Identification of 3α,4β,7α-trihydroxy-5β-cholanoic acid in human bile: reflection of a new pathway in bile acid metabolism in humans

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Abstract The chemical synthesis, nuclear magnetic resonance, and mass spectrometric characteristics of the first C-4 hydroxylated bile acid analogues are described. The data definitively confirm, for the first time, the identity of 3α,4β,7α-trihydroxy-5β-cholanoic acid in human fetal gallbladder bile. In addition, 3α,4β,7α-12α-tetrahydroxy-5β-cholanoic was identified in the feces from healthy newborn infants many days after birth, indicating a hepatic origin for C-4 hydroxylation of bile acids. To our knowledge bile acids hydroxylated at the C-4 position of the steroid nucleus have never been previously recognized in any mammalian species. The finding of this novel bile acid which accounts for 5–15% of the total biliary bile acids in early gestation indicates that C-4 hydroxylation is a unique and important metabolic pathway in early human development.—**Dumaswala, R., K. D. R. Setchell, L. Zimmer-Nechemias, T. Iida, J. Goto, and T. Nambara.** Identification of 3α,4β,7α-trihydroxy- 5β-cholanoic acid in human bile: reflection of a new pathway in bile acid metabolism in humans. *J. Lipid Res.* 1989. **30:** 847–856.

Supplementary key words bile acid metabolism • C-4 hydroxylation • mass spectrometry • fetal bile • chemical synthesis

Recent studies of human fetal gallbladder bile have served to highlight the fact that there are differences between fetal and adult pathways for bile acid synthesis from cholesterol (1, 2). In general, while the major pathways for cholic and chenodeoxycholic acid are well developed by the fetus in early gestation (1–4), the fetal liver has the additional capacity to synthesize an array of bile acids not typical of adult life (1, 2, 5–10). The most important of these pathways that have been identified are 1β- and 6α-hydroxylation (9, 10). Recent analyses of human fetal gallbladder bile (1,2) and neonatal gastric aspirates containing refluxed bile (6,8) have indicated the presence of a quantitatively important trihydroxy bile acid of unknown structure. This unknown bile acid comprised between 5 and 15% of the total biliary bile acids in early human development (2). This paper presents data that confirm the structure of this biliary bile acid as 3α,4β,7α-trihydroxy-5β-cholanoic acid. Definitive confirmation of the structure of this unique C-4 hydroxylated cholanoic acid was made possible by the chemical synthesis of several C-4 hydroxy bile acids including 3α,4β,7α-trihydroxy-5β-cholanoic acid. To our knowledge these data provide the first conclusive evidence for the occurrence of naturally occurring bile acids having a 4β-hydroxy group.

MATERIALS AND METHODS

Collection of bile and isolation of 3α,4β,7α-trihydroxy-5β-cholanoic acid from bile

Human fetal gallbladder bile was obtained following autopsy of 28–34 week gestation still-born fetuses, and aspiration of the gallbladder. This material was obtained under a protocol approved by the Children's Hospital Medical Center Institutional Review Board (no. 87-5-4). Bile acids were extracted from bile using octadecylsilane-bonded silica cartridges exactly as described elsewhere (11). This extract was then subjected to HPLC analysis (12, 13). HPLC was carried out using a Varian 5000 Series HPLC System equipped with a Shandon ODS

Abbreviations: HPLC, high performance liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; TLC, thin-layer chromatography.

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column (25 cm × 4.6 mm, 5 μm particle size) with an isocratic mobile phase consisting of methanol–0.1 M ammonium acetate 75/25 (v/v), pH 5.3, at a flow rate of 0.7 ml/min. The eluting components detected by UV (λ = 205 nm) were collected in 1-ml fractions immediately following injection of the bile extract. Removal of the mobile phase buffer was achieved by diluting the extract with distilled water to give a final concentration of 5% methanol, and passing the diluted sample through a cartridge of octadecylsilane-bonded silica (11). After washing the cartridge with distilled water the bile acids in each fraction were recovered by elution with methanol (3 ml). These extracts were taken to dryness over nitrogen and the bile acids were hydrolyzed with 10 units of cholylglycine hydrolase (*Clostridium perfingens*) in the presence of 0.02 M 2-mercaptoethanol in a 0.1 M phosphate buffer (pH 5.8) for 16 h at 37°C. The unconjugated bile acids were extracted from the hydrolysates by passage through a cartridge of octadecylsilane-bonded silica, and recovered with methanol (3 ml). Location of 3α,4β,7α-trihydroxy-5β-cholanoic acid in the HPLC fractions was achieved by GLC–MS analysis after preparation of the methyl ester–trimethylsilyl (Me–TMS) ether derivatives. Repeated HPLC analysis of bile samples afforded a means of collecting a sufficient amount of this bile acid for characterization studies.

Derivatization

Methyl ester–trimethylsilyl (Me–TMS) ethers: The methyl ester derivative was first prepared essentially as described by Fales, Jaouni, and Babashak (14) using ethereal diazomethane freshly prepared from the reaction between sodium hydroxide and N-methyl-N-nitroso-*p*- toluenesulfonamide (Diazald, Aldridge Chemical Co., UK). The excess reagents were removed by evaporation under a stream of nitrogen and the trimethylsilyl (TMS) ether derivative was prepared (15) by the addition of 50 μl of a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (v/v/v). The derivatized sample was further purified through a small column of Lipidex 5000 (16).

Gas–liquid chromatography–mass spectrometry

The Me–TMS ether derivatives were chromatographed on a 30 meter × 4 mm DB-1 (film thickness 0.25 μm) fused silica capillary column (J and W Scientific, Folsom, CA) using a temperature-programmed operation between 225°C and 295°C with increments of 2°C/min with initial and final isothermal periods of 5 min and 20 min, respectively. Helium was used as carrier gas with a flow rate of 1.8 ml/min measured from the column end at ambient temperature.

GLC–MS analysis was carried out using a Finnigan 4635 quadrupole GLC–MS–DS instrument (Finnigan Inc., San Jose, CA) housing an identical GLC column with the same chromatographic conditions. Electron impact (70 eV) ionization mass spectra were recorded over the mass range 50–800 daltons by repetitive scanning (2.0 sec/cycle) of the eluting components. Data were stored and processed using the Super Incos Data System (Finnigan Inc., San Jose, CA).

High resolution mass spectrometric analysis of the principal nomimal masses in the electron impact (70 eV) ionization mass spectrum of the Me–TMS ether derivative of the bile acid isolated from human fetal bile was carried out using a VG-70S-250 magnetic sector mass spectrometer (VG Analytical, Manchester, UK). The GLC–MS conditions were exactly as described for low resolution analysis.

NMR spectroscopy

^{1}H-NMR spectra were obtained on a JEOL FX-90Q (90 MHz) Fourier transform spectrometer with $CDCl_3$ containing 1% tetramethylsilane as the solvent; chemical shifts are expressed (ppm) relative to tetramethylsilane.

Chemical synthesis of C-4 hydroxylated bile acids

Methyl 3α,4β,7α-trihydroxy-5β-cholanoate and methyl 3β,4β,7α-trihydroxy-5β-cholanoate were synthesized from methyl chenodeoxycholate in seven steps according to the chemical route shown in **Fig. 1**. The individual steps were carried out as follows.

Step 1—Preparation of methyl 7α-hydroxy-3α-tosyloxy-5β-cholanoate (2). A solution of tosyl chloride (13.5 g) dissolved in dry pyridine (15 ml) was added to a solution of methyl chenodeoxycholate (1, 21.5 g) in dry pyridine (135 ml) and the mixture was allowed to stand overnight at room temperature. The solution was added dropwise to a mixture of ice chips in water with stirring. The precipitated solid was filtered, washed with water, and recrystallized from benzene hexane to give the product (methyl 7α-hydroxy-3α-tosyloxy-5β-cholanoate, 2) as colorless thin plates; yield, 25.63 g (86%). mp 135–137°C. ^{1}H-NMR δ:0.64(3H, s, 18-CH_3), 0.87(3H, s, 19-CH_3), 2.44(3H, s, phenyl CH_3), 3.66(3H, s, $COOCH_3$), 3.80(1H, m, 7-H), 4.29(1H, brm, 3-H), 7.31 and 7.79(each 2H, d, J = 9.0 Hz, *p*-disubstituted phenyl).

Step 2—Preparation of methyl 7α-hydroxy-5β-chol-3-enoate (3). A solution of the 3α-monotosylate (2, 2.9 g) in 2,6-lutidine (30 ml) was refluxed for 3.5 h under a stream of N_2. Most of lutidine was evaporated under a reduced pressure, and the residue was extracted with dichloromethane. The extract was successively washed with distilled water, 10% HCl, and distilled water, dried with Drierite, and evaporated to dryness. Repeated fractional crystallization of the residue from aqueous methanol gave methyl 7α-hydroxy-5β-chol-3-enoate (3) as colorless prisms: yield, 1.80 g (89%). Slight melting at 98°C, mp 110–113°C (literature mp 117–120°C (17). ^{1}H- NMR δ: 0.67(3H, s,

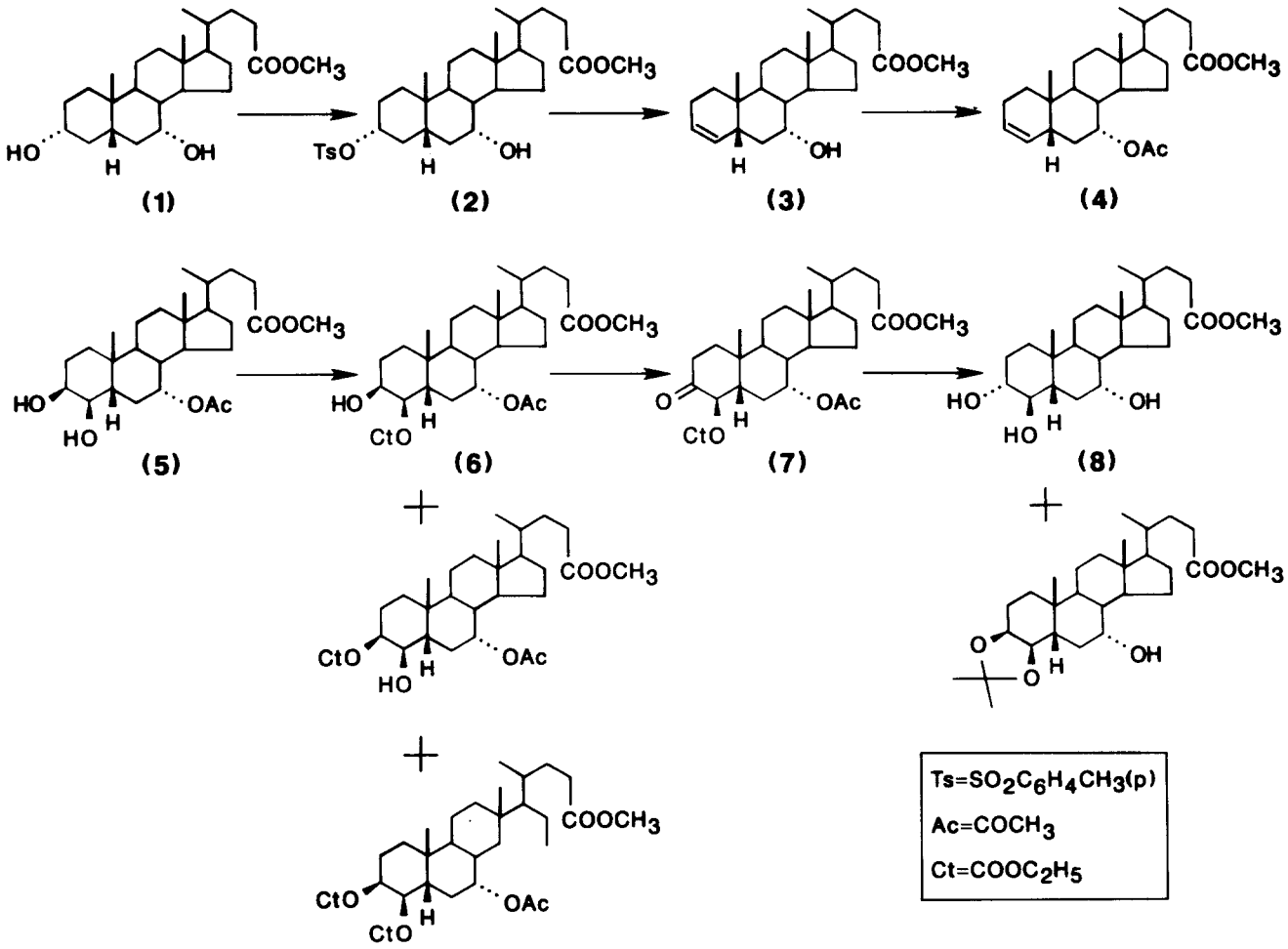

Fig. 1. Schematic of the route for chemical synthesis of methyl 3α,4β,7α-trihydroxy-5β-cholanoate.

18-CH_3), 0.92(3H, d, J = 6.3 Hz, 21-CH_3), 0.97(3H, s, 19-CH_3), 3.66(3H, s, $COOCH_3$), 3.71(1H, brm, 7-H), 5.70(2H, brs, 3- and 4-H).

Step 3—Preparation of methyl 7α-acetoxy-5β-chol-3-enoate (4). To a stirred solution of the 7α-hydroxy-Δ^3 ester (3, 6.0 g) in dry pyridine (50 ml), acetic anhydride (8 ml) and 4-dimethylaminopyridine (1.86 g) were added. The mixture was stirred for 2 h at room temperature, water was then added, and the oily product that separated was extracted with dichloromethane. The combined extract was successively washed with distilled water, 10% HCl, and distilled water to neutrality, dried with Drierite, and evaporated. The residual oil was purified by column chromatography on silica gel (120 g) and elution with benzene gave a 97% yield (6.44 g) of the desired product, methyl 7α- acetoxy-5β-chol-3-enoate (4), that was homogeneous as judged by TLC and ^{1}H-NMR, yet failed to crystallize. ^{1}H-NMR δ:0.66(3H, s, 18-CH_3), 0.92(3H, d, J = 5.4Hz, 21-CH_3), 0.97(3H, s, 19-CH_3), 1.99(3H, s, 7-$OCOCH_3$), 3.66(3H, s, $COOCH_3$), 4.79(1H, m, 7-H), 5.23-5.60(2H, m, 3- and 4-H).

Step 4—Preparation of methyl 7α-acetoxy-3β,4β-dihydroxy-5β-cholanoate (5). To a solution of the 7α-acetoxy-Δ^3 ester (4, 5.37 g) dissolved in 54 ml of tert-butyl alcohol-tetrahydrofuran-water 10:3:1 (v/v/v); N-methylmorpholine N-oxide (4.03 g) and osmium tetroxide (110 mg) were added. After standing for 12 h at room temperature, distilled water was added, and the reaction product was extracted with dichloromethane. The extract was washed with 10% HCl and water, dried with Drierite, and evaporated to dryness. The resulting dark brown oil was chromatographed on a column of neutral alumina (150 g, activity II) and eluted with benzene-ethyl acetate 1:1 (v/v). Recrystallization of the product from aqueous methanol gave the product methyl 7α-acetoxy-3β,4β-dihydroxy-5β-cholanoate (5) as colorless thin plates, which partially melted at 105-106°C, resolidified, and remelted at 139-140°C; yield, 4.90 g (85%). ^{1}H-NMR δ:0.65(3H, s, 18-CH_3), 0.92(3H, d, J = 6.3 Hz, 21-CH_3), 0.98(3H, s, 19-CH_3), 2.06(3H, s, 7-$OCOCH_3$), 3.66(3H, s, $COOCH_3$), 3.99(1H, m, 3-H), 4.11(1H, brm, 4-H), 4.91 (1H, m, 7-H).

Step 5—Preparation of methyl 7α-acetoxy-4β-cathyloxy-3β-hy-

droxy-5β-cholanoate (6). The 7α-acetoxy-3β,4β-dihydroxy ester (5, 4.0 g) was dissolved in dioxane (60 ml) and dry pyridine (9 ml) and cooled with stirring in an ice-bath. Ethyl chloroformate (8 ml) was added dropwise and the mixture was left to stand at room temperature overnight. Distilled water was then added, and the reaction product was extracted with dichloromethane. The combined extract was washed with distilled water, 10% HCl, and distilled water, dried over Drierite, and evaporated. The oily residue was chromatographed on a column of silica gel (130 g) and eluted with benzene–ethyl acetate 8:2 (v/v). Recrystallization of the product from aqueous acetone gave methyl 7α-acetoxy-3β,4β-dicathyloxy-5β-cholanoate (1.02 g, 19%) as colorless fine needles; mp 86–87°C. ^{1}H-NMR θ: 0.65 (3H, s, 18-CH_3), 0.92 (3H, d, J = 5.4 Hz, 21-CH_3), 1.03 (3H, s, 19-CH_3), 1.30 and 1.31(each 3H, t, J = 7.2 Hz, 3- and 4-$OCOOCH_2C\underline{H}_3$), 2.02(3H, s, 7-$OCOCH_3$), 3.66 (3H, s, $COOCH_3$), 4.16 and 4.17(each 2H, q, J = 10.8 Hz, 3- and 4-$OCOOC\underline{H}_2CH_3$), 4.92 (1H, m, 7-H), 5.28(2H, brm, 3- and 4-H).

Continued elution with benzene–ethyl acetate 7:3 (v/v) afforded the desired product, methyl 7α-acetoxy-4β-cathyloxy-3β-hydroxy-5β-cholanoate (6), which crystallized slowly from aqueous methanol as colorless needles; yield, 1.05 g (23%); mp 95–96°C. ^{1}H-NMR δ:0.65(3H, s, 18-CH_3), 0.92(3H, d, J = 5.4 Hz, 21-CH_3), 1.02(3H, s, 19-CH_3), 1.30(3H, t, J = 7.2 Hz, 4-$OCOC\underline{H}_2CH_3$), 2.04(3H, s, 7-$OCOCH_3$), 3.66(3H, s, $COOCH_3$), 4.15 (2H, q, J = 10.8 Hz, 4-$OCOOC\underline{H}_2CH_3$), 4.16(1H, m, 3-H), 4.90(1H, m, 7-H), 5.14(1H, dd, $J_{1.2}$ = 11.7 Hz, $J_{2.4}$ = 3.6 Hz, 4-H).

Further elution with benzene–ethyl acetate 1:1 (v/v) gave methyl 7α-acetoxy-3β-cathyloxy-4β-hydroxy-5β-cholanoate (1.14 g, 25%), which crystallized from aqueous acetone as colorless thin plates; mp 171–172°C. ^{1}H-NMR δ: 0.65(3H, s, 18-CH_3), 0.92(3H, d, J = 5.4 Hz, 21-CH_3). 0.99(3H, s, 19-CH_3), 1.31(3H, t, J = 7.2 Hz, 3-$OCOOCH_2C\underline{H}_3$), 2.05(3H, s, 7-$OCOCH_3$), 3.66(3H, s, $COOCH_3$), 4.15(1H, brm, 4-H), 4.19(2H, q, J = 10.8 Hz, 3-$OCOOC\underline{H}_2CH_3$), 4.91(2H, m, 3- and 7-H).

The most polar fraction eluted with ethyl acetate afforded the 3β,4β-dihydroxy ester (5; yield 0.84 g; 21%).

Step 6—Preparation of methyl 7α-acetoxy-4β-cathyloxy- 3-oxo-5β-cholanoate (7). Jones' reagent (2.5 ml) was added slowly and dropwise to a magnetically stirred solution of the 7α-acetoxy-4β-cathyloxy-3β-hydroxy ester (6, 1.0 g) in acetone (10 ml) cooled in an ice-bath at 10°C. The mixture was further stirred at room temperature for 30 min and poured into a methanol–water mixture. The reaction product was extracted with dichloromethane, and the organic extract was washed with water, dried with Drierite, and evaporated. The residue was recrystallized from aqueous methanol to give the product, methyl 7α-acetoxy-4β-cathoxy-3-oxo-5β-cholanoates (7), as colorless fine needles; yield, 856 mg (86%); mp 134–135°C. ^{1}H—NMR θ: 0.69(3H, s, 18-CH_3), 0.93(3H, d, J = 5.4 Hz, 21-CH_3), 1.07(3H,s, 19- CH_3), 1.32(3H, t, J = 7.2 Hz, 4-$OCOOCH_2C\underline{H}_3$), 2.02(3H, s, 7- $OCOCH_3$), 3.66 (3H, s, $COOCH_3$), 4.20(2H, q, J = 10.8 Hz, 4- $OCOOC\underline{H}_2$-CH_3), 5.02(1H, m, 7-H), 5.58(1H, d, J = 11.7 Hz, 4-H).

Step 7—Preparation of methyl 3α,4β,7α-trihydroxy-5β-cholanoate (8). Sodium borohydride (650 mg) was added slowly to a stirred solution of the 7α-acetoxy-4β-cathyloxy-3-oxo ester (7, 650 mg) in methanol (30 ml) at room temperature. After further stirring for 1 h, the reaction mixture was diluted with 10% HCl and extracted with dichloromethane. The organic layer was washed to neutral pH with water, dried with Drierite, and evaporated. After the treatment of the residual oil by hydrolysis with 10% methanolic KOH, methyl esterification with methanol and *p*-toluenesulfonic acid, followed by acetonide formation with acetone (60 ml) and *p*-toluenesulfonic acid (60 mg), the crude product was chromatographed on a column of silica gel (15 g). Elution with benzene–ethyl acetate 6:4 (v/v) yielded a component that was identified as methyl 3β,4β,7α-trihydroxy-5β-cholanoate-3,4-acetonide (303 mg); mp 113–114°C.

A more polar fraction that eluted with ethyl acetate was recrystallized from aqueous methanol to give the desired final product methyl 3α,4β,7α-trihydroxy-5β-cholanoate (8) as colorless needles; yield, 94 mg (18%); mp 128–130°C. ^{1}H-NMR δ:0.66(3H, s, 18-CH_3), 0.92(3H, d, J = 4.5 Hz, 21-CH_3), 0.94(3H, s, 19-CH_3), 3.26(1H, brm, 3-H), 3.66(3H, s, $COOCH_3$), 3.88(1H, m, 7-H), 4.15 (1H, dd, $J_{1.2}$ = 10.8 Hz, $J_{2.4}$ = 9.0 Hz, 4-H).

RESULTS AND DISCUSSION

In a previous report describing the bile acid composition of gallbladder bile from the human fetus (1) we observed the presence of a quantitatively important bile acid of unknown structure. This bile acid was shown to account for between 5 and 15% of the total biliary bile acids during early gestation (weeks 17–19) and our evidence indicated its structure to be consistent with a trihydroxy-cholanoic acid (2). The same compound had earlier been found in gastric aspirates of 3–5-day-old newborn infants with high intestinal obstruction (6, 8), a condition that leads to bile reflux into the stomach. Following the separation of biliary bile acids according to their mode of conjugation, this bile acid was found to be almost exclusively conjugated to taurine (2).

In an attempt to elucidate the structure of the unknown bile acid, bile was collected from still-born fetuses and the unknown trihydroxy bile acid was isolated by HPLC. This compound, which was more hydrophilic than taurocholate, could be identified by GLC-MS from the characteristic ion (*m/z* 181) given by the Me-TMS ether derivative that was prepared following collection and en-

zymatic hydrolysis of individual HPLC fractions. Previous studies, using a lipophilic anion exchange gel to separate bile acids according to their mode of conjugation, had shown this novel bile acid to be mainly conjugated to taurine (2). In its isolation, therefore, a mild enzymatic hydrolysis only was required to release the free bile acid, thereby eliminating the potential for artifact formation.

The electron impact ionization (70 eV) mass spectrum of the Me-TMS ether derivative of the biliary bile acid (**Fig. 2**) revealed a molecular ion (M^+) at *m/z* 638, indicating a trihydroxy-cholanoate structure. This is further supported by the fragment ion at *m/z* 253 which arises from the ABCD-rings after loss of three nuclear trimethylsiloxy groups (-[3 × 90] da) and the side chain (-115 da). Sequential loss of the trimethylsiloxy groups from molecular ion yields the fragment ions at *m/z* 548 (M-90), *m/z* 458 (M-[2 × 90]) and *m/z* 369 (M-[2 × 90 + 89]). This latter loss is characteristically observed with vicinal hydroxyl groups where the last trimethylsiloxy group is lost without a hydrogen (-89 da).

The most significant feature of this mass spectrum is the relatively intense ion of *m/z* 181. This unique ion has not been observed in any of the 111 mass spectra of Me-TMS ether derivatives of bile acids recently compiled as a reference library (18). We previously suggested that this ion may arise from a disubstituted A or B ring structure where two of the hydroxyls were on adjacent carbon atoms (1). The fragmentation pattern of this mass spectrum is not typical for bile acids having hydroxy groups at the C-1, C-2, C-5, or C-6 positions (18-21). To our knowledge, there have been no previous reports in any mammalian species of bile acids having hydroxy groups at the C-4 position, but by elimination this appeared the only possibility for this unique bile acid and we tentatively suggested a 3,4,7-trihydroxy bile acid as the most likely candidate.

The origins of the unique ion *m/z* 181 are unclear; however high resolution mass spectrometric analysis (**Table 1**) indicated its chemical composition to be consistent with $C_{10}H_{17}O_1Si_1$ fragment, and we postulate that this fragment is formed following the loss of one of the trimethylsiloxy groups on the A-ring and cleavage across the C-6, 7 and C-9, 10 bonds. This cleavage is probably facilitated by the presence of the adjacent trimethylsiloxy groups at C-3 and C-4, and from comparison of the mass spectra (See Figs. 3 and 4) of a series of C-4 hydroxy bile acid analogues that were chemically synthesized (T. Iida et al., unpublished data) it appears that the presence of a C-7 trimethylsiloxy group is essential to give rise to this unique ion. The ion at *m/z* 329, arising from loss of 129 daltons from the *m/z* 458, is typically seen with an accompanying ion at *m/z* 129 in the mass spectra of 3-hydroxy-Δ^5 steroids (18, 20). In this compound, this fragment most probably derives by a similar mechanism after the prior loss of the 4β-trimethylsiloxy group.

From these mass spectral data we postulated that this unique bile acid was a 3,4,7-trihydroxy-cholanoic acid isomer. It was of particular interest that the biliary bile acid did not form an acetonide or cyclic boronate derivative. These derivatives are formed by vicinal hydroxy groups and this finding, therefore, indicated that the configura-

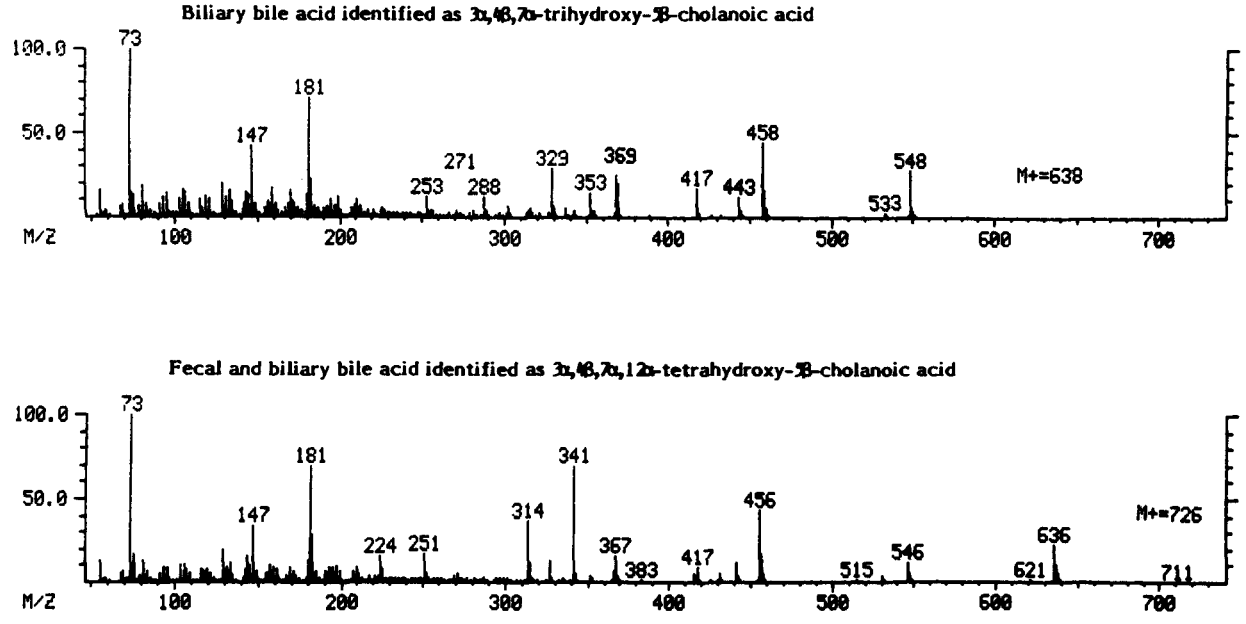

Fig. 2. Electron impact (70 eV) ionization mass spectra of the Me-TMS ether derivatives of the bile acids identified in fetal bile and newborn infant feces as 3α,4β,7α-trihydroxy-5β-cholanoic and 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic acids.

TABLE 1. High resolution GLC-MS analysis of the principal fragments in the electron impact (70 eV) ionization mass spectrum of Me-TMS ether of C-4 hydroxy bile acid

Nominal Mass	Observed Mass	Elemental Composition	Fit
638	not measured		
548	not measured		
458	458.325134	$C_{28}\ H_{46}\ O_3\ Si_1$	3.5 mmu
369	369.294037	$C_{25}\ H_{37}\ O_2$	
368	368.270721	$C_{25}\ H_{36}\ O_2$	0.8 mmu
181	181.105026	$C_{10}\ H_{17}\ O_1\ Si_1$	0.2 mmu

tion of this bile acid was not 3α,4α,7α- or 3β,4β,7α-, leaving only 3α,4β,7α- and 3β,4α,7α-trihydroxy structures as possible candidates. As the majority of bile acids in human fetal gallbladder bile are of a 3α-hydroxy configuration (1, 2) the former stereochemistry (3α,4β,7α) appeared the most probable structure. Confirmation of the position of the hydroxyl groups on the steroid nucleus was achieved following chemical synthesis of several C-4 hydroxy analogues.

Methyl 3α,4β.7α-trihydroxy-5β-cholanoate (8) was synthesized from methyl chenodeoxycholate (1) in a complex seven-step chemical synthesis outlined in Fig. 1.

Methyl chenodeoxycholate (1) was converted to its 3-monotosylate (2) by the general tosylation procedure with tosyl chloride-pyridine. Dehydrotosylation of 3α-tosyloxy-5β-cholanoates in refluxing collidine or lutidine proceeds preferentially towards C-4 to yield the Δ^3-olefine (17, 22, 23) although varying proportions of the Δ^2- and Δ^3-isomers can arise (23). In our experience, methyl cholate 3-monotosylate yields an olefin mixture of the Δ^2- and Δ^3-isomers (14% and 59%, respectively, after chromatographic purification), whereas methyl chenodeoxycholate 3-monotosylate affords almost exclusively the desired Δ^3-compound which is evidenced by a ^{1}H-NMR signal appearing at 5.70 ppm as a broad singlet (T. Iida, T. Momose, J. Goto, and T. Nambara, unpublished data). Elimination of the 3α-tosyloxy group by 2,6-lutidine under reflux condition gave the 7α-hydroxy-Δ^3 compound (3) in 89% yield; ^{1}H-NMR δ, 5.70 (2H, s, 3- and 4-H). After acetylation of 3 with acetic anhydride-4-dimethylaminopyridine in pyridine, the product (4) was transformed into the 7α-acetoxy-3β,4β-dihydroxy ester (5) by the treatment with a catalytic amount of osmium tetroxide and an excess of N-methylmorpholine N-oxide in *tert*-butylalcohol–tetrahydrofuran–water 10:3:1 (v/v/v); yield, 85%; ^{1}H-NMR δ, 3.99 (1H, m, 3-H), 4.11 (1H, brm 4-H), 4.91 (1H, m, 7-H). Carbethoxylation of 5 with ethylchloroformate in dioxane and pyridine produced a mixture of 3,4-

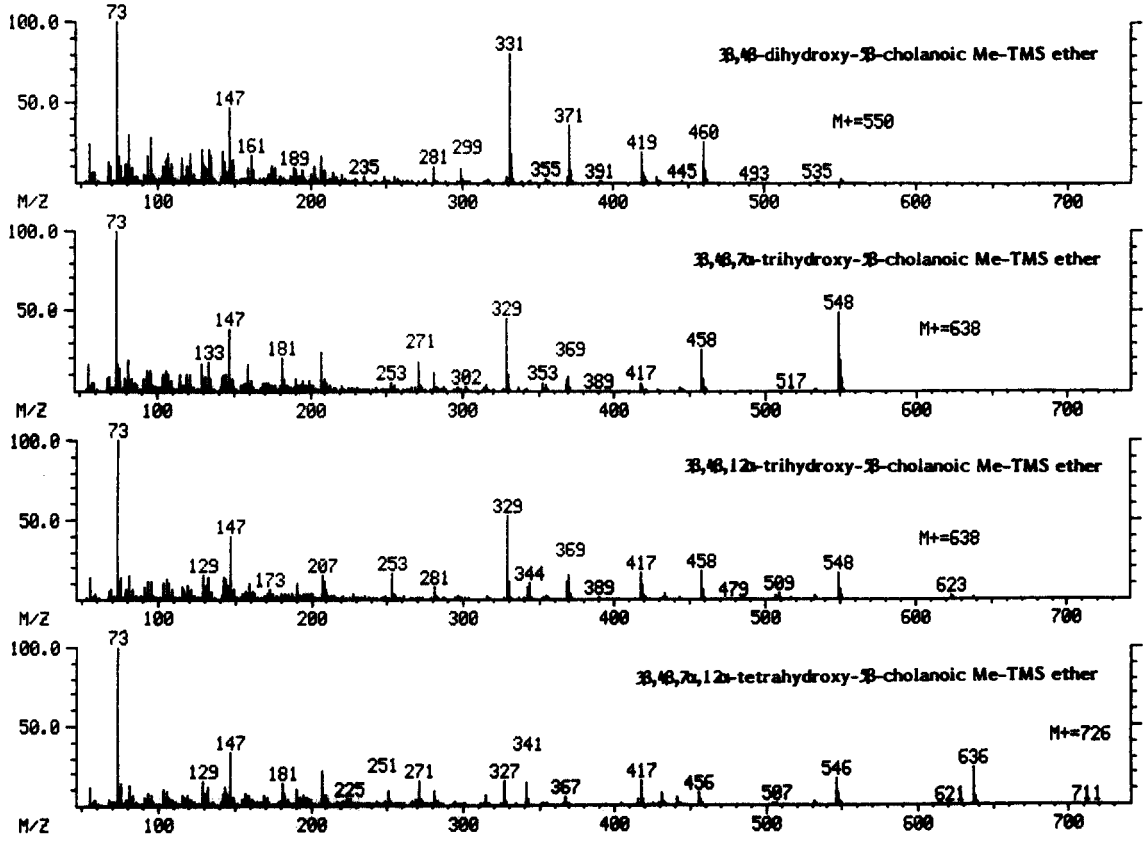

Fig. 3. Electron impact (70 eV) ionization mass spectra obtained for the Me-TMS ether derivatives of authentic standards of a series of 3β,4β-hydroxylated analogues of cholanoic acids.

dicathylate, 3β-monocathylate, 4β-monocathylate (6), and 5 , and purification by silica gel chromatography afforded the desired product (6) in 23% yield; ^{1}H- NMRδ, 4.16 (1H, m, 3-H), 4.90 (1H, m, 7-H), 5.14 (1H, dd, J = 11.7 Hz and J = 3.6 Hz, 4-H). The 4-monocathylate (6) was then oxidized with Jones' reagent to give the 7α-acetoxy-4β-cathyloxy-3-oxo compound (7) in 86% yield; ^{1}H-NMR δ, 5.02 (1H, m, 7-H), 5.58 (1H, d, J = 11.7 Hz, 4-H). Subsequent reduction of 7 with sodium borohydride gave a mixture of methyl 7α-acetoxy- 4β-cathyloxy-3β-hydroxy-5β-cholanoate and its 3α-epimer. This mixture was hydrolyzed with methanolic potassium hydroxide, esterified with methanol- *p*-toluenesulfonic acid, and then treated with acetone-*p*-toluenesulfonic acid to give a mixture of methyl 3β, 4β,7α-trihydroxy-5β-cholanoate 3,4-acetonide and the desired methyl 3α,4β,7α-trihydroxy-5β-cholanoate (8). Separation of the two compounds was efficiently achieved by silica gel chromatography; yield of 8 was 18%; mp 128-130°C (from aqueous methanol); ^{1}H-NMR δ, 0.66 (3H, s, 18- CH_3), 0.92 (3H, d, J = 4.5 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 3.26 (1H, brm, 3-H), 3.66 (3H, s, $COOCH_3$), 3.88 (1H, m, 7-H), 4.15 (1H, dd, J = 10.8 Hz and J = 9.0 Hz, 4-H).

The electron impact ionization mass spectra of the Me-TMS ether derivatives of 3α,4β,7α-trihydroxy-5β-cholanoic and 3β,4β,7α-trihydroxy-5β-cholanoic acids both revealed the diagnostically significant ion *m/z* 181 (**Fig. 3**). However, this ion was not observed in the mass spectrum of 3α,4β-dihydroxy-5β-cholanoic, 3β,4β-dihydroxy-5β-cholanoic, 3α,4β,12α-trihydroxy-5β-cholanoic and 3β,4β,12α-trihydroxy-5β-cholanoic acids (Fig. 3 and **Fig. 4**). This fragment is, however, observed in both 3,4,7,12-tetrahydroxy isomers. The relative intensity of *m/z* 181 ion in the 3α,4β,7α-trihydroxy-5β-cholanoic acid was significantly higher than in the 3β,4β,7α-trihydroxy analogue (as was the case for the corresponding tetrahydroxy isomers) and, excluding the ubiquitous TMS ions, was the base peak in the mass spectrum.

The mass spectrum and GLC retention values (**Table 2**) for the biliary bile acid and the pure standard of 3α, 4β,7α-trihydroxy-5β-cholanoic acid were identical, thereby providing definitive evidence for the structure of this novel bile acid as 3α,4β,7α-trihydroxy-5β-cholanoic acid. As a cautionary note we should point out that the relative retention value for this compound has been reported to differ depending upon the GLC column used. For exam-

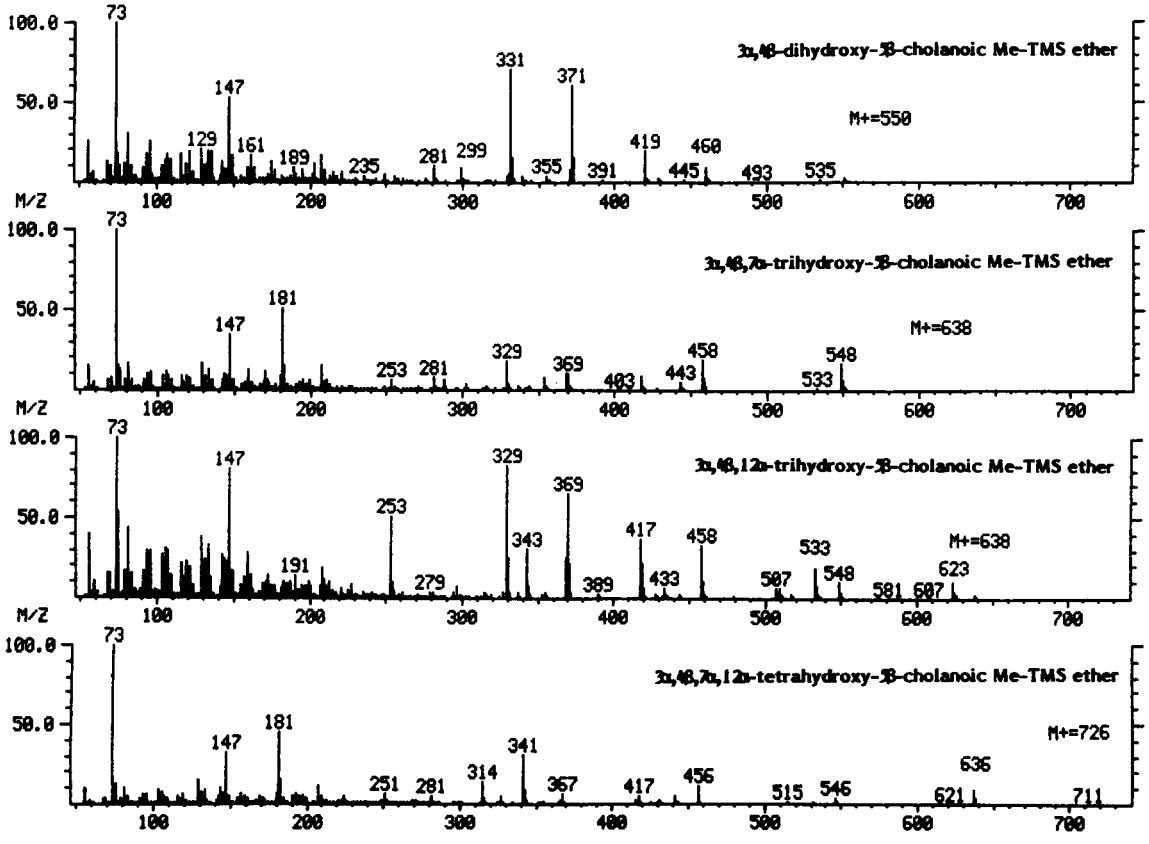

Fig. 4. Electron impact (70 eV) ionization mass spectra obtained for the Me-TMS ether derivatives of authentic standards of a series of 3α,4β-hydroxylated analogues of cholanoic acids.

TABLE 2. GLC retention indices[a] of reference bile acids as methyl ester-trimethylsilyl-ether derivatives

Bile Acid	t_R (MU)[b]
3α,4β-dihydroxy-5β-cholanoic	33.39
3β,4β-dihydroxy-5β-cholanoic	32.81
3α,4β,7α-trihydroxy-5β-cholanoic	33.84
3β,4β,7α-trihydroxy-5β-cholanoic	34.09
3α,4β,12α-trihydroxy-5β-cholanoic	34.00
3β,4β,12α-trihydroxy-5β-cholanoic	33.03
3α,4β,7α,12α-tetrahydroxy-5β-cholanoic	34.62
3β,4β,7α,12α-tetrahydroxy-5β-cholanoic	33.34
3α,4β,7α-trihydroxy-5β-cholanoic identified in fetal bile	33.84
3α,4β,7α,12α-tetrahydroxy-5β-cholanoic identified in fetal bile and newborn feces	34.62

[a]Bile acids were chromatographed on a 30 m × 4 mm i.d. DB-1 fused silica capillary column (film thickness 0.25 μm) using helium as carrier gas (2 ml/min) and operated with temperature programming 225°C–295°C with increments of 2°C/min and initial and final isothermal periods of 5 and 20 min, respectively.

[b]Retention times are expressed relative to an homologous series of n-alkanes and referred to by the Methylene Unit (MU) value. For reference, cholic acid has a retention value of 32.27 MU and a comprehensive list of GLC retention indices is published elsewhere (22).

ple, we first reported its value to be 33.84 MU (1). This is consistent with values reported here and identical to the authentic standard run under identical GLC conditions. In a recent study, however, when a different GLC column with the same selective phase (DB-1) from the same manufacturer was used, the retention values obtained for both the biliary bile acid and the authentic sample were 34.15 MU (2). For each GLC column these retention values

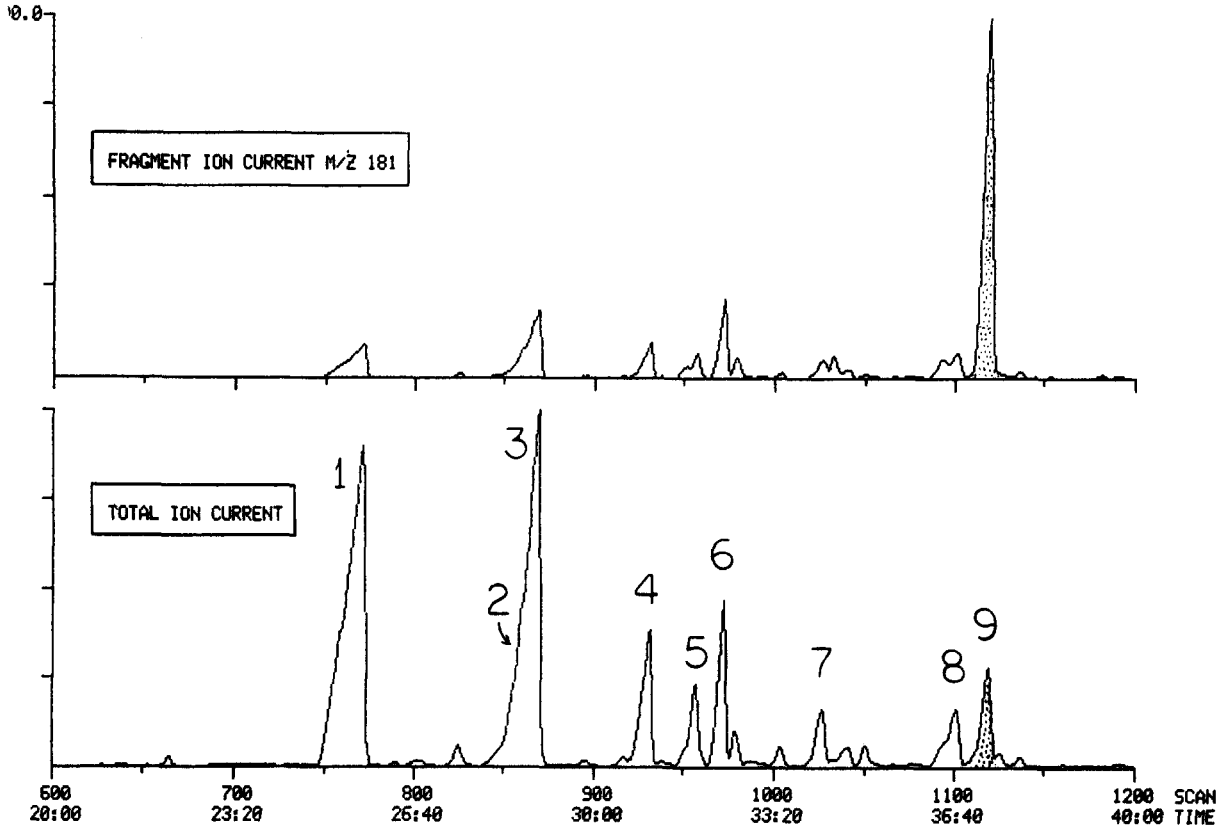

Fig. 5. Total ion current chromatogram (upper trace) and fragment ion current chromatogram (lower trace) of the ion *m/z* 181 (characteristic of bile acids with a 3,4,7-tri-OTMS structure) obtained from the GLC-MS analysis of the Me-TMS ethers of unconjugated bile acids isolated from the feces of a 4-day-old healthy full-term infant. The principal compounds identified are denoted by numbers as follows: 1. cholesterol; 2. chenodeoxycholic acid; 3. cholic acid; 4. 3α,6α,12α-trihydroxy-5β-cholanoic acid; 5. 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid; 6. 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid; 7. unknown dihydroxy-oxo-bile acid; 8. 2β,3α,7α,12α-trihydroxy-5β-cholanoic acid; 9. 3α,4β,7α,12α-trihydroxy-5β-cholanoic acid. The peak shaded corresponds to the C-4 hydroxylated tetrahydroxy bile acid and its full mass spectrum is shown in Fig. 2.

were reproducible, but they highlight the problems of relying upon retention indices for structural elucidation studies and serve to confirm that it is imperative to analyze the authentic standard with the unknown under identical GLC conditions (18, 20). The reason for these differences is unknown, but it probably reflects differing degrees of selectivity between GLC columns that are introduced during the manufacturers preparation and no doubt accounts for some of the reported discrepancies in the literature values of retention indices for different bile acid derivatives (20, 24).

This is not the only C-4 hydroxylated bile acid to be found in human fetal bile. Previously published (2) fragment ion current chromatograms of the *m/z* 181 ion indicated the presence of a minor compound with a longer retention time (34.62 MU) than 3α,4β,7α-trihydroxy-5β-cholanoic acid (33.84 MU). This compound is also found in meconium and in significant proportions in the feces of infants during the first week after birth (25). For example, **Fig. 5** shows the total ion current chromatogram and fragment ion current chromatogram for *m/z* 181 of Me-TMS ether derivatives of the unconjugated bile acids isolated by previously described techniques (26) from the feces of a healthy 4-day-old full-term infant. Quantitatively, this compound was found to comprise approximately 10% of the level of cholic acid, the major fecal bile acid at this age. The mass spectrum confirmed this compound to be a tetrahydroxy-cholanoic acid structure (Fig. 2). The molecular ion is at *m/z* 726, and the fragment ions at *m/z* 636 (M-90), m/z 546 (M-[2 × 90]) *m/z* 456 (M-[3 × 90]), and *m/z* 367 (M- [3 × 90 + 89]) reflect the consecutive losses of four ring hydroxyls. The ion *m/z* 251 confirms these hydroxy groups to be in the steroid nucleus. The loss of side-chain is evident from the fragment at *m/z* 341 (*m/z* 456-115) and the origins of the *m/z* 181 ion have been discussed previously. The mass spectrum and retention time of the Me-TMS ether derivative were found to be identical to that of the authentic bile acid 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic acid (shown in Fig. 4) that was synthesized via the same chemical route as that described here for 3α,4β,7α-trihydroxy-5β-cholanoic acid. The presence of this bile acid in biological samples collected many days after birth (6, 8, 25) indicates that C-4 hydroxylation must be a primary reaction occurring in the fetal and newborn liver and that these unique bile acids are not of maternal origin.

To our knowledge this is the first time that bile acids hydroxylated at the C-4 position have been identified in any mammalian species. Although a systematic search has not yet been made to determine the distribution of C-4 hydroxylated bile acids, in other species it appears that C-4 hydroxylation may be a unique feature of bile acid metabolism in early human life. In view of the quantitative importance of this bile acid in fetal gallbladder bile (2) and in feces, the physiological and physiochemical properties of this unique bile acid warrant future investigation.

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