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Regiospecific Oxidoreductions Catalyzed by a New *Pseudomonas paucimobilis* Hydroxysteroid Dehydrogenase

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Abstract: The preparative-scale regio- and stereo-specific oxidation of hydroxy groups and reduction of keto functions at C(3) of several C_{24} bile acids, catalyzed by a new 3α-hydroxysteroid dehydrogenase (3α-HSDH), is reported. The crude enzyme, isolated from the cells of *Pseudomonas paucimobilis*, revealed the presence of a further enzymatic fraction containing a secondary alcohol dehydrogenase (SADH), that has been used to recycle the cofactor.

INTRODUCTION

Nicotinamide cofactor-dependent oxidoreductase is a class of enzymes extensively used in organic synthesis.[1] In particular, hydroxysteroid dehydrogenases (HSDH), which catalyze the reversible oxidoreduction of the hydroxy-keto groups of steroids,[2] have been employed for preparative-scale transformations of bile acids[3] and related important pharmaceutical derivatives. In this respect, significant results have been obtained in the regiospecific oxidation of cholic acid and in the regio- and stereo-specific reduction of dehydrocholic acid at each of the three possible positions.[3c] The natural specificity of the particular enzyme is designated by its name and enzymes selective for positions C(3), C(7), C(12) and C(20) have been reported.[4] As expected, the hydroxy or keto groups at the different bile acid positions show variable reactivity.[5] Thus for oxidation reactions the reactivity order is C(7)>C(12)>C(3), whereas for reduction the order is C(3)>C(7)>C(12). The position C(3) appears to be particularly tractable, showing great tendency to reduction but poor propensity to oxidation.

As a part of our interest in the development of new enzymes for organic synthesis,[6] we have initiated a screening to search for new alcohol dehydrogenases from microrganisms which are able to elaborate bile acid hydroxy and keto functions.[7] A list of substrates is given below.

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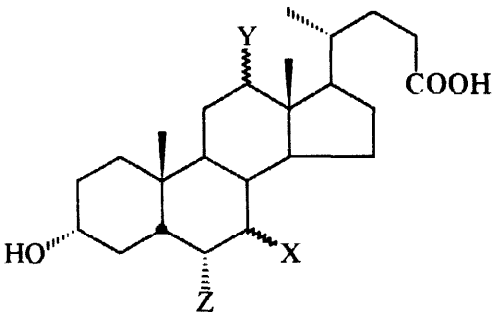
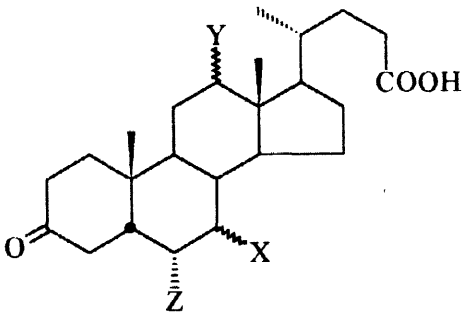


1: X = Y = α-OH, Z = H (cholic acid)
2: Y = α-OH, X = Z = H (deoxycholic acid)
3: X = α-OH, Y = Z = H (chenodeoxycholic acid)
4: X = β-OH, Y = Z = H (ursodeoxycholic acid)
5: X = Y = H, Z = α-OH (hyodeoxycholic acid)
6: X = O, Y = α-OH, Z = H
7: X = Z = α-OH, Y = H (hyocholic acid)
8: X = Y = Z = H (lithocholic acid)
9: X = α-OH, Y = β-OH, Z = H
10: X = Y = O, Z = H
11: X = α-OH, Y = O, Z = H



12: X = Y = α-OH, Z = H
13: X = Z = H, Y = α-OH
14: X = α-OH, Y = Z = H
15: X = β-OH, Y = Z = H
16: X = Y = H, Z = α-OH
17: X = O, Y = α-OH, Z = H
18: X = Z = α-OH, Y = H
19: X = Y = Z = H
20: X = α-OH, Y = β-OH, Z = H
21: X = Y = O, Z = H
22: X = α-OH, Y = O, Z = H

Preliminary results have been obtained using *Pseudomonas paucimobilis*, isolated from samples withdrawn from Venice lagoon. The choice of this microrganism has been made on the basis of a screening of a series of bacteria having similar origin.[7]

RESULTS AND DISCUSSION

Pseudomonas paucimobilis was tested in the oxidation reactions of **1-6**, added to the culture after the growth of the microrganism, and the most significant results are collected in Table 1. All substrates were oxidized in preparative-scale to the corresponding 3-keto derivatives **12-17** in high yield (60-100%) and excellent regioselectivity, independently from number, stereochemistry and oxidation state of the substituents. This confirmed the very high activity and specificity of *Pseudomonas paucimobilis* for the

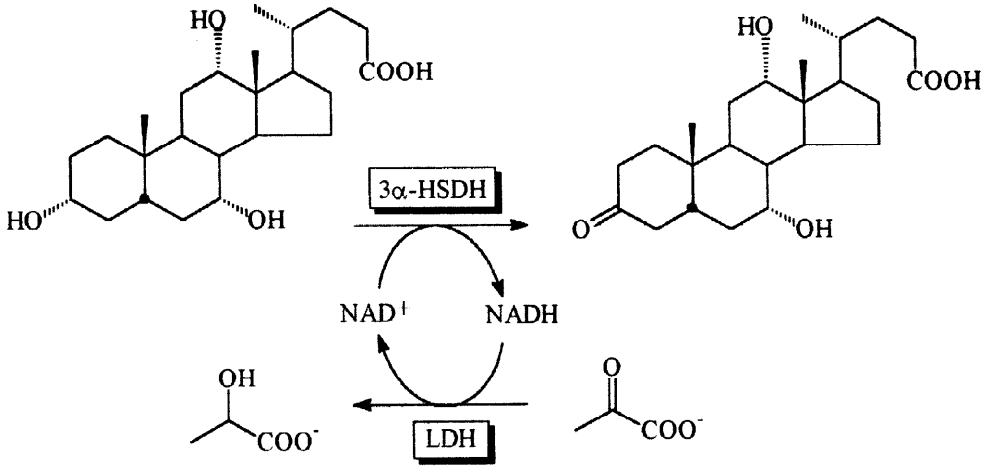
oxidation at the most reluctant position C(3). Despite the good results obtained, the major limitation in the use of whole cells in the transformations mentioned above is the banishment within the oxidative direction.

Table 1. Microbial oxidation of bile acids **1-6** with *Pseudomonas paucimobilis*.

substrate	time (h)	product	yield (%)
1	24	12	95
2	72	13	60
3	24	14	100
4	24	15	90
5	24	16	90
6	24	17	90

To overcome this drawback, from the cells of *Pseudomonas paucimobilis*, treated with lysozyme in TEA buffer, we have isolated and partially purified a new enzyme that has been classified as 3α-hydroxysteroid dehydrogenase (3α-HSDH) by comparison with the commercially available HSDH from *Pseudomonas testosteroni*.[8] The crude enzyme contains NAD dependent hydroxysteroid dehydrogenase activity and, depending on the experimental conditions adopted, catalyzes both the oxidation of hydroxyl function and the reduction of carbonyl groups at position 3 of bile acids.

The general oxidation protocol, using cholic acid **1** as model substrate, is shown in Scheme 1.



Scheme 1

As the bile acid is regiospecifically oxidized to the 3-keto compound NADH is formed, which in turn is reconverted to NAD^+ in the presence of pyruvate and lactic dehydrogenase (LDH). Table 2 collects the most notable results obtained in the enzymatic oxidation of trihydroxy compound **1**, dihydroxy compounds **2**,**3**,**7** and mono-hydroxy compound **8**.

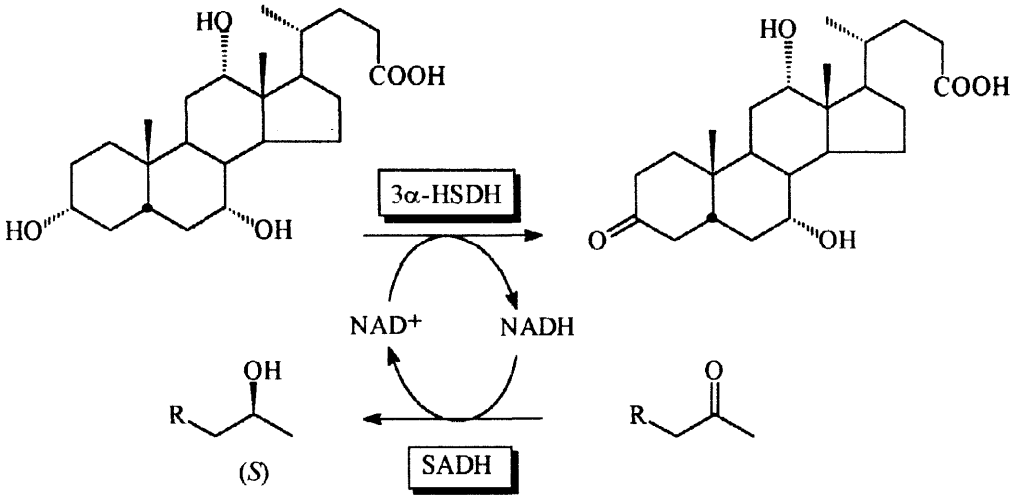
Table 2. Enzymatic oxidation[a] of selected bile acids catalyzed by the 3α-HSDH from *Pseudomonas paucimobilis*.

substrate	**1**	**2**	**3**	**7**	**8**[b]
product (yield %)	**12** (56)	**13** (72)	**14** (73)	**18** (60)	**19** (70)

[a] Reaction time 48 h. [b] The reaction has been carried out in a two-phase system, see text.

The procedure of Scheme 1 can be easily modified and extended to substrates poorly soluble in aqueous media. This is the case of lithocholic acid **8**, the oxidation of which has been carried out in a two-phase system consisting of cyclohexane/ethyl acetate 10:7 and water phosphate buffer pH 8.5, under moderately fast stirring. The oxidation of the steroid is achieved in satisfactory yield (56-73%), with no apparent decrease of the system efficiency.

The partial purification of the *Pseudomonas paucimobilis* 3α-HSDH revealed the presence of a second enzymatic fraction, characterized as a secondary alcohol dehydrogenase (SADH).[9] The new SADH was specifically linked with NADH as coenzyme and catalyzed the reduction of carbonyls to secondary hydroxyl functions. Taking into account that this enzyme is naturally present in the crude enzymatic extract from *Pseudomonas paucimobilis* used in the oxidation, it has been employed to perform the recycling of the coenzyme, in place of the pyruvate/lactic dehydrogenase counterpart. The modified catalytic cycle, based on a single crude enzymatic extract, is shown in Scheme 2.

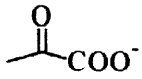
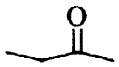


R = H, CH_3, 2-furyl

Scheme 2

Different ketones were used to optimize the recycling system and the results obtained in the oxidation of cholic acid **1** are collected in Table 3.

Table 3. 3α-HSDH catalyzed oxidation of **1** using different recycling systems.

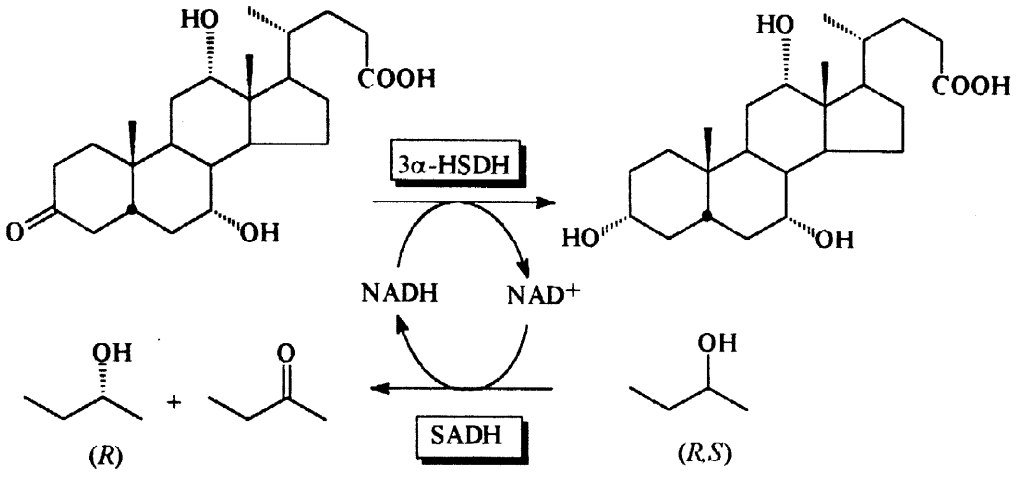
recycling system	time (h)	3-ketocholic acid **12** (yield%)
 / LDH	24	75
 / SADH	24 72	21 46
 / SADH	24	70
 / SADH	24 48	73 90

As shown in Table 3, the activity of the couple 2-butanone/SADH is comparable to that of pyruvate/lactic dehydrogenase and consequently several advantages may be envisaged in the new procedure: (i) it is economically convenient because SADH is already present in the crude enzyme solution and 2-butanone is a low cost and easily available reagent; (ii) it is equally effective, since the activity of SADH is comparable with that of lactic dehydrogenase; (iii) it is highly stereospecific, if a prochiral ketone is used. This is the case of 2-butanone and 2-furylpropanone, that are reduced in 98% enantiomeric excess to (*S*)-2-hydroxy butane and 2-((*S*)-2-hydroxypropyl)furan, respectively. The latter substrate is particularly important as representative of the class of heteroaryl derivatives possessing masked, furtherly manipulatable functionalities.[7]

As for many enzyme transformations, both the new 3α-hydroxysteroid dehydrogenase and the SADH from *Pseudomonas paucimobilis* are equally effective in the opposite direction, catalyzing the reduction of the C(3) carbonyl function of various bile acids to the corresponding hydroxyl derivatives and the recycling of the coenzyme via oxidation (Scheme 3). Table 4 collects the results obtained with different 3-keto bile acids.

Table 4. Enzymatic reduction of selected bile acids catalyzed by the 3α-HSDH from *Pseudomonas paucimobilis*. Reaction time 48 h.

substrate	**12**	**17**	**20**	**21**	**22**
product (yield %)	**1** (100)	**6** (100)	**9** (82)	**10** (100)	**11** (80)



Scheme 3

Together with the good product yield, the regio- and stereo-specificity of the overall process is worthy of note.[10] The bile acids tested, in fact, are quantitatively reduced at C(3) to the corresponding α-hydroxy derivatives, despite the presence of other keto functions. This is the case of the 3-ketocholic acid **12**, of the 3,7-diketocholic acid **17** and of the 3,7,12-triketo derivative (dehydrocholic acid) **21** transformed into **1**, **6** and **10**, respectively. In addition, only the (*S*)-2-butanol, used to recycle NADH, is stereoselectively oxidized to 2-butanone, with consequent kinetic resolution of the (*R*) enantiomer, that is the alcohol with opposite stereochemistry to that in Scheme 2.

We are currently extending the catalytic cycles of Schemes 2 and 3 to the stereoselective reduction and kinetic resolution of bis- and poly-functional ketones and secondary alcohols possessing heterocyclic rings, as synthetic equivalents of important functions.[7]

EXPERIMENTAL SECTION

Gas chromatographic analyses were performed on a Carlo Erba GC 6000 Vega series 2. The reaction products (derivatized with trifluoroacetic anhydride and hexafluoroisopropanol) were analyzed by GLC on fused silica capillay column Megadex SE 52 (25 m X 0.32 mm) from Mega s.n.c., helium carrier gas 0.55 atm, temp. 250° C for 5 min, 250-300° C (5° C/min), 300° C for 3 min. TEA buffer is composed of 50 mM triethanolamine-HCl buffer (pH 7.5) containing 0.1 mM EDTA and β-mercaptoethanol 1 mM. All cholanic acids 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 **1-22** are characterized by comparison with authentic samples.[5]

***Pseudomonas paucimobilis* oxidations of bile acids 1-6. General procedure.** A sterilized nutrient broth (100 mL), prepared dissolving glucose (2 g), peptone (10 g), yeast extract (5 g) and KH_2PO_4 (0.2 g) in 1 L of water, was inoculated with a culture of the bacterium *Pseudomonas paucimobilis* (10 mL grown for 24 h). The mixture was incubated for 24 h at 28° C on a reciprocatory shaker. To the resulting suspension of grown cells the sodium salt of the appropriate bile acid (100 mg) in water (1 mL) was added. After further 24 h of incubation (72 h for the acid **2**) the suspension was centrifuged to eliminate the cells. The surnatant was acidified with 5% HCl to pH 3-4 and extracted with ethyl acetate (2 X 50 mL). The organic layer was dried over anhydrous Na_2SO_4, the solvent removed under vacuum and the residue was chromatographed (silica, cyclohexane/ethyl acetate/acetic acid 50:50:1) to give the corresponding 3-keto derivative (see Table 1).

Preparation of the enzyme. *Pseudomonas paucimobilis* was cultivated in 1 L medium (prepared as above) for 48 h at 28° C with reciprocal shaking. Wet cells (6 g) were harvested by centrifugation (8000 rpm/15 min), washed with 0.15 M NaCl (200 mL), suspended in TEA buffer (30 mL) and treated with lysozyme (30 mg) at 20° C for 8 h and recentrifuged. Surnatant (enzyme solution) was used for the enzymatic reactions without further treatment or as acetone powder.[11]

Enzyme assays. Enzyme assays were carried out at 22° C by monitoring the absorbency change at 340 nm of the diphosphopyridine dinucleotide coenzyme (NAD or NADH) involved in the redox reaction. The oxidation of 3-hydroxy derivatives and the reduction of the 3-keto derivatives as sodium salts were carried out in 100 mM phosphate buffer, pH 7.5, containing 0.06 mM NAD (or NADH) and 0.9 mM substrate. The reactions were started by addition of few μL of enzyme to 1 mL of the reaction mixture and followed for 1 min taking readings at 0.1 min intervals. One unit (1 U) of enzyme activity is defined as amount of enzyme that transforms 1 μmol of substrate/min under standard assay condition.

3α-HSDH-catalyzed oxidations of the bile acids 1-3, 7 and 8. General procedure. A portion of the enzyme solution (10 U of 3α-HSDH) was added to 1 L solution containing 0.9 mM sodium salt of the proper 3α-hydroxy bile acid, 0.06 mM NAD, 10 mM LDH and 10 mM sodium pyruvate in 0.1 M phosphate buffer, pH 7.5. After 48 h the reaction mixture was acidified with 5% HCl to pH 3-4 and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, the solvent removed under vacuum and the residue was chromatographed (silica, cyclohexane/ethyl acetate/acetic acid 50:50:1) to give the corresponding 3-oxo derivatives (see Table 2).

The reaction for the lithocholic acid was carried out in two-phase system by adding a mixture of cyclohexane/ethyl acetate 10:7 to the reaction in 1:1 ratio with respect to phosphate buffer.

Recycle of the cofactors. NADH formed during oxidation of the 3α-hydroxy bile acid may be recycled using the second enzyme (SADH, secondary alcohol dehydrogenase) present in the crude enzyme solution (3α-HSDH:SADH 3:1 ratio). The oxidation of the cholic acid was carried out as above using various ketones as substrate (70 mM). The results are summarized in Table 3.

3α-HSDH-catalyzed reductions of bile acids 12,17,20-22 with SADH recycle. A portion of the enzyme solution (4.8 U) was added to 1L solution containing 0.9 mM sodium salt of the 3-oxo bile acid, 0.06 mM NAD and 50 mM 2-butanol in 0.1 M phosphate buffer, pH 7.5. After 48 h the reaction mixture was acidified with 5% HCl to pH 3-4 and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, the solvent removed under vacuum and the residue was chromatographed (silica, cyclohexane/ethyl acetate/acetic acid 50:50:1) to give the corresponding 3-hydroxy derivatives (see Table 4).

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11. See reference 1, p.63.