Synthesis of 19-Hydroxylated Bile Acids and Identification of 3α,7α,12α,19-Tetrahydroxy-5β-cholan-24-oic Acid in Human Neonatal Urine

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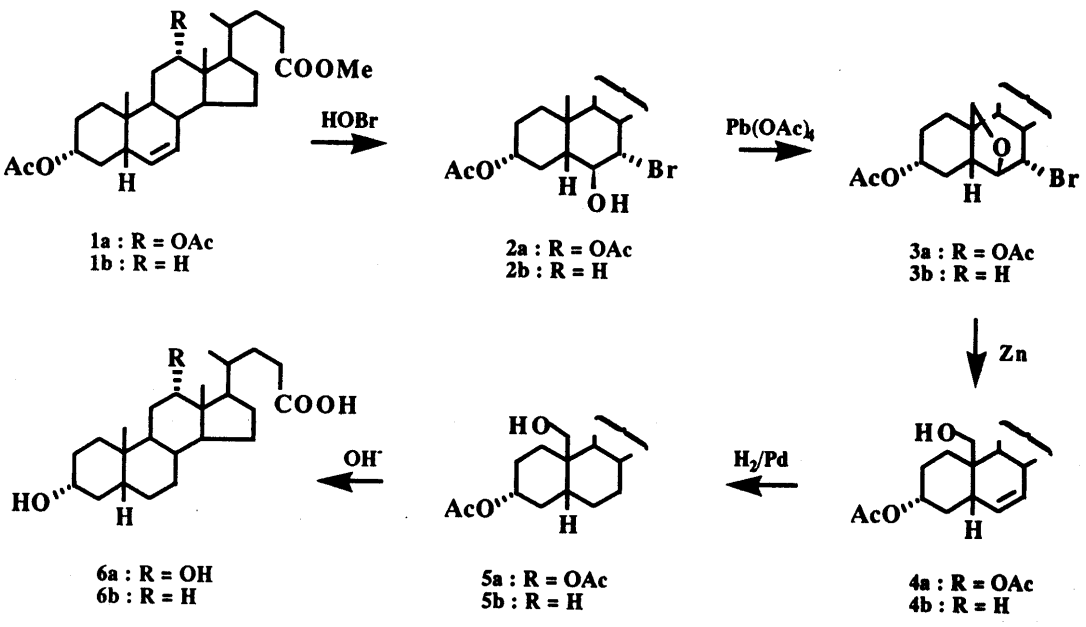
The synthesis of 19-hydroxylated bile acids (3α,19-dihydroxy-, 3α,7α,19-trihydroxy-, 3α,12α,19-trihydroxy- and 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acids) was described. These synthesized 19-hydroxylated bile acids were used as standard samples for the analysis of bile acids in human urine by gas chromatography–mass spectrometry. 3α,7α,12α,19-Tetrahydroxy-5β-cholan-24-oic acid was identified in neonatal urines (0.1—1.5 μg/ml and 1.5—7% of total bile acids).

Key words bile acid; synthesis; 19-hydroxylated bile acid; gas chromatography–mass spectrometry; selected ion monitoring; neonatal urine

Recently, the biological fate of bile acid in fetal and neonatal liver has been noted ever since the "hyper-hydroxylated" bile acids such as 1β,3α,7α,12α-,[2)] 3α,6α,7α,12α-,[3,4)] 2β,3α,7α,12α-,[5,6)] and 3α,4β,7α,12α-tetrahydroxy-5β-cholan-24-oic acids[7)] were found in the biological fluids of neonates and of patients with liver malfunction. These observations suggested that primary bile acids, cholic and/or chenodeoxycholic acids were further hydroxylated before excretion in the fetus and newborn stages. Thus, the metabolic pathway of bile acids in fetal and neonatal liver is dramatically different from that of an adult. The physiological role and function of such 1β-, 2β-, 4β- and 6α-hydroxylations have not been made clear, but are considered to be one of the forms for the excretion of bile acids in fetal and neonatal stages.[2-4,6)] Gustafsson *et al.*[8,9)] reported on the metabolic study of 3α-hydroxy-5β-cholan-24-oic acid by its incubation with human fetal liver, and they tentatively identified 3α,19-dihydroxy-5β-cholan-24-oic acid by gas chromatography–mass spectrometry (GC-MS). This observation also indicated the possibility of various hydroxylation for bile acids in fetal liver. However, this unique hydroxylation of bile acid in early life has not been quantitatively studied because of the lack of authentic specimens. We now describe here a synthesis of four 19-hydroxylated bile acids as reference compounds for GC-MS analysis as well as the identification of 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acid (**11a**) in neonatal urine.

Results and Discussion

Synthesis of 19-Hydroxylated Bile Acids The bile acids hydroxylated at the C-19 position (3α,19-dihydroxy-, 3α,7α,19-trihydroxy-, 3α,12α,19-trihydroxy- and 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acids (**6b**, **11b**, **6a**, **11a**)) were synthesized as shown in Charts 1 and 2. The key step of this approach is to functionalize the C-19



1a : R = OAc
1b : R = H

2a : R = OAc
2b : R = H

3a : R = OAc
3b : R = H

6a : R = OH
6b : R = H

5a : R = OAc
5b : R = H

4a : R = OAc
4b : R = H

Chart 1

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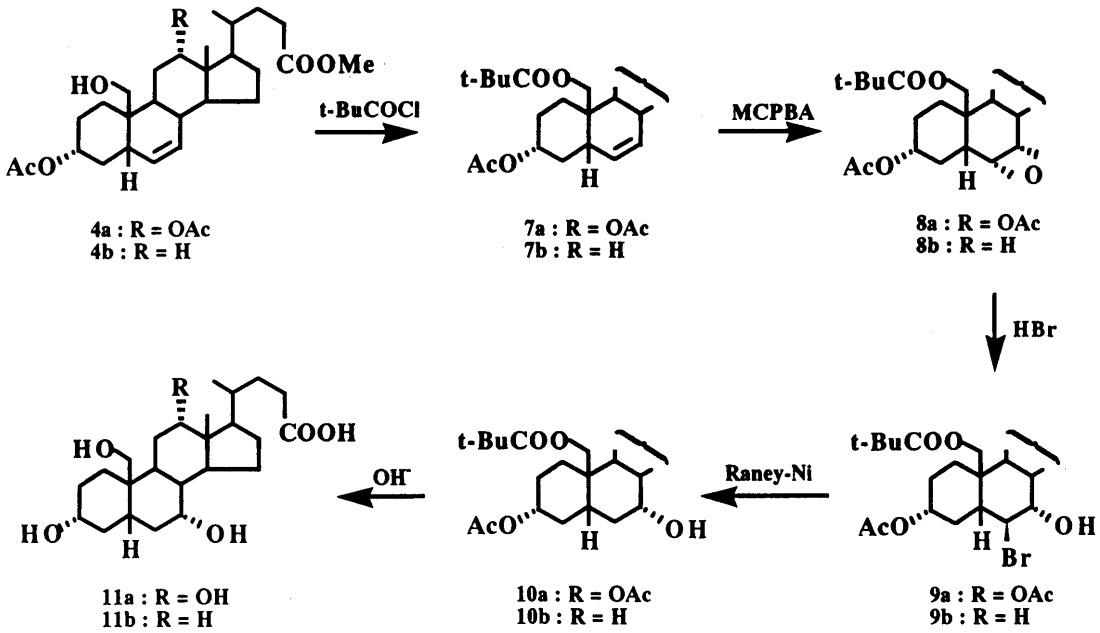

Chart 2

angular methyl group by hypoiodite reaction. It is generally known that the 6β-oriented hydroxyl group in the steroidal ring can be utilized for the functionalization of the angular methyl group.[10,11] Therefore, the preparation of a 6β-hydroxylated compound was initially attempted. Methyl 3α,12α-diacetoxy- and 3α-acetoxy-5β-chol-6-en-24-oates (**1a, b**), derived from their corresponding methyl cholate and chenodeoxycholate according to the reported method,[3,12] were chosen as starting materials for the transformation into 6β-hydroxylated compounds. Hydroxybromination of these compounds with *N*-bromoacetamide afforded two bromohydrins (**2a**: 69%, **2b**: 73%). The hydroxyl group at the C-6 position of **2** was favorably located for the functionalization of the C-19 angular methyl group.[11] The hypoiodite reaction of these bromohydrins (**2a,b**) gave the desired 6,19-epoxy compounds (**3a, b**) in yields of 91% and 84%. The structures of these compounds were established on the basis of their ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra. They clearly showed the disappearance of 19-methyl signals and newly appearing resonances of 19-methylene protons around 3.3 and 3.9 ppm as a pair of doublets. These compounds (**3a, b**) were then transformed into 19-hydroxylated compounds (**4a, b**) by treatment with zinc powder. The ^{1}H-NMR spectrum of **4a** showed the signals of two 19-protons at 3.45 and 3.70 ppm as a pair of doublets, and of olefinic protons at 5.50 ppm. Another 19-hydroxy compound (**4b**) also showed a similar spectrum to **4a**. The catalytic hydrogenation of these compounds (**4a, b**) gave saturated compounds (**5a, b**), which were finally hydrolyzed with methanolic potassium hydroxide to afford 3α,12α,19-trihydroxy- and 3α,19-dihydroxy-5β-cholan-24-oic acids (**6a**: 77%, **6b**: 84%), respectively.

The synthesis of 3α,7α,12α,19-tetrahydroxy- and 3α,7α,19-trihydroxy-5β-cholan-24-oic acids (**11a, b**), considered to be potential metabolites of primary bile acids by 19-hydroxylation, was further carried out from the above synthetic intermediates (**4a, b**) as shown in Chart 2. The most likely way of introducing a hydroxyl group into the C-7α position is by hydroboration and subsequent oxidation. This approach, however, gave only a complex mixture. Therefore, α-epoxidation and reductive cleavage of **4** were carried out. After protection of the 19-hydroxyl group of compound **4a** by acetylation, epoxidation with *m*-chloroperbenzoic acid gave α- and β-epoxides in the ratio of about 1 : 1. The orientation of both epoxides was determined by their ^{1}H-NMR spectra. In the β-epoxide, the signals of 6- and 7-protons appeared at 2.82 and 2.99 ppm as doublets, respectively. This assignment was tentatively settled in comparison with the peak width of each signal. The *J* value (1—2 Hz) between the 5- and 6-protons resulted to broaden the peak width of the signal of the 6-proton. On the other hand, these protons of the α-epoxide appeared at *ca.* 3.1 ppm as a pair of doublets (data are not shown). These coupling patterns were in good agreement with the expected values calculated from the dihedral angles by the Karplus equation. To avoid the unfavorable β-epoxidation, the compounds (**4a, b**) were first treated with pivaloyl chloride to give the pivaloyl esters (**7a, b**). The protection provided by this bulky group may hinder the β-side attack of the reagent. Indeed, the reaction of **7** with *m*-chloroperbenzoic acid gave only α-epoxides (**8a**: 94%, **8b**: 73%). These α-epoxides were then treated with hydrobromic acid to give 6β-bromo-7α-hydroxy compounds (**9a**: 71%, **9b**: 67%) by the transfission of epoxy linkage.[12,13] The structures of these bromohydrins (**9a, b**) were also confirmed by their ^{1}H-NMR spectra, in which the signals appeared as a broad singlet around 4.1 ppm with the $W_{1/2}$-value (5 Hz) because of small *J*-values. These indicated the presence of 6α- and 7β-oriented protons. These bromohydrins were next subjected to reductive debromination with Raney nickel[12] to give 7α-hydroxy compounds (**10a, b**). Finally, these compounds (**10a, b**) were hydrolyzed with methanolic potassium hydroxide to give 3α,7α,12α,19-tetrahydroxy-

and 3α,7α,19-trihydroxy-5β-cholan-24-oic acids (**11a, b**), respectively.

Determination of 19-Hydroxylated Bile Acids The synthesized 19-hydroxylated bile acid (**6b**) was first applied for the identification of 3α,19-dihydroxy-5β-cholan-24-oic acid, reported from analysis of the MS spectrum by Gustafsson *et al.*[8] The MS spectrum of the methyl ester–trimethylsilyl ether (Me-TMS) derivative (Fig. 1) was completely identical with the reported data. This result confirms the presence of hydroxylation at the C-19 position of 3α-hydroxy-5β-cholan-24-oic acid in fetal liver. Other synthesized 19-hydroxylated bile acids were applied as reference compounds for the analysis of bile acids in biological fluids by GC-MS. Fig. 2a shows the MS

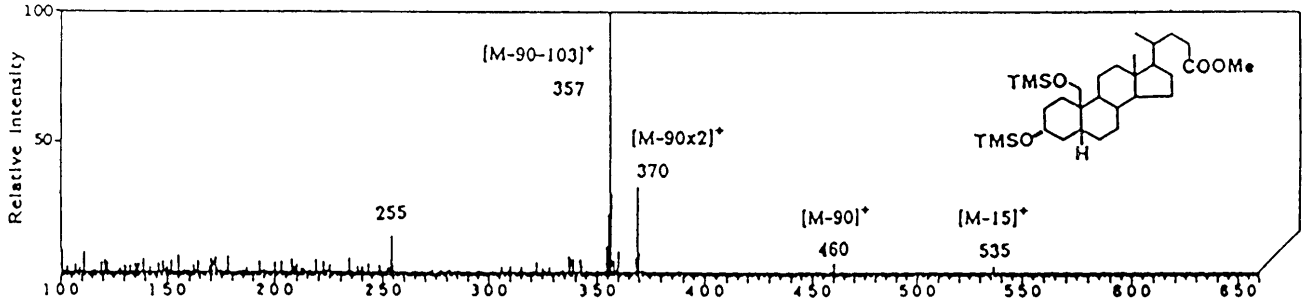

Fig. 1. Mass spectrum of the Me-TMS Derivative of Synthesized 3α,19-Dihydroxy-5β-cholan-24-oic Acid (See Ref. 8)

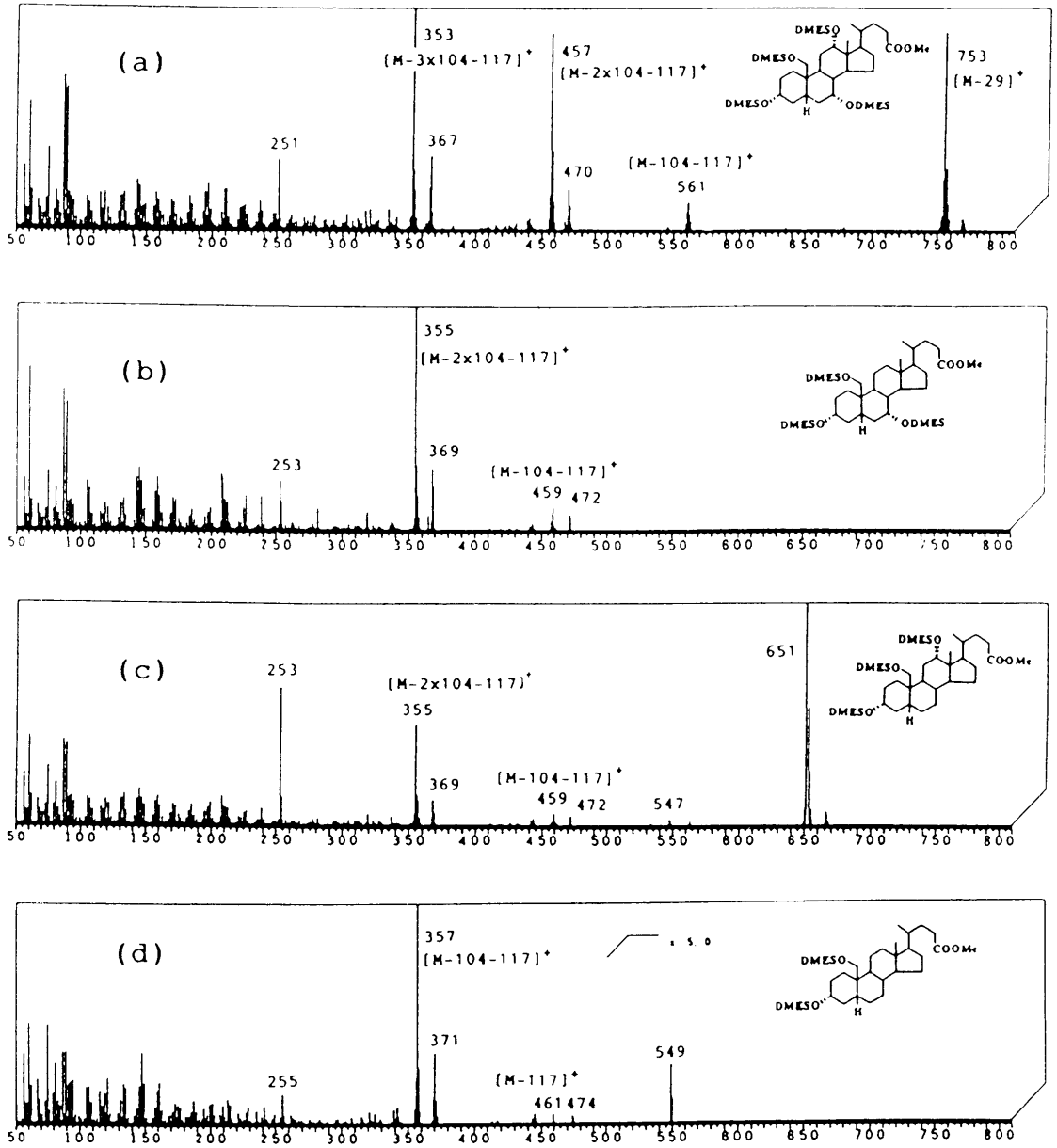

Fig. 2. Mass Spectra of the Me-DMES Derivatives of the Synthesized 3α,7α,12α,19-Tetrahydroxy-5β-cholan-24-oic Acid (**11a**, a), 3α,7α,19-Trihydroxy-5β-cholan-24-oic Acid (**11b**, b), 3α,12α,19-Trihydroxy-5β-cholan-24-oic Acid (**6a**, c) and 3α,19-Dihydroxy-5β-cholan-24-oic Acid (**6b**, d)

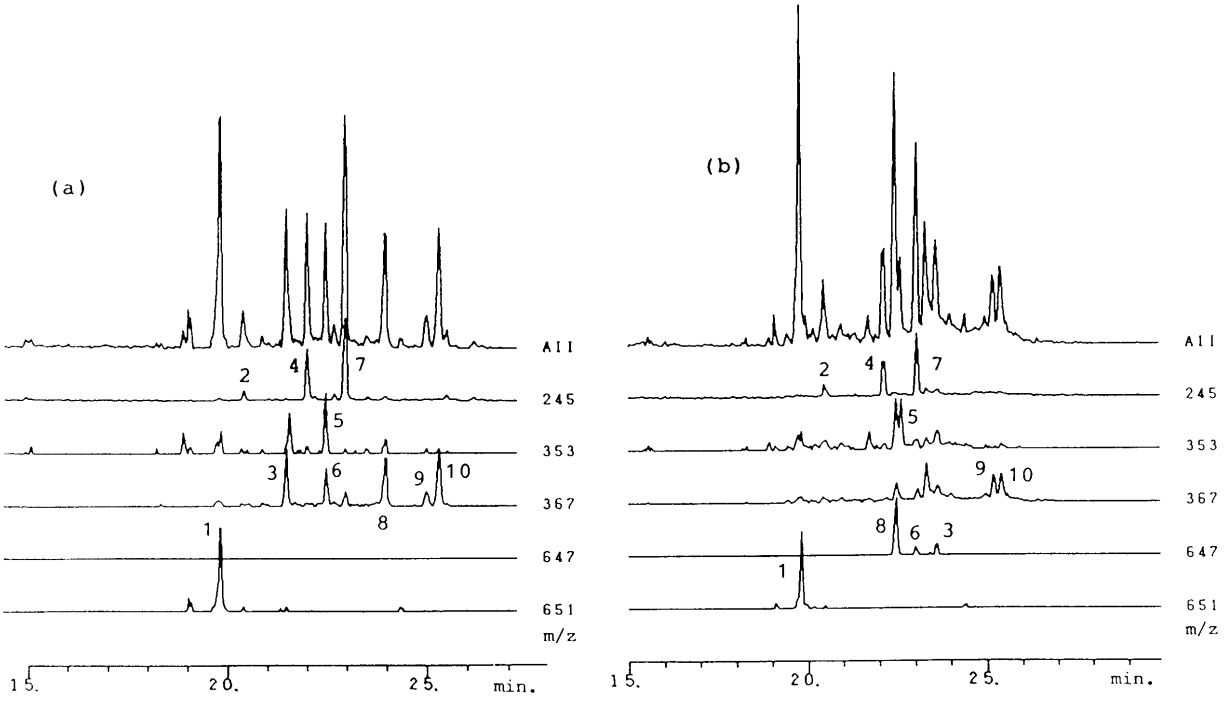

Fig. 3. Mass Chromatograms of the Me-DMES Derivatives of Bile Acids Obtained from Neonatal Urine, Before (a) and After (b) Treatment with *n*-Butylboronic Acid

1: 3α,7α,12α-trihydroxy-, 2: 1β,3α,12α-trihydroxy-, 3: 3α,6β,7β,12α-tetrahydroxy-, 4: 1β,3α,7α-trihydroxy-, 5: 3α,7α,12α,19-tetrahydroxy-, 6: 3α,6α,7α,12α-tetrahydroxy-, 7: 1β,3α,7α,12α-tetrahydroxy-, 8: 3,4,7,12-tetrahydroxy-(tentatively), 9: 3α,4β,7α,12α-tetrahydroxy-, 10: 2β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acids.

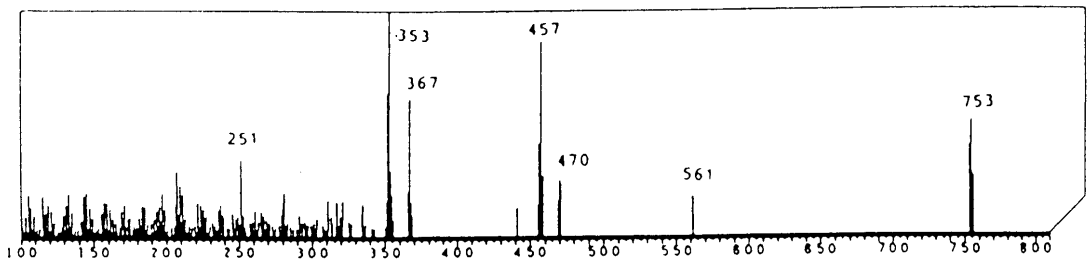

Fig. 4. Mass Spectrum of the Me-DMES Derivative of 3α,7α,12α,19-Tetrahydroxy-5β-cholan-24-oic Acid (**11a**) Isolated from Neonatal Urine

spectrum of a methyl ester–dimethylethylsilyl ether (Me-DMES) derivative of synthesized **11a**. The characteristic fragment ions at *m/z* 561, 457 and 353 correspond to the elimination of dimethylethylsilanols (DMESOH) and the following bond cleavage between C-10 and C-19. These unique fragment ions are often observed in the MS spectra of bile acids by the loss of angular methyl and hydroxyl groups, however, the relative intensities for other fragment ions are not usually significant.[14] Thus, we concluded that these fragment ions are very characteristic and are effective for a quantitative determination of the 19-hydroxylated bile acids. In fact, the Me-DMES derivatives of the other synthesized 19-hydroxylated bile acids (**11b**, **6a** and **6b**) also showed the corresponding fragment ions in their MS spectra (Fig. 2b—d). Other fragment ions of the Me-DMES derivative of **11a** at *m/z* 753, 470, 367 and 251 are assigned to the $[M-Et]^+$, $[M-3\times DMESOH]^+$, $[M-3\times DMESOH-DMESO]^+$ and $[M-4\times DMESOH\text{-side chain}]^+$, respectively. The selected ion monitoring (SIM) method using the above characteristic ion at *m/z* 353 was first carried out for the detection of **11a** in healthy neonatal urine. Figure 3a shows the mass chromatogram of Me-DMES derivatives of bile acids in neonatal urine prepared by the use of monitoring ions at *m/z* 245 (1β-hydroxylated bile acids), 353 (3,7,12,19-tetrahydroxy-bile acid), 367 (2,3,7,12-, 3,4,7,12- and 3,6,7,12-tetrahydroxy bile acids), and 651 (cholic acid). The retention time (22.66 min) of peak 5 was identical with that of the synthetic standard sample, however, peak 6 (3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid, 22.59 min) at *m/z* 367 interfered with the measurement of the mass spectrum. In order to separate peak 5 and peak 6, the derivatization with *n*-butylboronic acid, known as the formation of cyclic boronate[15] with 1,2-*cis* diol, was undertaken. Figure 3b shows the result of the above derivatization. No effect on peak 5 was observed, but peak 6 was shifted to 23.00 min by the formation of cyclic boronate with 6,7-hydroxy groups. Peak 8, which newly appeared close to peak 5, was tentatively assigned as one of the isomers of 3,4,7,12-tetrahydroxy-5β-cholan-24-oic acid.[16] By this method the mass spectrum of peak 5 was obtained without concomitant interference. Figure 4 shows the MS spectrum of the Me-DMES derivative of 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acid found in neonatal urine, which is identical with that of the synthesized compound, as

shown in Fig. 2a. A quantitative determination of this bile acid was also carried out by the SIM method using the monitoring ion at m/z 353. A novel bile acid, 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acid (**11a**), was determined in 10 samples by the analysis of 11 samples from normal neonatal urines (2—9 d after birth). The amounts of the above bile acid in neonatal urine ranged from 0.1 to 1.5 μg/ml, which were relatively significant proportions (1.5—7%) of total bile acids. Since this unique bile acid was not detected in the urine of healthy infants and adults, it is considered to be a characteristic bile acid excreted only in the early neonatal stage.

To the best of our knowledge, this is the first identification and determination of **11a** in human neonatal urine. This result suggests that the hydroxylation to the C-19 position of bile acid proceeds, as well as 1-, 2-, 4- and 6-hydroxylation, in neonatal periods. The quantitative study of these hydroxylated bile acids in the fetal and neonatal periods are now under investigation and the details will appear elsewhere.

Experimental

Synthesis of 19-Hydroxylated Bile Acids All melting points were determined with a micro hot-stage apparatus (Mitamura Riken Co., Tokyo, Japan) and are uncorrected. Infrared (IR) spectra were obtained using an IRA-102 spectrometer (JASCO, Tokyo, Japan) and are expressed in cm^{-1}. Optical rotations were measured with a DIP-360 digital polarimeter (JASCO, Tokyo, Japan). ^{1}H-NMR spectra were recorded at 400 MHz with a JNM-EX 400 spectrometer (JEOL Co., Tokyo, Japan). Chemical shifts are given as the value with tetramethylsilane as an internal standard. The abbreviations used are s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. ^{1}H-NMR data are summarized in Table 1 and other physiochemical data are shown in Table 2. Column chromatography was done using silica gel CQ-2 (74—147 μm, Fuji Gel Hanbai Co., Tokyo, Japan). The usual work-up refers to washing the organic layer with water, drying it over sodium sulfate, and removing the solvent under reduced pressure.

Methyl 3α,12α-Diacetoxy-7α-bromo-6β-hydroxy-5β-cholan-24-oate (2a) and Methyl 3α-Acetoxy-7α-bromo-6β-hydroxy-5β-cholan-24-oate (2b) *N*-Bromoacetamide (4.5 g, 36 mmol) was added to a stirred solution of **1**[3,13] (**a**; 5.86 g, **b**; 5.16 g, 12 mmol) in dioxane (50 ml) containing 0.46 M perchloric acid (12 ml) at 4 °C. After 15 min, 5% sodium bisulfate was added until the color of the bromine disappeared. The mixture was diluted with water and extracted with ether. The usual work-up gave a crude product, which was purified by column chromatography eluted with *n*-hexane/ethyl acetate (3:1 for **a**, 4:1 for **b**). **2a** (4.84 g, 69%, colorless needles from *n*-hexane/ethyl acetate) and **2b** (3.85 g, 73%, colorless needles from methanol) were obtained.

Methyl 3α,12α-Diacetoxy-7α-bromo-6β,19-epoxy-5β-cholan-24-oate (3a) and Methyl 3α-Acetoxy-7α-bromo-6β,19-epoxy-5β-cholan-24-oate (3b) Lead tetraacetate (3.3 g, 7.4 mmol) and calcium carbonate (2.4 g) were added to a stirred mixture of the bromohydrin **2** (**a**; 3.6 g, **b**; 3.24 g, 6.15 mmol) and iodine (1.0 g) in dry benzene (60 ml) and cyclohexane (50 ml). The reaction was carried out by irradiation with a 500 W tungsten lamp for 20 min at 80 °C. The mixture was diluted with ethyl acetate and filtered through a pad of Celite. The filtrate was washed with 5% sodium thiosulfate and the usual work-up gave an oily crude product, which was purified by column chromatography eluted with *n*-hexane/ethyl acetate (2:1). **3a** (3.25 g, 91%, colorless amorphous powder) and **3b** (2.72 g, 84%, colorless leaflets from *n*-hexane/ethyl acetate) were obtained.

Methyl 3α,12α-Diacetoxy-19-hydroxy-5β-chol-6-en-24-oate (4a) and Methyl 3α-Acetoxy-19-hydroxy-5β-chol-6-en-24-oate (4b) Zinc powder (9 g) was added to a stirred solution of the ether **3** (**a**: 2.4 g, **b**: 2.2 g, 4.1 mmol) in acetic acid (30 ml) at 100 °C. After 30 min, the mixture was diluted with dichloromethane and filtered over a pad of Celite. The filtrate was washed with water, 5% sodium bicarbonate, dried and evaporated. The crude product was purified by column chromatography eluted with *n*-hexane/ethyl acetate (2:1 for **4a**, 3:1 for **4b**). **4a** (1.45 g, 70%) and **4b** (1.44 g, 78%) were obtained as colorless needles from *n*-hexane/ethyl acetate.

Methyl 3α,12α-Diacetoxy-19-hydroxy-5β-cholan-24-oate (5a) and Methyl 3α-Acetoxy-19-hydroxy-5β-cholan-24-oate (5b) A mixture of **4** (**a**: 400 mg, **b**: 480 mg) and 10% Pd/C (280 mg) in ethanol (10 ml) was stirred at room temperature in a hydrogen atmosphere for 10 h. The crude product was purified by column chromatography eluted with *n*-hexane/ethyl acetate (1:1). **5a** (360 mg, 90%, colorless amorphous powder) and **5b** (382 mg, 80%, colorless needles from *n*-hexane/ethyl acetate) were obtained.

3α,12α,19-Trihydroxy-5β-cholan-24-oic Acid (6a) and 3α,19-Dihydroxy-5β-cholan-24-oic Acid (6b) A solution of the ester **5** (**a**: 240 mg, **b**: 300 mg) in 2 M methanolic KOH (15 ml) was refluxed for 12 h. After removal of the solvent, the residue was dissolved in water and acidified with 2 M HCl. The precipitates were collected and recrystallized. **5a** (182 mg, 77%, colorless leaflets from ethyl acetate). $[\alpha]_D^{23}$: +58.5° (c = 1.36, MeOH). **6b** (252 mg, 84%, colorless needles from ethyl acetate).

Table 1. ^{1}H-NMR Data for Compounds **2**—**11**[a]

	3-H	6-H	7-H	12-H	18-H	19-H	21-H
2a	4.57 (m)	4.17 (br s)	4.16 (br s)	5.12 (br s)	0.79 (s)	1.12 (s)	0.83 (d, 6)
2b	4.58 (m)	4.18 (br s)	4.16 (br s)		0.70 (s)	1.13 (s)	0.93 (d, 6)
3a	4.65 (m)	4.30 (t, 4)	4.12 (t, 4)	5.12 (br s)	0.85 (s)	3.30, 3.94 (d, 9)	0.86 (d, 6)
3b	4.65 (m)	4.26 (t, 4)	4.05 (t, 4)		0.75 (s)	3.26, 3.93 (d, 9)	0.93 (d, 6)
4a	4.65 (m)	5.50 (br s)	5.50 (br s)	5.10 (br s)	0.79 (s)	3.45, 3.70 (d, 9)	0.82 (d, 6)
4b	4.65 (m)	5.48 (br s)	5.48 (br s)		0.69 (s)	3.55, 3.62 (d, 9)	0.92 (d, 6)
5a	4.70 (m)			5.06 (br s)	0.73 (s)	3.57, 3.82 (d, 9)	0.82 (d, 6)
5b	4.73 (m)				0.64 (s)	3.53, 3.89 (d, 9)	0.90 (d, 6)
6a	3.90 (m)			4.16 (br s)	0.73 (s)	3.57, 3.82 (d, 10)	1.16 (d, 6)
6b	4.00 (m)				0.62 (s)	3.72, 4.12 (d, 9)	0.96 (d, 6)
7a	4.14 (m)	5.50 (br s)	5.50 (br s)	5.10 (br s)	0.75 (s)	3.80, 4.10 (d, 11)	0.82 (d, 6)
7b	4.65 (m)	5.48 (br s)	5.48 (br s)		0.67 (s)	3.86, 4.08 (d, 11)	0.99 (d, 6)
8a	4.64 (m)	3.10 (dd, 4, 5)	3.13 (dd, 4, 5)	5.07 (br s)	0.77 (s)	3.87, 3.96 (d, 10)	0.83 (d, 6)
8b	4.66 (m)	3.07 (dd, 4, 5)	3.11 (dd, 4, 5)		0.68 (s)	3.90, 3.94 (d, 11)	0.90 (d, 6)
9a	4.52 (m)	4.10 (br s)	4.10 (br s)	5.11 (br s)	0.80 (s)	3.95, 4.67 (d, 11)	0.83 (d, 6)
9b	4.55 (m)	4.02 (br s)	4.07 (br s)		0.72 (s)	3.99, 4.70 (d, 11)	0.93 (d, 6)
10a	4.53 (m)		3.88 (br s)	5.10 (br s)	0.75 (s)	3.91, 4.25 (d, 11)	0.82 (d, 6)
10b	4.55 (m)		3.87 (br s)		0.66 (s)	3.98, 4.26 (s, 11)	0.92 (d, 6)
11a	3.89 (m)		4.16 (br s)	4.27 (br s)	0.84 (s)	3.93, 4.28 (d, 11)	1.24 (d, 6)
11b	3.94 (m)		4.07 (br s)		0.72 (s)	3.88, 4.23 (d, 11)	1.04 (d, 6)

a) Spectra were measured in $CDCl_3$ except that 6 and 11 were recorded in pyridine-d_6. Chemical shifts are in δ values and are followed by multiplicities and J-values (in Hz).

Table 2. Physiochemical Data of Compounds **2**—**11**

	mp (°C)	IR (Nujol) (cm^{-1})	Formula	Analysis (%) Calcd C	Calcd H	Found C	Found H
2a	94—96	3450, 1735, 1700	$C_{29}H_{40}BrO_7$	59.48	7.75	59.21	7.49
2b	169—171	3530, 1745, 1720	$C_{27}H_{43}BrO_5$	61.47	8.22	61.21	7.99
3a	57—59[a]	1740, 1720	$C_{29}H_{43}BrO_7$	59.69	7.43	59.47	7.22
3b	122—124	1735, 1720	$C_{27}H_{41}BrO_5$	61.71	7.86	61.58	7.75
4a	114—115	3450, 1730, 1720	$C_{29}H_{44}O_7$	69.02	8.79	68.98	8.57
4b	146—148	3500, 1730, 1720	$C_{27}H_{42}O_5$	72.61	9.48	72.45	9.33
5a	64—68[a]	3500, 3350, 1730	$C_{29}H_{46}O_7$	68.75	9.15	68.66	8.97
5b	182—183	3350, 1730	$C_{27}H_{44}O_5$	72.28	9.89	72.10	9.78
6a	138—140	3350, 2650, 1690	$C_{24}H_{40}O_5$	70.55	9.87	70.30	9.93
6b	220—222	3350, 2700, 1710	$C_{24}H_{40}O_5$	73.43	10.27	73.36	9.99
7a	72—77[a]	1740, 1630	$C_{34}H_{52}O_8$	69.36	8.90	69.15	8.72
7b	107—109	1720	$C_{32}H_{50}O_6$	72.41	9.50	72.37	9.67
8a	169—172[a]	1760, 1720	$C_{34}H_{52}O_9$	67.52	8.67	67.36	8.54
8b	172—174	1720	$C_{32}H_{50}O_7$	70.30	9.22	70.09	9.33
9a	104—108[a]	3500, 1725	$C_{34}H_{53}BrO_9$	59.56	7.79	59.33	7.68
9b	94—99[a]	3500, 1730	$C_{32}H_{51}BrO_7$	61.23	8.19	60.91	8.01
10a	79—82[a]	3550, 1730	$C_{34}H_{54}O_9$	67.30	8.97	67.17	9.14
10b	69—72[a]	3500, 1730	$C_{32}H_{52}O_7$	70.04	9.55	69.90	9.34
11a	275—277	3450, 2650, 1700	$C_{24}H_{40}O_6 \cdot 1/2H_2O$	66.48	9.53	66.75	9.54
11b	129—130	3350, 2650, 1700	$C_{24}H_{40}O_5 \cdot 1/2H_2O$	69.03	9.90	69.08	10.02

a) Amorphous powder.

$[\alpha]_D^{23}$: +35.8° (c=0.62, MeOH).

Methyl 3α,12α-Diacetoxy-19-trimethylacetoxy-5β-chol-6-en-24-oate (7a) and Methyl 3α-Acetoxy-19-trimethylacetoxy-5β-chol-6-en-24-oate (7b) Trimethylacetyl chloride (0.4 ml, 2.8 mmol) was added dropwise to a stirred solution of **4** (**a**: 1.20 g, **b**: 1.13 g, 2.5 mmol) in dry pyridine (10 ml) under ice-cooling. After 3 h, the reaction mixture was poured into ice-water and extracted with ether twice. The combined extract was washed with 2 M HCl and then 5% sodium bicarbonate. The usual work-up gave a crude product, which was purified by column chromatography eluted with *n*-hexane/ethyl acetate (4 : 1 for **7a**, 6 : 1 for **7b**). **7a** (1.20 g, 82%, colorless amorphous powder) and **7b** (1.01 g, 76%, colorless needles from methanol) were obtained.

Methyl 3α,12α-Diacetoxy-6α,7α-epoxy-19-trimethylacetoxy-5β-cholan-24-oate (8a) and Methyl 3α-Acetoxy-6α,7α-epoxy-19-trimethylacetoxy-5β-cholan-24-oate (8b) A solution of **7** (**a**: 0.98 g, **b**: 0.88 g, 1.7 mmol) and *m*-chloroperbenzoic acid (0.67 g, 3.9 mmol) in dichloromethane (30 ml) was stirred for 4 h under ice-cooling and then the reaction mixture was washed with 5% sodium sulfite. After the usual work-up, the crude product was purified by column chromatography eluted with *n*-hexane/ethyl acetate (3 : 1 for **8a**, 5 : 1 for **8b**). **8a** (0.94 g, 94%, colorless amorphous powder) and **8b** (0.65 g, 73%, colorless needles from *n*-hexane/ethyl acetate) were obtained.

Methyl 3α,12α-Diacetoxy-6β-bromo-7α-hydroxy-19-trimethylacetoxy-5β-cholan-24-oate (9a) and Methyl 3α-Acetoxy-6β-bromo-7α-hydroxy-19-trimethylacetoxy-5β-cholan-24-oate (9b) 25% Hydrobromic acid in acetic acid (0.9 ml) was added to an ice-cooled solution of **8** (**a**: 0.85 g, **b**: 0.77 g, 1.4 mmol) in dichloromethane (10 ml). The reaction mixture was stirred for 2 h at room temperature and then diluted with dichloromethane. The mixture was washed with water and 5% sodium bicarbonate. The usual work-up gave an oily crude product, which was purified by column chromatography eluted with *n*-hexane/ethyl acetate (3 : 1). **9a** (0.68 g, 71%) and **9b** (0.59 g, 67%) were obtained as colorless amorphous powders.

Methyl 3α,12α-Diacetoxy-7α-hydroxy-19-trimethylacetoxy-5β-cholan-24-oate (10a) and Methyl 3α-Acetoxy-7α-hydroxy-19-trimethylacetoxy-5β-cholan-24-oate (10b) Freshly prepared Raney-Ni W-2 (7 ml, 10% suspended solution in ethanol) was added to a stirred solution of **9** (**a**: 620 mg, **b**: 562 mg, 0.90 mmol) in 70% ethanol (45 ml) containing three drops of acetic acid. The reaction mixture was refluxed with stirring for 10 min. The catalyst was removed by filtration and the filtrate was evaporated. The residue was worked-up in the usual manner. The crude product was purified by column chromatography eluted with *n*-hexane/ethyl acetate (2 : 1 for **10a**, 3 : 1 for **10b**). **10a** (480 mg, 88%) and **10b** (420 mg, 85%) were obtained as colorless amorphous compounds.

3α,7α,12α,19-Tetrahydroxy-5β-cholan-24-oic Acid (11a) and 3α,7α,19-Trihydroxy-5β-cholan-24-oic Acid (11b) A solution of **10** (**a**: 400 mg, **b**: 367 mg, 0.67 mmol) in 2 M methanolic KOH (15 ml) was refluxed for 12 h. After evaporation of the solvent, the residue was dissolved in water and acidified with 2 M HCl. The mixture was extracted with ethyl acetate three times. The usual work-up gave a crude product, which was purified by recrystallization. **11a** (200 mg, 70%) as colorless prisms from ethyl acetate/methanol. $[\alpha]_D^{20}$: +40.4° (c=0.95, MeOH). **10b** (180 mg, 64%) as colorless prisms from ethyl acetate. $[\alpha]_D^{20}$: +21.1° (c=0.59, MeOH).

GC-MS GC-MS was performed on a GCMS 9100 MK spectrometer equipped with a data processing system, GCMSPAK 1500 (Shimadzu Co., Kyoto, Japan). An Ulbon HR-1T (25 m × 0.25 mm i.d., Shinwakako Co., Kyoto, Japan), fused-silica capillary column bonded with methyl silicone, was used as a gas chromatographic column coupled to the mass spectrometer. The ionization energy was set at 70 eV and the carrier gas was helium at a linear velocity of 45 cm/s. The column oven temperature program was 210 °C (3 min, hold) followed by temperature ramps at 10°C/min to 260 °C and then 2 °C/min to 310 °C.

Sample Preparation from Urine for GC-MS Analysis One microgram of 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid as an internal standard was added to each 1 ml of healthy neonatal urine (n=11, 2—9 d after birth). The above urine samples were loaded on a Bond Elut C18 cartridge column (3 ml, Analytichem, Harbor City, CA), washed with water (3 ml) and then extracted with MeOH (3 ml). The extract was subjected to solvolysis and then hydrolysis as previously reported.[3] The free bile acid fraction prepared by the method reported by Goto *et al.*[17] was first treated with diazomethane in ether at room temperature and then dimethylethylsilylimidazole (30 μl, 60 °C, 45 min) to give Me-DMES derivatives, which were further purified by passing them through a silica gel column (30 mm × 8 mm i.d.) eluted with *n*-hexane/ethyl acetate (3 : 1, 4 ml). After evaporation of the solvent, the residue was redissolved in *n*-hexane (50 μl) and 1 μl was applied to GC-MS analysis. The Me-TMS derivatives were also prepared by the same procedure as above using trimethylsilylimidazole at 45 °C in the place of dimethylethylsilylimidazole. The cyclic boronate derivatives were prepared with 40 μl of 0.75% *n*-butylboronic acid in pyridine at 60 °C for 50 min before treatment with dimethylethylsilylimidazole.

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