Steroids 74 (2009) 483–486

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Biotransformation of methyl cholate by *Aspergillus niger*

Amal Al-Aboudi[a,*], Mohammad Y. Mohammad[b], Salim Haddad[a], Rawhi Al-Far[a,c], M. Iqbal Choudhary[b], Atta-ur-Rahman[b]

[a] *Chemistry Department, University of Jordan, 1194 Amman, Jordan*
[b] *H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan*
[c] *Faculty of Science and IT, Al-Balqa'a Applied University, Salt, Jordan*

ARTICLE INFO

Article history:
Received 5 September 2008
Received in revised form
23 December 2008
Accepted 6 January 2009
Available online 17 January 2009

Keywords:
Biotransformation
Methylcholate
Aspergillus niger
Methyl 3α,7α,12α,15β-tetrahydroxy-5β-cholan-24-oate
Methyl 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oate

ABSTRACT

Biotransformation of methyl cholate using *Aspergillus niger* was investigated. This led to the isolation of two derivatives: methyl 3α,7α,12α,15β-tetrahydroxy-5β-cholan-24-oate identified as a new compound, and a known compound methyl 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oate. The structure elucidation of the new compound was achieved using 1D and 2D NMR, MS and X-ray diffraction.

1. Introduction

Cholic acid is one of the main components of bile acids in humans. Besides their importance as end products of cholesterol metabolism, bile acids are essential in the digestion and re-sorption of fat, fatty acids and lipid-soluble vitamins. Cholic acid and its derivatives have received a great interest in pharmaceutical industry [1], due to their numerous therapeutic applications in cholestatic liver disease [2] and in dissolution of cholesterol gallstones, thus avoiding the need for surgery [3]. In addition to their applications in pharmaceutical industry, they have been used as chiral templates in asymmetric synthesis [1] and have been observed to act as organogelators, offering potential applications, not only in medicine, but also in cosmetics, material sciences and environmental clean up [1].

Microbial transformation of bile acids has been used in the preparation of pharmaceutically significant compounds [4]. In particular, it has been successfully used to enhance the selectivity and to reduce the number of preparation steps. Biotransformation is considered as the method of choice for selective oxidation of the hydroxy groups at C-3, C-7 or C-12 [5]. The method is also used for epimerization of the hydroxy groups at C-3 [6] and C-7 [7]. Regio- and stereo-specific hydroxylation of methylene carbons of bile acids, an inaccessible reaction by chemical means, can be achieved in one step by using microbial transformation [4].

Although there are many studies describing biotransformation of cholic acid and many of its derivatives using bacterial and fungi species, most of the studies employed bacterial strains [4]. Only few studies were reported on biotransformation of cholic acid by Fungal species. Carlstrom et al. reported that biotransformation of cholic acid using Fungal species produced traces of hydroxylated metabolites [8], while other bile acid derivatives (i.e. lithocholic, deoxycholic, chenodeoxycholic and ursodeoxycholic acid) gave good yields of different hydroxylated metabolites [9,10].

In this study, the ability of the *Aspergillus niger* to transform methylcholate has been investigated for the first time. Two metabolites, a new and a known one, have been isolated and characterized by different spectroscopic and X-ray diffraction techniques.

2. Experimental

2.1. General methods

Methylcholate was purchased from Merck. Silica gel (70–230 mesh, Merck) was used for column chromatography (CC). Pre-coated silica gel plates (Merck, F254; 20 × 20, 0.25 mm

* Corresponding author. Tel.: +962 6 5355000/22022; fax: +962 6 5355560.
E-mail address: alaboudi@ju.edu.jo (A. Al-Aboudi).

0039-128X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2009.01.002

Table 1
^{1}H NMR and ^{13}C NMR data for compounds **1**–**3**.[a].

Position	Compound **1**		Compound **2**		Compound **3**	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	0.99, 1.81	35.35	0.99, 1.82	35.18	1.30, 1.75	37.45
2	1.45, 1.59	30.38	1.46, 1.60	29.80	2.35[b]	31.06[b]
3	3.41	71.92	3.40	71.51	3.57	70.86
4	1.66, 2.29	39.59	1.65, 2.30	39.14	1.34, 2.48	39.48
5	1.42	41.71	1.43	41.78	1.60	46.61
6	1.53, 1.95	34.68	1.52, 2.02	34.40	2.85 dd (J = 5.5, 13.2), 2.02	45.19
7	3.94	68.49	4.12	67.15	–	213.71
8	1.80	39.59	1.85	34.57	2.72 ψt J = 12	45.59
9	2.25	26.42	2.32	26.25	2.35	35.88
10	–	34.78		35.24	–	34.56
11	1.58	28.19	1.58	27.90	1.60, 1.70[c]	29.40[c]
12	3.81	73.10	3.85	73.26	3.95	72.54
13	–	46.46	–	47.12		46.48
14	2.00	41.51	1.82	46.16	1.90	45.41
15	1.12, 1.76	23.25	4.30 t, J = 5.8	69.05	1.76, 1.20[c]	29.66[c]
16	1.32, 1.90	27.52	1.35, 2.38	40.46	1.20, 1.34[c]	29.74[c]
17	1.86	47.05	1.82	46.46	1.60	46.61
18	0.65	12.51	0.91	14.31	0.88	14.67
19	0.86	22.48	0.93	21.81	1.22	22.74
20	1.43	35.29	1.81	34.74	1.58	34.51
21	0.95 d, J = 5.9	17.34	0.98 d, J = 6.4	16.36	0.94 d J = 6.3	17.45
22	1.35, 1.79	30.95	1.80	30.84	1.19, 1.85	34.11
23	2.21, 2.37	31.12	2.32	30.43	2.25[b]	30.82[b]
24	–	174.86	–	175.00	–	174.72
25	3.64	51.53	3.68	50.70	3.65	51.64

[a] Assignments were based on 2D NMR spectra (COSY, HMBC and HMQC).
[b] Exchangeable assignments.
[c] Exchangeable assignments.

and 0.50 mm) were used for TLC. Spots were detected by spraying with anisaldehyde/sulfuric acid, followed by heating at 140 °C. ^{1}H, ^{13}C and 2D NMR spectra ($CDCl_3$ solutions) were recorded using Bruker Avance-300 or Bruker Avance-500 spectrophotometers. Chemical shifts (δ) are reported in ppm with TMS as an internal standard, and coupling constants (J) in Hz. EIMS were recorded on Jeol JMS-600H mass spectrometer. High resolution mass spectra (HRMS) were measured in positive ion mode by electrospray ionization (ESI) technique on a Bruker APEX-4 instrument. The samples were dissolved in acetonitrile, and infused using a syringe pump with a flow rate of 2 μL/min. External calibration was conducted using Arginine cluster in a mass range m/z 175–871. All reagents used were of analytical grades.

2.2. Organism

Cultures of *A. niger* were obtained from the American Type Culture Collection (ATCC 10549). All cultures were grown on Sabouraud dextrose agar (SDA) and stored at 4 °C.

2.3. Incubation of methylcholate (*1*)

A. niger was grown in shake cultures at 30 °C in two conical flasks (250 mL), each containing 100 mL of a sterile medium comprising of (per dm^3) glucose (10 g), peptone (5 g), KH_2PO_4 (5 g), yeast extract (5 g), glycerol (5 mL), and NaCl (5 g). The medium solution was adjusted to pH 5.5. The medium was sterilized by autoclaving at 121 °C for 15 min. Incubations were initiated by suspending the surface growth from slants in sterile medium. After 2 days of incubation in the above-described medium, methylcholate (**1**) (0.08 g), dissolved in 2 ml of acetone, was equally distributed in the conical flasks containing the medium. Culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions but without the substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and were incubated under the same conditions. The first metabolite was detected by TLC after 32 h of incubation, and its amount continued to increase with the progress of fermentation. The second metabolite was detected after 104 h.

The experiment was repeated under the same conditions using 23 conical flasks and 1.0 g methylcholate, dissolved in 23 ml acetone. After 5 days, the fermentation products were filtered and extracted three times with ethyl acetate. The residual mycelium was washed with methanol. The combined ethyl acetate and methanol solutions were dried over Na_2CO_3 and evaporated under vacuum to yield 3.4 g of a gummy mixture.

2.4. Isolation of metabolites

The mixture was chromatographed on a column containing 160 g silica gel, packed in ethyl acetate/benzene (75:25, v/v). The polarity of the eluent was increased gradually to pure ethyl acetate then to 10:90 (v/v) methanol/ethyl acetate. Fractions collected from the column were further purified either by crystallization or by thin layer chromatography. This led to the isolation of two compounds; methyl 3α,7α,12α,15β-tetrahydroxy-5β-cholan-24-oate (**2**, 420 mg) and methyl 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oate (**3**, 50 mg).

2.5. Spectral data of the isolated compounds

The ^{1}H NMR and ^{13}C NMR data of compounds **1-3** are summarized in Table 1. 2D-experiments (COSY, HMBC and HMQC) were used to assign the chemical shifts of compounds (Fig. 1).

2.6. Crystallographic data of compound *2*

The structure of compound **2** was unambiguously determined by single-crystal X-ray diffraction technique. Compound **2** was recrystallized from acetonitrile. A colorless crystal with dimensions 0.5 mm × 0.4 mm × 0.1 mm was selected for the crystallographic measurements. $C_{25}H_{42}O_6$. C_2H_3N (F.W. 479.64), monoclinic with

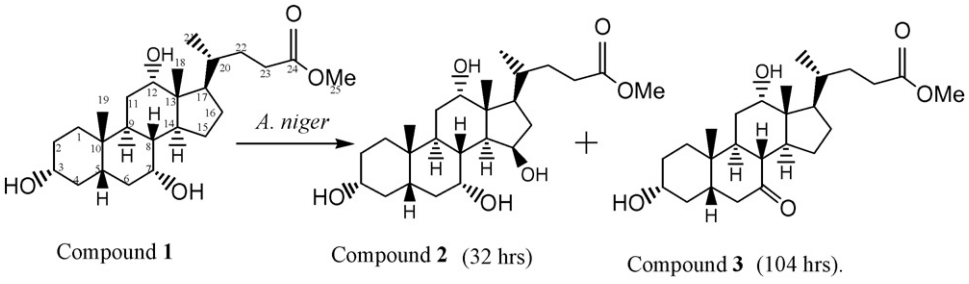

Fig. 1. Biotransformation of methyl cholate **1** using *A. niger*.

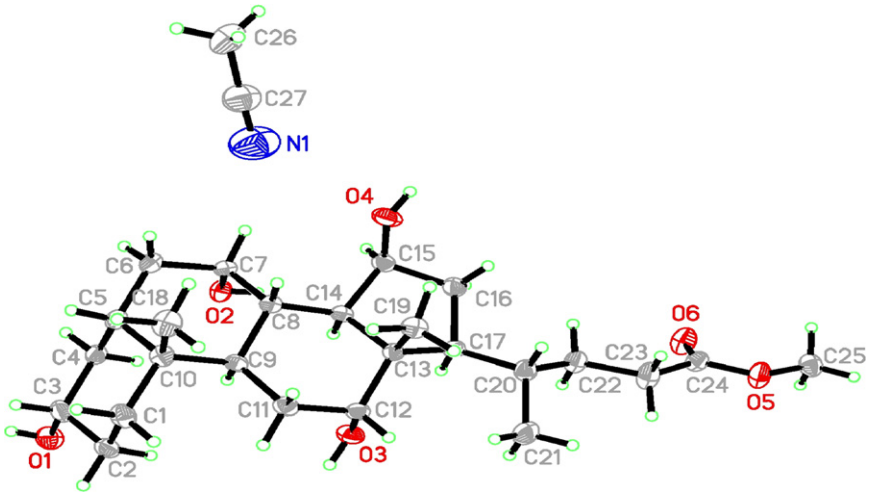

Fig. 2. ORTEPII program plot of compound **2** unit with thermal ellipsoids at 30% probability.

Table 2
Crystal data and structure refinement for 'Methyl 3 α,7α,12 α,15β-tetrahydroxy-5β-cholan-24-oate.acetonitrile'.

Identification code	RF-BAU	
Empirical formula	C27 H45 N O6	
Formula weight	479.64	
Temperature	293(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	$P2_1$	
Unit cell dimensions	$a = 9.756(2)$ Å	$\alpha = 90^\circ$
$b = 8.3630(17)$ Å	$\beta = 97.16(3)^\circ$	
$c = 16.242(3)$ Å	$\gamma = 90^\circ$	
Volume	1314.8(5) Å^3	
Z	2	
Density (calculated)	1.212 Mg/m^3	
Absorption coefficient	0.084 mm^{-1}	
$F(000)$	524	
Crystal size	0.2 mm × 0.15 mm × 0.15 mm	
Theta range for data collection	2.53–27.87°	
Index ranges	$-12 \le h \le 12$, $-7 \le k \le 10$, $-21 \le l \le 21$	
Reflections collected	5853	
Independent reflections	5369 [R(int) = 0.0322]	
Completeness to theta = 27.87°	98.5%	
Absorption correction	Numerical	
Max. and min. transmission	0.988 and 0.982	
Refinement method	Full-matrix least-squares on F^2	
Data/restraints/parameters	5369/1/307	
Goodness-of-fit on F^2	0.980	
Final R indices [$I > 2\backslash S(I)$]	$R1 = 0.0456$, $wR_2 = 0.1110$	
R indices (all data)	$R1 = 0.0587$, $wR_2 = 0.1194$	
Absolute structure parameter	1.0(9)	
Largest diff. peak and hole	0.201 and −0.245 eÅ^{-3}	

space group = $P2_1$, $a = 9.756(2)$ Å, $b = 8.3630(17)$ Å, $c = 16.242(3)$ Å, $\beta = 97.16(10)^\circ$, $V = 1314.8(5)$ Å^3, $Z = 2$, $D_{calc} = 1.212$ mg/m^3, $F(000) = 524$, Mo Kα = 0.71073 Å. Unit cell dimensions were determined by least squares fit of 556 reflections. Diffractometer used Mercury CCD (2 × 2 bin mode) and the data acquired with Crystal Clear software [11]. The intensity data within (θ) range of 2.53–27.87° were collected at 293(2) K. A total of 5369 reflections were collected, of which 4295 reflections were judged observed on the basis of $I > 2\sigma(I)$. The structure was solved by the direct methods with all non-hydrogen atoms were refined anisotropically by a full-matrix least-square calculation on F^2 using the SHELXTL V5.03 package [12]. Hydrogen atoms were constrained to side on their parent atoms with 1.2 times Ueq of the parent atom. The final factors were $R_1 = 0.0456$ and $wR_2 = 0.1110$. Fig. 2 is an ORTEPII program plot of the molecular unit with thermal ellipsoids at 30% probability. Crystallographic data for compound **2** is presented in Table 2.

2.7. Supplementary material

CCDC contains supplementary crystallographic data. This data can be obtained free of charge via http://www.ccdc.cam.ac.uk/data_request/cif, by e-mailing data_request@ccdc.cam.ac.uk, or by contacting the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB2 1EZ, UK. Fax: +44 1223 336033.

3. Results and discussion

Screening scale experiments have shown that *A. niger* was capableScreening scale experiments have shown that *A. niger* was capable of converting methylcholate (**1**) into two metabolites. Large

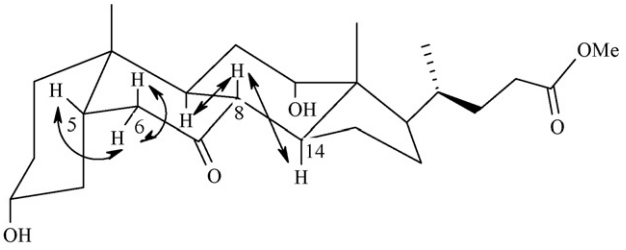

Fig. 3. Key COSY correlations in compound **3**.

scale fermentation was thus carried out to produce sufficient quantities of the metabolites for structure elucidation (Fig. 1). Two sets of controls were used to ensure the authenticity of metabolites. Metabolites were isolated from the culture medium by successive ethyl acetate extraction. The extract was evaporated under vacuum. The residue was fractionated by silica gel column chromatography.

Methyl 3α,7α,12α,15β-tetrahydroxy-5β-cholan-24-oate (**2**) was isolated as a colorless solid from the silica gel column. EIMS of compound **2** showed a weak molecular ion peak at *m*/*z* 438 ($C_{25}H_{42}O_6$), 16 u higher than the substrate, while the base peak was at 402, which may result from the loss of two water molecules. High resolution mass spectrometry showed a molecular ion peak at *m*/*z* 461.290753 corresponding to the formula $C_{25}H_{42}O_6$ + Na (cal. 461.287360). Comparing the NMR spectral data of compound **2** with that of the parent compound **1** suggested that an extra hydroxy group was present either at C-15 or C-16 as the two upfield CH_2's at δ 23.2 and 27.5 characteristic for C-15 and C-16 in methylcholate were absent and all chemical shifts of ring D were shifted downfield. The downfield shift of 7β-H ($\Delta\delta$ = 0.18 ppm) indicated hydroxylation at C-15. Moreover, the downfield shift of the 18-methyl signal ($\Delta\delta$ = 0.26 ppm) suggested β-hydroxylation at ring D due to the pseudo 1,3-diaxial relationship between the methyl and the hydroxy group, whereas such a shift is not observed in the case of α-hydroxylation [13]. In addition the ^{1}H and ^{13}C NMR spectra of compound **2** are in good agreement with those reported [13] for methyl 3α,7α,15β-trihydroxy-5β-cholan-24-oate.

The 2D NMR techniques (COSY-45, HMQC and HMBC) supported hydroxylation at C-15. The COSY spectrum showed cross peaks between H-14 (δ 1.82) and H-15 (δ 4.30) and between H-15 (δ 4.30) and H-16 (δ 2.38). The HMBC spectrum showed interactions between H-15 (δ 4.30) and C-14 (δ 46.16); H-17 (δ 1.82) and C-16 (δ 40.46); H_3-21 (δ 0.98) and C-17 (δ 46.46). The position and stereochemistry of hydroxylation were finally confirmed with single-crystal X-ray diffraction (Fig. 2). All the bond angles and bond lengths were found to be in the normal range. The hydroxyl group at C-15 position was found to be β-oriented with respect to C-3, C-7 and C-12 hydroxy substituents. It is worth mentioning that 15β-hydroxylation was not reported in the biotransformation of any of the bile acids using *A. niger*, but it was reported from microbiological transformation of lithocholic acid (3α-hydroxy-5β-cholanic acid) by *Cunninghamella* sp. [14], and deoxycholic acid by *Pencillium* sp. [8].

Methyl 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oate (**3**) was also isolated by column chromatography and purified by TLC. Oxidation of one of the hydroxy groups to a keto group was inferred from the presence of two C**H**OH moieties, instead of three, in the ^{1}H NMR spectrum of compound **3**, resonating at 3.57 and 3.95. Also, ^{13}C NMR spectrum revealed the presence of an extra carbonyl group at δ 213.17. The position of the oxidation was assigned at C-7 on the basis of the splitting pattern in the region 2.7–2.9 ppm. It is well known that oxo-hydroxy bile acids exhibit clear and specific signals in this region, due to the deshielding effect of the carbonyl group on the near-by protons. Compound **3** showed a pseudo triplet at δ 2.72 and a doublet of doublet at δ 2.85. The upfield pseudo triplet was assigned to H-8 as it was coupled with the two vicinal axial protons at C-9 (δ 2.35) and C-14 (δ 1.90) with coupling constants very close in value (Fig. 3). The downfield doublet of doublet was assigned to the axial proton at C-6. The observed splitting is the result of two coupling constants; a larger one with the geminal proton (δ 2.02) and a smaller coupling constant is with the equatorial proton at C-5 (δ 1.60) [15]. The data were in good agreement with the reported values of 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic [15]. Compound **3** is believed to be a direct product of 7α oxidation of compound **1**, indicating the presence of 7α-hydroxysteroid dehydrogenase (7α-HSDH) in *A. niger*, so this organism can be used to convert methyl cholate to the corresponding 7-oxo derivative in a high regio-specific manner. There are many publications describing using either hydroxysteroid dehydrogenases [16] or whole cell biotransformations that regio-specifically oxidize cholic acid at each of the three possible positions. Most of the microorganisms reported are anaerobic bacteria [4,5]. Moreover, Bovara et al showed that cholic acid has a much lower affinity for 12α-HSDH than 7α-HSDH [17], which might be the reason for not obtaining the 12-oxo methyl cholate in this study. It is worth noting that compound **3** did not react with 7α-HSDH. A possible explanation is the presence of 15β-hydroxy that prevents the steroid from properly fitting in the enzyme binding site.

Acknowledgements

The first author would like to thank the Deanship of Scientific Research at the University of Jordan for financial support. Thanks are also due to Al-Balqa' Applied University, Salt, Jordan for the use of the X-ray diffractometer.

References

[1] Virtanen E, Kolehmainen E. Use of bile acids in pharmacological and supramolecular applications. Eur J Org Chem 2004;16:3385–99.
[2] Colombo C, Setchell KDR, Podda M, Curcio L, Ronchi M, Giunta A. The effects of ursodeoxycholic acid therapy in liver disease associated with cystic fibrosis. J Pediatr 1990;117:482–9.
[3] Crosignani A, Setchell KDR, Invernizzi P, Larghi A, Rodrigues CMP, Podda M. Clinical pharmacokinetics of therapeutic bile acids. Clin Pharmacokinet 1996;30:333–58.
[4] Bortolini O, Medici A, Poli S. Biotransformations on steroid nucleus of bile acids. Steroids 1997;62:564–77.
[5] Fantin G, Ferrarini S, Medici A, Pedrini P, Poli S. Regioselective microbial oxidation of bile acids. Tetrahedron 1998;54:1937–42.
[6] Canzi E, Maconi E, Aragozzini F, Ferrari A. Cooperative 3-epimerization of chenodeoxycholic acid by *Clostridium innocuum* and *Eubacterium lentum*. Curr Microbiol 1989;18:335–8.
[7] Hirano S, Masuda N. Epimerization of the 7-hydroxy group of bile acids by the combination of two kinds of microorganisms with 7α- and 7β-hydroxysteroid dehydrogenase activity, respectively. J Lipid Res 1981;22:1060–8.
[8] Carlstrom K, Kirk D, Sjovall J. Microbial synthesis of 1β- and 15β-hydroxylated bile acids. J Lipid Res 1981;22:1225–34.
[9] Hayakawa S. Microbial transformation of bile acids, a unified scheme for bile acid degradation and hydroxylation of bile acids. Zeitschrift Allgemeine Mikrobiologie 1982;22:309–26.
[10] Sawada H, Kulprecha S, Nilubol N. Microbial production of ursodeoxycholic acid from lithocholic acid by *Fusarium equiseti* M41. Appl Environ Mirob 1982;44:1249–52.
[11] Rigaku. Crystal clear. Rigaku Corporation, Tokyo, Japan; 2000.
[12] Bruker, SHELXTL (including XPREP, XCIF, XL, XP, XS) Version 6.10. Bruker AXS Inc., Madison, WI, USA; 2001.
[13] Iida T, Hikosaka M, Kakiyama G, Shiraishi K, Schteingart C, Hagey L, et al. Potential bile acid metabolites. 25. Synthesis and chemical properties of stereoisomeric 3α,7α,16- and 3α,7α,15-trihydroxy-5β-cholan-24-oic acids. Chem Pharm Bull 2002;50:1327–34.
[14] Bettarello L, Bortolini O, Fantin G, Guerrini A. Mixed oxo-hydroxy bile acids as actual or potential impurities in ursodeoxycholic acid preparation: a ^{1}H and ^{13}C NMR study. Il Farmaco 2000;55:51–5.
[15] Kulprecha S, Nihira T, Shimomura C, Yamada K, Nilubol N, Yoshida T, et al. 15β-Hydroxylation of lithocholic acid by *Cunninghamella* sp. Tetrahedron 1984;40:2843–6.
[16] Riva S, Bovara R, Pasta P, Carrea G. Preparative-scale regio- and stereospecific oxidoreduction of cholic acid and dehydrocholic acid catalyzed by hydroxysteroid dehydrogenases. J Org Chem 1986;51:2902–6.
[17] Bovarar R, Carrea G, Riva S, Secundo F. A new enzymatic route to the sun thesis of 12-ketoursodeoxycholic acid. Biotechnol Lett 1996;18:305–8.