METHOXYLATION OF 3β,7α-DIHYDROXYCHOL-5-EN-24-OIC ACID, A KEY INTERMEDIATE OF CHENODEOXYCHOLIC ACID BIOGENESIS, COMPARED WITH THAT OF ITS 7β-EPIMER

Teruo HARANO, Keiko HARANO and Kazumi YAMASAKI

Department of Biochemistry, Kawasaki Medical School,

Kurashiki 701-01, Japan.

Received 4-10-78

ABSTRACT

The conventional methods of gas liquid chromatography or mass spectrometry failed to be useful for the identification of the biliary 3β,7α-dihydroxychol-5-en-24-oic acid, a key intermediate of chenodeoxycholic acid biogenesis. It has been preliminarily reported that this acid in human bile was successfully identified by gas chromatography-mass spectrometry, after the methoxylation of its allyl alcohol group. Physical as well as spectral properties of the methoxylation products derived from the acid were reported, compared with those from its 7β-epimer.

INTRODUCTION

Yamasaki and his associates (1) have recently proposed an alternative pathway of chenodeoxycholic acid biogenesis, in which 3β,7α-dihydroxychol-5-en-24-oic acid is a key intermediate. When a ^{14}C-labeled precursor of the bile acid, such as cholesterol, 7α-hydroxycholesterol or mevalonic acid, was administered to a rat with a bile fistula, the intermediate was isolated from the fistula bile and identified as such by isotope dilution experiments (2). However, it proved too difficult to isolate and identify this intermediate in bladder bile by the conventional methods of gas liquid chromatography and mass spectrometry, because not only its content in bile is very small in quantity, but also it has an allyl alcohol group in the molecule, which proved to be acid- as well as heat-labile.

We have lately made a preliminary report that this acid was successfully isolated from human bile and identified as such by gas chromatogra-

phy-mass spectrometry (3).

The present paper deals with the fundamental investigations on which our successful results were based.

EXPERIMENTAL

Methods-----Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Specific rotatory powers were measured in methanol solution at room temperature with a Yanagimoto OD-50 photometric polarimeter. Thin layer chromatography (TLC) was carried out on a silica gel (E. Merck, Kieselgel H, 0.25 mm thick): solvent system, isooctane-ethyl acetate-acetic acid (20: 40: 1, by vol.); color was developed with the phosphomolybdic acid reagent (2) at room temperature. Column chromatography was carried out on a column of silica gel (E. Merck, Kieselgel type 60, 230 mesh), using chloroform, and eluates were checked by TLC or by GLC. Gas liquid chromatography (GLC) was carried out with a Shimadzu GC-4BPTF gas chromatograph: glass column (4 mm x 2 m) packed with 0.75% SE-52; flow rate of carrier gas (nitrogen), 88 ml/min; column temperature, 230°C; injection temperature, 250°C. Mass spectra were recorded on a Hitachi RMU-6MG mass spectrometer, using a direct insertion probe: electrom impact energy, 70 eV; temperature, 220°C. NMR spectra were measured in deuterochloroform solution on a Hitachi spectrometer (60 MHz), using tetramethylsilane as an internal reference. The detection of proton signal of hydroxyl group was carried out by adding deuterium oxide after the above measurements. Ultraviolet (UV) and infrared (IR) spectra were determined on a Hitachi 323 and 285 spectrophotometers, respectively.

Materials-----Methyl 3β,7α-dihydroxychol-5-en-24-oate (Ia).

This was prepared from methyl 3β-acetoxychol-5-en-24-oate according to the method reported by Yamaga (4). The last named ester (800 mg) was dissolved in freshly distilled carbon tetrachloride (30 ml) and N-bromosuccinimide (500 mg) was added. The mixture was refluxed under ultraviolet irradiation for 8 min and the resulting precipitate was filtered off. Alumina (10 g, once washed with ethyl acetate) was added to the filtrate and kept under stirring at room temperature for 2 hr. Alumina was filtered off; after the solvent was evaporated in vacuo, the residue was hydrolyzed in ethanolic 2N potassium hydroxide solution. The free acid thus obtained was crystallized from ethyl acetate to give a yet impure sample, mp 210-214°C.

Methyl ester (Ia): After methylation with diazomethane, the above acid was chromatographed on a column of silica gel to give a pure sample, mp 168-169°C; $[\alpha]_D$ -85° [reported (5) mp 161-163°C; $[\alpha]_D$ -98.3°]. Anal. Calcd. for $C_{25}H_{40}O_4$: C, 74.23; H, 9.97%. Found: C, 74.26; H, 10.04%.

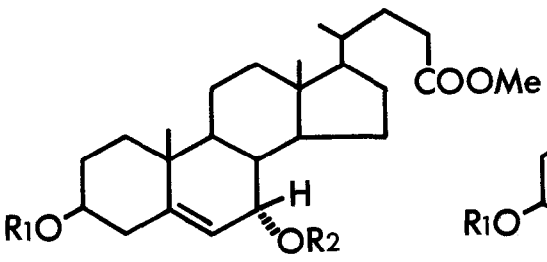
Free acid: The ester was hydrolyzed in ethanolic alkali and the free acid obtained was recrystallized from ethyl acetate, mp 214°C; $[\alpha]_D$ -89° [reported (5) mp 206-210°C; $[\alpha]_D$ -87.4° (dioxane)]. Yield, 100 mg. Anal. Calcd. for $C_{24}H_{38}O_4$: C, 73.81; H, 9.81%. Found: C, 73.52; H, 9.89%.

Methyl 3β,7β-dihydroxychol-5-en-24-oate (IIa).

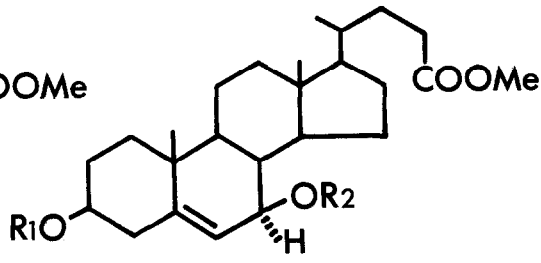
Methyl 3β-acetoxy-7-oxochol-5-en-24-oate (1 g, mp 181-181.5°C) was

prepared from methyl 3β-acetoxychol-5-en-24-oate by oxidizing with tert-butyl chromate (6). It was reduced with sodium borohydride in aqueous dioxane and hydrolyzed in ethanolic alkali. The free acid obtained was recrystallized from ethyl acetate to give a pure sample, mp 218-219°C; $[\alpha]_D$ +14° [reported (5) mp 223-225°C; $[\alpha]_D$ +10.4°]. Yield, 800 mg. Anal. Calcd. for $C_{24}H_{38}O_4$: C, 73.81; H, 9.81%. Found: C, 73.65; H,9.81%.

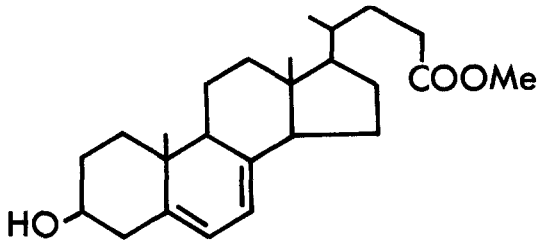
Methyl ester (IIa) was obtained by methylation with diazomethane and melted at 144°C; $[\alpha]_D$ +17.8° [reported (5) mp 144-146°C; $[\alpha]_D$+10.7°(MeOH)]. Anal. Calcd. for $C_{25}H_{40}O_4 \cdot 1/2\ H_2O$: C, 72.60; H, 9.99%. Found: C, 72.29; H, 9.89%.



Ia $R_1 = R_2 = H$
Ib $R_1 = H$, $R_2 = CH_3$
Ic $R_1 = Ac$, $R_2 = CH_3$



IIa $R_1 = R_2 = H$
IIb $R_1 = H$, $R_2 = CH_3$
IIc $R_1 = Ac$, $R_2 = CH_3$



III

Preliminary experiments. Methyl 3β,7α-dihydroxychol-5-en-24-oate (Ia, 7α-Epimer) and its 7β-epimer (IIa)(10 mg each) were dissolved in methanol (5 ml) containing one drop of 2N hydrochloric acid respectively and incubated at room temperature. The reaction products of both epimers were pipetted up at various incubation times and checked by TLC and by GLC.

i) TLC-----As shown in Fig. 1. A, a less polar product (Rf 0.5)[methoxy-compound(s)] of the 7α-epimer was detected as early as at 5 min incubation and the starting material was almost invisible on a plate at 1 hr incubation. On the other hand, a similar product (Rf 0.5)[methoxy-compound(s)] of the 7β-epimer appeared on a chromatoplate much later and the starting material was detected even at 4 hr incubation (Fig. 1. B).

ii) GLC-----Both epimers were incubated as above and the reaction products were monitered at various incubation times by GLC without acetyla-

tion. As shown in Fig. 2. A and B, two distinct peaks, b(b') and c(c'), corresponded apparently to the respective compounds that were detected as single spots (Rf 0.5) on the chromatoplates and nearly in accordance with the results of TLC (Fig. 1. A and B), peak a (the 7α-epimer) promptly disappeared, while peak a' (the 7β-epimer) was observed even at 1 hr incubation.

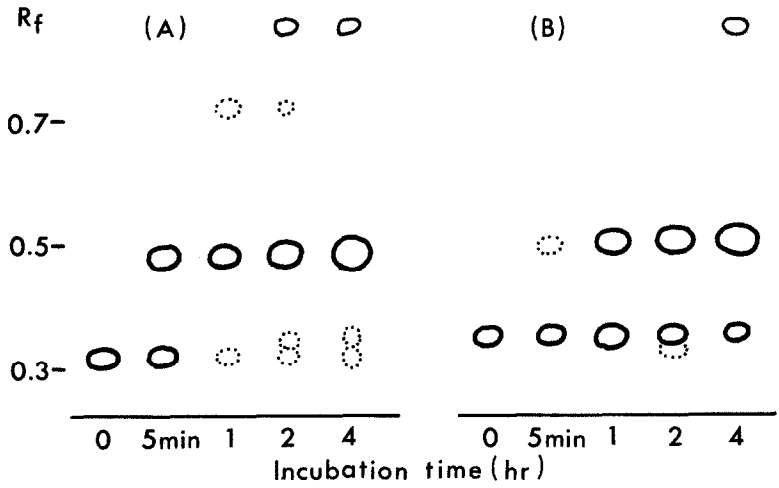


Fig. 1. TLC of the respective mixtures obtained from methyl 3β,7α-dihydroxychol-5-en-24-oate (A) and its 7β-epimer (B), when they were incubated in methanol-HCl for various times.

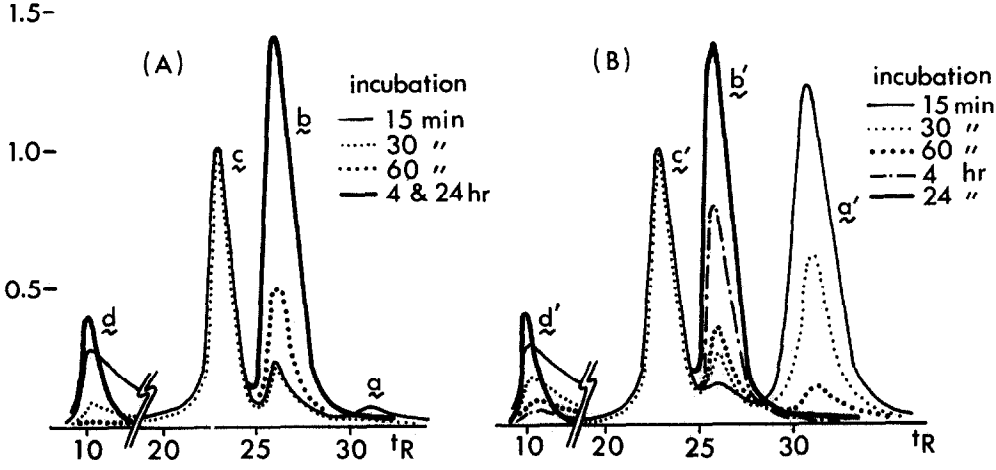


Fig. 2. GLC of the respective products obtained from methyl 3β,7α-dihydroxychol-5-en-24-oate (A) and its 7β-epimer (B) at various incubation times by the methanol-HCl treatment. Heights of peaks c and c' are taken as the standard (1.0) of the respective chromatograms.

According to the results of the preliminary experiments, methyl 3β, 7α-dihydroxychol-5-enoate and its 7β-epimer were incubated in methanol containing a trace of hydrochloric acid at room temperature and the respective major products isolated were analyzed.

Experiment I. Methoxylation of methyl 3β,7α-dihydroxychol-5-en-24-oate (Ia). Ia (100 mg) was dissolved in methanol (5 ml) and one drop of 2N hydrochloric acid was added. The mixture was incubated for one hr at room temperature and the reaction was stopped by addition of a saturated sodium bicarbonate solution. The mixture was extracted twice with ether and the extract was washed with water. After drying over anhydrous sodium sulfate, the solvent was evaporated under reduced pressure. The residue (99.6 mg) was chromatographed on a column of silica gel (30 g), using chloroform.

Methyl 3β-hydroxy-7α-methoxychol-5-en-24-oate (Ib). Each eluate was checked by TLC and by GLC to obtain two fractions. Repeated chromatographies of the less polar fraction gave a pure sample of Ib, mp 150-151°C; $[\alpha]_D$ -113° (MeOH). Yield, 52,6 mg. GLC of Ib gave a single peak at t_R 23 min. Anal. Calcd. for $C_{26}H_{42}O_4$: C, 74.60; H, 10.11%. Found: C, 74.51; H, 10.18%.

The eluates of the other fraction were combined and the solvent was evaporated. The residue was rechromatographed after acetylation, giving 5 mg of the same material (IIc, mp 89-90°C; $[\alpha]_D$ +6°) as described later (Exp. II).

Acetylation of Ib (Ic). Acetylation of Ib was carried out by heating in acetic anhydride-pyridine as usual and the product was recrystallized from methanol, mp 127-130°C; $[\alpha]_D$ -105°.

Free acid. Hydrolysis of Ib in ethanolic potassium hydroxide gave an acid, melting at 187-189°C. Anal. Calcd. for $C_{25}H_{40}O_4 \cdot H_2O$: C, 71.06: H, 10.01%. Found: C, 71.28; H, 9.86%.

Experiment II. Methoxylation of methyl 3β,7β-dihydroxychol-5-en-24-oate (IIa). IIa (70 mg) was incubated in 5 ml of methanol containing one drop of 2N HCl for 24 hr and worked up in a similar manner to that described above (Exp. I). The reaction product (66 mg) was chromatographed on a column of silica gel (30 g), using chloroform to remove the less polar fractions, which contained Ib and III (see below).

Methyl 3β-acetoxy-7β-methoxychol-5-en-24-oate (IIc). The major eluates thus obtained were combined and the solvent was evaporated *in vacuo*. After acetylation with acetic anhydride (2 ml) in pyridine (5 ml), the residue was rechromatographed on a column of silica gel (20 g), using chloroform to give 20 mg of a pure sample IIc, mp 89-90°C, $[\alpha]_D$ +6°. GLC of IIc showed a single peak at t_R 33 min.

Hydrolysis of IIc followed by methylation. Hydrolysis of IIc in ethanolic potassium hydroxide gave a free acid, which melted at 177-179°C. Anal. Calcd. for $C_{25}H_{40}O_4 \cdot H_2O$: C, 71.06; H, 10.01%. Found: C, 70.87; H, 9.72%.

Methyl ester (IIb). The above acid was methylated with diazomethane and melted at 95-98°C; $[\alpha]_D$ +2° (MeOH); t_R 26 min. Anal. Calcd. for $C_{26}H_{42}O_4 \cdot 1/4\ H_2O$: C, 73.80; H, 10.12%.

Isolation of methyl 3β-hydroxychola-5,7-dien-24-oate (III). The product obtained in Exp. II was rechromatographed and the eluates less polar than those containing IIb were collected and combined. The solvent was evaporated *in vacuo* and the residue was again chromatographed to obtain the least polar eluates together with those containing Ib (13 mg). Concentration of these eluates combined gave a crystalline material (2.5

mg), corresponding to peak d'(Fig. 2. B), mp 114-115°C. UV λ_{max}^{EtOH} : 282 nm. IR $\nu_{max}^{Chlf.}$ cm^{-1}: 3500(OH), 1720(CO), 1660, 1610(-CH=CH-CH=CH-). NMR ($CDCl_3$, D_2O) δ: 0.71s(3H, 18-CH_3), 0.96s(3H, 19-CH_3), 1.56s(1H, OH), 3.63s(3H, $COOCH_3$), 5.5-5.9dd(J= ca. 6 Hz, olefinic 2H).

RESULTS and DISCUSSION

As shown in Figures 1 (A and B) and 2 (A and B), methyl 3β,7α-dihydroxychol-5-en-24-oate and its 7β-epimer were more or less readily methoxylated, as will be discussed later, by incubation in methanol-HCl (see Experimental) like 7α-hydroxycholesterol and its 7β-epimer (7,8). GLC of both resulting products gave two distinct, similar peaks at t_R 23 min (peaks c and c') and at t_R 26 min (peaks b and b') together with more or less indefinite peaks at t_R 10 min (peaks d and d') and at t_R 31 min (peaks a and a'), respectively. It should be noted that the methoxylation of the 7α-epimer proceeded much faster than that of the 7β-epimer. The preliminary data of GLC (Fig. 2) indicated that the products corresponding to peaks b and c are the same as those corresponding to peaks b' and c', because the respective t_R values were coincident with each other. And it was also indicated that peaks c and c' appeared as the major peaks at earlier stages of incubation, while they decreased and peaks b and b' conversely increased in height as the incubation time was prolonged, both ratios b to c and b' to c' reaching approximately the same value 2:1 after 46 hr incubation. When the starting epimers were subjected to GLC without the methanol-HCl treatment (methoxylation), they were more or less decomposed: The 7α-epimer gave only an indefinite peak at t_R 10 min, while the 7β-epimer showed a peak at t_R 33 min (probably the unchanged starting material) together with a plateau between t_R 10 min and t_R 30 min.

This is why the conventional methods of GLC or mass spectometry fail-

ed to be useful for the identification of the biliary 3β,7α-dihydroxychol-5-en-24-oic acid (9).

Methyl 3β-hydroxy-7α-methoxychol-5-en-24-oate (Ib) and its 7β-epimer (IIb). The physical as well as spectral data of Ib and IIb are summarized in Tables I and II.

Table I. Physical and spectral data obtained from the products Ib and IIb.

	Ib(peak c)	IIb(peak b')
mp	150-151°C	95-98°C
$[\alpha]_D$	-113°(MeOH)	+2°(MeOH)
t_R	23 min	26 min
MS(m/e)	418(M^+), 400[$(M-H_2O)^+$], 386[$(M-MeOH)^+$], etc.	418(M^+), 400[$(M-H_2O)^+$], 386[$(M-MeOH)^+$], etc.

		7α-OMe	3β-OH	3β-OAc		7β-OMe	3β-OH	3β-OAc
IR(cm^{-1})	Ib	1070	3600, 1040	---	IIb	1080	3600, 1040	---
	Ic	1070	---	1030	IIc	1080	---	1030

Table II. NMR data of Ia-c and IIa-c, compared with those of methyl 3β-hydroxychol-5-en-24-oate and its acetate for reference (δ-values).

Compds.	18-Me	19-Me	3β-OH	3β-OAc	7α-OMe	7β-OMe	est-Me	6-H	D_2O***
Ia	0.68	0.98					3.67	5.59d (J=6Hz)	-
Ib	0.66	0.97	1.60		3.33		3.63	5.74d (J=5Hz)	+
Ic	0.69	0.98		2.01	3.33		3.64	5.77d (J=5Hz)	-
IIa	0.70	1.05					3.68	5.35s	-
IIb	0.69	1.03	1.53			3.26	3.64	5.46s	+
IIc	0.68	1.03		2.00		3.24	3.64	5.47s	-
Reference									
*3β-OH-Δ^5	0.69	1.00	1.82				3.63		+
**3β-OAc-Δ^5	0.68	1.01		2.04			3.65		-

*Methyl 3β-hydroxychol-5-en-24-oate; **methyl 3β-acetoxychol-5-en-24-oate; ***see the text; s, broad singlet; d, broad doublet.

The mass spectra of Ib and IIb (Fig. 3. A and B) show very similar patterns of fragmentation ions to each other, having the same character-

istic ions m/e 400[$(M-H_2O)^+$] and 386[$(M-MeOH)^+$]. The IR spectra (Table I) show that both products (Ib and IIb) retained at least one of the acylable hydroxyl groups in the respective starting epimers (Ia and IIa), because the absorption bands which had been observed at ν 3600 cm^{-1} on their spectra disappeared on acetylation. It is certain that these acylable hydroxyl groups in both epimers are 3β-OH of the molecules, because both IR spectra (Table I) show the same ν values of 3β-OH (ν 1040 cm^{-1}) and 3β-OAc (ν 1030 cm^{-1}) as those reported previously (10). Accordingly there remain the hydroxyl groups at C-7 in both epimers that were methoxylated by the methanol-HCl treatment, as was expected.

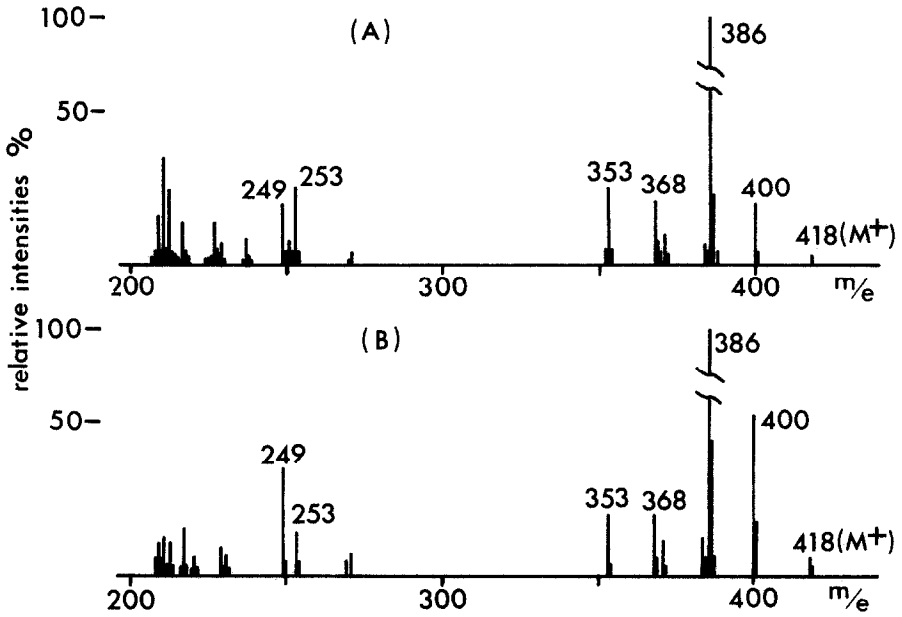


Fig. 3. Mass spectra of methyl 3β-hydroxy-7α-methoxychol-5-en-24-oate (A: Ib) and its 7β-epimer (B: IIb).

This was confirmed by the NMR spectra of Ib,c and IIb,c, where their spectra are compared with those of methyl 3β-hydroxychol-5-en-24-oate and its acetate for reference (Table II). The signals corresponding to

3β-OH and 3β-OAc taken from Ib,c and IIb,c were found in nearly the same regions (δ 1.5-1.6 and δ 2.0 ppm), as those of the reference compounds. Two methyl signals bound to oxygen were observed in the region between δ 3.00 and δ 4.00 ppm on the respective spectra of Ib,c and IIb,c (Fig. 4).

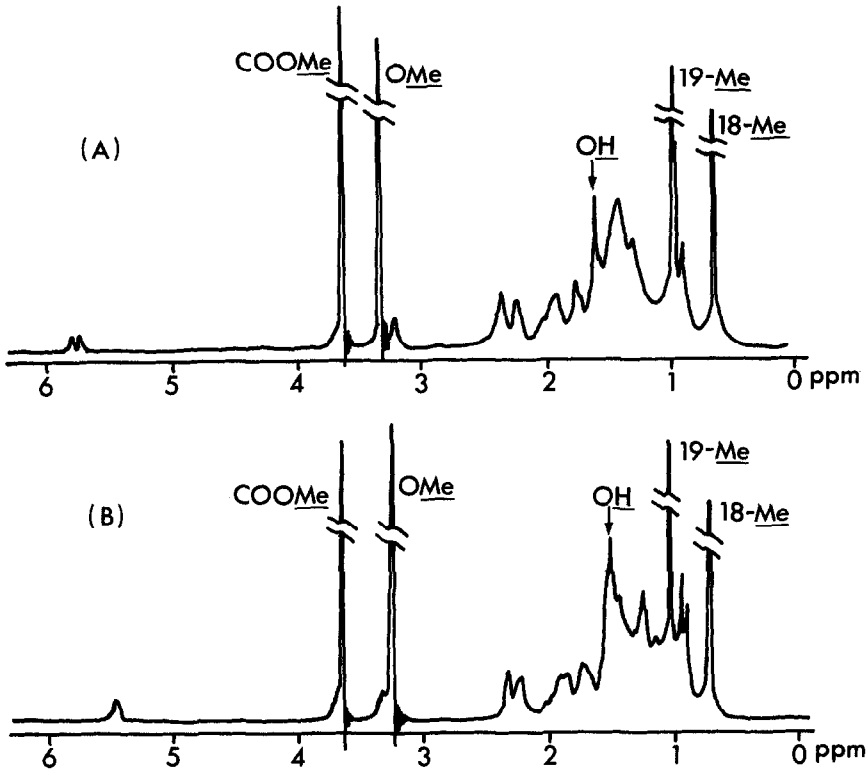


Fig. 4. NMR spectra of methyl 3β-hydroxy-7α-methoxychol-5-en-24-oate (A: Ib) and its 7β-epimer (B: IIb).

The signals at δ 3.3 ppm arose from the C-7 methoxyl function of the respective compounds, while the other low field signals (δ 3.6 ppm) were attributed to their methyl ester function, as is the case with the reference compounds (11). The signals (δ 5.5-5.7 ppm) of olefinic proton at C-6 are of interest, because the coupling constant of 6H-7βH (J=5 Hz) in Ib,c was found in agreement with that calculated for 3He'-2H (12) of a cyclohexene ring, while that of 6H-7αH in IIb,c was found to be almost

zero (13). The last mentioned findings were well accordant with the $[\alpha]_D$ values of Ib and IIb (Table I). From these data it is concluded that the structures of the major products (peak c and peak b') obtained from Ia and IIa by the methanol-HCl treatment are assigned to methyl 3β-hydroxy-7α-methoxychol-5-en-24-oate (Ib) and its 7β-epimer (IIb), respectively.

Methyl 3β-hydroxychola-5,7-dien-24-oate (III). The least polar product corresponding to peak d' on the gas liquid chromatogram (Fig. 2. B) was obtained in a small amount (2.5 mg), when IIa (70 mg) was incubated in methanol-HCl (Exp. II). The UV spectrum of the product showed an absorption maximum at 282 nm, indicative of a homoannular diene system (calcd. λ_{max} 283 nm)(14). The presence of such a conjugated diene system in the molecule was confirmed by the absorption bands at 1660 and 1610 cm^{-1} on the IR spectrum. The methoxyl signal at δ 3.3 ppm, observed on the NMR spectrum of IIb, disappeared on the spectrum of III (see Exp. II). Hence the homoannular diene system was formed due to dehydration or demethoxylation at C-7 by the methanol-HCl treatment. The coupling constant of the olefinic proton at C-6,7 (J= 6 Hz) was well accordant with the above deduction. The data of the IR (ν 3500 cm^{-1}) as well as the NMR (δ 1.56 ppm) spectra (Exp. II) show that the C-3β-OH was retained in the molecule of III.

From these data the structure methyl 3β-hydroxychola-5,7-dien-24-oate (III) was assigned to the product corresponding to peak d' (Fig. 2. B).

REFERENCES

1) Yamasaki, K., Ayaki, Y. and Yamasaki, H., J. Biochem., 70, 715-718(1971)
2) Ayaki, Y. and Yamasaki, K., J. Biochem., 71, 85-89(1972)
3) Harano, K., Harano, T., Yamasaki, K. and Yoshioka, D., Proc. Japan Acad., 52, 453-456(1976)

4) Yamaga, N., J. Biochem., 70, 125-131(1971)
5) Yamasaki, K., Ikawa, S., Kinoshita, D., Usui, T. and Nakada, F., Yonago Acta Med., 11, 159-164(1967)
6) Heusler, K. and Wettstein, A., Helv. Chim. Acta, 35, 284(1952)
7) Henbest, H. B. and Jones, E. R. H., J. Chem. Soc., 1948, 1798
8) Kulig, M. J., Teng, J. I. and Smith, L. L., Lipids, 10, 93(1975)
9) It was once reported that two Lifschutz-positive compounds were obtained from fistula bile of the hen and that they were identified as 3β,7α-dihydroxychol-5-en-24-oic and 3β,7α-dihydroxychol-4-en-24-oic acids by GLC, compared with the respective authentic samples(1). Later experiments suggested, however, that the respective peaks (t_R: 5-6 min) concerned corresponded to the peaks of the decomposed compounds derived from the respective unsaturated bile acids.
10) Jones, R. N. and Roberts, G., J. Am. Chem. Soc., 80, 6121(1959); Page, J. E., J. Chem. Soc., 1955, 2017
11) Bhacca, N. S. and Williams, D. H., in Application of NMR spectroscopy in organic chemistry-Illustrations from the steroids field, Holden-Day, Inc. San Francisco (1966) p. 40
12) He': quasi-equatorial proton
13) Corey, E. J. and Sneen, R. A., J. Am. Chem. Soc., 77, 2505(1955)
14) Fieser, L. F. and Fieser, M., in Steroids, Van Nortrand Reinhold Co. (1959) pp. 15-24

Trivial names used here: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; 7α-hydroxycholesterol, 5-cholestene-3β,7α-diol; mevalonic acid, 3,5-dihydroxy-3-methyl-pentan-1-oic acid.