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7α-OH epimerisation of bile acids via oxido-reduction with *Xanthomonas maltophilia*

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Abstract

The microbial 7α-OH epimerisation of cholic, chenodeoxycholic, and 12-ketochenodeoxycholic acids (7α-OH bile acids) with *Xanthomonas maltophilia* CBS 827.97 to corresponding 7β-OH derivatives with scarcity of oxygen is described. With normal pressure of oxygen the 7-OH oxidation products are obtained. No biotransformations are achieved in anaerobic conditions. The microbial 7α-OH epimerisation is achieved by oxidation of 7-OH function and subsequent reduction. Partial purification, in fact, of the enzymatic fraction revealed the presence of two hydroxysteroid dehydrogenases (HSDH) α- and β-stereospecific together with a glycocholate hydrolase. On the basis of these results a further application is the microbial reduction of 6α-fluoro and 6β-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl esters to the corresponding 7α-OH and 7β-OH derivatives.

Keywords: Bile acids; Microbial epimerisation; Ursodeoxycholic acid; 6α-Fluoro-3α,7α-dihydroxy-5β-cholan-24-oic acid; 6β-Fluoro-3α,7β-dihydroxy-5β-cholan-24-oic acid

1. Introduction

Chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) and the 7-OH epimer ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) have important pharmaceutical applications related to their ability to solubilize cholesterol gallstones [1]. 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 [2,3]. However ursodeoxycholic acid is industrially prepared from chenodeoxycholic acid by a sequence of chemical reactions, the last two steps of which involve the selective α/β inversion of the 7-OH carbon center [4]. In a recent review, we have reported that a selective control in the reduction of the 7-keto functionality of bile acids (BAs) can efficiently be achieved by employing enzymatic biotransformations, mainly with anaerobic bacteria [5]. In particular, in a preliminary work we have described the microbial 7-OH epimerisation of cholic acid (CA, **1**) and chenodeoxycholic acid (CDCA, **2**) to ursocholic acid (UCA, **9**) and ursodeoxycholic acid (UDCA, **10**), respectively, via oxidation of the 7α-hydroxy group and subsequent stereospecific reduction of the keto functionality thus formed into the corresponding 7β-hydroxy group mediated by the aerobic bacter *Xanthomonas maltophilia* [6]. With the aim of extending the application of enzymatic biotransformations in the BA field, we have studied the epimerisation of two new bile acids, namely 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid (12-oxo-CDCA, **3**) and 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid (hyocholic acid, HCA, **4**) (Scheme 1). Moreover, the possibility to apply the microbial reduction to 6-fluoro bile acid derivatives for obtaining the 7β-hydroxy group has prompted us to the application of this methodology for the efficient preparation of these 6-substituted bile acids. Accordingly, we have explored the stereoselective 7-keto → 7β-hydroxy conversion of 6α- and 6β-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid esters (**12**, **13**) belonging to a new, interesting class of BA derivatives (Scheme 2). One of

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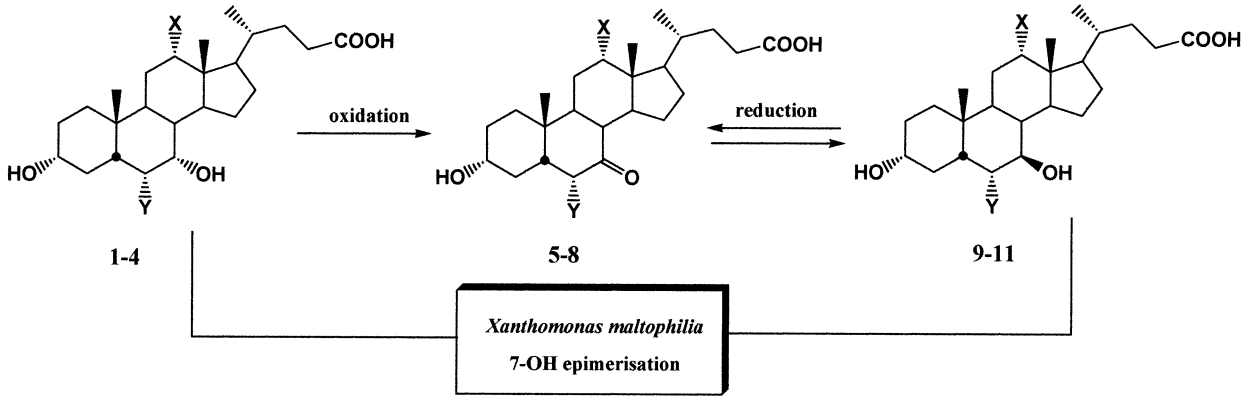
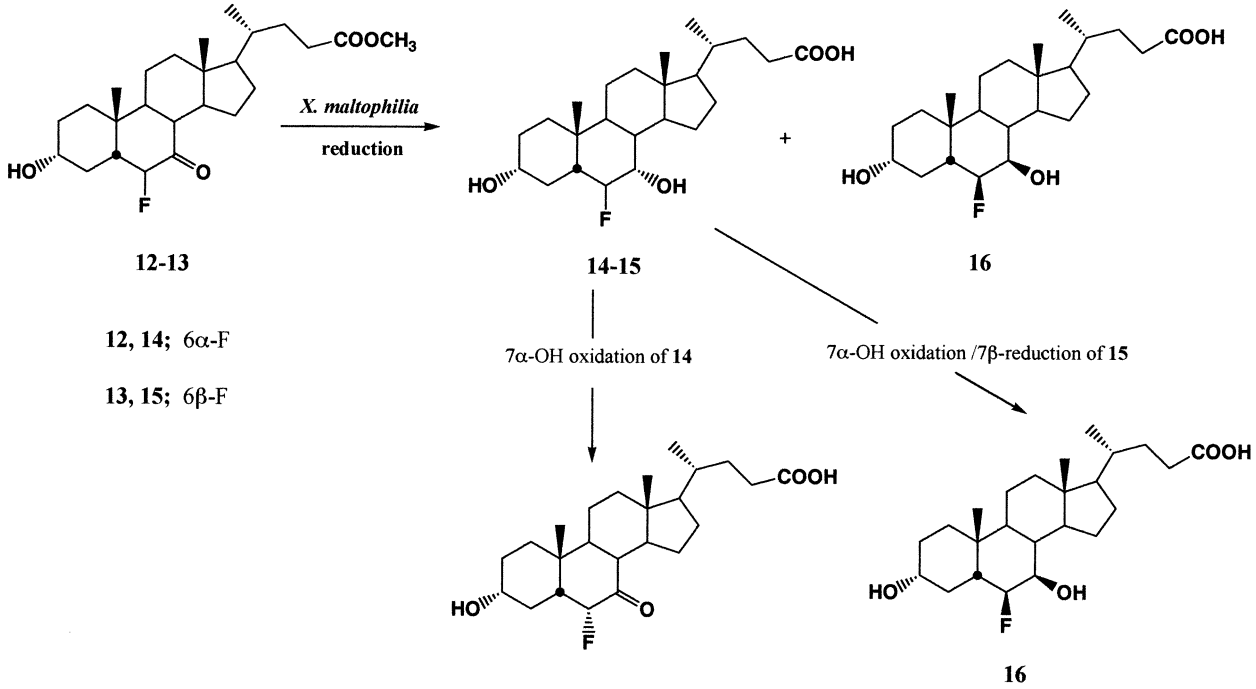


1; X = OH Y = H (CA)
2; X = H Y = H (CDCA)
3; X = O Y = H (12-oxo-CDCA)
4; X = H Y = OH
5; X = OH Y = H
6; X = H Y = H
7; X = O Y = H
8; X = H Y = OH
9; X = OH Y = H (UCA)
10; X = H Y = H (UDCA)
11; X = O Y = H (12-oxo-UDCA)

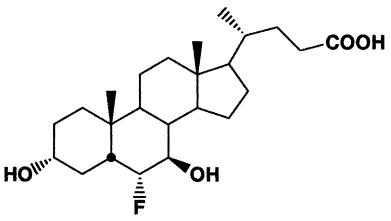
Scheme 1.

the four possible diastereoisomers, 6α-fluoro-3α,7β-dihydroxy-5β-cholan-24-oic acid (6-FUDCA) [7,8] has been shown to be endowed with a favourable pharmacological profile, mainly due to its slightly increased hydrophilicity, resistance to bacterial 7-dehydroxylation and good enrichment in bile with respect to UDCA (Fig. 1).

The only reported synthesis of 6-FUDCA includes the 6-fluorination of methyl 7-ketolithocholate chosen as starting product, followed by a long and tedious reaction sequence which accomplishes the transformation in several steps of the 7-keto function into the corresponding β-hydroxy group [7]. While the first goal can efficiently be



Scheme 2.



6-FUDCA

Fig. 1.

obtained with an electrophilic fluorination reaction which employs Selectfluor™ as reagent, the selective metal reduction of the 7-keto group into the β-hydroxy function cannot be employed, due to the presence of the fluorine atom at C-6 which is removed under the required reaction conditions. The required 7β-hydroxy group is obtained by a synthetic sequence which involves the initial formation of the 7α-hydroxy group by sodium borohydride reduction of 6α-fluoro-7-ketolithocholic acid (**12**) followed by the inversion of the configuration at C-7. The results obtained by the enzymatic biotransformations mediated by *Xanthomonas maltophilia* on the above reported substrates are described herein.

2. Experimental

Sodium salt of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), chenodeoxycholic (3α,7α-dihydroxy-5β-cholan-24-oic acid), 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid, and hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid) have been supplied by ICE industry.[1] 6α-Fluoro and 6β-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl esters are prepared according to the literature procedure [7]. TLC analyses are performed on silica gel with ethyl acetate/cyclohexane/acetic acid 50/50/1 as eluent. Gas chromatographic analyses were 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 X 0.32 mm) from Mega s.n.c.: helium as carrier gas (0.55 atm); temp. 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 acid are the following: ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid) **9**, 8.58; cholic acid **1**, 9.27; 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid **5**, 10.91; 3α,7β-dihydroxy-12-oxo-5β-cholan-24-oic acid **11**, 11.33; 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid **3**, 11.53; 3α-hydroxy-7,12-dioxo-5β-cholan-24-oic acid **7**, 14.01; dehydrocholic acid (as internal standard) 16.17. Retention times (in min) for the series of chenodeoxycholic acid are the following: chenodeoxycholic acid **2**, 5.95; ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) **10**, 6.20; 3α-hydroxy-7-oxo-5β-cholan-24-oic acid **6**, 8.10; 3,7-dioxo-5β-cholan-24-oic acid (as internal standard) 9.98. Retention times (in min) for the series of hyocholic acids are the following: hyocholic acid **4**, 6.86; 3α,6α-dihydroxy-7-oxo-5β-cholan-24-oic **8**, 9.28; 6α-hydroxy-3,7-dioxo-5β-cholan-24-oic (as internal standard) 12.11. Retention times (in min) for the series of 6α-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl ester, after derivatization with diazomethane and trifluoroacetic anhydride, are the following: 6α-fluoro-3α,7α-dihydroxy-5β-cholan-24-oic acid **14**, 13.80; 6α-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl ester **12**, 14.44; 6α-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid **17**, 14.44; dehydrocholic acid methyl ester (as internal standard), 19.20. The compounds **12** and **17** are characterized by TLC. ^{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. Mass spectra were obtained on a Mariner (applied bio-systems)

2.1. Epimerisation of bile acids 1–4 with Xanthomonas maltophilia CBS 827.97

2.1.1. Method A

The culture medium is prepared dissolving in 1 l of water glucose (10 g/l), bactotryptone (5 g/l), yeast extract (2.5 g/l) and KH_2PO_4 (0.2 g/l). The culture medium (100 ml), previously sterilized at 120°C for 20 min, is inoculated with a spore suspension of *Xanthomonas maltophilia* and grown for 48 h under stirring at 30°C. To a grown culture is added the proper bile acid as sodium salt (1 g) adjusting the pH to 8 with 10% NaOH and the incubation is continued for the appropriate time (see Table 1) without stirring (pressure of oxygen about 1–2%). The reaction is monitored by TLC and the crude reaction products are analyzed by GLC [9] and compared with authentic samples. The suspension is removed by centrifugation, the mixture is acidified with 5% HCl and extracted with ethyl acetate. Drying with anhydrous Na_2SO_4 and chromatography (silica, ethyl acetate/cyclohexane/acetic acid 50:50:1) afford the pure products (see Table 2).

2.1.2. Method B

The reactions are carried out as above maintaining the incubation under stirring (normal pressure of oxygen) even after the addition of the bile acids.

2.2. Partial purification of Xanthomonas maltophilia hydroxysteroid dehydrogenases (HSDH)

The growth of *Xanthomonas maltophilia* was carried out in a medium (1 l) containing glucose (15 g), yeast extract (5

[1] ICE (Industria Chimica Emiliana) industry (Reggio Emilia, Italy) extracts, and purifies bile acids from raw materials (ox, and pig bile).

Table 1
Oxido-reduction of bile acids **1–4**, **12**, and **13** with *Xanthomonas maltophilia*

Bile acid	Method[a]	Time (h)	7β-OH bile acid (yield %)	7-CO bile acid (yield %)	7α-OH bile acid (yield %)
1	**A**	24	**9** (80)	**5** (10)	—
	B	24	**9** (10)	**5** (85)	—
2	**A**	24	**10** (27)	**6** (23)	**2** (40)
	A	48	**10** (10)	**6** (44)	**2** (40)
	B	24	—	**6** (80)	**2** (12)
3	**A**	96	**11** (80)	**7** (10)	—
	B	96	**11** (10)	**7** (85)	—
4	**A**	96	—	**8** (25)	**4** (66)
	B	96	—	**8** (80)	**4** (10)
12	**A**	24	—	—	**14** (95)
	A	48	—	**17** (95)	—
13	**A**	24	**16** (46)	—	**15** (46)
	A	72	—	—	**15** (92)

[a] **A**, product incubation without stirring (scarcity of oxygen); **B**, product incubation with stirring (normal pressure of oxygen).

g), soy peptone (3 g), Nutriferm L90 (15 g), and sodium cholate (0.25 g) at pH 6.5 for 24 h at 28°C with constant slow shaking. Wet cells (5 g) were harvested by centrifugation and washed with 0.9% NaCl solution. Wet cells (5 g), suspended in 50 ml of 20 mM Na-phosphate buffer (Na-P) pH 7.5 containing 1 mM EDTA, 2 mM mercaptoethanol and supplemented with egg lysozyme (15 mg) and 1 mM phenylmethylsulfonyl fluoride (PMSF), were stirred overnight at 4°C. Cell debris was removed by high speed centrifugation (200.000 *g* for 20 min) and nucleic acids by precipitation with protamine sulfate (2 mg/ml). After pH adjustment to pH 8.3, crude extract is chromatographed on DEAE-sepharose column equilibrated with 20 mM Na-P buffer at pH 8.3. Cholylglycine hydrolase (CGH) and 7α-HSDH were eluted with NaCl linear gradient to a final concentration of 250 mM NaCl. 7β-HSDH is not bound by the resin and is eluted by rinsing the column with the equilibration buffer. The acetone powder of the partially purified 7α-HSDH and 7β-HSDH were prepared [10].

2.3. Enzyme assay

7α- and 7β-hydroxysteroid dehydrogenase activities were assayed by monitoring NADH formation at 340 nm and 25°C by an Uvicon 930 spectrometer (Kontron Instruments). The standard reaction mixture (total volume 1 ml) contained 25 mM Na-phosphate buffer pH 7.5, 0.8 mM NAD^+ and 5 mM sodium cholate (7α-HSDH activity assay) or 5 mM sodium ursocholate (7β-HSDH activity assay). Specific activities of the partially purified 7α- and 7β-hydroxysteroid dehydrogenase acetone powder were 12.8 IU/mg and 5 IU/mg, respectively. One international unit (IU) of enzyme is defined as the amount required to yield 1 μmol of NADH per minute.

Table 2
Selected spectroscopical data of bile acids **14–16** and **17** (as methyl ester **12**)

Compound	Selected ^{1}H NMR signals (ppm)[a]	Selected ^{13}C NMR signals (ppm)[a]	HRMS(ESI)
14	0.55 (3H, s, 18-Me), 0.80 (3H, s, 19-Me), 0.85 (3H, d, 21-Me), 3.30 (1H, m, 3-CH), 3.85 (1H, m, 7-CH), 4.53 (1H, ddd, $J_{H\text{-}F}$ = 45 Hz, $J_{H\text{-}H}$ = 3.5, 5.5 Hz, 6-CH)	175.83 (CO_2H), 94.20 and 90.66 (C-6, $J_{C6,F}$ = 178.4 Hz), 71.64 (C-3), 70.27 and 69.94 (C-7, $J_{C7,F}$ = 31.5 Hz)	Calcd for $C_{24}H_{40}FO_4$ $[MH]^+$ 411.2905; found 411.2904; $[MH]^+$ major fragmentation observed $[MH\text{-}H_2O]^+$ m/z 393
15	0.60 (3H, s, 18-Me), 0.90 (3H, d, 21-Me), 0.95 (3H, d, 19-Me), 3.35 (1H, m, 3-CH), 3.75 (1H, m, 7-CH), 4.35 (1H, dm, $J_{H\text{-}F}$ = 43 Hz, 6-CH)	175.66 (CO_2H), 98.56 and 95.12 (C-6, $J_{C6,F}$ = 173.2 Hz), 71.48 (C-3), 69.81 and 69.22 (C-7, $J_{C7,F}$ = 29.6 Hz)	Calcd for $C_{24}H_{40}FO_4$ $[MH]^+$ 411.2905; found 411.2906; $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$ m/z 393
16	0.70 (3H, s, 18-Me), 0.95 (3H, d, 21-Me), 1.03 (3H, d, 19-Me), 3.60 (2H, m, 3-CH and 7-CH), 4.73 (1H, dm, $J_{H\text{-}F}$ = 36 Hz, 6-CH)	177.16 (CO_2H), 93.63 and 90.16 (C-6, $J_{C6,F}$ = 177.1 Hz), 78.19 and 77.45 (C-7, $J_{C7,F}$ = 22.6 Hz), 70.54 (C-3)	Calcd for $C_{24}H_{40}FO_4$ $[MH]^+$ 411.2905; found 411.2905. $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$ m/z 393
12	0.60 (3H, s, 18-Me), 0.87 (3H, d, 21-Me), 1.18 (3H, s, 19-Me), 3.55 (1H, m, 3-CH), 3.60 (3H, s, CO_2Me), 5.18 (1H, dd, $J_{H\text{-}F}$ = 49.7 Hz, $J_{H\text{-}H}$ = 6.3 Hz, 6-CH)	205.52 (C=O), 174.35 (CO_2Me), 93.66 and 89.82 (C-6, $J_{C6,F}$ = 193.5 Hz), 69.72 (C-3)	Calcd for $C_{25}H_{40}FO_4$ $[MH]^+$ 423.2905; found 423.2906; $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$ m/z 405

[a] Obtained in $CDCl_3$ + CD_3OD.

2.4. Reduction of 6α-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl ester **12** with X. maltophilia

The reaction is carried out according to method A starting from 0.1 g of **12** dissolved in DMF (1 ml) added to a grown culture of *X. maltophilia* (10 ml). Aliquots were withdrawn periodically and monitored by GLC and TLC. After 24 h incubation TLC analysis confirmed no more starting product. Centrifugation, extraction with ethyl acetate, drying with anhydrous Na_2SO_4, and chromatography (silica, ethyl acetate/cyclohexane/acetic acid 50:50:1) of the reaction mixture afford the pure 6α-fluoro-3α,7α-dihydroxy-5β-cholan-24-oic acid (**14**): 0.092 g (95%): ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 0.55 (3H, s, 18-Me), 0.80 (3H, s, 19-Me), 0.85 (3H, d, 21-Me), 3.30 (1H, m, 3-CH), 3.85 (1H, m, 7-CH), 4.53 (1H, ddd, $J_{H\text{-}F}$ = 45 Hz, $J_{H\text{-}H}$ = 3.5, 5.5 Hz, 6-CH); ^{13}C-NMR ($CDCl_3$+ CD_3OD) δ 175.83 (CO_2H), 94.20 and 90.66 (C-6, $J_{C6,F}$ = 178.4 Hz), 71.64 (C-3), 70.27 and 69.94 (C-7, $J_{C7,F}$ = 31.5 Hz); HRMS (ESI) calcd for $C_{24}H_{40}FO_4$ $[MH]^+$ 411.2905; found 411.2904; $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$m/z 393. Anal. Calcd for $C_{24}H_{39}O_4F$: C%, 70.21; H%, 9.57; F%, 4.62. Found: C%, 71.02; H%, 9.39; F%, 4.60. The reaction was repeated and crude reaction products were monitored by GLC and TLC until 48 h incubation. At 24 h the quantitative yield of **14** was confirmed but at 48 h the 7-oxo derivative **17** was quantitatively produced. Compound **17** was transformed with diazomethane in the methyl ester **12** and the data are the following: ^{1}H-NMR ($CDCl_3$) δ 0.60 (3H, s, 18-Me), 0.87 (3H, d, 21-Me), 1.18 (3H, s, 19-Me), 3.55 (1H, m, 3-CH), 3.60 (3H, s, CO_2Me), 5.18 (1H, dd, $J_{H\text{-}F}$ = 49.7 Hz, $J_{H\text{-}H}$ = 6.3 Hz, 6-CH); ^{13}C-NMR ($CDCl_3$ + CD_3OD) δ 205.52 (C=O), 174.35 (CO_2Me), 93.66 and 89.82 (C-6, $J_{C6,F}$ = 193.5 Hz), 69.72 (C-3); HRMS (ESI) calcd for $C_{25}H_{40}FO_4$ $[MH]^+$ 423.2905; found 423.2906; $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$ m/z 405. Anal. Calcd for $C_{25}H_{39}O_4F$: C%, 71.06; H%, 9.30; F%, 4.49. Found: C%, 70.95; H%, 9.17; F%, 4.53.

2.5. Reduction of 6β-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl ester **13** with X. maltophilia

The reaction is carried out according to method A starting from 0.1 g of **13** dissolved in DMF (1 ml) added to a grown culture of *X. maltophilia* (10 ml). Aliquots were withdrawn periodically and monitored by TLC. After 24 h incubation TLC analysis confirmed no more starting product. Centrifugation, extraction with ethyl acetate, drying with anhydrous Na_2SO_4, and chromatography (silica, ethyl acetate/cyclohexane/acetic acid 50:50:1) of the reaction mixture afforded the pure 6β-fluoro-3α,7α-dihydroxy-5β-cholan-24-oic acid **15** (45 mg, 46%) and 6β-fluoro-3α,7β-dihydroxy-5β-cholan-24-oic acid **16** (45 mg, 46%). Compound **15** showed the following: ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 0.60 (3H, s, 18-Me), 0.90 (3H, d, 21-Me), 0.95 (3H, d, 19-Me), 3.35 (1H, m, 3-CH), 3.75 (1H, m, 7-CH), 4.35 (1H, dm, $J_{H\text{-}F}$ = 43 Hz, 6-CH); ^{13}C-NMR ($CDCl_3$ + CD_3OD) δ 175.66 (CO_2H), 98.56 and 95.12 (C-6, $J_{C6,F}$ = 173.2 Hz), 71.48 (C-3), 69.81 and 69.22 (C-7, $J_{C7,F}$ = 29.6 Hz); HRMS (ESI) calcd for $C_{24}H_{40}FO_4$ $[MH]^+$ 411.2905; found 411.2906; $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$·m/z 393. Anal. Calcd for $C_{24}H_{39}O_4F$: C%, 70.21; H%, 9.57; F%, 4.62. Found: C%, 70.64; H%, 9.41; F%, 4.66. Compound **16** showed the following: ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 0.70 (3H, s, 18-Me), 0.95 (3H, d, 21-Me), 1.03 (3H, d, 19-Me), 3.60 (2H, m, 3-CH and 7-CH), 4.73 (1H, dm, $J_{H\text{-}F}$ = 36 Hz, 6-CH); ^{13}C-NMR ($CDCl_3$ + CD_3OD) δ 177.16 (CO_2H), 93.63 and 90.16 (C-6, $J_{C6,F}$ = 177.1 Hz), 78.19 and 77.45 (C-7, $J_{C7,F}$ = 22.6 Hz), 70.54 (C-3); HRMS (ESI) calcd for $C_{24}H_{40}FO_4$ $[MH]^+$ 411.2905; found 411.2905. $[MH]^+$ major fragmentation observed $[MH - H_2O]^+$·m/z 393. Anal. Calcd for $C_{24}H_{39}O_4F$: C%, 70.21; H%, 9.57; F%, 4.62. Found: C%, 70.83; H%, 9.29; F%, 4.58. The reaction was repeated and crude reaction products were monitored by TLC until 72 h incubation. At 24 h the 1:1 mixture of **15** and **16** was confirmed but at 72 h only **16** (92%) was recovered.

3. Results and discussion

The incubation of cholic acid (CA, **1**) and of 12-oxo-chenodeoxycholic acid (12-oxo-CDCA, **3)** with a grown and non-stirred culture of *Xanthomonas maltophilia* CBS 827.97 (method A) affords the corresponding 7β-OH derivatives ursocholic acid (UCA, **9**) and 12-oxo-ursodeoxycholic acid (12-oxo-UDCA, **11**) in 80% yield, after 24 and 96 h respectively (Scheme 1, Table 1).

On the other hand, when the incubation of the BAs **1** and **3** is carried out under vigorous stirring (method B), the 7-oxo derivatives **5** and **7** are obtained in 85% yield, after 24 and 96 h respectively, together with small amount of the 7β-OH products **9** and **11** in 10% yield.

Worst results are obtained in the incubation of chenodeoxycholic acid (CDCA, **2**) with scarsity of oxygen (method A). After 24 h only 27% of the 7β-OH ursodeoxycholic acid (UDCA, **10**) is produced together with 23% of the corresponding 7-keto derivative **6**. Surprisingly, prolonged incubation (48 h) of **2** increased the amount of the oxidation product **6** (44%) and decreased the percentage of UDCA (10%). In these conditions the 7β-OH oxidation is probably faster than the reduction. On the other hand the incubation of CDCA **2** under vigorous stirring (method B) affords the oxidation product **6** in 80% yield after 24 h. The 7-keto derivative **8** is, however, the only product obtained by incubation of hyocholic acid **4** with *X. maltophilia* both with scarsity of oxygen (method A, 25% yield) and vigorous stirring (method B, 80% yield). On the other hand the starting materials were recovered unaltered when the incubation of BAs **1–4** was carried out in anaerobic conditions. On the basis of these results we can suggest the presence of two hydroxysteroid dehydrogenases (HSDH) α- and β-ste-

reospecific. The 7β-HSDH is activated by the scarcity of oxygen during the biotransformation producing the epimerisation products **9–11** to the disadvantage of the oxidation ones **5–7.** Only in the case of hyocholic acid **4** is the 7β-HSDH inhibited probably because of the presence of the 6α-OH function. Partial purification [11] of the enzyme responsible for 7-OH inversion confirmed the presence of two strictly NADH-dependent hydroxysteroid dehydrogenases α- and β-stereospecific together with a cholylglycine hydrolase. The obtained acetone powders of 7α-HSDH and 7β-HSDH, in fact, oxidize the CA **1** and the UCA **9**, respectively, to the corresponding 7-oxo derivative **5**. The preliminary enzymatic results are confirmed by the *X. maltophilia* reductions of 6α-fluoro and 6β-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acids methyl esters **12** and **13** in scarcity of oxygen (method A). The 24 h incubation of 6α-fluoro derivative **12** affords the hydrolized 7α-OH bile acid **14** (6-FCDCA) in 95% yield (Scheme 2).

Also in this case the presence of the 6α-F group inhibits the 7β-HSDH because no 6-FUDCA **18** has been obtained. If the reaction is prolonged up to 48 h, the 7α-OH function is again oxidized to give the 6α-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid **17** in quantitative yield. On the contrary, the incubation of 6β-fluoro-3α-hydroxy-7-oxo-5β-cholan-24-oic acid methyl esters **13** with *X. maltophilia* affords after 24 both the 7α- and 7β-hydroxy derivatives **15** (6β-FCDCA) and **16 (**6β-FUDCA), in 46% yield. In this case, a prolonged incubation up to 72 h does not afford any oxidation product but only the further transformation of 7α-OH **16** to 7β-OH **17**, probably through a slow oxidation of the 7α-OH and a fast 7β-reduction, because no 7-CO derivative has been detected. All new 6-F,7-OH derivatives **14–16,** together with the oxidation product **17** (as methyl ester **12**), are fully characterized. Selected significant data are summarized in Table 2.

In conclusion, the described microbial transformations represent an interesting procedure for the selective reduction of the 7-keto functionality of bile acids to 7β-hydroxy derivatives. This methodology, however, enables the alternative preparation of three diastereoisomeric 6-fluoro substituted bile acids thus allowing the possibility to study their pharmacokinetic and metabolic profile.

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