UNIVERSITÀ DEGLI STUDI DELL'INSUBRIA

DOTTORATO IN BIOTECNOLOGIE

Scuola di Dottorato in Scienze Biologiche e Mediche

XXIV CICLO

NEW MICROBIAL HYDROXYSTEROID DEHYDROGENASES AND THEIR SYNTHETIC APPLICATION FOR THE SELECTIVE MODIFICATION OF BILE ACIDS

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Anno accademico: 2010/2011

Abstract

Hydroxysteroid dehydrogenases (HSDHs), mainly obtained from bacterial sources, have been widely employed for the regio- and stereoselective oxidoreduction of the hydroxyl-keto groups of steroids, bile acids and their derivatives. In particular they might be suitable biocatalysts for the industrial synthesis of bile acids derivatives of pharmacological interest such as ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid).

Aim of this work was i) to investigate a new enzymatic process for the synthesis of UDCA; ii) to develop new cofactor regeneration systems to be coupled to HSDHs-catalyzed reactions; iii) to clone and overexpress in *E. coli* new HSDHs suitable for the modification of bile acids up to an industrial level.

Specifically, the preparative-scale HSDHs-catalyzed one-pot enzymatic synthesis of 12-ketoursodeoxycholic acid (3α,7β-dihydroxy-12-oxo-5β-cholanoic acid), a key intermediate for the synthesis of ursodeoxycholic acid, from cholic acid has been investigated. This goal has been achieved by alternating oxidative and reductive steps in a one-pot system and employing HSDHs with different cofactor specificity. To provide the necessary driving force to opposite reactions (i.e., oxidation and reduction) acting concurrently on different sites of the same substrate molecule, suitable cofactor regeneration systems were coupled to these reactions. However, due to a limited cofactor specificity of some of the enzymes used, an undesired reaction equilibrium was established resulting in by-products formation. This problem was overcome by uncoupling the oxidative and reductive biocatalysts (**Paper I**).

Moreover, a new laccase/mediator system for $NAD(P)^+$ cofactor regeneration in HSDHs-catalyzed oxidations has been developed. This system has been successfully applied in aqueous, buffered reactions (space-time yield of 2.35 g L^{-1} h^{-1}) and in biphasic systems (space-time yields of up to 27.47 g L^{-1} h^{-1}), demonstrating to be very efficient, to have high stability, to tolerate solvents, and to be simple to employ (**Paper II**).

Finally, NADPH-dependent 7α- and 7β-hydroxysteroid dehydrogenases (7α-HSDH and 7β-HSDH) from *Clostridium absonum* were cloned and overexpressed in recombinant form in *E. coli*. The enzymes were further

characterized from a functional and a kinetic point of view, demonstrating that both of them, in suitable bioconversion conditions, could be promising candidates for further applications in the epimerization reaction of bile acids at the C-7 position (**Paper III**).

Papers included in this thesis*

Paper I: Monti D, Ferrandi EE, Zanellato I, Hua L, Polentini F, Carrea G, Riva S. "One-pot multienzymatic synthesis of 12-ketoursodeoxycholic acid: subtle cofactor specificities rule the reaction equilibria of five biocatalysts working in a row." *Adv. Synth. Catal.* **351**:1303-1311 (2009).

Paper II: Ferrandi E.E., Monti D., Patel I., Kittl R., Haltrich D., Riva S. and Ludwig R. "Exploitation of a Laccase/Meldola's blue system for NAD$^+$ coenzyme regeneration in hydroxysteroid dehydrogenase-catalyzed oxidations." In preparation

Paper III: Ferrandi E.E., Bertolesi G. M., Polentini F., Negri A., Riva S., Monti D. "In search of sustainable chemical processes: cloning, recombinant expression and functional characterization of the 7α- and 7β-hydroxysteroid dehydrogenases from *Clostridium absonum*." Accepted by Applied Microbiology and Biotechnology.

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Introduction

Industrial Biotechnology, i.e., the application of biotechnology for the processing and production of chemicals, materials, and energy, is having an increasingly important role in the development of a sustainable, bio-based economy at a European level. Beside biotechnological applications in sectors such as food and feed, paper and pulp, textiles and energy, easy access to building blocks and materials, that were only affordable before by laborious routes or not at all, could be provided to the chemical industry by Industrial Biotechnology processes (Schmid A et al. 2001).

Biocatalysis, in the framework of Industrial Biotechnology, is considered a key component for the development of a sustainable chemistry in Europe by the European Commission, CEFIC and Biotech Industries Association (http://www.suschem.org/). Biocatalysis involves the use of enzymes' special ability to solve problems in the pharmaceutical field and in fine chemistry. Enzymes are very attractive for industrial purposes because of their high turnover numbers (up to 10^7 s^{-1}) and their extraordinary selectivity. Moreover, enzymes can be used under mild conditions (temperature around 30°C at ambient pressure) and in aqueous systems, thus limiting the use of organic solvents and reducing the environmental impact. Biocatalysis can also help the shortening of synthetic routes, avoiding protection group chemistry, and reduce by-products formation. This may result effective in lowering operation time and costs as well (Hollmann F et al. 2011).

In recent years, biocatalysis has gained significant attention thanks to the fact that the number of available enzymes is continuously increasing. Moreover, protein engineering, by improving enzyme properties, allows to overcome limitations for synthetic applications of the enzymes found in Nature.

Up to now, more than 100 industrial enzymatic processes have been introduced (Strohmeier GA et al. 2011). Large-scale examples include the preparation of semisynthetic penicillins catalyzed by penicillin amidases and the exploitation of nitrile hydratase to catalyze the hydration of acrylonitrile into acrylamide (Zaks A et al. 2001).

The development of a biocatalytic process requires the identification and, in some cases, the improvement, of one or more suitable biocatalysts, and the set-up of an economically feasible bioconversion. Therefore, the input of many different specialists, such as biochemists, microbiologists, organic chemists and chemical engineers, is needed in most cases. The economical feasibility of a

biocatalytic process depends on several factors, first of all the availability of an highly efficient and stable biocatalyst. To limit enzyme consumption, some biocatalysts are used in immobilized form as heterogeneous catalysts that can be recovered and reused. However, homogeneous processes that make use of suspended cells or recombinant enzymes that are sufficiently inexpensive to permit single use have been developed as well.

Many biocatalytic processes are based on bioconversions catalyzed by cofactor-independent enzymes, such as hydrolases and isomerases. Specifically, hydrolytic enzymes, e.g., esterases, lipases and proteases, have been widely applied to the preparation of enantiopure compounds from racemic or diastereomeric mixtures. These enzymes are often active even in anhydrous organic solvents, thus allowing their exploitation not only in the natural reaction direction, i.e., hydrolysis, but also in the reverse synthetic direction by means of either reverse hydrolysis or transesterification mechanisms.

However, many synthetically practical enzymatic reactions require cofactors, whose regeneration from its reaction by-product is necessary in order to develop an economically and industrially feasible process. For example, the cost of NAD^+ is of around 1,500 euros per kilogram and that of $NADP^+$ around 6,000 euros per kilogram. The reduced forms NADH and NADPH are considerably more expensive than the oxidized ones. Nevertheless, in combination with suitable regeneration systems, various cofactor-dependent reactions have been applied on preparative or industrial scale.

In this respect, enzyme belonging to the oxidoreductases class, such as dehydrogenases, monooxygenases, dioxygenases, oxidases, peroxidases, are currently receiving increasing interest for their synthetic application at an industrial level. The reactions catalyzed by oxidoreductases are numerous and comprise oxidation of hydroxyl, aldehyde and keto groups, oxidation of primary and secondary amines, hydroxylation of aromatic or non-activated carbon atoms, dehydrogenation of carbon-carbon single bonds, heteroatom oxygenation, Baeyer-Villiger oxidation, and double bond epoxidation. Reduction reaction of aldehydes, ketones, carboxylic acids and double and triple carbon-carbon bonds can be catalyzed as well. A wide array of cofactors may be involved in oxidoreductases-catalyzed reactions, such as $NAD(P)^+$, cytochrome, quinone or similar compounds, and flavin. Synthetic applications of isolated NAD(P)H-dependent oxidoreductases, e.g., dehydrogenases and enoate reductases, are most likely to be developed thanks to the existence of different strategies for the regeneration of these cofactors (Schmid A et al. 2001).

This Thesis deals with the biocatalytic application of a selected class of NAD(P)H-dependent oxidoreductases, hydroxysteroid dehydrogenases.

The work done during this Ph.D was performed at the ICRM-CNR (Milano, Italy) and supported by Prodotti Chimici e Alimentari S.p.A (PCA) (Basaluzzo (AL), Italy), a world leader company in the production of bile acids for pharmacological applications. Specifically, PCA produces ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid), the first-choice drug used in the treatment of cholesterol gallstones, by a ton-scale chemical synthesis. However, this route is not completely satisfactory because of the lengthy protocol, the low yields and the use of polluting substances. The biocatalytic routes herein investigated, using enzymes such as hydroxysteroid dehydrogenases (HSDHs), could be valid alternatives in order to shorten the synthesis, increase the yield of UDCA and reduce the environmental impact. The work described in this Thesis is helping PCA in introducing biocatalytic methods in the synthesis of UDCA (and possibly in that of other bile acid derivatives) at an industrial level.
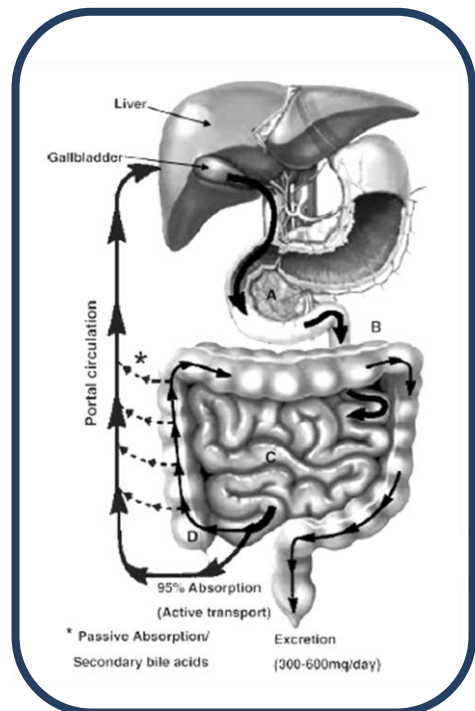
BILE ACIDS

Structure and biosynthesis of bile acids

Physiological functions of bile acids

Bile acids applications

Structure and biosynthesis of bile acids

Bile acids are polycyclic molecules belonging to the steroids family and characterized by the presence of a short aliphatic side chain with a terminal carboxylate group linked to the rigid steroid nucleus (Fig. 1). The latter is formed by a cyclopentane ring (designated as D ring) fused to three six-member rings (designated as A, B and C rings). In higher vertebrates, because the A and B rings are fused in a *cis* configuration, the steroid nucleus is curved and presents a convex hydrophobic surface (face β) and a concave hydrophilic surface (face α). This, coupled to the presence of the negatively charged side chain, gives a strong amphiphilic nature to these molecules, from which the well-known detergent properties arise (Mukhopadhyay S and Maitra U 2004).
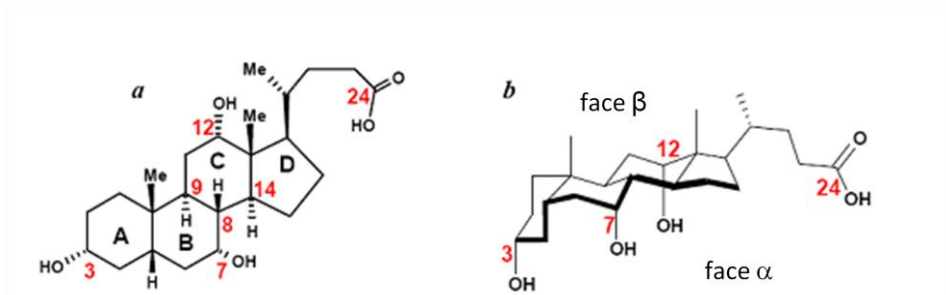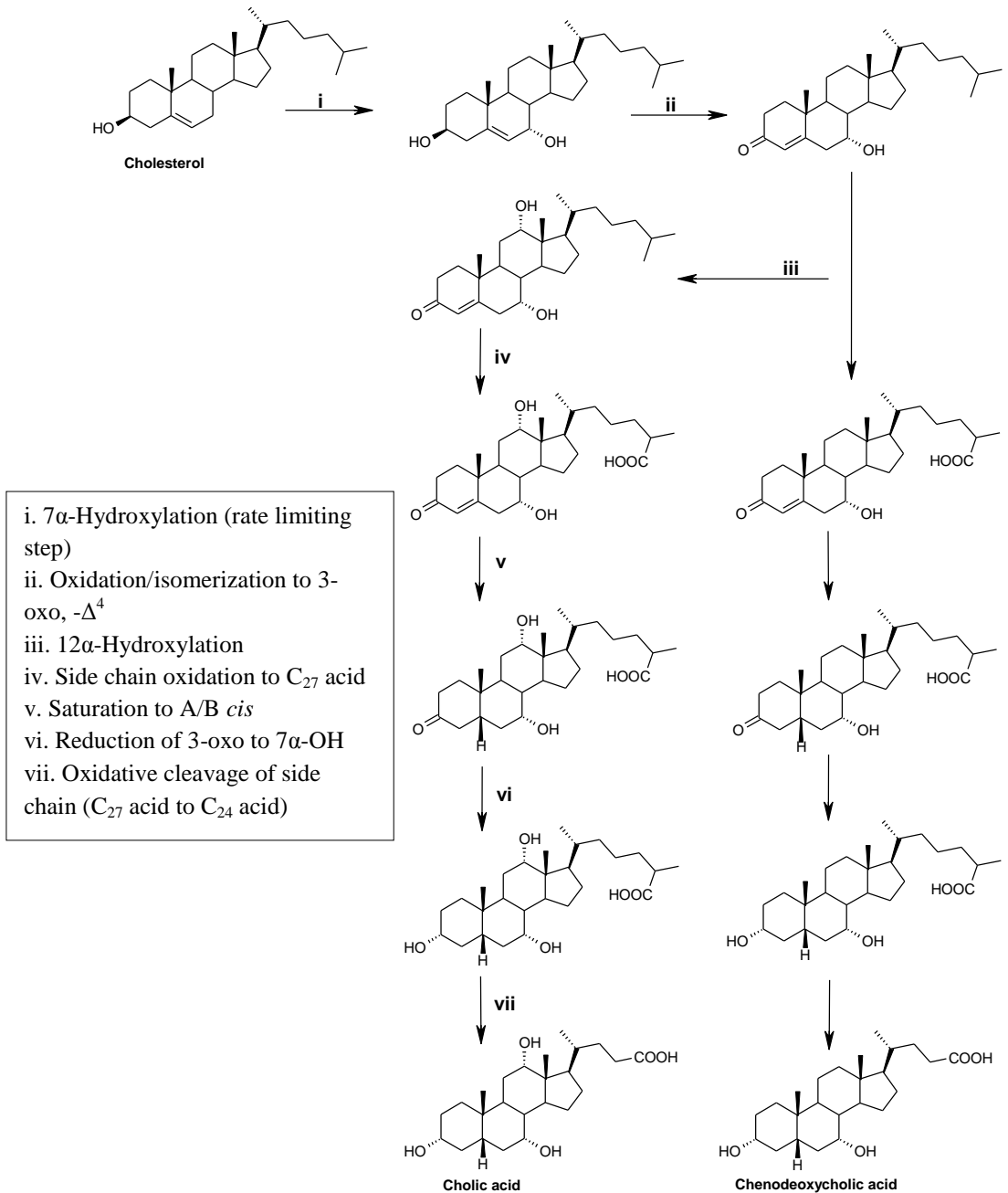

Figure 1. Structure of bile acids (specifically cholic acid). a) chemical structure, b) tridimensional structure (Mukhopadhyay S and Maitra U 2004).

Primary bile acids, i.e. cholic (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid), are, together with cholesterol, phospholipids and bilirubin, the principal constituents of bile. They are synthesized in the liver from cholesterol, secreted in the bile canaliculi and subsequently stored in the gall bladder where they are concentrated during the interdigestive period.

The hepatic synthesis of bile acids accounts for the majority of cholesterol breakdown in the body. In humans, ca. 500 mg of cholesterol is converted to bile acids every day (Thomas C et al. 2008).

The biosynthetic pathway of CA and CDCA, summarized in Figure 2, is a multienzymatic process which results in both the modification of the ring structure of cholesterol and the oxidation and shortening of the side chain.

i. 7α-Hydroxylation (rate limiting step)
ii. Oxidation/isomerization to 3-oxo, -Δ^4
iii. 12α-Hydroxylation
iv. Side chain oxidation to C_{27} acid
v. Saturation to A/B *cis*
vi. Reduction of 3-oxo to 7α-OH
vii. Oxidative cleavage of side chain (C_{27} acid to C_{24} acid)

**Figure 2**. Primary bile acids biosynthesis.

In this pathway, two different hydroxylations occur, catalyzed by microsomal mixed function oxidases. The first of these hydroxylations, catalyzed by a cholesterol 7α-hydroxylase at C-7, is the rate limiting step and control the whole pathway. The second hydroxylation reaction, at C-12, leads to the formation of the cholic acid backbone. Finally, the dehydrogenation and the subsequent reduction of the hydroxyl group at C-3 inverts its configuration and combines the rings A and B in a *cis*-type configuration. (Mukhopadhyay S and Maitra U 2004).

Before being secreted into the bile, bile acids in the liver are usually conjugated *via* an amide bond to the amino acids glycine and taurine. This process makes bile acids impermeable to cell membranes and permits high concentrations to persist in bile and intestinal content. The conjugates of cholic acid with glycine and taurine are called glycocholate and taurocholate, respectively.

After food ingestion, release of cholecystokinin from the duodenum stimulates the gall bladder to contract, causing bile to flow into the duodenum, where it contributes to the solubilization and digestion of lipid-soluble nutrients. Bile acids are then absorbed by high-affinity active transport from the terminal ileum, complexed with plasma proteins in the bloodstream, and transported back to the liver *via* the portal vein - a process termed enterohepatic circulation. The liver extracts bile acids with great avidity and reconjugates deconjugated bile acids before resecreting them into the bile, thus completing enterohepatic circulation. Absorption of bile acids from the distal intestine leads to the accumulation of a large mass of bile acid molecules, termed the *bile acid pool*, which is about 2 to 3 g in weight in adults. Therefore, bile acid synthesis, which, as reported above, averages about 0.5 g/d, is "amplified" by the enterohepatic cycling of bile acids. (Hofmann AF 1999a, Ridlon JM et al. 2006).

Bile acids that are not reabsorbed in the small intestine (about 400-800 mg/d) pass into the large intestine where they encounter populations of facultative and strictly anaerobic bacteria. The intestinal flora consists mainly of microorganisms belonging to the families Lactobacillaceae and Bacteroidaceae, and to a lesser extent, of microorganisms such as *Escherichia coli*, coliforms and Clostridia. These intestinal bacteria express enzymes that transform primary bile acids into different derivatives, called secondary bile acids. The relationship between bile acids and intestinal microorganisms is a two-way process, that is, bile acids can affect bacteria and *vice versa*. (Prahba V and Ohri M 2006).

Bile acids modifications by intestinal bacteria include deconjugations, oxidations/reductions of hydroxyl groups at C-3, C-7, and C-12, epimerizations of hydroxy groups and 7α/β-dehydroxylations (Fig. 3). Deconjugations and 7α/β-dehydroxylations of bile acids increase their hydrophobicity and their pK_a, thereby permitting their recovery via passive absorption across the colonic epithelium. In this way intestinal bacteria contribute to the recovery of bile salts that escape active transport in the distal ileum.
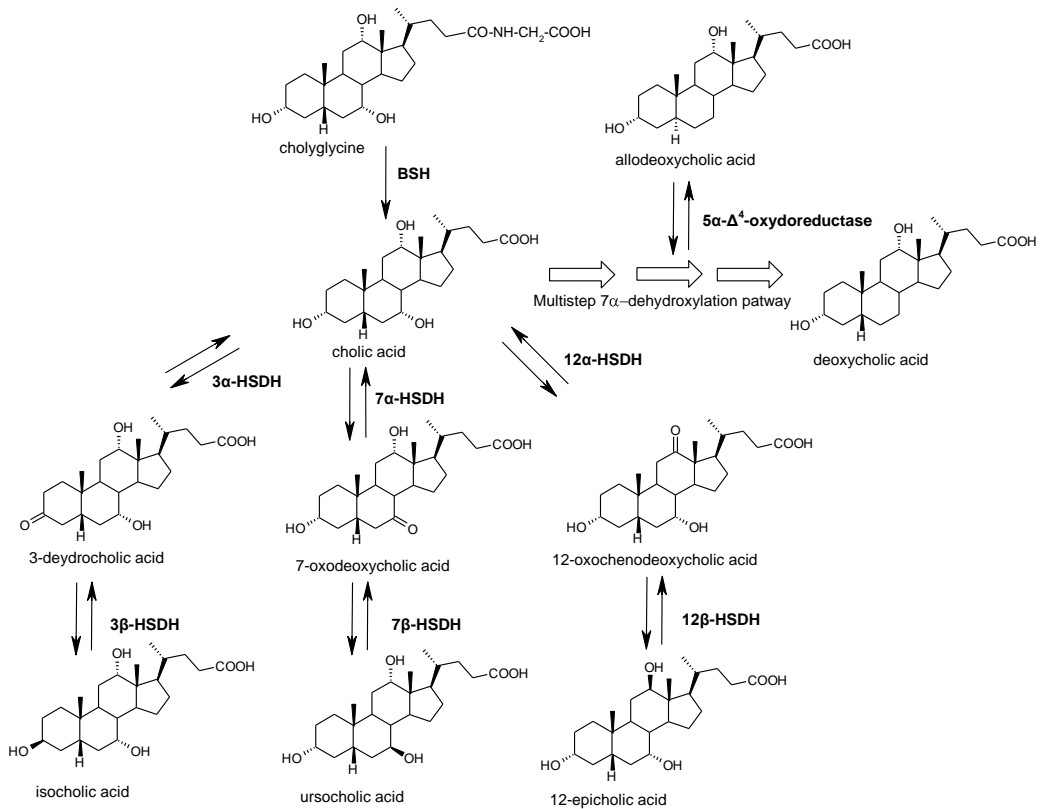
Figure 3. Bacterial bile salt biotransformations in the human intestinal tract. BSH, bile salt hydrolase; HSDH, hydroxysteroid dehydrogenase.

Deconjugation, i.e. the enzymatic hydrolysis of the C-24 N-acyl amide bond linking bile acids to their amino acid conjugates, is catalyzed by bile salt

hydrolases (BSHs), enzymes belonging to the choloylglycine hydrolase family (EC 3.5.1.24). These enzymes have been isolated and/or characterized from several genera of intestinal bacteria, such as *Bacteroides*, *Lactobacillus*, *Clostridium* and *Bifidobacterium*.

Oxidations and epimerizations of hydroxyl groups at C-3, C-7, and C-12 are catalyzed by hydroxysteroid dehydrogenases (HSDHs), a group of oxidoreductases belonging to the short chain dehydrogenase/reductase (SDR) superfamily (Kavanagh et al. 2008). Epimerization, i.e. the reversible change in stereochemistry from α to β configuration (or *vice versa*) through a keto intermediate, requires the concerted effort of two position-specific, stereochemically distinct HSDHs of intraspecies or interspecies origin. These enzymes will be further discussed in the following.

Both deconjugation and epimerization of hydroxyl groups decrease toxicity of bile acids and they could represent strategies acquired by intestinal bacteria to counteract bile salt toxicity (Ridlon JM et al. 2006).

Unlike bile acid oxidation and epimerization, 7α/β dehydroxylation appears limited to deconjugated bile acids. In fact, removal of glycine/taurine from bile acid conjugates by BSH enzymes is a prerequisite for 7α/β -dehydroxylation by intestinal bacteria. 7α-dehydroxylation is the most quantitatively important bacterial bile salt biotransformation in the human colon. Lithocholic acid (LA, 3α-hydroxy-5β-cholan-24-oic acid) and deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid), formed from CA and CDCA dehydroxylation, respectively, are indeed the most abundant bile acids in human feces. Removal of the 7-hydroxy group proceeds through a multi-step biochemical pathway. This pathway has been extensively investigated by Hylemon and coworkers who reported that genes codifying for the enzymes involved in the 7α-dehydroxylation route are part of a policystronic bile acid inducible operon in *Clostridium* sp. strain TO-931, *Clostridium scindens* VPI 12708, and *Clostridium hylemonae* (Ridlon JM et al. 2010, Wells JE and Hylemon PB 2000, Mallonee DH and Hylemon PB 1996).

Physiological functions of bile acids

Physiological functions of bile acids have been well established.
First of all, bile acids promote the elimination of the excess of cholesterol, bile acids synthesis being indeed the primary pathway of cholesterol catabolism.

Moreover, micellar solubilization of cholesterol in bile enables cholesterol to move from the hepatocytes to the intestinal lumen, and ultimately to be eliminated via the fecal route(Hofmann AF 1999a).

In addition to the homeostatic function on cholesterol, bile acids play an important role in the solubilization and transport of lipids in the digestive process. In fact, the surface area of fat particles can be greatly increased through the formation of mixed micelles, thus making them more widely available to the degradative action of lipases. Moreover, mixed micelles of bile acids are responsible for the solubilization and absorption of fat-soluble vitamins, such as vitamin E (Mukhopadhyay S and Maitra U 2004, Traber MG 1996). Bile acids contribute to the intestinal absorption of many dietary lipids also by activating the pancreatic lipase BAL (bile acid activated lipase), which catalyzes the conversion of triglycerides in glycerol and fatty acids (Wang CS et al. 1999, Terzyan S et al. 2000).

In addition to lipid digestion, bile salts may be involved in other processes. For example, it has been recently reported that conjugated bile acids affect the digestion and assimilation of dietary proteins by accelerating the hydrolysis catalyzed by pancreatic proteases, such as trypsin and chymotrypsin. The related mechanism is still unknown, but it has been hypothesized that binding of bile acids to hydrophobic pocket(s) of the proteases may destabilize their structure, making additional interior domains of the dietary protein available for luminal endoprotease action (Gass J et al. 2007).

Other physiological functions of bile acids include stimulation of bile flow and of biliary phospholipid secretion. Bile acids are actively transported into the semipermeable biliary canaliculi, where they reach high concentration and induce bile flow by means of their osmotic properties. The presence of phospholipids in bile results in a greater fraction of bile acids existing in the form of mixed micelles and a lower monomer concentration of bile acids, thereby preventing bile acids from damaging the bile duct epithelium (Hofmann AF 1999a and Hofmann AF 1999b).

Finally, bile acids have recently been shown as versatile signaling molecules endowed with systemic endocrine functions. It has been demonstrated that they are ligands for G-protein-coupled receptors such as TGR5 and also modulate several nuclear hormone receptors including farnesoid X receptor (FXR) and pregnane X receptor (PXR). Through activation of these diverse signaling pathways, bile acids have been shown to regulate not only their own synthesis

and enterohepatic recirculation, but also triglyceride, cholesterol, energy and glucose homeostasis (Thomas C et al. 2008, Staudinger JL et al. 2001).

Bile acids applications

Currently, the main applications of bile acids are in the field of pharmacology, in particular they are used in the treatment of bile acid deficiencies, some liver diseases, and in the dissolution of cholesterol gallstones.

There are two main rationales for administering bile acids in therapy. The first is bile acids replacement, where the aim is to correct a deficiency of bile acids caused by defective biosynthesis (inborn errors of metabolism) or defective intestinal conservation (short bowel syndrome). The second is bile acids displacement where the aim is to change the composition of the circulating bile acids without any great change in tissue concentrations or secretion. This is achieved in the treatment of cholestatic liver disease and cholesterol gallstone disease (Hofmann AF and Hagey LR 2008, Hofmann AF 2009).

Cholesterol gallstones are hard, stones-like formations, ranging in size from a few millimeters to several centimeters, that can accumulate in the gall bladder. Most of these gallstones are produced in the presence of a cholesterol-saturated bile, a factor favoring the precipitation of cholesterol itself, first with the formation of minute crystals and then as clusters of larger and larger size. The prevalence of cholesterol gallstones disease in adults ranges from 10% to 15% (Di Ciaula A et al. 2010).

The bile acids-mediated dissolution was introduced in the early '70s, the drug of first choice being ursodeoxycholic acid (UDCA, 3α,7β-dihydroxy-5β-cholan-24-oic acid, Fig. 4). UDCA is the major bile acid in nutrias, bears, and beavers, while it is present only in small amount in human bile (3-4 %). UDCA appears to be almost as effective as its enantiomer chenodeoxycholic acid (CDCA), but it is devoid of hepatotoxicity (Hofmann AF and Hagey LR 2008).
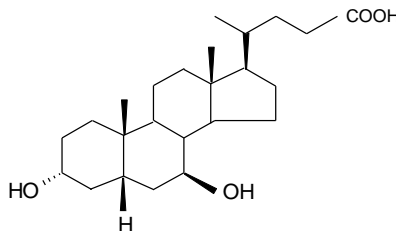
Figure 4. Ursodeoxycholic acid (UDCA)

The best indication of UDCA in cholesterol gallstones dissolution therapy is represented by symptomatic patients with small (< 5 mm), mobile, not calcified, cholesterol-enriched (i.e., more than 80%) gallstones, and contained within a functioning gall bladder with a patent cystic duct. This event comprises about 15 % of patients with gallstones and avoids the removal of the gallstones by surgical treatment.

The mechanisms by which this effect occurs are many: decreasing secretion of cholesterol into the bile *via* a reduction in intestinal absorption and hepatic synthesis of cholesterol itself, increasing the total pool of bile acids that favor the micellar solubilization of cholesterol, forming a liquid-crystalline mesophase which allows a solubilization of cholesterol superior than that achieved in the equilibrium phase (Di Ciaula A et al. 2010, Jain UK et al. 1992).

UDCA has been proven to be very effective in the treatment of many other cholestatic diseases. In fact, it is currently used in the standard treatment of primary biliary cirrhosis (PBC), a chronic autoimmune inflammatory disease of the liver with a striking female preponderance (Bhandari BM et al 2011). In PBC, treatment with UDCA improves liver tests and increases the time interval to liver transplantation (Hofmann AF and Hagey LR 2008). Moreover, UDCA is the most studied drug for the treatment of primary sclerosing cholangitis (PSC), a chronic cholestatic disease characterized by inflammation and progressive obliterative fibrosis of the intra and /or extra-hepatic biliary tree which leads to bile stasis and hepatic fibrosis. Up to now, the efficacy of UDCA in PSC treatment is still controversial, some clinical trials suggesting that UDCA only slows disease progression (Triantos CK et al. 2011, Hofmann AF and Hagey LR 2008). On the contrary, UDCA is clearly useful in the syndrome of cholestatic disease of pregnancy which is likely to be caused by a defect in canalicular transport (Glantz A et al. 2005). In cholestasis of pregnancy, UDCA has been shown to improve liver tests and fetal outcome (Hofmann AF and Hagey LR 2008).

Also in the case of cholestatic diseases, UDCA positive effects may be the result of its multiple mechanisms of action. For example, while the high concentration of citotoxic bile acids, such as CDCA, can differentially induce either necrosis or apoptosis in cholestatic diseases, UDCA halts apoptosis by preventing the formation of mitochondrial pores, membrane recruitment of death receptors and endoplasmic-reticulum stress. In addition, UDCA reduce

intracellular bile acids level and decrease bile acids citotoxicity by inducing changes in the expression of metabolizing enzymes and transporters which result, for example, in a decrease in bile acids synthesis, in an increase in bile acid hydroxylation and in an improved renal excretion. Its capability to positively modulate ductular bile flow helps to preserve the integrity of bile ducts and UDCA also prevents the endocytic internalization of canalicular export pumps involved in bile flow generation, a common feature in cholestasis.

Finally, UDCA has immunomodulatory properties that limit the exacerbated immunological response occurring in autoimmune cholestatic diseases by counteracting the overexpression of MHC antigens and perhaps by limiting the production of cytokines by immunocompetent cells (Roma MG et al. 2011).
Additionally, it has also been recently demonstrated that UDCA protects liver cells from the immune reactions caused by the transplantation of hematopoietic cells (McDonald GB 2006). Potential applications of UDCA and of its derivatives as a neuroprotectant in Huntington's disease and Alzheimer's disease have been suggested as well (Sharma R et al. 2011).
Moreover, evidences from experimental animal models and human studies suggest that UDCA exerts chemopreventive activity against colorectal carcinogenesis and, in particular, it has been shown that it may inhibit tumor development in some experimental genetic and chemical models of intestinal tumorigenesis (Serfatya L et al. 2010).

UDCA is currently produced by a chemical synthesis using as starting substrate cholic acid, which is found in large quantities in the bile of mammals.
The hydroxy groups at C-3, C-7 and C-12 have different reactivity and therefore it is possible to catalyze regiospecific oxidation/reduction and acylation reactions. The reduction of the oxo-derivatives with $NaBH_4$ leads preferentially to the formation of axial hydroxy group, while the use of sodium alkoxides, e.g., sodium methoxide, in the presence of hydrogenation catalysts leads mainly to equatorial hydroxy groups.

Industrial-scale production of UDCA is carried out by the seven-steps chemical process shown in Figure 5 (Hofmann AF 1963; Samuelsson B 1960). However, the yields are limited to about 30% and the stereo- and regioselectivity is never absolute, several steps of purification and crystallization being required to obtain the product with a high degree of purity. In addition, the chemical

synthesis involves the use of highly polluting and toxic substances such as CrO_3, pyridine and methanol, the residues of which must be completely eliminated from the final product.

To overcome the low yields of the above mentioned process, several changes to the method have been proposed, such as the use of *tert*-amyl alcohol and potassium instead of methanol and sodium (Batta AK et al. 1991). According to this procedure, a product with a 90% purity degree and a yield of 95% can be recovered, but only after multiple steps of crystallization with ethyl acetate to remove the co-product chenodeoxycholic acid.

Many approaches have been also proposed in order to obtain a safer process, such as the indirect electrooxidation of chenodeoxycholic acid to 7-ketolithocholic acid (7KLCA, 3α-hydroxy-7-oxo-5β-cholan-24-oic acid) (Zhao H et al 2010).

Alternative synthetic pathways including biotransformation steps, either by whole cells or isolated enzymes, will be presented and discussed in the following chapters.
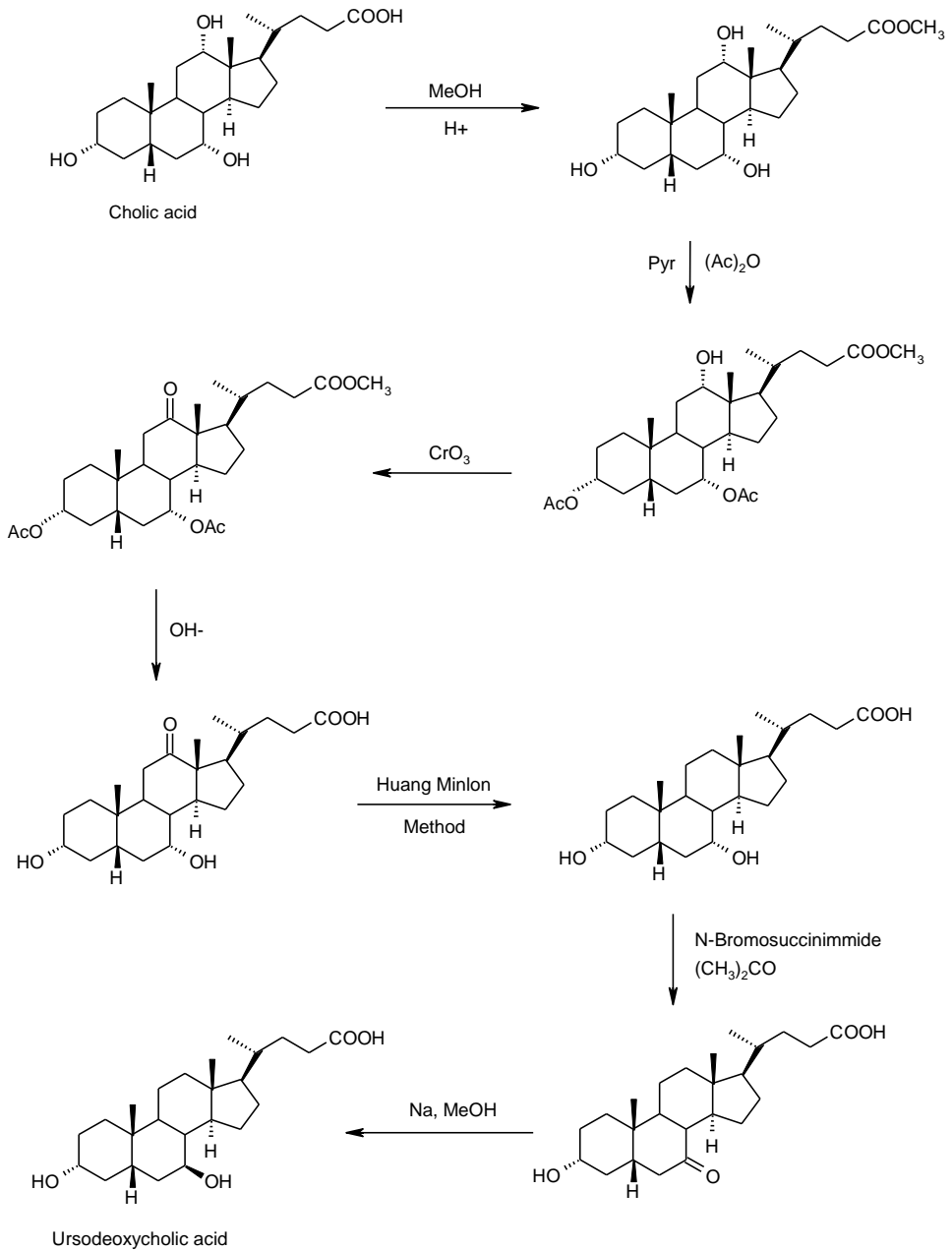
Figure 5. Chemical synthesis of ursodeoxycholic acid.

Recently, bile acids-controlled signaling pathways have become the source of promising novel drug targets thanks to the newly discovered activity of these compounds as ligands of the FXR, PXR, and TGR5 receptors. Both natural bile acids and semisynthetic derivatives are currently under investigation for the treatment of metabolic disorder, hepatic disease, diabetes, and obesity through the activation of FXR or TGR5 receptors. (Thomas C. et al. 2008).

Moreover, recent studies have revealed that the PXR receptor can function as a master regulator in the control of the expression of drug-metabolizing enzymes, such as enzymes of the cytochrome P450 3A (CYP3A) family, and of members of the drug transporter family, including multiple drug resistance 1 (MDR1). Therefore, PXR downregulation by bile acids analogues could be a novel therapeutic approach for the augmentation of sensitivity to anticancer agents or to overcome resistance to them, *e.g*., in the treatment of endometrial cancer (Masuyama H et al 2007).

In addition to pharmaceutical applications, several applications of bile acid as "building blocks" for the construction of chiral artificial receptors and supramolecular hosts for molecular recognition have been proposed as well (Tamminem J and Kolehmainen E 2001). Bile salts are used for the structural rigidity of the steroid skeleton, which allows the formation of stable cavities, and because both hydroxyl groups and the side-chain carboxylate can be readily derivatized. Moreover, the *cis* A/B junction allows ring A of bile acids to bend inward and produce a concave curvature where all of the OH groups line on the inside (Mukhopadhyay S and Maitra U 2004). Some examples of artificial receptors are the anion receptor characterized by a marked selectivity for the fluoride ion (Davis AP et al. 1997), and the porphyrin conjugated cyclocholate selective for organic-soluble glycoside (Davis AP and Wareham RS 1999), depicted in Figure 6a and 6b, respectively.

a)

b)

c)
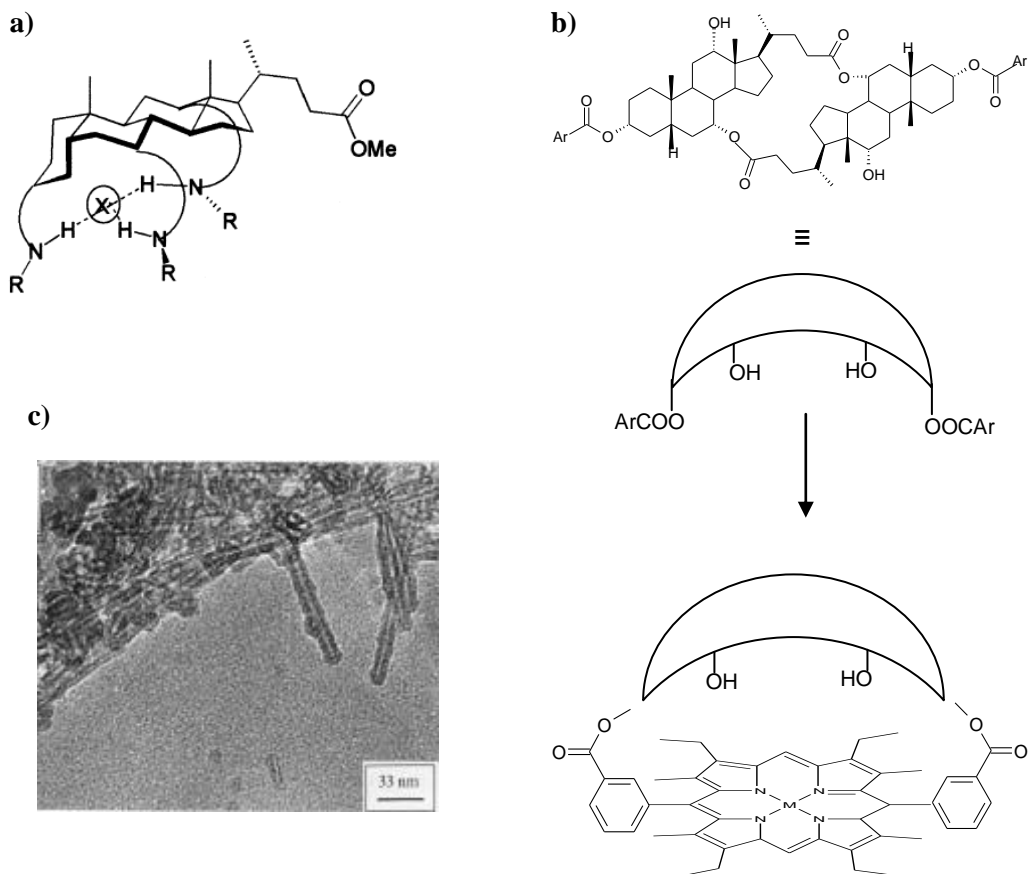
Figure 6. a) Acyclic cholic acid-based receptor for anions (Mukhopadhyay S and Maitra U 2004); b) porphyrin-conjugated cyclocholate; c) titanium nanotubes made from hydrogel derived from tripodal cholamide gelator (Gundiah G et al 2003).

Bile acids have also been used for the optical resolution of several classes of racemic organic compounds by forming crystalline enantioselective inclusion complexes with organic compounds (Bortolini O et al. 2005).

A new and interesting application of bile acids is in the chemical synthesis of polymers which can be used for drug delivery, as gelificator agents or for other applications such as chromatographic supports and photoresists. (Ropponem J et al. 2005, Zhu XX and Nichifor M 2002). In some cases, bile acids can create

gels in aqueous solutions and esterification of bile acids with different organic substances allows to change the physical characteristics of the gel. Hydrogels derived from bile acids are of considerable interest due to their excellent water-holding ability. Dye-intercalation studies on gels show that they could be potential materials for drug delivery. It is noteworthy that these hydrogels are thermoreversible and biodegradable, unlike traditional polymeric gels (Mukhopadhyay S and Maitra U 2004).

Additionally, these gels can be used as templates to prepare inorganic nanotubes as shown in Figure 6c. (Gundiah G et al 2003).

Finally, the high specificity and capacity of the bile acid transport systems during the enterohepatic circulation forms the basis of the current research efforts to elaborate drug-bile acid conjugates for specific drug targeting to the liver and for improving the intestinal absorption of poorly absorbed or non-absorbed drugs, *e.g.*, peptides. For example, Kramer and Wess joined various drug molecules to positions 3, 7, and/or 12 in the steroid nucleus via linker moieties of different functionality, length, and polarity. One of these conjugates is shown in Figure 7, where oxaprolylpeptide, a drug for liver fibrosis, has been conjugated to a cholic acid derivative. This conjugate showed a greatly improved ileal absorption compared to the parent drug (Tamminem J and Kolehmainen E 2001, Enhsen A et al. 1998).
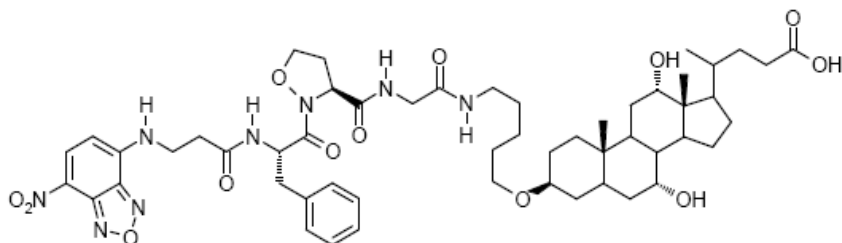

Figure 7. A oxaprolylpeptide-bile acid conjugate

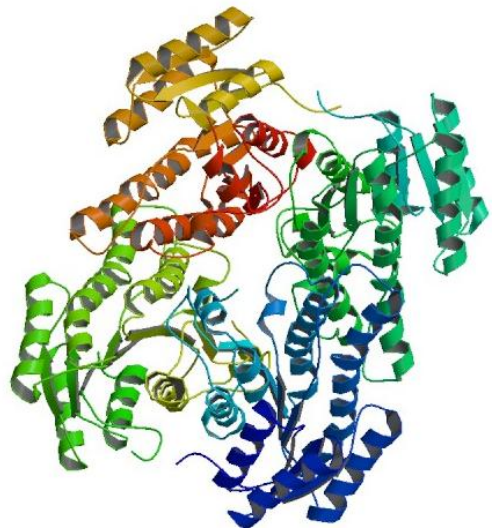# HYDROXYSTEROID DEHYDROGENASES

Hydroxysteroid dehydrogenases (HSDH)

HSDHs in bile acids transformations

7α-Hydroxysteroid dehydrogenases

7β- Hydroxysteroid dehydrogenases

12α-Hydroxysteroid dehydrogenases

Hydroxysteroid dehydrogenases from *C. absonum*

Hydroxysteroid dehydrogenases

Hydroxysteroid dehydrogenases (HSDHs) are NAD(P)H-dependent enzymes which catalyze the oxidation/reduction of hydroxyl groups of steroids or bile acids in vivo (Ridlon JM et al. 2006). They belong to the short chain dehydrogenase/reductase (SDR) superfamily (Kavanagh KL et al. 2008), which constitutes one of the largest enzyme superfamilies with over 46,000 members present in sequence databases. The low sequence similarity among different members of this class suggests an early differentiation to enzymes with different catalytic properties (Persson B et al 2009).

Typically, HSDHs are homodimers or homotetramers, each monomer consisting of about 250 residues with a core of β-sheets sandwiched between α-helices. The NAD(P)H cofactor binding domain is located at the N-terminus of the monomer, while the catalytic domain is located at its C-terminus. The contact area between monomers is mainly formed by two α helices (αE, αF) on each subunit.

Despite a moderate sequence similarity, enzymes belonging to this family show similar secondary and tertiary structures. Moreover, they share common sequence motifs, such as those defining the cofactor binding site (TGxxxGxG, comprised in a Rossmann fold motif) and the active site, the latter represented for example in *E. coli* 7α-HSDH by the catalytic triad ($S(x)_{12}Y(x)_3K$) (Tanaka N et al. 1996, Tanaka N et al. 2001).

HSDHs-catalyzed bile acids modifications have been studied using enzymes from different microbial sources. Good microbial producers of HSDHs may have evolved these peculiar enzymatic activities as detoxification mechanisms toward bile acids present in mammals intestines (Ridlon JM et al. 2006). The regulation of their expression varies depending on the enzyme and the source organism. Specifically, the expression can be either constitutive or induced by bile acids or growth phase dependent (Coleman JP et al. 1994).

Hydroxysteroid dehydrogenases in bile acids transformation

NAD(P)H-dependent HSDHs have been extensively used to catalyze the selective oxido-reduction of the hydroxy-keto groups of a variety of bile acids (Bovara R et al. 1996).

For example, enzymes belonging to this class have been used as biocatalysts for redox reactions involving the hydroxy groups of cholic acid (CA) (Fig. 8) and the keto groups of dehydrocholic acid (DHCA, 3,7,12-trioxo-5β-cholan-24-oic acid, Fig. 9).

Specifically, CA has been regiospecifically oxidized at each of the three possible positions (3α-OH, 7α-OH and 12α-OH, respectively) using either the 3α-HSDH from *P. testosteroni*, the 7α-HSDH from *E. coli*, or the 12α-HSDH from *Clostridium* sp. (all these enzymes were commercially available at the time of the investigation).
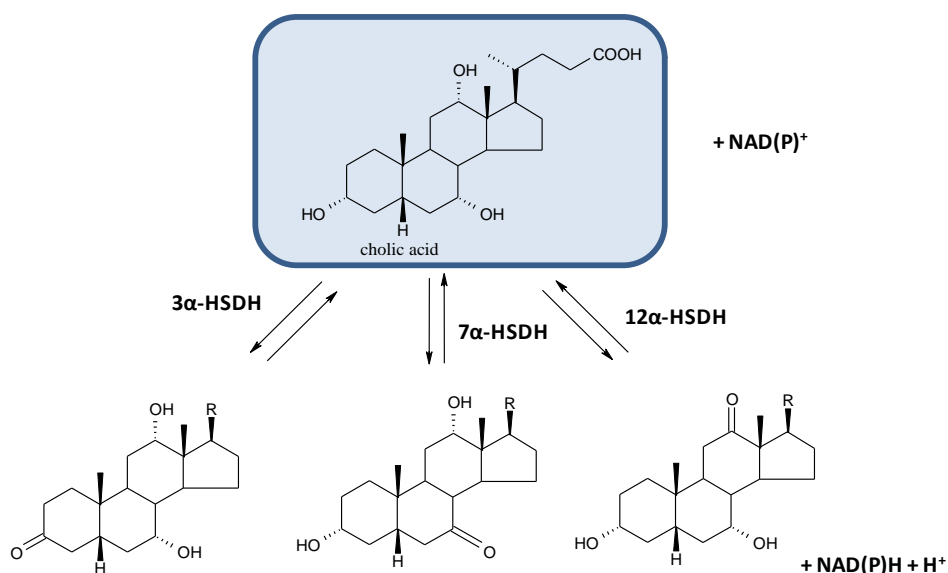
Figure 8. Enzymatic oxidation of cholic acid

Analogously, DHCA has been regio- and stereospecifically reduced at each of the three possible positions using 5 different HSDHs (the same used in cholic acid oxidation plus the commercially available 3β-HSDH from *P. testosteroni* and a 7β-HSDH, partially purified from *C. absonum*) (Fig. 9).
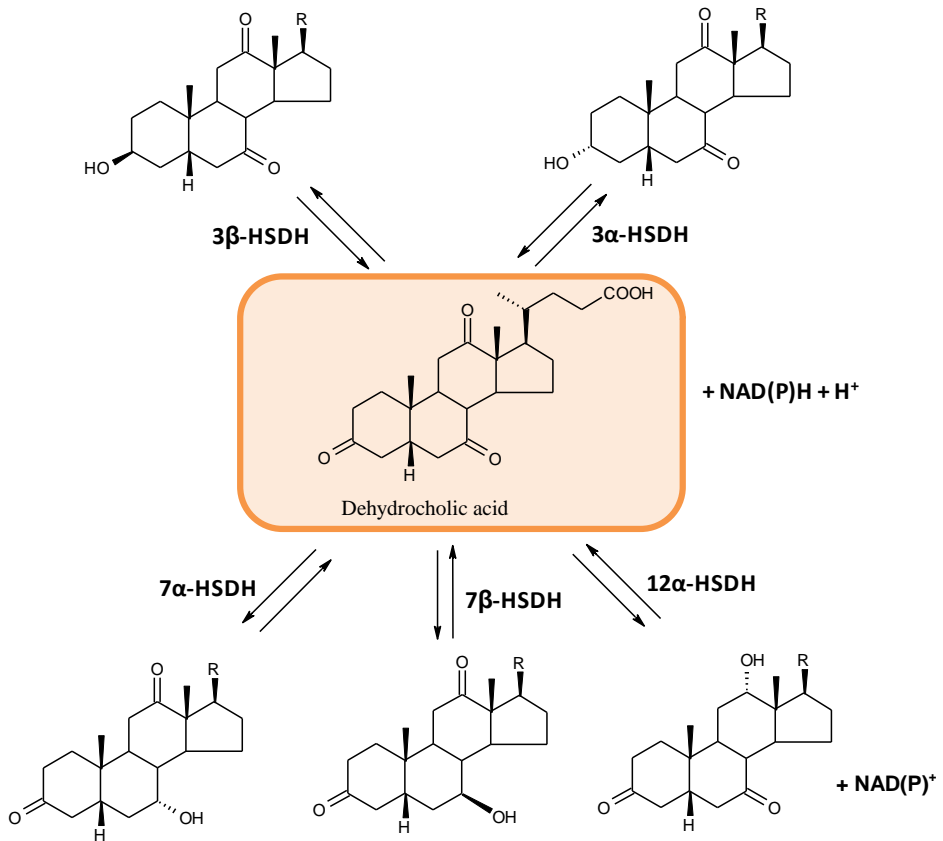
Figure 9. Enzymatic reduction of dehydrocholic acid.

These reactions were carried out on a preparative scale, in potassium phosphate buffer. As reactions catalyzed by HSDHs are reversible, they were coupled to suitable cofactor regeneration systems to obtain complete conversions. Products were then recovered by acidic precipitation with ca. 80-90 % yields and purity over 97 % (Riva S et al.1986).

Bioconversions catalyzed by HSDHs can be performed either with free enzymes or with enzymes immobilized on suitable supports. Enzyme immobilization usually improves enzyme stability, especially in those reactions carried out in the presence of organic solvents such as in biphasic systems. For example, cholic acid methyl ester was successfully oxidized to the corresponding 12-keto derivative by a 12α-hydroxysteroid dehydrogenase immobilized on Eupergit C

in a biphasic system butyl acetate/potassium phosphate buffer (Carrea G et al.1988).

More recently, HSDHs have been also employed in the preparation of a 39 members array of cholic acid derivatives, thus demonstrating to be valuable biocatalysts also in the modification of non natural bile acids (Fig. 10) (Secundo F et al. 2003).
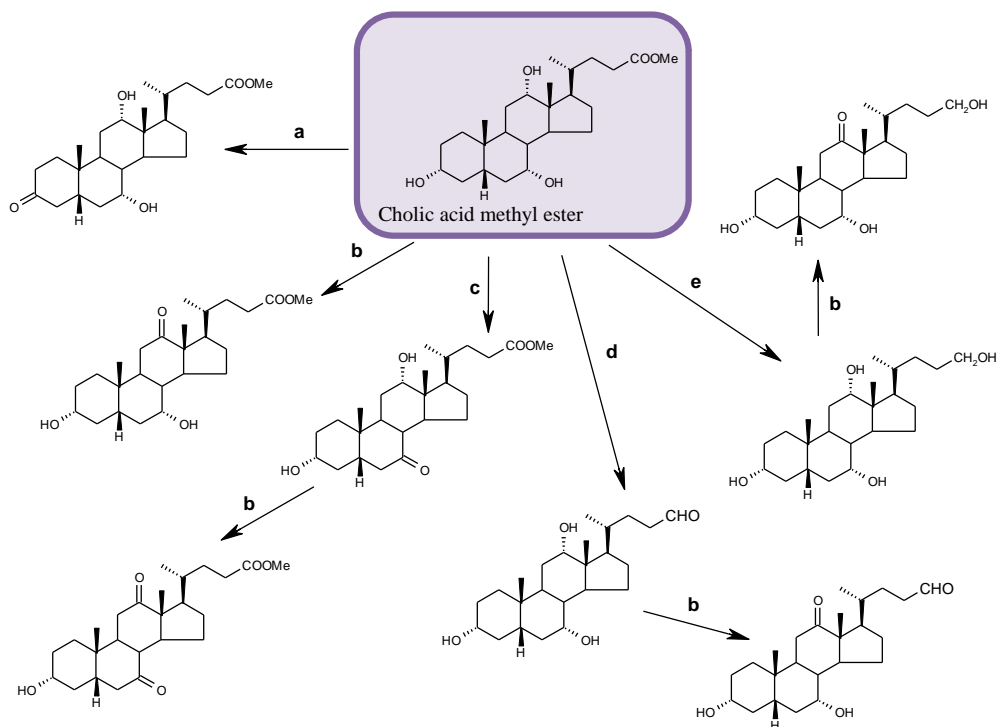
Figure 10. Production of cholic acid derivatives by regioselective chemo-enzymatic oxidation-reduction steps and acylation steps. a: 3α-HSDH, MeOH/H$^+$; b: 12α-HSDH; c: 7α-HSDH, MeOH/H$^+$; d: DIBAH/THF; e: LiAlH4/THF;

Moreover, the 7α-HSDH from *B. fragilis* has been successfully used for the stereoselective reduction of some aromatic and bulky aliphatic α-ketoesters and in the reduction of various benzaldehyde analogues (Zhu D et al 2006, Liu Y et

al 2011). HSDHs could be therefore considered as valuable biocatalysts even for the enantioselective reduction of non-steroidal carbonyl compounds.

As this Thesis work was focused on the development and synthetic application of selected HSDH activities, i.e., 7α-HSDH, 12α-HSDH, and 7β-HSDH, the state-of-the-art about these specific HSDHs is summarized below.

7α-Hydroxysteroid dehydrogenases

Up to now, various 7α-HSDHs (EC: 1.1.1.159) have been cloned, e.g., from *Escherichia coli* (Yoshimoto T et al. 1991), *Bacteroides fragilis* (Bennett M et al. 2003), *Clostridium sordellii* (Coleman JP et al. 1994) and *Eubacterium* sp. strain VPI 12708 (Baron S et al. 1991). The literature reports also the partial characterization of 7α-HSDHs from *Bacteroides tetaiotamicron* (Sherrod JA and Hylemon PB 1977) *Clostridium perfringens* (Macdonald IA et al. 1976), *Clostridium absonum* (Macdonald IA and Roach P 1981), *Clostridium limosum* (Sutherland JD and Williams CN 1985), *Clostridium bifermentans* (Sutherland JD et al. 1987), *Clostridium baratii* (Lepercq P et al. 2004), *Xanthomonas maltophilia* (Pedrini P et al . 2006), and *Acinetobacter calcoaceticus lwoffii* (Giovannini PP et al. 2008). Moreover, a search of primary structures of 7α-HSDH in the UniProt Knowledgebase database (Swiss-Prot and TrEMBL, http://www.expasy.org/sprot/) results in more than 50 sequences codifying for putative 7α-HSDHs.

To this list, the 7α-HSDH from *C. absonum* recently cloned and overexpressed in *E. coli* by our group (**Paper III**) must be added.

The expression of these enzymes can be either constitutive or inducible (Coleman JP et al. 1994). In organisms such as *E. coli* and *Eubacterium* sp. strain VPI 12708, 7α-HSDH is expressed constitutively (Prabha V et al. 1990, Macdonald IA et al. 1973, Franklund CV et al 1990, Baron S et al. 1991), while, for example, the 7α-HSDH from *Clostridium limosum* is induced by addition of primary bile acids or deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid) to the culture medium at the time of inoculation (Sutherland JD and Williams CN 1985). In *Bacteroides* spp., the production of 7α-HSDH activity is both growth phase dependent and bile acid inducible (Bennett M et al. 2003, Hylemon PB and Sherrod JA 1975, Sherrod JA and Hylemon PB 1977). In *C. sordellii*, maximal expression levels are reached during the early stationary

phase in both the absence and the presence of bile acids in culture medium, but, in presence of bile acids, enzyme synthesis is improved of about 10 fold in the respect of that observed in uninduced cell (Coleman JP et al. 1994).

The best characterized 7α-HSDH is the one from *E. coli*, whose crystal structure was solved at a resolution of 2.3 Å in a binary complex with NAD$^+$ (www.rcsb.org, PDB ID: 1AHH) and at 1.8 Å in the ternary complex with NADH and the reaction product 7-oxoglycochenodeoxycholic acid (PDB ID: 1FMC) (Fig. 11) (Tanaka N et al. 1996).
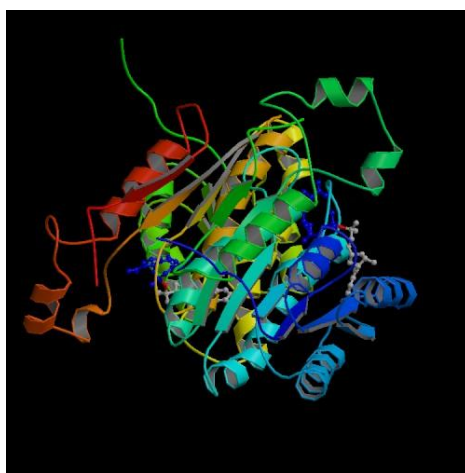
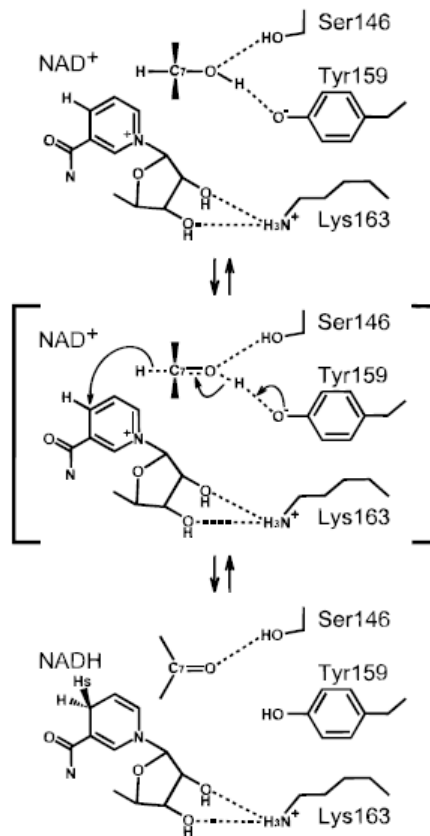**Figure 11**. Crystal structure of the 7α-HSDH from *E. coli* (PDB ID: 1AHH).

Structural analyses indicate a possible involvement of the Tyr159 and Ser146 residues, highly conserved in the SDR family proteins, in the interaction with the hydroxy groups of the substrate, whereas another highly conserved amino acid, Lys163, interacts *via* hydrogen bonds with the hydroxy groups in 2 'and 3' of NADH ribose (Fig. 12).
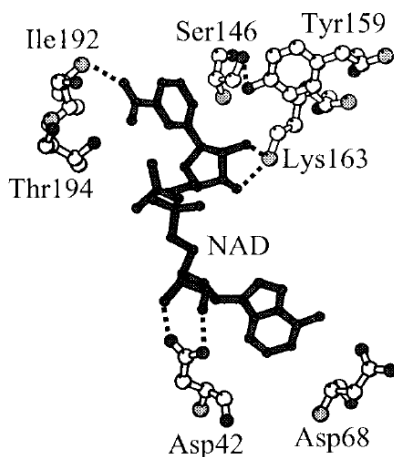
Figure 12. NAD$^+$ binding to the active site in the binary complex of 7α-HSDHs (Tanaka N et al. 1996).

On the basis of structural observations, a catalytic mechanism for the enzyme from *E. coli* that may be valid also for other 7α-HSDHs has been proposed (Fig. 13) (Tanaka N et al. 1996, Tanabe T et al. 1998).

In the first step, Lys163, lowering the pK$_a$ value of Tyr159, allows the deprotonation of the latter residue. The resulting deprotonated phenolic group forms a hydrogen bond with the hydroxyl group in position 7 of the substrate. The hydroxyl group of Ser146 may then form a hydrogen bond with the substrate, stabilizing its position. Subsequently, the deprotonated tyrosine residue acts as a base by extracting a hydrogen atom from the hydroxy group of the substrate. Concurrently, NAD$^+$ accepts a hydrogen released from the position 7 of the steroid skeleton on the β face of its nicotinamide ring.

Figure 13. Proposed catalytic mechanism for 7α-HSDH (Tanaka N et al. 1996).

Recently, the crystal structure of the 7α-HSDH from *Brucella melitensis* has been solved at a resolution of 2.2 Å (PDB ID: 3GAF).

Figure 14 shows the multiple alignment of sequences of some bacterial 7α-HSDH (CLOSO, *Clostridium sordellii* ATCC 9714; EUBSP, *Eubacterium* sp. strain VPI 12708; BACFR, *Bacteroides fragilis* ATCC 25285; ECOLI, *Escherichia coli* strain HB101). As shown, 7α-HSDH primary structures do not exhibit a high level of identity, a greater conservation being observed in the N-terminal region where a few amino acids necessary for catalysis, in particular residues essential for binding of cofactor (KX$_4$TX$_4$GIG sequence), are located.
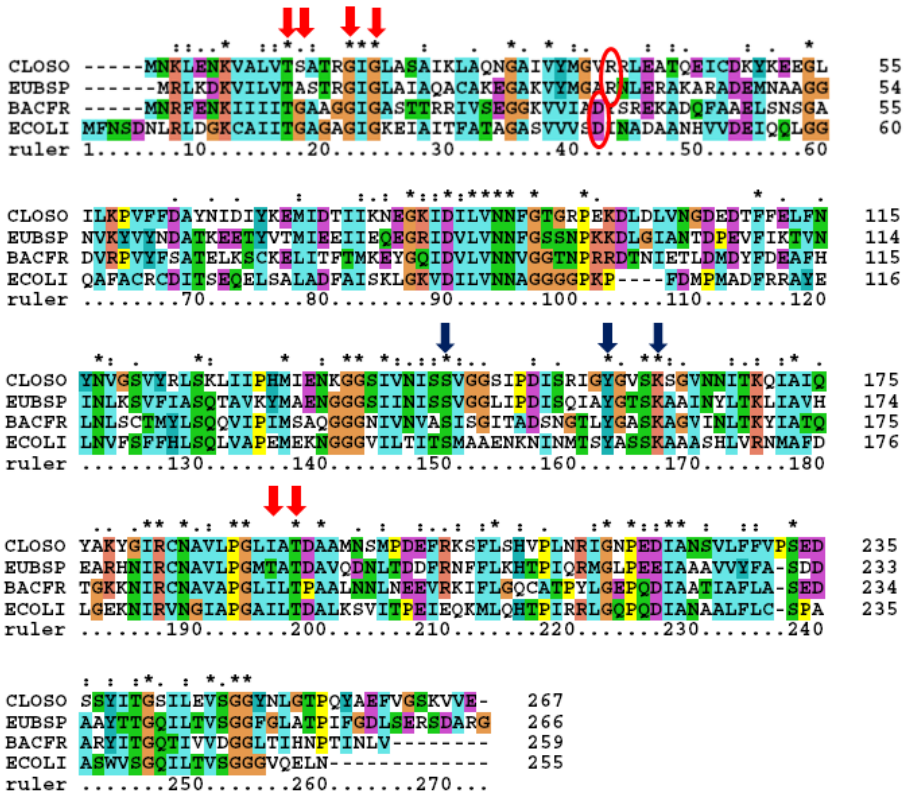
Figure 14. Multiple sequence alignment of some bacterial 7α-HSDH (CLOSO, *Clostridium sordellii* ATCC 9714; EUBSP; *Eubacterium* sp. strain VPI 12708; BACFR, *Bacteroides fragilis* ATCC 25285; ECOLI, *Escherichia coli* strain HB101). Blue arrows indicate the active site, whereas red arrows indicate amino acids supposed to be involved in cofactor binding. Amino acid underlined by red circles are involved in cofactor specificity.

Among cloned or partially purified 7α-HSDHs, the only enzymes investigated up-to-now for synthetic applications are the one from *E. coli* (Riva S et al. 1986, Bovara R et al. 1993, Secundo F et al. 2003), that for a certain period has been commercially available from Sigma-Aldrich, the one from *C. absonum* (Bovara R et al. 1996), that from *Bacteroides fragilis* (Zhu D et al. 2006, Liu Y et al. 2011) and the enzyme from *Acinetobacter calcoaceticus lwoffii* (Giovannini PP et al. 2008).

At least in non-operative conditions, the most robust biocatalyst among those studied seems to be the thermostable 7α-HSDH from *Bacteroides fragilis*. In

fact, this enzyme can be partially purified from the crude cell extract by a thermoprecipitation treatment without loss of activity (Bennet M et al. 2003). We have successfully used this enzyme in **Paper I** for the one-pot enzymatic synthesis of 12-ketoursodeoxycholic acid (12-KUDCA, 3α,7β-dihydroxy-12-oxo-5β-cholan-24-oic acid) and in **Paper II** for the development of a new cofactor regeneration system either in aqueous or in biphasic systems.

7β-Hydroxysteroid dehydrogenases

Much less information is available from the literature regarding 7β-HSDH (EC 1.1.1.201) activities. The production of 7β-HSDH activity has been described for some clostridia, e.g., *C. absonum*, *C. limosum*, and *C. baratii* (Macdonald IA et al. 1981, Sutherland JD and Williams CN 1985, Lepercq P et al. 2004), and microorganisms belonging to the intestinal bacterial flora, e.g., *Eubacterium aerofaciens* and *Peptostreptococcus productus* (Hirano S and Masuda N 1982, Masuda N et al. 1983), and *Ruminococcus* sp. (Akao T et al. 1987), or present in the soil such as *Xanthomonas maltophilia* (Pedrini P et al. 2006). 7β-HSDH activity has been recently found also in rabbit liver microsomes (Shiotsuki H et al. 2006).

Up to now, the unique cloned and overexpressed 7β-HSDH is the NADPH-dependent enzyme from *Collinsella aerofaciens* (Liu L et al. 2011). Cloning of this gene has been achieved by cloning and expression in *E. coli* of nine genes coding for short-chain dehydrogenases displayed in the genome of *C. aerofaciens*. The only one capable to convert ursodeoxycholic acid into 7-keto-lithocholic acid has then been identified.

The evolutionary tree based on the alignment of *C. aerofaciens* 7β-HSDH with different HSDHs from either microbial or mammalian source (Fig. 15) shows that this prokaryotic 7β-HSDH is closer to the animal 11β-HSDH subgroup instead of the microbial 7α-HSDH and 3α-HSDH subgroups.
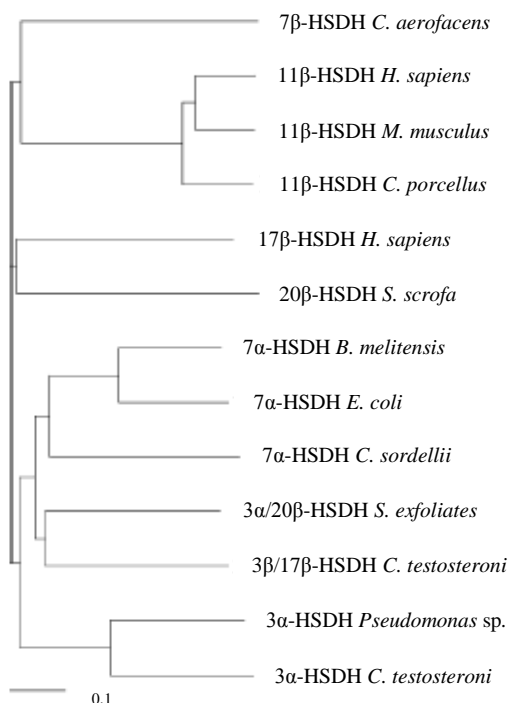
Figure 15. Phylogenetic tree based on alignment of the *C. aerofacens* 7β-HSDH protein sequence, with other HSDHs (Liu L et al. 2011).

The recombinant 7β-HSDH from *C. aerofaciens* has been purified and characterized. This enzyme shows a pH optimum of 9-10 in the oxidation of ursodeoxycholic acid and a pH optimum in the range of 4 to 6 in the reduction of 7-ketolithocholic acid (7KLCA). Maximum activity is reached at around 30°C, whereas above 50°C both enzyme activity and stability are very low (Liu L et al. 2011).

In the same period, our group has cloned and overexpressed in *E. coli* the NADPH-dependent 7β-HSDH from *C.absonum* (**Paper III**)*,* the most used 7β-HSDH for synthetic applications (Bovara R et al. 1996, Bovara R et al. 1993, Carrea G et al. 1992, Riva S et al.1986). No 7β-HSDH is currently commercially available.

12α-Hydroxysteroid dehydrogenases

12α-HSDHs (EC 1.1.1.176) are mainly found among clostridia, e.g., *Clostridium perfringens* (Macdonald IA et al. 1976), *Clostridium leptum* (Harris JN end Hylemon PB 1978), *Eubacterium lentum* (Macdonald IA et al. 1979a), and *Clostridium* group P strain C 48–50 (Macdonald IA et al. 1979b), and in *Bacillus sphaericus* (Tamasawa N et al. 1988). A 12α-HSDH activity has been recently described in *Acinetobacter calcoaceticus lwoffii* as well (Giovannini PP et al. 2008). These enzymes are constitutively expressed.

Up to now, only the NADPH-dependent 12α-HSDH from *Clostridium* group P strain C 48–50 (Braun M et al. 1991) has been cloned and overexpressed in *E. coli* (Aigner A et al. 2009). The gene sequence has been obtained by sequencing the whole genome of the *Clostridium* strain and comparison with the N-terminal sequence of 12α-HSDH previously determined by Braun M et al. It has been found that this enzyme can exist in two forms, a long version (270 aa) and a N-terminally truncated short version (258 aa). Moreover, substrate inhibition by cholic acid has been observed, these effects being limited by a single amino acidic mutation (37D12). Further attempts to change 12α-HSDH cofactor specificity from NADPH to NADH by substitutions G37D and R38L have not been successful. This enzyme is commercially available from ASA.

The NADH dependent 12α-HSDH from *B. sphaericus* is commercially available from Genzyme, but there are no available information about its sequence.

Hydroxysteroid dehydrogenases from *Clostridium absonum*

Hydroxysteroid dehydrogenases produced by *Clostridium absonum* (*Ca*7α-HSDH and *Ca*7β-HSDH) have been widely used for the modification of bile acids (Bovara R et al. 1996, Bovara R et al. 1993, Riva S et al. 1986 and **Paper I**).

These enzymes catalyze *in vivo* the epimerization of the 7α-OH of primary bile salts (Fig. 16). The resulting 7β-OH bile salts are less toxic to cell membranes compared to the 7α-OH primary bile salts because they are more hydrophilic (Macdonald IA et al. 1983).
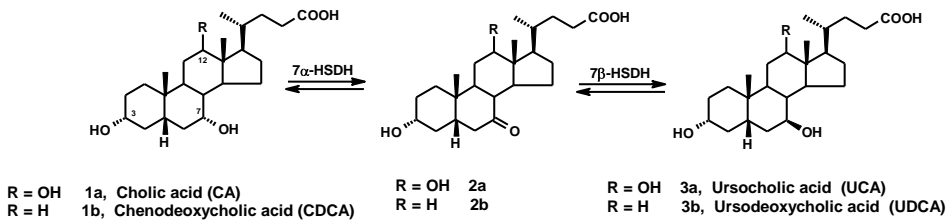
Figure 16. Epimerization of bile acids catalyzed by *Ca*7α- and *Ca*7β-HSDH.

Clostridium absonum was isolated for the first time in the soil among some strains of "perfringens-like" clostridia (Nakamura N. et al. 1973), and it is a Gram-positive spore-forming anaerobic rod-shaped bacterium.

The production of hydroxysteroid dehydrogenases by this microorganism has been extensively studied. Specifically, it has been shown that *Ca*7-HSDHs production has to be stimulated by inducers added to the culture medium. It is noteworthy that a low release of *Ca*7α-HSDH activity, but not of *Ca*7β-HSDH, was observed even in uninduced cells (Macdonald IA and Roach P 1981).

Induction of *Ca*7α-HSDH and *Ca*7β-HSDH in cultures grown to stationary phase has been achieved by the addition of metabolizable bile salt inducers such as chenodeoxycholic acid (CDCA), 7-ketolithocholic acid (7KLCA), and cholic acid (CA), or by the addition of non-metabolizable bile salts, such as 12-ketochenodeoxycholic acid (12KCDCA, 3α,7α,12-oxo-5β-cholan-24-oic acid) and deoxycholic acid (DCA).

The addition of metabolizable bile salt inducers has to be done 2.5-3 h after inoculation, because addition prior to or after this period markedly reduces the enzymes production. On the other hand, addition of non-metabolizable inducers could be done 2.5-3 h after inoculation or at earlier times with no significant difference in enzyme production levels. The best enzyme yield was achieved by adding CDCA (0.4 mM final concentration) at 2.5-3 h after inoculation. Studies on different volumes of growth showed that an increase in that volumes (i.e., from 100 mL to 1 L) resulted in yields higher (about 40% more), the best induction time in these conditions being at 1.5 h after inoculum (Macdonald IA and Sutherland JD 1983).

It was also observed that the end product of CDCA epimerization, UDCA, was very effective in blocking the induction mediated by CDCA or DCA, while the end product of CA epimerization, ursocholic acid (UCA, 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid), was less effective (Macdonald IA and Sutherland JD

1983)**.** Moreover the addition of rifampicin at the same time with the inducer completely suppressed the expression of the two enzymes, thus suggesting a mechanism of induction based on the *de novo* synthesis of mRNA coding for proteins (Macdonald IA et al. 1983).

The separation of *Ca*7β-HSDH and *Ca*7α-HSDH has been described by Macdonald and coworkers by a Procion red affinity chromatography. Subsequent purification of *Ca*7β-HSDH has been performed by a HPLC-TSK 3000 gel filtration column. Approximately, a 35-fold purification of *Ca*7β-HSDH was achieved, but SDS-PAGE analysis showed 6 major protein bands and unambiguous identification of the active band was not possible at that time (Macdonald IA et al. 1983).
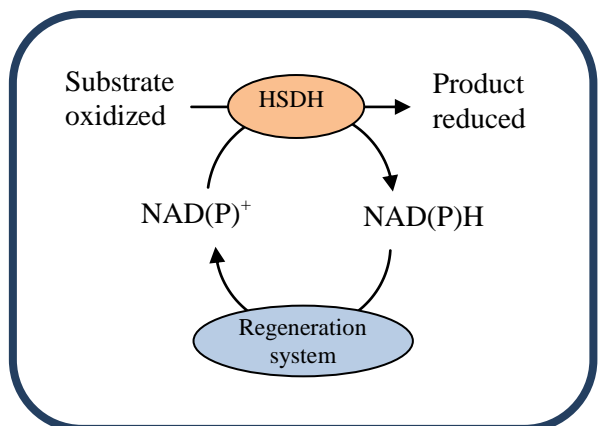
HSDH-CATALYZED BIOTRANSFORMATIONS

Methods and strategies used in cofactor regeneration

Cofactor regeneration by enzymatic systems

Applications of cofactor regeneration systems in HSDH-catalyzed reactions

HSDHs in multienzymatic processes

As previously stated, HSDHs are NAD(P)H-dependent enzymes. Due to their high cost, cofactors cannot be used in stoichiometric amounts in oxidation or reduction reactions, especially in the case of industrial applications. Furthermore, as reactions catalyzed by HSDHs are reversible, the conversion of the substrate into the desired product is dependent on the equilibrium constants of the two opposite reactions. To overcome the above problems, a cofactor regeneration system can be exploited. In fact, it allows the use of catalytic amounts of the cofactor and, at the same time, it unbalances the system towards product formation by removing one of the products of the coupled reaction (i.e., the oxidized/reduced cofactor).

Methods and strategies used in cofactor regeneration

Generally speaking, no regeneration method is ideal and a combination of factors determines the best method for a certain application. From the point of view of the cost, the efficiency of such a procedure may be estimated by the molar amount of synthesized product per molar amount of consumed cofactor during the course of the complete reaction. This ratio is called total turnover number (TTN) (Leonida MD 2001). To make a process economically viable, the TTN should be in the order of hundreds up to thousands. The TTN rises steeply by decreasing cofactor concentration at the expense of the volumetric productivity, since the cofactor concentration gradually becomes limiting for the total reaction system (Wichmann R and Racki D 2005). At the same time, enzyme consumption (ecn), i.e., the amount of biocatalyst needed for producing a defined amount of product, must be considered as well. This parameter can be expressed also as productivity of the system, i.e., the amount of product that can be produced using a defined amount of biocatalyst.

Several methods of cofactor regeneration have been studied: chemical, photochemical, electrochemical, biological and mixed methods (Leonida MD 2001). Direct methods, such as chemical, photochemical and electrochemical methods, might be the simplest methods to regenerate cofactors but each of these has major disadvantages.
Chemical methods, e.g., the direct oxidation or reduction of NAD(P)(H) by an inexpensive chemical reagent (Fig 17a), such as dithionite, piridinium salts and flavins (Jones JB et al. 1972, Jones JB and Taylor KE 1976), have been studied

in the '70s , but they have been set aside because they are not efficient due to the instability of the reagents and slow reaction rates. Photochemical methods (Fig 17b), e.g., the direct oxidation/reduction of NAD(P)(H) by a photosensitizer/electron carrear system activated by irradiation, are more efficient than chemical methods, TTN being as high as 1125. However, they are still not advantageous because the photocatalysts could be photochemically degraded and possible side reactions could occur (Willner I and Mandler D 1989).

Electrochemical regeneration of NAD(P)$^+$/NAD(P)H cofactors is an attractive alternative as it avoids the use of any additional reagent, it is simple and presents high enough regeneration numbers for preparative-scale applications (Wichmann R and Racki D 2005). Electrochemical strategies can be differentiated into two main approaches: (i) cofactors can be regenerated either directly on the surface of an electrode (Fig 17c) or (ii) by a mediator which is regenerated back on the surface of the electrode (Fig 17d). The first method has major drawbacks in NADH oxidation. In fact direct anodic oxidation of NADH at an inert electrode surface requires a relatively high overvoltage causing electrode fouling by strong absorption of NAD$^+$ and the formation of enzymatically inactive forms of NAD$^+$ (Gründig B et al. 1995). These limitations can be solved through the use of chemically modified electrodes that contain mediators which substantially lower the high overvoltage for NADH oxidation (method ii). To accelerate the electroanalytic oxidation of NADH, mediators such as *o-* or *p*-quinones, quinoid redox dyes such as indamines, phenazines, phenoxazines, phenothiazines, *o-* or *p*-phenylindiamines, aminophenols, flavin adenine dinucleotide (FAD), pyrroloquinoline quinone (PQQ), and metal ions like oxometalates, poly-metallophthalocyanines and ruthenium complexes (Hollmann F and Schmid A 2004, Gorton L 1986) have been investigated. Electrochemical reduction of NAD(P)$^+$ has been achieved by direct catodic electroreduction (TTN = 350 for the electrode direct NADH regeneration coupled to a glucose dehydrogenase/glucose system (Fassouane A et al 1990) and TTN = 220 for the direct electrode NADH regeneration coupled to a L-lactate dehydrogenase/L-lactate system (Biade AE et al. 1992)) or using mediators such as methyl viologen (Fisher RJ et al. 2000).
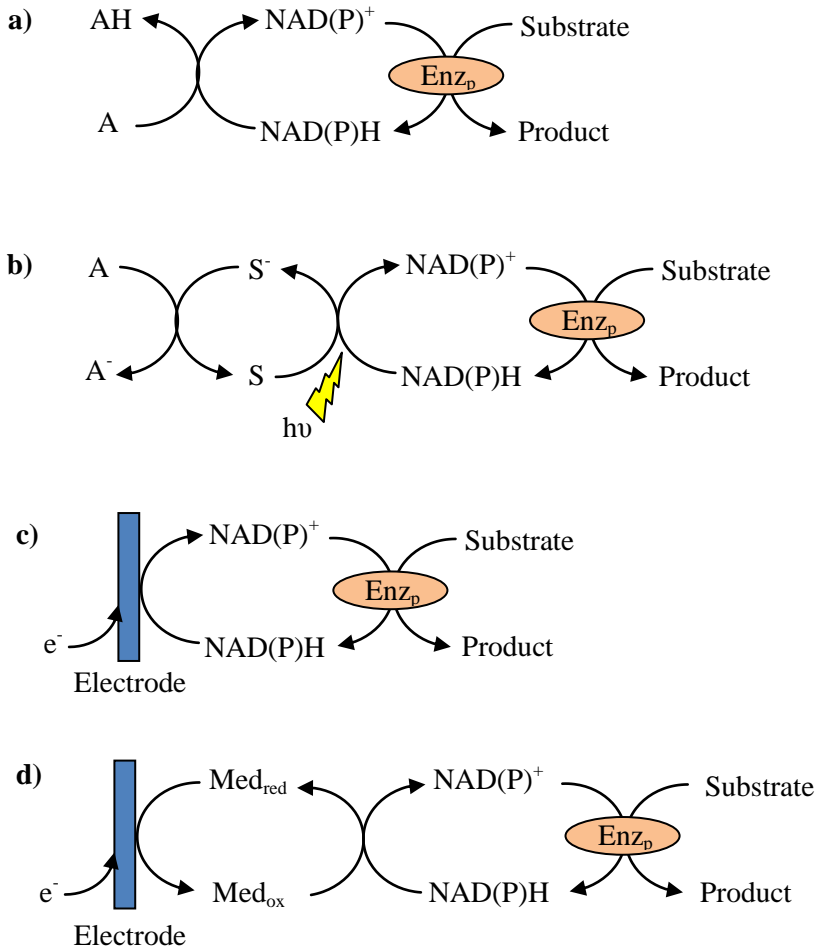
Figure 17. Examples of NAD(P)$^+$ regeneration systems: a) chemical regeneration, b) photochemical regeneration through a reductive quenching mechanism, c) electrochemical regeneration d) electro-enzymatic regeneration. Enz$_p$= enzyme catalyzing the desired reaction, S= photosensitizer, A= electron acceptor, Med$_{red}$= Reduced form of the mediator, Med$_{ox}$= oxidized form of the mediator.

As an alternative, biological methods, including both the use of whole cells and that of enzymes, have been investigated.

In the case of whole cells, the enzyme that catalyzes the desired reaction can be naturally produced by the cells or it could be recombinant. Cofactors are

anyway regenerated by the systems present inside the cell (Fig. 18a). This system present major drawbacks, such as the difficulty of recovering the desired product, the need to use low substrate concentrations in large volumes due to low tolerance of cells to the substrate, low productivity and the possible formation of side products (Hollmann F et al. 2011).

Instead, the use of isolated enzyme for cofactors regeneration may be very advantageous. Enzymatic regeneration can be achieved by two different ways (Fig. 18b and c). The first one is by means of the use of substrate-coupled reaction systems, in which one enzyme that uses both the reduced and oxidized forms of a cofactor is applied to catalyze both the desired synthesis of the product from one substrate and the cofactor regeneration reaction with a second substrate (Fig 18b). Since the same enzyme is required to catalyze two separated reactions simultaneously, it is usually difficult to achieve thermodynamically-favorite reaction conditions for both reactions in the same reaction medium.

The second approach has been adapted for the majority of cofactor regeneration processes and consists in coupling the desired enzymatic reaction to another reaction catalyzed by a second enzyme that regenerate the cofactor (Fig 18c). These systems are very advantageous as offering greater efficiency and ease of use and they do not require any special equipment as in the case of photochemical or electrochemical methods. Moreover, if the coupled reaction is not reversible, the reaction of interest will be thermodynamically favored and can be driven towards the complete formation of the desired product.

Among the cofactor regeneration systems mentioned herein, the enzymatic approach is particularly preferred for industrial processes due to its high selectivity and efficiency (Liu W and Wang P 2007).

These systems will be further discussed in the following paragraph.
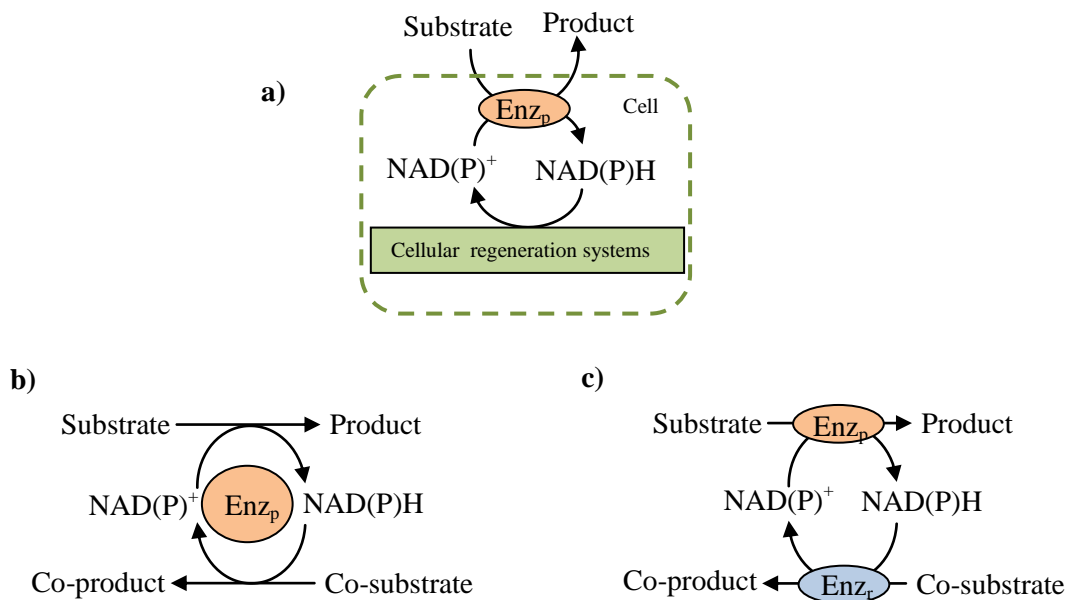
Figure 18. Examples of NAD(P)$^+$ regeneration systems by biological methods: a) whole cell regeneration, b) substrate-coupled regeneration, c) enzyme-coupled regeneration. Enz$_p$= enzyme catalyzing desired reaction, Enz$_r$= enzyme catalyzing the regeneration reaction

Cofactor regeneration by enzymatic systems

Different enzymatic systems have been developed for cofactor recycling, depending both on the cofactor used (either NAD(H) or NADP(H)) and the direction of the reaction (oxidation or reduction).

Concerning the enzymatic regeneration of NAD(P)H, i.e. the reduced form of the cofactor that is used in reduction reactions (e.g., reduction of the 7-keto bile acid derivatives catalyzed by 7β-HSDH), the most commonly employed systems are the glucose/glucose dehydrogenase (GDH) (Fig. 19a) and formate/formate dehydrogenase (FDH) systems (Fig. 19b):
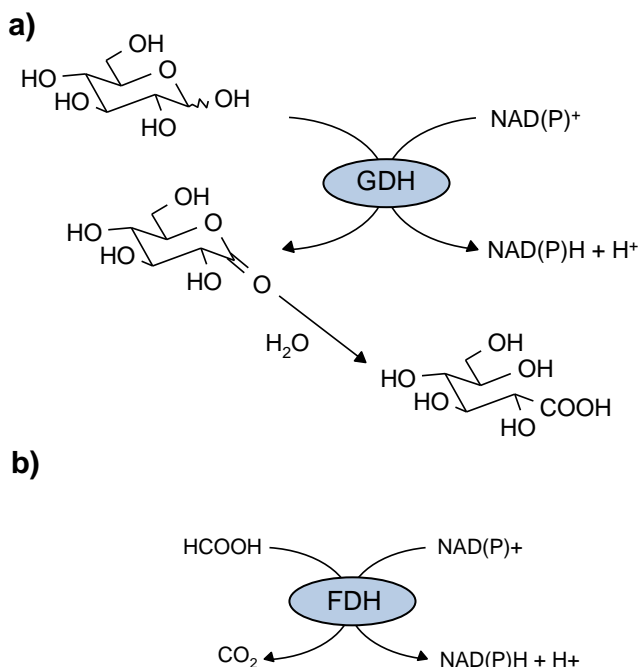
Figure 19. Enzymatic cofactor regeneration system for NAD(P)H regeneration. GDH= Glucose dehydrogenase, FDH= Formate dehydrogenase

These systems are very advantageous, in fact both cosubstrates, glucose and formate, are inexpensive and both reactions are irreversible as glucose is oxidized to gluconolactone, which hydrolyzes spontaneously, while formate is oxidized into carbon dioxide that escapes into the environment. Moreover, in the case of formate dehydrogenase, formate/CO_2 can be used as a buffering system, thus allowing to avoid the use of traditional buffers containing salts, such as phosphate and organic acids, that are considered powerful environmental pollutants and must be separated from the solutions before disposal of wastes. TTN obtained by using the formate/FDH system (up to 10^3) are usually lower compared to those obtained by the glucose/GDH system (up to 10^6) (van der Donk WA and Zhao H 2003).

In the case of enzymatic regeneration of NAD(P)$^+$, i.e. the oxidized form of the cofactor that is used in oxidation reactions (e.g., in the oxidation of 7α-OH bile acid derivatives catalyzed by 7α-HSDH), the most frequently employed systems

are α-ketoglutarate/glutamate dehydrogenase (GlDH) and acetone/alcohol dehydrogenase (ADH) for NAD(P)$^+$ regeneration (Figure 20a and b) and pyruvate/lactate dehydrogenase (LDH) for NAD$^+$ regeneration (Fig 20c) (van der Donk WA and Zhao H 2003):

a)

b)

c)
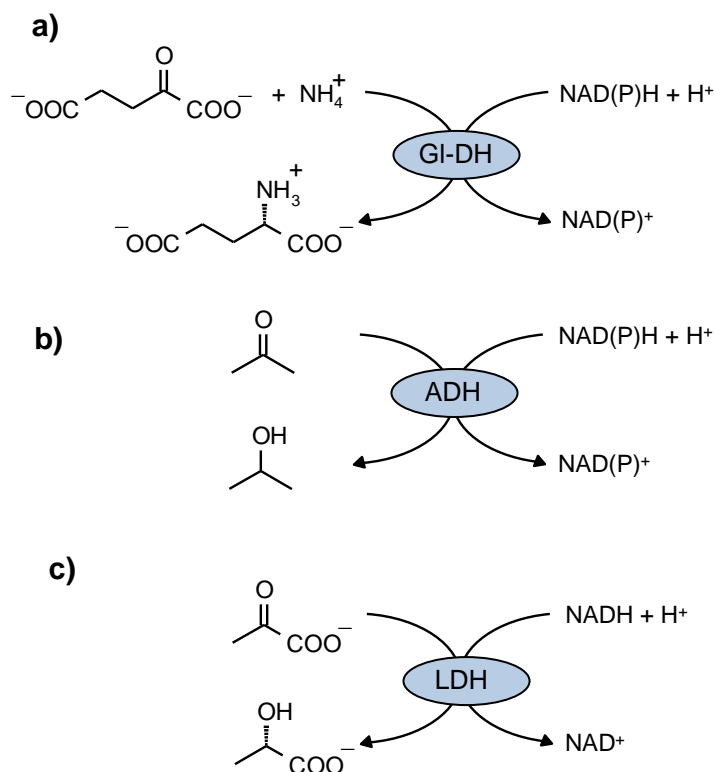
Figure 20. Enzymatic cofactor regeneration system for NAD(P)$^+$ regeneration. Gl-DH= L-Glutammate dehydrogenase, ADH= Alcohol dehydrogenase, LDH= L-lactate dehydrogenase.

Applications of cofactor regeneration systems in HSDH-catalyzed reactions

As far as reduction reactions concerns, the glucose/GDH system has been mainly employed for cofactor regeneration on a lab scale (Bovara R et al 1996, Bovara R et al. 1993, Carrea G et al. 1992, Riva S et al.1986), while only

preliminary investigations have been carried out in the presence of the formate/formate dehydrogenase system.

Various enzymatic regeneration systems have been instead applied to HSDHs-catalyzed oxidations.

In particular, the α-ketoglutarate/ GlDH and the lactate/LDH systems have been widely employed to regenerate the $NADP^+$ cofactor (Secundo F et al. 2003, Bovara R et al 1996, Bovara R et al. 1993, Riva S et al.1986, Carrea G et al.1984) and the NAD^+ cofactor (Giovannini PP et al 2008, Fossati E et al. 2006, **Paper I**), respectively.

The use of LDH from rabbit muscles (commercially available from Sigma-Aldrich) for NAD^+ regeneration in the oxidation of cholic acid to 12-KCDCA catalyzed by the NADH-dependent 12α-HSDH from *Bacillus sphaericus* (Genzyme) has been recently patented. Experiments were carried out on a few ml scale, but using cholic acid concentrations up to 60 g/l (Fossati E et al. 2006). In the same oxidation reaction, the commercially available GlDH from *Proteus* sp. was coupled to the NADPH-dependent 12α-HSDH from *Clostridium* sp. (from ASA) to regenerate $NADP^+$ cofactor. After preliminary work, this reaction has been scaled-up to 15 L by the company "Prodotti chimici e alimentari S.p.A." (Basaluzzo (AL), Italy) in the '90s. To allow their reuse, both enzymes were immobilized on solid support. Cholic acid oxidation to 12-KCDCA acid at 40 g/l concentrations gave very promising results as quantitative yields for 50 cycles of reaction were achieved (unpublished results). Unfortunately the high cost of both the cosubstrate and the glutamate dehydrogenase made this system difficult to implement on an industrial production.

In the search for efficient and cheap alternatives to the use of the α-ketoglutarate/GlDH system for the *in situ* regeneration of $NADP^+$ during the oxidation of cholic acid to 12-KCDCA acid, Fossati and coworkers explored the acetone/alcohol dehydrogenase (ADH) system. Because of the reversibility of the reduction reaction of the cosubstrate, a high concentration of acetone (more than 30 equivalents) was used to push the desired reaction to completion. The use of a large amount of acetone, however, turned out to be problematic because it compromised the stability of the enzymes. Moreover, despite the high concentration of the cosubstrate, the best reaction, carried out on a preparative scale (10 ml total volume, cholic acid concentration 40 g/l, acetone concentration 25% (v/v)), did not exceed 92% conversion. So the system was

not successful from an industrial point of view, unless TTN was up to 1,000 (Fossati E et al. 2005).

In 2011 this system was further investigated by Braun and coworkers who identified kinetic models for the simulation of batch processes for 12-KCDCA acid production integrated with the cofactor regeneration. Based on the identified process, model batch process optimization was performed *in silico* to minimize enzyme costs. Optimization allowed to identify best enzyme (12α-HSDH from *Clostridium* sp.) concentrations and cofactor concentrations in order to reach the maximal possible conversion of 100 mM cholic acid within 48 h. Thanks to this tool, conversion of 100 mM cholic acid coupled to the acetone/ADH regeneration system was improved but complete conversion has not been achieved yet (Braun M et al. 2011).

Recently, it was reported the use of activated ketones (e.g., methyl acetoacetate, chloroacetone) as cosubstrate in systems with cofactor recycling by ADH (Bisogno FR et al. 2009, Lavandera I et al. 2008). The advantage of these systems would be that the reduction reactions of activated ketones are practically irreversible, and therefore low amounts of cosubstrate, about 1.5 equivalents, might be sufficient to push the reactions to which they are coupled to the complete oxidation. Patents have already been filed on these systems. For example, a system has been patented for the production of enantiomerically pure (*S*)-2-butanol by coupling the enantioselective oxidation of a racemic mixture of 2-butanol catalyzed by an ADH to the reduction of methyl acetoacetate (Pfaller R and Schneider C 2007). Inspired by these works, we have recently submitted a patent application for the exploitation of similar cofactor regeneration systems in different oxidation reactions of bile acids catalyzed by HSDHs (data not shown).

Moreover, Aksu and coworkers have recently proposed a new cofactor regeneration method, coupling the electrochemical (Fig. 21) approach to the enzymatic approach (Aksu S et al. 2009). In this work, the reduced cofactor accepts electrons from a mediator, specifically 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), which is subsequently *in situ* regenerated by a laccase, an oxidase widely used in textile industry. The terminal acceptor of electrons is oxygen that is reduced to water by the laccase. The mediator is inexpensive and it is used in low catalytic amounts.

This system was applied to the oxidation of several alcohols by ADHs, and TTN of >300 and >16000 were obtained for the nicotinamide cofactor and the mediator, respectively.
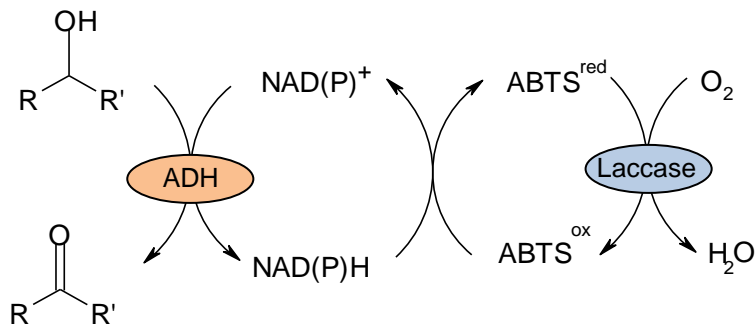
Figure 21. Laccase/mediator system coupled to an ADH-catalyzed alcohol oxidation

In **Paper II** we investigated the exploitation of a different mediator, Meldola's blue, in the oxidation of cholic acid to the corresponding 7-ketoderivative catalyzed by the 7α-HSDH from *B.fragilis*. A commercially available laccase from *Trametes* sp. was employed for mediator reoxidation. The system was successfully used also in a biphasic system where the methyl ester derivative of cholic acid had been dissolved in a suitable organic solvent.

HSDHs in multienzymatic processes

Multienzymatic processes, which involve the use of two or more enzymes to catalyze reactions in a defined pathway, are becoming very attractive for the production of many compounds at an industrial level (Santacoloma PA et al. 2011)

The enzymatic reactions of a multienzymatic process can be performed either in two or more sequential steps that require the isolation of intermediates, or in a "one-pot" fashion, i.e., without the isolation of intermediates.

In the first case, the first reaction of the process is set up, the product is isolated and added to a second reaction mixture to be converted into a second product.

In one-pot processes instead the first reaction is set up, the product is not isolated, but, for example, other reagents and/or catalysts are subsequently added to the reaction mixture for the conversion of the first product into a
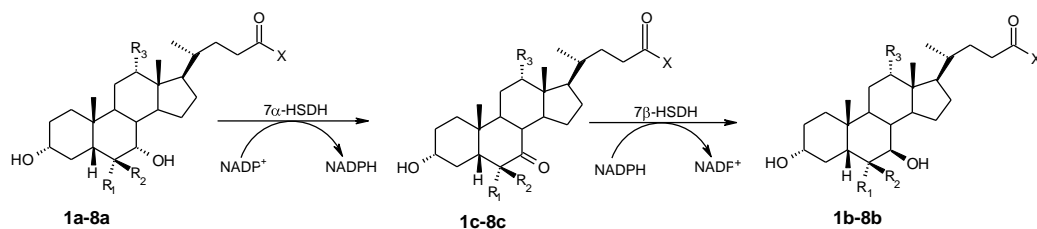
second product. A particular case of one-pot processes are the so-called "cascade" processes where all reagents and catalysts are present right from the beginning and the different enzymatic reactions proceed simultaneously.

One-pot processes, by avoiding time-consuming or yield-reducing isolation and purification of the intermediates and minimizing the amounts of chemicals/solvents required, result effective in reducing operation time, costs and environmental impact. Moreover the whole process can be developed without having to optimize the individual steps.

However, these processes are challenging as it is often difficult to find reaction conditions that are suitable at the same time for the different reactions involved in the process. Due to the diverging reaction conditions, even much more challenging are those one-pot processes that involve concomitant oxidation and reduction steps (Schrittwieser JH et al. 2011). In fact, while oxidation and reduction reactions occur simultaneously in living cells with great efficiency, it's not easy to find conditions that allow to drive both reactions to completion *in vitro*.

One of the first examples of sequential use of HSDHs for bile acid modification was from Bovara and coworkers. It reported the epimerization of bile acids and some of their derivatives by using the NADH dependent 7α-HSDH from *E. coli* and the NADPH-dependent 7β-HSDH partially purified from *C. absonum* (Bovara R et al. 1993) (Fig. 22).

After the oxidative step the product was recovered and used for the subsequent reductive step. Reactions were coupled to a lactate/LDH system for NAD^+ regeneration and to a glucose/GDH system for NADPH regeneration. Epimerization of compounds from **1a** to **7a** were carried out at a substrate concentration of 12.5 mM in potassium phosphate buffer and the obtained yield was between 85 % and 94 %. Epimerization of compound **8a** was performed in a biphasic system with a 25 mM substrate concentration in the organic phase.

R$_1$	R$_2$	R$_3$	X	
H	H	OH	OH	1
H	H	H	OH	2
H	OH	H	OH	3
OH	H	H	OH	4
H	H	OH	NHCH$_2$COOH	5
H	H	OH	NHCH$_2$CH$_2$SO$_3^-$	6
H	H	H	NHCH$_2$CH$_2$SO$_3^-$	7
H	H	OH	OCH$_3$	8

Figure 22. Epimerization of different bile acids and their derivatives

The sequential use of the 7α-HSDH and 7β-HSDH from *C. absonum* for the α/β inversion of C-7 hydroxyl of cholic acid was further investigated by the same group in order to obtain ursodeoxycholic acid directly from cholic acid (Bovara R et al 1996).

In this case, cholic acid hydroxy groups at C-7 and C-12 were simultaneously oxidized by the NADPH-dependent 7α-HSDH from *C. absonum* and the NADPH-dependent 12α-HSDH from *Clostridium* sp., respectively. Thus, through a stereo- and regioselective reduction catalyzed by the NADPH-dependent 7β-HSDH from *C. absonum*, the previously isolated intermediate was transformed into the 12-ketoursodeoxycholic acid and then in ursodeoxycholic acid by a chemical reduction (Wolff-Kishner method) (Fig. 23). In the first step oxidative reactions were coupled to a α-ketoglutarate/GlDH system for NADP$^+$ regeneration, whereas in the second step the reductive reaction was coupled to a glucose/GDH system for NADPH regeneration. Reactions were carried out in aqueous solution and substrate concentration was 12.5 mM.

The 12α-HSDH used was commercially available from ASA, while 7α-HSDH was a crude extract from *C. absonum* and 7β-HSDH was partially purified from

the same source. It is noteworthy that, according to this approach, preparations of crude 7α-HSDH and 12α-HSDH may be successfully used without any formation of byproducts, as these enzymes are highly regioselective and the preparations are free from enzyme contamination that could result in undesired products (e.g., 3α-HSDH activities).
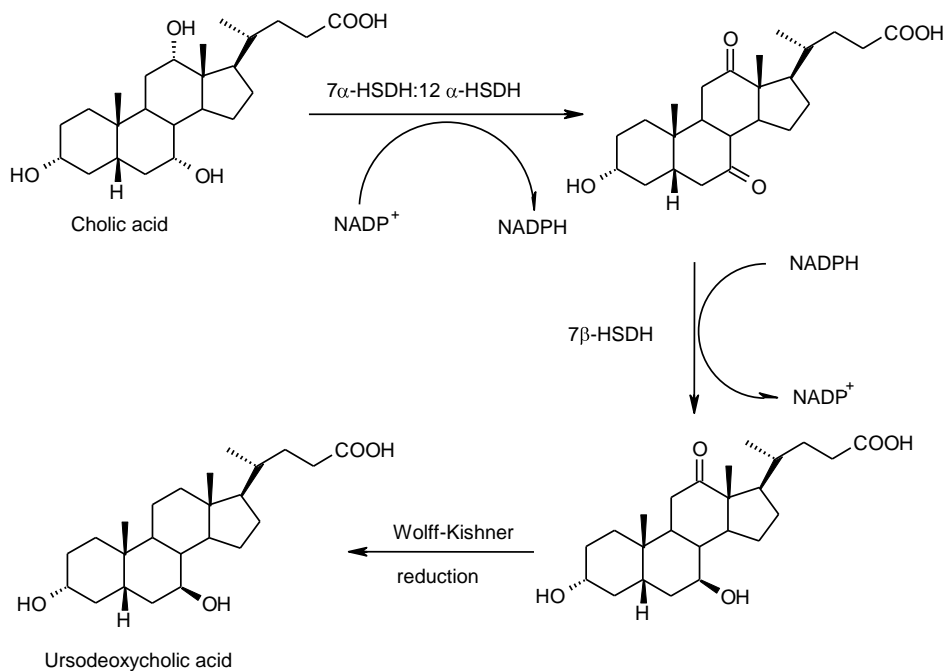
Figure 17. Chemoenzymatic synthesis of ursodeoxycholic acid

On the contrary, the reduction reaction of the 7-oxo intermediate requires the use of a preparation of 7β-HSDH completely devoid of any 7α-HSDH activity, whose presence would lead to the formation of the corresponding 7α-hydroxy by-product (12-KCDCA).

Reactions were quantitative and both the recovery of the final product (88 %) and its purity (>98 %) were satisfactory.

This topic has been further investigated in **Paper I** by using a multienzymatic system with five different dehydrogenase activities and different reactions set-ups (i.e., sequential, one-pot and cascade processes).

THESIS OVERVIEW

Thanks to the exploitation of molecular biology techniques and to the advances of bioprocesses engineering, biocatalysis is making an increasingly important contribution to the development of sustainable industrial chemical processes. In fact, the number of available enzymes is continuously increasing, and directed evolution and metabolic engineering technologies have led to the production of stable biocatalysts with customized activity and selectivity (Zaks A 2001). Moreover, enzymes belonging to different classes have been shown to be usable in a wide variety of applications and even in large-scale processes. This is particularly important in the pharmaceutical industry where high reaction selectivity on complex substrates is critical (Schmid A et al. 2001).

Hydroxysteroid dehydrogenases (HSDHs) are NAD(P)H-dependent enzymes which belong to the SDR (short chain dehydrogenase/reductase) superfamily (Kavanagh KL et al. 2008). They are homotetrameric or homodimeric proteins, with a subunit molecular weight of approx. 30 kDa (Tanaka N et al. 1996), and catalyze the regio- and stereoselective oxidation/reduction of hydroxyl groups of steroids or bile acids *in vivo* (Ridlon JM et al. 2006). HSDHs from both mammalian and microbial sources have been identified, the latter having been widely employed in the selective modification of bile acids and their derivatives, even on preparative scale (Bovara R et al. 1996).

Bile acids are amphiphilic steroidal molecules synthesized in the liver from cholesterol. *In vivo*, they play an important role in the digestion and adsorption of fats, fatty acids and lipid-soluble vitamins (Mukhopadhyay S and Maitra U 2004).
Bile acids and their derivatives are used in a variety of application, especially in the pharmaceutical field. In fact, they are currently used in the treatment of bile acid deficiencies, some liver diseases, and in the dissolution of cholesterol gallstones (Hofmann AF and Hagey LR 2008, Hofmann AF 2009). Moreover, bile acids are of interest for their ability of forming gels, thus making them potential materials for drug delivery (Mukhopadhyay S and Maitra U 2004). Additionally, their particular shape and amphiphilic properties make these compounds ideal as "building blocks" to be used as chiral artificial receptors and supramolecular hosts for molecular recognition (Tamminem J and Kolehmainen E 2001).

Among bile acids, ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid) is the most used in the pharmaceutical field, being indeed the first-choice drug in the treatment of cholesterol gallstones and being also widely used for the treatment of other liver diseases, such as primary biliary cirrhosis (PBC) and primary sclerosis cholangitis (PSC) (Hofmann AF and Hagey LR 2008).

UDCA is currently synthesized from cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), a primary bile acid purified in huge amounts from bovine bile, by a ton-scale chemical synthesis, but this route is not completely satisfactory because of the lengthy protocol, the low yields and the use of polluting substances. The use of enzymes such as hydroxysteroid dehydrogenases could be a valid alternative in order to shorten the synthesis, increase the yield of UDCA, and limit the environmental impact of the production process.

In the development of a biocatalytic process employing HSDHs, it must be taken into account that, as previously stated, HSDHs catalyzed reactions require NAD(P)H cofactors, which are very expensive. Therefore, especially in the case of industrial applications, a suitable cofactor regeneration system is necessary in order to use cofactors in catalytic amounts and no longer stoichiometrically. Furthermore, as reactions catalyzed by HSDHs are reversible, the conversion of the substrate into the desired product is dependent on the equilibrium constants of the two opposite reactions and the cofactor regeneration system helps unbalancing the system towards product formation.

During this PhD work, the preparative-scale HSDHs-catalyzed one-pot enzymatic synthesis of 12-ketoursodeoxycholic acid (3α,7β-dihydroxy-12-oxo-5β-cholanoic acid), a key intermediate for the synthesis of ursodeoxycholic acid, from cholic acid, was first investigated. This goal has been achieved by using HSDHs with different cofactor specificity in oxidation and reduction reactions occurring in the same reaction mixture and properly driven by different cofactor regeneration systems. It was demonstrated that all the involved biocatalysts should be exclusively selective for the respective cofactors in order to perform this multienzymatic synthesis in a cascade process. In this specific case, the establishment of undesired reaction equilibria (likely due to a limited cofactor specificity of some of the enzymes used) leading to a non-specific bioconversion process and, consequently, to reduced product yields

and purity, was overcome by uncoupling the oxidative and reductive biocatalysts. However, we may foresee that, thanks to the ongoing advances in biocatalysts engineering, new enzymes with exclusive NADPH or NADH-specificity will become available in the near future (**Paper I**).

As mentioned above, cofactor regeneration is a critical issue in HSDHs catalyzed reactions. Although NAD(P)H regeneration by enzymatic methods is quite satisfactory, $NAD(P)^+$ regeneration is still challenging as the enzymatic methods reported in literature are actually limited by the high co-substrate costs or their reactivity. Moreover, exploitation of alternative electrochemical methods gave scarce results, mainly due to low total turnover numbers (TTN). In the second part of this PhD work we investigated a new $NAD(P)^+$ regeneration system, in which a $2H^+/4e^-$ redox mediator, Meldola's Blue (MB), used in low, catalytic amounts, accepts electrons from NAD(P)H and is thus converted into its reduced form. Subsequently, a laccase, an oxidase widely used in textile industry, reduces molecular oxygen as terminal $4H^+/4e^-$ acceptor to H_2O and concomitantly re-oxidizes NAD(P)H *via* MB at high turnover rates. This system was employed in the gram-scale 7α-hydroxysteroid dehydrogenase catalyzed oxidation of cholic acid into its 7-keto derivative. The system was tested both in aqueous and biphasic systems, and demonstrate to be cheap, very efficient, to have high stability, to tolerate solvents, and to be simple to employ (**Paper II**).

Finally, it must be remarked that, while biocatalyzed transformations of bile acids by isolated HSDHs-catalyzed reactions have been widely investigated on a lab-scale, the lack of commercially available biocatalysts has generally limited the scale-up of the studied reactions to an industrial level. In the last part of this PhD work, my efforts were devoted to the cloning of the NADPH-dependent 7α- and 7β-hydroxysteroid dehydrogenases (7α-HSDH and 7β-HSDH) from *Clostridium absonum*. The enzymes, once overexpressed in recombinant form in *E. coli*, have been submitted to a detailed kinetic and functional characterization in oxidoreductive reactions of industrial interest for the synthesis of bile acids derivatives. The results showed that both enzymes, in suitable bioconversion conditions, could be promising candidates for further applications in the epimerization reaction of bile acids at the C-7 position, e.g. the conversion of cholic acid into ursocholic acid (3α,7β,12α-trihydroxy-5β-

cholan-24-oic acid) or chenodeoxycholic acid (3α,7α,-diihydroxy-5β-cholan-24-oic acid) into UDCA (**Paper III**).

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Acknowledgements

I would like to express my sincere gratitude to:

Dr. Daniela Monti for generously sharing with me her experience within and outside the field of biocatalysis, for the friendship, for the excellent guidance and great advice, and for helping me in the thesis-writing process

Dr. Sergio Riva for his valuable teachings and suggestions

Prof. Loredano Pollegioni for giving me the opportunity to do this PhD

Dr. Roland Ludwig for welcoming me in his lab in Vienna, for his brilliant ideas and enthusiasm, for the fruitful collaboration, and for the big big help in writing paper II. Thanks also to all students and technicians who helped me during my stay in Vienna

Prodotti Chimici e Alimentari S.pA. for the financial support, and in particular to Mr Fausto Polentini for his great collaboration and for having strongly believed in this project

All the people from the ICRM-CNR lab for the memorable laughs and interesting discussions

Finally, sincere thanks for all the support and the fundamental encouragement to Andrea and my parents.

Grazie a tutti! Thank you all! Vielen Dank an alle!