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Regio- and stereoselective reductions of dehydrocholic acid

Giancarlo Cravotto[a,*], *Arianna Binello*[a], *Luisa Boffa*[a], *Ornelio Rosati*[b], *Marco Boccalini*[c], *Stefano Chimichi*[c,*]

[a] *Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, via P. Giuria 9, 10125 Torino, Italy*
[b] *Dipartimento di Chimica e Tecnologia del Farmaco, Università di Perugia, via del Liceo, 06123 Perugia, Italy*
[c] *Dipartimento di Chimica Organica, Università di Firenze, via della Lastruccia 13, 50019 Sesto F.no (FI), Italy*

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ABSTRACT

Dehydrocholic acid (DHCA), an unnatural bile acid, is manufactured by oxidation of cholic acid. Its biotransformation by two basidiomycetes (*Trametes hirsuta* and *Collybia velutipes*) is reported. These mycelia showed different affinities for the substrate and selectivities of attack: *T. hirsuta* in particular regio- and stereoselectively reduced the 3-keto group to yield 3α-hydroxy-7,12-diketo-5β-cholan-24-oic acid (7,12-diketolithocolic acid) as the main product. A number of different chemical reductions were carried out on DHCA; among them hydrogenation with Raney Nickel in water under high-intensity ultrasound proved highly regio- and stereoselective, yielding 7,12-diketolithocolic acid exclusively. ^{1}H and ^{13}C resonances were assigned in details thanks to a series of 1D and 2D NMR runs including DEPT, NOESY, H–H COSY, gHSQC and gHMBC.

1. Introduction

Cholic acids and their derivatives hold a great interest for pharmaceutical industry owing to their numerous therapeutic employs, as in cholestatic liver disease and for dissolving cholesterol gallstones [1], to protect hepatocytes and other cell types from apoptosis [2–4] and as antimicrobials [5,6]. With the aim to increase the solubility of cholesterol in the bile a new family of compounds, fatty-acid/bile acid conjugates (FABACs), has recently been synthesized [7–9]. As they are able to prevent the formation of cholesterol gallstones in hamsters and mice, they hold a potential for use in humans.

Dehydrocholic acid (DHCA) (**1**) [10] is usually obtained from cholic acid by oxidation of the C-3, C-7 and C-12 hydroxy groups, a process carried out industrially with sodium hypochlorite under heating. In this way a pure product can also be recovered from complex mixtures of bile acids (leftovers from the industrial production of ursodeoxycholic acid) that would otherwise be useless. DHCA finds therapeutic applications as a cholagogue, hydrocholeretic, diuretic, laxative and as a diagnostic aid. Within 6–12 h of oral administration it acts on the smooth muscle of the intestinal wall to cause a vigorous bowel movement. Besides this laxative effect, it increases the water content and volume of bile. Unlike natural bile acids and their conjugates, DHCA does not readily form with fats and phospholipids the micelles that play an essential role in the absorption of these nutrients.

Considering that DHCA can easily be obtained at low cost, we investigated the possibility of modifying its structure to yield pharmacologically interesting derivatives. Published work has shown that the biotransformation of bile acids has a potential as an aid to synthesis [11–15]. This method has been

* *Corresponding authors*. Tel.: +39 011 6707684; fax: +39 011 6707687.
E-mail address: giancarlo.cravotto@unito.it (G. Cravotto).
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employed to prepare a number of useful cholic acid derivatives [16,17], some of which are impossible or very difficult to obtain by ordinary chemical reactions.

For the present study we chose the basidiomycetes *Trametes hirsuta* and *Collybia velutipes* that in our hands had afforded some interesting regio- and stereoselective modifications of terpene hydrocarbons [18]. In order to ascertain whether biocatalysis would yield DHCA derivatives that cannot be easily obtained by chemical synthesis, we also carried out several chemical reductions on DHCA. Methods employed were catalytic hydrogenation (Pd/C, Raney Nickel) and reduction with $NaBH_4$ [19] or with $HCOO^-$ NH_4^+ under microwaves (MW) [20,21].

2. Experimental

2.1. General procedures

DHCA was provided by PCA S.p.A. (Prodotti Chimici Alimentari, Basaluzzo, AL, Italy).

Other reagents and solvents were from Carlo Erba Reagenti and Acros Organics. Reactions were monitored by TLC on Fluka F_{254} 0.25 mm plates, which were visualized by spraying with molybdic acid and heating. For column chromatography silica gel 60 Merck was used.

IR spectra were recorded on a Shimadzu FT-IR 8001 spectrophotometer. ^1H NMR, ^{13}C NMR, DEPT (distortionless enhancement by polarisation transfer), ^1H–^1H COSY (correlation spectroscopy), NOESY 1D (nuclear Overhauser effect spectroscopy), gHSQC (heteronuclear single-quantum correlation), and gHMBC (heteronuclear multiple bond correlation) spectra were recorded at 399.95 MHz for ^1H, and at 100.58 MHz for ^{13}C with a Varian Mercuryplus 400 instrument in pyridine-d_5, $CDCl_3$ or CD_3OD solutions; chemical shifts (δ) are reported in ppm from tetramethylsilane as secondary reference standard, coupling constants in Hz. Low-resolution mass spectra (LRMS) were taken on a Finnigan-MAT TSQ70 (chemical ionization) with isobutane as the reactant gas. Gas chromatographic analyses were performed on a Shimadzu GC-14B chromatograph equipped with a flame ionization detector. MW-promoted reactions were carried out in a MILESTONE ATC-FO 300 MicroSYNTH oven. Sonochemical reactions were performed in a high-power reactor developed in the authors' laboratory (frequency 19 kHz) [22].

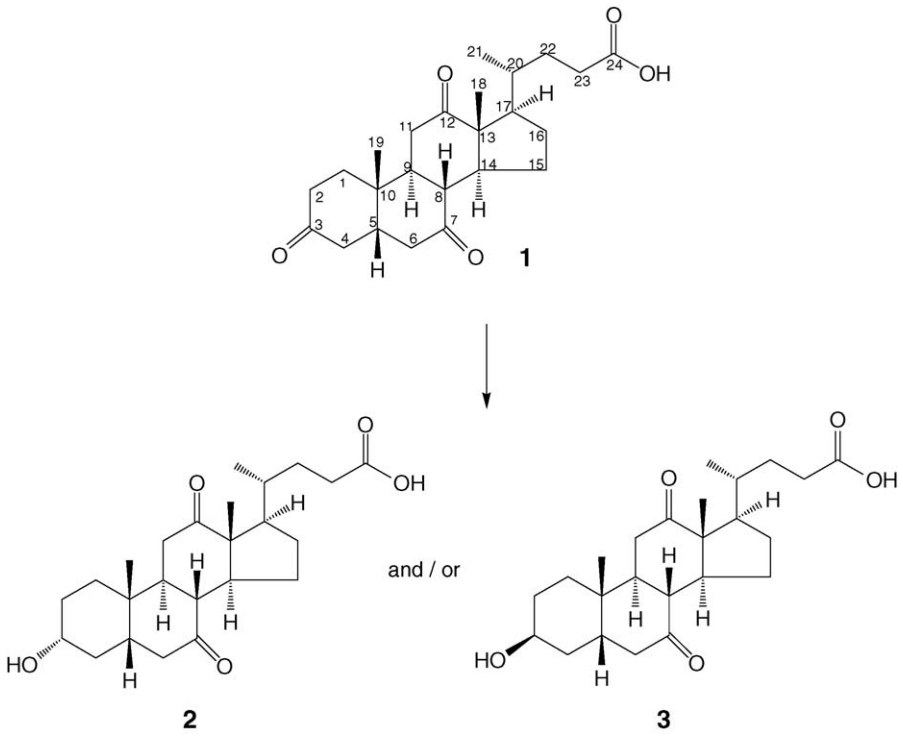
2.2. Mycelia

The basidiomycetes selected for the present study belong to the following families: Tricholomataceae (*C. velutipes*) and Polyporaceae (*T. hirsuta*). Mycelia, obtained from the Department of Plant Biology, University of Turin, Italy, are registered by MUT (Mycotheca Universitatis Taurinensis) with the following MUT accession numbers: *C. velutipes* 3638, *T. hirsuta* 3639.

2.3. Culture, fermentation and incubation procedure

Mycelia were grown on Petri dishes for 14 days at 24 °C to provide precultures. The culture medium contained 20 g glucose, 20 g malt extract and 20 g agar (Merck) per litre of distilled water. pH was not adjusted.

300-ml Erlenmeyer flasks containing 100 ml of liquid culture medium (20 g/l glucose and 20 g/l malt extract) were inoculated with fragmented disks (16 mm in diameter) of mycelia taken from 7-day old precultures.



Scheme 1 – Bioelaboration of DHCA (1) by *Trametes hirsuta* (2, 60%) and *Collybia velutipes* (2, 40% and 3, 20%).

In order to isolate fungal metabolites in sufficient amounts for identification (at least 15 mg), ten 300-ml Erlenmeyer flasks containing fragmented mycelia suspended in 100 ml of liquid medium were inoculated with 50 mg of substrate and kept at 24 °C for 7 days on a rotary shaker (Dubnoff BSD) at 90 rpm. Better yields were obtained in this way rather than using one 1-l Erlenmeyer flask. As DHCA (**1**) is poorly soluble in water, its sodium salt was used for better bioavailability. Each experiment was repeated three times. Blanks without mycelium were run in parallel to rule out the occurrence of spontaneous chemical transformations. Substrate toxicity was evaluated for each mycelium by monitoring the variation of fungal biomass during incubations. Dry weight determinations on 7-day-old cultures showed that mycelial growth was not affected by the presence of DHCA sodium salt (Scheme 1).

2.4. *Extraction and isolation of metabolites*

At the end of incubations, cultures were filtered and filtrates were centrifuged in the cold (7000 rpm for 20 min at 5 °C), then acidified with HCl 1 N and extracted with $CHCl_3$. The solvent was evaporated to yield crude extracts that were chromatographed on a silica gel column with a $CHCl_3$/MeOH gradient. Elution was monitored by TLC, eluent $CHCl_3$/MeOH (9:1).

2.4.1. *3α-Hydroxy-7,12-diketo-5β-cholan-24-oic acid (7,12-diketolithocholic acid)* (**2**)

White solid; mp: 178–180 °C (Lit. mp: 185–187 °C) [23]; IR (KBr) 3350 (OH), 2950, 2864, 1728 (COOH), 1702 (CO), 1699 (CO), 1333, 1263, 1186, 1051, 802 cm^{-1}; ^1H NMR (400 MHz, pyridine-d_5): δ 3.77 (m, 1H, H-3), 2.93 (dd, J = 12.6, 5.5 Hz, 1H, H-6α), 2.90 (pt, J = 12.0 Hz, 1H, H-8), 2.70 (pt, J = 12.6 Hz, 1H, H-11α), 2.65 (m, 1H, H-23), 2.54 (m, 1H, H-23), 2.45 (m, 1H, H-15), 2.37 (m, 1H, H-9), 2.19 (m, 1H, H-17), 2.18 (dd, J = 12.6, 1.8, 1H, H-11α), 2.12 (m, 1H, H-22), 2.04 (dd, J = 12.6, 2.2 Hz, 1H, H-6α), 1.95 (m, 1H, H-16), 1.91 (m, 1H, H-4β), 1.82 (m, 1H, H-5), 1.81 (m, 2H, H-14 and H-2β), 1.64 (m, 1H, H-1α), 1.60 (m, 1H, H-22), 1.56 (m, 1H, H-4α), 1.46 (m, 1H, H-2α), 1.44 (m, 1H, H-20), 1.34 (m, 1H, H-16), 1.26 (m, 1H, H-15), 1.23 (s, 3H, Me-19), 1.13 (m, 1H, H-1β), 1.08 (d, J = 6.8 Hz, 3H, Me-21), 1.00 (s, 3H, Me-18); ^{13}C NMR (100.58 MHz, pyridine-d_5): δ 212.21 (C12), 209.61 (C7), 176.48 (C24), 69.96 (C3), 57.09 (C13), 52.25 (C14), 48.98 (C8), 46.22 (C17), 45.82 (C9), 45.79 (C6), 45.53 (C5), 38.67 (C11), 38.32 (C4), 36.09 (C10), 36.03 (C20), 34.52 (C1), 32.24 (C23), 31.46 (C22), 30.60 (C2), 28.00 (C16), 25.40 (C15), 22.48 (C19), 19.21 (C21), 11.88 (C18). CIMS (m/z): 405 $(M+H)^+$, 387 $(M+H)^+$-H_2O.

2.4.2. *3β-Hydroxy-7,12-diketo-5β-cholan-24-oic acid* (**3**)

White solid; mp 260–262 °C (Lit. mp: 270–272 °C) [24]; IR (KBr) 3400 (OH), 2932, 1728 (COOH), 1708 (CO), 1698 (CO), 1541, 1385, 1103, 1087, 914, 731 cm^{-1}; ^1H NMR (400 MHz, pyridine-d_5): δ 4.13 (m, 1H, H-3), 1.29 (s, 3H, 19-Me), 1.06 (d, J = 8.8 Hz, 3H, 21-Me), 1.02 (s, 3H, 18-Me); ^{13}C NMR (100,58 MHz, pyridine-d_5): δ 212.68 (C12), 210.21 (C7), 176.48 (C24), 65.10 (C3), 23.09 (C19), 19.23 (C21), 11.92 (C18). CIMS (m/z): 405 $(M+H)^+$, 387 $(M+H)^+$-H_2O.

2.5. *$NaBH_4$ reductions of DHCA (general procedure)*

In a 50-ml round-bottomed flask DHCA (200 mg, 0.50 mmol) was dissolved in a sufficient amount of solvent (MeOH, EtOH or $CHCl_3$), and $NaBH_4$ (56.5 mg, 1.49 mmol) was added. The reaction was magnetically stirred for 10 h at room temperature and monitored by TLC using $CHCl_3$/MeOH (9:1) as eluent. The reacted mixture was diluted with H_2O, acidified with 0.5 N HCl and extracted with $CHCl_3$. The organic phase was washed with H_2O and brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. Products were purified by column chromatography using a $CHCl_3$/MeOH elution gradient (99:1 → 8:2).

2.6. *Hydrogenation of DHCA in PARR reactor*

In the reaction vessel (160 ml) of a PARR reactor a solution of DHCA (200 mg, 0.50 mmol) in MeOH (30 ml) and Pd/C 10% (50 mg) were added. The reaction was carried under pressure (H_2, 30 bar) and vigorous stirring at 105 °C for 15 h. The reaction was monitored by TLC, eluent $CHCl_3$/MeOH (9:1). Crude product was purified by column chromatography (gradient 99:1 → 8:2), beside the recovered starting material (94 mg) a diastereomeric mixture of **2** (12%) and **3** (11%) was isolated.

2.7. *Reduction of DHCA under MW*

In a 50-ml two-necked round-bottomed flask DHCA (0.78 g, 1.94 mmol), $HCOO^-$ NH_4^+(0.7 g, 11.10 mmol) and a catalytic amount of Pd/C were added to a mixture of CH_2Cl_2 (10 ml) and propylene glycol (5 ml). The mixture was refluxed 1.5 h under MW irradiation (50 W). The reaction was monitored by TLC ($CHCl_3$/MeOH 8:2). The reacted mixture was filtered on a celite pad over a sintered-glass funnel (G3) and evaporated to dryness. To improve extraction yields the crude product was subjected to Fischer esterification by heating under reflux (3 h) with absolute EtOH and a catalytic *p*-toluenesulfonic acid, then transferred to a separatory funnel and extracted with $CHCl_3$. The organic phase was washed with H_2O and saturated brine, dried over anhydrous Na_2SO_4 and evaporated to dryness yielding 72 mg of ethyl cholate (85%).

2.8. *Hydrogenation of DHCA sodium salt with Raney Nickel*

In a 100-ml two-necked round-bottomed flask equipped with a gas inlet, 100 mg of sodium dehydrocholate were dissolved in 25 ml of water (resulting pH = 8.8), Raney Nickel (20 mg) was added and the mixture was vigorously stirred under H_2 atmosphere (gummy balloon) at 30 °C for 20 h. Under high-intensity ultrasound at the same temperature the reaction time was cut down to 2 h. The reaction was performed under H_2 atmosphere in a 1-mm thick Teflon® tube inserted in a sonochemical reactor (19 kHz, 70 W) thermostatted by two Peltier modules and was monitored by TLC, eluent $CHCl_3$/CH_3OH 9:1. The reacted mixture was filtered, acidified with HCl 1 N and extracted with $CHCl_3$. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. The only product isolated by column chromatography ($CHCl_3$/CH_3OH 97:3) was 3α-hydroxy-7,12-diketocholic acid (**2**) (76 mg, yield = 80%).

2.9. Derivatization and GLC analyses

Esterification (methyl ester): 50 mg of bile acid were dissolved in methanol (1 ml), then methansulfonic acid (80 μl) and anhydrous Na_2SO_4 were added. The mixture was irradiated with MW for 2 min at 50 W. The reacted mixture was diluted with H_2O, brought to alkaline pH with 2 N NaOH and extracted with $CHCl_3$. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. All the methyl esters were obtained in high yield (90–96%).

Silylation: 1 mg of bile acid methyl ester was dissolved in $CHCl_3$ (500 μl) and an excess of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (3 eq.) was added. The mixture was stirred about 1 h at 60 °C, the reaction being monitored by TLC, eluent $CHCl_3/CH_3OH$ mixtures.

The products were used for GC analysis without any further purification.

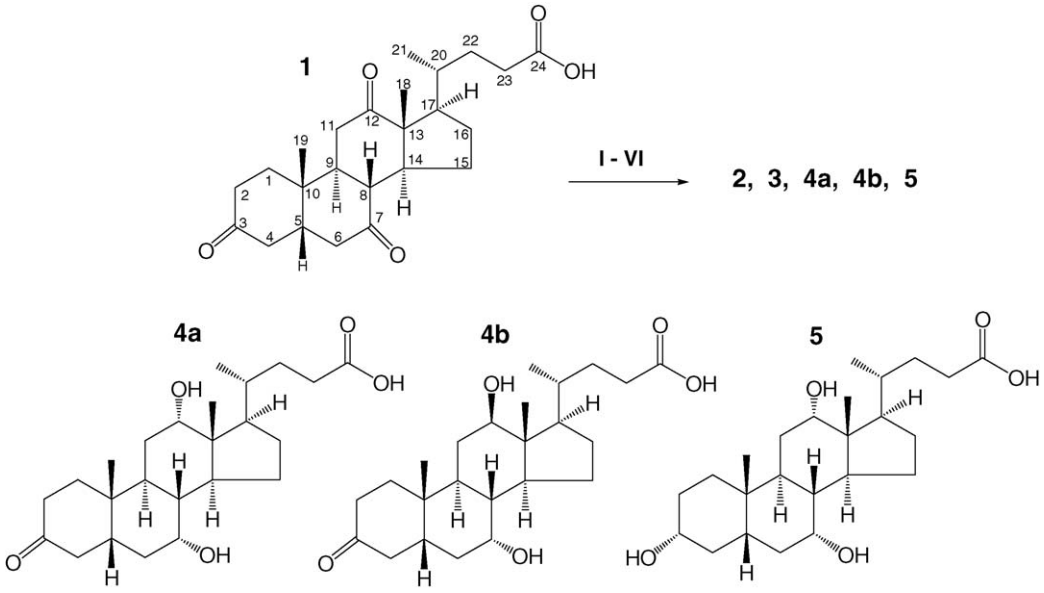
Analyses: the derivatized samples were analyzed by GLC on fused capillary column as previously described by Lepercq et al. [12]. A pool of standards was chromatographed for products identification and quantification.

3. Results and discussion

Biotransformation results for DHCA sodium salt (Scheme 1) showed that both mycelia were able to reduce the keto groups, particularly at the position 3. *T. hirsuta* afforded 3α-hydroxy-7,12-diketo-5β-cholan-24-oic acid (7,12-diketolitocholic acid) (**2**) as main product (about 60% yield). *C. velutipes* showed a lesser stereospecificity (40% yield for **2**), also producing 3β-hydroxy-7,12-diketo-5β-cholan-24-oic acid (**3**) in 20% yield. Bioelaboration by *C. velutipes* led to other products of higher polarity than DHCA, but their amounts were much too low to allow identification.

Results from chemical reductions were very different (Scheme 2). As previously reported by Fantin et al. [19], $NaBH_4$ in MeOH afforded a mixture of 7α,12α-dihydroxy-3-keto-5β-cholan-24-oic acid (**4a**) and 7α,12β-dihydroxy-3-keto-5β-cholan-24-oic acid (**4b**). Using ethanol as solvent we found lesser yields of these compounds while the major product was cholic acid (**5**), as observed when working in $CHCl_3$.

The reduction of DHCA with $HCOO^-$ $NH_4^+$and catalytic Pd/C under MW irradiation [21] rapidly converted it to cholic



Scheme 2 – Chemical reductions of DHCA.

Table 1 – Chemical reduction of DHCA (1) in different conditions

Entry	Reducing agent and conditions	Time (h)	Yield (%)			
			2	4a	4b	5
I	$NaBH_4$, MeOH, Stirring rt	10	–	70	20	–
II	$NaBH_4$, EtOH, Stirring rt	10	–	23	15	42
III	$NaBH_4$, $CHCl_3$, Stirring rt	10	–	19	–	61
IV	Pd/C, MeOH, Parr reactor, 105 °C	15	12[a]	–	–	–
V	$HCOO^-$ NH_4^+, CH_2Cl_2/prop. glycol, MW 50 W rfx	1.5	–	–	–	85
VI	Raney Nickel, H_2O, US 70 W 30 °C	2	80	–	–	–

[a] In mixture with **3** (11%).

acid. Identification of products was done by comparison with published data. Yields and reaction times are listed in Table 1. It is remarkable that hydrogenation of DHCA with Ni/Raney in water proved highly regio- and stereoselective affording **2** in high yield.

Although DHCA is a starting material for the synthesis of 5β-cholanic acid derivatives, no ^{1}H NMR data for this compound exist in the literature with the exception of a single reference for carbon-13 assignments in $CDCl_3$ [25]. Thus, we proceeded to study the proton and carbon NMR spectra in two solvents ($CDCl_3$ and, more conveniently, pyridine-d_5); obviously methanol could not be used because of the reactivity of DHCA.

In order to assign all the ^{1}H and ^{13}C resonances we carried out a series of 1D and 2D NMR experiments runs including DEPT, NOESY, H–H COSY, gHSQC and gHMBC (Table 2).

For our immediate aim, the structural determination of compounds arising from the reduction of DHCA, a sound C=O attribution in the latter was mandatory. Thus, we started from the HMBC spectrum of **1** attributing to the 12-CO the quaternary carbon resonating at δ 211.98 owing to its correlation peak (^3J coupling) with a methyl group (δ 1.02 ppm, singlet) that must be assigned to the 18-Me; the same carbon showed correlations to both protons at position 11 (δ 2.82 and 2.22 ppm, 11-CH_2 at δ 38.92 from HSQC).

As regards the 7- and 3-C=O carbon atoms, we noticed that whereas the signal at higher frequency (δ 209.12 ppm) showed correlations to protons belonging to a CH_2 and a CH group, the signal at δ 208.86 ppm was connected to two CH_2 groups. On this basis the resonances could be attributed to the carbonyl groups at positions 7 and 3, respectively. This attribution was confirmed by the connectivity between the 7-C=O and H-8, easily recognized from its correlation with the previously assigned C-11.

Once enabled to ascertain the regiochemistry of the reduction reaction, we turned to its stereoselectivity. To this end, NOE experiments were carried out irradiating the new 3-, or 7- or 12-H (easily recognizable in the ^{1}H NMR spectrum); they allowed the distinction between α and β epimers of a monoreduced compound by preventive identification of H-5 (for 3-OH) or H-8 (for 7- or 12-OH).

For example, if H-8 has been previously identified in compound **1**, H-5 can be unambiguosly assigned from the gHMBC experiment, as C-5 (CH unit from DEPT spectrum) will show correlations (see Table 2) to both 19-Me and 6-CH_2, thus allowing the attribution. The same holds for the monoreduced compounds **2** and **3**. On these grounds we found that reduction of DHCA sodium salt with *T. hirsuta* afforded 7,12-diketolithocholic acid (**2**) whose stereochemistry (3α-OH) was confirmed by NOE experiments carried out in $CDCl_3$, CD_3OD

Table 2 – ^{1}H and ^{13}C NMR spectral data and HMBC most significant correlations for DHCA (1) (400 MHz)

Carbon	DEPT	^{1}H NMR[a] (δ)		^{13}C NMR (δ)		HMBC[b]	Observed NOEs[c]
		Pyridine-d_5	$CDCl_3$	Pyridine-d_5	$CDCl_3$		
1	CH_2	1.80α; 1.43β	2.00α; 1.65β	35.26	35.26[d]	2	19,1β, 11α, 2; 1α, 19, 5, 2
2	CH_2	2.28 and 2.09	2.29	36.67	36.46[d]		
3	C	–		208.86	209.08	2, 4	
4	CH_2	2.38 and 2.26	2.23 and 2.16	43.11	42.76	6β	
5	CH	2.18	2.37	46.88	46.83[d]	19, 6	
6	CH_2	2.93β (dd, J = 13.0, 5.9); 1.98α (dd, J = 13.0, 2.4)	2.96β (dd, J = 13.1, 6.2); 2.07α	45.22	44.95		19.6α, 5
7	C	–		209.12	208.68	6, 8	
8	CH	3.02 (pt, J = 11.7)	2.93 (pt, J = 12.5)	49.05	48.97[d]	6α, 11α, 9	18, 19, 11β
9	CH	2.57	2.39	45.40	45.54	19, 1β, 11, 8	
10	C	–		36.22	35.99	6α, 9, 8	
11	CH_2	2.82β (pt, J = 12.2); 2.22α	2.88β (pt, J = 12.7); 2.18α	38.92	38.61	9	18, 19, 8, 11α
12	C	–		211.98	211.96	18, 11	
13	C	–		57.16	56.88	18, 14, 17	
14	CH	1.96	1.88	52.06	51.73[d]	18, 8	
15	CH_2	2.45 and 1.32	2.34 and 1.29	25.38	25.11	14, 8	
16	CH_2	1.95 and 1.35	2.08 and 1.38	27.97	27.59		
17	CH	2.20	2.08	46.28	45.62[d]	18, 21	
18	CH_3	1.02	1.11	11.88	11.84	14, 17	8, 11β
19	CH_3	1.29	1.43	21.60	21.89	9	
20	CH	1.46	1.34	36.02	35.46	21, 23	
21	CH_3	1.08 (d, J = 6.8)	0.89 (d, J = 6.6)	19.19	18.58	22	
22	CH_2	2.13 and 1.59	1.90 and 1.46	31.40	30.18[d]	21, 23	
23	CH_2	2.64 and 2.57	2.48 and 2.34	32.17	31.11[d]	22	
24	C	–		176.34	179.22	22, 23	

[a] ^{1}H directly attached to ^{13}C as determined from HSQC experiment.
[b] ^{1}H–^{13}C long range correlation (HMBC) corresponding to two- or three-bond connectivities.
[c] Through-space interactions.
[d] Revised assignment with respect to literature data [25].

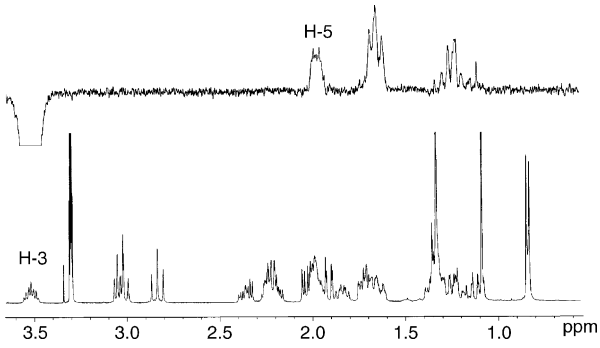


Fig. 1 – NOESY 1D (CD_3OD) experiments proving the α position of 3-OH in compound 2.

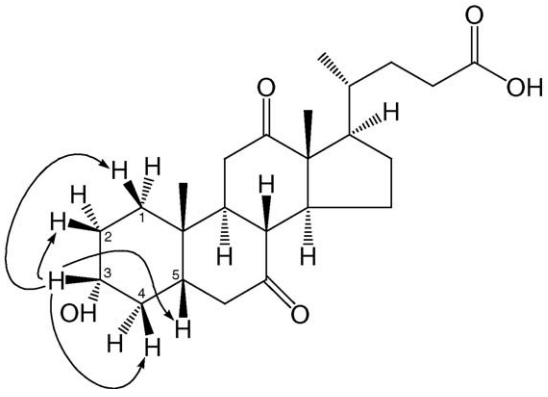


Fig. 2 – Compound 2: NOE effects after irradiation of H-3 in $CDCl_3$, CD_3OD and pyridine-d_5.

or pyridine-d_5 irradiating H-3 and observing a positive NOE effect on H-5 (Fig. 1). NOE effects after irradiation of H-3 in three different solvents are reported in Fig. 2.

In conclusion, our results show that biotransformation by *C. velutipes* and hydrogenation with Raney Nickel are both effective procedures for the regio- and stereoselective reduction of the 3-keto function of bile acids. Compounds obtained in this way could be used as intermediates for selective functionalization of the position 3, as in FABACs, leading to new molecules that may be used for the prevention and treatment of cholesterol gallstone disease.

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