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7α- and 12α-Hydroxysteroid dehydrogenases from *Acinetobacter calcoaceticus lwoffii*: a new integrated chemo-enzymatic route to ursodeoxycholic acid

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ABSTRACT

We report the very efficient biotransformation of cholic acid to 7-keto- and 7,12-diketocholic acids with *Acinetobacter calcoaceticus lwoffii*. The enzymes responsible of the biotransformation (i.e. 7α- and 12α-hydroxysteroid dehydrogenases) are partially purified and employed in a new chemo-enzymatic synthesis of ursodeoxycholic acid starting from cholic acid. The first step is the 12α-HSDH-mediated total oxidation of sodium cholate followed by the Wolf–Kishner reduction of the carbonyl group to chenodeoxycholic acid. This acid is then quantitatively oxidized with 7α-HSDH to 7-ketochenodeoxycholic acid, that was chemically reduced to ursodeoxycholic acid (70% overall yield).

1. Introduction

Bile acids (BAs), their conjugates and salts are natural products and fundamental constituents of bile [1]. Among them chenodeoxycholic and 7-OH epimer ursodeoxycholic acid (UDCA) have important pharmaceutical applications related to their ability to solubilize cholesterol gallstones [2,3]. 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 the relatively inexpensive cholic acid that is therefore used as starting materials for the synthesis of chenodeoxycholic acid [4,5] and ursodeoxycholic acid [6,7] through different synthetic pathways [7–9]. 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 classic chemical reactions [1]. Among them, two steps are particularly crucial: the regioselective oxidation of the 12α-OH group [4] and the selective α/β inversion of the 7-OH carbon center [6]. Chemically the regioselective oxidation of 12α-OH can be obtained after the problematic protection of the 3- and 7-OH groups, lowering the oxidation yields, while the α/β inversion can be achieved through the regioselective oxidation of the 7α-OH function and the subsequent radicalic reduction with Na in ethanol [7] or with Li in liquid NH_3 [10].

Hoffmann [5] has described a seven-step sequence that represents the pure chemical approach to this problem. More recently, Sawada et al. [11,12] reported the preparation of

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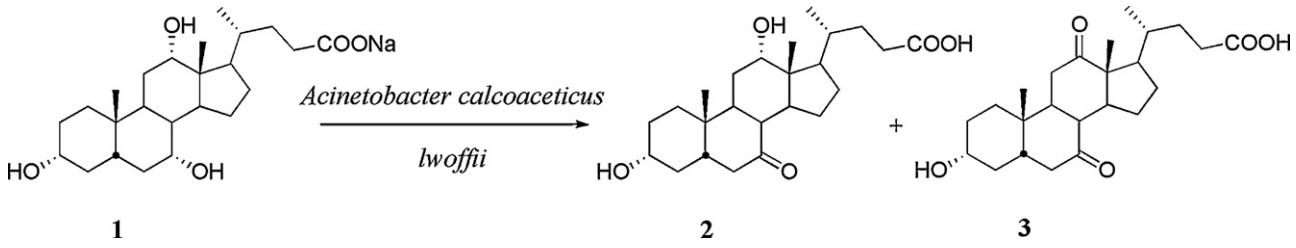


Scheme 1

chenodeoxycholic acid by bioconversion of dehydrocholic acid mediated by bacteria. Additionally, in an our previous work this problem was resolved chemically by selective reduction of dehydrocholic acid [13]. On the other hand, various NAD-dependent 12α-hydroxysteroid dehydrogenases were purified from *Eubacterium lentum* [14] and *Clostridium* group P, strain C48–50 [15,16] and have been efficiently utilized in the synthesis of 12-ketochenodeoxycholic acid [17,18] and 12-ketoursodeoxycholic [19,20].

In this paper we report the oxidation of C_7–OH and C_{12}–OH groups of sodium cholate **1** (Scheme 1) by *Acinetobacter calcoaceticus lwoffii*, a strain isolated from a soil sample of ICE industry [21], and further partial purification of 7α-HSDH and 12α-HSDH. These enzymes are used in a new integrated chemo-enzymatic synthesis of ursodeoxycholic acid **7** starting from sodium cholate **1** (Scheme 2).

2. Experimental

Sodium salt of cholic acid **1** and chenodeoxycholic acid **5a** have been supplied by ICE industry [21].

NAD^+, phenylmethyl sulphonyl fluoride (PMSF), β-mercaptoethanol, egg white lysozyme, DEAE–sepharose, and LDH from rabbit muscle were obtained from Sigma.

Melting points are uncorrected and were determined on a 510 Büki melting point instrument. ^1H and ^{13}C NMR spectra were obtained with a Varian Gemini 300 spectrometer operating at 300 MHz (^1H) and 75 MHz (^{13}C), with Me_4Si as internal standard.

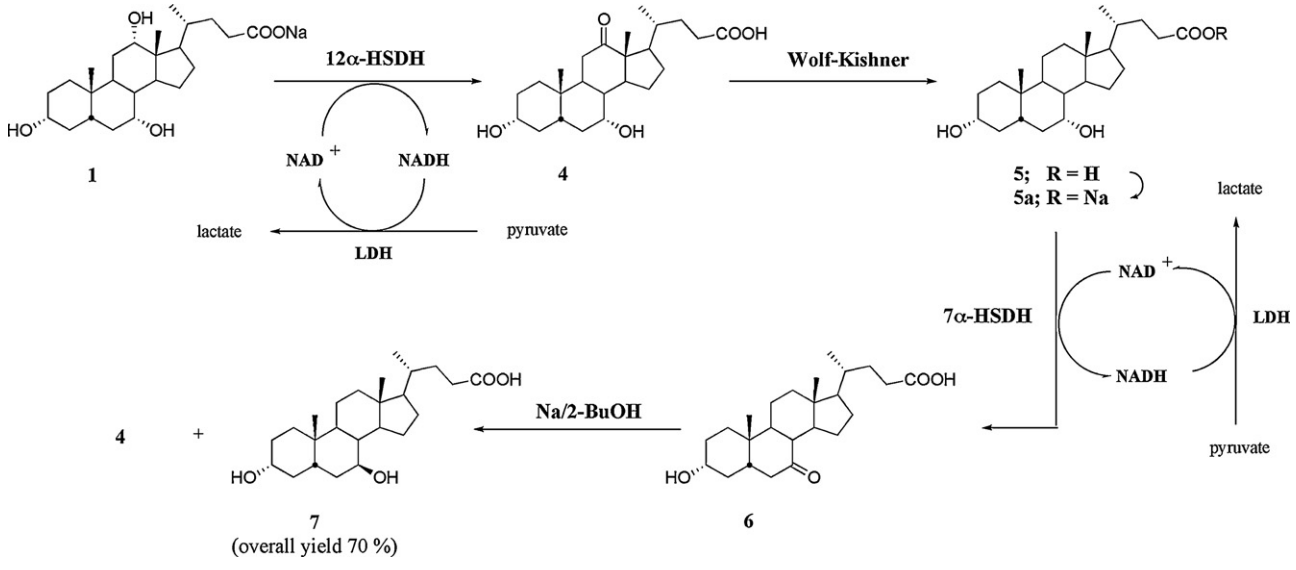
TLC were performed on precoated silica gel plates (thickness 0.25 mm, Merck) and silica gel (Fluka, Kiesegel 60, 70–230 mesh) was used for preparative column chromatography.

Gas chromatographic analyses were performed on a Carlo Erba HRGC 5160 Mega series chromatograph. The reaction products, previously derivatized with trifluoroacetic anhydride and hexafluoroisopropanol, were 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 and chenodeoxycholic acids were reported in a previous paper [22].

2.1. Biotransformation of sodium cholate 1 with A. calcoaceticus lwoffii

A loopful of *A. calcoaceticus lwoffii* from a culture on plate count agar was inoculated in plate count broth (100 mL) containing glucose (1 g/L), yeast extract (2.5 g/L) and tryptone (5 g/L). After 48 h at 30 °C and 100 rpm, sodium cholate **1** (0.1 g) was added and the culture was maintained in the same conditions for 48 h. The biomass was removed by centrifugation (10,000 rpm, 15 min) and 20% HCl was added to reach pH 2. The acidified solution was extracted with ethyl acetate (3× 60 mL), the



Scheme 2

organic layer dried over anhydrous Na_2SO_4 and the solvent evaporated to afford 100 mg of crude oxo-derivatives mixture. The chromatographic separation on column (silica gel, ethyl acetate/cyclohexane/acetic acid 50/50/1 as eluent) gave 22 mg (24%) of 3α,12α-dihydroxy-7-keto-5β-cholan-24-oic acid **2** [6] and 68 mg (72%) of 3α-hydroxy-7,12-diketo-5β-cholan-24-oic acid **3** [23].

3α,12α-Dihydroxy-7-keto-5β-cholan-24-oic acid **2**: mp 195–198 °C; ^{1}H NMR (CD_3OD): (selected data) δ 0.77 (s, 3H, H-18); 1.06 (d, J = 6.2 Hz, 3H, H-21); 1.26 (s, 3H, H-19); 2.60 (dd, J = 12 Hz, 1H, H-8β); 3.02 (dd, J = 13 and 6.6 Hz, 1H, H-6β); 3.58 (br, 1H, H-3β); 4.04 (s, 1H, H-12β).

3α-Hydroxy-7,12-diketo-5β-cholan-24-oic acid **3**: mp 188–190 °C; ^{1}H NMR (CD_3OD): (selected data) δ 0.89 (d, J = 6.2 Hz, 3H, H-21); 1.14 (s, 3H, H-18); 1.38 (s, 3H, H-19); 3.57 (br, 1H, H-3β).

2.2. *Partial purification of A. calcoaceticus lwoffii enzymes*

2.2.1. *Cell free extract preparation*

A culture of *A. calcoaceticus lwoffii* was grown in plate count broth (10 mL) for 48 h at 30 °C and 100 rpm. This culture was used to start a 1 L culture. After 48 h growth in the same conditions, the cells were harvested by centrifugation (10,000 rpm, 15 min) and washed with 150 mM NaCl (20 mL). The wet cells (2 g) were added to 10 mL of 50 mM triethanolamine buffer at pH 7.5 containing 1 mM β-mercaptoethanol and 0.1 mM EDTA (TEA-buffer). Lysozyme (6 mg) and phenylmethylsulfonyl fluoride (1 mg) were added to the suspension. After 3 h at 30 °C with gently shaking the cell free extract was centrifuged (18,000 rpm, 15 min, 10 °C) to remove the cells debries.

2.2.2. *Enzymes assay*

During the oxidation of sodium chenodeoxycholate and sodium deoxycholate, 7α- and 12α-HSDH activities were respectively assayed at 22 °C monitoring NADH formation (absorbance change at 340 nm). The reaction was started adding 10 μL of enzyme solution to 1 mL of 50 mM phosphate buffer (pH 8.5), containing either 0.3 mM NAD^+ and 2.4 mM sodium chenodeoxycholate for 7α-HSDH or sodium deoxycholate for 12α-HSDH, respectively. One international unit (U) of enzyme is defined as the amount required to produce 1 μmol of NADH per minute.

2.2.3. *Separation of 7α- and 12α-HSDH*

Cell free extract (10 mL) containing 0.5 U/mL of 7α-HSDH and 0.4 U/mL of 12α-HSDH was loaded on a DEAE–sepharose column (1 cm × 4 cm) equilibrated with TEA-buffer. The column was washed with the same buffer until no protein was detected in the eluted fractions. The non-bonded 7α-HSDH can be recovered from these fractions and used without further purification to catalyze the chenodeoxycholate oxidation. This enzyme was stable for a week at 4 °C (maintaining 80% of activity after 7 days). DEAE–sepharose column was washed with TEA-buffer containing 0.1 M NaCl to remove the residual 7α-activity and then 12α-HSDH was eluted with 0.2 M NaCl (Fig. 1). The fractions containing 12α-HSDH activity were pooled and the resulting protein solution was fractionated with ammonium sulphate in order to collect insoluble proteins between 65 and 95 % saturation. After centrifugation (18,000 rpm, 15 min, 5 °C), the pellet containing 12α-HSDH was dissolved in 0.5 mL of TEA-buffer and used to catalyze the sodium cholate oxidation. This enzyme solution can be stored at 4 °C for 3 days without significant decrease of activity (Table 1).

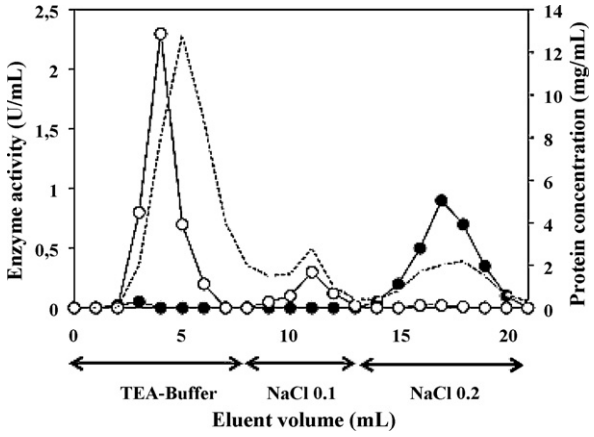


Fig. 1 – Elution profile of 7α- and 12α-HSDH from DEAE–sepharose column: (●) 12α-HSDH; (○) 7α-HSDH; (- - -) protein.

2.3. *Oxidation of sodium cholate 1 with partially purified 12α-HSDH*

12α-HSDH (0.5 mL, 2.8 U) was added to a 12 mM sodium cholate in 50 mM phosphate buffer solution (100 mL at pH 8.5), containing 0.2 mM NAD^+, 60 mM sodium pyruvate, 1 mM β-mercaptoethanol, and 0.1 mM EDTA. After the absorbance of the solution (at 340 nm) reached 2.0, lactate dehydrogenase (15 U) was added and the reaction was kept at 22 °C overnight. The reaction mixture was acidified to pH 2 with 20% HCl, extracted with ethyl acetate (3× 150 mL) and the combined organic layers were dried over anhydrous Na_2SO_4. They were then evaporated under reduced pressure to afford the crude 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid **4** [24] (0.51 g, 98%). The product was used without further purification: mp 219–221 °C; ^{1}H NMR (CD_3OD): (selected data) δ 0.90 (d, J = 6.3 Hz, 3H, H-21); 1.10 (s, 3H, H-18); 1.13 (s, 3H, H-19); 2.62 (dd, J = 13.3, 1H, H-11β); 3.42 (br, 1H, H-3β); 3.94 (brs, 1H, H-7β).

2.4. *Wolf–Kishner reduction of 12-ketocholic acid 4*

Chenodeoxycholic acid **5** was prepared from the 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid **4** according to the Wolf–Kishner modified procedure [25]. Eighty-five percent KOH (4 g) and 80% hydrazinium hydroxide (5 mL) were added to a solution of **4** (4 g, 10 mmol) in ethylene glycol monomethylether (35 mL) and H_2O (3 mL). The solution was heated at 110 °C for 4 h, distilled until the temperature of the reaction mixture raised to about 135 °C and this temperature was maintained for additional 8 h. The reaction mixture was cooled to 20 °C, diluted with H_2O (12 mL) and acidified to pH 2 with 40% H_2SO_4. The crude chenodeoxycholic acid **5** (3.6 g,

Table 1 – Separation and partial purification of 7α-HSDH and 12α-HSDH

Step	Volume (mL)	Protein (mg)	7α-HSDH (U)	12α-HSDH (U)	Specific activity[a]		Purification[b]	
					7α-HSDH	12α-HSDH	7α-HSDH	12α-HSDH
Cell extract	10	145	5	4	0.035	0.027		
DEAE–sepharose (TEA buffer)	8	37	4	nd	0.11		3.1	
DEAE–sepharose [0.2 M NaCl followed by $(NH_4)_2SO_4$ (65–95%)]	0.5	9	nd	2.8		0.3		11.1

[a] Specific activity (U/mg).
[b] S.A. of the purified HSDH/S.A. of the same HSDH in the cell extract.

90%) was collected by filtration and, after transformation into the corresponding sodium salt (obtained with 10% NaOH in H_2O/CH_3OH 90/10), submitted to the next step without further purification (TLC and ^1H NMR are identical to commercial sample).

2.5. *Oxidation of sodium chenodeoxycholate with partially purified 7α-HSDH*

7α-HSDH (8 mL, 4 U) was added at 22° C to a 12 mM sodium chenodeoxycholate **5a** in 50 mM phosphate buffer solution (130 mL at pH 8.5), containing 0.2 mM NAD^+, 60 mM sodium pyruvate, 1 mM β-mercaptoethanol, and 0.1 mM EDTA. After the absorbance of the solution (at 340 nm) reached 2.0, lactate dehydrogenase (20 U) was added and the reaction was kept at 22 °C overnight. The work-up of the reaction mixture was carried out as above for the oxidation of cholic acid to afford the crude 3α-hydroxy-5-keto-5β-cholan-24-oic acid **6** [5] (0.65 g, 97%). The product was used without further purification: mp 206–210 °C; ^1H NMR (CD_3OD): (selected data) δ 0.73 (s, 3H, H-18); 0.98 (d, J = 6.2 Hz, 3H, H-21); 1.28 (s, 3H, H-19); 2.60 (dd, J = 10.9 Hz, 1H, H-8β); 3.03 (dd, J = 12.1, 4.8 Hz, 1H, H-6β); 3.58 (br, 1H, H-3β).

2.6. *Reduction of 7-ketochenodeoxycholic acid 6*

Ursodeoxycholic acid **7** was prepared from the 7-ketoacid **6** according to a modified procedure described by Samuelsson [7]. A solution of the 7-ketoacid (1 g, 2.5 mmol) and sodium (2 g) in 20 mL of anhydrous 2-butanol was refluxed for 3 h. The reaction mixture, diluted with water and acidified with HCl, was extracted with ethyl acetate (2× 20 mL). The organic layer was dried over Na_2SO_4 and evaporated *in vacuo*. The residue, chromatographed on silica gel (C_6H_6/dioxane/acetic acid 75/20/2, as eluent), gave 0.82 g (82%, overall yield 70%) of pure ursodeoxycholic acid **7** [7] and 0.15 g (15%) of chenodeoxycholic acid **5**. Ursodeoxycholic acid **7**: mp 199–202 °C; ^1H NMR (CD_3OD): (selected data) δ 0.76 (s, 3H, H-18); 1.00 (d, J = 6.2 Hz, 3H, H-21); 1.00 (s, 3H, H-19); 3.55 (br, 2H, H-3β and H-7α).

3. Results and discussion

For a long time our research group has been interested in the synthesis [26,27] and biotransformation of bile acids [28–30]. In this field, many microrganisms have been isolated, characterized and screened in biotransformations of different bile acids. In particular, *A. calcoaceticus lowffii*, which was isolated from a soil sample, showed a good activity in the oxidation of the C_7 and C_{12} positions of cholic acid, affording the mixture of 7-keto **2** and 7,12-diketocholanic acid **3** in 1/3 ratio and practically quantitative yields (24 and 72%, respectively) (Scheme 1).

The same products have been obtained using the crude cell free extract with a catalytic amount of NAD^+ and using LDH as cofactor recycling. This confirmed the presence of NAD-dependent 7α-HSDH and 12α-HSDH and encouraged us to achieve an easy procedure to separate the two enzymes in order to employ them in the chemo-enzymatic synthesis of ursodeoxycholic acid.

Table 1 shows the results of a typical procedure for 7α- and 12α-HSDH partial purification.

Ion-exchange chromatography is one of the most employed techniques for the early steps of enzymes purification. The cell free extract, which was obtained from 2 g of *A. calcoaceticus lwoffii* wet cells, was loaded on a DEAE–sepharose column equilibrated at pH 7.5 with TEA-buffer. The column was washed with the same buffer to obtain a protein solution containing the 7α-HSDH (4 U) free from 12α-HSDH. The 7α-HSDH and the 12α-HSDH activities were measured using chenodeoxycholic acid and deoxycholic acid, respectively.

On the other hand, partially purified 12α-HSDH (2.8 U) was eluted from the DEAE column with TEA-buffer containing 0.2 M NaCl, after rinsing with the same buffer containing 0.1 M NaCl. This simple procedure allowed the complete separation of the two HSDSs, obtaining, from 1 L of culture, two enzymatic solutions able to oxidize at least 0.5 g of the corresponding substrates. This chromatographic step allowed the purification of 7α-HSDH and 12α-HSDH (3 and 11 folds, respectively) and could be a good starting point for the complete purification of the enzymes (Fig. 1).

7α-HSDH was stable for a week, maintaining 80% of its activity after 7 days when stored at 4 °C. 12α-HSDH, on the

other hand, can be precipitated with $(NH_4)_2SO_4$ (65–95% saturation) and dissolved in 0.5 mL of TEA-buffer and stored at 4 °C for 3 days without significant decrease of activity.

Since a key step of the synthesis of ursodeoxycholic acid from cholic acid is the regioselective oxidation of the 12α-OH function and the oxidation aptitude follows the order $C_7 > C_{12} > C_3$, it is necessary to protect the C_7– and C_3–OH functions to obtain this goal. The partially purified 12α-HSDH, first, and 7α-HSDH, after, were efficiently used in a new integrated chemo-enzymatic synthesis of ursodeoxycholic acid **7** starting from sodium cholate **1** (Scheme 2).

The first step was the treatment of sodium cholate **1** with the partially purified 12α-HSDH in the presence of a catalytic amount of NAD^+. The reduction of sodium pyruvate by lactate dehydrogenase from rabbit muscle maintained an high NAD^+ concentration that afforded, after 12 h, 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid **4** with an excellent yield (98%).

The subsequent Wolf–Kishner modified reduction [25] of the crude 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid **4** gave, after 12 h at 110-135 °C, very good yield (90%) of the crude chenodeoxycholic acid **5**. This was transformed in the corresponding sodium chenodeoxycholate **5a** and used in the next step without further purification.

On the other hand, the chenodeoxycholate **5a** was very efficiently transformed into the 3α-hydroxy-7-keto-5β-cholan-24-oic acid **6** (97%) by the partially purified 7α-HDSH, using the same cofactor recycling system as for the 12α-OH oxidation.

Finally, the 7-keto function of **6** was chemically reduced [6] with sodium in 2-butanol to give ursodeoxycholic acid **7** (82%) together with chenodeoxycholic acid **4** (15%). The overall yield of this new integrated chemo-enzymatic synthesis of ursodeoxycholic acid (70%) was considerable.

4. Conclusions

In conclusion, the numerous screenings of microbial oxidations carried out by our research group on different bile acids allowed us to identify bacteria able to oxidize regioselectively the OH functions of cholic acid. In particular, *A. calcoaceticus lwoffi* showed the presence of 7α- and 12α-HSDHs, that were also very easily separated. This permits a simple and efficient application of these partially purified enzymes to the synthesis of ursodeoxycholic acid. The key steps of this new chemo-enzymatic route are the quantitative oxidation of the 12-OH function of cholic acid and of the 7-OH function of chenodeoxycholic acid carried out by 12α-HSDH and 7α-HSDH, respectively.

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