ELSEVIER

www.elsevier.nl/locate/farmac

Il Farmaco 55 (2000) 51–55

IL FARMACO

Mixed oxo-hydroxy bile acids as actual or potential impurities in ursodeoxycholic acid preparation: a ^{1}H and ^{13}C NMR study

Lisa Bettarello, Olga Bortolini, Giancarlo Fantin *, Alessandra Guerrini

Dipartimento di Chimica, Università di Ferrara, via Borsari 46, I-44100 Ferrara, Italy

Received 19 July 1999; accepted 28 October 1999

Abstract

Some distinctive unprecedented ^{1}H NMR signals and the complete ^{13}C NMR resonances are assigned for the entire set of mixed oxo-hydroxy bile acid isomers, obtained by selective oxidation of the hydroxy groups at positions (3,7), (3,12) and (3,7,12) of chenodesoxycholic acid, desoxycholic acid and cholic acid, respectively. Partially or totally oxidized products are the major actual or potential impurities formed during the preparation of the pharmaceutically active ursodeoxycholic and chenodeoxycholic acids.

Keywords: Bile acids; Nuclear magnetic resonance; ^{1}H NMR; ^{13}C NMR

1. Introduction

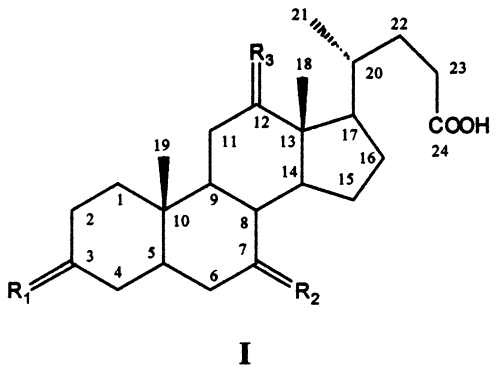
Chenodeoxycholic acid (structure **I** of Fig. 1, $R_1 = R_2 = \alpha$-OH, $R_3 = H$) and especially its 7-hydroxy epimer ursodeoxycholic acid (**I**: $R_1 = \alpha$-OH, $R_2 = \beta$-OH, $R_3 = H$) are widely used as therapeutic agents for the dissolution of cholesterol gallstones [1,2] and in the therapy of bile reflux gastritis [3]. Moreover, the recent introduction of ursodeoxycholic acid for the treatment of cholestatic liver diseases [4–6] is emerging as a powerful new approach for primary biliary cirrhosis [4], primary sclerosis cholangitis [5] and cystic fibrosis [6]. Both cheno- and ursodeoxycholic acids are prepared on a large scale from raw, low-cost materials with a high bile acid content. In particular, bovine bile, upon alkaline hydrolysis to release the free bile acids, is the most commonly employed biological material. Its major component is cholic acid (**I**: $R_1 = R_2 = R_3 = \alpha$-OH), which is subsequently used as starting reagent for the synthesis of the two pharmaceutical active compounds. The synthetic protocols at hand involve a sequence of chemical and/or biochemical transformations, mainly based on oxidation and reduction steps [7]. In order to control these oxido-reductions and monitor the presence of related by-products, rapid, precise and simple analytical techniques are required. In this respect, the successful application of chromatographic methods (GC, HPLC) [8,9] coupled with suitable detectors (MS, ELSD) [8,10] has been exhaustively demonstrated. In particular cases, however, the complexity of the molecules under examination requires a detailed structural analysis that, in the present case, has been obtained by NMR spectra. NMR spectroscopy represents one of the most important methods for the determination of steroid structure, particularly concerning stereochemical problems.

In this paper we report and discuss some diagnostic and distinctive unpublished ^{1}H NMR signals, together with the complete ^{13}C chemical shift correlation, for the entire set of mixed oxo-hydroxy bile acid, having α-hydroxyl and/or carbonyl groups at position 3, and/or 7, and/or 12 (Fig. 1). Partially or totally oxidized products are, in fact, the major actual or potential impurities formed during the preparation of these pharmaceutically active compounds. Comparisons between experimental and calculated ^{13}C chemical shift values are also discussed.

* Corresponding author. Tel.: +39-0532-291 173; fax: +39-0532-240 709.

E-mail address: fnn@dns.unife.it (G. Fantin)

0014-827X/00/$ - see front matter
PII: S0014-827X(99)00121-4



I

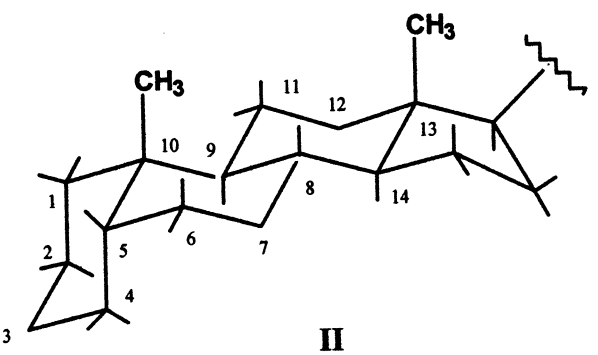
	R1	R2	R3
1	H,H	H,H	H,H
2	O	α-OH,H	H,H
3	α-OH,H	O	H,H
4	O	H,H	α-OH,H
5	α-OH,H	H,H	O
6	O	α-OH,H	α-OH,H
7	α-OH,H	O	α-OH,H
8	α-OH,H	α-OH,H	O
9	O	O	α-OH,H
10	O	α-OH,H	O
11	α-OH,H	O	O

Fig. 1.

2. Results and discussion

The ^{1}H NMR spectra of bile acids, structure **I**, are typified in two major zones [11] e.g. the region 0.5–2.5 ppm or the methylene–methine envelope, due to overlapping resonance peaks of the perhydrophenanthrene ring protons and the region 3–5 ppm formed by the resonances of protons geminal to hydroxy functions, i.e. R_1, R_2, $R_3 = H$, OH. The signals of the angular methyls 18-CH_3, 19-CH_3 and the chain methyl 21-CH_3, occurring in the methylene–methine envelope, are the most distinctive feature of these derivatives, since they are affected by the pattern of substitution of the steroid molecule [12]. Oxo-hydroxy bile acids, however, exhibited additional, very clear and specific signals in the range 2.5–3.6 ppm, which may be explained on the basis of the deshielding effect induced by the carbonyl groups on selected adjacent protons [13,14]. As shown in Fig. 2 and reported in Table 1, for compounds **2**–**11**, the specific resonances always take the form of a doublet of doublets, becoming pseudo triplets (ψt) when the two coupling constants are close in value. The chemical shifts, the multiplicity of the signals and the coupling constants with geminal and adjacent nuclei pertaining to the specific resonances are equally included in Table 1. The updated resonance assignments have been obtained by ^{13}C selective proton decoupling experiments [15] with the aid of the complete interpretation of the ^{13}C NMR spectra of **2**–**11**, here reported and discussed as well. Despite the importance of these compounds as potential metabolites, in fact, the comprehensive assignment of ^{13}C NMR resonances of mixed oxo-hydroxy bile acids has never been published.

For the 3-oxo compounds **2**, **4** and **6** only one proton is detected in the region 2.5–3.6 ppm corresponding to the axial proton bond to C-4. Its multiplicity takes the form of a pseudo triplet in virtue of the coupling with the geminal proton and with the vicinal proton bond to C-5, structure **II** [16]. In this case, the value of the two coupling constants is similar ca. 14 Hz, in agreement with what usually found for $^2J_{\text{geminal}}$ and $^3J_{\text{axial/axial}}$ in ring systems [14] and thus supporting the axial configurational assignment. A similar behavior is observed in the ^{1}H NMR spectra of the 12-oxo derivatives **5** and **8**, where the axial proton at C-11 caused the peculiar resonance. The pseudo triplet is explained as overlapping of two doublets with almost equal coupling constants (ca. 12 Hz): a $^2J_{\text{geminal}}$ and a $^3J_{\text{axial/axial}}$, caused by the proton at C-9.



II

Unlike the two previous cases, the 7-oxo bile acids **3** and **7** showed two well-defined signals in the analyzed region: a pseudo triplet and a doublet of doublets. The upfield pseudo triplet is attributable to the proton at C-8, whose multiplicity is generated by the two vicinal *trans* diaxial protons at C-9 and C-14 with coupling constants very close in value, whereas the downfield resonance is a canonical doublet of doublets pertaining to the axial proton at C-6. The observed splitting is the result of two specific coupling constants: a larger one due to the geminal proton and a smaller one caused by the equatorial proton at C-6 [17].

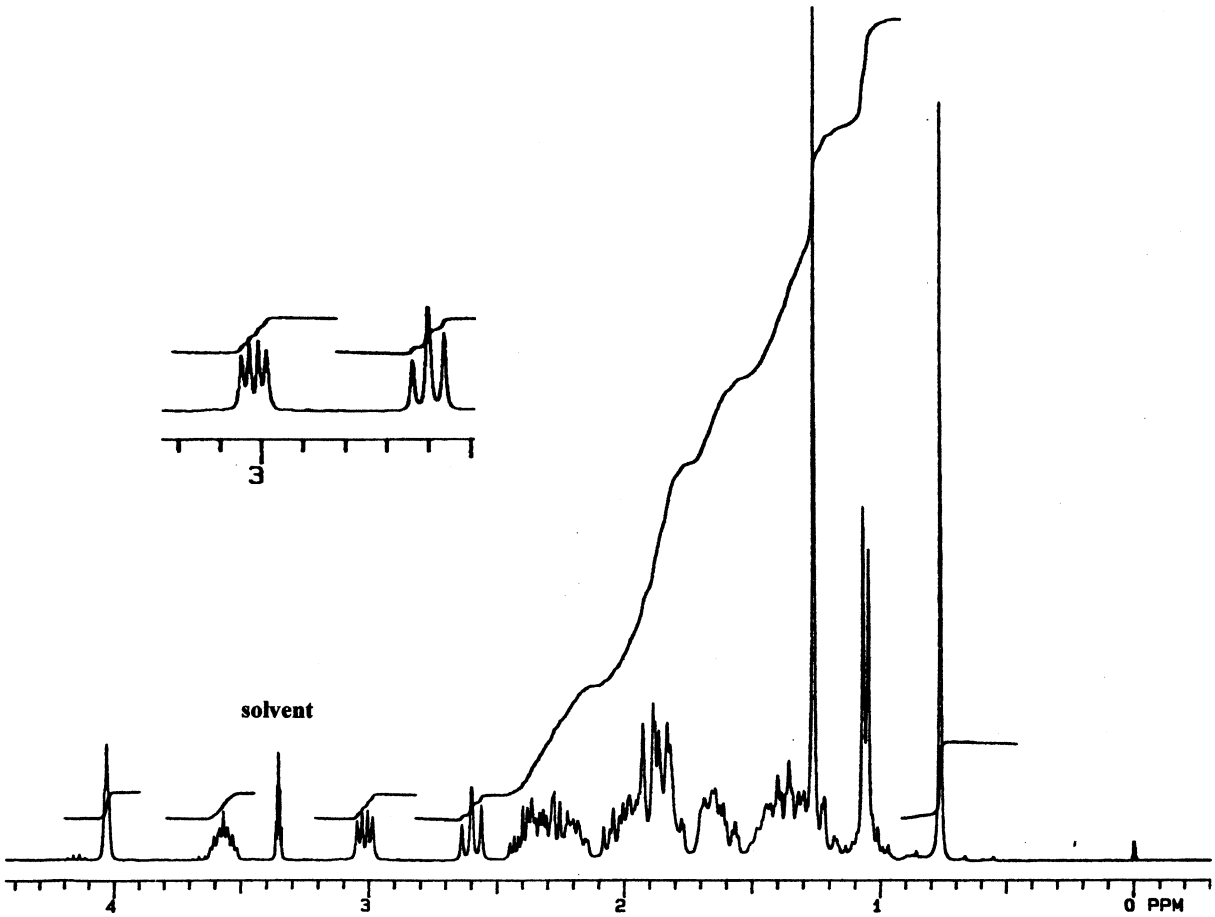


Fig. 2. ^{1}H NMR spectrum of 3α, 12α-dihydroxy-7-oxo-5-β-cholan-24-oic acid **7**, in CD_3OD. Region 2.5–3.2 ppm in the inset.

The analysis of the 2.5–3.6 ppm region in the ^{1}H NMR spectra of the dioxo bile acids **9**–**11** reserved a peculiar and unexpected behavior related to the fact that only with the 3,12- and 7,12-dioxo compounds **10**, **11** are the protons involved and the related multiplicity patterns are the precise overlap of what observed individually for 3-oxo-, 7-oxo- and 12-oxo- compounds. In the case of the 3,7-dioxo derivative **9**, the presence of the 7-oxo group seems to nullify the deshielding effect on proton at C-4. Accordingly, the specific resonances of **9** are caused by two nuclei: the proton at C-6, in axial configuration as proved by a $^{2}J_{\text{geminal}}$ (12.6 Hz) and a $^{3}J_{\text{axial/equatorial}}$ (5 Hz) with the proton at C-5, and the proton at C-8. On the contrary, the specific resonances of **10** and **11** are those separately found for the 12-oxo compound, added together with the effect of the 3- and 7-oxo groups, respectively.

Furthermore, the chemical shifts and multiplicities collected in Table 1 for free bile acids are equally diagnostic also with ester derivatives. It should be noted, however, that the chemical shifts listed in the Table are slightly dependent on the solvent used.

The ^{13}C NMR chemical shifts for the examined compounds including the unfunctionalized bile acid **1**, obtained in CD_3OD [18], are collected in Table 2. The signals pertaining to C-15, C-16 and from C-18 to C-24,

Table 1
Observed chemical shifts, multeplicities and coupling constants in ^{1}H NMR of oxo-hydroxy bile acids **2**–**11** in CD_3OD

Compound	Proton [a]	δ (ppm)	Multiplicity [b]	J (Hz)
2	4 a	3.52	ψt	14.6
3	8	2.55	ψt	10.9
	6 a	2.98	dd	12.1; 4.8
4	4 a	2.87	ψt	14.0
5	11 a	2.62	ψt	12.2
6	4 a	3.58	ψt	14.2
7	8	2.55	ψt	12.0
	6 a	2.98	dd	13.0; 6.6
8	11 a	2.68	ψt	12.6
9	8	2.62	ψt	11.2
	6 a	3.00	dd	12.6; 5.0
10	11 a	2.72	ψt	12.2
	4 a	3.40	ψt	15.0
11	11 a	2.88	ψt	12.6
	8	3.08	ψt	11.9
	6 a	3.09	dd	14.0; 5.6

[a] Suffix a for axial.
[b] ψt means pseudo-triplet.

Table 2
^{13}C NMR chemical shifts (ppm) for oxo-hydroxy bile acids **1**–**11** in CD_3OD

Carbon	Compound										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	38.8	37.6 [a]	36.4	37.8 [a]	36.9	37.8 [a]	35.0	36.5	36.2	37.6 [a]	35.0
2	22.5	37.9 [a]	30.5	38.0 [a]	30.8[a]	37.6 [a]	30.5	31.2 [a]	37.4	37.4 [a]	31.7 [a]
3	28.1	217.3	71.6	217.2	72.1	217.6	71.7	72.3	213.7	215.4	71.3
4	28.6	46.6	38.1	43.2	36.2	46.6	38.2	40.2	43.8	46.3	38.1
5	45.1	44.8	47.5	45.9	42.9	49.9	47.2 [a]	42.6	48.9	44.2	46.2
6	28.4	36.7	46.3	27.6	28.1	34.9	46.3	35.8	45.8	35.0	46.5
7	27.8	69.0	216.1	26.6	27.1	68.8	215.9	68.4	213.1	68.5	213.2
8	37.2	40.9	50.8	37.0	36.9	40.9	50.7	40.5	50.7	40.5 [b]	49.8
9	42.0	34.4	44.3	34.7	45.8	28.3	37.4	38.6	37.3	39.1 [b]	47.4 [b]
10	36.5	35.0	36.2	35.5	36.4	36.0	35.8	37.0	36.0	37.1	36.7
11	22.0	22.0	22.8	30.0	39.0	29.7	30.4	39.0	30.8	39.0	39.3
12	41.5	40.6	40.3	74.0	219.6	74.0	72.9	218.6	72.8	217.1	216.6
13	44.0	43.7	43.8	47.6	58.9	47.6	47.5	57.9	47.6	58.4	58.3
14	58.0	51.5	50.4	49.3	60.6	42.9	41.8	55.1	41.9	55.1	53.9
15	25.3	24.4	25.8	24.7	25.2	24.0	25.2	24.7	25.3	26.7	26.0
16	29.3	29.1	29.1	28.5	28.4	28.6	28.6	28.5	28.7	28.5	28.5
17	57.4	57.4	56.3	48.1	47.9	48.0	47.4 [a]	47.7	47.3	47.8	47.0 [b]
18	12.8	12.0	12.3	13.0	11.9	12.8	13.2	11.9	13.3	12.0	11.9
19	25.0	22.2	23.3	22.6	23.1	22.0	23.2	22.7	22.4	21.7	22.5
20	36.7	36.4	35.1	36.6	36.8	36.7	36.4	36.8	36.6	36.9	36.8
21	19.0	18.6	19.0	17.4	18.8	17.4	17.5	19.0	17.7	19.1	19.0
22	32.1	31.8	31.9 [a]	31.9	32.2 [a]	32.0	31.9	32.2[a]	32.1	32.4	32.2
23	32.3	32.2	32.2 [a]	32.2	31.7 [a]	32.2	32.2	31.8 [a]	32.3	31.9	30.2[a]
24	178.0	178.8	178.7	178.9	178.8	179.1	178.8	178.7	178.2	178.8	179.0

[a] Assignment along a vertical column may be interchanged.
[b] Assignment along a vertical column may be interchanged.

which are far enough from electron-withdrawing carbonyl and hydroxy groups, were assigned by correlation with the spectrum of 5β-cholanoic acid **1**. On the other hand, the ^{13}C resonances of the carbonyl (C=O) or carbinol (C–OH) functional groups at C-3, C-7, and C-12 were easily identified, the former being located in the range 213.0–215.6 ppm and the latter situated from 68.4 to 74.0 ppm. It is worth noting the fact that the chemical shifts referred to the carbinol carbons are very close to the expected values, calculated by adding the effects of the monosubstituted compounds [19–23] on unfunctionalized bile acid **1**, while the corresponding C=O signals, with the exception of **9**, are deshielded by ca. 1.8–4.0 ppm, likely due to hydrogen bonding between oxygen atoms and the solvent [24].

The remaining carbon resonances were assigned on the basis of single-frequency off-resonance decoupling (SFORD) spectra, known empirical shift rules such as that related to α-, β-, γ- and δ-substituent effects and spectral comparison between related compounds [19–23]. The complete set of ^{13}C resonance attribution has been confirmed by DEPT experiments. The observed chemical shifts are in good agreement with the theoretical values calculated as described above, confirming the additivity of the substituent effect and indicating that carbonyl and/or hydroxyl groups at C-3, C-7, and C-12 of the 5 β-colanoic nucleus have negligible steric and electronic interactions [19].

In conclusion, ^1H NMR spectra of all the possible isomers of mixed oxo-hydroxy derivatives from cholic, deoxycholic and chenodeoxycholic acid highlighted the occurrence of a fingerprint region situated in the range 2.5–3.6 ppm. The chemical shift and the multiplicity of the signals characterize the number and position of the oxidation sites. The complete ^{13}C NMR resonances are assigned for the entire set of bile acids.

3. Experimental

All spectra were recorded on a Varian Gemini 300 VT spectrometer operating at 300.1 MHz and 75.463 MHz for ^1H and ^{13}C nuclei, respectively. All experiments were carried out using 10–60 mg samples dissolved in 0.5 ml of CD_3OD in 5 mm tubes at 25°C.

The chemical shifts were given in parts per million (ppm) using as internal standard either tetramethylsilane (TMS) for ^1H measurements or the CD_3OD signal for ^{13}C measurement; (δ (CD_3OD) = 49.0 ppm).

Instrumental settings: (^{1}H): spectral width 4000 Hz, pulse width 7 μs (45°), acquisition time 3 s, number of transients 16–64; (^{13}C): spectral width 20 000 Hz, pulse width 5.5 μs (35°), acquisition time 0.7 s, number of transients 15 000–70 000, number of data points 32 K.

^{13}C DEPT acquisition and processing data were obtained by the Gemini standard pulse program using the following parameters: spectral width 20 000 Hz, pulse width 14.1 μs (90°), acquisition time 1.8 s, pulse delay (PD) 2 s, average one bond ^{1}H–^{13}C coupling constants 150 Hz.

The synthesis of the compounds investigated (**2**, **4**, **6**, **10** [25], **3**, **5**, **7**, **11** [26] and **8**, **9**) [27] has been described elsewhere.

References

[1] J.L. Thistle, A.F. Hofmann, Efficacy and specificity of chenodeoxycholic acid therapy for dissolving gallstones, New Engl. J. Med. 289 (1973) 655–661.

[2] A. Ward, R.N. Brogden, R.C. Heel, T.M. Speight, G.S. Avery, Ursodeoxycholic acid. A review of its pharmacological properties and therapeutic efficacy, Drugs 27 (1984) 95–131.

[3] S. Scalia, P. Pazzi, G. Stabellini, M. Guarneri, HPLC assay of conjugated bile acids in gastric juice during UDCA therapy of bile reflux gastritis, J. Pharm. Biomed. Anal. 6 (1988) 911–917.

[4] R. Poupon, R.E. Poupon, Y. Calmus, Y. Chretien, F. Ballet, F. Darnis, Is ursodeoxycholic acid an effective treatment for primary biliary cirrhosis?, Lancet 1 (1987) 834–836.

[5] U. Beuers, U. Spengler, W. Kruis, U. Aydemir, B. Weibecke, W. Heldwein, M. Weinzierl, G.R. Pope, T. Sawerbruch, G. Paumgartner, Ursodeoxycholic acid lowers the pool size of other dihydroxy bile acids in healthy subjects, but not in patients with cholestatic liver disease, Hepatology 16 (1992) 707–714.

[6] C. Colombo, K.D.R. Setchell, M. Podda, L. Curcio, M. Ronchi, A. Giunta, The effects of ursodeoxycholic acid therapy in liver disease associated with cystic fibrosis, J. Pediatr. 117 (1990) 482–489.

[7] O. Bortolini, A. Medici, S. Poli, Biotransformations on steroid nucleus of bile acids, Steroids 62 (1997) 564–577.

[8] O. Bortolini, G. Fantin, A. Medici, P. Pedrini, Key fragmentations for the interpretation of mass spectra of disubstituted bile acids of bovine and porcine origin, Rapid Commun. Mass Spectrom. 11 (1997) 1286–1288 and references therein.

[9] G.C. DiDonato, B.M. Warrack, Ion spray liquid chromatographic/mass spectrometric characterization of bile acids, Biol. Mass Spectrom. 22 (1993) 101–111.

[10] A. Roda, C. Cerrè, P. Simoni, C. Polimeni, C. Vaccari, A. Pistillo, Determination of free and amidated bile acids by high-performance liquid chromatography with evaporative light-scattering mass detection, J. Lipid Res. 33 (1992) 1393–1402.

[11] S. Brane, D.N. Kirk, The Bile Acids, Chemistry, Pyisiology and Metabolism, vol. 4, Plenum, New York, 1988 and references therein.

[12] R.F. Zurcher, Progress in Nuclear Magnetic Resonance Spectroscopy In Organic Chemistry, Pergamon, Oxford, 1969.

[13] O. Bortolini, A Carpi, G. Fantin, A Guerrini, A Medici, A reappraisal of ^{1}H NMR spectra of oxo bile acids, Ann. Chim. (Rome) 89 (1999) 319–322.

[14] L.M. Jackman, S. Sternhell, Applications of Nuclear Magnetic Resonance Spectoscopy in Organic Chemistry, Pergamon, Oxford, 1969.

[15] N.S. Bhacca, F.W. Wehrli, N.H. Fischer, Carbon-13 magnetic resonance study of terpenoids. An application of heteronuclear selective decoupling experiments to the spectral assignments of nonproton-bearing carbon-13 resonances of a germacranolide, melampodin, J. Org. Chem. 38 (1973) 3618–3622.

[16] The stereoview of structure **II** has been adapted from the crystal structure of cholic acid: L. Lessinger, Cholic acid monohydrate $C_{24}H_{40}O_5 \cdot H_2O$, Cryst. Struct. Comm. 11 (1982) 1787–1792. AM1 calculations have shown that the structure is slightly distorted in the presence of oxo groups. Functions at C-3, C-7, C-12 and side chain have been omitted for clarity.

[17] Note that the nomenclature axial–equatorial refers to ring B and the configurational assignment of the proton at C-6 as axial is proposed by analogy with other protons under discussion. The experimental results and the dihedral angle of ca. 90°, however, are consistent with both axial and equatorial configuration for the proton at C-6.

[18] ^{13}C NMR chemical shifts in $CDCl_3$ of compound **1** are already reported, see Ref. [22].

[19] J.W. Blunt, J.B. Stothers, ^{13}C NMR spectra of steroids. A survey and commentary, Org. Magn. Reson. 9 (1977) 439–464 and references therein.

[20] W.B. Smith, in: G.A. Webb (Ed.), Annual Reports in NMR Spectroscopy, vol. 8, Academic Press, New York, 1978, p. 199 and references therein.

[21] D. Leibfritz, J.B. Roberts, Nuclear magnetic resonance spectroscopy. Carbon-13 spectra of cholic acids and hydrocarbons included in sodium deoxycholate solutions, J. Am. Chem. Soc. 95 (1973) 4996–5003.

[22] T. Iida, T. Tamura, T. Matsumoto, F. Chang, Carbon-13 NMR spectra of hydroxylated bile acid stereoisomers, Org. Magn. Reson. 21 (1983) 305–309.

[23] T. Iida, T. Tamura, T. Matsumoto, F. Chang, Carbon-13 NMR spectra of oxobile acid methyl esters, J. Jpn. Oil Chem. Soc. 32 (1983) 232–233.

[24] E. Breitmaier, W. Voelter, Carbon-13 NMR Spectroscopy, third ed., VCH, Weinhem, 1987, pp. 120–121.

[25] G. Fantin, M. Fogagnolo, A. Medici, P. Pedrini, U. Cova, Synthesis of 7- and 12-hydroxy and 7,12-dihydroxy-3-keto-5β-cholan-24-oic acids by reduction of 3,7-, 3,12 and 3,7,12-oxo derivatives, Steroids 58 (1993) 524–526.

[26] G. Fantin, S. Ferrarini, A. Medici, P. Pedrini, S. Poli, Regioselective microbial oxidation of bile acids, Tetrahedron 54 (1998) 1937–1942.

[27] S. Riva, R. Bovara, P. Pasta, G. Carrea, Preparative-scale regio- and stereospecific oxidoreduction of cholic acid and dehydrocholic acid catalyzed by hydroxysteroid dehydrogenase, J. Org. Chem. 51 (1986) 2902–2906.