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Natural Occurrence of 3β, 7α-Dihydroxychol-4-en-24-oic Acid in Hen Bile

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A new C_{24}-bile acid containing allyl alcohol grouping, 3β, 7α-dihydroxychol-4-en-24-oic acid, was isolated from hen bile and identified by comparing with the authentic sample synthesized by TLC, IR spectra and dilution experiments of ^{3}H-acetyl methyl ester of the compound. Its biochemical significance in chenodeoxycholic acid biogenesis was discussed.

From the bile of domestic fowls, so far, five bile acids have been isolated, chenodeoxycholic (*1–3*),** cholic (*4*),** allocholic (*5*),** 3α-hydroxy-7-oxocholan-24-oic (*6*) and 3-oxochol-4, 6-dien-24-oic acids (*7*), of which chenodeoxycholic acid is the major component.

In this paper we report the isolation of a new C_{24}-bile acid having allyl alcohol grouping from hen bile and its identification as 3β, 7α-dihydroxychol-4-en-24-oic acid in comparison with the specimen synthesized.***

MATERIALS AND METHODS

Materials—The following dihydroxychol-5-en-24-oic acids were prepared and purified as described previously (*8–10*):

3α, 7α-Dihydroxychol-5-en-24-oic acid: 3α, 7α-Dihydroxychol-5-en-24-oic acid was prepared by the bromination of methyl 3α-acetoxycholen-24-oate with N-bromosuccinimide in boiling CCl_4 followed by the treatment of the product with aluminum oxide. mp 208–209°C, $[\alpha]_D^{25}$ −139.2° (dioxane); methyl ester, mp 165–170°C.

3α, 7β-Dihydroxychol-5-en-24-oic acid: 3α, 7β-Dihydroxychol-5-en-24-oic acid was obtained by KBH_4 reduction of 3α-acetoxy-7-oxochol-5-en-24-oate. mp 200–203°C, $[\alpha]_D^{24}$ +26.3° (dioxane); methyl ester, mp 167–170°C.

3β, 7α-Dihydroxychol-5-en-24-oic acid: 3β, 7α-Dihydroxychol-5-en-24-oic acid was synthesized from methyl 3β-acetoxycholen-24-oate in the same way as was done its 3α-epimer (see above). mp 206–210°C, $[\alpha]_D^{24}$ −82° (methanol); methyl ester, mp 161–163°C.

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* The present address: Central research institute, Teijin Ltd., 1995, Hirayama, Hino-city, Tokyo. Reprint requests should be addressed to Dr. K. Yamasaki. ** The following trivial names are used: chenodeoxycholic and cholic acids, 3α, 7α-dihydroxy- and 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acids; allocholic acid, 3α, 7α, 12α-trihydroxy-5α-cholan-24-oic acid. The last named was reported once as tetrahydroxynorsterocholanic acid (Yamasaki, K. 1951) (*5*). *** The outline of this report was presented at the 42nd general meeting of Japan. Biochem. Soc., Hiroshima, October 8, 1969.

3β, 7β-Dihydroxychol-5-en-24-oic acid: 3β, 7β-Dihydroxychol-5-en-24-oic acid was obtained by KBH_4 reduction of 3β-acetoxy-7-oxochol-5-en-24-oate. mp 220–223°C, $[\alpha]_D^{24}+12°$ (methanol); methyl ester, mp 144–146°C.

3β, 7α-Dihydroxychol-4-en-24-oic acid: 7α-Hydroxy-3-oxochol-4-en-24-oic acid (mp 230–231°C) (*11*) was methylated with diazomethane. To the solution of this ester (200 mg dissolved in 20 ml of methanol), sodium borohydride (120 mg) was added, and the mixture was kept at room temperature for 24 hr. The reaction mixture was acidified with 10 ml of acetic acid, diluted with 50 ml of water, and then extracted repeatedly with ether. The combined ether extracts were washed twice with NaCl-saturated water and dried. After the evaporation of the solvent, the residue was dissolved in hot ethanol (90%). To this solution, digitonin (700 mg in 50 ml of 90% ethanol) was added. The mixture was diluted with water until it began to be slightly turbid and then kept at 5°C for 24 hr. The amorphous precipitate collected was washed with 20 ml of ether containing 2 drops of methanol and then with hot ether.

The digitonide (about 200 mg) dissolved in 5 ml of pyridine was diluted with 100 ml of ether and kept overnight at 5°C. The resulting precipitate was filtered off and washed with hot ether. The filtrate was combined with the washings and filtered through a layer of charcoal. The filtrate was concentrated and diluted with ether. After the ethereal layer was washed with dilute HCl and water, it was dried and evaporated to dryness. The residue was hydrolyzed with 5% methanolic potassium hydroxide overnight at room temperature and the free acid was purified in the usual way. After several recrystallizations from aqueous methanol, fine needles (87 mg) of the title acid were obtained. mp 229–231°C (dec.)*; $[\alpha]_D^{23}+40.8\pm0.5°$C (*c*, 0.25, methanol). Found: C, 74.03; H, 9.66. $C_{24}H_{38}O_4$ requires C, 73.79; H, 9.79%. Methyl ester, mp 134–136°C.

The amount of the 3α-epimer of this dihydroxy acid obtained from the mother liquor was too small for further investigation.

Phosphomolybdic acid reagent: Phosphomolybdic acid reagent was prepared as reported before (*8*, *9*).

Lifschütz reagent: Lifschütz reagent was a mixture of conc. H_2SO_4 and glacial acetic acid (10 : 90, v/v) (*12*, *13*).

Methods-Thin-layer chromatography(TLC): Plates of Kieselgel G (Merck AG, Darmstadt, Germany) (*ca.* 0.25 mm thick) were used unless otherwise stated. Solvent systems: a, isooctane-ethyl acetate-acetic acid (20 : 20 : 4, v/v); b, isooctane-ethyl acetate-acetic acid (20 : 40 : 4, v/v); c, benzene-chloroform-acetone (50 : 2 : 2, v/v); d, ether-heptane (30 : 70, v/v); e, isooctane-ethyl acetate-acetic acid (40 : 15 : 1.5, v/v); f, benzene-chloroform-acetone (50 : 3 : 5, v/v).

RESULTS

Preparation of "Lifschütz Positive" (LP) Fraction—Hen bile (1.5 liters) obtained from a slaughter-house was worked up mostly in the same way as previously reported (*5*): The bile sample was hydrolyzed with 2 N KOH at 130°C for 3 hr. The hydrolysate was acidified and chenodeoxycholic acid was removed as completely as possible by the repeated $BaCl_2$-NH_4OH precipitation. About 120 g of fairly pure chenodeoxycholic acid were obtained.

The filtrate of the above final precipitate (Ba-salt) was acidified to give 11.5 g of brown resinous material ("cholic acid" fraction). This was extracted with ethyl acetate and the solvent was evaporated to dryness. The residue (2.2 g) was recrystallized repeatedly from ethanol to give 1.5 g of fairly pure cholic acid.

The mother liquors from the cholic acid recrystallization were combined and evaporated to dryness. The residue was dissolved in a minimum amount of hot ethyl acetate. On cooling, semicrystalline material (600 mg) of a mixture of allocholic acid and cholic acid was deposited (the identification of the former compound will be reported elsewhere). The filtrate of this crop is designated as "Lifschütz positive" (LP) fraction.

* When the melting point was measured with a microscopic melting point apparatus, this material was found to melt at 251–253°C (dec.).

Isolation and Identification of the Lifschütz-Positive Compound I (LPC-I)—On TLC in solvent system a, LP-fraction gave two violet-colored spots with Lifschütz reagent, the lower spot, Lifschütz positive compound I (LPC-I) and the upper one, Lifschütz positive compound II (LPC-II) (Fig. 1-A), the latter of which was also found in the "chenodeoxycholic acid" fraction (Fig. 1-B). No further investigation of LPC-II was attempted.

Based on these results, LP-fraction was subjected to column chromatography with Kieselgel G (Gel for column chromatography, Merck AG, Darmstadt, Germany) (50 g) and eluted with ethyl acetate (*ca.* 1.2 liters).

The latter half of the eluate was found to contain most of LPC-I (500 mg). This fraction was rechromatographed with the same gel (13 g). The column was eluted with ligroin-isooctane-ethyl acetate-acetic acid (1 : 1 : 0.1, v/v), and fractions of 2 ml were collected. All the fractions, which showed a spot of the same *Rf* as that of LPC-I on TLC in solvent system a, were combined (elution volume: 32–48 ml) and worked up in the usual way, 80 mg of a crude LPC-I sample being obtained.

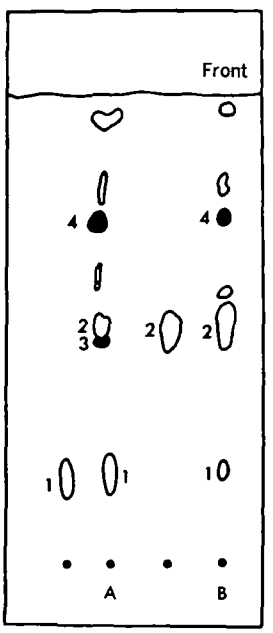

Fig. 1. Thin-layer chromatograms of Lifschütz positive fraction (A) and chenodeoxycholic acid fraction (B). 1, cholic acid; 2, chenodeoxycholic acid; 3, LPC-I; 4, LPC-II; solvent system a.

Since this sample was still contaminated with a large amount of chenodeoxycholic acid, it was purified by reversed phase column chromatography according to Norman and Sjövall (*14*). A column (23 mm×130 mm) was packed with 13.5 g of hydrophobic Celite and developed with solvent system F_1 at flow rate of 1–2 ml/min. Fractions of 4.3 ml were collected, and those containing LPC-I (checked by TLC in solvent system a, elution volume: 52–58 ml) were combined and evaporated to dryness under reduced pressure.

The residue (6 mg) was methylated with diazomethane and subjected to a preparative thin-layer chromatography. The plate was developed with solvent system b and the zone corresponding to LPC-I was scraped off and eluted with methanol. After the solvent was removed in a stream of nitrogen, the residue was dissolved in ether. The ethereal solution was washed with water, dried and evaporated to give purified methylated LPC-I. Most part of this ester was hydrolyzed with 5% methanolic KOH and the free acid was crystallized in needles from ethanol.

The behaviors of this compound (LPC-I) on reversed phase column chromatography as well as on TLC were very similar to those of chenodeoxycholic acid, though LPC-I seemed to be somewhat more polar than that. This suggested that LPC-I was one of the epimers of 3,7-dihydroxychol-4-en-24-oic or -5-en-24-oic-acid.

Therefore, LPC-I was compared on TLC with the reference samples of 3β,7α-dihydroxychol-4-en-24-oic acid and the four epimers of 3,7-dihydroxychol-5-en-24-oic acid (see *Materials*). As shown in Fig. 2-A, the behavior of LPC-I on ordinary TLC (solvent system b) coincided well with those of 3β,7α-dihydroxychol-4-en-24-oic acid and 3β,7α-dihydroxychol-5-en-24-oic acid. On a $AgNO_3$-impregnated Kieselgel G plate (*15*) (solvent system b), however, the behavior of methyl ester of LPC-I coincided only with that of methyl 3β,7α-dihydroxychol-4-en-24-oate but not with 3β,7α-

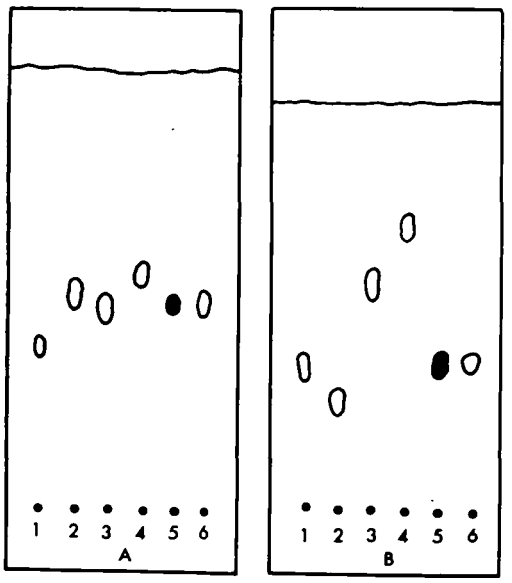

Fig. 2. Thin-layer chromatograms of dihydroxycholen-24-oic acids and their methyl esters on usual plate (A) and $AgNO_3$-impregnated plate (B), respectively; solvent system b. Samples on plate A: 1, 3α, 7α-dihydroxychol-5-en-24-oic acid; 2, 3α, 7β-dihydroxychol-5-en-24-oic acid; 3, 3β, 7α-dihydroxychol-5-en-24-oic acid; 4, 3β, 7β-dihydroxychol-5-en-24-oic acid; 5, LPC-I; 6, 3β, 7α-dihydroxychol-4-en-24-oic acid. Methyl esters of these were placed on plate B.

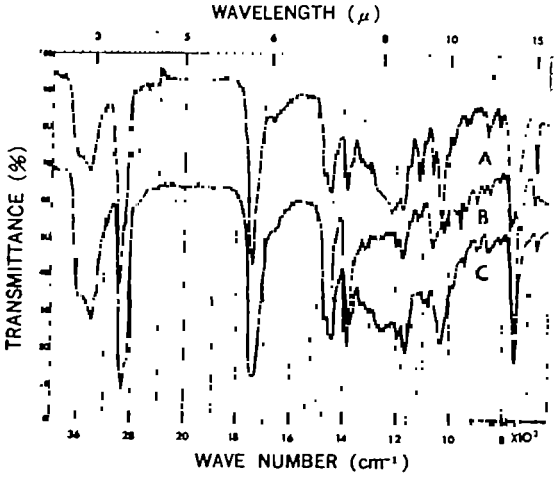

Fig. 3. Infra-red spectra in potassium bromide. A, methyl 3β, 7α-dihydroxychol-4-en-24-oate; B, methyl 3β, 7α-dihydroxychol-5-en-24-oate; C, LPC-I (methylated).

dihydroxychol-5-en-24-oate (Fig. 2-B). This was confirmed on another plate in solvent system a.

The free LPC-I melted at 251–253°C (dec.) (a microscopic melting point apparatus) and showed no mp depression on admixture with the synthesized sample of 3β, 7α-dihydroxychol-4-en-24-oic acid [mp 251–253°C (dec.)] (see *Materials*). These findings strongly suggested that LPC-I was 3β, 7α-dihydroxychol-4-en-24-oic acid. Moreover, the infra-red spectrum of methylated LPC-I (C) was similar to that of methyl 3β, 7α-dihydroxychol-4-en-24-oate (A), but different from that of methyl 3β, 7α-dihydroxychol-5-en-24-oate (B) (Fig. 3).

For further confirmation of these findings, the following experiment was carried out: A portion of the free acid was methylated with diazomethane and then acetylated by heating 0.2 ml of tritium labeled acetic anhydride* (the Radiochemical Center, England, RK-2) and 0.1 ml of pyridine at 100°C for 30 min. The resulting mixture was worked up in the usual way to give brown colored syrup of tritium labeled acetyl ester of LPC-I.

Since crystallization of methyl 3β, 7α-diacetoxychol-4-en-24-oate (5 mg) was unsuccessful, it was mixed with the above tritium labeled acetyl methyl ester in chloroform and spotted on a Kieselgel G plate. The plate was developed with solvent system c. After scanning with an autoradio-chromatogram scanner (Aloka Thin-Layer Chromatogram Scanner Model TRM-IB), the plate was exposed to the iodine vapor. The iodine-marked spot coincided well with the main peak of radioactivity (Fig. 4-a). Another spot of this compound also showed a positive test of the Lifschütz reaction. The area of this spot was scraped off and eluted with chloroform. This solution was washed with water and then evaporated. The residue dissolved in chloroform was spotted on another plate, developed again with the same solvent system and worked up in the same manner as described above. TLC was performed with three different solvent systems (d, e and f). Each iodine-marked area was scraped off and the collected gel was

* Acetic anhydride-T (25 mCi) was diluted with 10 ml of freshly distilled acetic anhydride.

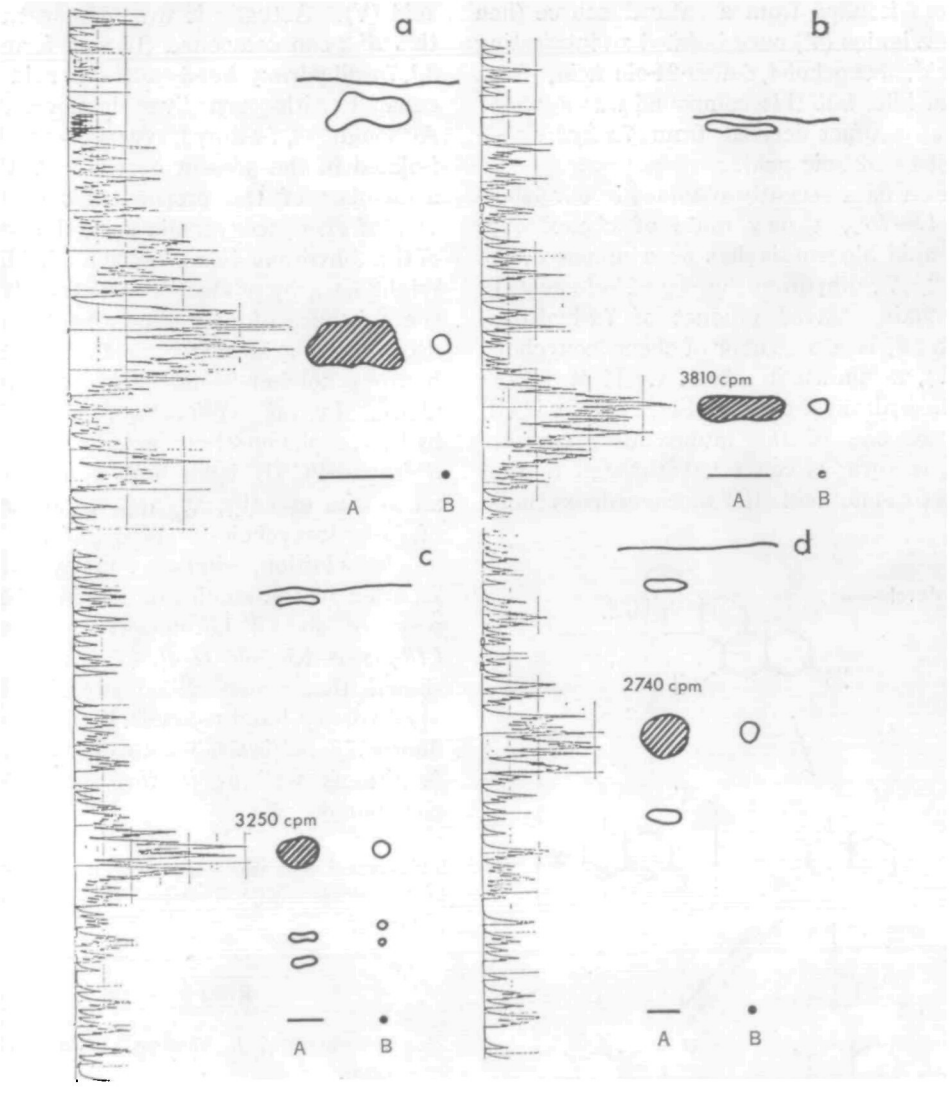

Fig. 4. Thin-layer chromatograms and autoradiochromatograms of the mixture of methyl T-acetylated LPC-I and methyl 3β,7α-diacetoxychol-4-en-24-oate. Plates, a–d, were treated with solvent systems c, d, e and f, respectively (see text). A: the mixed material; B: methyl 3β,7α-diacetoxychol-4-en-24-oate.

eluted with chloroform and diluted to 10 ml, the radioactivity of which (1-ml each aliquot) was measured with a windowless gas flow counter (Toshiba UDS-2420B). The radioactivity of the corresponding areas was scarcely decreased by repeated chromatographies, as shown in Fig. 4-b–d. Accordingly, LPC-I was definitely identified as 3β,7α-dihydroxychol-4-en-24-oic acid.

DISCUSSION

For the first time, a new C_{24}-bile acid with a 3-hydroxy-4-ene grouping in the molecule

has been isolated from a natural source (hen bile). Wiggins (*7*) once isolated an interesting bile acid, 3-oxochol-4,6-dien-24-oic acid, from chicken bile, but this compound was assumed to be an artifact derived from 7α-hydroxy-3-oxochol-4-en-24-oic acid.

From data recently obtained in our laboratory (*8–10*), a new route of chenodeoxycholic acid biogenesis has been proposed,* in which 3β,7α-dihydroxychol-5-en-24-oic acid (I), a side-chain cleaved product of 7α-hydroxycholesterol, is a precursor of chenodeoxycholic acid (V), as shown in Chart 1. If it is the case, 7α-hydroxy-3-oxochol-4-en-24-oic acid (II) might be one of the immediate precursors which, in turn, is converted through a saturated 3-oxo-bile acid (III) to chenodeoxycholic acid (V). Actually it was demonstrated that this Δ^4-3-oxo compound (II) was formed when 3β,7α-dihydroxychol-5-en-24-oic acid was incubated with carp liver homogenate (*16*). Although 3β,7α-dihydroxychol-4-en-24-oic acid isolated in the present experiment (IV) is not a member of the proposed biogenetic pathway of chenodeoxycholic acid, the occurrence of this 3-hydroxy-4-ene compound in bile, which might be a by-product, could strongly support the existence of such a new route of chenodeoxycholic acid biogenesis through 3β,7α-dihydroxychol-5-en-24-oic acid as mentioned above. In this connection, both 3β,7α-dihydroxychol-4-en-24-oic acid-^{14}C (IV) and its Δ^5-isomer-^{14}C (I) have been demonsterated in an *in vivo* experiment (hen) to be metabolites of 7α-hydroxycholesterol-^{14}C (*17*).

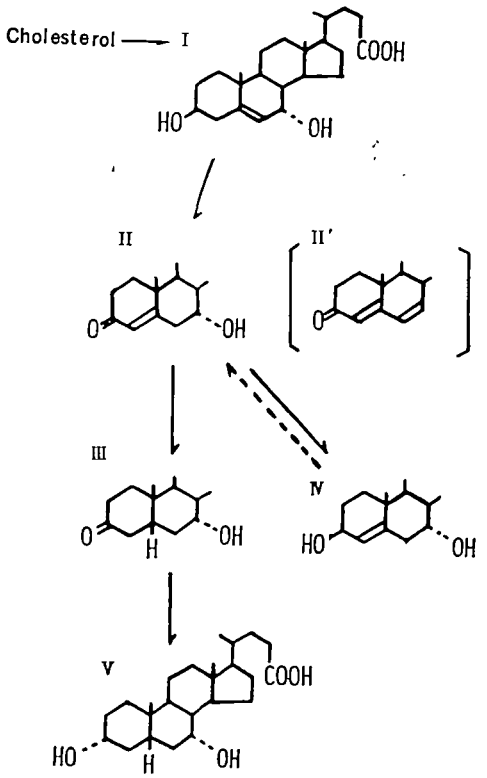

Chart 1. A biogenetic pathway of chenodeoxycholic acid proposed.

In addition, similar findings have been reported in the studies on the metabolic pathways of steroid hormones: Neeman *et al.* (*18*) and Ringold *et al.* (*19*) have recently shown that some Δ^4-3-oxosteroids, such as 11β-hydroxy-4-androstene-3,17-dione and 6β-fluoro-17β-androsten-3-one were reduced in part *in vivo* as well as *in vitro* to Δ^4-3-hydroxy compounds.

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