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Regioselective Microbial Oxidation of Bile Acids

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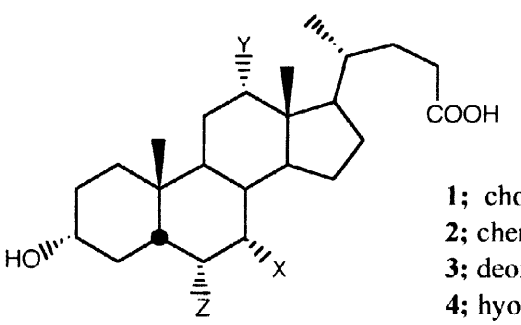
Abstract: High regioselectivity in the microbial oxidation of C_7, C_3 and C_{12} hydroxyl groups of cholic, chenodeoxycholic, deoxycholic and hyocholic acids **1-4** is reported. The tested microrganisms have been isolated from 50 enviromental samples withdrawed from an industry that extracts and purify bile acids.

Bile acids, their conjugates and salts are natural products, fundamental constituents of bile.[1] The primary bile acids in human bile are cholic acid and chenodeoxycholic acid mainly present as glycine and taurine conjugates while deoxycholic acid is commonly known as secondary bile acid. Chenodeoxycholic acid and its 7-hydroxy epimer ursodeoxycholic acid have important pharmaceutical applications related to their ability to solubilize cholesterol gallstones and are prepared in large scale from raw materials with high bile acid content. Chemical sequences in the synthesis of these compounds involve oxidations and reductions. During the last decade biotranformations have been successfully used to enhance the selectivity and to reduce the number of steps.[2] Excellent results have been obtained in this field using, for the oxidoreduction, NAD(P)-dependent hydroxysteroid dehydrogenases that regiospecifically oxidize cholic acid at each of the three possible position.[3] On the other hand various microrganisms (most of them anaerobic) give regiospecific oxidations of cholic, chenodeoxycholic and deoxycholic acids[2] while oxidation of hyocholic acid has not been studied.



1-4

1; cholic acid **Y = X = OH, Z = H**
2; chenodeoxycholic acid **X = OH, Y = Z = H**
3; deoxycholic acid **Y = OH, X = Z = H**
4; hyocholic acid **X = Z = OH, Y = H**

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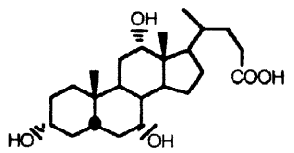
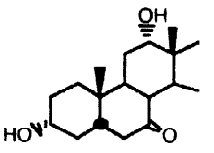
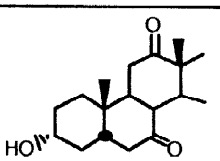
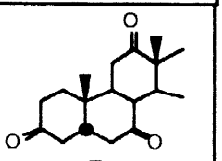
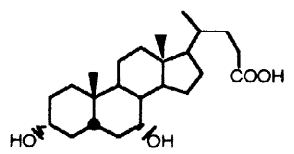
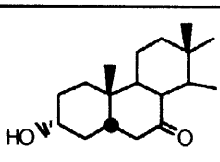
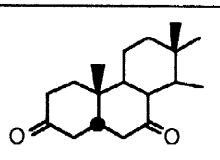
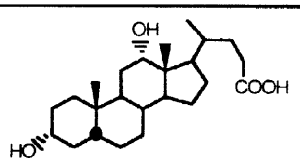
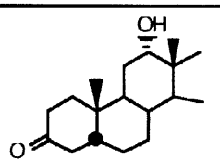
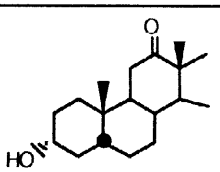
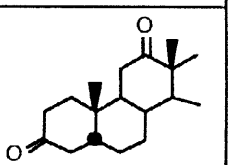
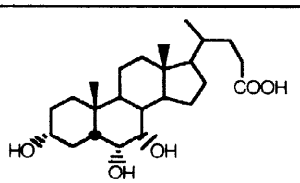
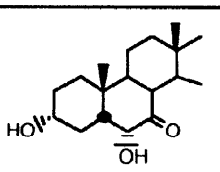
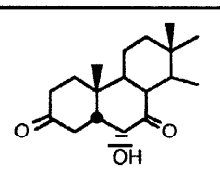
Table 1. Microbial oxidation of bile acids **1-4**.

Microrganism[a]	Bile acid	Products		
	 **1**	 **5**	 **6**	 **7**
ICE BS6		87%		
ICE BS7		75%		
ICE BS11		52%	30%	
ICE B37		14%	80%	
ICE B49		27%	30%	
ICE B25				25%
ICE B26				41%
	 **2**	 **8**	 **9**	
ICE BS6		99%		
ICE BS11		99%		
ICE B37		97%		
ICE B23		43%	56%	
ICE B18			93%	
	 **3**	 **10**	 **11**	 **12**
ICE B23		91%		
ICE B37			96%	
ICE B49			84%	
ICE BS11			98%	
ICE B25				96%
	 **4**	 **13**	 **14**	
ICE BS6		90%		
ICE BS7		96%		
ICE B23		34%	50%	

[a] The identification of the strains, most of them are *Pseudomonas* or *Acinetobacter*, is given in the experimental section: the microrganisms belong to ICE private collection.

In this paper we have isolated and classified several microrganisms from 50 enviromental samples withdrawn from ICE industry[4] that extracts and purifies bile acids from raw materials (ox and pig bile). These

microrganisms (mostly bacteria) have been screened in oxidation reactions of cholic, chenodeoxylic, deoxycholic and hyocholic acids **1-4**. The most significative results are summarized in Table 1.

Cholic acid **1** is selectively oxidized at C_7 position by BS6 and BS7 strains (85% and 75% yields of **5**, respectively) while a mixture of the 7-oxo and 7,12-dioxo derivatives **5** and **6** is obtained with BS11, B37 and B49 strains. On the other hand, bacteria B25 and B26 afford the dehydrocholic acid **7** (25 and 41% yields, respectively).

Excellent selectivity for the C_7 position is also showed in the oxidation of chenodeoxycholic acid **2** with bacteria BS6, BS11 and B37: the yields of the 7-oxo derivative **8** are in all cases > 95%. The strain B23, however, gives in about 1:1 ratio the 7-oxo and the 3,7-dioxo derivatives **8** and **9** while B18 produces exclusively the 3,7-dioxo deivative **9** in 93% yield.

Deoxycholic acid **3** (the hydroxyl group at C_7 is not present) is regioselectively oxidized with B23 strain at C_3 position to afford the 3-keto acid **10** in 91% yield. The C_{12} hydroxyl group is almost quantitatively oxidized (84-98% yield) to 12-keto acid **11** with B37, B49 and Bs11 strains while bacterium B25 produces 3,12-diketocholanic acid **12** (96%).

The oxidation of the hyocholic acid **4** with BS6 and BS7 strains gives the 7-oxo compound **13** in excellent yield (90-96%) by while the bacterium B23, analogously to the oxidation of chenodeoxycholic acid, affords the 3-keto and the 3,7-diketo derivatives **13** and **14**, 34% and 50% yields, respectively.

On the basis of these results and of the negative ones (all bacteria are tested in the oxidation of bile acids **1-4**) we can summarize the regioselectivity of these microrganisms with respect to the oxidation of the hydroxyl group in C_3, C_7 and C_{12} in Table 2.

Table 2. Regioselectivity of the ICE strains in the oxidation of bile acids **1-4**

C_7-Oxidation	C_3-Oxidation	C_{12}-Oxidation
Xanthomonas maltophilia BS6		
Pseudomonas fluorescens BS7		
Pseudomonas fluorescens B18	*Pseudomonas fluorescens* B18	
Bacillus mycoides B23	*Bacillus mycoides* B23	
Acinetobacter calcoaceticus lwoffii BS11		*Acinetobacter calcoaceticus lwoffii* BS11
Acinetobacter calcoaceticus lwoffii B37		*Acinetobacter calcoaceticus lwoffii* B37
Acinetobacter calcoaceticus acidovorans B49		*Acinetobacter calcoaceticus acidovorans* B49
Pseudomonas fluorescens B25	*Pseudomonas fluorescens* B25	*Pseudomonas fluorescens* B25
Pseudomonas fluorescens B26	*Pseudomonas fluorescens* B26	*Pseudomonas fluorescens* B26

The bacteria BS6 and BS7 oxidize regioselectively the C_7 hydroxyl group with almost quantitative yield. Bacterium B18 has probably the same velocity in the oxidation of the C_7 and C_3 hydroxyl group affording only the 3,7-dioxo derivative, while B23, although with the same regioselectivity, produces the 3-oxo and 3,7-dioxo derivative in about 1:1 ratio. The strains BS11, B37 and B49 are the microrganisms of choice for the oxidation of the C_7 and C_{12} hydroxyl groups affording almost quantitative yields the 7-oxo derivative from chenodeoxycholic acid (C_{12} hydroxyl group absent) and the 12-oxo acid from deoxycholic acid (C_7 hydroxyl group absent). This feature is confirmed by the oxidation of the cholic acid where the 7-oxo and 7,12-dioxo derivatives are obtained in various ratios.

Only the strains B25 and B26 show almost the same selectivity for all C_3, C_7 and C_{12} hydroxyl groups giving the compound completely oxidized from cholic and deoxycholic acids

Acknowledgements

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Experimental

Gas chromatographic analyses were performed on a Carlo Erba GC 6000 Vega series 2. The reaction products (derivatized with trifluoroacetic anhydride and hexafluoroisopropanol) are analyzed by GLC on fused silica capillary column Megadex SE 52 (25 m X 0.32 mm) from Mega s.n.c.: carrier gas: helium 0.55 atm; temp. 250°C for 5 min, 250-300° C (5°C/min), 300°C for 3 min. Retention time in min: for oxidation of **1**, cholic acid **1** 7.26, 7-CO **5** 9.15, 3,7-CO **6** 10.59, 3,7,12-CO **7** 14.36; for the oxidation of **2**, chenodeoxycholic acid **2** 6.27, 7-CO **8** 8.58, 3,7-CO **9** 10.61; for the oxidation of deoxycholic acid 3, deoxycholic acid **3** 7.41, 3-CO **10** 9.08, 12-CO **11** 10.34, 3,12-CO **12** 12.32; for the oxidation of hyocholic acid **4**, hyocholic acid **4** 8.93, 7-CO **13** 11.44, 3,7-CO **14** 14.03.

All cholanic acid in this work are of 5β series: the older name cholanic is used in place of the newer IUPAC-suggested cholan-24-oic acid. The bile acids **1-12**[1] are characterized by comparison with an authentic sample.

Isolation of microrganisms from environmental samples collected within I.C.E. factory. General procedure. Fifty water samples (mother liquor and water from soil) have been collected within I.C.E. chemical factory. A small amount (1 ml) of each sample has been diluted in a sterile saline solution (9 mL) and 1 ml of each diluted sample has been streaked on an agar medium: Plate Count Agar (Oxoid) distributed in Petri dishes. The dishes have been incubated at room temperature for 24 hours.

Isolation medium (Plate Count agar) has developed the growth of different types of colonies. Colonies have been picked and transferred to fresh agar medium to obtain the purification of cultures. Purified colonies have been transferred to maintenance agar slants, incubated at room temperature for 48 hours and stored at + 4°C.
The microorganisms have been identified by means of API TEST (Api System, bioMérieux). Tests have demonstrated that the strains are pure and belong mainly to genera *Pseudomonas* and *Acinetobacter*.
The ICE strains BS7, B25, B26 and B18 are *Pseudomonas fluorescens*, ICE BS11 and B37 are *Acinetobacter calcoaceticus lwoffi*, ICE B49 is *Acinetobacter calcoaceticus acidovorans* and ICE BS6 is *Xanthomonas malthophilia*.

Screening of microbial oxidation of bile acids 1-4 on analytical scale. A nutrient broth was prepared dissolving bactotryptone (5 g), yeast extract (2.5 g), glucose (10 g) and KH_2PO_4 (0.2 g) in 1 L of distilled water. A solution of the appropriate bile acid is prepared dissolving the sodium salt (0.2 g) in 2 mL of distilled water. A sterilized nutrient broth (8 mL), with 2 mg of the selected bile acid as sodium salt (20 μL of water solution), was inoculated with a loopful of the selected bacterium (20 ICE strains are tested). The mixture was incubated for 1 day at 30°C on a reciprocatory shaker. To the resulting suspension of grown cells the sodium salt of bile acid (8 mg, 80 μL of water solution) was added. The incubation was continued for 24 h and the reaction mixture (1 mL) was acidified with 5% HCl to pH 3-4 and extracted with 1 mL of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and analyzed by t.l.c.. For GC analyses the mixture was derivatized with trifluoroacetic anhydride and hexafluoroisopropanol.

Microbial oxidation of hyocholic acid 4 with *Bacillus mycoides* B23 on preparative scale. A sterilized nutrient broth prepared as above (80 mL), with 20 mg of hyocholic acid **4** as sodium salt (200 μL of water solution), was inoculated with a loopful of the bacterium B23. The mixture was incubated for 32 h at 30°C on a reciprocatory shaker. To the resulting suspension of grown cells the sodium salt of **4** (80 mg, 800 μL of water solution) was added. After a further incubation of 32 h, the suspension was centrifugated to eliminate the cells. The surnatant was acidified with 5% HCl to pH 3-4 and extracted with ethyl acetate (2 X 80 mL). The organic layer was dried over anhydrous Na_2SO_4, the solvent removed under vacuum and the residue chromatographed (silica, ethyl acetate/ acetic acid 50:1) to give 3α,6α-dihydroxy-7-oxo-cholanic acid **13**[5] (33 mg, 34%) and 6α-hydroxy-3,7-dioxo-cholanic acid **14** (49 mg, 50%).
The compound **13** showed the following: mp 183-185°C (lit[5] 184-186°C); ^1H NMR ($CDCl_3$, 300 MHz) δ 0.66 (s, 3 H), 0.95 (d, 3H, J = 6.3 Hz), 1.24 (s, 3 H), 3.55 (m, 1 H, 3β-H), 4.50 (d, 1 H, 6β-H, J = 6.3 Hz).

The compound **14** showed the following: mp 95-97°C ; $[\alpha]_D^{25}$ = -156.5 (*c* 0.2, $CHCl_3$); IR ($CHCl_3$) 2950, 1710 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) δ 0.74 (s, 3 H), 0.96 (d, 3 H, *J* = 6.5 Hz), 1.35 s, 3 H), 4.62 (d, 1 H, 6β-H, *J* = 5.6 Hz); MS (as 6α-trifluoroacetyl derivative) m/z (relative abundance %) 650 (M^+, 44), 632 (9), 537 (30), 536 (92), 465 (100), 399 (16), 385 (14), 359 (26), 245 (14).

Anal. cald for $C_{24}H_{40}O_5$: C, 70.55; H, 9.87. Found: C, 69.95; H, 9.90.

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