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Xanthomonas maltophilia CBS 897.97 as a source of new 7β- and 7α-hydroxysteroid dehydrogenases and cholylglycine hydrolase: Improved biotransformations of bile acids

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ABSTRACT

The paper reports the partial purification and characterization of the 7β- and 7α-hydroxysteroid dehydrogenases (HSDH) and cholylglycine hydrolase (CGH), isolated from *Xanthomonas maltophilia* CBS 897.97. The activity of 7β-HSDH and 7α-HSDH in the reduction of the 7-keto bile acids is determined. The affinity of 7β-HSDH for bile acids is confirmed by the reduction, on analytical scale, to the corresponding 7β-OH derivatives. A crude mixture of 7α- and 7β-HSDH, in soluble or immobilized form, is employed in the synthesis, on preparative scale, of ursocholic and ursodeoxycholic acids starting from the corresponding 7α-derivatives. On the other hand, a partially purified 7β-HSDH in a double enzyme system, where the couple formate/formate dehydrogenase allows the cofactor recycle, affords 6α-fluoro-3α, 7β-dihydroxy-5β-cholan-24-oic acid (6-FUDCA) by reduction of the corresponding 7-keto derivative. This compound is not obtainable by microbiological route.
The efficient and mild hydrolysis of glycinates and taurinates of bile acids with CGH is also reported. Very promising results are also obtained with bile acid containing raw materials.

1. Introduction

Bile acids (BAs), their conjugates and salts are natural products, fundamental constituents of bile [1]. The primary bile acids in human bile are cholic acid (CA) and chenodeoxycholic acid (CDCA), mainly present as glycine and taurine conjugates, while deoxycholic (DCA) and lithocholic (LCA) acids are commonly known as secondary bile acids, produced from cholic and chenodeoxycholic acids, respectively, by intestinal bacteria through hydrolysis of the side-chain amide bonds and C-7 dehydroxylation [1,2]. Among them chenodeoxycholic and the 7-OH epimer ursodeoxycholic acid (UDCA) have important pharmaceutical applications related to their ability to solubilize cholesterol gallstones [3,4]. Both these acids are prepared on a large scale from raw, low cost materials with high bile acid content as bovine bile. Its major component is cholic acid that is, therefore, used as starting materials for the synthesis of chenodeoxycholic acid [5,6]. The first step of the chemical procedure, that affords these valuable drugs, is the alkaline hydrolysis of the conjugates (i.e. taurinates and glycinates) obtained from bovine bile followed by a sequence of chemical reactions, the last two steps of which involve the selective α/β inversion of the 7-OH carbon center [7,8]. This inversion is chemically achieved through the regioselective oxidation of

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the 7α-OH function and the subsequent radicalic reduction with Na in ethanol [9].

Regarding the first reaction, the enzymatic hydrolysis of the conjugates should be certainly interesting for pharmaceuticals because it would avoid the use of concentrated bases and high temperature. Up to now, this hydrolytic activity has been reported in anaerobic and microaerophile bacteria present in mammalian intestines [10]. The hydrolases, that have high affinity towards bile acids conjugates, have been isolated from *Lactobacillus* [11,12], *Clostridium perfringens* [13] and *Bifidobacterium longum* [14] and hydrolyze at acidic pH with a moderate thermostability.

Regarding the selective α/β inversion, moreover, in a recent review a selective control in the reduction of the 7-keto functionality of bile acids can efficiently be achieved by employing enzymatic biotransformations, mainly with anaerobic bacteria [15]. The epimerization of the CDCA to UDCA can occur via oxido-reduction, through the intermediate 7-ketolithocholic acid (7KLCA), or via α-dehydroxylation-β-hydroxylation through the LCA [16] (Scheme 1).

Generally, the inversion is achieved through oxido-reduction reactions mediated by faecal flora [17,18], by pure *Clostridium* cells [19–21] or by mixture of *Clostridium absonum* and *Eubacterium* sp. [21]. The enzymes responsible of these reactions are NAD or NADP-dependent 7α- and 7β-hydroxysteroid dehydrogenases (HSDH) or 7α- and 7β-hydroxysteroid hydroxylases. In the literature, the purification NADP-dependent 7α- and 7β-HSDH from *C. absonum* have been reported [10,21,22].

Only recently the 6-FUDCA [23,24] has shown to be endowed with a favourable pharmacological profile, mainly due to its slightly increased hydrophily, resistance to bacterial 7-dehydroxylation and good enrichment in bile with respect to UDCA.

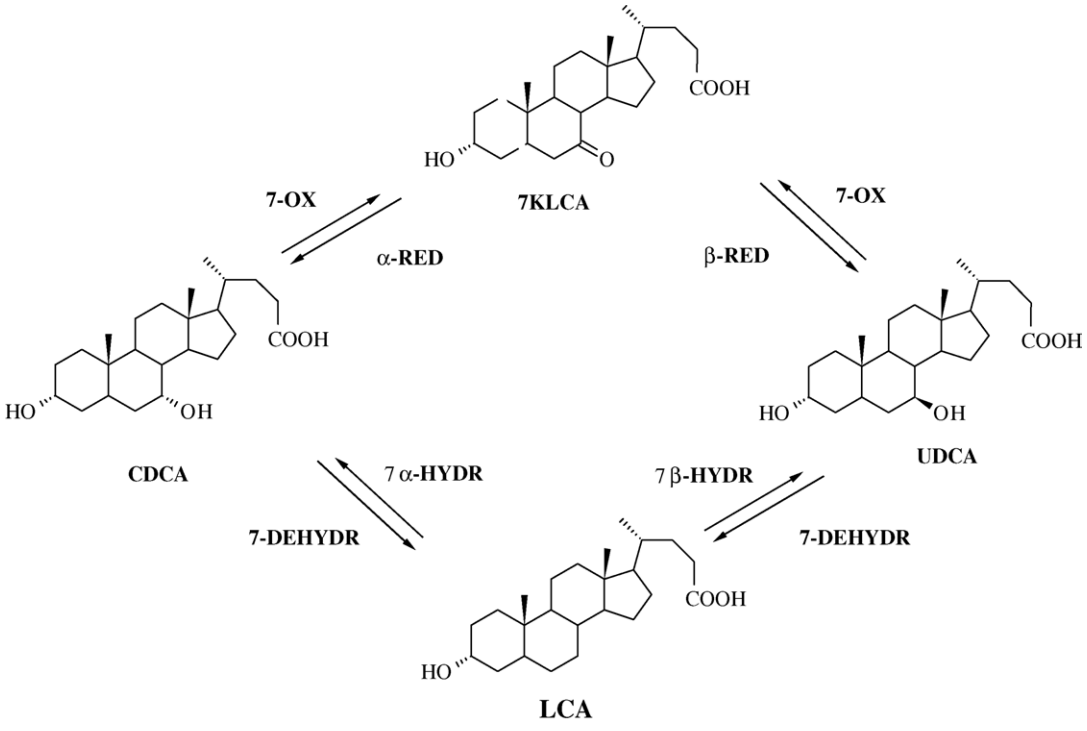
In our previous works regarding the microbiological approach to the synthesis of pharmaceutically interesting BAs, we have isolated, from a bile acids contaminated soil, a bacterium, *Xanthomonas maltophilia* CBS 827.97, able to hydrolyze the bile acid conjugates [25] and to achieve the microbial 7α/7β OH-inversion of various bile acids via oxidation of the 7α-hydroxy group and subsequent stereospecific reduction of the keto functionality into the corresponding 7β-hydroxy group [26,27]. We have also described the isolation and characterization of the cholylglycine hydrolase (CGH) from *X. maltophilia* [28].

In this paper, we report the partial purification and characterization of the 7β-HSDH and 7α-HSDH and the further improving application of these enzymes to the synthesis of UCA, UDCA and 6-FUDCA. The efficient hydrolysis with CGH of various bile acid conjugates, also present in raw materials, is also described.

2. Experimental

Sodium salt of 7-ketocholic acid **1**, 3,7-diketocholic acid **2**, 7,12-diketocholic acid **3**, 7-ketohyocholic acid **4**, and 7-keto-chenodeoxycholic acid **6** and glycinates and taurinates of cholic acid **13**, chenodeoxycholic acid **14**, deoxycholic acid **15**, and hyodeoxycholic acid **16** have been supplied by ICE industry [29].

NAD^+, phenylmethyl sulphonyl fluoride (PMSF), egg white lysozime, protamine sulphate, DEAE-sepharose, Cibacrom-Blue 3GA agarose and Phenyl-sepharose were Sigma products while hydroxylapatite was supplied by Calbiochem. 6-Fluoro-7-keto-hyocholic acid **5** is prepared according to the literature procedure [23].



Scheme 1

TLC analyses are performed on silica gel with ethyl acetate/cyclohexane/acetic acid 50/50/1 as eluent.

Gas chromatographic analyses are performed on a Carlo Erba HRGC 5160 Mega series chromatograph. The reaction products, previously derivatized with trifluoroacetic anhydride and hexafluoroisopropanol, are analyzed by GLC on fused capillary column SE52 (25 m × 0.32 mm) from Mega s.n.c.: helium as carrier gas (0.55 atm); temperature 250 °C for 5 min, 250–300 °C (5 °C/min) and then 300 °C for 3 min.

Retention times (in min) for the series of cholic, chenodeoxycholic, hyocholic and 6α-fluoro-3α-hydroxy-7-keto-5β-cholan-24-oic acids are reported in a previous paper [27].

HPLC analyses are performed on HPLC modular chromatographic system that consists of PU-980 Intelligent HPLC Pump, LG-1580-02 Ternary Gradient Unit (Jasco, Tokyo, Japan) and a light scattering detector (Sedex 55, Sedere). Separations are performed on a 5 μm Tracer extrasil ODS 2 column (150 mm × 0.46 mm i.d., Teknokroma, Barcelona, Spain) fitted with a Tracer ODS guard column (Teknokroma, Barcelona, Spain), eluted with gradient system (methanol, solvent A; 15 mM ammonium acetate (pH 4.3 with acetic acid), solvent B; 20 min at 64 A/36 B (v/v), linear gradient from 64 A/36 B (v/v) to 75 A/25 B (v/v) in 10 min, 20 min at 75 A/25 B (v/v), at flow-rate of 1.0 mL/min.

Retention times (in min) are the following: taurocholic acid **13b**, 5.78; glycocholic acid **13a**, 9.46; taurochenodeoxycholic acid **14b**, 11.24; taurodeoxycholic acid **15b**, 12.91; glycochenodeoxycholic acid **14a**, 20.03; glycodeoxycholic acid **15a**, 23.21; cholic acid **13**, 31.67; chenodeoxycholic acid **14**, 40.29; deoxycholic acid **15**, 40.97.

^{1}H and ^{13}C NMR spectra were obtained with a Varian Gemini 300 spectrometer. Chemical shifts are given in parts per million from Me_4Si as internal standard. IR spectra were obtained on a Perkin-Elmer Model 297 grating spectrometer and on a FTIR Nicolet 510 P spectrometer.

6-FUDCA 11 [30] showed the following: mp 148–150 °C; IR ($CHCl_3$) 1725 cm^{-1}; ^{1}H NMR ($CDCl_3/CD_3OD$) δ 0.68 (s, 3H, 18-Me), 0.85–2.1 (m, 28H, methylene–methyne envelope), 2.22 (ddd, 1H, J = 15, 8.5, 6.2 Hz), 2.36 (ddd, 1H, J = 15, 10, 5.4 Hz) 3.45–4.0 (m, 5H), 4.55 (ddd, 1H, J = 49, 8.5, 6.2 Hz); ^{13}C NMR ($CDCl_3/CH_3OD$) δ 177.27 (CO_2H), 95.31 (d, J = 173 Hz, C-6), 73.12 (dd, J = 17 Hz, C-7), 70.44 (C-3), 55.75, 54.84, 46.13 (d, J = 15 Hz), 43.62, 40.93 (d, J = 7 Hz), 39.77, 39.08, 35.40 (d, J = 8 Hz), 35.19, 35.01, 30.92, 30.82, 30.18 (d, J = 5 Hz), 29.54, 28.36, 26.37, 23.21, 20.98, 18.21, 11.98. Anal Calcd for $C_{24}H_{39}O_4F$: C%, 70.21; H%, 9.57; F%, 4.62. Found: C%, 70.89; H%, 9.50; F%, 4.75.

7β,12α-Dihydroxy-3-keto-5β-cholan-24-oic acid **8** showed the following: IR (nujol), 3400, 1700 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ 0.72 (s, 3H, 18-Me), 0.8–2.6 (m, 24H, methylene–methyne envelope) 0.97 (d, 3H, J_{H-H} = 5.6 Hz, 21-Me), 1.02 (s, 3H, 19-Me), 3.62 (m, 1H, 7-CH), 4.02 (br s, 1H, CH-12); ^{13}C NMR ($CDCl_3$), δ 12.72, 17.25, 22.25, 26.15, 27.86, 29.10, 30.72, 30.90, 32.08, 33.84, 35.00, 36.09, 36.16, 36.76, 42.92, 43.27, 44.22, 45.78, 47.24, 47.37, 70.60, 72.44, 178.31, 212.90. Anal Calcd for $C_{24}H_{38}O_5$: C%, 70.90; H%, 9.42. Found: C%, 70.44; H%, 9.63.

12α-Hydroxy-3,7-dioxo-5β-cholan-24-oic acid **7** showed the following [31]: ^{1}H NMR (CD_3OD) δ 0.79 (s, 3H, 18-Me), 1.15 (s, 3H, 19-Me), 2.62 (ψt, 1H, J_{H-H} = 11.2 Hz, 8-CH), 3.00 (dd, 1H, J_{H-H} = 12.6 Hz, J_{H-H} = 5.0 Hz, 6α-CH), 4.02 (br s, 1H, 12-CH); ^{13}C NMR (CD_3OD) δ 13.3 (C-18), 17.7 (C-21), 22.4 (C-19), 25.3 (C-15), 28.7 (C-16), 30.8 (C-11), 32.1 (C-22), 32.3 (C-23), 36.0 (C-10), 36.2 (C-1), 36.6 (C-20), 37.4 (C-2), 37.3 (C-9), 41.9 (C-14), 43.8 (C-4), 45.8 (C-6), 47.3 (C-17), 47.6 (C-13), 48.9 (C-5), 50.7 (C-8), 72.8 (C-12), 178.2 (C-24), 213.1 (C-7), 213.7 (C-3).

Centriflow CF25/CF50 membrane cone Amicon was used for ultrafiltration. Spectrophotometric analyses were performed on an Uvicon 930 Spectrometer (Kontron Instrument). Glycine concentration was measured using a cadmium-ninhydrine procedure [32]. Protein concentration was determined following the Lowry method [33].

2.1. *Purification of Xanthomonas maltophilia enzymes*

The cell extract was prepared as reported [27]. Enzymatic solutions of 7α- and 7β-HSDH were obtained after chromatography, on DEAE-sepharose column, of cell extracts starting from wet cells (9 g).

2.1.1. *Partial purification and enzyme assay of 7α- and 7β-HSDH*

Both the solutions containing 7α- and 7β-HSDH were fractionated with ammonium sulphate in order to collect insoluble proteins between 55 and 75% saturation (Tables 1 and 2). Pellet containing 7α-HSDH was dissolved in 5 mL of 50 mM sodium phosphate (Na-P) buffer at pH 7.5 and used for kinetics determinations. This enzyme is stable at 4 °C for 2 days.

7β-HSDH purification was carried out dissolving the corresponding precipitate in Na-P buffer pH 7.5 (30 mL) and dialyzing against the same buffer (1.0 L). The dialyzed solution was loaded on a Hydroxylapatite column (1 cm × 3 cm) equilibrated with 20 mM Na-P buffer pH 7.5. The enzyme is eluted with 130 mM Na-P buffer after rinsing with 55 mM Na-P buffer. The pooled active fractions are diluted to a final phosphate concentration of 20 mM and loaded on a Cibachrom Blue 3GA agarose column (1 cm × 2 cm) equilibrated with 20 mM Na-P

Table 1 – Purification procedure for 7β-HSDH

Step	Volume (mL)	Protein (mg)	Activity (U)	Sp. activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	89	356	142	0.4	–	100
DEAE-sepharose[a]	160	224	134	0.6	1.5	94
$(NH_4)_2SO_4$ (55–75%)	30	20	96	4.8	12	67
Hydroxylapatite	4.5	1	45	45	112	31
Cibachrom Blue 3GA agarose	1	0.2	14	70	175	10

[a] Not bounded to the resin.

Table 2 – Procedure for partial purification of 7α-HSDH

Step	Volume (mL)	Protein (mg)	Activity (U)	Sp. activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	89	356	249	0.8	–	100
DEAE-sepharose	30	8	150	18.8	23	60
$(NH_4)_2SO_4$ (55–75%)	5	4	132	33	41	46

buffer pH 7.5 (Table 1). 7β-HSDH was eluted with Na-P buffer pH 7.5 containing 1.0 mM NAD^+. Starting from 9 g of *X. maltophilia* wet cells is possible to obtain 4 mg of partially purified 7α-HSDH (33 U/mg, 46% yield) and 0.2 mg of purified 7β-HSDH (70 U/mg, 10% yield) with a purification factor of 41- and 175-fold, respectively.

7β- and 7α-Hydroxysteroid dehydrogenase activities were assayed at 22 °C monitoring NADH formation (absorbance change at 340 nm) during the oxidation of ursocholic acid and cholic acid, respectively. The reaction was started adding 10–20 μL of enzyme solution to 1 mL of 20 mM Na-P buffer pH 7.5 (for 7α-HSDH) or 8.5 for (7β-HSDH) containing 5 mM NAD^+, 5 mM of the suitable bile acid, 1 mM EDTA and 2 mM β-mercaptoethanol. One international unit (IU) of enzyme is defined as the amount required to produce 1 μmol of NADH per minute.

The values of K_m and V_{max} for substrates, obtained from Lineweaver–Burk plots, are referred to the oxidation of cholic and ursocholic acid at pH 7.5, respectively. These experiments are carried out using 0.8 mM NAD^+ and a 8–100 mM range for substrates. The values of K_m and V_{max} for NAD^+ were obtained using 5 mM substrate and a 10–100 mM range for NAD^+. The kinetic parameters for 7α-HSDH are: V_{max} 33 U/mg, K_m (for substrate) 1 mM and K_m (for NAD^+) 0.15 mM. The kinetic parameters for 7β-HSDH are: V_{max} 70 U/mg, K_m (for substrate) 2.5 mM and K_m (for NAD^+) 1 mM. The rate of enzymatic oxidations is affected by pH. The oxidations of cholic and ursocholic acids have the optimum of pH at 7.5 and 9.5, respectively. Both enzymatic reactions show an optimal value of temperature at 28 °C.

Molecular weight of 7β-HSDH, determined by gel filtration on Sephacryl S-200 using as standards myoglobin (17 kDa), albumin from bovine serum (67 kDa) and lactic dehydrogenase (134 kDa), was 130 kDa.

2.1.2. *Purification of CGH [28]*

Cholylglycine hydrolase eluted together with 7α-HSDH, from DEAE-sepharose column [27] was further purified obtaining 0.3 mg of CGH (101 U/mg, 16% yield) with a purification factor of 168-fold. The cholylglycine hydrolase activity was assayed monitoring the released glycine, by a cadmium-ninhydrin procedure [32], during the hydrolysis of sodium glycocholate (5 mM) in 20 mM Na-P buffer pH 8 at 25 °C.

2.2. *Determination of 7β- and 7α-HSDH activity on 7-keto bile acids 1–6*

To a solution (1 mL) of 20 mM Na-P buffer at pH 7.0, containing 0.15 mM NADH, 1 mM EDTA, 2 mM β-mercaptoethanol and 5 mM of the proper 7-keto bile acid, 5 μL of purified 7β-HSDH or 7α-HSDH were added and the absorbance (340 nm) change during the first minute was measured (Table 3).

The reduction of the 7-keto bile acids **1–6** with 7β-HSDH is carried out on analytical scale, with saturating concentration of NADH, starting from a 2.5 mM solution of the selected 7-keto bile acid sodium salt, in 20 mM phosphate buffer at pH 7 (20 mL) with 2 mM β-mercaptoethanol, 1 mM EDTA and 2.5 mM NADH, adding a partial purified 7β-HSDH (0.15 IU) at room temperature. After 48 h the reaction mixture was acidified, extracted with ethyl acetate and analyzed by GLC. The yields of β-reduction were: ursocholic acid **7** (80%), 3-ketoursocholic acid **8** (60%), 12-ketoursocholic acid **9** (90%), 6F-ursodeoxycholic **11** (70%) and ursodeoxycholic acid **12** (75%). No 7β-hydroxy hyocholic acid **10** was obtained.

2.3. *Epimerization on preparative scale of cholic and chenodeoxycholic acids to ursocholic and ursodeoxycholic acids 7 and 12*

2.3.1. *Epimerization with soluble enzyme*

A shorter procedure for the cell lysis was used: 5 g of wet cells grown and washed as described above, were lyophilized and suspended in 50 mM triethanolamine at pH 7.5, containing 0.1 mM EDTA and 1.0 mM β-mercaptoethanol (TEA-buffer, 150 mL). After standing 30 min at 4 °C, the suspension was centrifuged (15000 rpm, 15 min) and the supernatant (containing about 1 U/mL of 7α- and 0.6 IU/mL of 7β–HSDH) was used without further purification.

To a 12 mM solution of cholic acid (1 L) in TEA-buffer, containing 0.2 mM NAD^+, the enzyme solution (130 mL) was added. The mixture was gently stirred at room temperature for 20 h. GLC analysis of the reaction mixture showed the epimerization to ursocholic acid (81%) together with unreacted cholic acid (12%) and 7-keto derivative (7%).

The epimerization rose up to 87% by adding 2-hexanol (5.1 g, 50 mmol) and maintaining the reaction in the same conditions for additional 5 h. The mixture was acidified to pH 3–4 with 20% HCl solution, extracted with ethyl acetate, dried over anhydrous Na_2SO_4 and evaporated. Chromatography of the residue on silica gel (ethyl acetate/cyclohexane/acetic acid 50/50/1 as eluent) afforded ursocholic acid (85%) and cholic acid (15%).

Table 3 – Enzymatic activity of 7β- and 7α-HSDH

Substrate	7β-HSDH (U)	7α-HSDH (U)
7-Ketocholic acid **1**	18	1.4
3,7-Diketocholic acid **2**	11.5	11.4
7,12-Diketocholic acid **3**	12.5	8
7-Keto hyocholic acid **4**	2	1.1
6-F-7-ketohyocholic acid **5**	13	7
7-Ketochenodeoxycholic acid **6**	15	1.9

2.3.2. *Epimerization with immobilized enzyme*

Previously washed Sepharose-CL6B (15 mL) was suspended in cold (0 °C) water (15 mL) and cyanogen bromide (1.0 g, 9 mmol) was added. After stirring at 4 °C for 1 h, maintaining the pH at 11 by addition of 20% NaOH, the resin was washed with cold water. The enzyme solution (15 mL), obtained as above described, was concentrated by ultracentrifugation (3 mL) using Centriflo CF25/CF50A membrane and added to a suspension of the activated Sepharose in cold (0 °C) 100 mM KH_2PO_4 buffer (pH 8.0) containing 1 mM β-mercaptoethanol and 0.1 mM EDTA. The suspension was stirred for 16 h, filtered and the resin, washed with cold water, was suspended in 100 mM Tris–HCl buffer pH 7.8 for 2 h, filtered and washed with water.

The enzymes-loaded resin was suspended in TEA-buffer (40 mL) containing cholic acid **13** (0.3 g, 0.7 mmol) and NAD^+ (4 mg, 0.6 mmol). The suspension was gently shaken at room temperature. After 5 days, ursocholic acid **7** reached to 75–80% yield by GLC analysis. The suspension was filtered and the solution treated as described to obtain ursocholic acid (80%), cholic acid (12%) and 7-keto derivative (8%).

The resin was employed for another epimerization reaction that affords 75% of ursocholic acid in 7 days (by GLC analysis). A third cycle is possible obtaining the same yield after 8–9 days, instead the fourth cycle after 12 days produces 65% of ursocholic acid.

The epimerization of chenodeoxycholic acid **14** (0.23 g, 0.58 mmol) was carried out obtaining 75% of ursodeoxycholic acid **12** with similar procedure and using the same quantity of enzymes-loaded resin. Also in this case the resin was employed for other two cycles of epimerization giving 74 and 68% of ursodeoxycholic acid after 7 and 10 days, respectively.

2.4. *Synthesis of 6-FUDCA on preparative scale*

Partially purified 7β-HSDH, obtained in the first step of the above described procedure (DEAE-sepharose column) was used. To a solution (100 mL) of 10 mM 6-fluoro-7-ketohyocholic acid 5, 50 mM sodium formate, 0.5 mM NAD^+, 1 mM EDTA and 2 mM β-mercaptoethanol in 50 mM TEA-buffer pH 7.0, *Candida boidinii* FDH (3 IU) was added. After 20 min at 22 °C (absorption at 340 nm about 1.5 OD), 7β-HSDH (about 10 mL) was added and the mixture gently stirred for 24 h. Acidification to pH 3–4 with 20% HCl solution, extraction with ethyl acetate, drying over anhydrous Na_2SO_4, evaporation under reduced pressure and chromatography of the residue on silica gel (ethyl acetate/cyclohexane/acetic acid 50/50/1 as eluent) afforded 6-FUDCA **11** in 90% yield.

2.5. *Determination of CGH activity on BAs glycinates 12a–16a*

CGH activity assay was performed measuring glycine release, from cholylglycine in Na-P buffer, by a cadmium-ninhydrine method [32]. To a 5 mM bile acid glycinate solution (0.1 mL) CGH (0.2–5 μg) was added. The mixture was incubated at 25 °C then diluted with water (0.4 mL) and ninhydrine cadmium solution (1.0 mL). The resulting mixture was heated at 84 °C for 5 min and the absorbance (507 nm) was measured (Table 4).

Table 4 – Activity of cholylglycine hydrolase (GCH)

Substrate	Activity (U/mg)
Glycoursodeoxycholic acid **12a**	30
Glycocholic acid **13a**	100
Glycochenodeoxycholic acid **14a**	90
Glycodeoxycholic acid **15a**	95
Glycohyodeoxycholic acid **16a**	140

Hydrolysis of glycinates **13a–16a** and taurinates **13b–16b** with CGH was carried out starting from the proper glycinates or taurinates (5 mM) in 25 mM phosphate buffer (pH 8, 2 mL) with 2 mM β-mercaptoethanol, 1 mM EDTA and 0.4 IU of purified CGH at room temperature. The reaction mixture is monitored by HPLC at 20 min and at 180 min. The results are summarized in Table 5.

The hydrolyses of commercially available bovine bile with CGH were carried out at room temperature in 60 mM phosphate buffer (pH 8, 6 mL) with 2 mM β-mercaptoethanol, 1 mM EDTA, 5.2 mg/mL of bile Hages (10.3 mg/mL of bile Portugal) and 0.72 IU of CGH. The reaction mixture is monitored by HPLC at 20, 60 and 120 min. The results are summarized in Table 6.

3. Results and discussion

X. maltophilia 7β- and 7α-HSDH are constitutive enzymes because identical enzymatic activity has been obtained by growth in presence of bile acids or their conjugates. Interesting feature is the 7β-HSDH activity dependence on O_2 pressure during the bacterium growth [26,27]. Vigorous shaking produces cells that show a low 7β-HSDH activity, while moderate shaking of the culture medium and 4 h standing afford a biomass with ten times higher activity. A possible explanation is that the 7β-HSDH expression could be controlled by the intracellular oxygen or that the enzyme could be denatured by a too high O_2 concentration.

Typical procedures of 7β- and 7α-HSDH partial purification are reported in Tables 1 and 2, respectively. It is worth mentioning that the ion-exchange chromatography on DEAE-sepharose allows the separation of dehydrogenases, and, moreover, is necessary to purify CGH [28].

Table 5 – Hydrolysis of BAs glycinates and taurinates 13–16 with GCH

Glycinates and taurinates	Time (min)	Bile acid (yield %)
13a	20	**13** (100)
13b	20	**13** (100)
14a	20	**14** (90)
	180	**14** (100)
14b	20	**14** (100)
15a	20	**15** (100)
15b	20	**15** (100)
16a	20	**16** (39)
	180	**16** (100)
16b	20	**16** (18)
	180	**16** (100)

Table 6 – CGH hydrolysis of commercially available bovine bile

Bovine bile	Bile acid conjugates (%)[a]	Time (min)	Bile acid (100% yield)
Bile Hages[b]	Taurocholic acid **13b** (35%)	20	Cholic acid **13**
	Glycocholic acid **13a** (33%)	120	Cholic acid **13**
	Taurochenodeoxycholic acid **14b** (10%)	20	Chenodeoxycholic acid **14**
	Glycodeoxycholic acid **15a** (7%)	20	Deoxycholic acid **15**
	Taurodeoxycholic acid **15b** (6%)	20	Deoxycholic acid **15**
	Glycochenodeoxycholic acid **14a** (2%)	20	Chenodeoxycholic acid **14**
Bile Portugal[c]	Taurocholic acid **13b** (35%)	20	Cholic acid **13**
	Glycocholic acid **13a** (33%)	120	Cholic acid **13**
	Taurochenodeoxycholic acid **14b** (10%)	20	Chenodeoxycholic acid **14**
	Glycodeoxycholic acid **15a** (7%)	20	Deoxycholic acid **15**
	Taurodeoxycholic acid **15b** (6%)	20	Deoxycholic acid **15**
	Glycochenodeoxycholic acid **14a** (2%)	20	Chenodeoxycholic acid **14**

[a] Relative concentration determined by HPLC.
[b] Lyophilized bile with 54% of bile acid conjugates.
[c] Concentrated bile containing 24% of bile acid conjugates.

Starting from 9 g of *X. maltophilia* wet cells is possible to obtain, together with CGH (0.3 mg, 101 U/mL), 0.4 mg of 7α-HSDH (33 U/mg, yield 46%) and 0.2 mg of 7β-HSDH (70 U/mg, yield 10%) with a purification factor of 41- and 175-fold, respectively. At this purification step, 7α-HSDH is stable at 4 °C for 2 days and then the activity decreases (20–30% per day). On the other hand, 7β-HSDH shows, in its native form, a molecular weight of 130 kDa, determined by gel filtration chromatography, and maintains an unaltered activity for 7 days at 4 °C.

7β-HSDH displays a 2.5 mM K_m for ursocholic acid, obtained from Lineweaver–Burk plot and the reaction has an optimum at pH 9.5. On the other hand the oxidation of cholic acid, catalyzed by 7α-HSDH, has an optimum at pH 7.5 and the enzyme shows a 1.0 mM K_m for this substrate.

Concerning this, several 7α- and 7β-HSDH from bacterial source have been described and most of them are $NADP^+$ dependent and exhibit high affinity for the substrate [34–37] (20–500 μM). On the contrary, *X. maltophilia* HSDHs are characterized by a strictly dependence from NAD^+ (K_m 0.15 mM and 1.0 mM, respectively) and exhibit a lower affinity for substrate. Although this reduces the applications of *X. maltophilia* HSDHs for analytical quantitation of BAs, their tolerance for high concentration of substrate without reaching saturation and the lack of significant product inhibition are important for preparative applications.

Partially purified 7β- and 7α-HSDHs were tested as catalysts for reduction of the 7-keto derivatives **1**–**6**.

Table 3 reports the reaction rates.

The 7β-HSDH shows generally a comparable activity towards 7-keto BAs **2**–**5** with respect to 7α-HSDH, while the activity is higher with respect to 7-ketocholic acid **1** and 7-ketochenodeoxycholic acid **6**. On the other hand both enzymes show low activity towards the 7-ketohyocholic acid **4**.

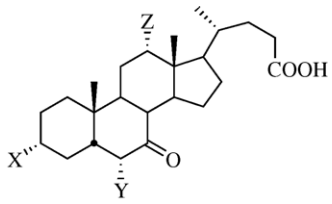
These data are confirmed by the reduction of 7-keto derivative **1**–**6** on analytical scale with 7β-HSDH and stoichiometric amount of NADH to the corresponding 7β-OH derivative **7**–**12** (Chart 1).

The 7β-HSDH is very efficient in the reduction of the 7-keto derivatives **1**–**6** (yield 60–90% after 48 h). In the case of ursocholic acid **7** (80%) and 12-ketoursocholic acid **9** (90%) the yields are comparable with those obtained in our previous work [27] by *X. maltophilia* oxido-reduction of the corresponding 7α-derivatives. Good is also the yield of 3-ketoursocholic acid **8** (60%). Very interesting is, on the other hand, the yield of ursodeoxycholic acid (UDCA) **12** (75%) obtained by reduction of the corresponding 7-keto derivative **6**. This result is very promising for further applications because of the importance of UDCA as drug. On the other hand, no 7β-hydroxy hyocholic acid **10** is obtained from the corresponding 7-keto derivative **4**.

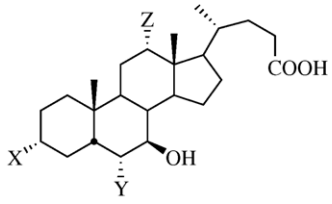
In this case, the bulky 6-OH function probably inhibits the formation of enzyme-substrate complex. In fact when the OH-group of **4** is substituted by the smaller fluorine (derivative **5**) or hydrogen (derivative **6**) the reaction proceeds with higher rate. This feature is confirmed by enzymatic activity of 7β-HSDH on the same compounds (Table 3). Moreover, 6α-fluoroursodeoxycholic acid (6-FUDCA) **11**, important for higher resistance to 7-dehydroxylation with respect to UDCA, is produced in 70% yield by 7β-HSDH reduction. This result is very important because no 6-FUDCA has been obtained by *X. maltophilia* oxido-reduction of 6α-fluoro-hyocholic acid. Ever the activities reported in Table 3 show that, while the enzymatic activity of 7β-HSDH is higher than 7α-HSDH towards 7-keto derivatives **1** and **6**, the same activity is lower with respect to 6F-7-keto bile acid 5. This allows the use of the mixture of 7β- and 7α-HSDH for the preparation of UCA and UDCA, but 6-FUDCA is not obtained owing the higher rate of 7α-reduction. From here, the reduction catalyzed by 7β-HSDH is the only possibility to obtain this potential interesting drug in mild condition and high yield.

On the basis of these results, a mixture of 7α- and 7β-HSDH, obtained as a supernatant by lysis of lyophilized cells and used without further purification, is employed for the synthesis on preparative scale of ursocholic and ursodeoxycholic acid starting from cholic **13** and chenodeoxycholic acid **14**, respectively (Scheme 2).

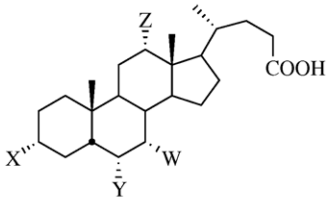
The presence of both enzymes allows to use NAD^+ in catalytic amount and, after 20 h, ursocholic **7** (81%) and ursodeoxycholic acids **12** (76%) are obtained. The epimerization yields increase further (87% of 7 and 82% of **12**, respectively) by adding 2-hexanol to the reaction. The reaction equi-



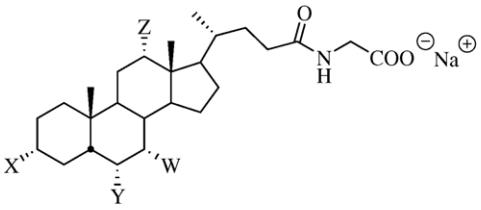
1; X = Z = α-OH, Y = H
2; X = =O, Z = α-OH, Y = H
3; X = α-OH, Z = =O, Y = H
4; X=Y= α-OH, Z = H
5; X = α-OH, Y = F, Z = H
6; X = α-OH, Y =Z= H



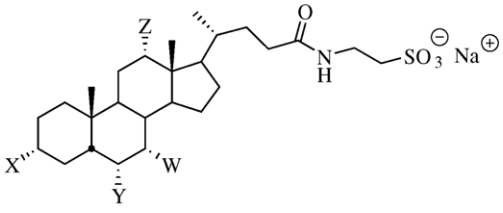
7; X = Z = α-OH, Y = H
8; X = =O, Z = α-OH, Y = H
9; X = α-OH, Z = =O, Y = H
10; X=Y= α-OH, Z = H
11; X = α-OH, Y = α-F, Z = H
12; X = α-OH, Y =Z= H



13; X = Z = W = α-OH, Y = H
14; X = W = α-OH, Y =Z= H
15; X = Z = α-OH, Y = Z =H
16; X = Y = α-OH, Z = Y = H



a; glycinates



b; taurinates

Chart 1

librium is shifted by the efficient oxidation of 2-hexanol and this suggests the presence, in the cellular lysate, of a secondary alcohol dehydrogenase that produces NADH, that is used by 7β-HSDH to reduce the 7-CO function.

The same mixture of 7α- and 7β-HSDH is immobilized on Sepharose-CL6B and the enzyme-loaded resin is employed for the epimerization of the BAs **13** and **14** obtaining similar results after 5 days (80% of UCA and 76% of UDCA). On the other hand, the immobilized enzymes is used, after filtration, for other cycles of epimerization of cholic acid **13** obtaining similar yields of UCA progressively after longer time: second cycle, 75% of UCA after 7 days; third cycle, 75% of UCA after 8–9 days; fourth cycle, 65% of UCA after 12 days.

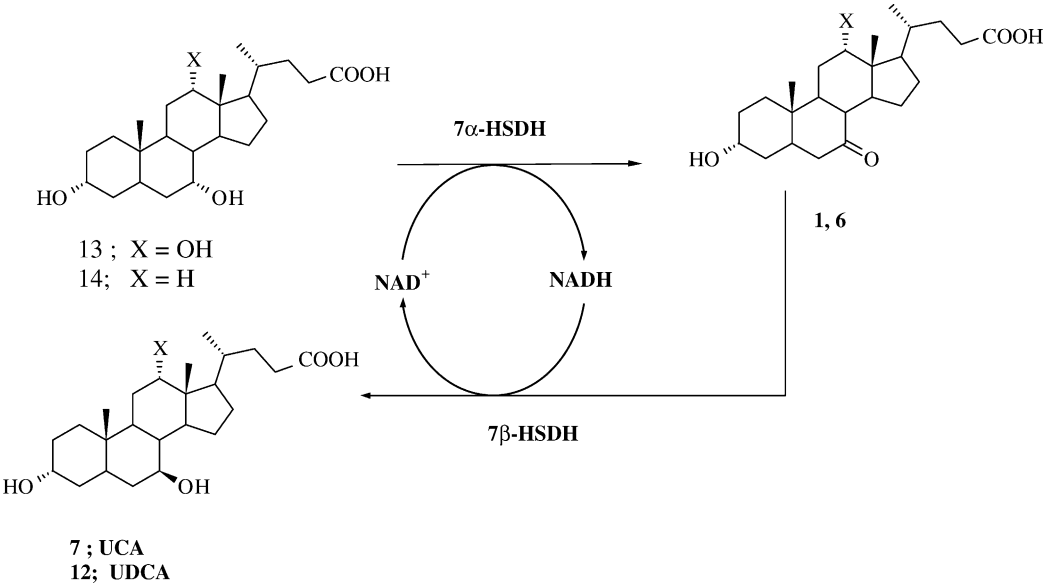
On the other hand, the interesting properties of 6-FUDCA **11** and the failure of its synthesis using *X. maltophilia* [27], prompt us to obtain **11** from the corresponding 7-keto derivative **5** in a double enzyme system where the couple formate/formate dehydrogenase (FDH) allows the cofactor recycle (Scheme 3).

For this purpose, a partially purified 7β-HDSH obtained from the first step of purification procedure (DEAE-sepharose column), is used for the reduction of 6-fluoro-7-keto-hyocholic acid **5** on preparative scale (0.5 g/L). The oxidation of formate catalyzed by NAD-dependent FDH from *C. boidinii* maintains an high NADH concentration that affords, after 24 h, 6-FUCDA **11** in very good yields (90%).

Together with 7β- and 7α-HSDH, also cholylglycine hydrolase (CGH), is purified from *X. maltophilia* as reported in a previous work [28]. CGH is thermally stable, since unaltered activity is shown after 75 min at 55 °C, and the optimum for hydrolysis of the BAs conjugates is pH 8 at 50 °C.

In the Table 4, the activity of CGH towards the BAs glycinates **12a–16a** (Chart 1) is reported.

The kinetic characterization has been made with glycocholic acid [28]. On the other hand the CGH hydrolyses effi-



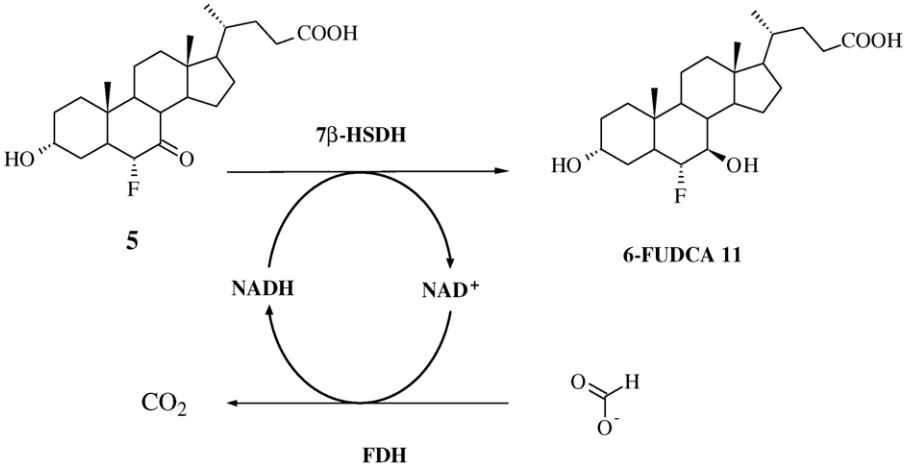
Scheme 2

ciently both pure glycinates and taurinates of bile acids **13–16** (Table 5).

For most of all conjugates the hydrolyses are complete in 20 min. Only hyodeoxycholic acid **16** is hydrolyzed more slowly (180 min) both as glycinate (**16a**) and taurinate (**16b**). This is in accordance with the lower activity of CGH toward hyocholic acid probably due to the bulky presence of 6α-OH.

The importance of industrial applications of this enzyme, due to the possibility of avoiding concentrated bases to hydrolyze commercial raw material containing bile acids, prompted us to test CGH on commercially available bovine bile. For this purpose a lyophilized bile, bile Hages, and a concentrated bile, bile Portugal, containing 54% and 24% of bile acids conjugates, respectively, have been chosen. The relative percentages of their BA glycinates and taurinates (identical for both starting materials) have been determined by HPLC and the hydrolyses with CGH monitored with the same method. The results are summarized in Table 6.

After 20 min at room temperature most conjugates are hydrolyzed. Surprisingly, 2 h are necessary for the complete hydrolysis of the glycocholic acid **13a** in both mixtures. This result is in contrast with the data obtained with the pure compound but anyway interesting for further industrial applications. The slower hydrolysis of **13a** is due to the enzyme inhibition by the same bile salts. It has been reported [28] that when glycocholic and taurocholic acids are present in the same reaction mixture, the hydrolysis of the first one is inhibited competitively by the presence of the second one (Ki = 2.5 mM). Moreover, taurine (Ki = 2.5 mM) and cholic acid (Ki = 4.7 mM) give uncompetitive inhibition in the hydrolysis of the glycocholic acid.



Scheme 3

4. Conclusions

X. maltophilia has proved to be a very interesting aerobic bacterium in bile acids biotransformation both as that and as source of stable enzymes potentially utilizable in pharmaceutical industry.

7β-HSDH is an efficient NADH-dependent reductase towards various 7-keto bile acid derivatives and a crude mixture of 7β- and 7α-HSDH allows obtaining on preparative scale UCA and UDCA in very good yields using cofactor in catalytic amount. Similar results, however, can be obtained immobilizing the enzymes and this is very promising for further applications. On the other hand, the most interesting outcome, that makes the use of 7β-HSDH competitive with that of the corresponding cells, is the efficient production of 6α-fluoro-7β-OH derivative **11** (6-FUDCA) in a double enzyme system where the couple formate/formate dehydrogenase allows the cofactor recycle. This compound is not obtainable by microbiological route where the double enzyme system is the 7-keto/7α-HSDH.

The enzymatic property of *X. maltophilia* includes, however, the presence of a thermally stable cholylglycine hydrolase, very efficient in the hydrolysis of bile acids glycinates and taurinates. This enzyme was successfully employed with commercial bile and this show a possible substitution of the chemical hydrolysis of bile acid conjugates, obtained with concentrated bases at high temperature.

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