cholic acid (**1**) and mono-hydroxy-derivatives (**11–15**) of dehydrocholic acid (**10**) synthetized by HSDHs,[38] and other bile acids [ursocholic acid (**2**), chenodeoxycholic acid (**3**), ursodeoxycholic acid (**4**), 7-ketolithocholic acid (**8**) and 12-ketoursodeoxycholic acid (**9**)] considered in this review.

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acid and characterized these enzymes biochemically. Following this first report, the number of identified microorganisms producing 3α-, 7α- and 12α-HSDH activities grew rapidly[7–15] and in the '80s the first microorganisms producing 7β-HSDHs were reported.[14,16,17] In particular, in 1981 Hirano and Masuda described the epimerization of the 7α-OH to the 7β-OH of bile acids using mixed cultures of microorganisms, mainly unknown, isolated from human feces, that produced 7α- and 7β-HSDHs.[16] Meanwhile, MacDonald and co-workers found that *Clostridium absonum* was a self-sufficient microorganism in catalyzing the epimerization studied by Hirano.[17] In 1982 MacDonald and Hirano published two papers related to the isolation of *Collinsella* (formerly *Eubacterium*) *aerofaciens,* the first identified microorganism capable of reducing 7-keto bile acids to 7β-OH bile acids without the formation of 7α-OH bile acids.[18,19]

The discovery of new microorganisms producing HSDHs was accompanied by examples related to their exploitation. The exquisite substrate specificity and stereoselectivity made these enzymes, at least in principle, particularly suitable for analytical purposes. The first proposed application regarded the use of HSDHs as analytical tools for the detection of bile acids. In 1969 Turnberg et al. described a method for the quantitative determination of bile salts in human bile using a purified 3α-HSDH from *C. testosteroni*.[20] Instead, in 1973 Haslewood et al. prepared a cell extract from *Escherichia coli* containing a 7α-HSDH and applied it to the estimation of 7α-hydroxy-bile acids and their conjugates in biological fluids.[21] Subsequently, HSDHs have been used for the detection of bile acids in the blood serum for the diagnosis of hepatopathy,[22–24] in the bile,[25,26] in the intestinal aspirates of patients affected by gastrointestinal disorders,[27] in the human feces,[28] and in the human urine to elucidate urinary bile acid patterns in patients with biliary atresia.[29] The contribution of our group in this area was the setup of a bioluminescent assay based on a multienzymatic system comprising an HSDH, an FMN-oxidoreductase and a luciferase that emits light in the presence of $FMNH_2$, in turn, formed by reduction of FMN at the expense of NADH. By co-immobilizing these enzymes on nylon tubes, as little as picomol of bile acids present in serum samples could be detected.[30,31]

Besides these applications in the analytical field, the availability of commercial preparations of HSDHs and the concomitant optimization of suitable protocols for the *in situ* enzymatic regeneration of the NAD(P)(H) cofactor[32] made our group interested in the synthetic exploitation of these enzymes. In the '70s, one of the main issues was related to the problem of the low solubility in water of most of the substrates, including the neutral steroids belonging to the androstane and pregnane families. The simple addition of water-soluble cosolvents was not always a good solution, as it did not solve problems related

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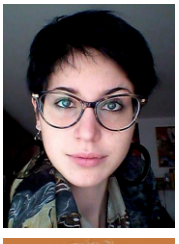


Susanna Bertuletti obtained her B. Sc. in Chemistry in 2016 and her M. Sc. in Chemical Sciences, with a focus on Organic Chemistry, in 2018 at the University of Milan. Since 2018, she is a Ph. D. student in Pharmaceutical Sciences at the University of Milan and she works in the laboratories of the Institute of Chemical Sciences and Technologies (SCITEC-CNR). Her Ph. D. is set in the framework of biocatalysis as a tool for pharmaceutical purposes.

Daniela Monti received her M. Sc. degree in Biological Sciences at the University of Milan in 1991 and her Specialization degree in Biotechnological Applications from the same institution in 1995. Since 2001 she is a research scientist at the Institute of Chemical Sciences and Technologies (SCITEC - CNR) and in 2020 she was appointed as Senior Researcher. Her research interests focus on the discovery and characterization of new biocatalysts and their application in organic synthesis.

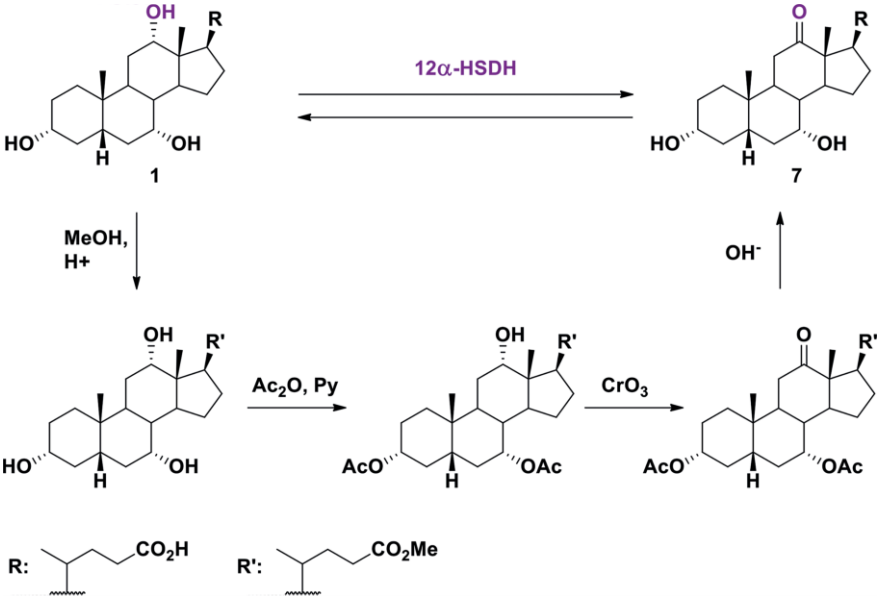
Sergio Riva is presently the Director of the Institute of Chemical Sciences and Technologies (SCITEC) of the Italian National Council of Research (CNR). In 1993 he was awarded the Ciamician medal by the Organic Chemical Division of the Italian Chemical Society for his research in Biocatalysis. His scientific activity is documented by more than 200 publications reporting on the isolation and characterization of different groups of enzymes and the synthetic exploitation of these biocatalysts.

to the progressive denaturation of the enzymes as well as to the enzyme inhibition by substrates and/or products. A solution to these problems came from the development of biphasic systems made by a water phase containing the enzyme(s) and the hydrophilic compounds (e.g., cofactors, salts) and a by water-immiscible solvent (i.e., ethyl acetate) containing most of the starting steroidal substrates and the formed products.[33] Once the proof of principle was established,[34] additional work was performed to better clarify the issues related to the solubility and partition of reagents and products between phases, the importance of fine-tuned shaking to assure the needed transfer between phases without affecting enzymes stability, the enzyme kinetics, the performances of different reactors.[35–37]

Specifically, as far as the nature of the organic solvent concerns, the lower the polarity is, the smaller the effect on the enzyme stability is. Ethyl acetate and butyl acetate proved to be the most suitable solvents to be used with HSDHs. The phase transfer is ensured by shaking or stirring. Usually moderate shaking (100–150 rpm) permits a satisfactory transfer without affecting too much the stability of the enzyme. With immobilized HSDHs, higher shaking rates can be used to allow better penetration of the substrate in the matrix. The use of a biphasic system might positively affect enzyme kinetics, specifically in the presence of substrate of product inhibition: as these compounds are mostly dissolved in the organic phase, their concentration in the buffer phase where the catalysis takes place is always low and therefore the inhibitory effect might be significantly reduced. In most cases, the apparent K_M of the steroid substrate is increased, whereas the *V*max of the reaction is not altered. Finally, concerning reactor types, free enzymes work better in a shaken vessel, the reaction rate being influenced by the amount of organic solvent dissolved in the aqueous phase and the strength of shaking. On the contrary, immobilized enzymes yield better performances in fixed-bed reactors. For large-scale synthesis, the latter system proved to be more suitable, allowing repeated cycles to be conducted (higher cofactor turnover number, higher enzyme stability and satisfactory mass transfer).

Coming to bile acids (like cholic acid, **1**, Figure 1), these compounds are water-soluble (up to 4 % w/w) at alkaline pHs compatible with HSDHs activity and stability, therefore they were identified as interesting substrates to confirm the high regio- and stereoselectivity of these enzymes. In a first report,[38] cholic acid (**1**) and dehydrocholic acid (**10**) were the substrates of choice to demonstrate the selectivity of a panel of HSDHs. Reactions were performed on a preparative scale and the transformations were always quantitative, allowing the isolation of the mono-keto derivatives of cholic acid (**5–7**) or the monohydroxy derivatives of dehydrocholic acid (**11–15**), respectively (Figure 1). Furthermore, by coupling the reduction of **10** with the oxidation of 1-*D*-glucose (catalyzed by an HSDH and glucose dehydrogenase, respectively) the regio- and stereoselective synthesis of mono-deuterated bile acids with an isotopic purity exceeding 94 % was obtained.[39] Moreover, the α/β inversion of the hydroxyl groups at position 3[40] or position 7[41] were also investigated. Inversions were quantitative and were obtained in two steps, requiring the isolation of the 3-keto or the 7-keto intermediates.

It must be emphasized that, from the '80s to date, HSDHs have been mainly investigated to develop new and more sustainable strategies for the synthesis of ursodeoxycholic acid (**4**), the drug of choice in the treatment of the cholesterol gallstones. The first studies included the use of mixed cultures of microorganisms producing 7α- and 7β-HSDHs, such as *C. absonum, C. aerofaciens* and *Bacteroides fragilis*. Unfortunately, these reaction systems did not allow the complete conversion of the bile acid substrate into the desired product, as the latter compound was always in equilibrium with the keto intermediate of the reaction.[17,18] Significantly better results were obtained using isolated enzymes. Scheme 1 compares the enzy-



Scheme 1. Comparison between the enzymatic and chemical synthesis of 12-ketochenodeoxycholic acid (**7**).

matic and chemical synthesis of 12-ketochenodeoxycholic acid (**7**). Using a glutamate dehydrogenase for the *in situ* regeneration of the oxidized form of the cofactor $NADP^+$, highly concentrated (up to 4 % w/w) cholic acid could be transformed into **7**.[42–44] The two enzymes proved to be significantly stable and, using an ultrafiltration membrane to keep the enzymes into the reactors, the reaction was scaled up to multigrams.

2. Developments in the Availability of Recombinant HSDHs and in the Process for the Production of Ursodeoxycholic Acid (1995–2014)

In the '90s the progress in molecular biology led to the widespread practice of gene cloning. The first two genes encoding for HSDHs, the 7α-HSDH from *E. coli* (Ec7α-HSDH)[45] and the 7α-HSDH from *Clostridium scindens* (formerly *Eubacterium* sp. VPI 12708),[46] were cloned and overexpressed in *E. coli* in 1991. The heterologous expression of enzymes was very advantageous as it allowed to overcome the problems related to the difficult cultivation of the wild type anaerobic bacteria which are the most common HSDHs producers and it allowed the recovery of the enzymes in high yield. Additionally, the recombinant production of Ec7α-HSDH paved the way to the crystallization of this enzyme and the determination of its protein structure (PDB: 1AHH) provided insights into the catalytic mechanism of HSDHs.[2] Specifically, in this 7α-HSDH, the mechanism involves three catalytic residues, namely Lys163, Tyr159, and Ser146. In the first step, Tyr159 is deprotonated thanks to the neighbor assistance of Lys163 that lowers its pK_a. Subsequently, the target hydroxyl-group of the steroid form a hydrogen bond with the deprotonated phenolic group of Tyr 159 and with the hydroxyl group of Ser146. Finally, the deprotonated tyrosine residue extracts a hydrogen atom from the hydroxyl group of the substrate and concurrently, the cofactor NAD^+ accepts a hydrogen atom from position 7 of the steroid skeleton on the β face of its nicotinamide ring, releasing the final 7-oxo-product. It has been found that the triad Lys/Tyr/Ser is conserved among HSDHs with only a few exceptions.[2,47]

The cloning of the aforementioned 7α-HSDHs was followed by the cloning of the first 3α-HSDH,[48] as well as of a 12α-HSDH,[49] and of a 7β-HSDH,[50] and the structure of *C. testosteroni* 3α-HSDH (Ct3α-HSDH) was solved as well (PDB: 1FJH).[51]

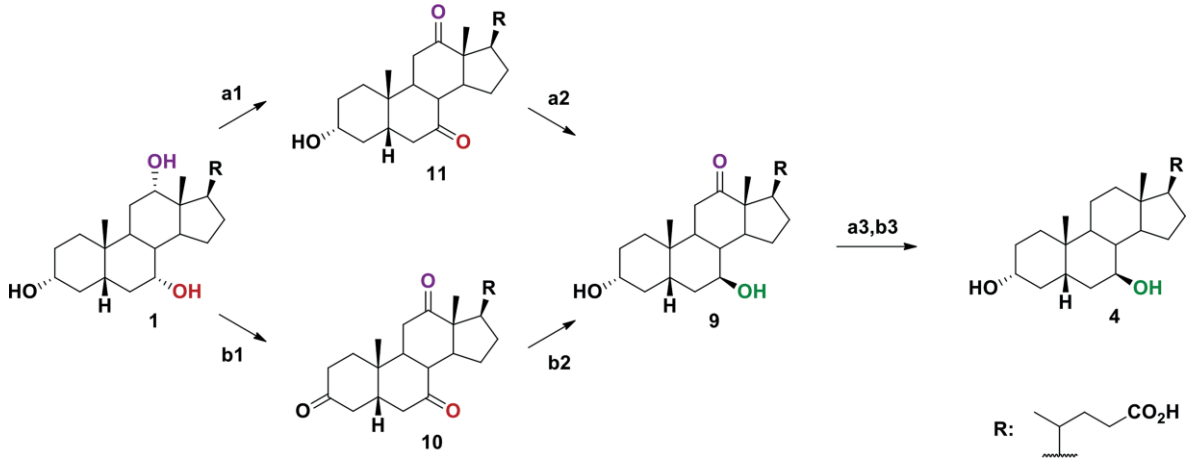
In the new millennium the expression of recombinant proteins *in frame* with affinity tags, e.g., His-tag, allowing the fast and easy purification of the target enzyme to homogeneity by affinity chromatography, has become of common use. This made the production of recombinant HSDHs even more advantageous,[50,52,53] since the synthetic exploitation of recombinant HSDHs was previously sometimes hampered by the presence of contaminant Ec-7αHSDH in *E. coli* cell extracts. This was particularly true for the preparations of recombinant 7β-HSDH or 12α-HSDH, which should have been completely devoid of 7α-HSDH activities when used for the regio- and stereoselective reduction of bile acids at C7 or C12, respectively.

In parallel, from the '90s the synthetic exploitation of HSDHs has been particularly focused on the development of multienzymatic processes for the selective modification of bile acids.

Multienzymatic processes can be performed either in sequential steps, implying the isolation of the reaction(s) intermediate(s), or in a "one-pot" fashion, where no intermediates are isolated. In the latter case reagents/catalysts are added in the same reaction medium either sequentially (telescopic process) or all together, in this way minimizing the required purification steps and the environmental impact, reducing the amounts of solvents and the overall reaction times.

The first examples of the sequential use of HSDHs to modify bile acids (and some of their derivatives) were reported by our group,[40,41] using the available 3α-HSDH, 3β-HSDH, 7α-HSDH, and 7β-HSDH to reverse, respectively, the C3 or the C7 stereochemistry of the hydroxyl group present in a large steroidal pool.

Different chemo-enzymatic strategies based on HSDHs to synthesize ursodeoxycholic acid (**4**) starting either from cholic



Scheme 2. a) HSDHs are used to oxidize the hydroxyl groups at C7 and C12 of cholic acid (**1**) (**a1**, 7α-HSDH and 12α-HSDH), as well as to catalyze the stereoselective reduction leading to the formation of 12-keto-ursodeoxycholic acid (**9**) (**a2**, 7β-HSDH).[56,57] Alternatively, **1** is chemically fully oxidized to dehydrocholic acid (**10**, **b1**), and then regio- and stereoselectively reduced at C3 and C7 using a 3α-HSDH and a 7β-HSDH (**b2**).[58,59] Both synthetic pathways end with a Wolff-Kishner reduction (**a3**,**b3**).

acid (**1**) or dehydrocholic acid (**10**) have been investigated and these multienzymatic processes have been even performed on preparative scales. Among the many approaches that have been reported,[54,55] the two most representative chemoenzymatic strategies to transform **1** into **4** are described in Scheme 2. Both these strategies included the stereoinversion of the C7 hydroxyl group of **1** *via* a sequence of regioselective oxidations and regio- and stereoselective reduction leading to the formation of 12-keto-ursodeoxycholic acid (**9**). The intermediate **6** was subsequently submitted to a Wolff-Kishner reduction to remove the 12-keto group.

According to Scheme 2**,** pathway a), our group proposed in 1996 a 2-step sequential process to transform **1** into **4**, comprising a one-pot oxidation at C7 and C12 mediated, respectively, by the partially purified 7α-HSDH from *Clostridium absonum* (Ca7α-HSDH) and by the commercially available 12α-HSDH from *Clostridium* group P, followed by the reduction at C7 catalyzed by the partially purified 7β-HSDH from *Clostridium absonum* (Ca7β-HSDH).[56] All the HSDHs where NADP(H)-dependent and therefore it was necessary to isolate the intermediate diketo-derivative **11**. In the first step, $NADP^+$ regeneration was accomplished using an α-ketoglutarate/glutamate dehydrogenase system, whereas in the second step the reductive reaction was coupled to a glucose/glucose dehydrogenase (GDH) system for NADPH regeneration.

Later on, thanks to the availability of new HSDHs, our group proposed the one-pot synthesis of **9** avoiding the isolation of the 7,12-diketo-intermediate **11**.[57] It is worth noting that such a one-pot process was rather challenging, as it implied concomitant oxidation and reduction reactions to happen in the same reaction flask. The hypothesis on which the process was based was that enzymes with different cofactor requirements (NADH or NADPH) would have allowed the separation of the reduction and oxidation systems so that the two reactions would have run independently without interfering with each other. Accordingly, **1** was transformed into **9** (Scheme 3) by employing together three HSDHs (the NAD(H)-dependent 7α-HSDH from *Bacteroides fragilis* (Bf7α-HSDH), produced in recombinant form and purified in our lab, a commercially available NAD(H)-dependent 12α-HSDH and the NADP(H)-dependent 7β-HSDH from the wild type *Clostridium absonum* purified in our lab). The regeneration of the cofactors was particularly crucial to shift the overall reaction equilibrium toward the desired final product and it was performed exploiting commercially available lactate DH (for NAD^+) and glucose DH (for NADPH), respectively. After 5 hours the reaction was complete and only the desired product **9** was present in the mixture. However, the process needed to be carefully controlled, as after 24 h it was noted that the reaction intermediate ursocholic acid (**2**) started to be produced again.

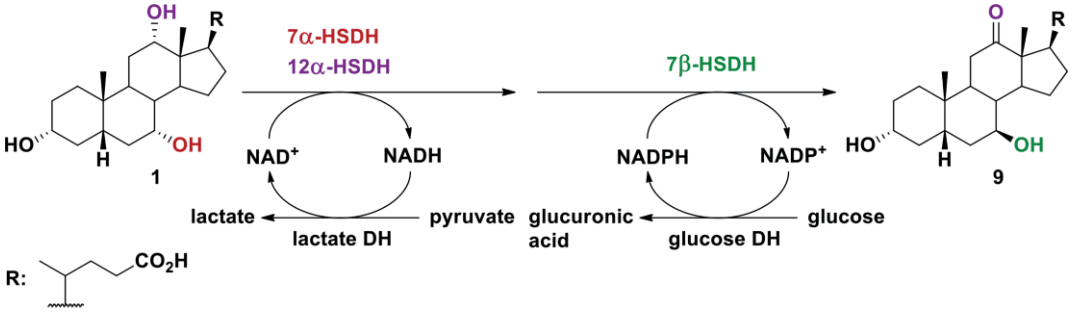
Alternatively, as shown in Scheme 2, pathway b), in 2012 Bakonyi et al.[58] and Liu et al.[59] proposed a two-step sequential process from **1** to **9** comprising the chemical oxidation of the cholic acid (**1**) to dehydrocholic acid (**10**) followed by the one-pot reduction at C3 and C7 employing the NADP(H)-dependent Ct3α-HSDH and the NADP(H)-dependent 7β-HSDH from *C. aerofaciens* (Scheme 2, b2).

The cofactors were regenerated by a glucose/GDH system in the process described by Bakonyi and by a formate/FDH system in the process described by Liu. In both cases, all the enzymes (HSDHs and DHs) were expressed in recombinant form in an *E. coli* strain knocked out for the Ec7α-HSDH gene (*E. coli* BL21 (DE3)Δ7α-HSDH). The lack of 7α-HSDH activities in this *E. coli* strain allowed the authors to use directly crude *E. coli* cell extracts harboring the desired recombinant HSDHs or DHs in the reaction, avoiding the biocatalyst purification steps. It is worth noting that the enzymatic reduction of **10** described by Bakonyi was scaled up to 1.8 L (starting from 100 mM **10**) and 80 g of pure **9** were obtained with a 99.5 % purity.[58]

In parallel, to boost the exploitation of HSDHs even on an industrial scale, many efforts have been focused on the attempt to develop new, cheap and more efficient cofactor regeneration systems.

In 2006 our group proposed to couple the oxidation of **1** (100 mM) to get 12-ketochenodeoxycholic acid (**7**), catalyzed by a NADP(H)-dependent 12α-HSDH, to the reduction of acetone to *i*PrOH catalyzed by an NADP(H)-dependent alcohol dehydrogenase (both commercially available).[60] However, even when a large amount of acetone was employed (up to 25 % v/v), the oxidation reaction was never complete and, in the best case, only 92 % substrate conversion was obtained.

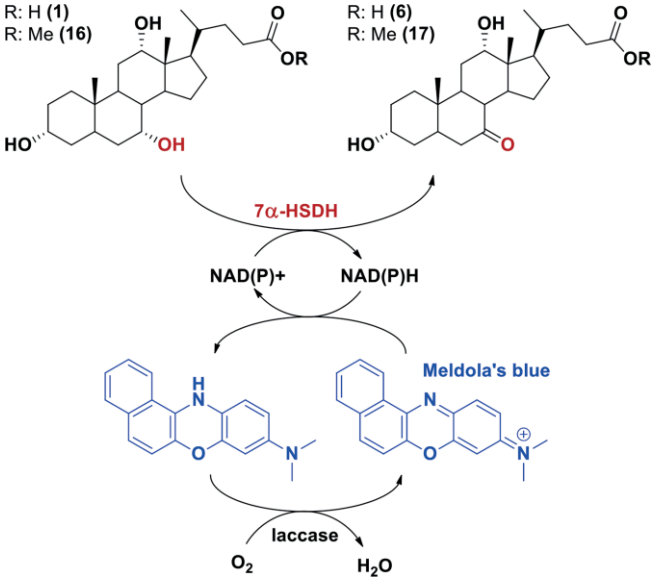
In subsequent work, our group investigated the use of different NAD(P)(H)-dependent alcohol dehydrogenases (ADHs) in combination with a β-keto ester, namely methyl acetoacetate, to regenerate $NAD(P)^+$, the reduction of this substrate being virtually irreversible due to the presence of an electron-withdrawing group near the keto group.[61] This reaction was coupled to the oxidation of various bile acids (substrate concentration up to 100 mM) catalyzed by different 7α-HSDH and 12α-



Scheme 3. One-pot synthesis of 12-ketoursodeoxycholic acid (**9**) mediated by HSDHs.

HSDH either commercially available or produced in recombinant form and purified. The reactions reached completion even in the presence of just a small excess of the co-substrate β-keto ester.[62]

A completely different $NADP^+$ regeneration system was proposed by our group in 2012,[63] inspired by the work described by Aksu and co-workers.[64] The cofactor NAD(P)H could be oxidized by a mediator, Meldola's blue, which in turn was oxidized by a laccase at the expense of molecular oxygen (Scheme 4). This system was successfully applied to the oxidation of cholic acid (**1**, 50 mM, 20.4 g) in an aqueous buffered solution (space-time yield of 2.35 g L^{-1} h^{-1}) or of cholic acid methyl ester (**16**, 200 mM, 5.9 g) in a biphasic system (space-time yields of up to 27.47 g L^{-1} h^{-1}), demonstrating to be very efficient, versatile, and simple. Both the reported HSDSs oxidation reactions were catalyzed by a recombinant 7α-HSDH.



Scheme 4. Oxidation of cholic acid (**1**) or cholic acid methyl ester (**16**) catalyzed by 7α-HSDH coupled with the Meldola's blu based-cofactor regeneration system.

Finally, it is worth mentioning that in 2013 a process was described for the reduction of 7keto-lithocholic acid (**8**), in a biphasic system coupled to the glucose/GDH cofactor regeneration system.[65] As quickly showed in the above-described examples of HSDHs applications, glucose/GDH cofactor regeneration system has been coupled with HSDHs catalyzed reduction reaction from the '80s. This system is advantageous as the co-substrate glucose is inexpensive and the reaction is irreversible as glucose is oxidized to gluconolactone, which hydrolyzes spontaneously. However, when it is applied in the presence of a highly concentrated bile acid solution the reaction mixture starts jellifying thus preventing the reaction to reach completion. The process described in this patent avoided the jellifying of the reaction mixture in the presence of highly concentrated 7keto-lithocholic acid (**8**) and the complete conversion to ursodeoxycholic acid (**4**) was observed without the formation of supramolecular assemblies. Remarkably the space-time yield of the process was as high as 480 g L^{-1} d^{-1}.

4. Novel HSDHs from (Meta)genomes Mining

In the last five years the number of HSDHs available has increased significantly. Concerning 7α- and 7β-HSDHs, very recently a number of new enzymes have been found, cloned and overexpressed in *E. coli* by Arends and co-workers (NAD(H)-dependent 7α- and 7β-HSDH),[54] Chen et al. (seven NAD(H)-dependent 7α-HSDHs and three NADP(H)-dependent 7β-HSDHs),[66] and by our group (three NAD(H)-dependent 7α-HSDHs, four NAD(H)-dependent 7β-HSDHs).[67] Among these HSDHs, it is worth mentioning the 7β-HSDH discovered in *Lactobacillus spicheri* (Ls7β-HSDH) by Arends et al.,[54] which is the first described 7β-HSDH dependent on the NAD(H) cofactor and the 7α-HSDH from *Brevundimonas* sp. identified by Chen et al. which showed very high activity toward chenodeoxycholic acid (**3**).[66] Among the HSDHs found by our group by mining the NCBI database and our in house database of metagenomic sequences from hot environments it is worth mentioning: the 7α-HSDH from *Deinococcus marmoris* (Dm7α-HSDH), showing high specific activity toward cholic acid (56 U mg^{-1}) and a peculiar thermophilicity, being active even at 80 °C; the 7β-HSDH from *Brucella* sp. (Bsp7β-HSDH) which displayed very high specific activity toward **4** (92 U mg^{-1}), as well as excellent expression yields (91 mg L^{-1}); the 7β-HSDH from the halophilic microorganism *Halomonas halodenitrificans* (Hh7β-HSDH), which showed an enzymatic activity influenced by the presence of salts with an increase of its specific activity of about 100 fold after incubation in presence of 1 M NaCl.[67] Interestingly, all the novel 7β-HSDHs discovered by our group, i.e., Sc7β-HSDH, Hh7β-HSDH, Bsp7β-HSDH, and Rs7β-HSDH, are phylogenetically distant from previously characterized 7β-HSDHs, including the NAD(H)-dependent Ls7β-HSDH (Figure 2), and were just annotated as generic SDRs in Genbank, thus hampering their easy identification by database search.

In recent years, the first 3D structures of an NADP(H)-dependent 7α-HSDH, i.e., Ca7α-HSDH (PDB: 5EPO),[68] and of a 7β-HSDH, i.e., Cae7β-HSDH (PDB: 5FYD; 5GT9),[69,70] have been disclosed. While Ca7α-HSDH resulted to be a homotetrameric enzyme, similar to the previously crystallized Ec7α-HSDH, Cae7β-HSDH turned out to be homodimeric. Both of these enzymes showed an overall three-dimensional structure of their monomeric units similar to the one of Ec7α-HSDH monomers, but with some differences in the C-terminal side. In particular, the C-terminus of Ca7α-HSDH includes an additional α-helix and it does not surround the active site, as it does in Ec7α-HSDH. Similarly, the C-terminal portion of Cae7β-HSDH resulted to be longer than the one of Ec7α-HSDH, including two α-helices linked by a loop. As a consequence, the structure of this C-terminal domain drastically changes the substrate-binding site, thus providing a possible explanation for the different stereopreference displayed by 7α- and 7β-HSDHs.

Concerning other HSDHs, only the NADP(H)-dependent 12α-HSDH from *Clostridium* group P strain C 48–50 (Csp12α-HSDH)[49] and few 3α-HSDHs resulted to have been cloned and overexpressed in *E. coli* before 2015.[48,71] A significant boost came by Devlin and Fischbach[72] and by Ridlon's group,[73,74] who found several new 12α-, 3α- and 3β-HSDHs by investigating the biosynthetic pathways that lead to the transformation

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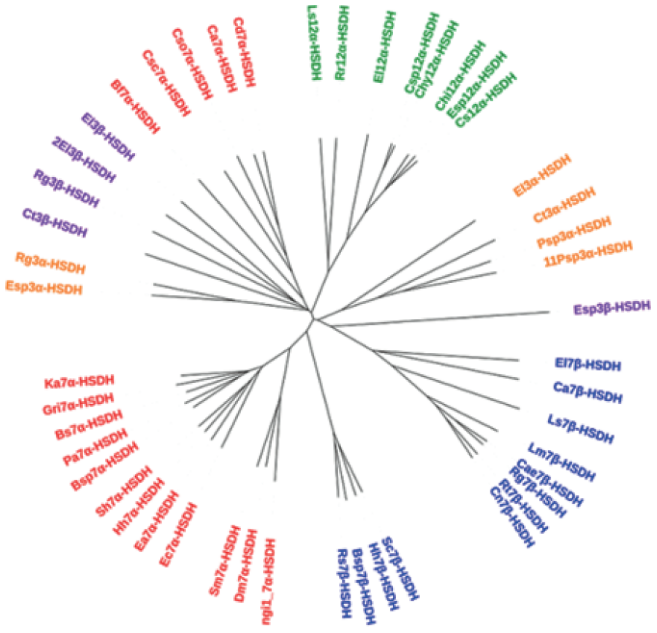


Figure 2. Unrooted phylogenetic tree of cloned and functionaly characterized HSDHs. 7α-HSDHs are indicated in red, 7β-HSDHs in blue, 12α-HSDHs in green, 3α-HSDHs in orange, and 3β-HSDHs in purple. The tree was generated with Clustal Omega[78] and visualized with iTOL.[79] Enzyme sources: Ec7α-HSDH: *Escherichia coli*;[45] Bf7α-HSDH: *Bacteroides fragilis*;[80] Ea7α-HSDH: *Erythrobacter atlanticus*;[66] Sm7α-HSDH: *Stenotrophomonas maltophilia*;[54] Sh7α-HSDH: *Shewanella* sp.;[66] Bsp7α-HSDH: *Bosea* sp.;[66] Bs7α-HSDH: *Brevundimonas* sp.;[66] Gri7α-HSDH: *Grimontia* sp.;[66] Ka7α-HSDH: *Kaistia adipata*;[66] Pa7α-HSDH: *Pseudoruegeria aquimaris*;[66] Dm7α-HSDH: *Deinococcus marmoris*;[67] Hh7α-HSDH: *Halomonas halodenitrificans*;[67] Ngi1_7α-HSDH: metagenome sample;[67] Cso7α-HSDH: *Clostridium sordellii*;[81] Csc7α-HSDH: *Clostridium scindens*;[46] Ca7α-HSDH: *Clostridium absonum*;[52] Cd7α-HSDH: *Clostridium difficile*;[82] Ca7β-HSDH: *Clostridium absonum*;[52] Cae7β-HSDH: *Collinsella aerofaciens*;[50] Rg7β-HSDH: *Ruminococcus gnavus*;[53] Rt7β-HSDH: *Ruminococcus torques*;[83] Cn7β-HSDH: *Clostridium nigeriense*;[66] Lm7β-HSDH: *Libanicoccus massiliensis*;[66] El7β-HSDH: *Eubacterium limosum*;[66] Ls7β-HSDH: *Lactobacillus spicheri*;[54] Sc7β-HSDH: *Stanieria cyanosphaera*;[67] Hh7β-HSDH: *Halomonas halodenitrificans*;[67] Bsp7β-HSDH: *Brucella* sp.;[67] Rs7β-HSDH: *Rhodobacter sphaeroides*;[67] El12α-HSDH: *Eggerthella lenta*;[84] Ls12α-HSDH: *Lysinibacillus sphaericus*;[67] Esp12α-HSDH: *Eggerthella* sp.;[73] Csp12α-HSDH: *Clostridium* sp.;[85] Rr12α-HSDH: *Rhodococcus ruber*;[86] Chi12α-HSDH: *C. hiranonis*;[74] Chy12α-HSDH: *C. hylemonae*;[74] Cs12α-HSDH: *C. scindens*;[74] Esp3α-HSDH: *Eggerthella* sp.;[73] El3α-HSDH: *Eggerthella lenta*;[72] Rg3α-HSDH: *Eggerthella lenta*;[72] Ct3α-HSDH: *Comamonas testosteroni*;[87] Psp3α-HSDH: *Pseudomonas* sp.;[71] 11Psp3α-HSDH: *Pseudomonas* sp.;[71] Esp3β-HSDH: *Eggerthella* sp. CAG:298;[73] El3β-HSDH: *Eggerthella lenta*;[72] 2El3β-HSDH: *Eggerthella lenta*;[72] Rg3β-HSDH: *Eggerthella lenta*;[72] Ct3β-HSDH: *Comamonas testosteroni*.[88]

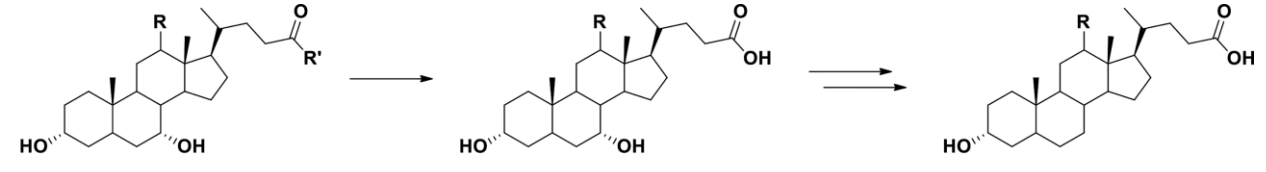
of primary bile acids to secondary bile acids in intestinal bacteria. (Scheme 5). Specifically, Devlin and Fischbach, looking for HSDHs involved in the isomerization of the 3α-OH group of deoxycholic acid (**18**), identified two 3α-HSDH and three 3β-HSDH genes in the genome of *Eggerthella lenta* and *Rhodococcus gnavus*.[72] In addition, Ridlon et al. found three NADP(H)-dependent 12α-HSDHs in the genome of three *Clostridia* (*C. scindens*, *C. hylemonae*, *C. hiranonis*)[74] that previously had been shown to be active in the modification of primary bile acids.[75–77] Moreover, a gene cluster encoding for a 12α-, a 3α- and a 3β-HSDH was identified in *Eggerthella* CAG:298.[73]

Notably, among the genes found by these authors, the 12α-HSDH from *E. lenta* (El12α-HSDHs)[89] has been later used by Tonin et al. for bile acid modification,[84] as described afterwards. Other new 12α-HSDHs have been found and employed for biocatalytic applications also by Shi et al.[86] and by our group (see below).[67] An overview of the phylogenetic relationships of the HSDHs that have been cloned, over-expressed in *E. coli* and functionaly characterized so far is given in Figure 2. As shown, most of the HSDHs are clustered based on their substrate and cofactor specificity, except for the 3α- and 3β-HSDHs which show an evident lack of similarity (for a nice discussion, see the paper from Devlin and Fischbach).[72] However, the prediction of the substrate scope of putative HSDHs from their sequences is still challenging.

5. Recent Examples of HSDHs Synthetic Exploitation (2015 – to Date)

The availability of novel and better performing enzymes has increased the interest in the synthetic exploitation of HSDHs. The main focus of the research performed in the recent years has been again the process optimization for the chemo-enzymatic synthesis of ursodeoxycholic acid (UDCA, **4**), specifically regarding two main crucial features of the preparation of **4**: the 12α-OH oxidation before the Wolff-Kishner reduction, and the 7α-OH epimerization to form the 7β-OH isomer. Subsequently, few meaningful examples of both the transformations are presented.

A very efficient approach to the oxidation of the 12α-OH of bile acid exploiting the new NAD(H)-dependent 12α-HSDH from *Rhodococcus ruber* (Rr12α-HSDH) has been recently reported by Shi and co-workers.[86] In their report, the oxidations



R: α–OH, R': $NH(CH_2)_2SO_3H$ (**1a**, TCA)
R: α–OH, R': $NHCH_2SO_3H$ (**1b,** GCA)
R: H, R': $NH(CH_2)_2SO_3H$ (**3a,** TCDCA)
R: H, R': $NHCH_2SO_3H$ (**3b,** GCDCA)

R: α–OH (**1**, CA)
R: H (**3**, CDCA)

R: α–OH (**18**, DCA)
R: H (**19**, LCA)

Scheme 5. Conversion of bile salts (taurocholic acid (**1a**), glycocholic acid (**1b**), taurochenodeoxycholic acid (**3a**), glycochenodeoxycholic acid (**3b**)) and primary bile acids (cholic acid (**1**), chenodeoxycholic acid (**3**)) into secondary bile acids (such as deoxycholic acid (DCA, **18**), lithocholic acid (LCA, **19**)) catalyzed by intestinal bacteria.

of the 12α-OH of several bile acids (i.e, **1**, **2**, **3**, **3a**, **3b**, **6** and **18**) were carried out on a preparative scale with substrate concentrations up to 200 mM, using a pyruvate/lactate dehydrogenase (LDH) cofactor regeneration system and lyophilized *E. coli* cells over-expressing Rr12α-HSDH and LDH, respectively. Remarkably, all the reactions reached >99 % conversions and in case of the oxidation of **1** to **7**, the space-time yield was significantly high (1632 g L^{-1} d^{-1}) and the product was successfully isolated in satisfactory yield (85 %).

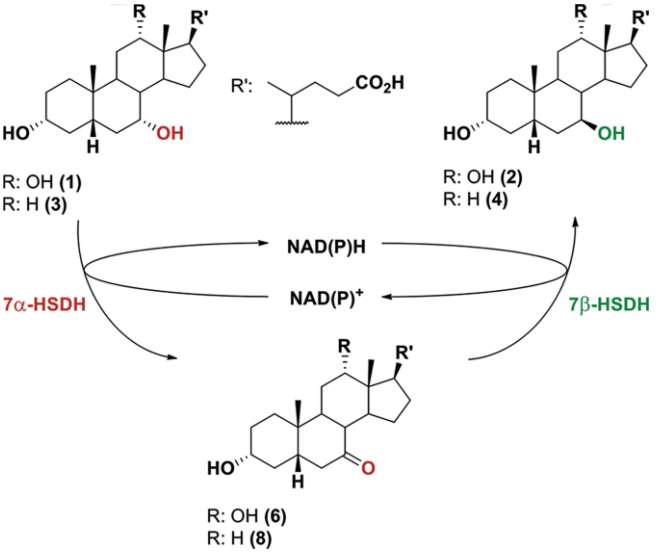
In another work, Arends and co-workers[84] employed either the purified NADP(H)-dependent Csp12α-HSDH or the NAD(H)-dependent El12α-HSDH to oxidize the same secondary 12α-OH bile acids using an alternative cofactor regeneration approach. Specifically, the cofactor $NAD(P)^+$ was regenerated using a NAD(P)H oxidase (NOX), an enzyme that reduces O_2 to H_2O while oxidizing NAD(P)H to $NAD(P)^+$. Unfortunately, complete conversions were obtained in 24–48 h only at a 10 mM concentration of **1** (4 g L^{-1}). As an increase of substrate concentration to 20 mM resulted in lower conversions, the authors tried to solve the problem by performing the reactions under O_2 pressure in an autoclave or by recirculating the substrate in an in-flow system, but the observed conversions were never quantitative. It seems that, to date, the NOX system coupled to HSDH oxidation (previously used also by Bakonyi et al. on analytical scale)[82] is not efficient enough when compared with other enzymatic approaches.

As far as the epimerization of 7α-OH to 7β-OH is concerned, various approaches have been investigated.

Recently, two different research groups carried out this epimerization on **1** or **3** in a cascade system using a combination of 7α-HSDH and 7β-HSDH showing the same cofactor specificity, either NADP(H)[83] or NAD(H)[54] (Scheme 6), exploiting the thermodynamics of the reactions which is favorable to the formation of the 7β-OH product. However, as it might be expected, these reactions did not proceed to completion, reaching conversions ranging from 80 %[83] to 92 %.[54] Similar conversions (81–87 %) to **4** were obtained by Pedrini and co-workers,[90] who investigated the use of cell extracts of *Stenotrophomonas* (formerly *Xanthomonas*) *maltophilia* containing NAD(H)-dependent 7α-HSDH and 7β-HSDH.[90] These numbers are not adequate for the development of a possible industrial process, as several product crystallization steps would be required to separate the product from the significant amounts of reaction intermediates.

It is worth noting that in one of these works the degree of conversion was improved by adopting a two-step reaction strategy, that is the coupling of the oxidation and the reduction reactions to suitable cofactor regeneration systems and heat-inactivating the enzymes involved in the oxidation step just before adding to the reaction mixture the enzymes involved in the reductive step.[83]

In a subsequent report, Zheng proposed to perform the epimerization of **3** in a flow system involving two modular column reactors.[91] In a first step, the 7α-OH group of **3** was oxidized by Ec7α-HSDH immobilized on an epoxy resin (first column reactor); subsequently, the 7-keto intermediate was reduced to give the desired product by the *Ruminococcus torques* 7β-HSDH



Scheme 6. Conversions of (**1**) into ursocholic acid (**2**), or (**3**) into (**4**), in a one-pot system.

immobilized on the same resin (second column reactor). The cofactors (NAD^+ and NADPH) were individually regenerated in each column by the respective co-immobilized enzymes, i.e., LDH and GDH. A quantitative production of **4** was obtained and remained stable for 12 h at a continuous substrate flow of 0.5 mL min^{-1} (space-time yield: 88.5 g L^{-1} d^{-1}). However, after 12 h, a drop of the conversions was observed, probably due to the insufficient stability of the immobilized enzymes.

Chen and co-workers also proposed a process for the one-pot epimerization of 7α-OH,[66] whose peculiarity was in the cofactor regeneration system coupled to the 7α-HSDH-catalyzed oxidation of **3** (Scheme 7). A flavin reductase mediated the re-oxidation of NADH to NAD^+ thanks to a catalytic amount of FMN (< 0.1 equiv.), that could auto-reoxidize in an aerobic atmosphere, in this way allowing the catalytic cycle to be pushed towards the formation of the 7-ketolithocolic acid intermediate (**8**). The reduction was then accomplished by a 7β-HSDH coupled with the NADP(H)-dependent ADH from *T. brockii* and in the presence of an excess of *i*PrOH. To demonstrate the efficiency of the proposed approaches for cofactor regeneration, initially, the authors carried out the two reactions in sequential steps obtaining high space-time yields. Subsequently, complete conversions were achieved either in a sequential one-pot mode (30 mM **3**, 92 % isolated yield on 50 mL scale) or in a concurrent one-pot mode (12.5 mM **3**, 78 % isolated yield) using *E. coli* recombinant cells co-expressing the oxidizing and the reducing apparatus. Interestingly, employing such *E. coli* cells the authors demonstrated that when the oxidative and the reductive step were performed separately in the presence of 10 mM substrate, the reactions reached completion without adding external cofactors, thus implying that endogenous NAD(P)H in the host cells was sufficient to get product formation.

In the same year, Xu et al. used *E. coli* cells harboring 7α-HSDH and 7β-HSDH to perform the epimerization of the 7α-OH of **3a** in a stirred tank fermenter on preparative scale.[92] The

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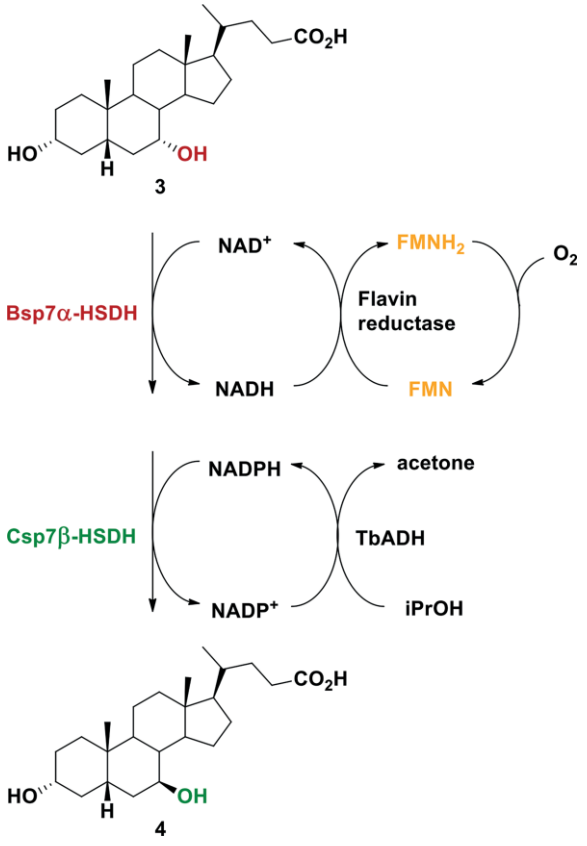

Scheme 7. Conversion of **3** into **4** in a one-pot system comprising HSDHs, a flavin reductase and an alcohol dehydrogenase.

approach was aimed to the valorization of waste, as inexpensive raw or refined chicken bile was employed as starting material for the biotransformation. The authors optimized the process by tuning the medium composition, analyzing the effect of dissolved oxygen on production, investigating the conditions for IPTG induction and for product recovery. The process was quite successful, as from 30 g of refined chicken bile or from 35 g of raw chicken bile, a dry powder product (29 g or 30 g, respectively), containing 35 % or 27 % of the 7β-OH epimer of **3a**, were obtained in a ratio with **3a** similar to that present into the medicinal material drainage bear bile powder.

All the practical examples reported above have been carried out using wild-type HSDHs. Even if some of them are really impressive, the main obstacles that hamper their industrial application are related to the enzymes' stability under process conditions and to substrate/product inhibition. Protein engineering can help to solve these problems. For example, Zheng et al. improved the activity, the half-life and the pH optimum of the *Ruminococcus torques* 7β-HSDH by combining different techniques such as error-prone PCR, DNA shuffling and site directed mutagenesis.[93] Specifically, the resulting best mutant (T189V/V207M) showed a 5.5 fold improvement in specific activity, a shift in the pH optimum toward weakly alkaline (more suitable for the solubilization of bile acids) and a 3-fold longer half-life at 40 °C. The authors employed the best mutant in a two-step cascade reaction for the synthesis of **4** starting from **3**. The space-time yield of the cascade process was 942 g L^{-1} d^{-1}, 7-fold higher than the value previously obtained with the wild-type enzymes.

In another work, Huang and co-workers obtained, by directed evolution, a double-mutant Ca7α-HSDH (Q255L/C260S) showing a 10 fold higher catalytic activity toward **3** and enhanced substrate tolerance.[94] The efficiency of this enzyme variant was demonstrated in the oxidation of the 7α-OH of a 100 mM solution of **3** by Q255L/C260S Ca7α-HSDH, which resulted in 99 % conversions in only 2 h, while only 85 % conversion in 16 h was achieved with the wild type enzyme.

Finally, it is worth noting that the HSDHs considered in the present review showed not only the ability to catalyze bile acids oxidoreductions, but they also had a certain degree of substrate promiscuity, meaning that they work also on molecules which are structurally distant from their natural substrates. Few examples in the literature are reported where HSDHs reduce bile acid derivatives,[95] as well as non-steroid carbonyl compounds.[96–100] Interestingly, in 2006 Zhu et al. found that the 7α-HSDH from *B. fragilis* was active towards some α-keto esters.[101]

Based on these reports, our group recently screened the activity of a large panel of 7α-, 7β- and 12α-HSDH toward different α-keto esters and bicyclic ketones.[67] Remarkably, all the tested enzymes showed to be capable of catalyzing the reduction of the selected α-keto esters with conversions up to >99 % and good-to-excellent ee for the (*R*)-enantiomer, while they demonstrated variable activity and selectivity toward more complex ketones.

As it has been shown in this minireview, hydroxysteroid dehydrogenases are a group of enzymes that have been and still are an interesting target for microbiologists, biochemists, molecular biologist, and organic chemists. More and more new HSDHs are becoming available and the research activity in this area is far from being completed. From our side, the identification of new HSDHs acting on hydroxyl groups located in different positions of the steroidic skeleton and the rationalization of the substrate promiscuity shown by these enzymes are two of the topics to which we will pay attention to shortly.

Keywords: Bile acids · Biocatalysis · Enzyme catalysis · Hydroxysteroid dehydrogenases

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Hydroxysteroid Dehydrogenases

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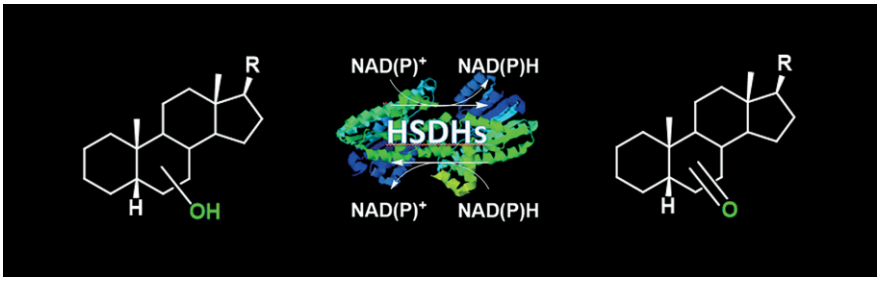
Hydroxysteroid Dehydrogenases: An Ongoing Story



This minireview provides an overview of the research progress in the discovery and synthetic applications of hydroxysteroid dehydrogenases (HSDHs), enzymes showing an exquisite regio- and stereoselectivity, as well as an interesting potential for the development of industrial biocatalyzed processes.

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Insights into the Substrate Promiscuity of Novel Hydroxysteroid Dehydrogenases

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Abstract: Hydroxysteroid dehydrogenases (HSDHs) are valuable biocatalysts for the regio- and stereoselective modification of steroids, bile acids and other steroid derivatives. In this work, we investigated the substrate promiscuity of this highly selective class of enzymes. In order to reach this goal, a preliminary search of HSDH homologues in in-house or public available (meta)genomes was carried out. Eight novel NAD(H)-dependent HSDHs, showing either 7α-, 7β-, or 12α-HSDH activity, and including, for the first time, enzymes from extremophilic microorganisms, were identified, recombinantly produced, and characterized. Among the novel HSDHs, four highly active (up to 92 U mg^{-1}) NAD(H)-dependent 7β-HSDHs showing negligible similarity towards previously described 7β-HSDHs, were discovered.
These enzymes, along with previously characterized HSDHs, were tested as biocatalysts for the stereoselective reduction of a panel of substrates including two α-ketoesters of pharmaceutical interest and selected ketones that partially resemble the structural features of steroids. All the reactions were coupled with a suitable cofactor regeneration system. Regarding the α-ketoesters, nearly all of the tested HSDHs showed a good activity toward the selected substrates, yielding the reduced α-hydroxyester with up to 99% conversions and enantiomeric excesses. On the other hand, only the 7β-HSDHs from *Collinsella aerofaciens* and *Clostridium absonum* showed appreciable activity toward more complex ketones, i.e., (±)-trans-1-decalone, but with interesting as well as different selectivity.

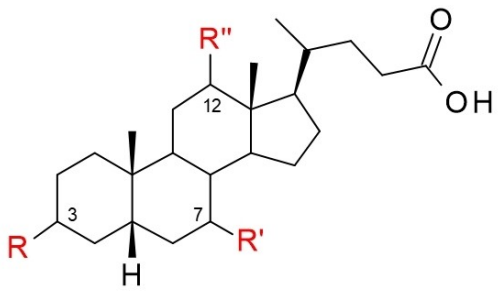
Keywords: Hydroxysteroid dehydrogenase; Bile acids; Substrate promiscuity; Enzyme discovery; Stereoselectivity

Introduction

Hydroxysteroid dehydrogenases (HSDHs) are a group of NAD(P)(H)-dependent oxidoreductases that are characterized by their ability to catalyze the oxidation/reduction of the hydroxyl/keto groups of bile acids and steroids.[1–3] Most of the HSDHs described so far show an exceptionally high selectivity that enables their application in the straightforward modification of their natural complex substrates, which is instead quite challenging by conventional chemical synthesis.

Specifically, HSDHs have been shown to oxidize the hydroxyl groups at different positions, e.g., at C-3, C-7, and C-12 of bile acids in a very high regiospecific manner (Scheme 1). Moreover, for each one of these positions, HSDHs usually show a practically absolute stereoselectivity by oxidizing only either the hydroxyl group above (β configuration) or below (α configuration) the plane of the steroid molecule.[4,5] The reactions catalyzed by HSDHs are reversible, thus these enzymes can be applied in the regioselective reduction of the corresponding keto derivatives as well.[4,6,7]

Thanks to these interesting features, HSDHs have been widely studied during the last years for their exploitation in the biocatalyzed synthesis of key intermediates of the drug ursodeoxycholic acid (UDCA), a largely applied therapeutic agent used for the dissolution of cholesterol gallstones and for the treatment of different hepatic diseases. These efforts



Cholic acid	R = R' = R'' = α-OH
Chenodeoxycholic acid	R = R' = α-OH; R'' = H
Deoxycholic acid	R = R'' = α-OH; R' = H
Ursocholic acid	R = R'' = α-OH; R' = β-OH
Ursodeoxycholic acid	R = α-OH; R' = β-OH; R'' = H

Scheme 1. Chemical structure of the most common bile acids, natural substrates of hydroxysteroid dehydrogenases.

have led to the characterization of different enzymes showing either 7α-, 7β- or 12α-HSDH activity,[2,3] as well as to the development of biocatalyzed processes for the preparation of UDCA intermediates.[8–11]

The ability of enzymes to act on substrates possessing chemical structures significantly different from their natural ones is a quite common feature.[12–14] This "substrate promiscuity" was also observed with HSDHs a long time ago by Davies and coworkers who reported the activity of a 3α,20β-HSDH from *Mortierella ramanniana* toward 3-hydroxybicyclo-heptan-6-ones.[15]

However, to date only few additional examples of HSDH promiscuous activities towards non-steroidal substrates have been described. For example, *Comamonas testosteroni* 3α-HSDH[16] and 11β-HSDH[17] were found able to reduce *p*-nitrobenzaldehyde, *p*-nitroacetophenone and metyrapone, while 17β-HSDH from the filamentous fungus *Cochliobolus lunatus*[18] was active toward quinones, menadione, *p*-nitrobenzaldehyde. Moreover, 3β,17β-HSDH from *Comamonas testosteroni* and 3α,20β-HSDH from *Streptomyces hydrogenans* showed activity toward muscarine precursors.[19] More recently, the catalytic promiscuous property of the 7α-HSDH from *Bacteroides fragilis* has been investigated by studying the enzyme substrate specificity and stereoselectivity in the reduction of different aromatic and aliphatic α-ketoesters to the corresponding α-hydroxyesters.[20] To the best of our knowledge, only these six papers have been published so far, describing, as just summarized, the results obtained with unrelated HSDHs (3α,20β-HSDH, 3α-HSDH, 11β-HSDH, 17β-HSDH, 3β,17β-HSDH, 3α,20β-HSDH and 7α-HSDH) on very different substrates (3-hydroxybicyclo-heptan-6-ones, metyrapone, *p*-nitroacetophenone, *p*-nitrobenzaldehyde, quinones, menadione, muscarine precursors, aromatic and aliphatic α-ketoesters).

In this work, we aimed at filling this gap by performing a more systematic investigation on HSDHs substrate promiscuity. This result was achieved by i) increasing the diversity of the available HSDHs by discovering novel enzyme homologues, ii) establishing their regio- and stereoselectivity in the respect of their natural substrates, i.e., bile acids, and iii) evaluating their possible substrate promiscuity toward a set of different non-steroidal substrates.

Results and Discussion

Discovery of Novel HSDHs

To the best of our knowledge, all the HSDHs characterized so far belong to the "short-chain dehydrogenases/reductases" (SDR) superfamily, a very large and highly divergent protein family with generally low sequence identity (20–30%), thus making quite challenging their functional annotation if a biochemical characterization is not performed.[21] On the other side, most of the known HSDHs are produced by gut or soil bacteria belonging to the phyla of Proteobacteria, e.g., *Escherichia*[22] and *Stenotrophomonas*[5] strains, Actinobacteria, e.g., *Collinsella*[23] and *Eggerthella*[8,24,25] strains, and Firmicutes, e.g., *Clostridium*,[26–30] *Ruminococcus*,[31,32] and *Lactobacillus*[5] strains, thus resulting in an overall limited diversity.

To improve the diversity of the HSDHs library already available in our lab, a bioinformatic screening was performed in search of HSDH homologues in in-house or public available (meta)genomes, with a special focus for those from extreme environments. Indeed, no HSDHs have been characterized so far from extremophilic microorganisms, e.g., thermophiles and halophiles. However, enzymes from these sources may have practical interest exceeding the scope of the present work, since, thanks to their higher robustness, they could be more suitable than mesophilic enzymes to industrial applications.[33–36]

The search of novel HSDH sequences was carried out by using either the Blastp tool (https://blast.ncbi.nlm.nih.gov/) for mining into the NCBI database (https://www.ncbi.nlm.nih.gov/) or the program LAST (http://last.cbrc.jp/) for the analysis of metagenomes of samples collected in hot terrestrial environments.[33,37,38] In both cases, only sequences of functionally characterized HSDHs (Table S1 in the Supporting Information) were used as queries during the multiple sequence alignments.

At the end of these analyses, ten SDR sequences were selected for cloning and characterization (Table 1).

Table 1. Expression yields and activity of the SDRs selected in this work.

Entry	Enzyme	Source	Identity with known HSDHs[a] (%)	HSDH activity[b]	Cofactor	Yield (U)[b,c]	Yield (mg)[c]	Specific activity ($U\,mg^{-1}$)
1	Dm7α-HSDH	*Deinococcus marmoris*	75.40 (Sm7α-HSDH)	7α-HSDH	NAD(H)	788	14	56
2	Hh7α-HSDH	*Halomonas halodenitrificans*	74.51 (Sh7α-HSDH)	7α-HSDH	NAD(H)	1986	64	31
3	Hh7β-HSDH	*Halomonas halodenitrificans*	38.89 (Bs7α-HSDH)	7β-HSDH	NAD(H)	955	95	10
4	Sc7β-HSDH	*Stanieria cyanosphaera*	38.40 (Gri7α-HSDH)	7β-HSDH	NAD(H)	261	7	38
5	Bsp7β-HSDH	*Brucella* sp.	36.00 (Pa7α-HSDH)	7β-HSDH	NAD(H)	8400	91	92
6	Rs7β-HSDH	*Rhodobacter sphaeroides*	39.68 (Bs7α-HSDH)	7β-HSDH	NAD(H)	2348	80	29
7	Ngi1_7α-HSDH	metagenome	69.05 (Sm7α-HSDH)	7α-HSDH	NAD(H)	24780	174	142
8	Is2-SDR	metagenome	38.49 (Bsp7α-HSDH)	n.a.[d]	NADP(H)	–	107	–
9	Ngi7-SDR	metagenome	35.50 (Csp12α-HSDH)	n.a.[d]	NAD(H)	–	326	–
10	Ls12α-HSDH	*Lysinibacillus sphaericus*	47.39 (El12α-HSDH)	12α-HSDH	NAD(H)	348	135	2.6

[a] For details about known HSDHs (source and Genbank accession numbers), see Table S1 in the Supporting Information.
[b] Activity assays were performed in the presence of NAD^+ as cofactor and respective substrates: 7α-HSDH, cholic acid; 12α-HSDH, deoxycholic acid; 7β-HSDH, ursodeoxycholic acid (see Experimental section for details).
[c] Yields refer to 1 L-scale enzyme production under optimized conditions (see Experimental section for details).
[d] n.a.: not active towards any of the tested bile acids (see Experimental section for details).

Two sequences (Dm7α-HSDH and Hh7α-HSDH, entries 1–2 in Table 1) showing about 75% similarity with known 7α-HSDHs (see also Figure S1 in the Supporting Information) were discovered for the first time in genomes from extremophilic bacteria. Dm7α-HSDH was annotated as a putative glucose 1-dehydrogenase in the genome of *Deinococcus marmoris* strain PAMC 26562, a radiation-resistant and psychro- and draught-tolerant bacterium isolated from an Antarctic rock sample.[39,40] Instead, Hh7α-HSDH was already annotated as a 7α-HSDH in the genome of the halophilic bacterium *Halomonas halodenitrificans*, but not yet characterized.

Since genes coding for HSDHs are often clustered in bacterial genomes, e.g., the gene coding for *Clostridium absonum* 7β-HSDH was found upstream that coding for a co-expressed 7α-HSDH,[29] the genome regions contiguous to Dm7α-HSDH and Hh7α-HSDH genes were checked. No putative dehydrogenase was found in proximity of the gene coding for Dm7α-HSDH.

Instead, a gene annotated as a SDR family oxidoreductase (Genbank: WP_027961749.1) was found upstream the Hh7α-HSDH gene. Interestingly, this sequence showed only a modest similarity to 7α-HSDHs (Table 1, entry 3, 28–38% identity at the deduced amino acid level), and no significative similarity to other HSDSs, including known 7β-HSDHs.

Even more curiously, when searching for homologues of this new sequence into the NCBI database, we found several uncharacterized SDRs from diverse sources with up to 79% identity. Three SDRs, i.e., one from plasmid material of the photosynthetic cyanobacterium *Stanieria cyanosphaera* PCC 7437 (Genbank: WP_015212061.1), one from a *Brucella* strain (Genbank: WP_004684107.1), and one from a *Rhodobacter sphaeroides* strain (Genbank: WP_011911126.1) were then selected for further studies (Table 1, entries 4–6, see also Figure S2 in the Supporting Information).

As far as the metagenomes mining concerns, three sequences were found, two of them from the metagenomes of samples collected at drilling wells in the Norwegian continental shelf (Ngi1_7α-HSDH and Ngi7-SDR, entries 7 and 9, Table 1) and one from the metagenome obtained from Icelandic hot spring sediments (Is2-SDR, entry 8, Table 1).

Ngi1_7α-HSDH (entry 7) showed about 70% similarity with known 7α-HSDHs. By performing a Blast analysis in the NCBI database, a putative 7α-HSDH

with 99.7% identity in the respect of Ngi1_7α-HSDH was found in the genome of a *Psychrobacter* sp. strain (Genbank: HAR74729.1).

It was more difficult to make hypotheses about the possible catalytic activity of the other two selected metagenomic sequences. In fact, Is2-SDR showed a quite low similarity with respect to both known HSDHs and sequences in the NCBI database [38% and 53% identity with the 7α-HSDH from *Bosea* sp. and with a putative D-threitol dehydrogenase from an *Aerophobetes* strain (Genbank: TKJ47585.1), respectively]. In the case of Ngi7-SDR, it showed a 35% identity with the 12α-HSDH from *Clostridium* sp., and was identical at the amino acidic level to an uncharacterized SDR family oxidoreductase from a *Pseudomonas pelagia* strain (Genbank: QFY56536).

Finally, a sequence from *Lysinibacillus* (formerly *Bacillus*) *sphaericus* showing about 47% identity with known 12α-HSDH was discovered and included in further studies (Ls12α-HSDH, entry 10, Table 1). In fact, from the same source, a commercially available NAD(H)-dependent 12α-HSDH (in the past from Genzyme Biochemicals Ltd., today from Creative Enzymes®) was widely used in previous studies,[11,41] but never cloned and produced in recombinant form.

Recombinant Production and Characterization of Novel HSDHs

The codon-optimized synthetic genes coding for the selected putative HSDHs were cloned into the pETite expression vector (Lucigen) in frame with a C-term His-Tag sequence. Protein over-expression in the corresponding recombinant *Escherichia coli* BL21 (DE3) strains was achieved by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) (see Experimental section for details).

As shown in Table 1, all the target enzymes could be produced in recombinant form in *E. coli* and homogeneous protein samples were obtained from the cell extracts with up to >300 mg L^{-1} yields by nickel-nitriloacetic acid (Ni-NTA) chromatography, as confirmed by SDS-PAGE analysis (Figure S3, Supporting Information).

The assessment of the catalytic activity of these novel SDRs toward different bile acids (Scheme 1) was first carried out by spectrophometric assays in oxido-reduction reactions in the presence of NAD(P)(H) cofactors. Subsequently, the respective HSDH activity was confirmed by setting up small-scale oxido-reduction reactions with suitable bile acids (see Experimental section, Figure S4 and Scheme S1 in the Supporting Information for details).

As expected from the previously reported sequence analysis, Dm7α-HSDH (Table 1, entry 1), Hh7α-HSDH (entry 2), and Ngi1_7α-HSDH (entry 7) showed NAD(H)-dependent 7α-HSDH activity, thus demonstrating for the first time the occurrence of HSDH activities in extremophilic microorganisms.

Interestingly, Dm7α-HSDH (entry 1) showed a thermophilic behavior by displaying an optimum temperature at 60 °C in the regioselective oxidation of cholic acid (see Figure S5 in the Supporting Information), which is, to our knowledge, the highest value reported so far for HSDHs. It is also noteworthy the broad temperature range at which this enzyme showed activity, since about 15% of the maximal activity recorded at 60 °C was retained at both 20 °C and 80 °C. The finding of a thermostable enzyme in a cold adapted microorganism looks contradictory at a first glance. However, despite their psychrophilic origin, the occurrence of thermophilic and thermostable enzymes in Antarctic microbes has been previously reported.[42–45] To a lower extent, also Ngi1_7α-HSDH (entry 7), whose origin, as previously mentioned, is likely close to psychrophilic bacteria, showed a moderate thermophilic behavior, with an optimum temperature at 40 °C (see Figure S5 in the Supporting Information). Remarkably, Dm7α-HSDH and Ngi1_7α-HSDH are phylogenetically related (Figure 1), and close also to the recently discovered 7α-HSDH from *Stenotrophomonas maltophilia* (Sm7α-HSDH), which showed a marked thermophilicity as well,[5] thus constituting a distinct clade from the other (mesophilic) 7α-HSDHs.

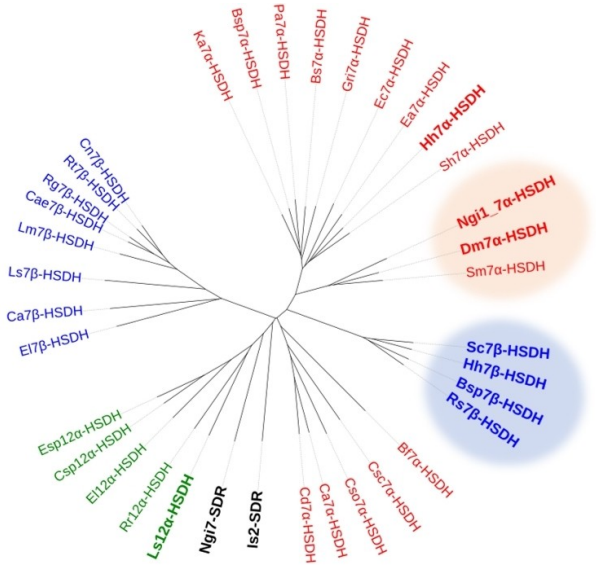


Figure 1. Unrooted phylogenetic tree of HSDHs. 7α-HSDHs are indicated in red, 7β-HSDHs in blue, 12α-HSDHs in green. Novel enzymes discovered in this work are in bold. Thermophilic 7α-HSDHs and the novel NAD(H)-dependent 7β-HSDHs are in light red and light blue background, respectively. The tree was generated with Clustal Omega[46] and visualized with iTOL.[47]

The third novel 7α-HSDH, Hh7α-HSDH (entry 2), showed, in agreement with the phylogenetic analysis (Figure 1), a mesophilic behavior (optimal temperature at 30 °C). Moreover, consistently with its origin, it showed a slight halophilic character. In fact, an about 40% decrease of activity was observed when removing by dialysis the salts contained in the elution buffer of the Ni-NTA chromatography.

Coming to the next four sequences (entries 3–6, Table 1), these enzymes showed unequivocally NAD(H)-dependent 7β-HSDH activity and were consequently named Hh7β-HSDH, Sc7β-HSDH, Bsp7β-HSDH, and Rs7β-HSDH, respectively. As shown in Figure 1, these 7β-HSDHs constitute a separate clade from that including other known 7β-HSDHs, which are all NADP(H)-dependent enzymes with the only exclusion of the recently discovered NAD(H)-dependent 7β-HSDH from *Lactobacillus spicheri* (Ls-7β-HSDH).[5] Moreover, the novel 7β-HSDHs showed a significantly higher specific activity (up to 92 U mg^{-1}) than Ls-7β-HSDH (3.1 U mg^{-1}),[5] thus suggesting a highly interesting potential for further biocatalytic applications in bile acid modifications.

Subsequent characterization of Hh7β-HSDH (entry 3) showed that the presence of salts had a stronger influence on its activity than that observed with its clustered enzyme Hh7α-HSDH (entry 2). In fact, dialyzed samples of Hh7β-HSDH showed about 2 orders of magnitude lower specific activity (0.07–0.16 U mg^{-1}) than that observed after the affinity chromatography step (10 U mg^{-1}). Interestingly, Hh7β-HSDH fully recovered its starting activity by addition of NaCl (1 M final concentration) to the dialyzed solution.

Sc7β-HSDH (entry 4) showed a rather limited solubility during the recombinant production with a detrimental effect on the expression yields, even under optimized conditions (Table S3, Supporting Information). However, this enzyme is quite thermophilic, showing an optimum of temperature at around 50 °C (Figure S4, Supporting Information).

Excellent yields and specific activity were obtained instead with Bsp7β-HSDH (entry 5) and Rs7β-HSDH (entry 6), both enzymes showing a mesophilic character.

As far as the metagenomic sequences Is2-SDR and Ngi7-SDR concern, both proteins were obtained with very good yields in soluble form (entries 8 and 9, Table 1). However, neither of the two showed catalytic activity toward any of the tested bile acids (see Experimental section for details). Indeed, both enzymes demonstrated to be functional dehydrogenases in the presence of NADPH (Is2-SDR) or NADH (Ngi7-SDR) as cofactors and different ketone substrates (see in the following).

Finally, Ls12α-HSDH (entry 10, Table 1) was obtained with excellent yield from recombinant production and confirmed as a NAD(H)-dependent 12α-HSDH, thus included, together with the other seven novel HSDHs, in the following study of the substrate promiscuity of this group of enzymes.

Substrate Scope of HSDHs

The demonstration of the catalytic activity of the eight novel HSDHs in oxidoreduction reactions of different bile acids paves the way to their exploitation as biocatalysts for the stereo- and regioselective preparation of steroid derivatives. Our group is currently performing specific investigations on this topic and the results will be reported in due time.

Additionally, we considered of synthetic interest a more detailed evaluation of the substrate promiscuity of this group of enzymes. Accordingly, the newly discovered 7α-, 7β-, and 12α-HSDHs were tested, along with our in-house collection of previously characterized HSDHs (Table 2, for details see also Table S1, Supporting Information), in the stereoselective reduction of a panel of substrates (**1**–**5**) (Scheme 2).

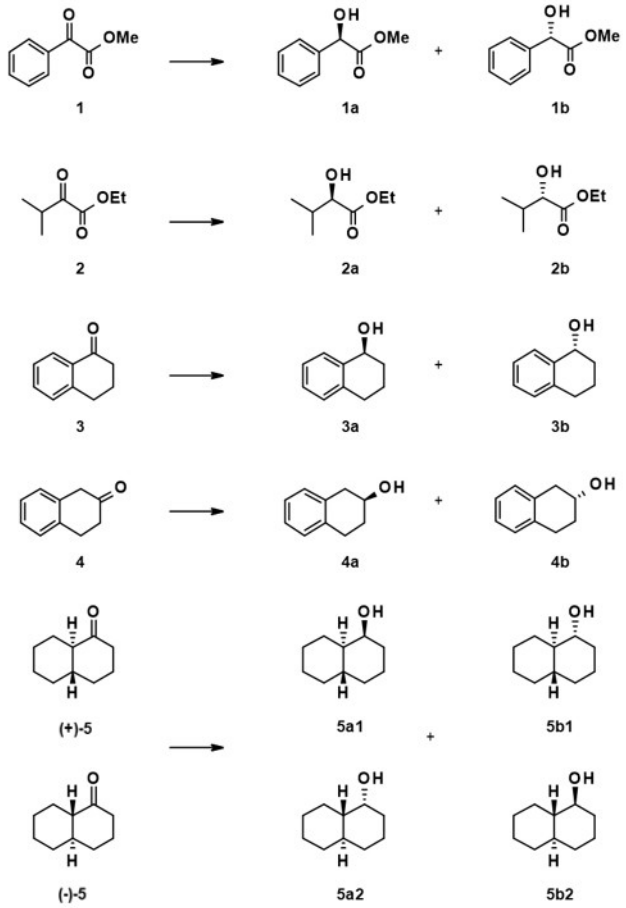
Specifically, the tested substrates included two α-ketoesters (**1**–**2**) of pharmaceutical interest,[48] chosen among the panel of ketoesters that, as previously discussed,[20] were selected by Zhu and co-workers to investigate the catalytic promiscuous properties of the

Table 2. Conversions and enantioselectivity of HSDH-catalyzed reduction of substrates **1** and **2**.

Entry	Enzyme[a]	Compound **1**		Compound **2**	
		c[b] (%)	ee$_R$[b] (%)	c[b] (%)	ee$_R$[b] (%)
1	Ec7α-HSDH	89.7	90.2	81.6	>99
2	Ca7α-HSDH	>99	88.3	42.3	91.1
3	Dm7α-HSDH	>99	82.9	>99	93.9
4	Hh7α-HSDH	95.9	97.9	81.0	>99
5	Ngi1_7αHSDH	26.1	>99	64.9	92.4
6	Ca7β-HSDH	>99	45.2	>99	89.3
7	Cae7β-HSDH	>99	23.3	>99	97.2
8	Sc7β-HSDH	61.7	92.5	92.0	94.6
9	Hh7β-HSDH	97.4	95.2	65.8	64.9
10	Bsp7β-HSDH	>99	89.6	>99	96.0
11	Rs7β-HSDH	75.4	>99	>99	95.5
12	Csp12α-HSDH	94.5	>99	93.9	85.7
13	Ls12α-HSDH	94.1	64.7	>99	93.6

[a] Sources of previously characterized HSDHs: Ec7α-HSDH (entry 1): *Escherichia coli*; Ca7α-HSDH (entry 2) and Ca7β-HSDH (entry 6): *Clostridium absonum*; Cae7β-HSDH (entry 7): *Collinsella aerofaciens*; Csp12α-HSDH (entry 12): *Clostridium* sp. (see Table S1, Supporting Information, for details).

[b] Conversions and ees were assigned via chiral HPLC for reduction of **1**, while chiral GC was used for reduction of **2** (see Experimental section for details).



Scheme 2. Substrates **1**–**5** and their possible reduction products.

B. fragilis 7α-HSDH, and three bicyclic ketones (**3**–**5**) partially resembling the structural features of steroids.

To allow the use of catalytic amounts of the cofactors and to provide the necessary driving force to product formation, the reactions were coupled with a suitable cofactor regeneration protocol exploiting a formate/formate dehydrogenase (FDH) system (see Experimental section and Scheme S2 in Supporting information).

Reaction conversions were estimated after 24–48 h by GC-MS (**1**, **3**–**5**) or by HPLC (**2**) analysis and enantiomeric excesses (ees) were evaluated by chiral GC (**1** and **5**) or chiral HPLC analysis (**2**).

For the sake of comparison, compounds **1**–**5** were also submitted to the action of the two new identified SDRs not showing HSDH activity (Is2-SDR and Ngi7-SDR, lines 8 and 9 of Table 1) and the results are reported in the Supplementary Materials (Table S4).

Regarding the α-ketoesters **1**–**2**, nearly all the tested HSDHs showed a good activity towards these substrates, yielding the corresponding reduced α-hydroxyesters, with high conversions and high enantiomeric excesses (Table 2).

Specifically, quantitative conversions of **1** were obtained with Ca7α-HSDH, Dm7α-HSDH, Ca7β-HSDH, Cae7β-HSDH and Bsp7β-HSDH (Table 2, entries 2, 3, 6, 7, and 10), while complete conversions of **2** were obtained with Dm7α-HSDH, Ca7β-HSDH, Cae7β-HSDH, Bsp7β-HSDH, Rs7β-HSDH and Ls12α-HSDH (Table 2, entries 3, 6, 7, 10, 11 and 13).

On the other hand, the most enantioselective HSDHs in the reduction of **1** were Ngi1_7α-HSDH, Rs7β-HSDH and Csp12α-HSDH (Table 2, entries 5, 11, and 12), while Ec7α-HSDH and Hh7α-HSDH (Table 2, entries 1 and 4) showed the best enantioselectivity in the reduction of **2**.

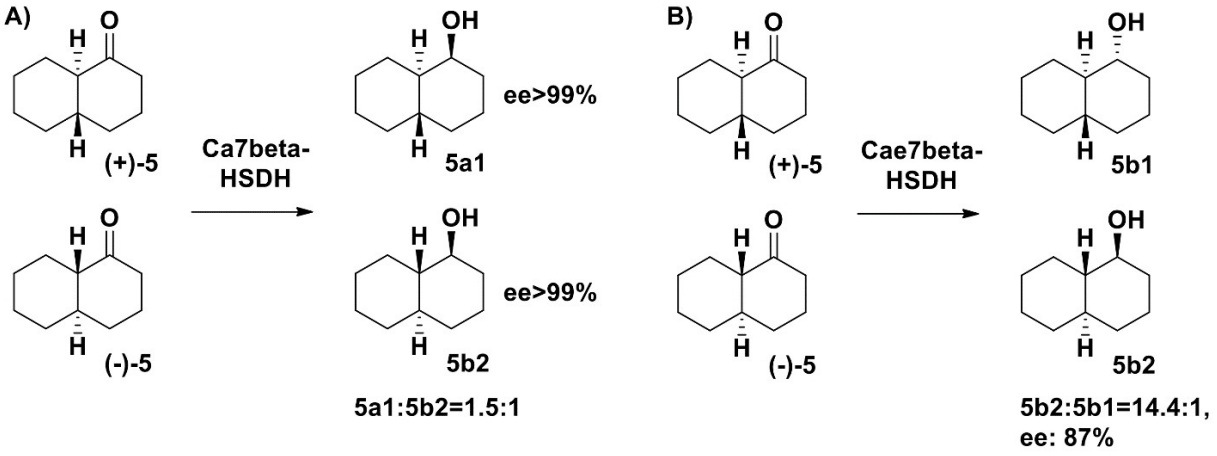
Interestingly, the stereoselectivity in the reduction of **1** was significantly lower than that observed with **2** when testing the NADP(H)-dependent Ca7β-HSDH and Cae7β-HSDH (entries 6 and 7, respectively), and, to a minor extent, the novel Ls12α-HSDH enzyme (entry 13). These results suggest that in these selected HSDHs the aromatic substituent in **1** may promote alternative substrate binding modes in either pro-*R* or pro-*S* fashion, whereas in the case of the aliphatic α-keto ester **2** only the pro-*R* binding mode is productive.

It is also worth of mentioning that reactions catalyzed by Hh7α-HSDH and Hh7β-HSDH (Table 2, entries 4 and 9) were carried out in parallel in the presence and in the absence of NaCl (0.4 M). As expected, conversions were very low in the absence of NaCl (< 5%, data not shown), while they were up to about 98% in the presence of salts, thus confirming the halophile behavior of these novel HSDHs.

The thermophilic Dm7α-HSDH (Table 2, entry 3) demonstrated to perform well also at sub-optimal temperatures, giving complete conversions of **1** and **2** at 25 °C.

The stereochemistry of the products obtained from the enzymatic reduction of **1** (the enantiomers **1a** or **1b**) was determined *via* analytical comparison with standards. All the HSDHs showed to be (*R*)-selective, compound **1a** being the preferred product. Instead, in order to assign the absolute configurations of the products obtained by the reduction of **2** (the enantiomers **2a** or **2b**), the reaction catalyzed by 7β-HSDH from *Collinsella aerofaciens* (Cae7β-HSDH, Table 2, entry 7), which combined quantitative conversion of **2** and high enantioselectivity, was scaled up (100 mg). Optical rotation measurements were carried out on the purified product (63% isolated yield), the resulting $[\alpha]_D^{22}$ value (−2.70°) being consistent with the value reported in literature for **2a**.[49] Thus, also in the case of the reduction of **2**, all the HSDHs demonstrated to be (*R*)-selective.

Concerning the reduction of the bicyclic substrates **3**–**5**, none of the tested enzymes showed activity towards the tetralones **3** and **4**, whereas only Ca7β-HSDH and Cae7β-HSDH were found able to catalyze the reduction of (±)-trans-1-decalone ((±)-**5**). These



Scheme 3. Enzymatic reductions of substrate (±)-**5** with A) Ca7β-HSDH and B) Cae7β-HSDH.

two enzymes, working under unoptimized reaction conditions, gave moderate conversions (18% and 35% after 48 h for Ca7β-HSDH and Cae7β-HSDH, respectively), but accompanied by interesting stereoselectivity (Scheme 3).

Considering that commercially available **5** is a racemate of the enantiomers (+)-**5** (4a*S*,8a*R*) and (−)-**5** (4a*R*,8a*S*), its chemical reduction can yield four different stereoisomers, i.e., **5a1** (1*S*,4a*S*,8a*R*), **5a2** (1*R*,4a*R*,8a*S*), **5b1** (1*R*,4a*S*,8a*R*), and **5b2** (1*S*,4a*R*,8a*S*) (Scheme 2). The four isomers were prepared by chemical reduction of **5** with $NaBH_4$, isolated and analyzed by NMR spectroscopy. In this way, it was possible to assign the relative stereochemistry of the diastereomers **5a** and **5b** (see Supporting Information for further details).Moreover, the four isomeric products (**5a1**, **5a2**, **5b1**, **5b2**) could be separated by chiral GC.

The same analysis of the samples obtained from the two enzymatic reductions indicated that Ca7β-HSDH catalyzed the formation of one enantiomer of each diastereomer (Scheme 3A, see also Scheme S3 in the Supporting Information for details). On the contrary, Cae7β-HSDH performed a kinetic resolution of the starting ketone. The unreacted substrate **5** showed an ee of 72%, whereas the product **5b** showed an ee of 87% (Scheme 3B). Therefore, Ca7β-HSDH and Cae7β-HSDH showed a different stereopreference for the enantiomers of (±)-**5**.

To assign the absolute configurations of the residual substrate and of the formed products, the reduction of (±)-**5** catalyzed by Cae7β-HSDH was scaled up to 100 mg.

The remaining substrate and the product (**5b**, 15% isolated yield; product **5a** was present in traces and therefore it was not isolated) were purified *via* flash chromatography, then submitted to NMR analysis in order to identify the relative stereochemistry and to optical rotation measurements to assess the absolute configurations (see Supporting Information for further details). The resulting $[\alpha]_D^{25}$ values (+44.45° and +2,75° for produced **5b** and residual **5**, respectively) were compared with the data reported in literature,[50] indicating that the residual ketone was enriched in the enantiomer (+)-**5** and the product was enriched in the enantiomer **5b** (1*S*,4a*R*,8a*S*). These data are summarized in Table 3.

Our previous investigations on the 3D structure of Cae7β-HSDH[51] showed that its substrate binding site, including a mobile substrate loop, is substantially different from that of other HSDHs, e.g., *E. coli* 7α-HSDH. Although the overall active site architecture results quite similar in this family of oxidoreductases, it is likely that substrate recognition and, consequently, stereoselectivity, are ruled by a network of very subtle

Table 3. Conversion and enantioselectivity of reduction of substrate (±)-**5** catalyzed by Cae7β-HSDH and Ca7β-HSDH.

Enzyme	c[a] (%)	ee[a] residual substrate (%)	ee[a] product **5b** (%)	product **5a** (%)
Cae7β-HSDH	35	72 (4a*S*,8a*R*)	87[b]	–
Ca7β-HSDH	18	9 (4a*R*,8a*S*)	>99[b]	>99[c]

[a] Conversions and ees were assigned via chiral GC (see Experimental section for details).
[b] Major product stereochemistry: (1*S*,4a*R*,8a*S*).
[c] Major product stereochemistry: (1*S*,4a*S*,8a*R*).

interactions between the substrate and the active site residues. Further investigations in this respect are currently under considerations in our lab.

Conclusion

As far as the mining of (meta)genomes for novel HSDHs is concerned, the results obtained in the first part of this work show that the occurrence of redox enzymes acting on steroids largely exceeds the range of microbes usually considered as possible HSDHs sources so far, i.e., gut and soil bacteria. In fact, functionally active HSDHs have been found for the first time in extremophilic bacteria as well as in photosynthetic cyanobacteria. This finding poses several interesting questions that were clearly out of the scope of this investigation, e.g., about the physiological role of these HSDHs in these microbes, as well as about how these enzymatic functions were acquired or evolved from common ancestral genes. However, since extremophilic enzymes are very often more robust than their mesophilic counterparts,[52] the discovery of these novel HSDHs could also be useful in the development of industrial biocatalytic processes for the stereo- and regioselective modification of steroid derivatives. We are currently working on this specific topic, as well as on the synthetic exploitation of the novel and highly active NAD(H)-dependent 7β-HSDHs for the preparation of bile acid derivatives.

Coming to the study of the activity of HSDHs toward non-steroidal substrates, we have confirmed that enzymes evolved to accommodate structurally complex and bulky substrates in their active sites, maintain their selectivity towards much simpler molecules, as observed with compounds **1**, **2** and **5**. These results corroborate the few scant previous literature reports, point out the importance of having in hands libraries of enzymes and encourage us to continue these studies in order both to acquire more information on the general substrate scope of HSDHs and to exploit molecular modeling approaches[53] to find suitable rationales for the observed selectivity.

Experimental Section

General

Methyl benzoylformate (purity 98%), ethyl 3-methyl-2-oxobutyrate (purity 97%), 1-tetralone (purity 97%), 2-tetralone (purity 98%) and (±)-trans-1-decalone (purity 98%) were purchased from Merck (Darmstadt, Germany) (catalog numbers M30507, 218456, T19003, T19208 and 156655, respectively) and used with no further purification. The analytical standards methyl D-mandelate (purity ≥99%, $[\alpha]_D^{20} = 144° \pm 2$, c = 2 in MeOH) and methyl L-mandelate (purity >99%) were from Fluka Chemika (catalog numbers 63456 and 63466, respectively). Formate dehydrogenase (FDH) from *Candida boidinii* and lactate dehydrogenase (LDH) from rabbit muscle were from Merck. The NADP(H) dependent FDH from methylotrophic bacterium *Pseudomonas* sp. 101 was a kind gift from Prof. Tishkov (M.V. Lomonosov Moscow State University). Unless otherwise stated, all other chemicals were of analytical grade and were purchased from Merck.

Reactions were monitored *via* TLC (thin-layer chromatography) on pre-coated silica gel 60 glass plates with fluorescent indicator UV_{254} and treated with an oxidizing solution [4-hydroxybenzaldehyde (6.3 g), H_2SO_4 (50% v/v in H_2O, 40 mL), MeOH (400 mL)]. The isolation of pure products was allowed via extraction with EtOAc and subsequent flash chromatography on silica gel 60 (70–320 mesh, Merck, eluent CH_2Cl_2).

Environmental sample collection, DNA extraction from samples, DNA sequencing, and generation of databases of metagenomic sequences were carried out as previously described.[54] The metagenomic sample "ngi" was collected in drilling wells in Svalbard island and DNA extraction and sequencing from this sample was performed as described previously for sample It3.[54]

Bacterial Strains

E. coli BL21(DE3) and *E. coli* HI-Control 10G were from Lucigen (Wisconsin, USA).

E. coli BL21(DE3)/pET24b-Ec7αHSDH producing the 7α-HSDH from *E. coli*, *E. coli* BL21(DE3)/pET24b-Cae7βHSDH producing the 7β-HSDH from *Collinsella aerofaciens*, *E. coli* BL21(DE3)/pETite-Ca7βHSDH producing the 7β-HSDH from *Clostridium absonum*, *E. coli* TOP10/pBAD-Ca7αHSDH producing the 7α-HSDH from *Clostridium absonum* were part of our in-house collection and were expressed as fusion proteins with a (6x)His-tag at the C-terminus.

Analytical Methods

At scheduled times, reaction samples (50 µl) were extracted with EtOAc and dried over Na_2SO_4 to obtain a 10 mM final concentration sample suitable to chiral GC (and/or GC-MS) analysis, or evaporated, resuspended in CH_3CN to 10 mM final concentration and analyzed by chiral RP-HPLC.

GC-MS analyses were performed using an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 µm) on a Finnigan TRACE DSQ GC/MS instrument (ThermoQuest, San Jose, CA). Elution conditions: 60 °C, 1 min; +10 °C min^{-1} until 300 °C; hold 1 min; flow rate: 1.0 mL min^{-1}; inlet temperature: 250 °C; ion source temperature: 250 °C; MS transfer line temperature: 250 °C. Retention times: (**1**): 9.53 min; (**1a**, **1b**): 9.38 min; ((−)-**5** and(+)-**5**): 9.57 min; (**5b1**, **5b2**): 9.25 min.

Chiral GC analyses were carried out on an Agilent Technologies 6850 Network GC system gas chromatograph equipped with split/splitless injector, FID detector and MEGA-DEX DAC Beta chiral capillary column (25 m × 0.25 mm × 0.25 µm). Elution conditions for **2** and **5**: 80 °C, 1 min; +1 °C min^{-1} until 90 °C; +10 °C min^{-1} until 180 °C; hold 5 min. Retention times: (**2**): 9.1 min; (**2a**, **2b**): 7.9 min, 8.8 min ((−)-**5** and(+)-**5**, respectively): 17.5 min, 17.6 min; (**5b1**, **5b2**): 17.7 min, 18.6 min. Elution conditions for **3** and **4**: 120 °C, 1 min; +5 °C min^{-1} until 180 °C; +10 °C min^{-1} until 200 °C; hold

5 min. Retention times: (**3**) 8.8 min; (**3a**, **3b**): 9.1 min, 9.3 min; (**4**) 10.5 min; (**4a**, **4b**): 10.0 min. Flow rate: 1.5 mL min^{-1}; detector temperature 200 °C; inlet temperature 250 °C.

Chiral HPLC analyses were performed on a Shimadzu LC-20AD high performance liquid chromatography system equipped with a Shimadzu SPD-20 A UV detector and a Phenomenex Lux Cellulose-1 5 µ chiral column. HPLC conditions: injection volume 10 µL; mobile phase H_2O + 0.1% trifluoroacetic acid:CH_3CN = 75:25 (isocratic elution); flow rate: 1.0 mL min^{-1}; detection 254 nm; temperature 30 °C. Retention times: (**1**): 4.0 min; (**1b**): 5.5 min; (**1a**): 7.0 min. Prior to performing HPLC analyses, the molar extinction coefficients of **1** and **1a**, **1b** were determined (see Supporting information for details), to be able to assess conversions while considering the different molar absorbivity of substrate and products.

The NMR spectra (^1H and ^{13}C) were acquired in $CDCl_3$ or in DMSO-d_6 at rt on a Bruker AV 400 MHz spectrometer with a z gradient at 400 MHz for ^1H-NMR analysis and 101 MHz for ^{13}C-NMR.

ESI-MS spectra were recorded on a Bruker Esquire 3000 PLUS instrument (ESI Ion Trap LC/MSn System), equipped with an ESI source and a quadrupole ion trap detector (QIT). The samples were dissolved in methanol to 10–2 g L^{-1} and then directly syringed in the ESI-MS at 4 µL min^{-1} rate. The analyses were performed in positive mode. The acquisition parameters were optimized as such: 4.5 kV needle voltage, 10 L h^{-1} N_2 flow rate, 40 V cone voltage, trap drive set to 46, 115.8 V capillary exit, 13000 (m/z) s^{-1} scan resolution over the 35–900 m/z mass/charge range, source temperature 250 °C.

Optical rotations were measured on a Jasco P-2000 polarimeter. The specific rotation was calculated as the $[\alpha]_\lambda^T = \alpha 100/cd$, where α represents the recorded optical rotation, c the analyte concentration (mg mL^{-1}), d the cuvette length (dm). As such, the specific rotation is expressed as (10^{-1} deg cm^{-2} g^{-1}). λ is reported in nm and T in °C. λ usually corresponds to sodium D line (589 nm), thus the optical rotation is referred to as $[\alpha]_D$. T, c and the solvent were chosen according to references reported in literature: c = 1 in $CHCl_3$ at 22 °C for **2**; c = 0.5 in EtOH at 25 °C for **5**; c = 0.75 in $CHCl_3$ at 25 °C for **5a** and **5b**.[49,50]

In Silico Screening for Novel HSDHs and Bioinformatic Analysis

Bioinformatic search for new HSDHs was performed by aligning query sequences (entry 1, 2, 4, 14–21, 33 Table S1 Supporting Information) with database metagenomic sequences using the program LAST (http://last.cbrc.jp/) with default settings[55] or with GenBank database sequences using standard protein Blast tool (https://blast.ncbi.nlm.nih.gov/).

Phylogenetic trees were created using the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/)[46] and visualized with iTOL webserver (http://itol.embl.de/).[47]

Gene Cloning and Generation of Recombinant Bacterial Strains

The codon-optimized genes coding for putative HSDHs (Table S1, Supporting Information, entries 11–13, 26–29, 31, 43, and 44,) and for Csp12α-HSDH (entry 33) were synthesized and cloned into the pUC57 vector by BaseClear (Leiden, The Netherlands).

Putative HSDH genes were amplified by PCR using primers reported in Table S2 (Supporting Information), for the subsequent cloning in the pETite C-His Kan vector *in frame* with C-term His-Tag sequence. PCR amplifications were carried out in 50 µl reaction mixtures containing 20 ng of pUC57 vector including the desired gene, primers (1 µM each), dNTPs (0.2 mM each), 2 U of Xtra Taq polymerase and 5 µl of buffer containing $MgCl_2$. All PCR reagents were from Genespin (Milan, Italy). PCR conditions were as follows: 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, primers T_m −5 °C for 30 s, 72 °C for 1 min, and then 72 °C for 10 min. PCR products were then purified from agarose gel (0.7% (w/v)) using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) before cloning. Purified sequences were subsequently cloned in the pETite C-His Kan plasmid using the Expresso T7 Cloning and Expression kit from Lucigen (Wisconsin, USA). Specifically, purified sequences were mixed with the pETite C-his linear plasmid and transformed in chemically competent *E. coli* HI-Control 10G cells following the manufacturer's instructions.

Csp12α-HSDH cloning into the pETite N-His Kan vector *in frame* with N-term His-Tag sequence was carried out by gene amplification with primers F10/R10 (Table S2, Supporting Information), using the PCR protocol described above. The amplified sequence was subsequently cloned in the pETite N-His Kan vector using the same kit described for the cloning in the pETite C-His Kan plasmid.

The resulting plasmids were purified from *E. coli* HI-Control 10G cells using the E.Z.N.A. Plasmid Mini kit II (Omega/VWR) and plasmid inserts were sequenced on both strands by Biofab Research (Rome, Italy) using primers T7 promoter and pETite reverse (Table S2, Supporting Information). Recombinant pETite plasmids were finally transformed in *E. coli* BL21 (DE3) chemically competent cells (Lucigen) for the expression of the corresponding genes.

HSDH/SDR and FDH Expression and Purification

Recombinant *E. coli* strains from our in-house collection (see bacterial strain section) and recombinant *E. coli* BL21(DE3) strains prepared as described above, were inoculated overnight in LB medium supplemented with the opportune antibiotic (Table S3, Supporting Information) (100 mL) and grown at 37 °C, 220 rpm. 25 mL of precultures were subsequently inoculated in 500 mL of LB medium containing the corresponding antibiotic and incubated at 37 °C, 220 rpm till the OD_{600} cell density reached 0.5–1. Gene expression was induced by the addition of IPTG (for IPTG final concentrations, see Table S3, Supporting Information) and the culture was transferred to 17–37 °C (see Table S3, Supporting Information) with shaking at 220 rpm and grown for 4–72 h (see Table S3, Supporting Information). In case of *E. coli* TOP10/pBAD-Ca7αHSDH, precultures were inoculated in TB medium containing rhamnose as protein expression inducer.

After recovery by centrifugation (5000 rpm, 30 min, 4 °C), cell pellet was resuspended in 20 mL wash buffer (20 mM

potassium phosphate (KP) buffer, pH 7.0, 500 mM NaCl, 20 mM imidazole) and cells were disrupted by sonication. Soluble protein fraction was separated from the cell debris by centrifugation (11,000 rpm, 30 min) and clear lysates were assessed for the presence of soluble protein by SDS-PAGE (12% T, 2.6% C).

For protein purification, Ni Sepharose 6 Fast Flow agarose resin (Ni-NTA) (GE Healthcare, Italy) was incubated with clear cell lysates containing soluble protein for 90 min at 4 °C under mild shaking. The mixture was then loaded onto a glass column (10 × 110 mm) and the resin was washed with 20 mL of wash buffer (20 mM imidazole, 500 mM NaCl, 20 mM KP buffer). His tagged proteins were eluted using a 3-step gradient (10 ml washing buffer with 100, 200, and 300 mM imidazole, respectively) and, if not stated otherwise, dialyzed against 5 L of a suitable buffer (Table S3, Supporting Information), at 4 °C for 16 h and stored at −80 °C. Protein content was measured using the Bio-Rad Protein Assay according to the method of Bradford and protein purity was verified by SDS-PAGE analysis (12% T, 2.6% C). The molecular weight protein standard mixture from Bio-Rad (Karlsruhe, Germany) was used as reference. Gels were stained for protein detection with Coomassie Brilliant Blue.

Activity Assays

Dehydrogenase activity of HSDHs and FDHs was determined spectrophotometrically by measuring the reduction of $NAD(P)^+$ at 340 nm (ε: 6.22 $mM^{-1}cm^{-1}$) in the presence of the opportune substrate. If not stated otherwise, cholic acid was used for 7α- and 12α-HSDHs, ursodeoxycholic acid was used for 7β-HSDHs, and ammonium formate was used for FDHs.

Specifically, assays were carried out in polyethylene cuvettes at 25 °C by adding the opportune purified dehydrogenase (1–20 µL) to the following assay mixtures (1 mL final volume):

HSDH assay: 2.5 mM substrate; 50 mM potassium phosphate buffer at pH 9.0; 0.20 mM $NAD(P)^+$.

FDH assay: 20 mM substrate; 50 mM potassium phosphate buffer at pH 7.0; 0.20 mM $NAD(P)^+$.

One unit of activity is defined as the enzyme activity that reduces 1 µmol of $NAD(P)^+$ per min under the assay conditions described above.

HSDHs optimal temperatures were determined by heating the assay solution in cuvettes in a water bath in the range 20–90 °C for 10 min before adding the enzyme. Results were compared to blanks.

Small-Scale Biotransformations of Bile Acids

Oxidation of cholic Acid to 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid or to 3α,7α-dihydroxy-12-oxo-5β-cholanoic acid. Cholic acid oxidation reactions were coupled with a pyruvate/LDH system to regenerate NAD^+. Specifically, reactions were carried out in a 1 mL solution containing 50 mM potassium phosphate buffer, pH 8.0, 10 mM cholic acid, 50 mM sodium pyruvate, 0.4 mM NAD^+, 0.5 U LDH from rabbit muscle, 2 U of opportune 7α-HSDH (entry 11–13, Table S1, Supporting Information) to obtain 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (Scheme S1, A) or Ls12α-HSDH to obtain 3α,7α-dihydroxy-12-oxo-5β-cholanoic acid (Scheme S1, B) at 25 °C for 24 h.

Reaction progress was monitored by TLC using chloroform-methanol-acetic acid, 10:1:0.08, as eluting system.

Reduction of 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid to ursocholic acid. 3α,12α-diHydroxy-7-oxo-5β-cholanoic acid reduction was coupled with a formate/FDH system to regenerate NADH. Specifically, reactions were performed in a 1 mL solution containing 50 mM potassium phosphate buffer, pH 8.0, 10 mM cholic acid, 50 mM NH_4HCO_2, 0.4 mM NADH, 0.5 U FDH from *Candida boidinii*, 2 U of opportune 7β-HSDH (entry 26–29, Table S1, Supporting Information) (Scheme S1, C) at 25 °C for 24 h.

Reaction progress was monitored by TLC using the same eluting system described for cholic acid oxidation (chloroform-methanol-acetic acid, 10:1:0.08), as eluting system.

Preparation of Standard Racemate Mixtures

The reductions of substrates **1–5** were performed following a standard protocol of reduction with $NaBH_4$.[56] To a stirred solution of 0.13 M substrate (1 eq, 100 mg) in MeOH (5 mL) at 0 °C, $NaBH_4$ (1 eq, 13 mg) was added. When the reaction mixture became clear, it was brought to rt and stirred for 2–4 h. The reaction was quenched with a saturated aqueous solution of NH_4Cl, then it was extracted with EtOAc (3x). The organic phase was dried over Na_2SO_4, filtrated and the solvent was evaporated under reduced pressure to yield the desired products (**1a** + **1b**, **2a** + **2b**, **3a** + **3b**, **4a** + **4b**, **5a1** + **5a2** + **5b1** + **5b2**) in quantitative yields. The products were characterized via ^{1}H-NMR analysis and chiral GC or HPLC analyses (see Supporting information, and analytical methods).

1a + **1b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49–7.30 (m, 1H), 5.20 (s, 1H), 3.79 (s, 1H).

2a + **2b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.42–4.15 (m, 1H), 4.04 (s, 1H), 2.72 (s, 1H), 2.19–2.00 (m, J = 13.7, 6.9, 3.5 Hz, 1H), 1.33 (t, J = 7.1 Hz, 1H), 1.05 (d, J = 6.9 Hz, 1H), 0.89 (d, J = 6.9 Hz, 1H).

3a + **3b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48–7.41 (m, 1H), 7.26–7.18 (m, 2H), 7.15–7.08 (m, 1H), 4.88–4.72 (m, 1H), 3.04–2.52 (m, 2H), 2.21–1.74 (m, 4H).

4a + **4b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.02 (m, 4H), 4.30–3.98 (m, 1H), 3.12 (dd, J = 15.9, 4.5 Hz, 1H), 3.07–3.06 (m, 1H), 2.99 (dt, J = 17.0, 5.8 Hz, 1H), 2.92–2.84 (m, 1H), 2.80 (dd, J = 16.3, 7.9 Hz, 1H), 2.15–2.04 (m, 1H), 1.92–1.79 (m, 1H).

5a1 + **5a2**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (q, J = 2.5 Hz, 1H), 1.92–1.74 (m, 2H), 1.74–1.55 (m, 4H), 1.56–1.43 (m, 3H), 1.44–1.16 (m, 5H), 1.15–0.85 (m, 3H).

5b1 + **5b2**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (ddd, J = 10.2, 9.1, 4.3 Hz, 1H), 2.18–2.07 (m, J = 6.1, 3.0 Hz, 1H), 2.04–1.91 (m, 1H), 1.87–1.59 (m, 4H), 1.61–1.17 (m, 7H), 1.12–0.81 (m, 5H).

Enzymatic Reduction of Substrates 1–5 Catalyzed by HSDHs

Reactions catalyzed by HSDHs were coupled with a formate/FDH system to regenerate NAD(P)H. For the initial activity screening the general reaction protocol was as follows: 76 mM NH_4HCO_2; 0.2 U mL^{-1} FDH; 0.4 mM NAD(P)$^+$; HSDH, 3.4 U mL^{-1}; 12.5 mM substrate; 5% v/v DMSO; 50 mM potassium phosphate buffer, pH 7.0 (total volume: 1 mL). Reaction catalyzed by Hh7α-HSDH and Hh7β-HSDH were performed in the presence and in the absence of 0.4 M NaCl. The mixtures were shaken at 25 °C and 100 rpm for 24 to 48 h and monitored over time *via* TLC (eluent CH_2Cl_2). Reaction conversions and enantiomeric excesses were evaluated by GC-MS, chiral GC or chiral HPLC analyses. The absolute configurations were assigned *via* analytical comparison with standards (products **1a** and **1b**) or *via* optical rotation measurements and confrontation with values reported in literature (products **2a** and **2b**, (+)-**5** and (−)-**5**, **5b1** and **5b2**).[49,50]

Reactions on substrates **2** and **5** catalyzed by Cae7β-HSDH were subsequently scaled up to semi-preparative scale (100 mg, 0.7 mmol in 50 mL total volume) following the protocol described above. The isolated products were characterized via ^{1}H- and ^{13}C-NMR, ESI-MS, chiral GC and $[\alpha]_D^{25}$ (see Supporting information, and analytical methods).

2a + **2b**: ^{1}H NMR (400 MHz, DMSO) δ 5.05 (d, J = 5.9 Hz, 1H), 4.04–3.89 (m, 2H), 3.64 (t, J = 5.4 Hz, 1H), 1.83–1.70 (m, 1H), 1.05 (t, J = 7.1 Hz, 3H), 0.74 (d, J = 6.9 Hz, 3H), 0.69 (d, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.90, 77.35, 77.04, 76.72, 74.99, 61.48, 41.00, 32.14, 18.74, 15.97, 14.23; $[M+Na]^+$: 167.1; conversion: 99.9%; isolated yield: 63.2%; ee (chiral GC): 97.1%; $[\alpha]_D^{22}$: −2.70°.

5: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.41–2.25 (m, J = 13.5, 5.0, 1.8 Hz, 2H), 2.10–2.00 (m, J = 16.3, 5.9, 2.8 Hz, 1H), 2.00–1.85 (m, 2H), 1.85–1.59 (m, 5H), 1.49–1.30 (m, 2H), 1.30–1.09 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 212.72, 77.35, 77.03, 76.71, 55.08, 44.96, 41.80, 34.38, 33.04, 26.48, 25.75, 25.43, 25.10; $[M+Na]^+$: 175.1; ee (chiral GC): 62.2%; $[\alpha]_D^{25}$: +2,75°.

5b1 + **5b2**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.29–3.11 (m, 1H), 2.23–2.06 (m, J = 6.0, 3.0 Hz, 1H), 2.02–1.91 (m, 1H), 1.84–1.58 (m, 4H), 1.58–1.43 (m, 2H), 1.41–1.13 (m, 4H), 1.11–0.77 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 77.33, 77.01, 76.70, 75.04, 50.46, 41.13, 35.82, 33.59, 33.43, 29.00, 26.34, 26.15, 24.03; $[M+Na]^+$: 177.1; conversion (GC-MS): 35.8%; isolated yield: 15%; ee (chiral GC): 89.1%; $[\alpha]_D^{25}$: +44.45°.

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Biocatalytic Approaches to the Enantiomers of Wieland–Miescher Ketone and its Derivatives

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This manuscript is dedicated to Prof. Franco Cozzi on the occasion of his 70th birthday.

Biocatalytic approaches have been investigated in order to isolate the enantiomers of Wieland–Miescher ketone (**1**) and of its alcoholic derivatives (*cis*-**2** and *trans*-**3**). Specifically, two enzymes from our in-house metagenomic collection of oxidoreductases, IS2-SDR and Dm7α-HSDH, catalyzed the kinetic resolution of the starting racemic ketone **1** or its complete conversion into two diastereomeric products, respectively. Moreover, the kinetic resolution of the racemic *cis*-alcohol (**2**) was very efficiently obtained (E≅2.000) by lipase PS catalyzed acetylation in dry acetone. All the products were isolated with ee ≥ 95 %. Simple chemical elaborations of some of them allowed to isolate the missing enantiomers.

Introduction

The so-called Wieland-Miescher ketone (**1**, Scheme 1) is an important intermediate widely used in the total synthesis of complex natural products, predominantly with a terpenoid structure.[1–3] It can be easily prepared in its racemic form from 2-methylcyclohexane-1,3-dione and methyl vinyl ketone *via* the Robinson annulation, a tandem process proposed in 1935 including a Michael addition and an intramolecular aldol condensation.[4]

The enantiomerically enriched form of **1** was firstly described in 1971, when two different groups of industrial chemists independently proposed an asymmetric version of the Robinson annulation catalyzed by proline.[5–7] Since then, the enantioselective synthesis of one of the enantiomers of **1** ((8a*S*)-**1a** and (8a*R*)-**1b**) has become a model target to evaluate the performances of new chiral organocatalyst,[8–10] as it has been recently carefully reviewed.[11,12]

The unselective reduction of the C_1 carbonyl moiety of **1** should theoretically afford a complex mixture of stereoisomeric alcohols (5-hydroxy-4a-methyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3H)-one) which exist as two couples of *cis*- and *trans*-enantiomers (compounds (4a*S*, 5*S*)-**2a**, (4a*R*, 5*R*)-**2b** and (4a*S*, 5*R*)-**3a**, (4a*R*, 5*S*)-**3b** in Scheme 1). These chiral derivatives, when obtained as enantiomerically enriched species, are known to be important building blocks for the synthesis of natural, bioactive products or compounds of pharmaceutical interest.[13–17] The presence of an enone group, in fact, easily allows to further manipulate their molecular skeleton and to transform them into structurally complex compounds.

From a chemical point of view, the reduction of racemic **1** with common hydride-based reducing agents (e.g., $NaBH_4$), due to the steric hindrance determined by the adjacent C_{8a} methyl group, usually gives access only to a racemic mixture of the *cis*-alcohols (**2a** and **2b**).[18] The two *trans*-alcohols **3a** and **3b** appear to be accessible only from the *cis*-isomers *via* low-efficient protocols of stereoinversion.[19]

Alternative "bio"-based strategies have been investigated to build a convenient entry to the enantiomers of **1** and/or to isolate compounds **2a**–**b** and **3a**–**b** as stereo-enriched species. On this respect, it deserves to be mentioned the outstanding results obtained by Lerner, Danishefsky and coworkers, who developed a catalytic antibody able to catalyze the enantioselective Robinson annulation.[20] The reaction was performed at a 110 mg scale and the *S*-enantiomer **1a** could be isolated in 94 % yield and 96 % optical purity.

More recently a biocatalyzed approach to the synthesis of **1**, *via* the Robinson annulation, has been proposed, exploiting the catalytic promiscuity[21] of a commercial lipase from porcine pancreas.[22] However, a more detailed investigation of the protein components of that crude preparation demonstrated that non-lipase contaminant enzymes, and specifically an α-amylase, were apparently responsible for the observed transformation. In any case, even under optimized conditions, the results both in terms of yield and enantiomeric excess of **1** were significantly lower when compared with a control organocatalyzed (with *L*-proline) reaction.[23]

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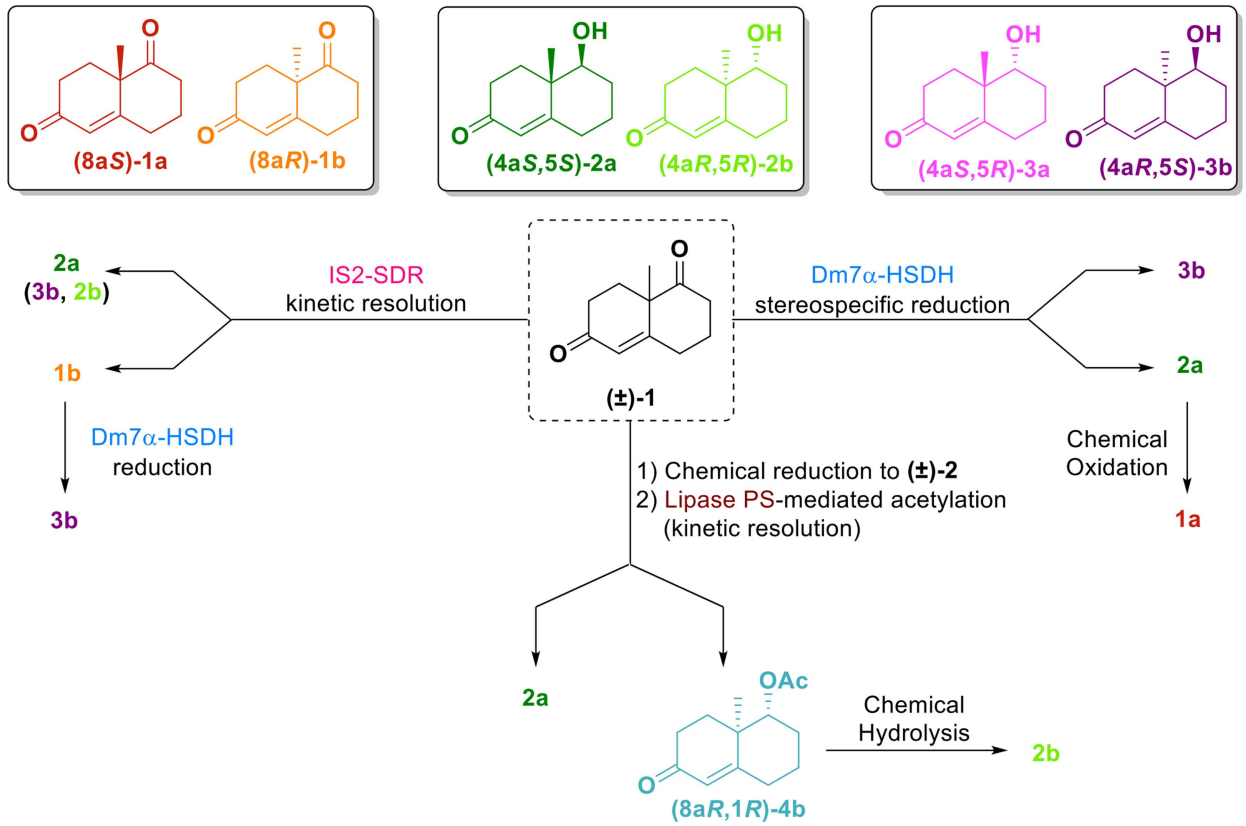

Scheme 1. (Chemo)enzymatic manipulation of racemic Wieland–Miescher ketone (±**1**) and of its alcoholic derivatives (±**2**).

A partial kinetic resolution of racemic **1** was obtained by submitting this compound to the action of a cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*, but the preferred enantiomer was irreversibly oxidized to the corresponding lactone.[18] Nevertheless, a biocatalyzed synthetic strategy, exploiting the intrinsic enantioselectivity of enzymes, might offer a simple and efficient solution to the separation of the two enantiomers of the easily obtainable racemic **1**. To our surprise, quite few reports can be found in the literature on this topic so far, most of them describing biotransformations with microbial whole cells. Stereoselective reduction of racemic **1** was firstly described by Prelog using whole cells of *Curvularia falcata*.[24–27] Later on, Sugai described the results obtained with yeast strains of *Torulaspora delbrueckii* and *Candida melibiosica*,[28,29] and Kodama with baker's yeast.[30] More recently Janeczko described the reductive performances of *Didymosphaeria igniaria* and *Coryneum betulinium* strains.[31]

Apparently, no examples of the stereoselective reduction of **1** catalyzed by isolated enzymes have been reported to date. Therefore, we considered racemic **1** an interesting substrate to evaluate the performance of our in-house collection of dehydrogenases, including new metagenomic and extremophilic enzymes.[32]

Moreover, complementary biocatalyzed strategies, i.e., the well-known kinetic resolution of racemic alcohols catalyzed by hydrolases, deserve to be explored to build alternative entries to Wieland-Miescher ketone derivatives as enantiomerically enriched species. On this respect, Shimizu *et al.* were able to obtain the stereoisomeric alcohol derivatives of **1** by conducting an enzymatic asymmetric hydrolysis (exploiting a library of hydrolases of different origins)[33] of the acetylated racemates of alcohols **2** and **3** obtained from **1** according to the above mentioned protocols.[18,19] Best results were obtained on the *cis* racemate **2** using a β-amilase from wheat. On the other hand, to our surprise again, we could not find examples related to the use of lipases in organic solvents[34] to catalyze the kinetic resolution, by esterification, of racemic **2** and **3**.

In the following we report the results that we have obtained investigating the performances of new oxidoreductases and a well-known lipase, together with a critical comparison with the literature data.

Results and Discussion

One of the most interesting properties of enzymes, from a synthetic point of view, is their substrate promiscuity, that is their ability to transform compounds with chemical structures significantly different from those of their natural substrates, still maintaining high levels of stereoselectivity. On this respect, we recently reported on the performances of a collection of new hydroxysteroid dehydrogenases (HSDHs), identified either by genome mining or by metagenomics approaches, in the reduction of a panel of substrates including, in addition to the

"natural" hydroxysteroids, α-ketoesters and selected ketones partially resembling the structural features of steroids.[32]

The results obtained in the reduction of racemic **1** *via* the preliminary screening of our panel of dehydrogenases is summarized in Table 1. Among the 15 (mostly new) enzymes tested, including 13 HSDHs (7α-HSDHs, 7β-HSDHs, and 12α-HSDHs) and two short-chain dehydrogenases/reductases (SDRs), only four accepted **1** as a substrate, two of them with significantly higher conversion values. The reactions with these two enzymes, IS2-SDR (a SDR identified from a metagenome obtained from Icelandic hot spring sediments) and Dm7α-HSDH (a 7α-hydroxysteroid dehydrogenase identified in the genome of the extremophilic bacterium *Deinococcus marmoris* strain PAMC 26562, a radiation-resistant and psychro- and draught-tolerant bacterium isolated from an Antarctic rock sample), were analyzed in more details as their chiral phase HPLC chromatograms showed two different reaction outcomes. As reported in Figure 1, while with Dm7α-HSDH a complete conversion of **1** into two products was obtained, IS2-SDR catalyzed a kinetic resolution of the starting racemic ketone.

The reactions were scaled-up in order to isolate and characterize the products, using the ancillary enzyme glucose dehydrogenase (GDH) to regenerate *in situ* the needed reduced cofactor (NADH for Dm7α-HSDH and NADPH for IS2-SDR). As summarized in the left upper part of Scheme 1, the kinetic resolution of **1** in the reduction catalyzed by IS2-SDR allowed

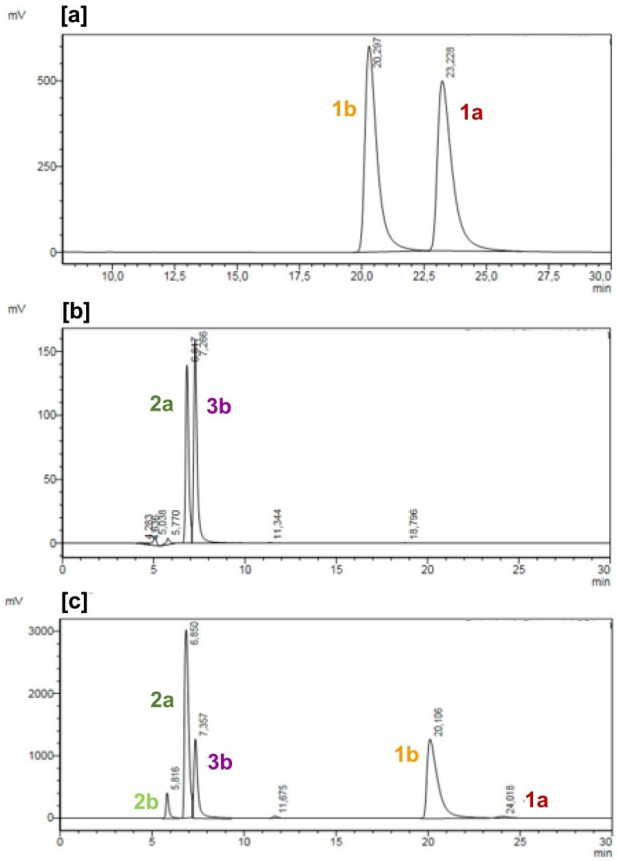

Figure 1. HPLC chromatograms of [a] racemic **1**; [b] Dm7α-HSDH stereoselective biocatalyzed reduction of racemic **1**; [c] IS2-SDR biocatalyzed kinetic resolution of racemic **1**.

Table 1. Screening of dehydrogenases for the reduction of racemic **1**.[a]

Entry	Enzyme[b]	Source[c]	Cofactor	Conversion[d]
1	Ca7α-HSDH	*Clostridium absonum*	NADP (H)	<1%
2	Dm7α-HSDH	*Deinococcus marmoris*	NAD(H)	>99%
3	Ec7α-HSDH	*Escherichia coli*	NAD(H)	6%
4	Hh7α-HSDH	*Halomonas halodenitrificans*	NAD(H)	<1%
5	NGI7α-HSDH	Norwegian metagenome	NAD(H)	<1%
6	Bsp7β-HSDH	*Bacillus* sp.	NAD(H)	<1%
7	Ca7β-HSDH	*Clostridium absonum*	NADP (H)	<1%
8	Cae7β-HSDH	*Collinsella aerofaciens*	NADP (H)	<1%
9	Hh7β-HSDH	*Halomonas halodenitrificans*	NAD(H)	<1%
10	Rs7β-HSDH	*Rhodobacter sphaeroides*	NAD(H)	<1%
11	Sc7β-HSDH	*Stanieria cyanosphaera*	NAD(H)	<1%
12	Csp12α-HSDH	*Clostridium* sp.	NADP (H)	<1%
13	Ls12α-HSDH	*Lysinibacillus sphaericus*	NAD(H)	<1%
14	IS2-SDR	Icelandic metagenome	NADP (H)	66%
15	NGI7-SDR	Norwegian metagenome	NAD(H)	12%

[a] Reaction conditions: 10 mM substrate; 50 mM phosphate buffer pH 7; 5% v/v DMSO; 50 mM glucose; 0.4 mM cofactor; 4 U mL^{-1} DH; 1 U mL^{-1} GDH; 0.4 M NaCl (only for entries 4 and 9); 25 °C, 100 rpm. [b] The library is composed both of HSDHs (entries 1–13) and of short chain reductases (SDRs, entries 14 and 15). [c] The recombinant enzymes were either from defined chemical sources or coming from metagenomic samples, as detailed in ref [32]. [d] Conversions were evaluated after 48 h by GC/MS and, for the entries 2, 3, 14, and 15, also by chiral phase HPLC analyses.

the isolation of the enantiomer **1b** with a 96.5% e.e ($[\alpha]_D^{22}$ –88.7°, c 1.0 in $CHCl_3$), in 32% yield. This result compares well, both in terms of selectivity and reaction outcome, with those obtained by Sugai with whole cells of *T. delbrueckii*,[28] by Kodama with baker's yeast,[30] and by Janeczko with *D. igniaria*.[31]

Later on this reaction was investigated in more details by submitting the two separate enantiomers to the catalytic action of IS2-SDR. It was confirmed that the enantiomer **1a** (8a*S* configuration) was the preferred one, the relative ratio of the reaction rates of its reduction *vs* that of **1b** being approximately 3 to 1. Moreover, the IS2-SDR-catalyzed reduction of **1a** was stereoselective and the *cis* alcoholic enantiomer **2a** was the only product. On the contrary, the slow reduction of **1b** gave predominantly the *trans* alcohol **3b**, accompanied by the *cis* diastereoisomer **2b** (relative ratio **3b** vs **2b**:4:1).

The stereospecific reduction of **1** was obtained in the reaction catalyzed by Dm7α-HSDH, which gave a 1:1 mixture of two diastereoisomers, whose preparative separation was quite troublesome and could be achieved by using a Biotage® SP1 system for flash chromatography. The two products proved to be the *cis*-alcohol **2a** derived from the enantiomer **1a** and the *trans*-alcohol **3b** derived from the enantiomer **1b** (Scheme 1, right upper part). Pure **3b** (>99% ee, $[\alpha]_D^{22}$ –111.6°, c 1.0 in

$CHCl_3$) was also obtained by Dm7α-HSDH-catalyzed reduction of the previously isolated enantiomer **1b** (Scheme 1, left upper part). In terms of stereoselectivity, the production of the *trans*-alcohol **3b** from the ketone enantiomer **1b** is in agreement with data reported by Sugai with whole cells of *C. melibiosica* (lower ee values were obtained)[28] and by Prelog with *C. falcata*.[24] A similar outcome but with much lower selectivity was observed by Janeczko in the biotransformations catalyzed by strains of *D. igniaria* and of *C. betulinium* whole cells.[31]

It is worthy to be reminded that the *trans*-alcohol **3** is not obtained using the classical chemical reductive reagents and only the *cis*-alcohol **2** could be isolated.[18,35] Moreover, once again it was possible to observe a significant selectivity of a HSDH, in this case devoted to the regio- and stereoselective modification of oxygenated substituent at the position C_7 of the steroid skeleton, with compounds not structurally related to a steroid. For additional examples of the substrate promiscuity of HSDHs see ref 33 and 36–41.

The easy availability of the racemic mixture of the *cis*-alcohol **2a** and **2b** prompted us also to evaluate an alternative biocatalyzed approach, exploiting the well-known enantioselectivity of hydrolases in the acylation of secondary alcohols in organic solvents.[34] As previously stated, to our surprise, apparently this substrate has never been considered in previous investigations, despite the fact that it is clearly a suitable target to be acylated by lipases, as shown by Franssen and coworkers with a structurally not so different compound.[42]

Compound **2** was dissolved in dry acetone containing a large excess (10% v/v) of the acylating agent vinyl acetate and different lipases were added. The reactions were shaken at 45 °C and monitored by TLC and chiral phase HPLC. As shown in Figure 2 we were pleased to find that the so-called lipase PS, adsorbed on celite, showed a really significant enantioselectivity. Actually, the value of enantiomeric ratio "E", that could be calculated from the values of degree of conversion and ee of the residual substrate according to the well-known formula proposed by Sih years ago,[43] was very high: approximately 2.000. This value makes this biocatalyzed acetylation really close to an ideal kinetic resolution process that spontaneously stops after the preferred enantiomer is consumed. And this was indeed the case, and the two pure *cis* enantiomers, the alcohol **2a** and the acetate **4b**, could be isolated and characterized.

The same protocol was applied to the diastereomeric mixture of alcohols **2a** and **3b** obtained by the reaction catalyzed by Dm7α-HSDH, but no conversion was observed with any of the lipases tested, leaving the above-described chromatographic protocol as the only way to separate these two compounds. The hydrolysis of the ester **4b** furnished the pure missing enantiomer of the alcohol, that is **2b**, while oxidation of the alcohol **2a** gave an easy and efficient entry to ketone **1a**.

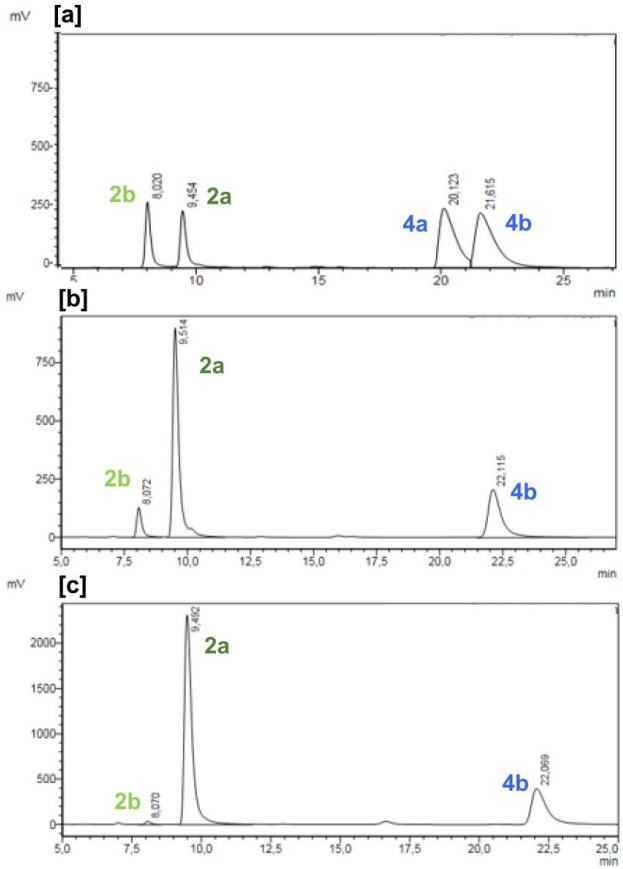

Figure 2. HPLC chromatograms of [a] racemic **2** + racemic **4**; [b] Lipase PS-biocatalyzed kinetic resolution of racemic **2** after 72 h; [c] After 120 h.

Conclusion

Wieland–Miescher ketone (**1**) has been chosen as a model synthon to evaluate the selectivity of a panel of dehydrogenases, including enzymes with different substrate scope as well as biological origin. The obtained results further exemplify the substrate promiscuity of this group of enzymes. Moreover, a more classical approach based on the kinetic resolution of a racemic mixture of the *cis* alcohol (**2**) catalyzed by lipase PS in acetone proved to be particularly efficient, very close to an ideal process in terms of enantioselectivity of the biocatalyst.

The dehydrogenase-catalyzed reactions were performed on a 50–100 mg scale, whereas the lipase-catalyzed kinetic resolution was obtained starting from 1 g of the substrate. All the reactions can be easily scaled-up as the DHs are available as recombinant overexpressed proteins, while lipase PS is a cheap commercially available enzyme. Specifically, the lipase-catalyzed reaction has not been optimized in terms of substrate concentration and substrate/biocatalyst ratio, therefore further improvements of productivity and solvent consumption are likely to be easily achieved at will.

The *trans* enantiomer **3a** is presently the only isomer that could not be produced by an enzyme-catalyzed reaction and this target will be the object of further future investigations.

Experimental Section

General

Wieland-Miescher ketone (8a-methyl-3,4,8,8a-tetrahydronaphthalene-1,6(2H,7H)-dione, purity 98.5%) was purchased from Fluorochem (Hadfield, UK). Amano lipase PS (adsorbed by us on celite)[44] was from Merck (Darmstadt, Germany). All reagents and solvents were purchased from Merck and used without further purification, unless otherwise stated.

Reactions were monitored *via* TLC (thin-layer chromatography) on pre-coated glass plates silica gel 60 with fluorescent indicator UV_{254} and treated with an oxidizing solution [4-hydroxybenzaldehyde (6.3 g), H_2SO_4 (50% v/v in H_2O, 40 mL), MeOH (400 mL)].

Abbreviations

HSDH: Hydroxysteroid Dehydrogenase; U: enzymatic unit; SDR: Short Chain Dehydrogenase/Reductase; BmGDH: Glucose Dehydrogenase from *Bacilllus megaterium*; IPTG: Isopropyl β-D-1-thiogalactopyranoside.

Enzyme Preparation

HSDHs/SDRs expression and purification

Expression and purification of HSDHs, SDRs, and BmGDH were carried out as previously described.[32]

Expression conditions of Dm7α-HSDH were optimized by expression trials carried out as follows: *E. coli* BL21(DE3) harboring the expression plasmid pETiteDm7aHSDH was inoculated overnight in 50 mL LB medium supplemented with 30 $\mu g\,mL^{-1}$ kanamycin (LB_{kan30}) and grown at 37 °C, 220 rpm. The overnight culture was subsequently inoculated in 1 L LB_{kan30} medium and incubated at 37 °C, 220 rpm. Protein expression was then induced with 1 mM IPTG at an OD_{600} of 0.5–1. The culture was transferred to 30, 25 or 17 °C with shaking at 220 rpm and grown for 24, 48 or 72 h, respectively. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C and resuspended in 20 mL lysis buffer (20 mM potassium phosphate (KP) buffer, pH 7.0, 500 mM NaCl, 20 mM imidazole). After disruption by sonication at 4 °C, the cell extract was centrifuged at 12000 rpm at 4 °C for 30 min and clear lysates were assessed for the presence of soluble protein by SDS-PAGE (12% T, 2.6% C). Protein purification was performed as previously described.[32] Cell incubation at 17 °C for 72 h after induction resulted in the best expression yields (i.e., 108 mg of pure Dm7α-HSDH (11.575 U) were obtained after protein purification).

Activity assays

Dehydrogenase activities of HSDHs and BmGDH were determined spectrophotometrically by measuring the reduction of $NAD(P)^+$ at 340 nm (ε: 6.22 $mM^{-1}cm^{-1}$), while the activity of IS2-SDR was measured by following the oxidation of NADPH at the same wavelength. Assays were carried out in polyethylene cuvettes at room temperature by adding the opportune purified dehydrogenase (1–20 μL) to the following assay mixtures (1 mL final volume):

HSDH assay: 2.5 mM substrate (cholic acid for 7α- and 12α-HSDHs, ursodeoxycholic acid for 7β-HSDHs); 50 mM potassium phosphate buffer, pH 9.0; 0.20 mM $NAD(P)^+$.

IS2-SDR assay: 10 mM methyl benzoylformate; 50 mM potassium phosphate buffer, pH 8.0; 0.20 mM NADPH.

BmGDH assay: 50 mM glucose; 50 mM potassium phosphate buffer, pH 7.0; 0.20 mM $NAD(P)^+$.

One unit (U) is defined as the enzyme activity that reduces/oxidizes 1 μmol of NAD(P)(H) per min under the assay conditions described above.

Analytical methods

At scheduled times, reaction samples (50 μL) were extracted with AcOEt and dried over Na_2SO_4 to afford 10 mM samples, submitted to GC-MS analysis, or evaporated, resuspended in *i*-PrOH to a 10 mM final concentration and analyzed by chiral HPLC.

GC-MS analyses were performed using an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm) on a Finnigan TRACE DSQ GC/MS instrument (ThermoQuest, San Jose, CA). Elution conditions: 60 °C, 1 min; + 10 °C min^{-1} until 300 °C; hold 1 min; flow rate: 1.0 mL min^{-1}; inlet temperature: 250 °C; ion source temperature: 250 °C; MS transfer line temperature: 250 °C. Retention times: (**1a**,**1b**): 13.7 min; (**2a**,**2b**): 14.6 min; (**3a**,**3b**): 14.8 min; (**4a**,**4b**): 15.4 min.

Chiral phase HPLC analyses were performed on a Shimadzu LC-20AD high performance liquid chromatography system equipped with a Shimadzu SPD-20 A UV detector and a Phenomenex Lux 3u Cellulose-2 chiral column (250 mm × 4.6 mm). HPLC conditions: injection volume 10 μL; mobile phase: *i*-PrOH: petroleum ether = 1:1; flow rate: 0.7 mL min^{-1}; detection λ: 254 nm; temperature: 33 °C. Retention times: (**1a**): 22.4 min; (**1b**): 19.1 min; (**2a**): 6.9 min; (**2b**): 5.9 min; (**3b**): 7.3 min; (**4a**): 16.1 min; (**4b**): 15.8 min. Prior to performing HPLC analyses, the molar extinction coefficients of **1**, **2** and **4** were determined, to be able to assess conversions while considering the different molar absorbivity of the molecules under analysis.

The nature of the obtained compounds, which have been previously characterized in the literature, was confirmed by means of ^{1}H-NMR, mass spectrometry and optical rotation measurements.

The NMR spectra were acquired in $CDCl_3$ or in DMSO-d_6 at rt on a Bruker AV 400 MHz spectrometer with a z gradient at 400 MHz for ^{1}H-NMR analysis and 101 MHz for ^{13}C-NMR.

ESI-MS spectra were recorded on a Bruker Esquire 3000 PLUS instrument (ESI Ion Trap LC/MSn System), equipped with an ESI source and a quadrupole ion trap detector (QIT). The samples were dissolved in methanol to 1–2 g L^{-1} and then directly syringed in the ESI-MS at 4 μL min^{-1} rate. The analyses were performed in positive mode. The acquisition parameters were optimized as such: 4.5 kV needle voltage, 10 L h^{-1} N_2 flow rate, 40 V cone voltage, trap drive set to 46, 115.8 V capillary exit, 13000 (m/z) s^{-1} scan resolution over the 35–900 m/z mass/charge range, source temperature 250 °C.

Optical rotations were measured on a Jasco P-2000 polarimeter. The specific rotation was calculated as the $[\alpha]\lambda T = \alpha 100/cd$, where α represents the recorded optical rotation, c the analyte concentration (mg mL^{-1}), d the cuvette length (dm). As such, the specific rotation is expressed as 10^{-1} deg cm^{-2} g^{-1}. λ is reported in nm and T in °C. λ corresponds to sodium D line (589 nm), thus the optical rotation is referred to as $[\alpha]_D$. T, c and the solvent were chosen according to references reported in literature (see below).

Determination of the molar absorbivity coefficients of compounds 1, 2 and 4

The molar extinction coefficients of ketone **1**, *cis*-alcohol **2** and *cis*-acetate **4** were determined at 254 nm. For each compound, a 10 mM solution in CH_3CN was prepared and it was used as a mother solution to test the absorption of each molecule in 1 mL quartz cuvettes in the concentration range of 0.1-25 μM in CH_3CN (where linearity of data was observed). ε_{254} (**1**): 6508 $M^{-1}cm^{-1}$; ε_{254} (**2**): 5889 $M^{-1}cm^{-1}$; ε_{254} (**4**): 2620 $M^{-1}cm^{-1}$ (for more details see Paragraph 1, Supporting information).

Characterization of commercially available racemic 1

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.87 (d, J = 1.8 Hz, 1H), 2.84–2.65 (m, 2H), 2.61–2.39 (m, 4H), 2.30–2.04 (m, 3H), 1.72 (qt, J = 13.3, 4.4 Hz, 1H), 1.46 (s, 3H). ESI-MS: $[M+Na]^+$: 201.0.

Synthetic chemistry

Preparation of standard racemate 2, (±)-4 a,5-cis-(5-hydroxy-4 a-methyl-4,4 a,5,6,7,8-hexahydronaphthalen-2(3H)-one)[18]

The reduction of substrate **1** was performed following a standard protocol with $NaBH_4$. To a stirred solution of 0.4 M substrate (1 eq, 100 mg) in EtOH (5 mL) at 0 °C, an ethanolic suspension (0.4 M) of $NaBH_4$ (1 eq, 13 mg) was added dropwise. After 4 hours, the reaction was quenched with a saturated aqueous solution of NH_4Cl, then it was extracted with AcOEt (3x). The organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure to yield a crude extract. A flash chromatography purification afforded the desired products (**2 a** + **2 b**) in up to 90% yield. The racemic product **2** was characterized by chiral HPLC (see analytical methods) and ^{1}H-NMR analyses. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.78 (d, J = 1.8 Hz, 1H), 3.42 (dd, J = 11.6, 4.3 Hz, 1H), 2.50–2.28 (m, 3H), 2.25–2.13 (m, 2H), 1.94–1.78 (m, 3H), 1.76–1.64 (ddd, J = 16.9, 13.3, 4.2 Hz, 1H), 1.48–1.33 (qt, J = 13.2, 4.0 Hz, 1H), 1.19 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 206.66, 199.56, 168.43, 125.50, 78.28, 41.63, 34.27, 33.72, 32.03, 30.31, 23.20, 15.27. ESI-MS: $[M+Na]^+$: 203.0.

Preparation of standard racemate 4, (±)-8 a,1-cis-(-8 a-methyl-6-oxo-1,2,3,4,6,7,8,8 a-octahydronaphthalen-1-yl acetate)[46]

The acetylation of racemate substrate **2** was performed according to a standard acetylation protocol with acetyl chloride. To a stirred solution of 0.1 M substrate (1 eq, 50 mg) in CH_2Cl_2 (2.8 mL), pyridine (1.2 eq, 27 μL) and acetyl chloride (1.2 eq, 24 μL) were added. The reaction was stirred overnight at room temperature. Then, CH_2Cl_2 was evaporated and the crude extract was resuspended in saturated aqueous $NaHCO_3$, then extracted (3×) with AcOEt. The organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure to give the desired products (**4 a** + **4 b**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (d, J = 1.8 Hz, 1H), 4.65 (dd, J = 11.8, 4.2 Hz, 1H), 2.44–2.30 (m, 3H), 2.30–2.21 (m, 1H), 2.08 (s, J, 3H), 1.98–1.66 (m, 5H), 1.55–1.41 (m, 1H), 1.27 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.82, 170.32, 166.67, 125.80, 79.17, 40.39, 33.98, 33.47, 31.76, 26.87, 22.95, 21.07, 16.64. ESI-MS: $[M+H]^+$: 223.1. ESI-MS: $[M+Na]^+$: 245.0.

Enzymatic reduction of racemic ketone 1 with HSDHs/SDRs

General protocol for the enzymatic reduction: the reactions catalyzed by HSDHs were coupled with a glucose/GDH system to regenerate NAD(P)H. For the initial activity screening of the whole library of enzymes, the general reaction protocol was as follows: 50 mM glucose; 0.2 U mL^{-1} GDH; 0.4 mM $NAD(P)^+$; 3.4 U mL^{-1} HSDH; 10 mM substrate; 5% v/v DMSO; 50 mM potassium phosphate buffer, pH 7.0 (total volume: 1 mL). Reactions catalyzed by Hh7α-HSDH and Hh7β-HSDH were performed both in the presence and in the absence of 0.4 M NaCl. The mixtures were shaken at 25 °C and 100 rpm for 24 to 48 h and monitored over time *via* TLC (eluent CH_2Cl_2 : AcOEt = 9 : 1). Reaction conversions and enantiomeric excesses were evaluated by GC-MS and chiral HPLC analyses. The isolation of pure products was obtained *via* extraction with AcOEt of the reaction mixtures and subsequent flash chromatography on silica gel 60 (70–320 mesh, Merck, eluent CH_2Cl_2/AcOEt mixtures). The absolute configurations of the residual substrate **1 b**, if any, and of the products **2 a** and **3 b** were assigned via optical rotation measurements and subsequent confrontation with values reported in literature.[24–27]

Enzymatic reduction of 1 catalyzed by IS2-SDR

Following the aforementioned general protocol on a 100 mg scale (10 mM substrate, 56 mL reaction volume) and stopping the reaction at 58% conversion, the following products were isolated after flash chromatography (eluent: CH_2Cl_2 : AcOEt = 9 : 1). **1 b**: ee: 96.7%; $[\alpha]_D^{22}$: −97.0° (c: 1.0 in $CHCl_3$);[47] isolated yield: 33 mg (32%); **2 a**: ee: 90.7%; isolated yield: 30 mg, (29%). NMR and ms spectra are in accordance with the proposed structures (see Supplementary).

Enzymatic reductions of 1 and 1 b catalyzed by Dm7α-HSDH

Following the general protocol on a 50 mg scale (10 mM substrate, 28 mL reaction volume) and bringing the reaction to full conversion from racemic **1**, the following products were isolated *via* flash chromatography on a Biotage SP1 working with 50 mg of crude mixture and a 10 g silica gel Biotage® cartridge in a gradient of AcOEt in DCM (10 CV 5% AcOEt; 5 CV from 5% to 15% AcOEt, 10 CV 15% AcOEt).

2 a: 20 mg, ee: 95.3%, isolated yield: 40.0%. NMR and ms spectra in accordance with the proposed structure (see Supplementary).

3 b: 25 mg, ee: >99%, isolated yield: 50.0%.^{1}H NMR (400 MHz, $CDCl_3$) δ 5.87 (br s, 1H), 3.65 (br s, 1H), 2.74–2.35 (m, 4H), 2.35–2.23 (m, 1H), 2.14–1.98 (m, 1H), 1.96–1.66 (m, 3H), 1.57–1.46 (m, 1H), 1.24 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.41, 167.68, 127.03, 75.33, 40.93, 34.02, 31.73, 30.80, 28.71, 21.83, 19.83; ee: >99%; $[\alpha]_D^{22}$: −111.6° (c: 1.0 in $CHCl_3$);[28] ESI-MS: $[M+Na]^+$: 203.0.[19]

Using the enantiomer **1 b** as a substrate (30 mg scale), the *trans*-alcohol **3 b** was obtained as sole product following, again, the general protocol. **3 b**: yield: 91%; ee: >99%.

Enzymatic acetylation of substrate 2 b catalyzed by Amano lipase PS

Racemic substrate **2** (1040 mg, 1 eq) was dissolved in dry acetone (80 mM substrate, 70 mL reaction volume). Then, excess vinyl acetate (10% v/v, 14 eq) and Amano lipase PS on celite (1000 mg, 1% wt, catalytic amount) were added. The reaction was shaken at 45 °C, 200 rpm for six days. The enzyme was filtered away and the solvent was evaporated to afford a crude mixture of **2 a** and **4 b**. The two products were separated *via* flash chromatography (CH_2Cl_2: AcOEt gradient from 9 : 1 to 7 : 3) to give the subsequent products: **2 a**: 660 mg, 50% isolated yield, ee: 97.3%; $[\alpha]_D^{22}$: +173.4° (c: 1.0 in $CHCl_3$).[33] **4 b**: 407 mg, 39% isolated yield, ee: >99%; $[\alpha]_D^{22}$: −69.5° (c: 1.0 in $CHCl_3$).[33]

Chemical hydrolysis of 4b to 2b

Substrate **4b** (100 mg, 0.450 mmol, 1 eq) was dissolved in EtOH (0.5 M). Then, a 1 M aqueous solution of NaOH was added dropwise (0.675 mL, 0.675 mmol, 1.5 eq). The reaction was stirred at room temperature for 2 h, then quenched with a saturated aqueous solution of $NaHCO_3$ and the aqueous phase thus obtained was extracted with AcOEt (3x). The organic phase was then dried over Na_2SO_4 and evaporated *in vacuo* to give the desired product **2b** (0.377 mmol, 90.0% yield). $[\alpha]_D^{22}$: $-180.5°$ (c: 1.0 in $CHCl_3$).[28] ee (HPLC): 98.5%.NMR and ms spectra are in accordance with the proposed structures (see Supplementary).

Chemical re-oxidation of 2a to 1a

2-Iodoxybenzoic acid (IBX,[45] 0.289 mmol) was dissolved 0.4 M in DMSO. Then, a 0.3 M solution of **2a** (0.172 mmol) in DMSO was added. The reaction was stirred for 17 h at room temperature, then quenched with an aqueous saturated solution of $NaHCO_3$. The phase thus obtained was extracted with AcOEt (3x). The organic phase was dried over Na_2SO_4 and evaporated *in vacuo* to afford a yellow oil. (0.168 mmol, 97.9% yield). $[\alpha]_D^{22}$: $+102.9°$ (c: 1.0 in $CHCl_3$);[48] ee (HPLC): 96.2%. NMR and ms spectra in accordance with the proposed structure (see Supplementary)

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biocatalysis · Dehydrogenases · Enzymes · Lipases · Wieland-Miescher ketone

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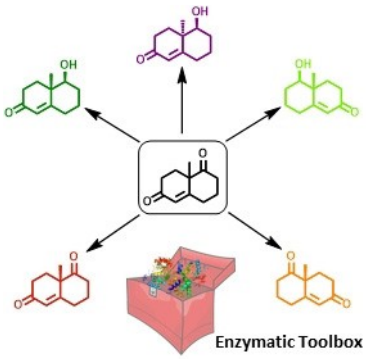
FULL PAPERS

A combination of different enzymatic activities and chemical protocols were exploited to access the two enantiomers of Wieland-Miescher ketone and three of its four alcohols derivatives as enantioenriched species. Specifically, the optimization of biotransformations of WM-ketone or of substrates (bio)chemically-prepared from it, mediated by hydroxysteroids dehydrogenases, short chain alcohol dehydrogenases and lipases, are described.



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1 – 8

Biocatalytic Approaches to the Enantiomers of Wieland–Miescher Ketone and its Derivatives

Special Collection

Synthesis of ω-Muricholic Acid by One-Pot Enzymatic Mitsunobu Inversion using Hydroxysteroid Dehydrogenases

Susanna Bertuletti,[a, b] Erica Elisa Ferrandi,[a] Daniela Monti,[a] Giovanni Fronza,[c] Ivan Bassanini,[a] and Sergio Riva*[a]

The biocatalyzed conversion of hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid) into ω-muricholic acid (3α,6α,7β-trihydroxy-5β-cholan-24-oic acid) has been obtained exploiting a small library of 7α- and 7β-HSDHs (hydroxysteroid dehydrogenases). The process has been optimized and performed avoiding the isolation of the 7-oxo intermediate using the appropriate coupled enzymes for the *in situ* cofactor regeneration. Moreover, the biocatalyzed reduction of 6,7-dioxolithocholic acid (3α-hydroxy-6,7-dioxo-5β-cholan-24-oic acid) was also investigated.

Introduction

Hyocholic acid (HCA, **1**, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid) is one of the major components of pig bile and is the only trihydroxylated bile acid that can be isolated from it.[1] Its structural characterization was described several decades ago, during the golden age of steroid chemistry,[2] and its trivial name as well as those of its cognate bile acids has been codified later on by Hofmann in a seminal paper specifically devoted to the nomenclature of these compounds.[3]

Muricholic acid is the trivial name for hyocholic acid isomers at C-6 (6β-OH, 7α-OH, α-muricholic acid) or at C-7 (6α-OH, 7β-OH, ω-muricholic acid) or both at C-6 and at C-7 (6β-OH, 7β-OH, β-muricholic acid). They account for ~35% of the total bile acid pool in mice, but both their isolation in pure form and their chemical syntheses are challenging, as it is reflected by their commercial costs from specialized suppliers. This is particularly the case for ω-muricholic acid (CAS number 6830-03-1), which is available at more than 250 USD for 1 mg (e.g., see in MolPort: https://www.molport.com/).

HCA and its derivatives can also be detected in human blood and urine and these molecules have been recently proposed as biomarkers for the early detection of severe metabolic disorders, such as type 2 diabetes mellitus.[4] Moreover, **1** might be considered as an interesting alternative starting material for the preparation of ursodeoxycholic acid (UDCA, **2**), presently synthesized on multiton scale per year from cholic acid (CA, **3**, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) *via* chenodeoxy-cholic acid (CDCA, **4**, 3α,7α-dihydroxy-5β-cholan-24-oic acid).[5] In this respect, one of the synthetic requirements is an efficient protocol for the α/β epimerization of the hydroxyl group at C-7.

Hydroxysteroid dehydrogenases (HSDHs) are a group of NAD(P)(H)-dependent dehydrogenases specifically devoted to the regioselective oxidation as well as to the regio- and stereoselective reduction of polyhydroxylated/ketonic steroids.[6] Their synthetic potential for the selective transformation of bile acids has been demonstrated in a series of papers published since the early eighties.[7]

Specifically, almost 30 years ago we described the α/β inversion of the C-7 hydroxyl of several bile acids, either in water (the free acids) or in biphasic systems (the corresponding methyl esters).[8]

As it is shown in Scheme 1 for the acids, it was a two-step procedure based on the HSDH-catalyzed oxidation of the C-7α OHs, the isolation of the corresponding 7-oxo derivatives, and their subsequent HSDH-catalyzed reduction to the expected C-7β OHs. To achieve these results, three HSDHs were employed: two 7α-HSDHs, the commercially available NAD(H)-dependent enzyme from *Escherichia coli* and the NADP(H)-dependent 7α- and 7β-HSDHs isolated by us from a *Clostridium* strain. When this protocol was applied to hyocholic acid (**1**), the expected 7-oxo derivative (**5**) was obtained, isolated, and characterized. However, compound **5** was the only 7-oxo bile acid intermediate that the 7β-HSDH available for us at that time failed to

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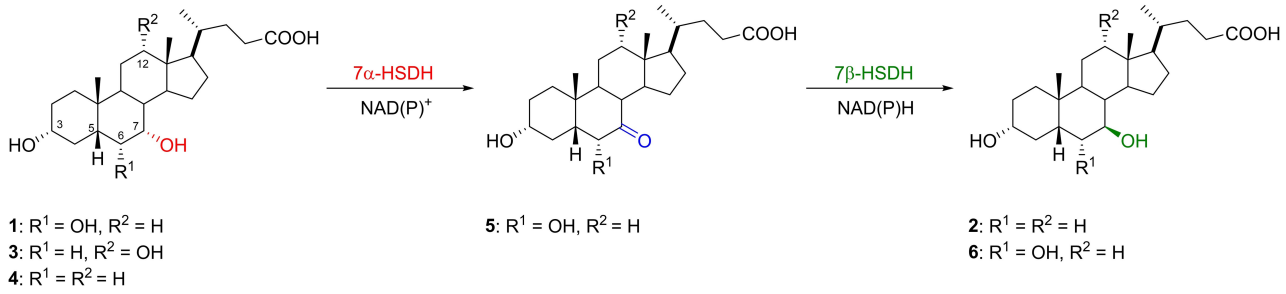


1: R^1 = OH, R^2 = H
3: R^1 = H, R^2 = OH
4: R^1 = R^2 = H

5: R^1 = OH, R^2 = H

2: R^1 = R^2 = H
6: R^1 = OH, R^2 = H

Scheme 1. Enzymatic stereoinversion of C-7 OH in a Mitsunobu-like approach.

reduce and the 7β-OH derivative **6**, that is ω-muricholic acid, could not be isolated.

In recent years, also exploiting metagenomic approaches, several new HSDHs became available to us, among which three novel 7α-HSDHs and four 7β-HSDHs (Table 1).[9] We thought that some of these new enzymes might have helped us to overcome the limitation that we encountered years ago and in the following we report on the obtained results.

Results and Discussion

Hyocholic acid (**1**) was submitted to the action of five different 7α-HSDHs (the two previously available[8] and three new enzymes, Table 1) with a suitable system for the *in situ* enzymatic regeneration of the oxidized cofactor $NAD(P)^+$. As expected, all these 7α-HSDHs were able to regioselectively and quantitatively oxidize **1**. The reactions were scaled up to 500 mg scale and the 7-oxo derivative **5** was isolated and characterized by NMR. Specifically, the location at carbon C-7 of the carbonyl group was shown by the proton and carbon correlation experiments, which allowed to recognize the CH(6)-CH(5)-C(10)-CH3(19) chain. Moreover, the value of 6.0 Hz of the vicinal coupling constant $J_{H5\text{-}H6}$, similar to that of hyocholic acid, indicated that the stereochemistry at C(5) and C(6) remained unchanged[8,10] (for more details, see the Experimental Part and the Supplementary Materials).

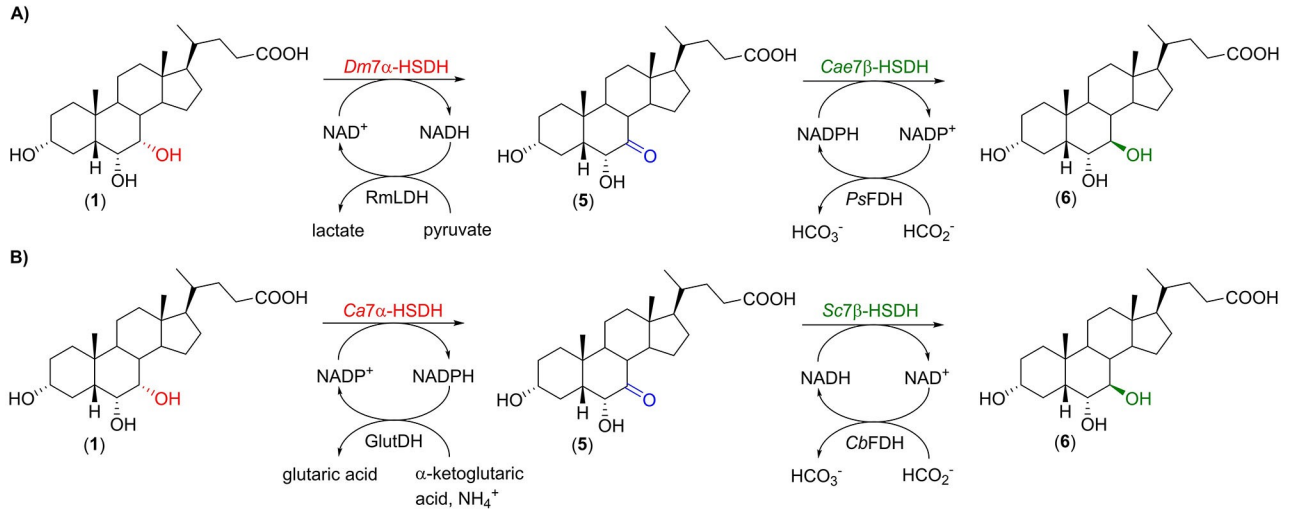
A similar preliminary screening of the enzymatic reduction of **5** with the available 7β-HSDHs (see Table 1) gave very positive results, as in all cases the formation of a more polar product was observed by TLC (data not shown). The reactions were thus scaled up to a 100–200 mg scale (see Experimental section) either with the NADP(H)-dependent 7β-HSDH from *Collinsella aerofaciens* (*Cae*7β-HSDH) or with the NAD(H)-dependent 7β-HSDH from *Stanieria cyanosphaera* (*Sc*7β-HSDH) and in both cases the quantitative conversion of **5** into a more polar product was observed, which could be isolated as a white powder simply by acidification of the water solution followed by filtration of the precipitate. Its structure was confirmed to be the expected 7β-OH derivative **6** (3α,6α,7β-trihydroxy-5β-cholan-24-oic acid) by NMR analysis. In fact, the configurational assignment of its H-6, H-7, and H-8 could be determined from the vicinal coupling constants $J_{H6\text{-}H7}$ and $J_{H7\text{-}H8}$. In hyocholic acid (**1**) the values of these coupling constants were 3.5 and 2.8 Hz, respectively, typical of an axial (H-6), equatorial (H-7) and axial (H-8) arrangement in rigid hydroxylated six-membered rings in a chair conformation.[10] In the product **6** the values of such coupling constants rose to 9.4 Hz, diagnostic for an axial orientation of all the three protons H-6, H-7 and H-8, thus confirming the β orientation of OH-7.

Looking for further improvements of this process, we considered the possibility of performing the 7α/β inversion of **1** into **6** avoiding the isolation of the 7-oxo derivatives **5**. The one-pot enzymatic epimerization of the C-7 OH of cholic acid (**3**) and/or chenodeoxycholic acid (**4**) has been studied since a long time. However, as it has been confirmed in detailed recent research reports,[11] the inversion could not be quantitative when using 7α-HSDHs and 7β-HSDHs with the same cofactor specificities (either both NAD(H)- or both NADP(H)-dependent enzymes) and the approximately 1 to 9 mixtures of 7α- and 7β-isomers were isolated.

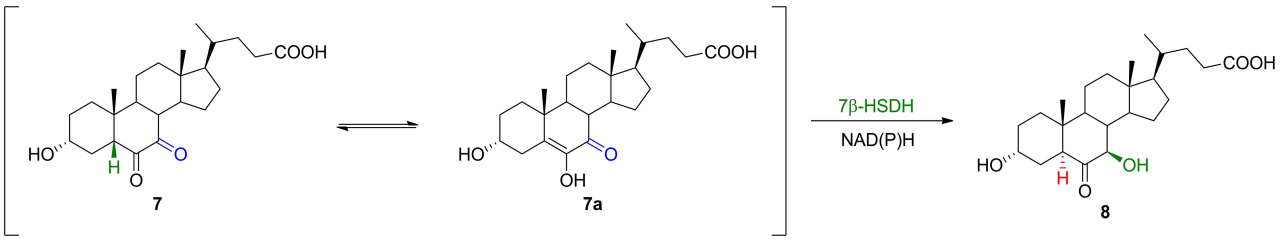
This "thermodynamic trap" could be overcome by exploiting enzymes with different cofactor specificities, as we reported years ago working on cholic acid.[12] By applying the same protocol to obtain the quantitative one pot stereoinversion of **1** into **6**, we considered both the options shown in Scheme 2, that is either catalyzing the oxidation of **1** with a NAD(H)-dependent 7α-HSDH and the reduction of the intermediate **5** with a NADP(H)-dependent 7β-HSDH (part A) or, on the contrary, the oxidation of **1** with a NADP(H)-dependent 7α-HSDH and the reduction of the intermediate **5** with a NAD(H)-dependent 7β-HSDH (part B). The thermodynamic driving force was given by the coupled enzymatic regeneration systems,

Table 1. 7α- and 7β-hydroxysteroid dehydrogenases employed in this study.[9]

Enzyme	Activity	Cofactor	Source
*Ca*7α-HSDH	7α-HSDH	NADP(H)	*Clostridium absonum*
*Dm*7α-HSDH	7α-HSDH	NAD(H)	*Deinococcus marmoris*
*Ec*7α-HSDH	7α-HSDH	NAD(H)	*Escherichia coli*
*Hh*7α-HSDH	7α-HSDH	NAD(H)	*Halomonas halodenitrificans*
Ng1-7α-HSDH	7α-HSDH	NAD(H)	Metagenome
*Bsp*7β-HSDH	7β-HSDH	NAD(H)	*Brucella* sp.
*Cae*7β-HSDH	7β-HSDH	NADP(H)	*Collinsella aerofaciens*
*Hh*7β-HSDH	7β-HSDH	NAD(H)	*Halomonas halodenitrificans*
*Rs*7β-HSDH	7β-HSDH	NAD(H)	*Rhodococcus sphaeroides*
*Sc*7β-HSDH	7β-HSDH	NAD(H)	*Stanieria cyanosphaera*



Scheme 2. One-pot stereoinversion of hyocholic acid (**1**) by C-7-OH epimerization to form ω-muricholic acid (**6**). A) NAD^+-based oxidizing system and NADPH-based reducing system; B) $NADP^+$-based oxidizing system and NADH-based reducing system.



Scheme 3. Keto-enolic tautomerism leading to C-5 epimerization and to the consequent change of the junction between the A and B steroid rings (from *cis* in **7** to *trans* in **8**).

exploiting enzymes with the same strict cofactor specificity of the coupled HSDH. Specifically, the two best performing 7β-HSDHs were employed, that is the NADP(H)-dependent *Cae*7β-HSDH for Scheme 2A and the NAD(H)-dependent *Sc*7β-HSDH for Scheme 2B.

The small-scale reactions (12.5 mM of the starting substrate **1**, total volume 1 mL) were monitored by TLC and, following derivatization, by GC-MS (see time evolution in GC-MS of one-pot 2B in the Supporting information). We were pleased to observe, in both cases, a complete conversion of **1** into **6**, without residual starting substrate **1** or ketonic intermediate **5**. The biotransformation shown in Scheme 2A was scaled up to 100 mg scale and the product was isolated again by acidification and filtration/extraction (see Experimental and/or Supplementary Materials for details). In this way an efficient and simple access to the rare ω-muricholic acid **6**[13] was optimized.

As a side investigation, in order to get useful information on the performances of our enzymes with compounds that might be obtained as intermediates *en route* of the conversion of hyocholic acid **1** into ursodeoxycholic acid (**2**), we submitted to the action of 7β-HSDHs the commercially available 6,7-dioxo derivative (**7**) (Scheme 3). Once again, to our satisfaction, all the five 7β-HSDHs were active on **7**. The reaction was scaled up to a 40 mg scale using *Cae*7β-HSDH and product **8** was isolated and characterized. NMR analysis confirmed the 7β-configuration of the obtained alcohol ($J_{H7\text{-}H8}$ of 9.5 Hz), but, to our surprise, the A and B ring showed a *trans* junction instead of the original *cis* configuration, i.e., the hydrogen atom at C(5) occurred *anti* with respect to the CH_3(19). A possible explanation was given by the NMR analysis of the starting diketone **7**, which, at least in DMSO solution, exists in the ketoenolic form **7a** (C(5)=C(OH)(6)-CO(7)-CH(8)). The double bond occurs between carbons C(5) and C(6), as indicated by the observation of the proton-carbon long range coupling constants CH(3)/C(5) and CH_3(19)/C(5) in the HMBC experiment. The enzymatic reaction reducing the carbonyl at C-7 to the correspondent hydroxyl derivative, allows the enolic double bond rearrangement to the C-6 oxo form, resulting in the formation of the more stable *trans* ring fusion of the cyclohexane rings (Scheme 3 and Figure 1). The conformational instability of the A–B ring fusion of 6-oxo steroids has been previously observed, as it has been reported, for instance in a recent patent.[14]

Conclusion

The one-pot biocatalyzed conversion of hyocholic acid (**1**, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid) into the rare ω-

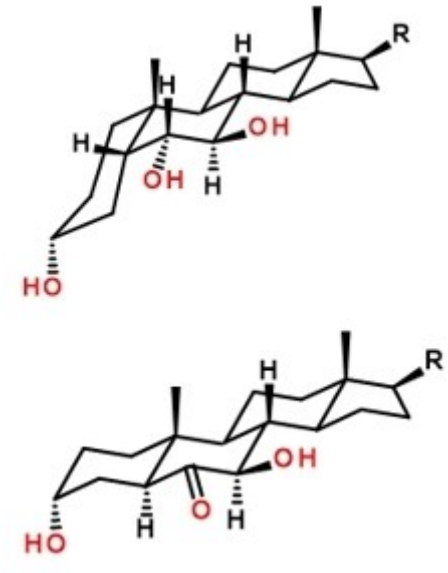


Figure 1. Chair representation of ω-muricholic acid (**6**, *cis* A–B ring junction) and of compound **8** (*trans* A–B ring junction).

muricholic acid (**6**, 3α,6α,7β-trihydroxy-5β-cholan-24-oic acid) has been obtained by exploiting a small library of new 7α- and 7β-HSDHs. It is an additional example of concomitant enzymatic oxidations and reductions running together and, thanks to the appropriate choice of the coupled enzymes for the *in situ* cofactor regeneration, performing a quantitative Mitsunobu-like stereoinversion of the C-7 OH of hyocholic acid.

Experimental Section

General

Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid) was kindly provided from Dipharma Francis S.r.l. (Baranzate, Italy). 3α-hydroxy-6,7-dioxo-5β-cholan-24-oic acid (**7**) was purchased from Steraloids Inc. (Newport, USA). L-glutamic dehydrogenase (GlutDH) from *Proteus* sp., lactate dehydrogenase (RmLDH) from rabbit muscle, and formate dehydrogenase from *Candida boidinii* (*Cb*FDH) were from Merck (Darmstadt, Germany). The NADP(H) dependent FDH from the methylotrophic bacterium *Pseudomonas* sp. 101 (*Ps*FDH)[15] was a kind gift from Prof. Tishkov (M.V. Lomonosov Moscow State University). All other reagents and solvents were purchased from Merck and used without further purification, unless elsewhere stated.

Reactions were monitored *via* TLC (thin layer chromatography, eluent $CHCl_3$: MeOH: AcOH = 9:1:0.02). on pre-coated glass plates silica gel 60 with fluorescent indicator UV_{254} and treated with Komarowki's reagent (an oxidizing solution of 16 mg L^{-1} 4-hydroxybenzaldehyde, 2.5% (w/v) H_2SO_4, 2.5% (v/v) water, and MeOH).

Abbreviations

HSDH: Hydroxysteroid Dehydrogenase; U: enzymatic units.

Enzyme Preparation

HSDHs expression and purification

Expressions and purifications of the HSDHs were carried out as previously described.[9]

Activity assays

Dehydrogenase activities of HSDHs and FDHs were determined spectrophotometrically by measuring the reduction of $NAD(P)^+$ at 340 nm (ε: 6.22 $mM^{-1}cm^{-1}$), while the activity of RmLDH was measured by following the oxidation of NAD(P)H at the same wavelength. The activity of GlutDH was indicated by Merck and thus not tested. Assays were carried out in polyethylene cuvettes at room temperature by adding the opportune amount of the purified dehydrogenase to the following assay mixtures (1 mL final volume):

HSDH assay: 2.5 mM substrate (cholic acid for 7α-HSDHs, ursocholic acid for 7β-HSDHs); 50 mM potassium phosphate buffer, pH 9.0; 0.20 mM $NAD(P)^+$.

FDH assay: 200 mM ammonium formate; 50 mM potassium phosphate buffer, pH 7.0; 0.20 mM $NAD(P)^+$.

RmLDH assay: 10 mM sodium pyruvate; 50 mM potassium phosphate buffer, pH 7.0; 0.20 mM NAD^+.

One unit (U) is defined as the enzyme activity that reduces/oxidizes 1 μmol of NAD(P)(H) per min under the assay conditions described above.

Analytical methods

Usually, TLC analyses were sufficient to determine whether a reaction on a bile acid was concluded or not. However, all the tested eluent mixtures were not capable to separate **1** and **6**. Therefore, at scheduled times, samples from the one-pot reactions (100 μL) were lyophilized, resuspended in MeOH to 1 mM concentration, and treated overnight with catalytic acetyl chloride to obtain the corresponding methyl esters. The derivatized samples were then submitted to GC-MS analysis.

GC-MS analyses were performed using an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm) on a Finnigan TRACE DSQ GC/MS instrument (ThermoQuest, San Jose, CA). Elution conditions: 300 °C, 15 min; flow rate: 1.0 mL min^{-1}; inlet temperature: 280 °C; ion source temperature: 280 °C; MS transfer line temperature: 280 °C. Retention times: (methyl ester of **5**): 9.3 min; (methyl ester of **1**): 11.9 min; (methyl ester of **6**): 11.2 min.

The nature of the obtained compounds was confirmed by means of ^{1}H-NMR, and ESI-MS spectrometry.

The samples were dissolved in deuterated dimethylsulfoxide, and their NMR spectra were acquired on a Bruker AV 400 or AV 500 instruments at 305 K. Since the spectra were quite complex only a partial analysis was performed. The assignments reported in the description of the spectra for each compound were based on the proton homonuclear experiments COSY and NOESY and on the proton-carbon heteronuclear experiments HSQC and HMBC. The spectra were also acquired after addition of D_2O to the solution to remove the hydroxyl protons thus allowing a more precise measurement of the coupling constants along the fragment C5-C6-C7-C8. The multiplicity of the carbon nuclei was determined by the DEPT 135 experiment which allows to distinguish among quaternary (s), methine (d), methylene (t) and methyl (q) carbons.

ESI-MS spectra were recorded on a Bruker Esquire 3000 PLUS instrument (ESI Ion Trap LC/MSn System), equipped with an ESI source and a quadrupole ion trap detector (QIT). The samples were dissolved in methanol to 1–2 $g\,L^{-1}$ and then directly syringed in the ESI-MS at 4 $\mu L\,min^{-1}$ rate. The analyses were performed in positive mode. The acquisition parameters were optimized as such: 4.5 kV needle voltage, 10 $L\,h^{-1}$ N_2 flow rate, 40 V cone voltage, trap drive set to 46, 115.8 V capillary exit, 13000 (m/z) s^{-1} scan resolution over the 35–900 m/z mass/charge range, source temperature 250 °C.

Characterization of commercially available 1 and 7

Compound 1

^{1}H NMR (DMSO-d_6), δ: 11.91 (1H, s br, COOH), 4.27 (1H, d, J = 5.0, OH-3), 4.15 (1H, s br, OH-6), 3.84 (1H, d, J = 2.9 Hz, OH-7), 3.60 (1H, dd, $J_{5,6}$ = 5.3, $J_{6,7}$ = 3.4 Hz, H-6), 3.58 (1H, ddd, $J_{6,7}$ = 3.4, $J_{7,8}$ = 2.8, $J_{5,7}$ = 1.0 Hz, H-7), 3.13 (1H, m, H-3), 0.89 (3H, d, J = 6.5 Hz, CH_3-21), 0.84 (3H, s, CH_3-19), 0.61 (3H, s, CH_3-18); ^{13}C NMR (DMSO-d_6), δ 174.8 (1C, s, COOH), 70.9 (1C, d, C-6), 70.4 (1C, d, C-3), 68.5 (1C, d, C-7), 55.5 (1C, d, C-17), 49.9 (1C, d, C-14), 48.0 (1C, d, C-5), 42.0 (1C, s, C-13), 39.3 (1C, t, C-12), 38.2 (1C, d), 35.6 (1C, t, C-1), 35.5 (1C, s, C-10), 34.9 (1C, d, C-20), 32.5 (1C, t), 32.2 (1C, d, C-9), 30.72 (1C, t), 30.66 (1C, t), 30.62(1C, t), 27.7 (1C, t), 23.1 (1C, q, CH_3-19), 23.0 (1C, t), 20.3 (1C, t), 18.1 (1C, q, CH_3-21), 11.6 (1C, q, CH_3-18). ESI-MS: $[m+Na]^+$: 431.2.

Compound 7

^{1}H NMR (DMSO-D_6), δ: 11.94 (1H, s br, COOH), 7.42 (1H, s, OH-6), 4.33 (1H, d, J = 3.3 Hz, OH-3), 3.96 (1H, m, H-3), 2.90 (1H, dt, J = 15.6, 2.6 Hz, H-4_{eq}), 2.05 (1H, dd, J = 15.6, 3.4 Hz, H-4_{ax}), 2.30 (1H, dd, J = 12.4, 10.1 Hz, H-8), 1.10 (3H, s, CH_3-19), 0.90 (3 h, d, J = 6.6 Hz, CH_3-21), 0.66 (3H, s, CH_3-18); ^{13}C NMR (DMSO-D_6), δ: 195.8 (1C, s, CO-7), 174.8 (1C, s, COOH), 142.9 (1C, s, C-5), 135.3 (1C, s, C-6), 64.4 (1C, d, C-3), 54.0 (1C, d, C-17), 50.1 (1C, d, C-14), 49.2 (1C, d, C-9), 43.7 (1C, d, C-8), 42.9 (1C, s, C-13), 38.3 (1C, t, C-12), 36.7 (1C, s, C-10), 34.7 (1C, d, C-20), 32.0 (1C, t, C-1), 30.8 (1C, t), 30.7 (1C, t), 30.1 (1C, t, C-4), 27.94 (1C, t), 27.91 (1C, t), 26.0 (1C, t), 20.4 (1C, t), 18.3 (1C, q, CH_3-21), 16.9 (1C, q, CH_3-19), 11.8 (1C, q, CH_3-18). ESI-MS: $[m+H]^+$: 405.3. ESI-MS: $[m+Na]^+$: 427.3.

Synthetic chemistry

Screening of 7α-HSDHs for the regioselective oxidation of 1 to 5

The reactions catalyzed by 7α-HSDHs were coupled with a lactate/LDH system to regenerate NADH and to α-ketoglutarate/GlutDH to regenerate NADPH. For the initial activity screening of the whole library of 7α-HSDHs, the general protocol was as follows: 12.5 mM **1**; 50 mM sodium pyruvate/α-ketoglutarate; 0.5 $U\,mL^{-1}$ LDH/GlutDH; 0.4 mM $NAD(P)^+$; 3.4 $U\,mL^{-1}$ HSDH; 50 mM potassium phosphate buffer, pH 8.0 (total volume: 1 mL). Additionally, the reaction catalyzed by *Hh*7α-HSDH was performed in the presence of 0.4 M NaCl. The mixtures were shaken at 25 °C and 100 rpm for 24 to 48 h and monitored over time *via* TLC (eluent $CHCl_3$: MeOH: AcOH = 9:1:0.02). Reaction conversions were evaluated by TLC analyses, showing that the reactions were mostly complete in 1 h.

Scale-up and isolation of 5

The reactions catalyzed by *Ca*7α-HSDH, *Dm*7α-HSDH, and *Ec*7α-HSDH were scaled-up to 100–500 mg following the aforementioned general protocol till full conversion was achieved (shown by TLC, eluent $CHCl_3$: MeOH: AcOH = 9:1:0.02). The product was recovered *via* precipitation by acidification of the reaction mixture and subsequent filtration to afford a white amorphous powder in quantitative yield. ^{1}H NMR (DMSO-D_6), δ: 11.88 (1H, s br, COOH), 4.56 (1H, d, J = 4.9 Hz, OH-6), 4.48 (1H, dd, $J_{5,6}$ = 6.0, $J_{6,8}$ = 1.0 Hz, H-6), 4.43 (1H, d, J = 4.8 Hz, OH-3), 3.28 (1H, m, H-3), 2.45 (1H, td, $J_{8,9}$ = $J_{8,14}$ = 11.3, $J_{6,8}$ = 1.0 Hz, H-8), 1.87 (1H, m, H-5), 1.16 (3H, s, CH_3-19), 0.88 (3H, d, J = 6.5 Hz, CH_3-21), 0.61 (3H, s, CH_3-18); ^{13}C NMR (DMSO-D_6), δ: 211.4 (1C, s, CO-7), 174.8 (1C, s, COOH), 73.6 (1C, d, C-6), 69.1 (1C, d, C-3), 54.2 (1C, d, C-17), 52.2 (1C, d, C-5), 48.6 (1C, d), 46.8 (1C, d, C-8), 42.4 (1C, d, C-9), 42.3 (1C, s, C-13), 38.5 (1C, t, C-12), 35.3 (1C, s, C10), 34.7 (1C, d, C-20), 34.1 (1C, t), 30.7 (1C, t), 30.6 (1C, t), 30.4 (1C, t), 29.8 (1C, t), 27.8 (1C, t), 24.1 (1C, t), 23.2 (1C, q, CH_3-19), 21.2 (1C, t), 18.2 (1C, q, CH_3-21), 11.8 (1C, q, CH_3-18). ESI-MS: $[m+Na]^+$: 429.2.[7]

Screening of 7β-HSDHs for the stereo- and regioselective reduction of 5 to 6

The reactions catalyzed by 7β-HSDHs were coupled with a formate/FDH system to regenerate NAD(P)H. For the initial activity screening of the whole library of 7β-HSDHs, the general reaction protocol was as follows: 12.5 mM **5**; 75 mM ammonium formate; 0.5 $U\,mL^{-1}$ FDH; 0.4 mM $NAD(P)^+$; 3.4 $U\,mL^{-1}$ HSDH; 50 mM potassium phosphate buffer, pH 8.0 (total volume: 1 mL). The reaction catalyzed by *Hh*7β-HSDH was performed in the presence of 0.4 M NaCl. The mixtures were shaken at 25 °C and 100 rpm for 24 to 48 h and monitored over time *via* TLC (eluent $CHCl_3$: MeOH: AcOH = 9:1:0.02). Reaction conversions and were evaluated by TLC analyses, showing that the reactions were mostly complete in less than 6 h.

Scale-up and isolation of 6

The reactions catalyzed by *Cae*7β-HSDH and *Sc*7β-HSDH were scaled-up to 100–200 mg following the aforementioned general protocol till full conversion was shown by TLC monitoring (eluent $CHCl_3$: MeOH: AcOH = 9:1:0.02). The product was recovered *via* precipitation by acidification of the reaction mixture and subsequent filtration to afford a white amorphous powder in quantitative yield. ^{1}H NMR (DMSO-D_6), δ: 11.72 (1H, s br, COOH), 4.42 (1H, s br, OH), 4.27 (1H, s br, OH), 3.88 (1H, d, J = 5.4 Hz, OH-7), 3.50 (1H, dd, $J_{6,7}$ = 9.4, $J_{5,6}$ = 5.5 Hz, H-6), 3.25 (1H, m, H-3), 3.07 (1H, t, $J_{6,7}$ = $J_{7,8}$ = 9.4 Hz, H-7), 0.88 (3H, d, J = 6.5 Hz, CH_3-21), 0.86 (3H, s, CH_3-19), 0.61 (3H, s, CH_3-18); ^{13}C NMR (DMSO-D_6), δ: 174.5 (1C, s, COOH, 74.5 (1C, d, C-7), 72.2 (1C, d, C-6), 70.2 (1C, d, C-3), 56.1 (1C, d), 54.9 (1C, d), 48.1 (1C, d), 43.5 (1C, s), 41.5 (1C, d), 40.0 (1C, t), 39.3 (1C, d), 35.4 (1C, t), 35.06 (1C, s), 35.04 (1C, d), 31.0 (2 C, t), 30.6 (1C, t), 30.3(1C, t), 28.3 (1C, t), 26.7 (1C, t), 23.7 (1C, q, CH_3-19), 21.2 (1C, t), 18.5 (1C, q, CH_3-21), 12.3 (1C, q, CH_3-18). ESI-MS: $[m+Na]^+$: 431.2.

One-pot stereoinversion of 1 to afford 6

Two different one-pot systems were tested on analytical scale (1 mL) for the C-7 stereoinversion of hyocholic acid (see Scheme 2A and B). A): 12.5 mM substrate **1**; 2 $U\,mL^{-1}$ *Dm*7α-HSDH; 50 mM sodium pyruvate; 0.5 $U\,mL^{-1}$ RmLDH; 0.4 mM NAD^+; 4 $U\,mL^{-1}$ *Cae*7β-HSDH; 75 mM ammonium formate; 0.5 $U\,mL^{-1}$ *Ps*FDH; 0.4 mM $NADP^+$; 50 mM potassium phosphate buffer, pH 8.0. B): 12.5 mM substrate **1**; 2 $U\,mL^{-1}$ *Ca*7α-HSDH; 50 mM α-ketoglutarate; 0.5 $U\,mL^{-1}$ GlutDH; 0.4 mM $NADP^+$; 4 $U\,mL^{-1}$ *Sc*7β-HSDH; 75 mM ammonium formate; 0.5 $U\,mL^{-1}$ *Cb*FDH; 0.4 mM NAD^+; 50 mM potassium phosphate buffer, pH 8.0. The mixtures were shaken at 25 °C and 100 rpm for 24 to 48 h and monitored over time *via* GC-MS. Reaction conversions were evaluated by GC-MS following derivatization.

Scale-up of one-pot stereoinversion B

The reaction catalyzed by *Ca*7α-HSDH and *Sc*7β-HSDH was scaled-up to 100 mg scale following the aforementioned one-pot B protocol and stopping the reaction at full conversion (24 h, monitored over time by GC-MS). The product **6** was recovered via precipitation by acidification of the reaction mixture and subsequent filtration to afford a white amorphous powder in quantitative yield. ESI-MS: $[m+Na]^+$: 431.2; $[m+K]^+$: 447.3. $[\alpha]_D^{25}$: +32.5° (c: 0.5 in EtOH).

Screening of 7β-HSDHs for the stereo- and regioselective reduction of 7

The reductions of the diketone **7** catalyzed by 7β-HSDHs were coupled with a formate/FDH system to regenerate NAD(P)H as follows: 12.5 mM **7**; 75 mM ammonium formate; 0.5 U mL^{-1} FDH; 0.4 mM NAD(P)$^+$; 4 U mL^{-1} HSDH; 50 mM potassium phosphate buffer, pH 8.0 (total volume: 1 mL). The reaction catalyzed by *Hh*7β-HSDH was performed in the presence of 0.4 M NaCl. The mixtures were shaken at 25 °C and 100 rpm for 24 to 48 h and monitored over time *via* TLC (eluent $CHCl_3$: MeOH: AcOH = 9:1:0.02). Reaction conversions were evaluated by TLC analyses, showing that the fastest reactions (i.e., those catalyzed by *Cae*7β-HSDH and *Sc*7β-HSDH) were complete in three hours.

Scale-up of the enzymatic reduction of 7 catalyzed by *Cae*7β-HSDH and isolation of 8

The reduction of **7** catalyzed by *Cae*7β-HSDH was repeated on a 40 mg scale, allowing the isolation and structural characterization of product **8**. ^{1}H NMR (DMSO-D_6), δ: 11.49 (1H, s br, COOH), 4.31 (1H, d, J = 5.0 Hz, OH-7), 4.29 (1H, s br, OH-3), 3.90 (1H, m, H-3), 3.78 (1H, dd, J = 9.5, 5.0 Hz, H-7), 2.68 (1H, dd, J = 12.3, 3.2 Hz, H-5), 1.50 (1H, m H-8), 0.89 (3H, d, J = 6.5 Hz, CH_3-21), 0.61 (3H, s, CH_3-18), 0.58 (3H, s, CH_3-19); ^{13}C NMR (DMSO-D_6), δ: 212.2 (1C, CO-6), 174.8 (1C, COOH), 78.3 (1C, d, C-7), 63.0 (1C, d, C-3), 56.1 (1C, d, C-14), 54.7 (1C, d, C-17), 51.3 (1C, d, C-9), 48.6 (1C, d, C-5), 45.8 (1C, d, C-8), 43.2 (1C, s, C-13), 40.6 (1C, s, C-10), 39.2 (1C, t, C-12), 34.8 (1C, d, C-20), 31.5 (1C, t, C-1), 30.8 (1C, t), 30.7 (1C, t), 27.9 (1C, t), 27.7 (1C, t), 27.5 (1C, t), 26.2 (1C, t), 20.7 (1C, t), 18.2 (1C, q, CH_3-21), 12.2 (1C, q, CH_3-19), 11.9 (1C, q. CH_3-18). ESI-MS: $[m+H]^+$: 407.2; $[m+Na]^+$: 429.2; $[m+K]^+$: 445.2.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: hyocholic acid · ω-muricholic acid · epimerization · one-pot reaction · hydroxysteroid dehydrogenases

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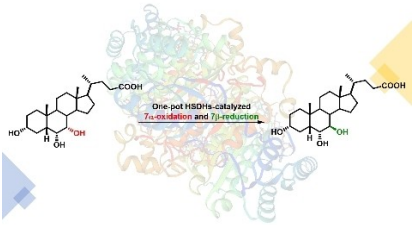

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1 – 7

Synthesis of ω-Muricholic Acid by One-Pot Enzymatic Mitsunobu Inversion using Hydroxysteroid Dehydrogenases

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